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 BsaI sites (shown in red). These sites are used to linearize the vector, while at the same time removing the eGFP gene.

ATTTTCAGGGCCCATGAGACCG...eGFP...GGTCTCACCCTAGTCGGGTACCCAC
TAAAGTCCCCCGGTACTCTGGC...eGFP...CCAGAGTGGCCAGCCACAGTGTT

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  CCGGTTCGGGTACCCAC
TAAAGTCCCCCGGT  CAGCCAGTGTT

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  CCGGTTCGGGTACCCAC
TAAAGTCCCCCGGT  CAGCCAGTGTT

ATTTTT  CCGGTTCGGGTACCCAC
TAAAGTCCCCCGGT  TGGT

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 BsaI digestion

**Materials**

- 1.5-ml microfuge tubes
- QIAquick Gel Extraction Kit
- 6X Loading dye solution
- Ethidium bromide (10 mg/ml)
- TBE buffer

**Chemicals**

- Agarose (electrophoresis grade)
- 10X New England Biolabs buffer 3 (supplied with the enzyme)

**Enzymes**

- BsaI

Mix in a 1.5-ml microfuge tube:

<table>
<thead>
<tr>
<th>µl</th>
<th>10X New England Biolabs buffer 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
<td>LIC vector DNA</td>
</tr>
<tr>
<td>µl</td>
<td>BsaI (10 units/µl)</td>
</tr>
</tbody>
</table>

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 hour 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 BsaI 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
- dTTP (100 mM)
- DTT (100 mM)
- 100X BSA

**Chemicals**

- T4 DNA polymerase
- 10X New England Biolabs buffer 2 (supplied with the enzyme)

**Enzymes**

- Mix in a 1.5-ml microfuge tube:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Ingredient</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 µl</td>
<td>10X New England Biolabs buffer 2</td>
</tr>
<tr>
<td>600 ng</td>
<td><em>Bsa</em>I-digested LIC vector</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>dTTP (100 mM)</td>
</tr>
<tr>
<td>1 µl</td>
<td>DTT (100 mM)</td>
</tr>
<tr>
<td>0.2 µl</td>
<td>100X BSA</td>
</tr>
<tr>
<td>0.4 µl</td>
<td>T4 DNA polymerase (3 units/µl)</td>
</tr>
<tr>
<td></td>
<td>Add sterile water to a volume of 20 µl</td>
</tr>
</tbody>
</table>

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: CAGGGCGCCTG-gene of interest
Reverse primer: GACCCGACGCCTA-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
- Enzymes
  - Pfu DNA polymerase (2.5 U/µl)
  - 10X Pfu polymerase buffer (supplied with the enzyme)

**Chemicals**

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

Mix in a 200-µl PCR tube:

<table>
<thead>
<tr>
<th>µl</th>
<th>10X Pfu polymerase buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>forward primer (100 pmol/µl)</td>
</tr>
<tr>
<td>0.5</td>
<td>reverse primer (100 pmol/µl)</td>
</tr>
<tr>
<td>*</td>
<td>dNTPs (10 mM each)</td>
</tr>
<tr>
<td>0.5</td>
<td>DNA template</td>
</tr>
<tr>
<td>1</td>
<td>Pfu DNA polymerase (2.5 units/µl)</td>
</tr>
</tbody>
</table>

* 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.
<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>2 min</td>
<td>95°C</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>30 sec</td>
<td>95°C</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>30 sec</td>
<td>56°C</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>* 72°C</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>10 min</td>
<td>72°C</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td></td>
<td>4°C</td>
<td>1</td>
</tr>
</tbody>
</table>

* 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 from 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
GTCCGCGGTAC...gene-of-interest...ATTGGCGCAGCCCAG
```

\[ \text{T4 DNA polymerase + dATP} \]

```
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:

$$\text{number of base pairs} \times 0.65 = \text{ng/pmol}$$

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:

<table>
<thead>
<tr>
<th>µl</th>
<th>10X New England Biolabs buffer 2</th>
<th>PCR product</th>
<th>dATP (100 mM)</th>
<th>DTT (100 mM)</th>
<th>100X BSA</th>
<th>T4 DNA polymerase (3 units/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td></td>
<td>0.2 pmol</td>
<td>0.5 µl</td>
<td>1 µl</td>
<td>0.2 µl</td>
<td>0.4 µl</td>
</tr>
</tbody>
</table>

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.

```
CAGGGCGCCATG...gene-of-interest...TAA
   AC...gene-of-interest...ATTGGCGCAGCCCAG
+
ATTTTT
TAAAAGTCCCCGCGGT

|  

ATTTTCAGGCGCCATG...gene-of-interest...TACCCGCTCGGTACACCAC
TAAAAGTCCCCGCGGTAC...gene-of-interest...ATTGGCGCAGCCCAGTGGTG
```

