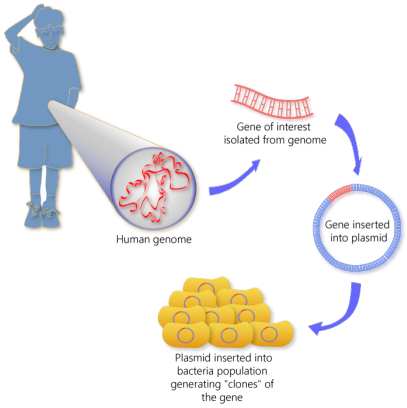
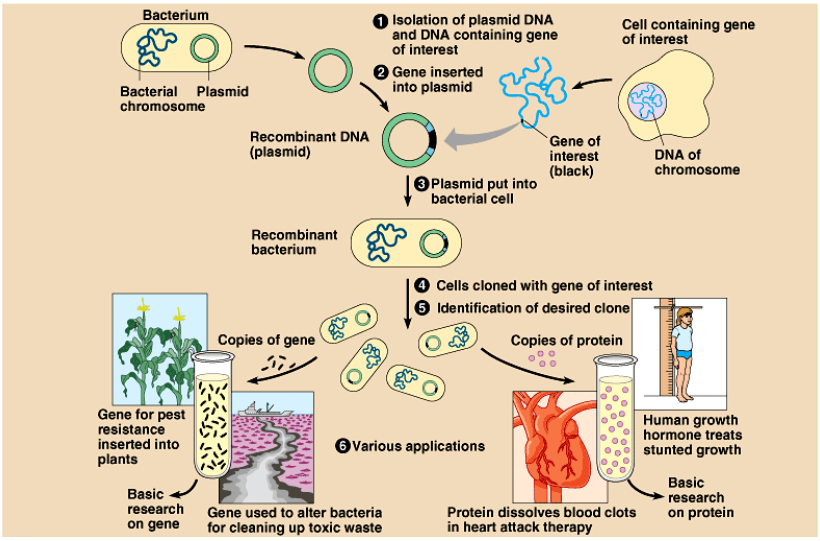
Techniques in Molecular Biology

Cloning – what is it?

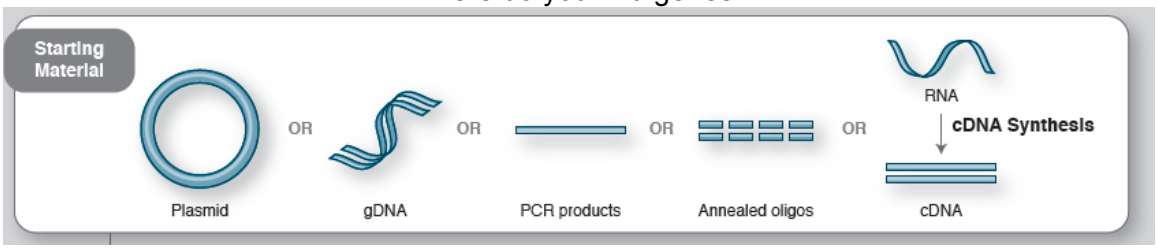
Dolly the sheep? Twins? For MoBio we are talking about moving gene(s) from one source or creating fusions of genes into a plasmid, vector or virus. Gene Cloning…



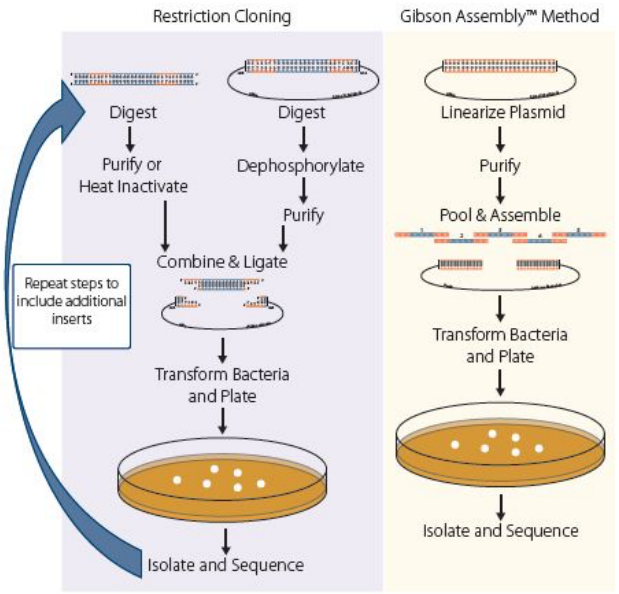
Many Uses of Cloned Genes



Where do you find genes?

