1. **<u>The 2.2-kb symmetric DNA Y-arms</u>**

A 2.2-kb symmetric DNA Y-arms can be made following the protocol **To obtain high annealing efficiency, see Section 2.**

a. PCR amplification of the two 1-kb DNA arms

Prepare pLB601 (pMDW2) plasmids using standard plasmid transformation procedure.

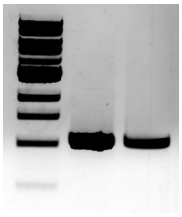
The following DNA oligos are required and can be ordered from IDT. Scale: 25 nmole for <72 nucleotides, 100 nmole for >= 72 nucleotides; Purification grade: Desalting.

1.1kb_pLB601_BstXI: ATTACCAGATCGCTGGAAGCTAGAGTAAGTAGTTC
1.1kb_pLB601_XhoI: TTAAAACTCGAGCTGATTTAACAAAAATTTAACG
1.1kb_pLB601_BstEII: TAATGGTTACCGGGAAGCTAGAGTAAGTAGTT
- Mix the following ingredients in separate 1.5-mL Eppendorf tubes. Add DNA polymerase last. Keep the tubes on ice after mixing. After adding all reagents and mixing, aliquot 50 uL reactions into 20 0.2-mL PCR tubes. Keep the PCR tubes on ice.

1-kb DNA arm 1, 20 reactions		**1-kb DNA arm 2, 20 reactions**	
1.1kb_pLB601_BstXI 100 uM	5 uL	1.1kb_pLB601_BstEII 100 uM	5 uL
1.1kb_pLB601_XhoI 100 uM	5 uL	1.1kb_pLB601_XhoI 100 uM	5 uL
pLB601 2 ng/uL	40 uL	pLB601 2 ng/uL	40 uL
dNTPs 25 mM (Roche, NEB, mixed)	8 uL	dNTPs 25 mM (Roche, NEB, mixed)	8 uL
Phusion DNAP (2 units/uL, NEB, M0530S)	8 uL	Phusion DNAP (2 units/uL, NEB, M0530S)	8 uL
Phusion buffer 5X	200 uL	Phusion buffer 5X	200 uL
dIH20	734 uL	dIH20	734 uL

Raise the temperature of the heat bock to 94 ^{o}C and assemble the PCR tubes into the heat block before running the following heat cycles.

98 ^{o}C (30 s) → <u>98 ^{o}C (15 s) → 53 ^{o}C (20 s) → 72 ^{o}C (31 s)</u> → 72 ^{o}C (10 min) → 4 ^{o}C (inf)
Repeat the underlined portion for 30 times



- Check the DNA on an agarose gel (0.8%, 110 V/10cm gel, 1X TAE, 30-40 min).

Gel electrophoresis of the two PCR products (~ 100 ng/lane) with the 1-kb DNA ladder. Both products have the expected size (~ 1.1 kb).
- To concentrate/clean-up the DNAs from these PCR products, use Purelink PCR spin columns following the protocol in the kit (Thermo Fisher, Cat# K310001). Use binding buffer B2.

- From 20 working reactions for each 1-kb DNA arms, combine and divide equally among 4 spin-columns and elute the DNA in 50 uL prewarmed Tris-Cl 10 mM pH 8.5 and 0.2 mM EDTA per each column. For each DNA, subsequently combine the four elutants. Use

a spectrophotometer to measure the DNA concentration. Typical yield for each column: 13.0 ug and 10.5 ug for the PCR reactions of the 1-kb DNA arm 1 and 2, respectively.

b. Digest the two 1-kb DNA arms with XhoI

Digest the PCR products from **Step a** (separately) as followed:

DNA arm 1		DNA arm 2	
1-kb DNA arm 1 from step b, 19.7 pmol	198 uL	1-kb DNA arm 2 from step b, 15.8 pmol	198 uL
XhoI (NEB, 20 units/uL, Cat# R0146S)	9.5 uL	XhoI (NEB, 20 units/uL, Cat# R0146S)	7.5 uL
CutSmart 10X	23 uL	CutSmart 10X	22.8 uL

Incubate at 37 ^{o}C in a water bath for 12-19 hours. Then heat kill at 65^{o}C for 20 min.

c. Klenow fill-in the two DNA arms' end

Klenow fill-in reactions were performed in the same tube as used in step (b). No DNA purification after step (b) is needed as Klenow exo^{-} is compatible with the CutSmart buffer. Use 33 uM nucleotides for Klenow fill-in reaction.

