Aug 22, 2024 Version 3

Automated Bar-Seq Library Preparation and Pooling V.3

DOI
dx.doi.org/10.17504/protocols.io.3byl49qdjgo5/v3

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Pooled, Growth-Based As…

Open Datasets Initiative
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DOI: dx.doi.org/10.17504/protocols.io.3byl49qdjgo5/v3

Protocol Citation: David Ross, Nina Alperovich 2024. Automated Bar-Seq Library Preparation and Pooling. **protocols.io** https://dx.doi.org/10.17504/protocols.io.3byl49qdjgo5/v3Version created by Open Datasets Initiative

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

Created: May 24, 2024

Last Modified: August 22, 2024

Protocol Integer ID: 106074

Disclaimer

The protocol outlined in this document was created as a part of the *Pooled, Growth-Based Assays for Protein Function Measurements* pipeline for Align to Innovate's Open Dataset Initiative. Align to Innovate is a non-profit research organization operating under open science principles with the goal of improving science research with programmable experiments. The Open Datasets Initiative is working to accelerate community-driven science with the use of automated labs to pioneer robust data collection methods and curated, high-fidelity, public biological datasets amenable to machine learning. This work was supported by Align to Innovate's Open Datasets Initiative which receives philanthropic funding in part from Griffin Catalyst.

Abstract

Protocol for automated Bar-Seq Library preparation

This protocol prepares 96 DNA samples, representing 24 samples from 4 different timepoints, for multiplexed Illumina sequencing. The process starts with two rounds of PCR, each followed by a bead-based cleanup. The first round of PCR attaches primers that serve as tags to identify the timepoint and sample (e.g., time-points 1-4 in the Pooled, Growth-Based Assay Protocol). The second round of PCR attaches flow-cell adapters required for Illumina sequencing. Following the PCR and cleanup steps, the protocol outlines a procedure for pooling all samples together to ensure balanced representation during sequencing. This process starts with quantifying each sample's DNA concentration and then diluting and pooling together samples from the same timepoint. The pooled samples' concentrations are then measured, and a small quantity of each timepoint sample is run on a gel to ensure the cleanup process was successful. The final step is to dilute and pool of all timepoint samples into one multiplexed tube for subsequent Illumina sequencing.

NOTES:

- Before implementing this protocol, prepare the magnetic bead suspension following the **Preparation of Sera-mag SpeedBeads protocol.**
- This protocol should be implemented after the **Automation Protocol for Plasmid DNA Extraction from E. coli** protocol
- A fragment analyzer can also be used instead of a gel to determine if the PCR clean-up process was a success

Materials

Starting samples:

- 96 DNA samples resulting from the Automation Protocol for Plasmid DNA Extraction from *E. coli* protocol

Reagents:

- Nuclease-free water (ThermoFisher Scientific 4387936)
- 80% Absolute Ethanol (Fisher Bioreagents BP2818500)
- Elution Buffer (Qiagen 19086)
- Phusion Flash PCR Mastermix (ThermoFisher Scientific F548L)
- Multiplexing Primers
- Universal Illumina Primers

Labware:

- Three 96-well DeepWell reagent plate (Abgene AB-0765) - one used as a reagent plate and two used as midi plates
- Two 96-well PCR plates (Bio-Rad HSP9635 or HSP9645)
- Three PCR plate lids (Agilent 202497-100)
- 96-well output plate (Eppendorf 30603303)

Primers:

	A	B	C
	Primer name	Description	Sequence
	BarSeq_1_F1	forward barSeq PCR 1 primer, with sample multiplex tag: CG TGTATCTT, row A	ACACTCTTTCCCTACACGACGCTCTTCCG ATCTNNNNNNNNCGTGTATCTTGGCCTAG ACGTGTGATAGACTCAGTCG
	BarSeq_1_F2	forward barSeq PCR 1 primer, with sample multiplex tag: AT TCATTGCA, row B	ACACTCTTTCCCTACACGACGCTCTTCCG ATCTNNNNNNNNATTCATTGCAGGCCTAG ACGTGTGATAGACTCAGTCG
	BarSeq_1_F3	forward barSeq PCR 1 primer, with sample multiplex tag: TC CTTCATAG, row C	ACACTCTTTCCCTACACGACGCTCTTCCG ATCTNNNNNNNNTCCTTCATAGGGCCTAG ACGTGTGATAGACTCAGTCG
	BarSeq_1_F4	forward barSeq PCR 1 primer, with sample multiplex tag: GA ATGCACGA, row D	ACACTCTTTCCCTACACGACGCTCTTCCG ATCTNNNNNNNNGAATGCACGAGGCCTA GACGTGTGATAGACTCAGTCG
	BarSeq_1_F5	forward barSeq PCR 1 primer, with sample multiplex tag: GG AATTGTTC, row E	ACACTCTTTCCCTACACGACGCTCTTCCG ATCTNNNNNNNNGGAATTGTTCGGCCTAG ACGTGTGATAGACTCAGTCG
	BarSeq_1_F6	forward barSeq PCR 1 primer, with sample multiplex tag: CC GGACCACA, row F	ACACTCTTTCCCTACACGACGCTCTTCCG ATCTNNNNNNNNCCGGACCACAGGCCTA GACGTGTGATAGACTCAGTCG
	BarSeq_1_F7	forward barSeq PCR 1 primer, with sample multiplex tag: GA CTTAGAAG, row G	ACACTCTTTCCCTACACGACGCTCTTCCG ATCTNNNNNNNNGACTTAGAAGGGCCTA GACGTGTGATAGACTCAGTCG
	BarSeq_1_F8	forward barSeq PCR 1 primer, with sample multiplex tag: TC TAGTCTTC, row H	ACACTCTTTCCCTACACGACGCTCTTCCG ATCTNNNNNNNNTCTAGTCTTCGGCCTAG ACGTGTGATAGACTCAGTCG