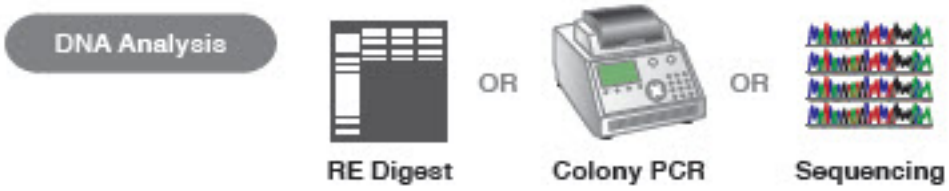


From custom DNA synthesized, cloning from another construct, genomic DNA or even using a cloning strategy of short oligonucleotides, there is no limit to genes to clone…

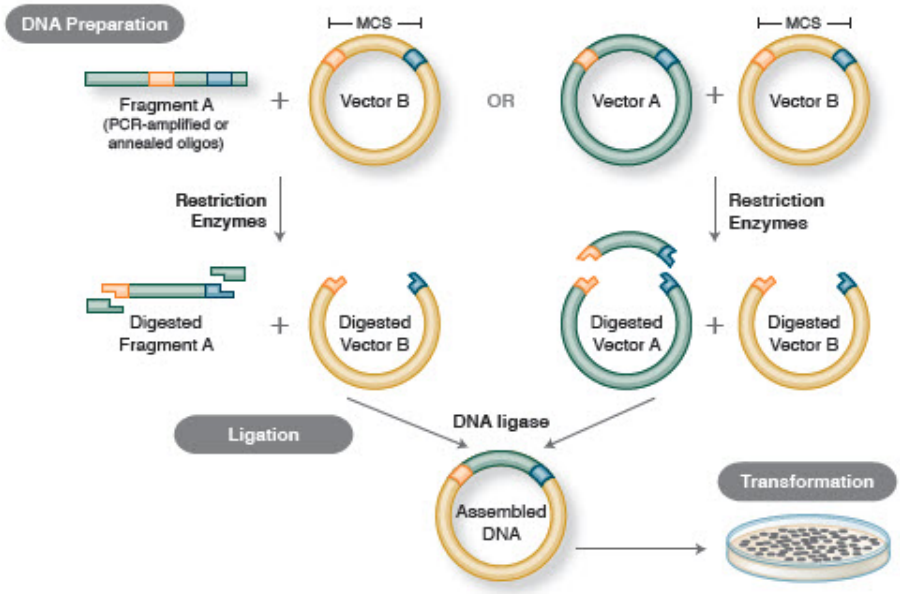


Typical Workflow

- Insert and Vector Preparation
- Ligate
- Transform
- Validate Insert



Traditional Cloning Restriction Enzyme Workflow



Moving a gene from one plasmid to another is referred to as subcloning

Restriction Enzymes
Review

Type II RE's
Typically recognize palindromic sites

- Bind and cut as a dimeric protein
- recognition sites range from 4-12 nts long
- cleavage of site typically blocked by methlyatioin

> 3500 knon Type II RE's with 241 specificities
Isochizomers recognize same site but cut differently

Palendromic Sites:
*Eco*RI: 5' – GAATTC – 3'
HindIII: 5' – AAGCTT – 3'
*Bcg*I: 5' – CGANNNNNNTGC – 3'
*Ava*II: 5' – GGWCC – 3'
*Sau*3AI: 5' – GATC – 3'

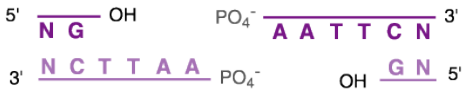
some do not cut within the recognition site

Sfi I: 5' – GGCCNNNNNGGCC – 3'
5' – GGCCN NNN/NGGCC – 3'
3' – CCGGN/NNN NCCGG – 5'

5' – GGCCNNNN–3' NGGCC – 3'
3' – CCGGN 3'–NNNNCCGG – 5'

*Csp*CI: 5' – CAANNNNNGTGG – 3'
5'– /NNNNNNNNNNNCAANNNNNGTGGNNNNNNNNNNNNN/–3'
3'–/NNNNNNNNNNNNNGTTNNNNNCACCNNNNNNNNNNN/ – 5'

