Inserting a DNA Sample into a Plasmid

DNA is cut with EcoRI at arrows.

Resulting DNAs have sticky (complementary) ends.

DNA is spliced by complementary base pairing and sealed with DNA ligase.
Ligation klebriger Enden:

Ligation glatter Enden:
DNA Ligase – enzyme catalysing formation of phosphodiesteric bond between group 3’-OH of one end of DNA molecule and group 5’-phosphate of the second end of DNA

DNA ligase

ATP (or NAD+)
Ligase cofactors

1. ATP
   - DNA ligases of bacteriophages (phage T4, T7)
   - DNA ligases of mammals

2. NAD+
   - DNA ligases of bacteria (Escherichia coli, Bacillus subtilis, Salmonella typhimurium)
T4 DNA ligase

T4 DNA Ligase catalyzes the joining of two strands of DNA between the 5’-phosphate and the 3’-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt ended configuration.

The enzyme has also been shown to catalyze the joining of RNA to either a DNA or RNA strand in a duplex molecule but will not join single stranded nucleic acids.

**Inactivation of T4 ligase:**
Heat to 70°C for 10 minutes.
Ligase of phage T4

- Requires ATP as a co-factor
- Optimal pH (7.2-7.8)
- Requires bivalent ions (Mg$^{2+}$, Mn$^{2+}$) and reducing factors ($\beta$-mercaptoethanol or ditiotreitol)
- Inhibitors: poliamines (spermin, spermidine), high concentration of ions (Na$^+$, K$^+$, Li$^+$, NH4$^+$)
- Can connect both cohesive and blunt ends (but for blunt ends reaction is slower and requires higher concentrations of enzyme)
What should be optimized for a successful ligation:

1. The ratio of the molar concentration of vector to insert.
   - Optimum ratios may vary from 8:1 to as high as 1:16 vector:insert, though generally fall in the range of 3:1 to 1:3.

2. Amount of DNA.
   - Usually 10-200 ng of plasmid is used for reaction.

3. Volume of reaction.
   - Usually a minimal volume is recommended (e.g. 10 μl).

4. Amount of ligase.
   - Each ligation reaction generally requires 1-10 units of high quality ligase.
What should be optimized for a successful ligation:

5. Incubation time and temperature.

The ligation incubation time and temperature may also need to be optimized. In general:
- **blunt-ended** ligations are performed at 4°C overnight;
- **sticky-end** ligations are performed for 1-3 hours (at 22°C or 16°C) or overnight at 4°C.

The optimal temperature for a ligation is a balance between the optimal temperature for T4 DNA Ligase enzyme activity (25°C) and the temperature necessary to ensure annealing of the fragment ends, which can vary with the length and base composition of the overhangs.

Shorter duplexes (linkers less than 16 bases long) require lower temperatures.

In general, ligation reactions performed at lower temperatures require longer incubation times.
What should be optimized for a successful ligation:

- For example, ligation of large inserts (>7-8 kbp) into pGEM® Vectors is facilitated by culturing the transformed bacteria at 30°C instead of 37°C.

7. Quality of bacteria.
- For simple retransformation low efficiency competent cells may be sufficient, but higher competency cells may be required for more difficult cloning experiments. To maximize the chances of obtaining the clone of interest, the highest competency bacterial cells available should be utilized (>1x10⁸ colony forming units/µg DNA).

8. Quality of DNA.
- Both the vector and insert DNA should be very clean. Contaminants can interfere with the ligase and inhibit the reaction if present.
<table>
<thead>
<tr>
<th>Ligase</th>
<th>None</th>
<th>2 U</th>
<th>2 U</th>
<th>0.2 U</th>
<th>0.02 U</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>--</td>
<td>5</td>
<td>45</td>
<td>45</td>
<td>45</td>
</tr>
</tbody>
</table>

Electrophoresis
Potential causes of low efficiency of fragment ligation

Deterioration of buffer components.

ATP, NAD and DTT included in the ligation buffer are easily degraded. The ligation reaction should be repeated with fresh buffer.

Restriction enzyme still active in the ligation mixture.

If the restriction endonuclease cannot be heat-inactivated at 65°C, phenol deproteinization and/or ethanol precipitation of the fragments after the restriction reaction should be performed.

Non-specific nuclease contamination.

The restriction endonuclease, ligase, or DNA preparation might contain non-specific nucleases due to improper handling. The source of the ligation mixture contamination can be identified by substituting each component one by one.
Potential causes of low efficiency of fragment ligation

Ligase concentration is too low.

In blunt end DNA ligation reactions, the concentration of ligase in the reaction mixture should be 100-500 u/ml.