	A	B	C
	BarSeq_1_R1	reverse barSeq PCR 1 primer, with sample multiplex tag: TA GAGTTGGA, column 1	CTCGGCATTCCTGCTGAACCGCTCTTCCG ATCTNNNNNNNNTAGAGTTGGATCGCAGA CGTTTTGCAGACTCCTG
	BarSeq_1_R2	reverse barSeq PCR 1 primer, with sample multiplex tag: AG AGCACTAG, column 2	CTCGGCATTCCTGCTGAACCGCTCTTCCG ATCTNNNNNNNNAGAGCACTAGTCGCAG ACGTTTTGCAGACTCCTG
	BarSeq_1_R3	reverse barSeq PCR 1 primer, with sample multiplex tag: AC TCTACAGG, column 3	CTCGGCATTCCTGCTGAACCGCTCTTCCG ATCTNNNNNNNNACTCTACAGGTCGCAG ACGTTTTGCAGACTCCTG
	BarSeq_1_R4	reverse barSeq PCR 1 primer, with sample multiplex tag: CG GTGACACC, column 4	CTCGGCATTCCTGCTGAACCGCTCTTCCG ATCTNNNNNNNNCGGTGACACCTCGCAG ACGTTTTGCAGACTCCTG
	BarSeq_1_R5	reverse barSeq PCR 1 primer, with sample multiplex tag: GC GTTGGTAT, column 5	CTCGGCATTCCTGCTGAACCGCTCTTCCG ATCTNNNNNNNNGCGTTGGTATTCGCAGA CGTTTTGCAGACTCCTG
	BarSeq_1_R6	reverse barSeq PCR 1 primer, with sample multiplex tag: TG TGCTAACA, column 6	CTCGGCATTCCTGCTGAACCGCTCTTCCG ATCTNNNNNNNNTGTGCTAACATCGCAGA CGTTTTGCAGACTCCTG
	BarSeq_1_R7	reverse barSeq PCR 1 primer, with sample multiplex tag: CC AGAAGTAA, column 7	CTCGGCATTCCTGCTGAACCGCTCTTCCG ATCTNNNNNNNNCCAGAAGTAATCGCAG ACGTTTTGCAGACTCCTG
	BarSeq_1_R8	reverse barSeq PCR 1 primer, with sample multiplex tag: CT TATACCTG, column 8	CTCGGCATTCCTGCTGAACCGCTCTTCCG ATCTNNNNNNNNCTTATACCTGTCGCAGA CGTTTTGCAGACTCCTG
	BarSeq_1_R9	reverse barSeq PCR 1 primer, with sample multiplex tag: AC TAGAACTT, column 9	CTCGGCATTCCTGCTGAACCGCTCTTCCG ATCTNNNNNNNNACTAGAACTTTCGCAGA CGTTTTGCAGACTCCTG
	BarSeq_1_R10	reverse barSeq PCR 1 primer, with sample multiplex tag: TT AGGCTTAC, column 10	CTCGGCATTCCTGCTGAACCGCTCTTCCG ATCTNNNNNNNNTTAGGCTTACTCGCAGA CGTTTTGCAGACTCCTG
	BarSeq_1_R11	reverse barSeq PCR 1 primer, with sample multiplex tag: TA TCATGAGA, column 11	CTCGGCATTCCTGCTGAACCGCTCTTCCG ATCTNNNNNNNNTATCATGAGATCGCAGA CGTTTTGCAGACTCCTG
	BarSeq_1_R12	reverse barSeq PCR 1 primer, with sample multiplex tag: CT CACACAAG, column 12	CTCGGCATTCCTGCTGAACCGCTCTTCCG ATCTNNNNNNNNCTCACACAAGTCGCAG ACGTTTTGCAGACTCCTG
	BarSeq_2_F	forward barSeq PCR 2 primer	AATGATACGGCGACCACCGAGATCTACAC TCTTTCCCTACACGACGCTCTTCCGATCT
	BarSeq_2_R	reverse barSeq PCR 2 primer	CAAGCAGAAGACGGCATACGAGATCGGTC TCGGCATTCCTGCTGAACCGCTCTTCCGAT CT