(a) 5' Staggered ends

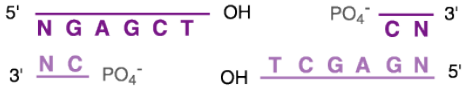


EcoRI

(b) Blunt ends

EcoRV

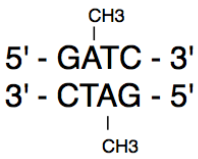
(c) 3' Staggered ends



SacI

Cleavage of site typically blocked by methylation

E. coli 'dam' methylates at A's of GATC sequence



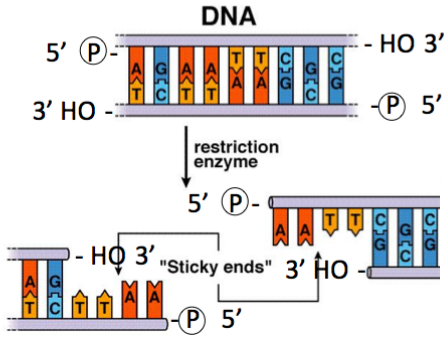
Methylated site

(N6-methyl-adenine)

DpnII (/GATC) cleavage blocked by methylated GATC

DNA Orientation

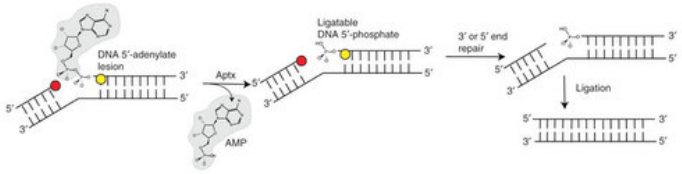
Don't forget which strand is where!



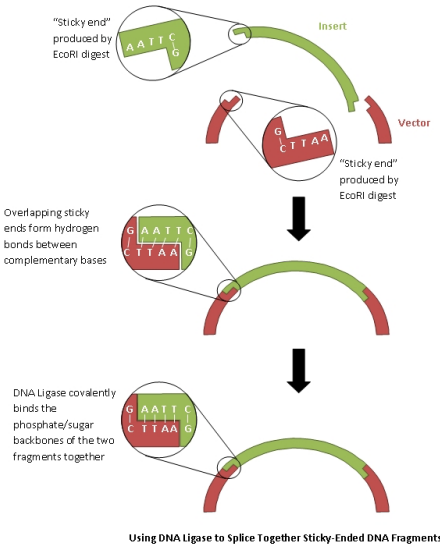
Ligation

Joining DNA fragments (insert to vector) is done by enzymes called DNA ligases

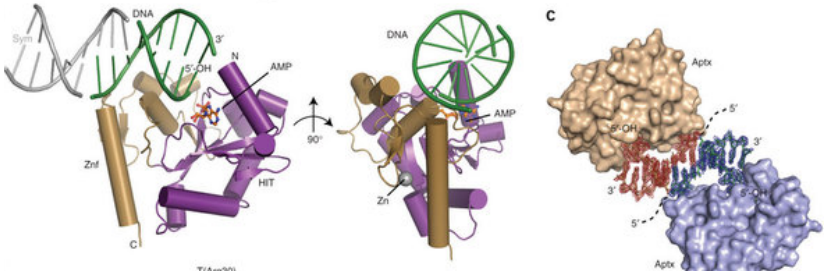
- Normal role of ligases is to repair single strand "nicks" or breaks in DNA and seal lagging end in replication

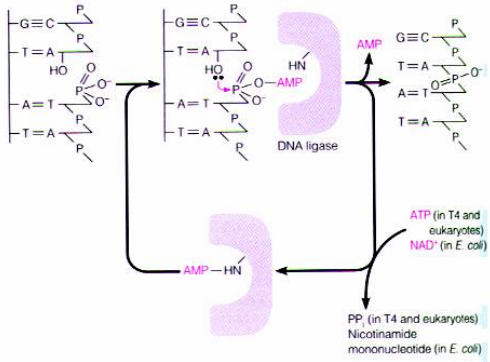


Sticky End Ligation



Using DNA Ligase to Splice Together Sticky-Ended DNA Fragments





Reaction Requires ATP and 5'-phosphate and 3' OH

Ligase reaction

- ATP, 3'-OH, 5' phosphate
- Reaction forms a covalent bond with lysine side change
- ATP -> AMP-DNA bond at 5' phosphate
- 3'OH attacks the AMP-DNA adduct to seal the sugar phosphate backbone

