Ligase Independent Cloning (LIC)

Ligase independent cloning (LIC) is a simple, fast and relatively cheap method to produce expression constructs. It makes use of the 3'--> 5'-activity of T4 DNA polymerase to create very specific 10-15 base single overhangs in the expression vector. PCR products with complementary overhangs are created by building appropriate extensions into the primers and treating them with T4 DNA polymerase as well. The annealing of the insert and the vector is performed in the absence of ligase by simple mixing of the DNA fragments. This process is very efficient because only the desired products can form.

1. Preparation of vector DNA

The EMBL-made LIC vectors (see appendix for vectors maps) all contain the gene encoding for eGFP flanked by two *Bsa*I sites (shown in **red**). These sites are used to linearize the vector, while at the same time removing the eGFP gene.

```
ATTTTCAGGGCGCCATGAGACCG..eGFP..GGTCTCACCGCGTCGGGTCACCAC
TAAAAGTCCCGCGGTACTCTGGC..eGFP..CCAGAGTGGCGCAGCCCAGTGGTG

                              |  BsaI
                              V

ATTTTCAGGGC                                 CCGCGTCGGGTCACCAC
TAAAAGTCCCGCGGT                                 CAGCCCAGTGGTG
```

Next the digested vector is treated with T4 DNA polymerase in the presence of dTTP. Because of the 3'--> 5' activity of the polymerase the bases are removed from both 3'-ends until the first thymidine (T) residue is reached.

```
ATTTTCAGGGC                                 CCGCGTCGGGTCACCAC
TAAAAGTCCCGCGGT                                 CAGCCCAGTGGTG

                              |  T4 DNA polymerase + dTTP
                              V

ATTTT                                       CCGCGTCGGGTCACCAC
TAAAAGTCCCGCGGT                                         TGGTG
```

This 2-step protocol leads to two specific overhangs in the LIC vector of 10 and 12 bases, respectively, which allow the specific, ligase-independent annealing reaction (protocol 3).

1.1 Linearization of the LIC vector by *Bsa*I digestion

Materials

1.5-ml microfuge tubes

QIAquick Gel Extraction Kit

Chemicals

agarose (electrophoresis grade)
6X Loading dye solution
ethidium bromide (10 mg/ml)
TBE buffer

Enzymes

*Bsa*I

10X New England Biolabs buffer 3 (supplied with the enzyme)

Mix in a 1.5-ml microfuge tube:

5 μl	10X New England Biolabs buffer 3
5 μg	LIC vector DNA
2.5 μl	*Bsa*I (10 units/μl)
	Add sterile water to a volume of 50 μl

1. Add the restriction enzyme last
2. Mix by briefly vortexing the solution and spin 1 min at 13,000 rpm in a microfuge centrifuge.
3. Incubate the digestion mix for 1 hour at 50°C.
4. In the meantime, prepare a 0.8% agarose gel.
Dissolve 0.4 g agarose in 50 ml TBE buffer by heating. After the solution has cooled down add 1-2 µl ethidium bromide solution and pour it into a prepared gel running chamber. After the gel has solidified fill the chamber with TBE buffer.
5. Add 10 μl 6X loading dye solution to the sample. Mix well and spin 1 min at 13,000 rpm in a microfuge centrifuge.
6. Load the sample on the agarose gel.
7. Run the gel for 1 hours at 100 V.
8. Analyze the gel on a UV lamp and cut out the band of the linearized LIC vector.
Expose the gel as briefly as possible to the UV lamp to avoid damage to the DNA.
9. Purify the vector DNA form the gel pieces using the QIAquick Gel Extraction Kit.
10. Elute the digested vector DNA in 50 μl elution buffer in a 1.5-ml microfuge tube.

The *Bsa*I digestion does not necessarily work 100%. It is important to cut out the band of the linearized LIC vector carefully to minimize the amount of undigested vector in the final preparation, as this will give false positive results later on.

