Pooled Cloning of Perturb-seq libraries with pPS

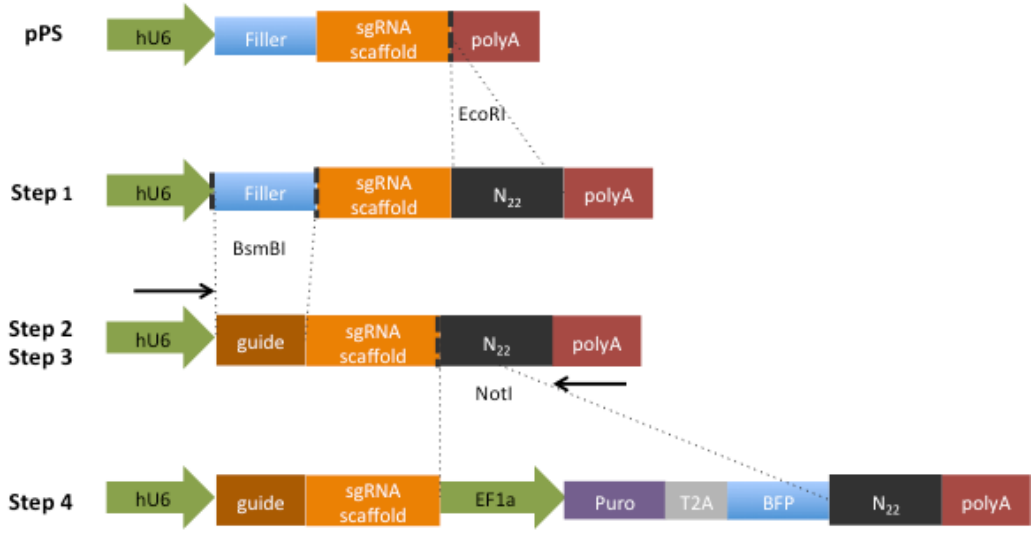
Perturb-seq leverages single-cell RNA sequencing to deeply profile the effects of many perturbations in parallel. This protocol describes how to clone pools of sgRNAs and guide barcodes (GBC) into pPS.

For more information see:
Dixit A, Parnas O *et al.* (2016). **Perturb-Seq: Dissecting Molecular Circuits with Scalable Single-Cell RNA Profiling of Pooled Genetic Screens**. *Cell* 167(7): 1853–1866.

Adamson B, Norman TM et al. (2016). **A Multiplexed Single-Cell CRISPR Screening Platform Enables Systematic Dissection of the Unfolded Protein Response**. *Cell* 167(7): 1867–1882.

Computational methods associated with pairing guide barcodes (GBC) and sgRNAs as well as GBC/cell barcode (CBC) pairing are provided here:
https://github.com/asncd/MIMOSCA/

Cloning Strategy



Reagents

Name	Description	Note
pPS	Backbone for pooled cloning of Perturb-seq libraries	Addgene # 85801
pBA439	Perturb-seq vector backbone	Addgene # 85967
sgRNA Oligo Pool	TATCTTGTGGAAAGGACGAAACACCG NNNNNNNNNNNNNNNNNNN GTTTAAGAGCTATGCTGGAAACAGCATAG	Ns denote guide sequences
GBC_Oligo	TTAAACGGGCCCTCTAGG NNNNNNNNNNNNNNNNNNNNN CAATTCGCCAGGGTTTTCCC	Ns denote random bases
GBG_Amp_F	GCTGATCAGCGGGTTTAAACGGGCCCTCTAGG	
GBG_Amp_R	CGCGTCGTGACTGGGAAAACCCTGGCGAATTG	
GuidePoolAmp_Fwd	GGCTTTATATATCTTGTGGAAAGGACGAAACACCG	
GuidePoolAmp_Rev	CTTATTTAAACTTGCTATGCTGTTTCCAGCATAGCTCTTAAAC	
IlluminaPoolSeq_Fwd	AATGATACGGCGACCACCGAGATCTACA NNNN CGATTTCTTGGCTTTATATATCTTGTGG	Ns denote sequencing barcode
IlluminaPoolSeq_Rev	CAAGCAGAAGACGGCATACGAGAT NNNNNNNN ACAGTCGAGGCTGATCAGC	Ns denote sequencing barcode
CassetteAmp_Fwd	TCGCCAGGGTTTTCCCAGTCACGACGCTTAATTAAGCTTGTGCCCCAGT	
CassetteAmp_Rev	TTGGGCTGGCAAGCCACGTTTGGTGGCGTGCCCGTCAGTGGG	
CustomRead1 Primer	CGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG	
CustomRead2 Primer	GCTGATCAGCGGGTTTAAACGGGCCCTCTAGG	
CustomIndex Primer	CCCGTTTAAACCCGCTGATCAGCCTCGACTGT	

