Type I enzymes:

- Complex, multisubunit, combination restriction-and-modification enzymes that cut DNA at random far from their recognition sequences.

- Originally thought to be rare, we now know from the analysis of sequenced genomes that they are common.

- They are of considerable biochemical interest but they have little practical value since they do not produce discrete restriction fragments or distinct gel-banding patterns.
**Type II enzymes:**

- Cut DNA at defined positions close to or within their recognition sequences.
- They produce discrete restriction fragments and distinct gel banding patterns, and they are the only class used in the laboratory for DNA analysis and gene cloning.
- The most common type II enzymes are those like Hha I or Hind III that cleave DNA within their recognition sequences. Most recognize DNA sequences that are **symmetric** but a few (e.g., BbvC I: CCTCAGC) recognize **asymmetric** DNA sequences. Some enzymes recognize **continuous sequences** (e.g., EcoR I: GAATTC), while others recognize **discontinuous sequences** (e.g., Bgl I: GCCNNNNNGGC) in which the half-sites are separated.
- The next most common type II enzymes, usually referred to as ‘type IIIs" are those like Fok I and Alw I that cleave outside of their recognition sequence to one side. In some of them the two enzymatic activities (endonuclease and methylase) reside in the same protein chain.
Type III enzymes:

- Large, restriction-and-modification enzymes.

- They cleave outside of their recognition sequences and require two such sequences in opposite orientations within the same DNA molecule to accomplish cleavage.

- They rarely give complete digests.

- No laboratory uses have been devised for them, and none are available commercially.
Homing endonucleases:

- They are double stranded DNases that have **large, asymmetric recognition sites** (12–40 base pairs).

- Homing endonuclease **recognition sites are extremely rare**. For example, an 18 base pair recognition sequence will occur only once in every $7 \times 10^{10}$ base pairs of random sequence. This is equivalent to only one site in 20 mammalian-sized genomes.

- However, unlike standard restriction endonucleases, homing endonucleases **tolerate some sequence degeneracy** within their recognition sequence. As a result, their observed sequence specificity is typically in the range of 10–12 base pairs.

- They do not have stringently-defined recognition sequences in the way that restriction enzymes do.
Homing endonucleases in practise:

**I-Ceu I** (from *Chlamydomonas eugametos*)

```
5’...TAACTATATACGGTCTCTAAGGTAGCGA...3’
3’...ATTGATATTGGCCAGGATTCCATCGCT...5’
```

**PI-Sce I** (from *Saccharomyces cerevisiae*)

```
5’...ATCTATGTCGGGTGCGGAGAAGAGGTAATGAAATGGCA...3’
3’...TAGATACAGCCACGCCTCTTTCCTCATATTACCTTACCGT...5’
```
Termini generated by restriction endonucleases

- Cohesive ends:
  - 5’-overhang, e.g. EcoRI
    
    5’...NNGAATTCCNN...3’
    3’...NNCTTAAGGNN...5’

    5’...NNG
    3’...NNCTTAAp
    GNN...5’

  - 3’-overhang, e.g. PstI
    
    5’...NNCTGCAGNN...3’
    3’...NNGACGTCNN...5’

    5’...NNCTGCA
    3’...NNGp
    ACGTCNN...5’

- Blunt ends, e.g. HaeIII:

    5’...NNGGCCCN...3’
    3’...NNCCGGNN...5’

    5’...NNGG
    3’...NNCCp
    GGNN...5’
**Isoschizomers**

- Restriction enzymes that cleave within the same target sequence:
  - in the same place, e.g. **MboI** vs. **Sau3AI**
    
    5’...NNGATCNN...3’
    3’...NNCTAGNN...5’
    5’...NNGATCNN...3’
    3’...NNCTAGNN...5’

- in distinct place (neoschisomers)
  
  e.g. **SmaI** vs. **XmalI**
  
  5’...NNCCCGGGGNN...3’
  3’...NNGGGGCCNN...5’
  5’...NNCCCGGGGNN...3’
  3’...NNGGGGCCNN...5’
Isocaudamers

- Restriction enzymes that generate compatible ends:

  - e.g. BamHI vs. Sau3AI
  
  5’...NGGATCCN...3’
  3’...NCCTAGGN...5’
  
  5’...NNGATCNN...3’
  3’...NNCTAGNN...5’

  - e.g. SalI vs. XhoI
  
  5’...NNGATCCN...3’
  3’...NCCTAGGN...5’
  
  5’...NNGATCNN...3’
  3’...NNCTAGNN...5’
Compatible ends

Two restriction enzymes that generate the same sticky ends:

SalI

5’...G
3’...CAGCT

vs.

