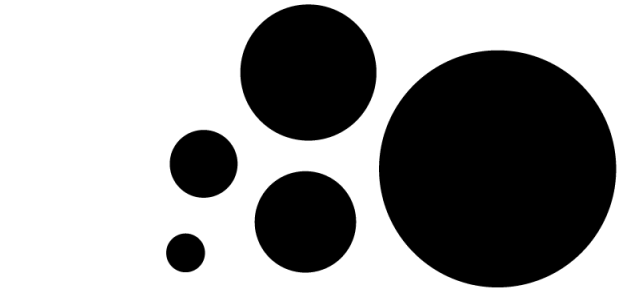


DENVER
BIOLABS

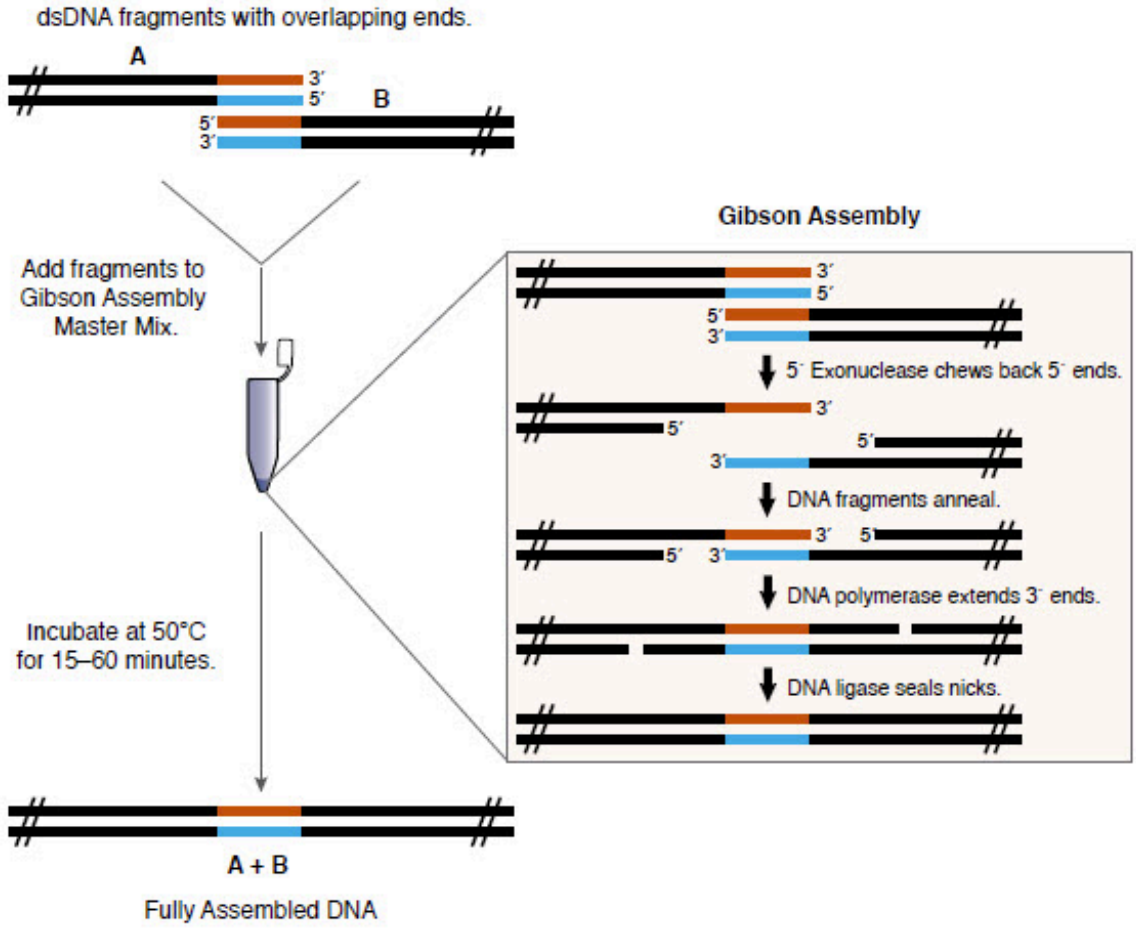
Bio-Bootcamp Protocol Guide
June 4th-5th, 2016

GIBSON ASSEMBLY

Gibson Assembly efficiently joins multiple overlapping DNA fragments in a single-tube isothermal reaction. The Gibson Assembly Master Mix includes three different enzymatic activities that perform in a single buffer:

- The exonuclease creates single-stranded 3´ overhangs that facilitate the annealing of fragments that share complementarity at one end (overlap region).
- The polymerase fills in gaps within each annealed fragment.
- The DNA ligase seals nicks in the assembled DNA.