- E. Coli DNA ligase can not ligate blunt fragments
- Bacteriophage T4 DNA ligase (T4 ligase) can

Standard Reaction:

- 25 ng Vector
- 75 ng insert
- Ligase buffer with ATP
- 0.5 – 1 ul T4 DNA Ligase
- QS water to 10 ul

Ligate for 2 hrs at room temp or 16oC overnight.
Use 1-5 ul of ligation mixture for transformation

Controls:

- Uncut vector (no ligase) check for cell competence and vector resistance and quality
- Cut vector (no ligase) Should not get transformants due to uncut vector
- Cut vector (with ligase) Transformant colonies are due to re-circularization – may need to CIP or SAP
- Insert or water (with ligase no vector) Positive colonies indicate contamination of plasmid or other issues

Optimizing ligation

Presence on contaminant or DNA can inhibit reaction

- Loss of 5'-phosphate is possible
- Work for intermolecular ligation
    - 3:1 insert to vector ratio is typical
    - Need to be high conc of DNA – low leads to side reactions (*intra*molecular reaction) Total DNA less than 10 ng/ul
    - May need to range ratio from 1:1 to 1:10
- These are molar ratio not mass (ng)
- A general conversion is (DNA ng)/size of DNA
- NOT true molar but used to give relative mass to size ratios used for ligating and many molec bio techniques

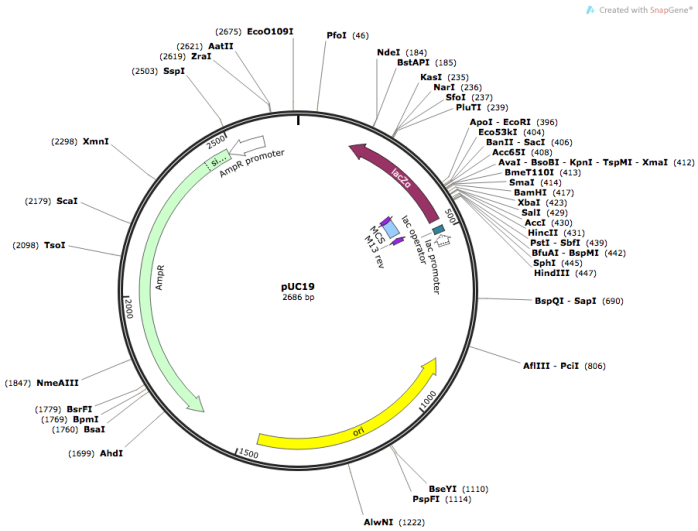
Low conc ligation

Possible outcomes (Fig in book)

Plasmid & Vectors

Double stranded DNA, exists naturally in bacteria

- labeled pXXX lower case p for plasmid
- Range from a few kbp to >100 kpb
- Possess their own ori site (origen of replication) and replicate under stringent or relaxed control
- High or low copy
- First artificial was designed in UC system pUC19
- Modern plasmids have many features including MCS with multiple reading frames