XhoI

TCGAG...3’
C...5’

5’...NNGTCGAGNN...3’
3’...NNCAGCTCNN...5’
Conditions of reaction

Buffer Composition:

Different restriction enzymes have differing preferences for ionic strength (salt concentration) and major cation (sodium or potassium). A battery of 3 to 4 different buffers will handle a large number of available enzymes, although there are a few that require a unique buffer environment.

In all cases, a major function of the buffer is to maintain pH of the reaction (usually at 8.0). Additionally, some enzymes are more fussy about having their optimal buffer than other enzymes.
Sometimes the presence of BSA in the reaction mix has a crucial influence on enzyme activity because it:

- stabilizes the enzyme,
- binds some impurities
- prevents enzyme adsorption on the test tube surface.

If BSA is recommended:

Add 0.1 mg/ml
Conditions of reaction

Incubation Temperature:

- Most restriction enzymes cut best at 37°C, but there are many exceptions.

- Enzymes isolated from thermophilic bacteria cut best at temperatures ranging from 50º to 65ºC.

- Some other enzymes have a very short half life at 37°C and its recommended that they be incubated at 25ºC.
Conditions of reaction

Reaction volume:

- Working with small volumes, excessive amounts of enzyme and/or glycerol should be avoided,

- Reaction volumes of 10-50 µl per microgram of DNA are the most commonly used.

Mixing:

- In order to achieve the uniform reaction conditions a restriction reaction mixture should be properly mixed up. Gentle pipetting of a reaction mixture for several times followed by quick spin down in microcentrifuge is preferential to its vortexing.
Conditions of reaction

Stopping a reaction:

- If the DNA digested shouldn't be used in further reactions, then no further manipulations of the digested DNA are to be performed, then the reaction can be stopped by adding 6X Loading Dye solution.

- If further reactions on the digested DNA should be performed, then it is necessary to inactivate the restriction endonucleases. As a rule, thermal inactivation (at 65°C or 80°C for 20 min) of the reaction mixture with RE is the most convenient method. For inactivation of the following thermostable enzymes:

  Adel, BcuI, BglII, BseLI, Cfr10I, TaaI, TaiI, TasI, TaqI, TauI and Tru1I,

phenol/chloroform deproteinization and ethanol or purification using columns is recommended.
Digestion with Multiple Enzymes

Often the two enzymes have different buffer requirements. To handle this:

- **Digest with both enzymes in the same buffer.** In many cases, even though a given buffer is not optimal for an enzyme, you can still get quite good cleavage rates.

- **Cut with one enzyme, then alter the buffer composition and cut with the second enzyme.** This usually applies to situations where one enzyme likes a low salt buffer and the other a high salt buffer, in which case you can digest with the first enzyme for a time, add a calculated amount of concentrated NaCl and cut with the second enzyme.

- **Change buffers between digestion with two enzymes.** In some cases, two enzymes will have totally incompatible buffers. In that case, perform one digestion, recover the DNA (by precipitation or using columns) and resuspend in the buffer appropriate for the second enzyme.
Troubles

Nature of DNA:

- Activity of restriction endonucleases is usually determined using phage lambda DNA as a substrate, but linear, supercoiled or agarose-embedded, number of recognition sequences, nature of nucleotides flanking the recognition sequence may influence enzyme activity.

- Contaminants such as phenol, chloroform, ethanol, detergents, EDTA, salts, that might partially or completely inhibit the activity of a restriction endonuclease.

- Usually supercoiled plasmid DNA, requires 5-10 u/µg to achieve complete digestion.
The efficiency with which a restriction enzyme cuts its recognition sequence at different locations in a piece of DNA can vary 10 to 50-fold. This is apparently due to influences of sequences bordering the recognition site, which perhaps can either enhance or inhibit enzyme binding or activity.

A related situation is seen when restriction recognition sites are located at or very close to the ends of linear fragments of DNA. Most enzymes require a few bases on either side of their recognition site in order to bind and cleave.

For PCR experiments that involve subsequent digestions it is important to know exactly how many extra nucleotides should be added to 5'-end of a PCR primer next to the introduced recognition site.
Troubles –PCR products

- Digestion is usually performed directly in the PCR mixture, without purification steps. The majority of restriction enzymes show sufficient activity (>20%) in PCR buffers, but direct digestion of amplified product is not recommended for AatII, BseSI, CfrI, Cfr9I, Cfr42I, HphI, ScaI, SmI, BglII, BplI, BspTI, EcoRI, Mva1269I, PauI and SalI.