The concentration of vector DNA can be determined using the absorbance at 260 nm (assuming A_{260} = 1 is 50 ng/μl).

1.2 T4 DNA polymerase treatment of the linearized LIC vector

In the annealing protocol 25-50 ng prepared LIC vector is used per reaction (see protocol 3). In the following protocol 600 ng *Bsa*I-digested LIC vector is treated with T4 DNA polymerase to produce enough vector for approx. 20 annealing reactions. This can be scaled up or down according to your own needs.

Materials

1.5-ml microfuge tubes

Chemicals

dTTP (100 mM)
DTT (100 mM)
100X BSA

Enzymes

T4 DNA polymerase

10X New England Biolabs buffer 2 (supplied with the enzyme)

Mix in a 1.5-ml microfuge tube:

2 μl	10X New England Biolabs buffer 2
600 ng	*Bsa*I-digested LIC vector
0.5 μl	dTTP (100 mM)
1 μl	DTT (100 mM)
0.2 μl	100X BSA
0.4 μl	T4 DNA polymerase (3 units/μl)
	Add sterile water to a volume of 20 μl

1. Add the polymerase last
2. Mix by briefly vortexing the solution and spin 1 min at 13,000 rpm in a microfuge centrifuge.
3. Incubate the reaction mixture for 30 min at 22°C (or room temperature).
4. Incubate for 20 min at 75°C to inactivate the polymerase.
5. Spin 1 min at 13,000 rpm in a microfuge centrifuge.

The LIC prepared vector solution obtained in this way can be used directly in the annealing reaction (protocol 3). For longer term storage of the prepared vector it would be better to purify it further using for instance the QIAquick PCR Purification Kit or Nucleotide Removal Kit (Qiagen). Take care that the final vector concentration is 10-20 ng/μl. The prepared vector can be stored at -20°C or lower.

2. Preparation of the insert

To create an insert with complementary overhangs to the EMBL-made LIC vectors the following primers have to be used:

Forward primer	**CAGGGCGCCA**TG-gene of interest
Reverse primer	**GACCCGACGCGGT**TA-gene of interest (rev. comp.)

The **forward primer** should contain the complementary overhang (shown in **red**), the ATG start codon (underlined), and a long enough overlap with the gene of interest to give a melting temperature of 60°C or more.

The **reverse primer** should contain the complementary overhang (shown in **red**), one or more stop codons (*e.g.* TAA as shown here underlined) if no C-terminal tag is used, and a long enough overlap with the reverse complement strand of the gene of interest to give a melting temperature of 60°C or more.

2.1 PCR amplification of the insert

Materials

200-µl PCR tubes
1.5-ml microfuge tube

QIAquick Gel Extraction Kit

Chemicals

agarose (electrophoresis grade)
dNTPs (10 mM each of dATP, dCTP, dGTP, dTTP)
ethidium bromide solution (10 mg/ml)
TBE buffer

Enzymes

Pfu DNA polymerase (2.5 U/µl)

10X Pfu polymerase buffer (supplied with the enzyme)

Mix in a 200-µl PCR tube:

5 µl	10X Pfu polymerase buffer
0.5 µl	forward primer (100 pmol/µl)
0.5 µl	reverse primer (100 pmol/µl)
*	dNTPs (10 mM each)
0.5 µl	DNA template
1 µl	Pfu DNA polymerase (2.5 units/µl)
	Add sterile water to a volume of 50 µl

* 20 ng for plasmid DNA
100 ng for genomic DNA

1. Add the polymerase last.
2. Mix by briefly vortexing the solution.
3. Perform the PCR as described below.

PCR protocol

Step	Time	Temperature	Cycles
Denaturation	2 min	95°C	1
Denaturation	30 sec	95°C	
Annealing	30 sec	56°C	30
Extension	*	72°C	
Extension	10 min	72°C	1
Hold		4°C	1

* use 1 min per kb for Pfu DNA polymerase

After the PCR it is important to remove the dNTPs completely from the reaction mixture. If the PCR template and the LIC vector have the same antibiotic resistance marker, the PCR product must be separated from the template. Both can be achieved by preparative agarose gel electrophoresis.