Step 1. Introducing Guide Barcode Pool into pPS

A. Prepare Backbone

1. Digest pPS with EcoRI.
2. Purify digested backbone with 0.75X AMPure XP SPRI.

B. Prepare Insert.

1. Amplify GBC by PCR:

PCR Reaction

	Volume (µL)
Q5 2X Master Mix	25
GBC_Amp_F @ 20µM	2.5
GBC_Amp_R @ 20µM	2.5
GBC_Oligo @ 5 nM	2.5
Water	17.5
Total	**50**

PCR Cycling Conditions

Temp (°C)	**Time (s)**	
98	30	
98	15	4 Cycles
66	15	
72	15	
98	15	20 Cycles
72	20	
72	120	
4	∞	

2. Verify 86 bp band on a gel.
3. Purify insert with 2X AMPure XP SPRI.

C. Assemble and Transform pPS-GBC

1. Gibson Assemble for 1 hour at 50°C:

Gibson Reaction

	Amount
NEB Gibson Assembly 2X Master Mix	10 µL
Digested pPS	500 ng
Amplified GBC Library	70 ng
Water	Fill to 20 µL
Total	**20 µL**

2. Purify assembled plasmid with 0.75X AMPure XP SPRI. Elute in 15 µL.
3. Electroporate entire volume into Lucigen Endura Competent Cells.
4. Estimate the number of transformed colonies by plating a serial dilution of transformation mixture as described in Wang, Lander, and Sabatini, 2016. *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot090803.
5. Expand the cells in LB with carbenicillin for 18 hours at 30°C.
6. If the number of transformed colonies exceeds 500x the desired number sgRNA-GBC pairs (see Step 2-C5), purify pPS-GBC pool with mini-,midi-, or maxi-prep kit.

Step 2. Cloning sgRNA Library into pPS-GBC

A. Prepare backbone

1. Digest pPS-GBC with BsmBI.
2. Purify digested backbone with 0.75X AMPure XP SPRI.

B. Prepare Insert.

1. Amplify sgRNA library by PCR:

PCR Reaction

	Volume (µL)
NEBNext 2X Master Mix	25
GuidePoolAmp_Fwd @ 5µM	5
GuidePoolAmp_Rev @ 5µM	5
Oligo Pool (1 ng/µL)	1
Water	14
Total	**50**

PCR Cycling Conditions

Temp (°C)	**Time (s)**	
98	30	
98	15	4 Cycles
68	15	
72	15	
98	15	12 Cycles
72	20	
72	120	
4	∞	

2. Verify 97 bp band on a gel.
3. Purify amplified sgRNA pool with 2X AMPure XP SPRI.

C. Assemble and Transform pPS-sgRNA-GBC

1. Gibson Assemble for 1 hour at 50°C:

Gibson Reaction

	Amount
Gibson Assembly 2X Master Mix	10 µL
Digested pPS-GBC	500 ng
Amplified sgRNA Library	70 ng
Water	Fill to 20 µL
Total	**20 µL**

2. Purify assembled plasmid with 0.75X AMPure XP SPRI. Elute in 15 µL.
3. Electroporate entire volume into Lucigen Endura Competent Cells.
4. Estimate the number of transformed colonies by plating a serial dilution of transformation mixture as described in Wang, Lander, and Sabatini, 2016. *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot090803.