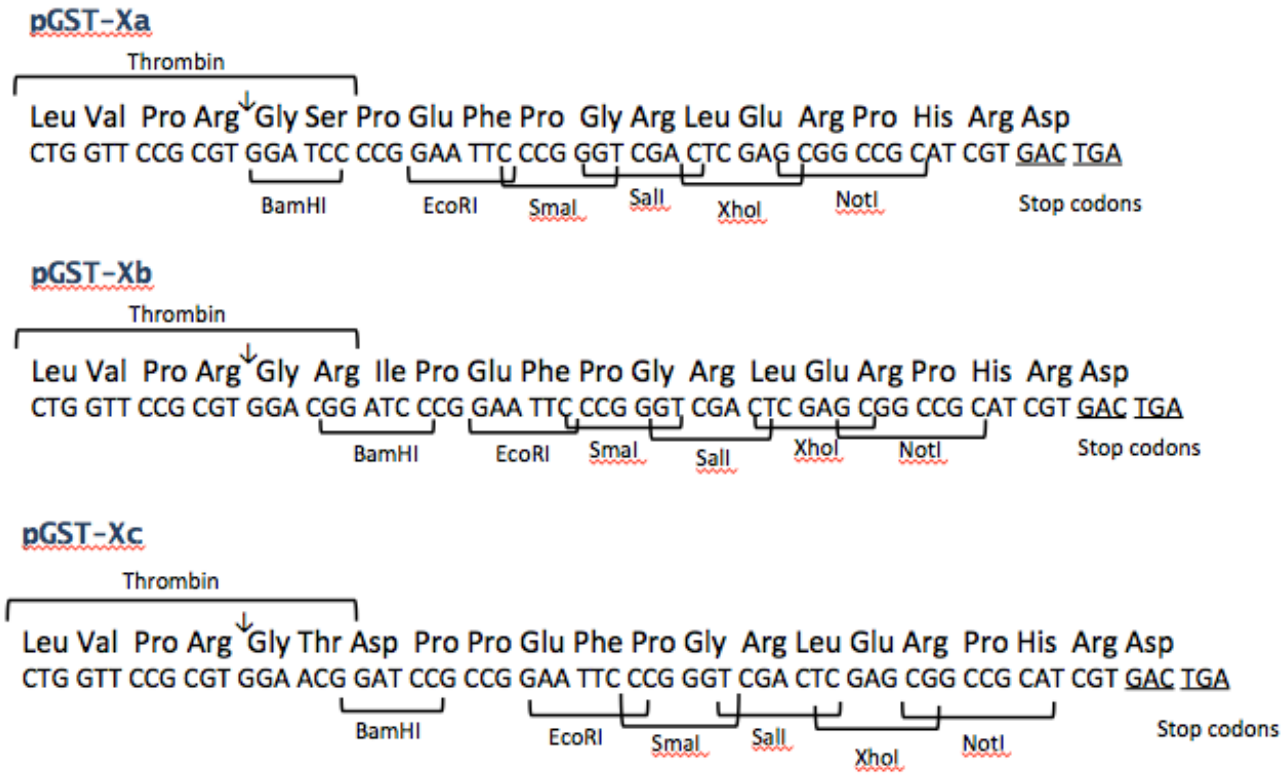


Your turn Reading in Frame

You have a sequence of a portion of a protein you wish to clone into a fusion protein called Glutathione S Transferase (GST) using a fictional GST plasmid.

- Which of the pGSTplasmids do you use? a, b or c?
- You are going to cut in with BamHI and EcoRI.
- Ensure you have the right reading frame for start and a stop at the end of the inserted sequence. Remember that GST is 5' to your insert. That is where the "#1 bp" is located
- Google search for "Restriction Sequence Translator" to find a program to map for where the RE cuts sites are
- Sequence
  GGATCCTGTAGATCTGCTGGCAGTTAAGAAGAAGCAGGAAACCAAACGTAGCATCAATGAGGA
  GATTCATACCCAGTTCCTGGATCATCTGCTGACTGGCATCGAGGACATCTGCGGTCACTATGG
  TCACCATCACGAATTC
- VDLLAVKKKQ ETKRSINEEI HTQFLDHLLT GIEDICGHYG HHH
- Find the map and defend your choice!
- Where is GST and The insert in terms of N to C terminus?

- Use the DNA to amino acid translator to find the coded amino acid sequence in the DNA sequence.
  - Where is the first coding base pair? Is it the same as the first bp in the DNA sequence?
  - What is the reading frame for the DNA sequence?
- Use an amino acid to DNA translator to find the DNA sequence and double check your work.
  - You may want to use the codon table to double check yourself
- Look up the restriction site sequence AND find where they cut the DNA sequence for BOTH the insert AND the vector.
  - DON'T just rely on a program that gives the bp number, convince yourself by looking for the sequence to confirm things…



How to add RE sites

You may need to add restriction enzyme sites
DNA and Linkers/Adaptors

- Bothe are short oligonucleotides, custom synthesized that contain a RE site.
- Use blunt end ligation to add to insert or vector DNA
- Using large amounts of linker in ligation increases likelihood of product (remember blunt end ligations are not efficient)

- **Linkers** – Blunt end ligation to generate new site
- **Adaptors** – oligos with partial complementation: one side matches vector/insert, second side of adaptor has new RE sequence

PCR extension is more common to add sites.