The end result is a double-stranded fully sealed DNA molecule that can serve as template for PCR, RCA or a variety of other molecular biology applications, including direct transformation. The method has been successfully used by Gibson's group and others to assemble oligonucleotides, DNA with varied overlaps (15–80 bp) and fragments hundreds of kilobases long.



Protocol:

1) Make sure vector of interest in linearized.

2) If synthesized gblock gene fragments from IDT are being used, be sure to dilute pellets, if not already done, with TE buffer (pH 7.5) to a concentration of 10 ng/µL.
(Homogenize by vortex and place on ice until ready to use or store @ -20ºC)

Use 50ng of linear plasmid and a 3 fold molar increase of DNA fragments for the assembly. Convert to pmoles via equation 1.
(Up to a 6 fold molar increase in fragments may be necessary when # of fragments increases above 4)

Equation 1

$$pmoles = \frac{(mass\ of\ DNA\ in\ ng) \times (1000pg/ng)}{(\#\ of\ sense\ strand\ bp) \times (650\ Daltons)}$$

ie: 50ng of pCAGEN (4798bp) = 0.016 pmoles. If 3, 643bp fragments are being assembled into pCAGEN; you would need 20.9ng of each fragment, which equates to ~2.1µL each of the 10ng/µL TE diluted IDT fragments.

3) Prepare the Gibson assembly on ice as specified below:

50ng linear vector
3 fold molar excess fragments
10 µL Gibson assembly master mix (-20ºC)
xx µL dH_2O
20 µL total volume

4) Incubate samples in a thermocycler at 50ºC for 15 minutes. For samples containing more than 3 fragments, incubate @ 50ºC for 30 minutes.
(Store samples at -20ºC or place immediately on ice for subsequent transformation)

5) Transform NEB 5-alpha competent *E. coli* cells (provided with kit) with 2µL of the assembly reaction using the following protocol for Bacterial Transformation.

BACTERIAL TRANSFORMATION

In molecular biology, transformation is the genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material (exogenous DNA) from its surroundings through the cell membrane(s). Transformation occurs naturally in some species of bacteria, but it can also be affected by artificial means in other cells. For transformation to happen, bacteria must be in a state of competence, which might occur as a time-limited response to environmental conditions such as starvation and cell density.

1. Thaw chemically competent cells on ice.
2. Transfer 50 µl of competent cells to a 1.5 ml microcentrifuge tube (if necessary).
3. If the chemically competent cells are from New England Biolabs, add 2 µl of assembled product to NEB competent cells.
4. Mix gently by pipetting up and down or flicking the tube 4–5 times. Do not vortex. Place the mixture on ice for 30 minutes. Do not mix.
5. Heat shock at 42°C for 30 seconds. Do not mix.
6. Transfer tubes on ice for 2 minutes.
7. Add 950 µl of room temperature SOC media* to tubes.
8. Place the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
9. Warm selection plates to 37°C.
10. Spread 100 µl of the cells onto the plates with appropriate antibiotics.
11. Incubate plates overnight at 37°C.

GENEJET PLASMID MINIPREP

Growth of Bacterial Cultures

• Pick a single colony from a freshly streaked selective plate to inoculate 1-5 mL of LB medium supplemented with the appropriate selection antibiotic. Incubate for 12-16 hours at 37°C while shaking at 200-250 rpm. Use a tube or flask with a volume of at least 4 times the culture volume.

• Harvest the bacterial culture by centrifugation at 8000 rpm (6800 × g) in a microcentrifuge for 2 min at room temperature. Decant the supernatant and remove all remaining medium.

Purification

• All purification steps should be carried out at room temperature.
• All centrifugations should be carried out in a table-top microcentrifuge at >12000 × g (10 000-14 000 rpm, depending on the rotor type).

