Oct 12, 2025

Guide Cloning

In 1 collection

DOI
https://dx.doi.org/10.17504/protocols.io.3byl46ddjgo5/v1

Nahum Smith[1]

[1]University of Washington

Nahum Smith
University of Washington

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.3byl46ddjgo5/v1

Protocol Citation: Nahum Smith 2025. Guide Cloning. **protocols.io** https://dx.doi.org/10.17504/protocols.io.3byl46ddjgo5/v1

Protocol status: Working
We use this protocol and it's working

Created: October 11, 2025

Last Modified: October 12, 2025

Protocol Integer ID: 229589

Keywords: guide cloning this protocol detail, guide cloning, cloning, protocol detail, protocol

Abstract

This protocol details guide cloning.

Materials

1. T4 DNA Ligase Buffer
2. T4 Polynucleotide Kinase (PNK)
3. Water
4. Oligos
5. BbsI-HF
6. PX45-sfGFP (50-100 ng/uL)

Phosphoannealing Oligo Duplexes

1h

1 Thaw deep well plates of paired oligos in a centrifuge pre-cooled at **4C at 500g for 20 minutes**.
If oligos are in separate tubes, pair Watson (W) and Crick (C) oligo tubes.

	A	B	C
	Reagent	1X (10 uL reaction)	100X (for full 96-well plate)
	T4 DNA Ligase Buffer	1uL	100 uL
	T4 Polynucleotide Kinase (PNK)	0.5 uL	50 uL
	Water	6.5 uL	650 uL
	Subtotal	8 uL	800 uL
	Oligos	1uL of each oligo 2uL if paired in well	X

1.1 Prepare phosphoannealing master mix on ice
Thaw T4 ligase buffer on ice
Do not use T4 **PNK** Buffer, use T4 **Ligase** buffer
Do not use T4 **Ligase** enzyme, use T4 **PNK**
Keep T4 PNK at -20C in a cool block
Add buffer first, then enzyme, then mix with water volume

2 Dispense 8 uL of mix to each well of a 96-well plate or sets of 8-strip tubes

3 Transfer 2 uL of 100uM paired oligos into each well, or 1 uL each of 100uM oligos from individual W and C tubes

4 Seal with foil seal and briefly spin down

5 Use **Phosphoannealing Oligos** protocol on Eppendorf thermal cyclers

1h

	A	B	C
	37C 30min	95C 5min	Ramp to 25C, -5C per min

6 Dilute 1 uL of phosphoannealing reaction into 199 uL water in a new 96-well plate, mixing thoroughly

Goldengate Diluted Oligo Duplexes into PX45-sfGFP

6.1 Prepare Goldengate master mix on ice

	A	B	C
	Reagent	1X (10 uL reaction)	100X
	T4 DNA Ligase Buffer	1 uL	100 uL
	T4 DNA Ligase	0.5 uL	50 uL
	BbsI-HF	0.5 uL	50 uL
	PX45-sfGFP		
	(50-100 ng/uL)	1 uL	100 uL
	Water	6 uL	600 uL
	Subtotal	9 uL	900 uL
	1:200 diluted oligos	1 uL	X

Thaw T4 ligase buffer on ice
Do not use T4 PNK enzyme, use T4 Ligase enzyme
Keep T4 ligase and BbsI-HF at -20C in a cool block
Add buffer first, then enzymes, then plasmid, then mix with water volume

7 Dispense 9 uL of Goldengate master mix to a new 96-well plate

8 Transfer 1 uL of diluted oligo duplexes to each well and mix thoroughly

9 Seal with foil seal and briefly spin down

10 Use BbsI T4lig GG protocol on Eppendorf thermal cyclers

	A	B	C	D	E
	50X		1X	1X	Hold
	37 C (cut)	16 C (ligate)	50 C (extra cut)	80 C (inactivate)	10 C
	1:00 min	1:00 min	30:00 min	5:00 min	Hold

37 C (cut) for 1:00 min, 16 C (ligate) for 1:00 min, 50 C (extra cut) for 30:00 min, 80 C (inactivate) for 5:00 min, then hold at 10 C.