Single Cut and Blunt Cut vs multi RE cut DNA

Cutting plasmid and insert with same vector can lead to self annealing / circularization

- Removing 5' phosphate from either vector or insert Complete ligation will take place during replication after transformation

Alkaline Phosphatase

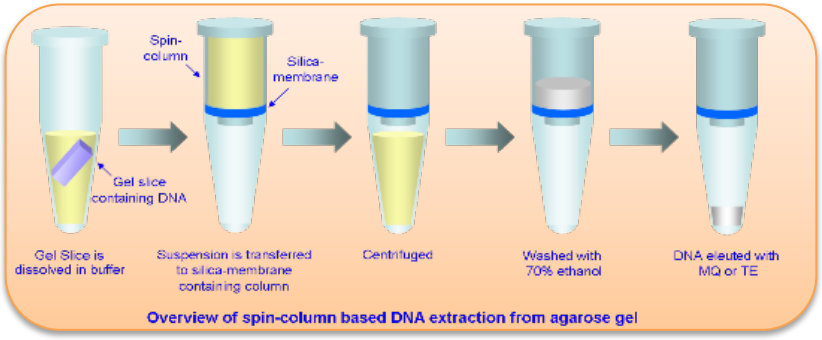
Unwanted ligation (most commonly from single enzyme digests) is prevented by alkaline phosphatase (AP) treatment

- Removal of 5' phosphate blocks ligase function
- Alkaline phosphatase (AP) or calf intestinal phosphatase (CIP) and Shrimp AP (SAP) function at higher pH
- Must be removed before continuing work. Done by phenol extraction or mini-spin column or gel extraction
- Only insert or vector are CIP treated
- Results in one backbone sealed, the other remains nicked until after replication

Gel Purification

Separate ligation products by size and purify from contaminates (salts, primers CIP, AP).

- Tricky and often do not work unless close attention is paid to details
- Lower % gel (0.7% or so)
- Select the right buffer and low melt agarose (see agarose handout)
- Visualize after UV light, cut agarose around band. (minimize exposure to limit dimerization and free-radical damage to DNA)
- Three common methods are:
  - to place gel slice in semi-permeable membrane (dialysis tubing) and subject to electrophoresis to migrate DNA into solution
  - Melt gel at low temps (~50°C) then recover DNA by spin column
  - Freeze to fracture gel and centrifuge to recover DNA