- None of the PCR mixture components, including primers, dNTP's, template DNA and Taq DNA polymerase affect RE activity.

- After three-fold dilution of the PCR mixture with RE reaction buffer, the DNA is digested by most REs, though in some cases a large excess of enzyme or prolonged incubation time is necessary.

- For cloning applications, direct digestion of the amplified fragment in the PCR mixture without purification is not recommended, since Taq DNA polymerase is still active and may result in blunting of 5'-sticky ends and the addition of an extra dA nucleotide to the blunt ends of restriction fragments.
Troubles – unexpected digestion pattern

The enzyme is contaminated with another restriction endonuclease

If the incorrect banding pattern persists, the enzyme might have become contaminated with another restriction endonuclease, due to improper handling. Sometimes the reaction buffer or DNA preparation may be the source of contamination. It is recommended to use tips with a filter.

Enzyme is not active

All restriction endonucleases should be stored at -20°C or on ice when not in freezer. A RE should always be the last to be added to the digestion reaction mixture.

DNA preparation tested is the mix of two different DNAs

The best solution is to re-transform bacteria and then check the DNA isolated from 4-6 colonies.
Almost all strains of E. coli bacteria used for propagating cloned DNA contain two site-specific DNA methylases:

- Dam methylase
- Dcm methylase

A number of restriction endonucleases will not cleave methylated DNA.

MboI and Sau3AI are isoschizomers that recognize and cleave the sequence GATC, which is the sequence recognized by Dam methylase. Digestion by MboI is inhibited, while digestion by Sau3AI is unaffected by methylation.
The recognition site for ClaI is ATCGAT, which is not a substrate for Dam methylase. *However*, if that sequence is followed by a C or preceded by a G, a Dam recognition site is generated and cleavage by ClaI is inhibited.
Troubles

High glycerol concentration:

- Some restriction endonucleases (Alw21I, BpiI, Bsp68I, BspTI, Eco32I, Eco91I, EcoRI, Hin6I, Hinfl, Mph1103I, Mva1269I, NcoI) are very sensitive to the concentration of glycerol in the reaction mixture.

- Since restriction enzymes are supplied in 50% glycerol, the enzyme should comprise not more than 1/10 of the final reaction volume (i.e. no more than 5% glycerol).
Reduced specificity of enzyme recognition may result from:

- Increasing **glycerol** concentration (>5%, enzymes are usually sold as concentrates in 50% glycerol)
- changing **pH** (>8.0), or low ionic strength (e.g. if you forget to add the buffer)
- replacing **Mg** with **Mn**
- reducing **NaCl** concentration
- Extremely high concentration of enzyme (>100 U/ug of DNA)
- Presence of **organic solvents** in the reaction (e.g. ethanol, DMSO)

*For example, Eco RI cleaves GAATTC at pH 7.3 and 100 mM NaCl in the presence of 5 mM Mg, but raising the pH, lowering the NaCl concentration, substituting Mn for Mg or adding organic solvents all tend to reduce the specificity of cleavage to AATT*
Incomplete cleavage

Star activity

1  2  3  4  5  6
Inserting a DNA Sample into a Plasmid

Plasmid

DNA is cut with EcoRI at arrows.

DNA to be inserted

Resulting DNAs have sticky (complementary) ends.

DNA is spliced by complementary base pairing and sealed with DNA ligase.

Recombinant DNA
Cohesive ends

Blunt ends

- Ligation
DNA Ligase – enzyme catalysing formation of phosphodiesteric bound between group 3’-OH of one end of DNA molecule and group 5’-phosphate of the second end of DNA
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 (β-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 (>1x10^8 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.
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 can not 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.
Self-ligation

pUC18

HindIII

T4 ligase

self-ligation
alkaline phosphatase (bacterial, calf intestine)

plasmid cannot ligate to itself

mix with cDNA + T4 ligase

vector

cDNA

ligation

nick

transformation

nicks repaired by bacterial host enzymes after transformation
Alkaline Phosphatase action

Plasmid DNA

- Restriction endonuclease cleavage
- Alkaline phosphatase treatment

HO -------------- OH

HO -------------- OH

T4 DNA ligase

Target DNA

- Restriction endonuclease cleavage

P

HO

P
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 irreversibly 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.
Thank you
Gateway system

Advantages of Gateway cloning:

- Method is rapid: no overnight incubations are necessary.

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

- Insert orientation is defined.

- 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.
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.