Transformation of Stellar Competent E. coli

11 Thaw [# of samples / 10] vials of Stellar Competent E. Coli on ice for 10 minutes
1 vial contains slightly over 100 uL of cells

11.1 Preheat thermal cycler to 42 C
Preheat LB + Amp agar plates in 37 C incubator
Preheat LB to 37 C (optional)

12 Dispense 10 uL of cells to each well of a new 96-well plate

13 Transfer 1 uL of each goldengate mix to each well of cells
Do not mix by pipetting, but ensure that the mix has been added to the middle of the cells, gently tap the side of the plate to mix

14 Seal with fold seal, do not spin down

15 Incubate cells on ice for additional 5 minutes

16 Heat-shock plate on thermal cycler with lid-open for 30 seconds

17 Incubate cells on ice for additional 2 minutes

18 Add 40 uL LB (20-37 C) to each well and mix

19 Add 45 uL LB to 2 new 96-well plates

20 Transfer 5 uL LB + Cells to dilution plate 1 and mix

21 Transfer 5 uL diluted LB + Cells from dilution plate 1 to dilution plate 2 and mix

22 Plate 5 uL of each well from dilution plate 2 on a new rectangular agar plate, or plate 50 uL on separate 10 cm agar plates
Allow rectangular agar plate to dry underneath open flame until liquid has absorbed into agar
Glass beads can spread media on 10 cm agar plates with no need for drying

23 Plate 2 uL of each well from dilution plate 2 on a second new rectangular agar plate

24 Grow overnight in 37 C incubator

Colony Selection and Stock Generation

25 Prepare a deep well plate for overnight growth
Mix 50 mL Terrific Broth with 50 uL Carbenicillin
Dispense 500 uL to each well of a new deep-well plate

26 Place agar plate with appropriate cell density on blue light block

27 Use pipette tip to pick non-GFP colonies from plate
Drop tip in corresponding well of media-filled deep-well plate
Colonies can also be picked and dropped immediately into 60 mL of Terrific Broth + 60 uL of Carbenicillin for overnight incubation if a Maxi-Prep is intended the following day.

28 Grow overnight at 37 C

29 The following day, prepare glycerol stocks
Mix 500uL of 50% glycerol in water with each well in deep well plate
Freeze glycerol stocks at -80 C. Glycerol stocks can be scraped with a pipette tip and dropped into 60 mL of Terrific Broth + 60 uL of Carbenicillin for overnight growth.

Plasmid Preparation and Sequence Verification

30 Centrifuge 50 mL of overnight growth at 3000 - 4000 rc f for 20 - 30 minutes
Media should be clear and pellet should be large at bottom of conical tube

31 Perform Zymo Maxi-Prep according to the manufacturer protocol
Elute with 400 uL 50 C water, not elution buffer
Proceed with endotoxin removal

32 Quant Plasmid Prep with Qubit Broad Range kit

33 Send approx 200 ng in 10 uL volume + 5 uL 5 uM U6 primer (5' GACTATCATATGCTTACCGT 3') to Genewiz (or equivalent Sanger sequencing service) for sequence verification
Store the prepared plasmid in the ‘awaiting confirmation’ sgRNA box.

34 The next day, upload sequences to Benchling

35 Use Benchling “Attach Primers” feature to automatically tag Watson strand sgRNA oligo sequences.
If the Sanger trace is poor quality, resequence
If there are visible BbsI cut sites in the sequence, dispose of the plasmid immediately
If there are no visible BbsI cut sites in the sequence, but the sequence has not been autotagged by the Attach Primers feature, please confirm the sequence by looking at other documents and spreadsheets and create a new Primer or Annotation for the correct guide sequence.
Only print a detailed label that references target and concentration if
Sequence confirmed
Acceptable concentration for transfection (typically 3e500 ng / uL)