4. During the PCR prepare a 0.8% agarose gel.
Dissolve 0.4 g agarose in 50 ml TBE buffer by heating. After the solution has cooled down add 1-2 µl ethidium bromide solution and pour it into a prepared gel running chamber. After the gel has solidified fill the chamber with TBE buffer.
5. Add 10 µl 6X loading dye solution to the PCR product.
6. Load the sample on the agarose gel.
7. Run the gel for 1 hours at 100 V.
8. Analyze the gel on a UV lamp and cut out the band of the PCR product.
9. Purify the DNA form the gel pieces using the QIAquick Gel Extraction Kit.
10. Elute the DNA in 30 µl elution buffer in a 1.5-ml microfuge tube.

2.2 T4 DNA treatment of the PCR product

In the next step, the PCR product is incubated with T4 DNA polymerase in the presence of dATP. Because of the 3'--> 5' activity of the polymerase the bases are removed from both 3'-ends until the first adenosine (**A**) residue is reached.

```
CAGGGCGCCATG...gene-of-interest...TAACCGCGTCGGGTC
GTCCCGCGGTAC...gene-of-interest...ATTGGCGCAGCCCAG

                                |   T4 DNA polymerase + dATP
                                V

CAGGGCGCCATG...gene-of-interest...TAA
          AC...gene-of-interest...ATTGGCGCAGCCCAG
```

For the annealing (protocol 3) 0.02 pmol of LIC prepared insert DNA is used. Below the T4 DNA polymerase treatment of the PCR product is set up with **0.2 pmol** to produce enough

material for 10 annealing reactions. This can be scaled up or down according to your own need.

The DNA concentration can be determined using the absorbance at 260 nm (assuming A_{260} = 1 is 50 ng/μl). To calculate the DNA concentration in pmol/μl apply:

number of base pairs x 0.65 = ng/pmol

For instance, for an insert of 1000 base pairs 0.2 pmol is equivalent to 130 ng.

Materials

1.5-ml microfuge tubes

Chemicals

dATP stock solution (100 mM)
DTT (100 mM)
100X BSA

Enzymes

T4 DNA polymerase

10X New England Biolabs buffer 2 (supplied with the polymerase)

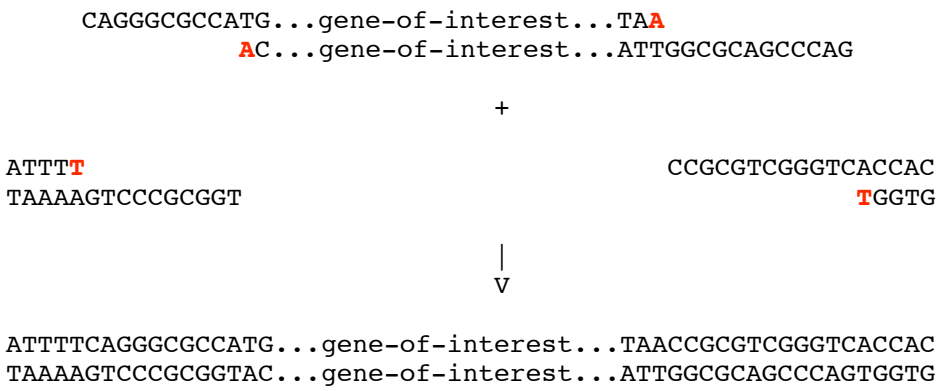
Mix in a 1.5-ml microfuge tube:

2 μl	10X New England Biolabs buffer 2
0.2 pmol	PCR product
0.5 μl	dATP (100 mM)
1 μl	DTT (100 mM)
0.2 μl	100X BSA
0.4 μl	T4 DNA polymerase (3 units/μl)
	Add sterile water to a volume of 20 μl

1. Add the polymerase last
2. Mix by briefly vortexing the solution and spin 1 min at 13,000 rpm in a microfuge centrifuge.
3. Incubate the reaction mixture for 30 min at 22°C (or room temperature).
4. Incubate for 20 min at 75°C to inactivate the polymerase.
5. Spin 1 min at 13,000 rpm in a microfuge centrifuge.