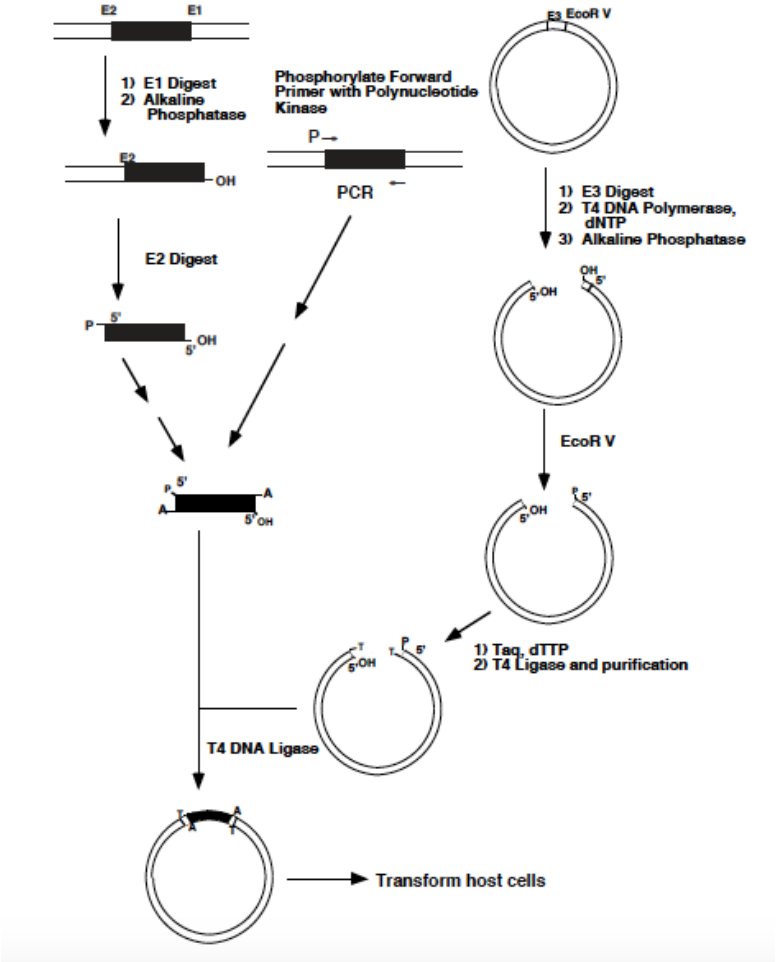


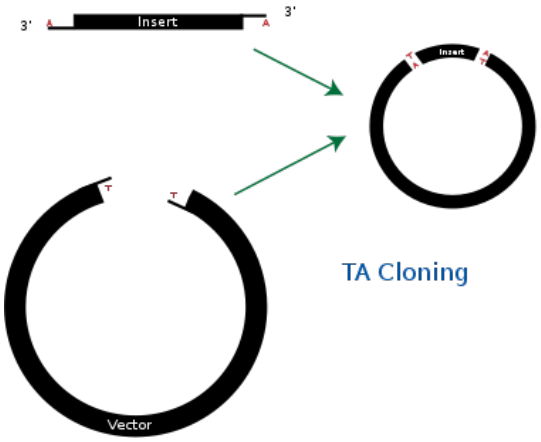
Ten Tips for Better Gel Purif

- Trim the gel slice as much as possible for better yield
- Minimize UV light exposure – work quickly
- If using phenol – remove all by evaporation or ethanol will not ppt
- Select high grade, low melt agarose
- Melting steps include chaotropic salts that alter downstream use – renature DNA by warming to 95oC then slowly cool
- Wash step with ethanol to remove residual salts
- If using silica to purify melted DNA, remove ethanol from wash steps. Spin sample without top to remove last bit of ethanol (PS if your DNA floats out of well, you have ethanol contamination)
- Elute DNA from slica column or membrane with hot buffer (70oC). Incubating for 5 min before elution helps too.

PCR aka TA Cloning

PCR cloning: Taq polymerase adds an A to 3' ends of amplicon resulting in a "tiny single bp overhang". The polymerase used must not have proofreading ability or a blunt end amplification will result.





The benefits to TA cloning are quick and efficient cloning. Using fast ligase reactions the whole process can be done in under 20 min.

- Some companies will sell TA prepared and cut vectors.
- May need to purify PCR product
- Non-directional so 50% of the product will be in the wrong direction.

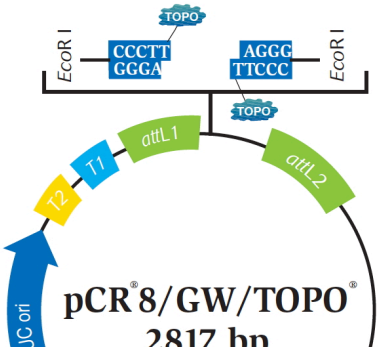
Directional TA cloning is performed by modifying one end of both the insert and the vector (hemi-phosphorylation)

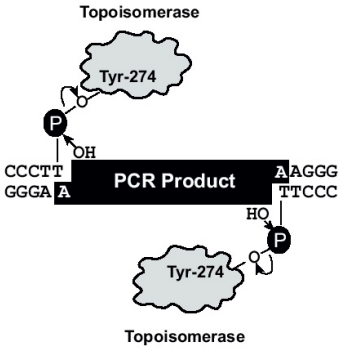
- Phosphorylating PCR primer using a DNA kinase creates hemi-P PCR insert.
- Vector is digested with one enzyme then CIP followed by a second cut

Topocloning

Topo-TA Cloning uses an enzyme (DNA topoisomerase I from Vaccinia virus), involved in controlling supercoiling.

- Topo enzyme takes place of ligase
- Enzyme binds and cuts double stranded DNA and remaines covalently bonded to P on DNA
- Kits provide vectors cut and bound to Topo
- Only need two components, prepared product
- Much faster than traditional ligase reactions



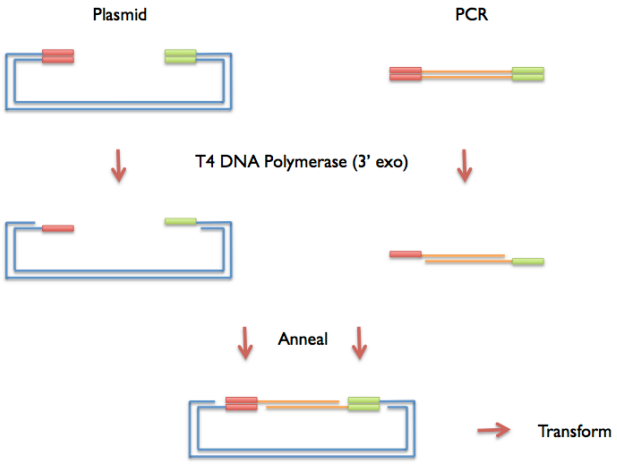


vector and TA PCR

Ligation Independent Cloning (LIC)

