Phusion high-fidelty DNA Polymerase

	50 µL	20 µL
Water	32.5 – X µL	13.3 – X
5X Phusion HF Buffer	10 µL	4 µL
dNTP Mix (10 mM each)	1 µL	0.5 µL
Forward primer (10 µM)	3 µL	1 µL
Reverse primer (10 µM)	3 µL	1 µL
Phusion (2 U/µL)	0.5 µL	0.2 µL
Template DNA	X (0.001-250 ng)	X (0.0004-100 ng)

	Temp, ˚C	Time	Cycles
Initial denaturation	98	30 s-3 min	1
Denaturation	98	5-10 s	25-40
Annealing	Lower Tm + 3	10-30 s	
Extension	72	15-40 s/kb	
Final Extension	72	5-10 min	1

DreamTaq

	50 µL	20 µL
Water	37.5 – X µL	15.4 – X
10X DreamTaq Buffer	5 µL	2 µL
dNTP Mix (10 mM each)	1 µL	0.5 µL
Forward primer (10 µM)	3 µL	1 µL
Reverse primer (10 µM)	3 µL	1 µL
DreamTaq (5 U/µL)	0.25 µL	0.1 µL
Template DNA	X (0.01 - 1000 ng)	X (0.004 - 400 ng)

	Temp, ˚C	Time	Cycles
Initial denaturation	95	1-3 min	1
Denaturation	95	30 s	25-40
Annealing	Tm - 3	30 s	
Extension	72	1 min/kb	
Final Extension	72	5-15 min	1

T4 DNA Ligase

	10 µL
Water	8.9 – X – Y µL
Vector DNA	X (10-50 ng)
Insert DNA	Y (1-5 amount vector)
10X Ligase Buffer	1 µL
T4 DNA Ligase (5 U/ µL)	0.1 µL

	Temp, ˚C	Time
Ligation	22	10-60 min

PrimeSTAR HS DNA Polymerase

	50 µL	**20 µL**
Water	32.5 – X µL	13.3 – X
5X PrimeSTAR Buffer	10 µL	4 µL
dNTP Mix (10 mM each)	1 µL	0.5 µL
Forward primer (10 µM)	3 µL	1 µL
Reverse primer (10 µM)	3 µL	1 µL
PrimeSTAR (2.5 U/µL)	0.5 µL	0.2 µL
Template DNA	X (0.01-200 ng)	X (0.004-100 ng)

	Temp, ˚C	**Time**	**Cycles**
Initial denaturation	98	30 s-3 min	1
Denaturation	98	10 s	25-40
Annealing	Tm	5-15 s	
Extension	72	1 min/kb	
Final Extension	72	5-10 min	1

FastDigest Restriction Enzyme

	50 µL	**20 µL**
Water	40 – X µL	16 – X µL
10X FastDiget Buffer	5 µL	2 µL
FastDigest	5 µL	2 µL
DNA	X (< 2 ng)	X (< 1 ng)

	Temp, ˚C	**Time**
Digestion	37	5-60 min
Inactivation	65	5 min

CPEC

	25 µL
dH_2O	17.75 – X µL
5X HF Phusion Buffer	5 µL
dNTP Mix (10 mM each)	1 µL
DMSO	0.75 µL
Phusion Polymerase (2 U/µL)	0.5 µL
DNA (1:1 of each piece)	X (0.01-1000 ng)

	Temp, ˚C	**Time**	**Cycles**
Initial denaturation	98	30 s	1
Denaturation	98	10 s	1-15
Annealing	55	10-30 s	
Extension	72	15 s/kb	
Final Extension	72	5-10 min	1

Yeast electroporation

10×TE buffer (pH 7.5):
100 mM Tris-HCl, 10 mM EDTA
10× LiAc: 1 M LiAc, pH 7.5 (adjusted using HAc).
Filter-sterilize or autoclave for 20 min.
1 M DTT: stored at -20°C

Day 1
Pick up single colonies on plates to 5 ml YPD medium. Culture at 30°C for 12-16 h.

Day 2

1. Inoculate into 50 ml YPD medium in flask. Culture at 30°C for 6-9 h.
   <<<<< Put sterilized water on ice >>>>>
2. When OD is 0.5-1.2, transfer cell culture into 50 ml cap tube (sterilized).
   <<<<< From here cells should be always on ice >>>>>
3. Collect cells by centrifugation (1100 g, 4°C, 5 min). Decant supernatant.
4. Re-suspend cells with 20 ml of sterilized H2O (ice-cold). Mix with pipetman. Centrifuge and decant supernatant.
5. Treat cells with 20 ml of 0.1 M LiAc (16 ml 1 M sorbitol + 2 ml 10×TE buffer + 2 ml 1 M LiAc) at 30°C for 30 min. Add 0.2 ml 1 M DTT and keep cells at 30°C for 15 min. Centrifuge and decant supernatant.
   <<<<< Put 1 M sorbitol on ice >>>>>
6. Wash cells twice with 20 ml of 1 M sorbitol (ice-cold). Centrifuge and decant supernatant.
7. Re-suspend cells with 100-200 µl of sterilized ice-cold 1 M sorbitol (final OD=100-200).
8. Take 50 µl of suspended cells into a new 1.5 ml tube on ice.
9. Add 5 µl fragment DNA (> 200ng/µl). Mix with pipetman and keep on ice for 15 min. Transfer all to a sterilized cuvette (green cap). Add 1 ml of cold 1 M sorbitol to new labeled tubes (used later).
10. Set the cuvette in the holder of Micro Pulser Electroporator. Chose “Manual”, and set voltage at 1.5 kV. Push the pulse button. Read “Time / ms”, if it is between 4.0-6.0, this process is successful.
11. Add 1 ml of cold 1 M sorbitol, immediately after the pulse. Mix well by pipetting up and down. (After this, it is OK to be at RT.) Transfer all to the sorbitol tube ASAP.
12. Incubate at 30°C for 1-3 h. Centrifuge (3000 g, 1 min) to ~150µl and then spread cells on selection plates. Make a negative control plate. Place the plates at 30°C air incubator.