Transfer Plasmid DNA to PCR Plate and Dilute with DI water

1 Pre-heat the on deck thermocycler (ODTC) for 1st PCR step

2 Remove lids from PCR-Plate 1, Sample-Plate, and Reagent Plate

3 Pipette 10uL nuclease-free water to each well in PCR plate 1

4 Transfer 35uL of extracted plasmid DNA to each well of PCR Plate 1
- This step is done 24 samples (one time point) at a time, with user instructions to load each plasmid sample input plate (without lids)
- aspirate 1 mm below liquid surface, with liquid following ON
- dispense 0.5 mm below liquid surface, with liquid following ON

5 User instruction to remove plasmid sample input Sample-Plate

Run First PCR - using primers to identify samples from each timepoint

6 Add 28.125 uL Master Mix with reverse primer for the appropriate timepoint and sample to each corresponding well of PCR plate 1
- dispense 0.5 mm below liquid surface, with liquid following On
- no mixing

7 Add 28.125 uL Master Mix with forward primer for the appropriate timepoint and sample to each corresponding well of PCR plate 1
- Mix 10x after dispensing
- dispense 0.5 mm below liquid surface, with liquid following On
- mix volume 70 uL
- mixing speed 75 uL/s
- mix position 1 mm (below dispense position)

8 Put a PCR plate lid 1 on the PCR plate 1.

9 Move PCR-Plate 1 to ODTC

10 Run First PCR Rx using conditions (101.25 uL volume) using the following cycles:

1. 98 C for 60 s
2. 3 cycles at:
   - 98 C for 10 s
   - 58 C for 20 s
   - 72 C for 20 s
3. 72 C for 60 s
4. 23 C for 10 s

First PCR Cleanup Part 1: Bind template plasmid DNA to the beads and keep the supernatant

11 Pipette 54 uL magnetic bead suspension into each well of Midi plate 1
- Bead ratio: 0.6x
- Mix the bead suspension 10x before pipetting
- Get new tips for each well
- Mix bead suspension again 2x before pipetting each well
- Pipette bead suspension into Midi plate during the Step 1 PCR

12 Move PCR plate 1 from ODTC; take lid off of PCR plate 1

13 Close ODTC; run PCR pre-heat method on ODTC (to get ready for Step 2 PCR)

14 Shake Midi plate 1 with bead suspension for 10 second at 1800 RMP

15 Transfer 90 uL of each sample from PCR plate 1 to Midi plate 1

15.1 Mix 10x after dispensing
- dispense 0.5 mm above bottom of well
- mix volume 108 uL
- mixing speed 100 uL/s
- mix position 2 mm (below liquid surface)
- liquid following ON

16 Incubate for 7 minutes at room temperature

17 Move Midi plate 1 to magnet base

18 Wait for 4 minutes

19 During 4 minute wait, move Midi Plate No. 2 to Hamilton Heater Shaker (HHS).

20 Transfer 134 uL of supernatant from Midi plate 1 to Midi plate 2

First PCR Cleanup Part 2: bind PCR product to beads and discard the supernatant

21 Pipette 80.4 uL bead suspension to Midi plate 2 (on HHS)
- Bead ratio: 0.6

21.1 Mix the bead suspension 10x before pipetting

21.2 Get new tips for each well and mix bead suspension again 2x before aspiration for each well

21.3 Mix 10x after dispensing
- dispense 0 mm below liquid level
- 10 mix cycles
- mix volume 193 uL
- mixing speed: 100 uL/s
- mix position 1 mm (below liquid surface)
- liquid following ON

22 Incubate for 7 minutes at room temp, without shaking

23 During 7 minute wait, throw Midi Plate No. 1 to waste

24 Move Midi plate 2 to magnet base

25 Wait for 5 minutes

26 Remove and discard most of the supernatant (209.4 uL)

27 Pipette 200 uL 80% ethanol into Midi plate 2 (on magnet)
- Jet dispense, from 20 mm height, using one tip for each row