DNA fragments obtained after cleavage with the following restriction endonucleases are ligated very poorly: BcnI, BfuI, Bme1390I, Bpu10I, Bpu1102I, Bsp68I, BspPI, Eam1105I, Eco72I, Eco81I, EheI, FspBI, MboII, MssI, MvaI, PsyI, SatI, SmI, XagI.

In such cases, a high concentration of ligase (20-40 u/1µg of fragments) and 10% PEG in the ligation reaction should be used. DNA phenol deproteinization should be performed both after the restriction and the ligation reactions.
The addition of polyethylene glycol (PEG) to ligation reactions can promote ligation of blunt-ended fragments by “macromolecular crowding”.

It is **not** recommended the use of PEG in ligations, however, due to extreme variability in the quality of PEG.

In addition, the use of PEG can lead to undesirable concatemerization when cloning cDNAs.
alkaline phosphatase
(bacterial, calf intestine)

plasmid cannot ligate to itself
mix with cDNA
+ T4 ligase

vector

vector

ligation

nick

transformation

nicks repaired by bacterial host enzymes after transformation
CIAP action

Plasmid DNA

- Restriction endonuclease cleavage
- Alkaline phosphatase treatment

Target DNA

- Restriction endonuclease cleavage

T4 DNA ligase
Dephosphorylation

To significantly reduce the background self-ligation of linearized plasmid, the vector can be dephosphorylated prior to the ligation reaction.

Generally, linearized plasmid DNA is treated with Calf Intestinal Alkaline Phosphatase (CIAP) for 60 minutes at 37°C at approximately 0.02 units per pmol DNA ends, followed by inactivation of the phosphatase and purification of the dephosphorylated DNA.

Phenol/chloroform/isoamyl alcohol extraction and gel-purification are recommended to purify the dephosphorylated linearized plasmid DNA as CIAP can be difficult to remove from the DNA, and will inhibit subsequent ligation reactions.
Calf Intestinal Alkaline Phosphatase (CIAP)

Alkaline Phosphatase isolated from calf intestinal mucosa catalyzes the hydrolysis of 5’-phosphate groups from DNA, RNA and both ribo- and deoxyribonucleoside triphosphates.

This enzyme is used to prevent recircularization and religation of linearized cloning vehicle DNA by removing phosphate groups from both 5'-termini.

For long term storage (infrequent use; 1-2 times per month), store at –70°C. For daily/weekly use, store at –20°C.
Calf Intestinal Alkaline Phosphatase (CIAP)

Dephosphorylation of 5´ Overhangs

1. Dilute sufficient CIAP for immediate use in CIAP 1X Reaction Buffer to a final concentration of 0.01 u/μl. Each pmol of DNA ends will require 0.01 u CIAP.
(1 μg of 1,000 bp DNA = 1.52 pmol DNA = 3.03 pmol of ends.)

2. Purify the DNA to be dephosphorylated by ethanol precipitation and resuspend the pellet in 40 μl of 10 mM Tris-HCl (pH 8.0).

3. Incubate with CIAP and CIAP Buffer at 37°C for 30 minutes.

4. Add another aliquot of diluted CIAP (equivalent to the amount used in Step 2) and continue incubation at 37°C for an additional 30 minutes.

5. Add 300 μl of CIAP stop buffer. Phenol:chloroform extract and ethanol precipitate by adding 0.5 volume 7.5M ammonium acetate (pH 5.5) and 2 volumes of 100% ethanol to the final aqueous phase.

Note: CIAP may be added directly to digested DNA. Add 5 μl CIAP 10X Reaction Buffer, 0.01 u CIAP/pmol of ends and deionized water to a final volume of 50 μl.

or: add 1 μl of CIAP directly to the restriction reaction
Calf Intestinal Alkaline Phosphatase (CIAP)

Dephosphorylation of 5'- Recessed or Blunt Ends

When 5'- recessed or blunt end DNA fragments are used as substrate, incubate at 37°C for 15 minutes and then at 56°C for 15 minutes. Then add a second aliquot of CIAP and repeat the incubations at both temperatures. The higher temperature ensures accessibility of the recessed end.

or: add 1 μl of CIAP directly to the restriction reaction

CIAP must be inactivated by Phenol:chloroform extraction and ethanol precipitation.
Unlike calf intestinal phosphatase, SAP is completely and irreversible inactivated by heat treatment for 15 min at 65°C.

Thus the restriction enzyme digestion, dephosphorylation, enzyme inactivation, and ligation can be performed in one single tube by just adding the appropriate reagents.

SAP is as well active on either 5’-protruding, 5’-recessive and blunt ends.
cDNA Library Construction

cDNA is a synthetic DNA molecule with no "specific" ends to the molecule.