Arm 1, filled with dig		Arm 2, filled with biotin	
DNA arm 1 digested with XhoI	230 uL	DNA arm 2 digested with XhoI	228 uL
Dig-dUTP 1 mM (11573152910, Sigma)	7.6 uL	Bio-dATP 0.4 mM (19524016, Thermo Fisher)	19 uL
dCTP 10 mM (11051458001, Sigma)	0.76 uL	dCTP 10 mM (11051458001, Sigma)	0.76 uL
dGTP 10 mM (11051466001, Sigma)	0.76 uL	dGTP 10 mM (11051466001, Sigma)	0.76 uL
dTTP 10 mM (11051482001, Sigma)	0.76 uL	dTTP 10 mM (11051482001, Sigma)	0.76 uL
Klenow exo^{-} (5 u/uL, NEB, M0212S)	1.2 uL	Klenow exo- (5 u/uL, NEB, M0212S)	1.2 uL

Incubate at 37 ^{o}C in a water bath for 1.5 hours. Heat kill at 75 ^{o}C for 20 min.

After Klenow fill-in, purify each DNA arm separately using 2 Pure Link PCR spin columns (K310001, Thermo Fisher). DNA is eluted using 100 uL prewarmed elution buffer (10 mM Tris-Cl pH 8.0 and 0.1 mM EDTA; prewarmed to 55 ^{o}C) equally divided for the 2 columns. For each DNA, subsequently combine the 2 elutants. I assumed the recovery yield is 90%. It is advised to check the concentration using a spectrophotometer.

d. Digest the end-labeled DNA arms to create suitable overhangs for ligation

Arm 1 (dig end)		Arm 2 (biotin end)	
Arm-1 17.7 pmol	98 uL	Arm-2 14.3 pmol	98 uL
Neb 3.1 10X buffer	26 uL	CutSmart 10X buffer	15 uL
BstXI 10 u/uL (R0113S, NEB)	13 uL	BstEII-HF 20 u/uL (R3162S, NEB)	5.5 uL
diH20	123 uL	diH20	31.5 uL

Incubate at 37 ^{o}C in a water bath for 3.5 hours.

Purify each DNA arm separately using 2 Pure Link PCR spin columns. DNA is eluted using 100 uL prewarmed elution buffer (10 mM Tris-Cl pH 8.0 and 0.1 mM EDTA; prewarmed to 55 ^{o}C) equally divided for the 2 columns. For each DNA, subsequently combine the eluants and check the DNA concentration using a spectrophotometer. The yield was ~ 9.17 pmol for Arm 1 and ~ 5.61 pmol for Arm 2. The DNA is stored in 4 ^{o}C.

e. Annealing the DNA adapters

The following DNA oligos are required and can be ordered from IDT. **p** indicates phosphorylation. Scale: 25 nmole for <72 nucleotides, 100 nmole for >= 72 nucleotides; Purification grade: Desalting..

Adapters

Upper-1: pGCAGTACCGAGCTCATCCAATTCTACATGCCGC

Lower-1p:
pGCCTTGCACGTGATTACGAGATATCGATGATTGCGGCGGCATGTAGAATTGGATGAGCTCGGTACTGCATCG

Upper-2: CGTTACGTCATTCTATACACTGTACAG

Lower-2p:
pGTAACCTGTACAGTGTATAGAATGACGTAACGCGCAATCATCGATATCTCGTAATCACGTGCAAGGCCTA

Mix the following ingredients in 0.2ml PCR tubes to achieve 10 uM final product assuming 100% annealing efficiency.

Adapter 1 (50 uL)		**Adapter 2 (50 uL)**	
Upper-1 3-invdT 100 uM	5 uL	Upper-2 77.8 uM	6.43 uL
Lower-1 85.2 uM	5.87 uL	Lower-2p 95.7 uM	5.22 uL
Annealing buffer 10X	5 uL	Annealing buffer 10X	5 uL
diH20	34.13 uL	diH20	33.35 uL

Assemble the tubes (1 tube for each adapter) into a PCR heat block and run the following heating-cooling task: Heat to 95 ^{o}C and hold for 10 minutes, and then slowly cool down to 26 ^{o}C using 70 steps with a rate of -1 ^{o}C/step**.** Hold at each step for 20 seconds.

The whole process will take ~ 40 minutes to finish. The DNA adapter is stored in 4 ^{o}C.

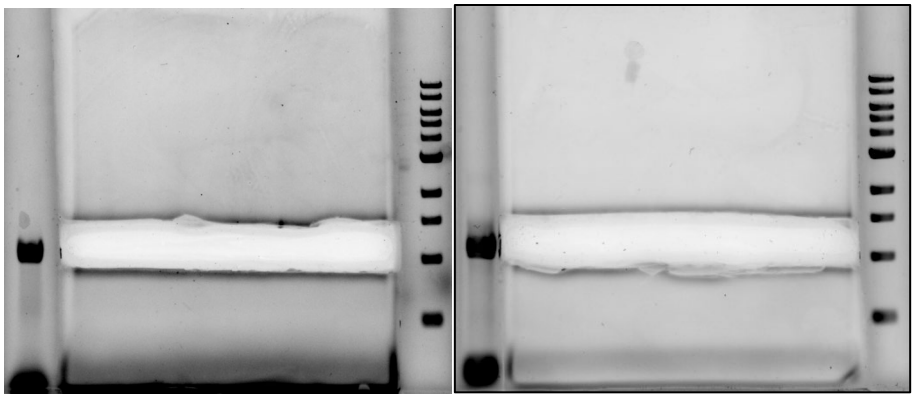
f. Ligate DNA Arm to the relevant DNA adapter and gel purify the ligated products