Note: To reduce sequencing required in Step 3, it may be advantageous to limit the number of colonies transformed to 1% of the expected sequencing reads (e.g. for 18 million reads expected from one run of a MiSeq, limit transformation to 180 thousand colonies). This is the number of of sgRNA-GBC pairs.

5. Expand the cells in LB with carbenicillin for 18 hours at 30°C.
6. If the number of transformed colonies is appropriate, purify pPS-sgRNA-GBC pool using a mini-,midi-, or maxi-prep kit.

Step 3. Next Generation Sequencing to Create sgRNA/GBC Dictionary

A. Prepare Sequencing Library

1. Amplify pPS-sgRNA-GBC pool by PCR:

PCR Reaction

	Volume (µL)
Q5 2X Master Mix	10
IlluminaPoolSeq_Fwd @ 5µM	2
IlluminaPoolSeq_Rev @ 5µM	2
pPS-sgRNA-GBC pool (1 ng/uL)	1
Water	5
Total	**20**

PCR Cycling Conditions

Temp (°C)	Time (s)	
98	30	
98	15	4 Cycles
64	15	
72	15	
98	15	22 Cycles
72	20	
72	120	
4	∞	

2. Gel purify 405bp band.

B. Sequence on MiSeq

Sequence R1: 21 bases, R2: 22 bases, I1: 8 bases, I2: 4 bases using custom R1, R2, and I1 primers. R1 corresponds to the guide sequence and R2 corresponds to the GBC.

Step 4. Insertion of the EF1α-Puro-T2A-BFP Cassette

A. Prepare backbone

1. Digest pPS-sgRNA-GBC Pool with NotI.
2. Purify digested backbone with 0.75X AMPure XP SPRI.

B. Prepare Insert.

1. Amplify the EF1α-Puro-T2A-BFP Cassette from the Perturb-seq vector backbone pBA439 (Addgene #85967) by PCR:

PCR Reaction

	Volume (µL)
Q5 2X Master Mix	25
CassetteAmp_Fwd @ 5µM	5
CassetteAmp_Rev @ 5µM	5
pBA439 (1 ng/uL)	1
Water	14
Total	**50**

PCR Cycling Conditions

Temp (°C)	**Time (s)**	
98	30	
98	15	4 Cycles
66	15	
72	60	
98	15	20 Cycles
72	60	
72	120	
4	∞	

2. Gel purify 2.6 kb band.
3. Purify insert with 0.75X AMPure XP SPRI.

C. Assemble and Transform pPS-sgRNA-Puro-BFP-GBC Pool

1. Gibson Assemble for 1 hour at 50°C:

Gibson Reaction

	Amount
Gibson Assembly 2X Master Mix	10 µL
Digested pPS-sgRNA-GBC Pool	400 ng
EF1α-Puro-T2A-BFP Cassette	150 ng
Water	Fill to 20 µL
Total	**20 µL**

2. Purify assembled plasmid with 0.75X AMPure XP SPRI. Elute in 15 µL.
3. Electroporate entire volume into Lucigen Endura Competent Cells.
4. Estimate the number of transformed colonies by plating a serial dilution of transformation mixture as described in Wang, Lander, and Sabatini, 2016. *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot090803.
5. Expand the cells in LB with carbenicillin for 18 hours at 30°C.
6. If the number of colonies exceeds 50x the number of sgRNA-GBC pairs, purify pPS-sgRNA-Puro-BFP-GBC pool using an endotoxin-free maxiprep kit.