3. Annealing of the insert and the LIC vector

The complementary overhangs that are created in the vector (protocol 1) and insert (protocol 2) are long enough for the very specific, enzyme -free annealing of the two DNA.



The annealing reaction is set up as follows:

- **0.02 pmol** of insert DNA.
- **25 - 50 ng*** of LIC prepared vector DNA.
- The control ligation is carried out with sterile water instead of the insert.

* The amount of LIC prepared vector DNA needed depends on the size of the vector and the molar ration of vector to insert (normally 1:2 or 1:3 is used).
Example: LIC prepared **pETM-11/LIC** has a size of 5318 bp. With a 1:2 molar ratio you need **0.01 pmol** vector in the annealing reaction. This is equivalent to **35 ng**.

Materials	*Chemicals*
1.5-ml microfuge tubes	EDTA (25 mM)

Mix in a 1.5-ml microfuge tube:

1 µl	LIC prepared vector DNA
2 µl	T4 polymerase treated insert DNA

1. Incubate the annealing mixture for 5 min at 22°C (or room temperature).
2. Add 1 µl EDTA (25 mM).
3. Mix gently by stirring the solution with the tip.
4. Incubate for a further 5 min at 22°C (or room temperature).

The annealing is complete within 5 min of incubation but reactions can be incubated up to 1 h with equivalent results.

4. Transformation of the annealing product into *E. coli* DH5α competent cells

Materials

1.5-ml microfuge tubes
chemically competent *E. coli* DH5α cells
SOC medium
LB-agar plates containing 50 μg/ml kanamycin

1. Thaw the appropriate amount of competent DH5α cells on ice.
2. Transfer 1 μl of the annealing mixture to a 1.5-ml microfuge tube and incubate on ice for at least 5 min.
3. Add 50 μl aliquots of competent cells.
4. Incubate the tubes for 30 min on ice.
5. Heat shock the cells for 45 sec at 42°C.
6. Place the tubes immediately on ice and incubate for at least 2 min.
7. Add 200 μl SOC medium to each tube and incubate for 1 hour at 37°C in a shaker/incubator.
8. Spin for 1 min at 5,000 rpm in a microfuge centrifuge.
9. Remove 150 μl of supernatant and resuspend the cells in the remaining medium.
10. Plate out the cell suspension on a LB agar plate containing 50 μg/ml kanamycin.
11. Incubate the plates overnight at 37°C.

5. Identification of positive constructs

Materials

1.5-ml microfuge tubes
15-ml Falcon tubes
LB medium
Qiaprep Spin Miniprep Kit

Chemicals

agarose (electrophoresis grade)
6X loading dye solution
dNTPs (10 mM each of dATP, dCTP, dGTP, dTTP)
kanamycin (30 mg/ml)

Enzymes

restriction enzymes (here *Sma*I and *Xba*I)
Pfu DNA polymerase

10X restriction enzyme buffer (supplied with the enzymes)

10X Pfu DNA polymerase buffer (supplied with the enzyme)

5.1 Preparation of plasmid mini-preps

1. Pick 3 colonies from the positive plate and inoculate 3 x 4 ml LB medium containing 30 μg/ml kanamycin in 15-ml Falcon tubes.
   The number of colonies picked depends on the ratio between the number of colonies on the positive and on the control plate (background). Usually the background is quite low and 3 colonies are sufficient but in some cases more colonies should be picked.
2. Incubate overnight at 37°C in a shaker/incubator.
3. Spin for 10 min at 4,000 rpm (table top centrifuge) and discard the supernatant.
4. Resuspend the pellets in the appropriate buffer to prepare plasmid mini-preps using the Qiaprep Spin Miniprep Kit (Qiagen).