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 ratio 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**

- 1.5-ml microfuge tubes
- EDTA (25 mM)

**Chemicals**

- LIC prepared vector DNA
- T4 polymerase treated insert DNA

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 \textit{E. coli} DH5α competent cells

\textit{Materials}

- 1.5-ml microfuge tubes
- Chemically competent \textit{E. coli} DH5α cells
- SOC medium
- LB-agar plates containing 50 µg/ml kanamycin

\textit{Procedure}

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

\textit{Materials}

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

\textit{Chemicals}

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

\textit{Enzymes}

- Restriction enzymes (here \textit{SmaI} and \textit{XbaI})
- 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 XbaI and SmaI sites could be used (see vector map in Appendix) but also other restriction sites are available.

Mix in a 1.5-ml microfuge tube:

<table>
<thead>
<tr>
<th>Volume (µl)</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 µl</td>
<td>10X New England Biolabs buffer 4</td>
</tr>
<tr>
<td>0.2 µl</td>
<td>100X BSA</td>
</tr>
<tr>
<td>5 µl</td>
<td>plasmid miniprep</td>
</tr>
<tr>
<td>1 µl</td>
<td>XbaI (20 units/µl)</td>
</tr>
<tr>
<td>1 µl</td>
<td>SmaI (20 units/µl)</td>
</tr>
</tbody>
</table>

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:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 µl</td>
<td>10X Pfu polymerase buffer</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>forward primer (100 pmol/µl)</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>reverse primer (100 pmol/µl)</td>
</tr>
<tr>
<td>1 µl</td>
<td>dNTPs (10 mM each)</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>plasmid miniprep DNA</td>
</tr>
<tr>
<td>1 µl</td>
<td>Pfu polymerase (2.5 units/µl)</td>
</tr>
</tbody>
</table>

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  
chemically competent *E. coli* DH5α cells  
QIAquick PCR Purification Kit  
QIAquick Gel Extraction Kit  
Qiagen (28706)  
Qiagen Spin Miniprep Kit  
Qiagen (27106)

Chemicals

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

Enzymes

*BsaI* (1000U)  
Pfu DNA polymerase  
T4 DNA polymerase (150U)  
restriction enzymes  
New England Biolabs (R0535S)  
Fermentas (EP0502)  
New England Biolabs (M0203S)  
New England Biolabs
### Appendix 2

**Available LIC vectors**

<table>
<thead>
<tr>
<th>Vector</th>
<th>Promoter</th>
<th>Selection</th>
<th>Tag</th>
<th>Protease cleavage site</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>pETM-11/LIC</td>
<td>T7/lac</td>
<td>Kan</td>
<td>N-His</td>
<td>TEV</td>
<td>pBR322</td>
</tr>
<tr>
<td>pETGB-1a/LIC</td>
<td>T7/lac</td>
<td>Kan</td>
<td>N-His</td>
<td>TEV</td>
<td>pBR322</td>
</tr>
<tr>
<td>pETZ2-1a/LIC</td>
<td>T7/lac</td>
<td>Kan</td>
<td>N-His</td>
<td>TEV</td>
<td>pBR322</td>
</tr>
<tr>
<td>pETTrx-1a/LIC</td>
<td>T7/lac</td>
<td>Kan</td>
<td>N-His</td>
<td>TEV</td>
<td>pBR322</td>
</tr>
<tr>
<td>pETNus-1a/LIC</td>
<td>T7/lac</td>
<td>Kan</td>
<td>N-His</td>
<td>TEV</td>
<td>pBR322</td>
</tr>
</tbody>
</table>
EMBL Hamburg Outstation

expression vector map

Source: Arie Geerlof
geerlof@embl-hamburg.de
pETM-11/LIC

T7 promoter --> lac operator  XbaI
GAAATTCAAGGACTTACTATAAGGGAATTGAGCCGATAAACATCTCTAGAAT
CTTTAATTATGCTGAGTATATCTCCCTAAACACTCGCTATTTGTTAAAGGGAGATCTTTA

rbs  His-tag  XbaI
AATTTGTTTTAAATTGAAAGGATATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCT
TTAAACTAAATTTGAAATCTCTCTCTATATGTTACTTTGTAGTGTTAGTTAGTTAGTTAGTTAGGG
MET

GFP

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  SpolI

Non Cutting Enzymes

AatII  Acc65I  AflII  AgeI  AhaIII  AscI
Asp718I  AsuII  AvrII  BalI  BamHI  BspMI
Bsu36I  Csp45I  CspI  CvnI  DraI  EagI
Eam1105I  Eco136II  Eco52I  EcoRI  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