Protocol A. Plasmid DNA purification using centrifuges
Step Procedure

1. Resuspend the pelleted cells in 250 µL of the Resuspension Solution. Transfer the cell suspension to a microcentrifuge tube. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2. Add 250 µL of the Lysis Solution and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear.
Note. Do not vortex to avoid shearing of chromosomal DNA. Do not incubate for more than 5 min to avoid denaturation of supercoiled plasmid DNA.

3. Add 350 µL of the Neutralization Solution and mix immediately and thoroughly by inverting the tube 4-6 times.
Note. It is important to mix thoroughly and gently after the addition of the Neutralization Solution to avoid localized precipitation of bacterial cell debris. The neutralized bacterial lysate should become cloudy.

4. Centrifuge for 5 min to pellet cell debris and chromosomal DNA.

5. Transfer the supernatant to the supplied GeneJET spin column by decanting or pipetting. Avoid disturbing or transferring the white precipitate.

6. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.

7. Add 500 µL of the Wash Solution (diluted with ethanol prior to first use as described on p.3) to the GeneJET spin column. Centrifuge for 30-60 seconds and discard the flow-through. Place the column back into the same collection tube.

8. Repeat the wash procedure (step 7) using 500 µL of the Wash Solution.

9. Discard the flow-through and centrifuge for an additional 1 min to remove residual Wash Solution. This step is essential to avoid residual ethanol in plasmid preps.

10. Transfer the GeneJET spin column into a fresh 1.5 mL microcentrifuge tube (not included). Add 50 µL of the Elution Buffer to the center of GeneJET spin column membrane to elute the plasmid DNA. Take care not to contact the membrane with the pipette tip. Incubate for 2 min at room temperature and centrifuge for 2 min.

11. Discard the column and store the purified plasmid DNA at 4°C.

RESTRICTION DIGEST

A given restriction enzyme cuts DNA segments within a specific nucleotide sequence, at what is called a restriction site. These recognition sequences are typically four, six, eight, ten, or twelve nucleotides long and generally palindromic (i.e. the same nucleotide sequence in the 5' - 3' direction). Because there are only so many ways to arrange the four nucleotides that compose DNA (Adenine, Thymine, Guanine and Cytosine) into a four- to twelve-nucleotide sequence, recognition sequences tend to occur by chance in any long sequence. Restriction enzymes specific to hundreds of distinct sequences have been identified and synthesized for sale to laboratories, and as a result, several potential "restriction sites" appear in almost any gene or locus of interest on any chromosome.

Standard Reaction Set-up

Component	Volume		
	Plasmid DNA	PCR product	Genomic DNA
Water*, nuclease-free (#R0581)	15 µL	17 µL	30 µL
10X FastDigest or 10X FastDigest Green Buffer	2 µL	2 µL	5 µL
DNA*	2 µL (up to 1 µg)	10 µL (~0.2 µg)	10 µL (5 µg)
FastDigest enzyme	1 µL	1 µL	5 µL
Total volume:	20 µL	30 µL	50 µL