To determine if the right size insert is present in the plasmid mini-preps they can be analyzed using one or both of the following protocols: digestion analysis (protocol 5.2) and/or PCR analysis (protocol 5.3).

5.2 Digestion analysis of the plasmid mini-preps

Since the LIC vector do not contain a multiple cloning site, you have to select 2 unique restriction sites in the vector backbone. For instance, with pETM-11/LIC the *Xba*I and *Sma*I sites could be used (see vector map in Appendix) but also other restriction sites are available.

Mix in a 1.5-ml microfuge tube:

2 μl	10X New England Biolabs buffer 4
0.2 μl	100X BSA
5 μl	plasmid miniprep
1 μl	*Xba*I (20 units/μl)
1 μl	*Sma*I (20 units/μl)
	Add sterile water to a volume of 20 μl

1. Add the restriction enzymes last
2. Mix by briefly vortexing the solution and spin 1 min at 13,000 rpm in a microfuge centrifuge.
3. Incubate the digestion mixture for 1-2 hours at 37°C.
4. In the meantime, prepare a 0.8% agarose gel.
   Dissolve 0.4 g agarose in 50 ml TBE buffer by heating. After the solution has cooled down add 1-2 µl ethidium bromide solution and pour it into a prepared gel running chamber. After the gel has solidified fill the chamber with TBE buffer.
5. Add 4 μl 6X loading buffer to the samples.
6. Load the samples on the agarose gel.
7. Run the gel for 1 hours at 100 V.
8. Analyze the gel on a UV lamp.

5.3 PCR analysis of the plasmid mini-preps

To determine if the right size insert is present in the plasmids mini-preps PCRs are performed using the forward and reverse primers for the gene of interest.

Mix in a 200-μl PCR tube:

5 μl	10X Pfu polymerase buffer
0.5 μl	forward primer (100 pmol/μl)
0.5 μl	reverse primer (100 pmol/μl)
1 μl	dNTPs (10 mM each)
0.5 μl	plasmid miniprep DNA
1 μl	Pfu polymerase (2.5 units/μl)
	Add sterile water to a volume of 50 μl

1. Add the polymerase last
2. Mix by briefly vortexing the solution.
3. Perform the PCR as described in "PCR experiments".
4. In the meantime, prepare a 0.8% agarose gel.
   Dissolve 0.4 g agarose in 50 ml TBE buffer by heating. After the solution has cooled down add 1-2 µl ethidium bromide solution and pour it into a prepared gel running chamber. After the gel has solidified fill the chamber with TBE buffer.
5. Add 10 μl 6X loading buffer to the samples.
6. Load the 10-20 μl of the samples on the agarose gel.
7. Run the gel for 1 hours at 100 V.
8. Analyze the gel on a UV lamp.

Appendix 1

Materials

200-μl PCR tubes	
1.5-ml microfuge tubes	
15-ml Falcon tubes	
SOC medium	Invitrogen (15544-034)
chemically competent *E. coli* DH5α cells	
QIAquick PCR Purification Kit	Qiagen (28106)
QIAquick Gel Extraction Kit	Qiagen (28706)
Qiaprep Spin Miniprep Kit	Qiagen (27106)

Chemicals

agarose (electrophoresis grade)	Invitrogen (15510-027)
dATP (100 mM)	Roth (K035.1)
dNTPs (10 mM of dATP, dCTP, dGTP, dTTP)	New England Biolabs (N0447S)
dTTP (100 mM)	Roth (K036.1)
DTT	Roth (6908.2)
EDTA	
ethidium bromide (10 mg/ml)	
kanamycin sulfate	Roth (T832.3)
6X loading dye solution	Fermentas (R0611)
10X TBE buffer	Roth (3061.2)
100X BSA	New England Biolabs (B9001S)