How can such molecules be joined (ligated) to vectors for cloning?

1. If the molecules are accurately blunt or flush ended they can be ligated directly into a restriction site in the vector which produces blunt ends.

   ![Diagram of cDNA library construction](image)
[2] Addition of restriction enzyme sites by synthetic DNA (oligonucleotide linkers)

synthetic HindIII oligonucleotide linker

\[ 5'-CCAAGCTTGG \]

palindromic = complementary so linkers dimerise instantly

\[ 5'-CCAAGCTTGG \]
\[ GGTTGGAACCC \]

phosphorylate 5' ends
T4 kinase

\[ 5'-CCAAGCTTGG \]
\[ GGTTGGAACCC \]

ligate to cDNA

\[ CCAAGCTTGG \]
\[ GGTTGGAACCC \]

restrict HindIII
triple ligation

cDNA fragments with HindIII sticky ends

restrict HindIII

ligate to vector and transform
cDNA

\[ \text{AAGCTTT} \overline{\text{TTTGGAA}} \]

\[ \text{AAGCTTT} \overline{\text{TTTGGAA}} \]

\[ \text{HindIII methylase} \]
\[ + \text{S-adenosyl methionine} \]
\[ \text{(methyl group donor)} \]

\[ \text{AAGCTTT} \overline{\text{TTTGGAA}} \]

\[ \text{cDNA with internal HindIII site protected} \]

\[ \text{ligate linkers as before} \]

\[ \text{CCAAGCTTTGG} \overline{\text{GGTTTCGAAACC}} \]

\[ \text{CCAAGCTTTGG} \overline{\text{GGTTTCGAAACC}} \]

\[ \text{restrict HindIII} \]

\[ \text{AGCTTTGG} \overline{\text{ACC}} \]

\[ \text{intact cDNA with HindIII sticky ends} \]

\[ \text{ligate to vector and transform} \]
RT - Polymerase Chain Reaction

Amplified total cDNA (BamHI / HindIII ends)

15-30 cycles

BamHI + HindIII

ligate to BamHI / HindIII cut vector
Terminal transferase

cDNA

AAAAAAA
TTTTTTT

terminal transferase + dCTP

cDNA (dC tails)

AAAAAAAAACCCCCCCCCCCC
TTTTTTTT

CCCCCCCCCC
TTTTTTT
B

pUC18

\[ \text{PstI} \]

\[ \begin{align*}
\text{CTGCAG} \\
\text{GACGTC}
\end{align*} \]

\[ \text{CTGCAG} \quad \text{G} \quad \text{ACGTC} \]

\[ \text{terminal transferase + dGTP} \]

\[ \begin{align*}
\text{GGGGGGACGTC} \\
\text{GGGGGGACGTC}
\end{align*} \]

\[ \text{mix vector and cDNA, anneal} \]

\[ \begin{align*}
\text{ACGCTGGGGGGG} \\
\text{CCCCCCCCCCC}
\end{align*} \]

\[ \begin{align*}
\text{AAAAAAAAACCCCCCCCC} \\
\text{TTTTTTTT}
\end{align*} \]

\[ \begin{align*}
\text{GGGGGGACGTC} \\
\text{gap}
\end{align*} \]

\[ \text{transformation (host repairs gaps)} \]
Gateway system

Adventages of Gateway cloning:

1. Method is rapid: no overnight incubations are necessary. The system incubates for one hour before it needs to be transferred to a medium.

2. Method is efficient: efficacy of recombination is typically in the upper 90% range.

3. Insert orientation is defined.

4. The method is universal: all types of DNA fragments may be cloned, whether that be PCR fragments, cDNA, or genomic DNA. It is also available for several organisms, from mammals and insects to E. coli.
Phage λ Recombination in *E. coli*

Phage λ

*attP*

*attB*

*E. coli*

Integration \(\downarrow\) Excision

Integrated prophage

*attL*

*attR*

*attP*: 243 bp

*attB*: 25 bp

*attL*: 100 bp

*attR*: 168 bp
To use Gateway system:
subclone your gene to EntryClone plasmid. Then it will be available for different cloning needs, thank to the att recombination sites used in combination with enzyme clonase mixes.
BP Protocol

BP Clonase™ Reaction Buffer 4 µl
attB-PCR product or expression clone 40-100 fmol
Donor vector (pDONR™) 300 ng
BP Clonase™ Enzyme Mix 4 µl
20 µl

1. Mix and incubate for one hour at 25°C.
2. Add Proteinase K solution and incubate for 10 min at 37°C.
3. Transform competent E. coli with 1 - 2 µl per 100 µl cells.
4. Grow for one hour and select on appropriate LB antibiotic-resistant plates.
Thank you