YPD: Yeast extract 10 g/l, peptone from meat 20g/l, glucose 20 g/l.
SC-URA: YNB without aa 6.9g/l, glucose 20g/l, URA-drop-out 0.77 g/l.
Delft: (NH4)2SO4 7.5 g/l, KH2PO4 14.4 g/l, MgSO4·7H2O 0.5 g/l, metal solution 1 ml/l, vitamin 1 ml/l.

Frozen-EZ Yeast Transformation II

Preparation of Competent Cells

Grow yeast cells at 30°C in 10 ml YPD broth until mid-log phase (~5 x 106 - 2 x 107 cells/ml or OD600 of 0.8-1.0). The following steps are accomplished at room temperature.

1. Pellet the cells at 500 x g for 4 minutes and discard the supernatant.
2. Add 10 ml EZ 1 solution to wash the pellet. Repellet the cells and discard the supernatant.
3. Add 1 ml EZ 2 solution to resuspend the pellet.

At this point, the competent cells can be used for transformations directly or stored frozen at or below -70°C for future use. It is important to freeze the cells slowly. To accomplish this, either wrap the aliquotted cells in 2-6 layers of paper towels or place in a Styrofoam box before placing in the freezer. DO NOT use liquid nitrogen to snap-freeze the cells.

Transformation

This part of the procedure is the same for both frozen stored (thawed at room temperature) and freshly prepared competent yeast cells.

1. Mix 50 µl of competent cells with 0.2-1 µg DNA (in less than 5 µl volume); add 500 µl EZ 3 solution and mix thoroughly.
2. Incubate at 30°C for 45 minutes. Mix vigorously by flicking with finger or vortexing (if appropriate for your DNA) 2-3 times during this incubation.
3. Spread 50-150 µl of the above transformation mixture on an appropriate plate. It is unnecessary to pellet and wash the cells before spreading.

Incubate the plates at 30°C for 2-4 days to allow for growth of transformants.

Gibson assembly method

(Optional) DpnI Digestion Protocol: When higher amounts of plasmid template must be used in the PCR reaction, it is recommended to digest the PCR product with DpnI (NEB #R0176) in order to destroy plasmid template before setting up the Gibson Assembly reaction. DpnI cleaves only E. coli Dam methylase-methylated plasmid DNA, but does not cleave the PCR product since it is not methylated. DpnI Digestion Protocol:

1. In a total 10 µl reaction, mix 5–8 µl of PCR product with 1 µl of 10X Cutsmart and 1 µl (20 units) of DpnI.
2. Incubate at 37°C for 30 minutes.
3. Heat-inactivate DpnI by incubating at 80°C for 20 minutes.
4. Proceed with the Gibson Assembly Cloning procedure.

Assembly Protocol

1. Set up the following reaction on ice:

	Recommended amount of fragment used for assembly		
	2-3 Fragment assembly	**4-6 Fragment Assembly**	**Positive control**
Total amount of fragments	0.02-0.5pmols* X µL	0.2-1pmols X µL	10 µL
Gibson Assembly mix (2X)	10 µL	10 µL	10 µL
Deionized H2O	10 – X µL	10 – X µL	0
Total volume	20 µL**	20 µL	20 µL

*50–100 ng of vectors with 2–3 fold of excess inserts. Use 5 times more of inserts if size < 200 bps

**If greater numbers of fragments are assembled, additional Gibson Assembly Master Mix may be required.

2. Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled. Following incubation, store samples on ice or at –20°C for subsequent transformation.
3. Transform NEB 5-alpha Competent E. coli cells (provided with the kit) with 2 µl of the assembly reaction, following the transformation protocol.

Gibson Assembly Transformation Protocol

1. Thaw chemically competent cells on ice.
2. Add 2 µl of the chilled assembly product to the competent cells. Mix gently by pipetting up and down or by flicking the tube 4–5 times. Do not vortex.
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at 42°C for 30 seconds. Do not mix.
5. Transfer tubes to ice for 2 minutes.
6. Add 950 µl of room-temperature SOC media to the tube.
7. Incubate the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Spread 100 µl of the cells onto the selection plates. Use Amp plates for positive control sample.
10. Incubate overnight at 37°C.