Enzymes

*Bsa*I (1000U)	New England Biolabs (R0535S)
Pfu DNA polymerase	Fermentas (EP0502)
T4 DNA polymerase (150U)	New England Biolabs (M0203S)
restriction enzymes	New England Biolabs

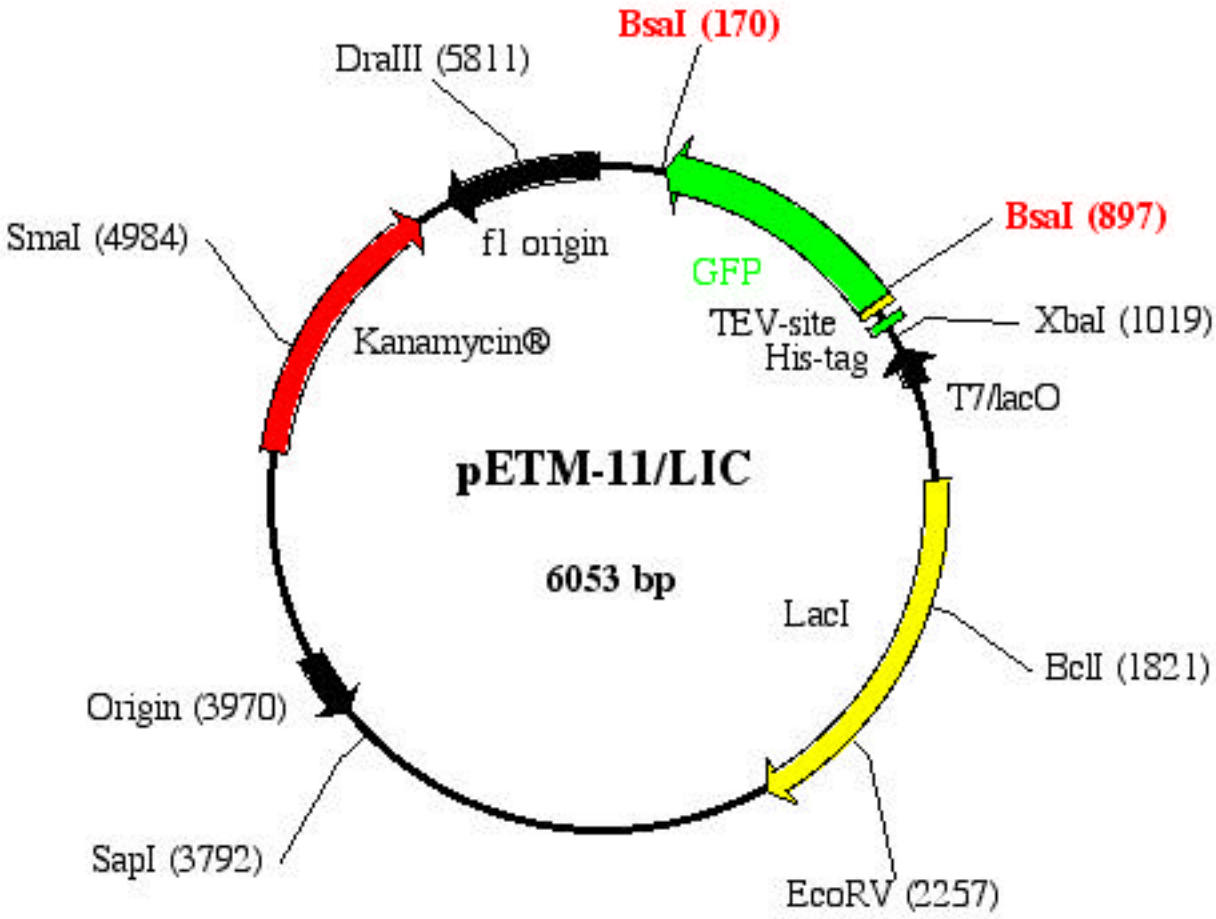
Appendix 2

Available LIC vectors

Vector	Promoter	Selection	Tag	Protease cleavage site	Origin
pETM-11/LIC	T7/lac	Kan	N-His	TEV	pBR322
pETGB-1a/LIC	T7/lac	Kan	N-His N-GB1	TEV	pBR322
pETZ2-1a/LIC	T7/lac	Kan	N-His N-Z-tag2	TEV	pBR322
pETTrx-1a/LIC	T7/lac	Kan	N-His N-TrxA	TEV	pBR322
pETNus-1a/LIC	T7/lac	Kan	N-His N-NusA	TEV	pBR322

EMBL Hamburg Outstation

expression vector map



Source: Arie Geerlof
geerlof@embl-hamburg.de

pETM-11/LIC

```
    T7 promoter  -->          lac operator    XbaI
GAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAAT
CTTTAATTATGCTGAGTGATATCCCCTTAACACTCGCCTATTGTTAAGGGGAGATCTTTA

                      rbs                  His-tag
AATTTTGATTTAACTTTAAGAAGGAGATATACCATGAAACATCACCATCACCATCACCCC
TTAAAACTAAATTGAAATTCTTCCTCTATATGGTACTTTGTAGTGGTAGTGGTAGTGGGG
                                   METLysHisHisHisHisHisHisPro

                                 TEV-site       BsaI
ATGAGCGATTACGACATCCCCACTACTGAGAATCTTTATTTTCAG GGCGCCATGAGACCG