Properties of FastDigest enzymes

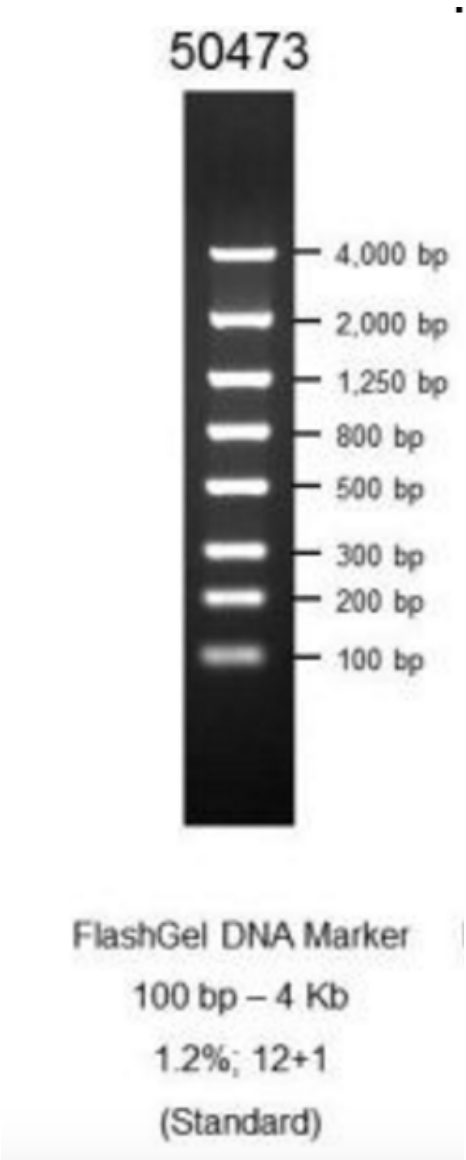
Enzyme	Specificity 5'→3'	Digestion time with 1µL of FastDigest enzyme, min			Thermal inactivation	Incubation without star activity, hours	Methylation effects
		Plasmid DNA	PCR product	Genomic DNA			
		1 µg/20 µL	~0.2 µg/30 µL	1 µg/10 µL			
FastDigest BamHI	G↓GATCC	5	5	5	80°C, 5 min	16	No effect
FastDigest BglII	A↓GATCT	20	30	20	No	16	No effect
FastDigest EcoRI	G↓AATTC	5	20	5	80°C, 5 min	0.5	CpG: may overlap - cleavage impaired
FastDigest EcoRV (Eco32I)	GAT↓ATC	5	5	5	No	16	No effect
FastDigest HindIII	A↓AGCTT	5	20	10	80°C, 10 min	16	No effect
FastDigest KpnI	GGTAC↓C	5	5	5	80°C, 5 min	16	No effect
FastDigest NdeI	CA↓TATG	5	60	30	65°C, 5 min	6	No effect
FastDigest NotI	GC↓GGCCGC	30	5	10	80°C, 5 min	16	CpG: completely overlaps - blocked
FastDigest PstI	CTGCA↓G	5	30	5	No	16	No effect
FastDigest SalI	G↓TCGAC	5	60	5	65°C, 10 min	16	CpG: completely overlaps - blocked
FastDigest SmaI	CCC↓GGG	5	5	5	65°C, 5 min	16	CpG: completely overlaps - blocked
FastDigest XbaI	T↓CTAGA	5	5	10	65°C, 20 min	16	Dam: may overlap - blocked
FastDigest XhoI	C↓TCGAG	5	5	10	80°C, 5 min	16	CpG: may overlaps - cleavage impaired

Standard Reaction Set-up

GEL ELECTROPHORESIS

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through a matrix of agarose. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. The DNA fragments can then be compared to a DNA ladder containing a mixture of known sizes of DNA fragments. The DNA ladder we will be using is shown below.

We will be using the LONZA Fast Gel System. I would recommend watching this video: https://www.youtube.com/watch?v=jMS_yRqjBVs



ISOHELIX DNA ISOLATION KIT

1. Swab inside of cheek for thirty seconds. Place the swab head into a suitable tube. If using Isohelix SK-1 or SK-2 swabs, use the tube provided.

2. Add 500µl LYS lysis buffer and vortex to cover the swab head.

3. Add 20µl Proteinase K solution, mix immediately by vortexing.

4. Incubate at 60^0C for a minimum of 10 minutes or up to 60 minutes to lyse the sample.

5. Add 750µl CB buffer, mix by vortexing thoroughly for 30 seconds.

6. Preheat the EB buffer at 70^0C (100µl per sample).

7. Add 1.25ml ethanol to the sample and vortex to mix.

8. Place an Xtreme DNA column onto a collection tube. Pipette 700µl of the sample into the column without touching the rim. Centrifuge at maximum speed (13.4K rpm, 12,000 x g) for 1 minute.
Discard the flow-through.

9. Repeat step 8 until all the sample has been loaded onto the column.

10. Wash the column by adding 750µl solution WB. Centrifuge at maximum speed (13.4K rpm, 12,000 x g) for 1 minute. Discard the flow-through.

11. Repeat the wash step by adding a further 750µl solution WB. Centrifuge at maximum speed (13.4K rpm, 12,000 x g) for 1 minute. Discard the flow-through.

12. Place the column in a clean collection tube and centrifuge at maximum speed (13.4K rpm, 12,000 x g) for 3 minutes to remove all traces of ethanol.

