Sam Zhao (Modified from Steve Long's protocol)
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PCR Reaction / PCR Conditions

For Phusion DNA Polymerase (Thermo Fisher)

Reagent		Standard	PCR Conditions	
5X Phusion HF buffer *	-	10 ul	98°C – 1 min	
10mM dNTP	-	1 ul	98°C – 15 s	30 cycles
Primers/each (10µM)	-	2.5 ul	55°C – 20 s	30 cycles
cDNA **OR**	-	0.5 ul	72°C –*	30 cycles
Genomic DNA **OR**	-	0.5 ul	72°C – 4 min	
Plasmid DNA (1:50 dilution of miniprep DNA ~5ng/ul)	-	1.0 ul	4°C - for ever	
Water	-	32.5-33 ul		
Phusion Turbo S	-	0.5 ul		
		50ul		

*7.5mM $MgSO_4$ in 5X Phusion HF buffer
*extension time: 30s per 1kb

For Deep Vent DNA Polymerase (NEB)

Reagent		Standard	PCR Conditions	
10X ThermoPol Buffer	-	5 ul	95°C – 2 min	
10mM dNTP	-	1 ul	95°C – 20 s	30 cycles
Primers/each (10µM)	-	2.5 ul	55°C – 20 s	30 cycles
cDNA **OR**	-	0.5 ul	72°C –*	30 cycles
Genomic DNA **OR**	-	0.5 ul	72°C – 6 min	
Plasmid DNA (1:50 dilution of miniprep DNA ~5ng/ul)	-	1.0 ul	4°C - for ever	
$MgSO_4$ (25mM)	-	3ul		
Water	-	34.5-35 ul		
Deep Vent	-	1 ul		
		50ul		

*extension time: 1min per 1kb

After PCR, run the products on 1% Agarose gel. If product is correct size, then cut off the band and use NucleoSpin Gel Clean-up Kit to purify DNA. For higher DNA yields,
* Use hot ddH_2O (~60℃) and incubate for ~4 min before elution.

Restriction Enzyme Digests(for E.coli cloning)

Digest with appropriate NEB Enzymes and Buffers (see Tools&Resources on the NEB website and then select Double Digest Finder to learn the information of specific restriction enzyme).

* Using larger reaction volume (~35-40 ul) is helpful
* For digest time, 2-3 hr is enough in most cases.

Gel-purify cut PCR product using NucleoSpin Gel Clean-up Kit.

Elute with 15-30ul ddH_2O.

Measure concentration and label tube.

Vectors

- To digest a vector for cloning, first digest with selected NEB enzymes, then CIP (removes phosphates from vector for better cloning) treat.

- Example: pNEH cut with EcoRI and SalI

DNA	1-2ug plasmid DNA (~5ul of a miniprep) or 20.25ul cleaned up PCR rxn
Enzyme 1 (EcoRI)	1ul
Enzyme 2 (SalI)	1ul
10X Buffer	2.5ul
100X Bsa	0.25 ul
Water	to 40ul
	40ul

- Digest for 2hrs at 37°C, then for vectors only add 0.2ul CIP (NEB) for 10min at 37°C.
- Immediately gel-purify the cut vector using a NucleoSpin Gel Clean-up Kit
- Elute with 15-30ul ddH_2O.
- Measure concentration and label tube.

Ligation and transformation (for E.coli cloning)

Ligation

- T4 ligase and T4 ligation buffer (10X)
- Mix the following contents in order and then react for ~12hr at 16°C:

o Vector	*
o Gene Insert	*
o Water	to 20ul
o 10X Ligation Buffer	2ul
o T4 Ligase	1ul
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	20ul

*Note that vector and gene insert should have a 1:4-1:10 molar ratio (i.e. 4 pieces of insert for each piece of vector). Always use 10-20ng of vector.

Example:
- 4Kb Vector and 1Kb Insert have a ratio of 1:4.
- If you use 15ng of vector [15*0.25=3.75]
- Then you would need to use 15ng of insert [3.75*4=15]

Transformation

- Thaw E.coli competent cells (XL2-Blue or TOP10) on ice.
- Pipet 5-20 ul ligation reaction directly into cells and mix by tapping gently. DO NOT mix by pipetting up and down.
- Incubate on ice for 30min.
- Heat shock cells in 42°C water bath for 45-60s.
- Return tube to ice.
- Add 350ul LB Media to cells and shake at 37°C for 1hr.
- Spread 50-200 ul from each transformation vial on separate, labeled LB plate with appropriate antibiotic and incubate at 37°C overnight.
- Select colonies and analyze by PCR or sequencing.

*Note that XL2-Blue cells are chloramphenicol resistant. If you are using chloramphenicol resistant vectors, such as pACYC Duet, please select TOP10 or other competent cells.

DNA Sequencing

- Visit website https://clims3.genewiz.com/default.aspx
- Create a username and password
- To submit a sample click on "Creat Sequencing Order"
- Fill in appropriate information for each sequencing item
- Click Submit
- Label the top of all microtubes with corresponding numbers provided
- Sample amounts:
    - Plasmid DNA 5-10 ul (50-70ng/ ul)
    - Primer 5-10 ul (10 uM)
- After receiving the sequencing results, carefully check both sequencing and tracing file. Normally, the available sequencing length is 600-700 nt. If needed, using software such as SeqMan or Vector NTI to assembly the forward and reverse DNA fragments.