TACTCGCTAATGCTGTAGGGGTGATGACTCTTAGAAATAAAAGTC CCGCGGTACTCTGGC
MetSerAspTyrAspIleProThrThrGluAsnLeuTyrPheGln|GlyAlaMET


ATGGTGAGCAAGGGCGAGGAGCTG....654bp...GCCGCCGGGATCACTCTCGGCATG
TACCACTCGTTCCCGCTCCTCGAC.....GFP....CGGCGGCCCTAGTGAGAGCCGTAC
MetValSerLysGlyGluGluLeu....218aa...AlaAlaGlyIleThrLeuGlyMet

                     BsaI                 C-His-tag
GACGAGCTGTACAAGTAAGGTCTCACCGCGTCGGGTCACCACCACCACCACCACTGAGAT
CTGCTCGACATGTTCATTCCAGAGTGGCGCAGCCCAGTGGTGGTGGTGGTGGTGACTCTA
AspGluLeuTyrLys***
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Single Cutters Listed by Site Order

80	Bpu1102I	1491	ApaBI	2871	BglI	4801	ClaI
80	EspI	1807	MluI	2889	MstI	4982	XmaI
181	Bsp1407I	1821	BclI	3653	Tth111I	4984	SmaI
1019	XbaI	2018	ApaI	3792	SapI	5110	PvuI
1085	BglII	2218	BssHII	4324	AlwNI	5110	XorII
1126	SgrAI	2257	EcoRV	4767	NruI	5811	DraIII
1282	SphI	2313	HpaI	4767	SpoI		

Non Cutting Enzymes

AatII	Acc65I	AflII	AgeI	AhaIII	AscI
Asp718I	AsuII	AvrII	BalI	BamHI	BspMI
Bsu36I	Csp45I	CspI	CvnI	DraI	EagI
Eam1105I	Ecl136II	Eco52I	Eco72I	EcoICRI	EcoRI
FseI	HindIII	I-PpoI	KpnI	MfeI	Mlu113I
MscI	MstII	NcoI	NdeI	NheI	NotI
PacI	PinAI	PmaCI	PmeI	PstI	RleAI
SacI	SacII	SalI	SauI	ScaI	SciI
SfiI	SnaBI	SpeI	SplI	SrfI	SstI
SstII	StuI	SunI	SwaI	XhoI	XmaIII