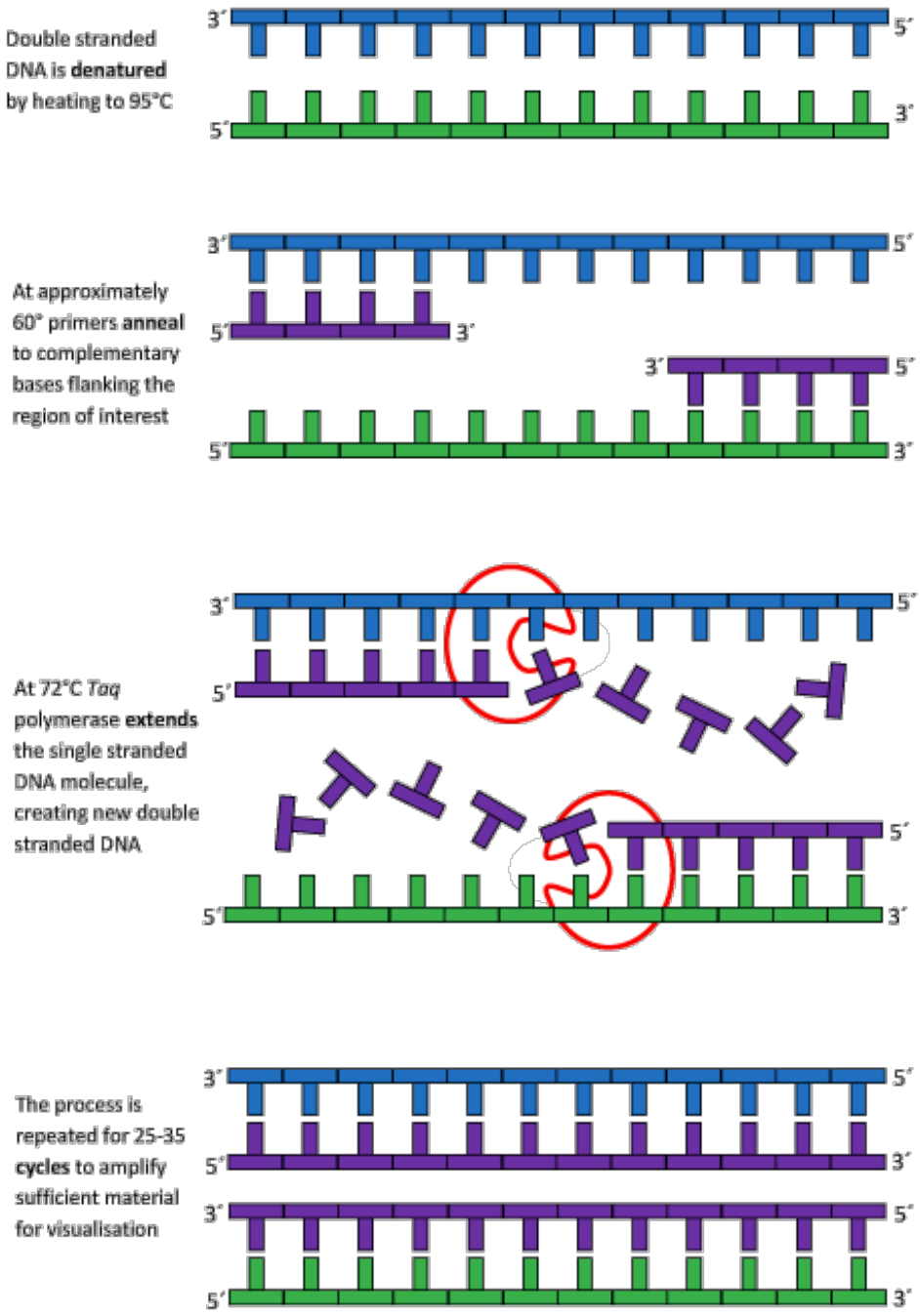
13. Place the column in a clean 1.5ml microcentrifuge tube. Add 100µl EB buffer pre-heated at 70^0C to the center of the membrane.

14. Incubate the column for 3 minutes then centrifuge at maximum speed (13.4K rpm, 12,000g) for 1 minute to elute the DNA.

15. Store the eluted DNA at -20^0C

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.



Protocol:

Component	Volume
Template DNA	variable(<1,000ng)
Forward Primer	200ng (y ul)
Reverse Primer	200ng (x ul)
PCR Master Mix	12.5 ul
H2O	to 25 ul
TOTAL VOLUME:	**25 ul**

-Add all components to a PCR tube.
-Run the following optimized program on a thermalcycler.

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95	1-3 min	1
Denaturation	95	30 s	25-40
Annealing	Tm-5	30 s	
Extension	72	1 min/kb	
Final Extension	72	5-15 min	1