Arm 1 + Adapter 1 (126 uL)		**Arm 2 + Adapter 2 (126 uL)**	
Arm 1 from step (d), 9.17 pmol	99 uL	Arm 2 from step (c), 5.04 pmol	99 uL
Adapter 1 from step (e), 5 uM	36 uL	Adapter 1 from step (d), 10 uM	12 uL
T4 10X buffer	15.7 uL	T4 10X buffer	15 uL
T4 ligase 400 u/uL	4 uL	T4 ligase 400 u/uL	4.2 uL
diH20	0 uL	diH20	19.8 uL

Keep the tube on ice during mixing. Incubate at 15.9 ^{o}C overnight in the PCR machine.

After DNA ligation, directly load the each entire DNA sample into a big wells (that can hold ~ 200 uL) in 1% agarose gels and run for ~ 40 min using 90V. You will run a separate gel for each Ligated Arm.

Carefully excise the dig-labeled ~ 1-kb DNA fragment (from Arm 1+Adapter 1 ligation, left image) and the biotin-labeled ~ 1-kb DNA (from Arm 2+Adapter 2 ligation, right image). Avoid shining the UV light to the DNA. Excise a minimal gel band size with only the DNA to increase DNA recovery yield and reduce contamination from the gel solution into eluted DNA.



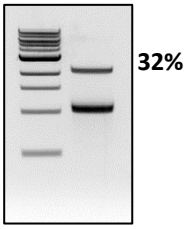
Elute each DNA using Zymo spin columns (D4001, Zymo Research). For each sample, the gel solution was divided into 4 equal sample volumes and was loaded into 4 Zymo columns. The DNA was eluted with ~ 8 uL prewarmed elution buffer (10 mM Tris-Cl pH 8.0 and 0.1 mM EDTA) per each column. For each DNA, subsequently combine the eluants and check the DNA concentration using a spectrophotometer. Typical yield after gel purification: ~ 4.4 ug for Arm 1 and ~ 3.0 ug for Arm 2, which indicates the recovery efficiency is 60 to 70%. The DNA arms is stored in 4 °C. However it is recommended to proceed to the DNA annealing step (step g).

g. Annealing the two arms to obtain the complete Y-arms

Mix the following ingredient in a 0.2ml PCR tube.

Purified Arm-1 173 nM	21 uL
Purified Arm-2 117 nM	31 uL
Annealing buffer 10X	5.78 uL

Put the tube in a PCR heat block. Heat the sample to 75 °C and hold for 10 minutes. Then slowly cool down from 75 °C to 50 °C using 250 steps (-0.1 °C per step). At each step, hold for 10 seconds. Then quickly step-wise cool down to 4 °C.



Check the annealed Y-arm on agarose gel. Use 1-kb DNA ladder for checking the DNA size (Lane 1). The top band (Lane 2) indicates the successfully annealed DNA Y-arms, which is ~ 2 kb. The bottom band indicates the unannealed arms (~ 1 kb). For this experiment, the annealing efficiency is ~ 32%. The DNA is stored in 4 °C and can be used for several years.

To increase the annealing efficiency, see **Section 2**

2. Technical note on optimizing the annealing of two arms (*perform if having low annealing efficiency in step g above*)

- The lab standard protocol for annealing the two arms is: 75 °C (10 min) -> 75 °C (-0.1 °C per 15 s) until the temperature is ~ 50 °C, then quickly step-wise cool the sample to 4 °C. The process takes about 1 hour to finish. The slow cooling allows two strand to anneal. 50 °C was chosen as it is well below the annealing temperature of the two ssDNA strands (~ 65 °C).

- The un-annealed fraction contains ssDNA arms with folded secondary structures that may prohibit efficient annealing during the cooling down process. If the 2nd structure happens to have a melting temperature below 50 °C, the quick cooling from 50 °C may not allow the 2 arms to anneal correctly.

- To optimize the annealing of two arms:

- Check all potential 2nd structures of the annealing part and identify their melting temperatures (T_m). An useful online tool can be found at: https://www.idtdna.com/UNAFold?
- Choose a temperature (**T***) that is slightly above the highest T_m of the lowest temperature 2nd structures but well below the T_m of the full annealing.
- Run an additional "**2nd structure fixing cycle**" as follows: **T*** (45 °C, 30 min), then slowly cool down to 20 °C at a rate of -0.5 °C per 10 s.

Using this simple method, one can readily increase the annealing efficiency. Shown on the right are examples of applying this method in producing 2.2-kb Y-arms. One T* incubation step increased the annealing efficiency from > 30% to > 60% (2.2 kb Y-arms, see top band; the lower band indicates two unannealed arms).