Alternative to traditional cloning using 3' -> 5' exo activity of T4 DNA Polymerase to create complementary 12 bp overhangs between vector and insert

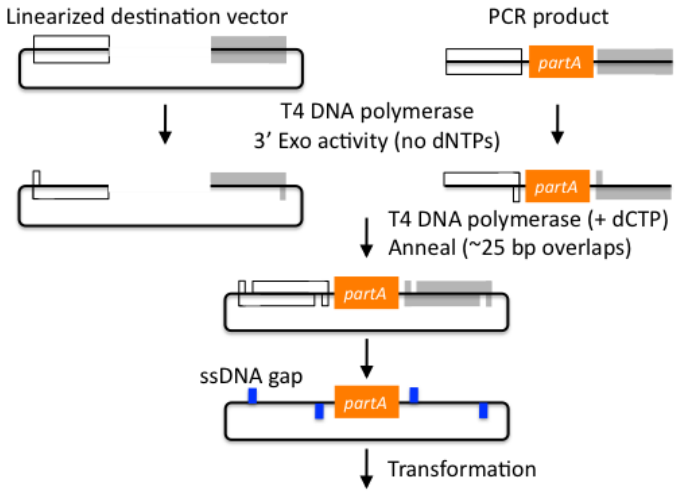
- No RE or ligases are used
- Use a single (often dGTP) nucleotide rest are NTP
- Exonuclease will digest one strand up to dG / C pair.
- Resulting long overlap must be planned with vector and insert or multiple sequences.
- Much more specific (longer complementation) than RE
- Nicks sealed by replication by transformation (DNA ligase)



Sequence and LIC (SLIC)

SLIC uses homologous recombination to repair gaps produced by the procedure after transformation

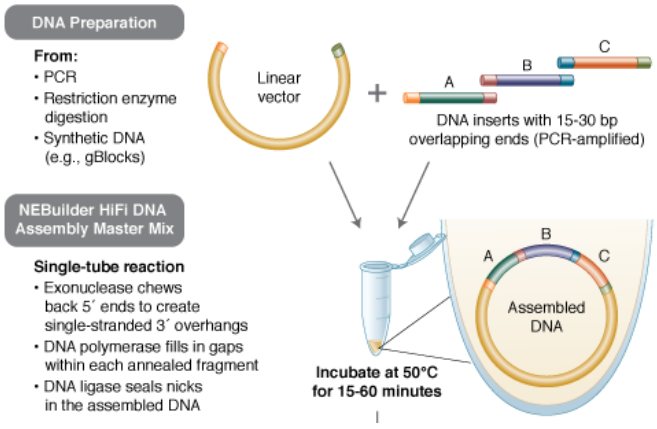
- PCR adds 5' and 3' homology regions and amplifies the fragment.
- Fragment and linearized plasmid are partially digested by T4 polymerase without dNTP to get exonuclease activity
- dNTP stops exonuclease reaction
- Overlaps can be pretreated with RecA to begin repair process before transformation
- Can be used to create multiple genes or "paste" pieces of genes together to make a new or fusion gene



Gibson Cloning

Originally described by Daniel Gibson of Craig Venter Inst. Three enzymes and exonuclease activity in isothermal conditions after PCR/digest creates seamless cloning or scarless cloning.

- One tube Rxn
- Combine several fragments
- Similar but not same as GGA



Gibson Cloning Procedure

- Design four PCR primers with overlap between insert/insert or insert/vector
- Adjacent segments should have identical sequences (A and B)
- PCR primers should contain 5' end that is the same as adjacent sequence and contains the 3' complementary ends
- Gel purification may not be needed
- Mix in equimolar concentration
- Three main components
  - ✓ ***T5 Exonuclease*** *- creates single-strand DNA 3' overhangs by chewing back from the DNA 5' end. Complementary DNA fragments can subsequently anneal to each other.*
  - ✓ ***Phusion DNA Polymerase*** *- incorporates nucleotides to "fill in" the gaps in the annealed DNA fragments.*
  - ✓ ***Taq DNA Ligase*** *- covalently joins the annealed complementary DNA fragments, removing any nicks and creating a contiguous DNA fragment.*
- Incubate for 1 hour at 50oC

to complete formation of new fragment