Cloning and DNA Manipulation

Restriction Enzyme Digestion

Source: https://www.neb.com/protocols/2012/12/07/optimizing-restriction-endonuclease-reactions (Retrieved: 28-08-2017)

1. Look up the enzyme properties and set up the following reaction:

Component:	**Volume:**
Restriction Enzyme	1µL
DNA	... µL (1 µg)
NEBuffer: CutSmart/1.1/2.1/3.1	5 µl (1X)
MQ	... µL
(CIP/rSAP/other)*	(2x 1µL)
Total Reaction Volume	**50 µl**
Incubation Time	1 hour (add 1µL CIP after 30 minutes)
Incubation Temperature	37ºC (enzyme-dependent)

2a. Run 5µL on gel to check for correct fragment sizes and heat inactivate the enzymes (65ºC/80ºC for 20 min, depending on the enzyme). PCR purify the samples afterwards.
2b. Run everything on gel and gel purify the band with the correct size.

* = Phosphatases (CIP/rSAP/other) may be added to prevent back-ligation of any of the parts, if applicable. Add 1µL to the initial mix and add an additional 1µL of CIP/rSAP after 30 minutes of incubation.

Retrieving DNA from iGEM plates

(Adapted from the iGEM website)

1. With a pipette tip, puncture the foil of the desired well.
2. Add 10µL MQ and pipette up and down a few times. Incubate for 5 minutes to make sure the DNA is fully resuspended.
3. Transform 1µL of this DNA into your desired strain by a transformation method of your choice and plate the culture on plates with the appropriate antibiotic. Incubate O/N at 37 ºC.
4. Pick a single colony and inoculate in LB with the appropriate antibiotic O/N at 37ºC, 200rpm.
5. Make glycerol stocks of the bacteria and miniprep the rest of the culture for use.

T4 Ligation

Source: https://www.neb.com/protocols/1/01/01/dna-ligation-with-t4-dna-ligase-m0202 (Retrieved: 28-08-2017)

1. Set up the following reaction (add DNA ligase last, vector:insert ratio of 3:1)*:

Component:	**Volume:**
T4 DNA Ligase Buffer (10x)	2 µL
Vector DNA (Backbone)	... µL (50ng)
Insert DNA (Construct)	... µL (37.5ng)
MQ	... µL
T4 DNA Ligase	1 µL
Total:	**20 µL**

2. Mix the solution by pipetting up and down. Centrifuge briefly to collect the liquid.
3a. For sticky ends, incubate at RT for 10 minutes/For blunt ends, incubate at RT for 2 hours.
3b. Alternatively, ligate O/N at 16ºC for tricky ligations.
3c. Alternatively, ligate O/N in an endless cycle of 30 seconds at 30ºC and 30 seconds at 10ºC [1].
4. Heat inactivate the ligase at 65ºC for 10 minutes.
5. Chill on ice and transform 2µL of the reaction into 50µL competent cells/Store at -20ºC until transformation.

* = Other vector:insert ratios may be preferable, use the NEBioCalculator (https://nebiocalculator.neb.com/#!/ligation) to estimate ratios. Multiple inserts can be used in one reaction, subtract the needed volumes from MQ in that case.

cDNA Synthesis using Reverse Transcriptase

Source: https://www.thermofisher.com/order/catalog/product/18080044 (Retrieved: 28-09-2017)
N.B. A 20µL reaction volume can be used for 1ng-5µg of total RNA or 1-500ng of mRNA. For further applications, it may be wise to remove the complementary RNA by adding 1µL (containing 2 units) of *E. coli* RNase H and incubate at 37ºC for 20 minutes.

1. Set up the following reaction:

Component:	**Volume:**
50-250ng random hexameric primers *or* 2pmole gene-specific primers	1µL
1ng-5µg total RNA *or* 1-500ng of mRNA	...µL
dNTP mix (10mM)	1µL
MQ	Fill to 12µL
Total:	**12µL**

2. Heat the mix to 65ºC for 5 min and quickly chill on ice.
3. Add the following components:

Component:	**Volume:**
5x First-Strand Buffer	4µL
0.1M DTT	2µL
RNase Inhibitors (40 units/µL)	1µL
Total (+earlier volume):	**19µL**

4. Mix the tube contents gently. Incubate at 42ºC for 2 minutes.
5. Add 1µL (containing 200 units) of Reverse Transcriptase and mix by pipetting up and down.
6. Incubate the mix at 25ºC for 10 min.
7. Increase the temperature to 42ºC and incubate for 50 more minutes.
8. Heat inactivate the enzyme by heating the mix to 70ºC for 15 minutes.

Golden Gate Assembly

N.B. If the T4 Ligase Buffer negatively affects the chosen enzyme, it can be replaced by any NEBuffer (1.1/2.1/3.1 or CutSmart). Take in mind that 1.5µL of a 10mM ATP solution should be added to the mix as well in order for the ligase to work.

1. Set up the following reaction (vector:insert ratio of 1:2, use 50-75ng of vector DNA):

Component:	**Volume:**
Vector DNA	... µL
Insert DNA*	... µL
DpnI	0.35 µL
Type IIS Enzyme	1 µL
T4 DNA Ligase	1 µL
T4 DNA Ligase Buffer	1.5 µL
BSA (1mg/mL)	1.5 µL
MQ	... µL (fill to 15 µL)
Total:	**15 µL**

Step:	**Temperature (ºC):**	**Time (minutes):**
Incubation	37	20
25x	37	3
	16	4
Heat inactivation	80	20
Cooling	16	1
Hold	12	∞

2. Incubate the mix according to the table on the right.
3. Use 1-2 µL of this mixture for transformation, or store at -20ºC until further use.

* = Other vector:insert ratios may be preferable, use the NEBioCalculator (https://nebiocalculator.neb.com/#!/ligation) to estimate ratios. Multiple inserts can be used in one reaction, subtract the needed volumes from MQ in that case. To estimate background colonies for transformation, make an identical mix, but leave the insert DNA out.

Gateway cloning

N.B. Generally, try to use the 4µL reaction volume. However, if the relevant PCR product concentrations are low(ish), resort to the 8µL end volume. To reach the maximum number of recombinants, extend the first incubation time to up to 18 hours.

1. Set up the BP mix as described in the table below:

Component:	**1x Volume:**	**2x Volume:**
PCR Product	... (150ng)	... (150ng)
pDONR207	... (150ng)	... (150ng)
TE Buffer	Add up to 4µL	Add up to 8µL
BP Clonase	1µL	2µL
Total:	**4µL**	**8µL**

2. Incubate for 1 hour at room temperature.
3. Add 1µL Protein Kinase to inactivate enzymes and incubate at 37ºC for 10 min.
4. Electroporate 1-2µL into DH10β and plate on LB+genta agar plates. Incubate overnight at 37ºC.

References

[1] A. H. Lund, M. Duch, and F. Skou Pedersen, "Increased cloning efficiency by temperature-cycle ligation," *Nucleic Acids Res.*, vol. 24, no. 4, 1996.