28 Remove ethanol supernatant and discard

29 Using smaller tips (e.g., 50 uL), remove remaining supernatant and dispose

30 Move Midi plate 2 to HHS

31 Wait 30 s to allow ethanol to finish drying

32 Pipette 55 uL DI water into Midi plate 2

32.1 Mix 2x after dispensing
- dispense 1 mm above bottom of well
- mix volume 41.25 uL
- mixing speed 100 uL/s
- mix position 0 mm
- liquid following OFF

33 Shake at 1800 RPM for 10 s

34 Shake at 400 RPM for 2 minutes

35 Incubate for 3 minutes at room temp without shaking

36 Move Midi plate 2 to magnet base

37 Start timer for 5 min incubation

37.1 While waiting, start next step by preparing the 2nd PCR reaction

Run 2nd PCR: using primers for Illumina multiplexing

38 Prepare PCR plate 2 by pipetting 54 uL of 2x master mix (containing both forward and reverse universal primers for Illumina sequencing) into each well

38.1 Wait for 5 minute timer from the 1st PCR cleanup to finish.

39 Transfer 45 uL of supernatant from Midi plate 2 to PCR plate 2

39.1 Mix 10x after dispensing
- dispense 0.5 mm below liquid surface
- mix volume 69.3 uL
- mixing speed 100 uL/s
- mix position 1 mm (lower than dispense position)
- liquid following ON

40 Put a PCR plate lid on PCR plate 2.

41 Move PCR-Plate 2 to ODTC

42 Run PCR plate 2 (99 µL) using conditions:
1. 98 C for 60 s
2. 15 Cycles at:
   - 98 C for 10 s
   - 72 C for 30 s
3. 72 C for 120 s
4. 10 C for 20 s

Note: during the 2nd PCR run, throw Midi plate 2 to waste and prepare Midi plate 3 following the instructions below.

Second PCR cleanup: Bind PCR product to beads and discard supernatant

43 Move Midi plate 3 to HHS (while 2nd PCR is running)

44 Pipette 89 uL bead suspension to Midi plate 3
- Bead ratio 1.0x

44.1 Mix the bead suspension 10x before pipetting

44.2 Get new tip for each well & mix beads suspension again 2x before pipetting each well

45 Wait for PCR to finish

46 Move PCR plate 2 from ODTC; take lid off of PCR plate 2

47 Shake Midi plate 3 with bead suspension for 10 second at 1800 RMP

48 Transfer 89 uL from PCR plate 2 to Midi plate 3

48.1 Aspirate 0.5 mm above bottom of well with liquid following OFF

48.2 Mix 10x after dispensing
- dispense 0.5 mm above bottom of well
- mix volume 133.5 uL
- mixing speed 100 uL/s
- mix position 2 mm (below dispense position)
- liquid following ON

49 Incubate for 7 minutes

50 Move Midi plate 3 to magnet base

51 Wait for 5 minutes

52 Remove and discard most of the supernatant (173 uL)

53 Pipette 200 uL 80% ethanol into Midi plate 4
- jet dispense, from 20 mm height, using one tip for each row

54 Wait 30 s

55 Remove ethanol supernatant and discard

56 Using smaller tips (e.g., 50 uL), remove remaining supernatant and dispose

57 Move Midi plate 3 to HHS

58 Wait 30 s to allow ethanol to finish drying

59 Pipette 50 uL Elution buffer into Midi plate 3

59.1 Mix 2x after dispensing
- dispense 1 mm above bottom of well
- mix volume 37.5 uL
- mix position 0 mm
- liquid following OFF

60 Shake at 1800 RPM for 10 s

61 Shake at 400 RPM for 2 minutes

62 Incubate for 3 minutes without shaking

63 Move Midi plate 3 to magnet base

64 Wait for 5 minutes
- Remove lid from Output plate during wait

65 Transfer 45 uL supernatant from Midi plate 3 to Output plate

66 Replace lid on Output plate

Quantify the amount of input plasmid DNA

67 Measure the DNA concentrations (C_n) for all input plasmid samples using Qubit or an equivalent DNA quantitative measurement.
- Note: The expected concentration ($C_{n's}$) for samples grown with zero selection antibiotic is typically around 0.5 ng/uL to 1 ng/uL, though samples from cultures grown with non-zero selection antibiotic may end up lower than that.

Run a gel for each sample from time point 1

68 Run a gel for each sample from time point 1, using the amplified product DNA for each, and compare the results with those obtained during BarSeq method development.
- The gels should not have any visible bands that are shorter than the expected amplicon size (315 bp)
- Shorter bands indicate incomplete PCR cleanup that could result in a very low sequence count from Illumina sequencing.
- If the gels results look correct, proceed to the next step

Quantify the amount of amplified DNA

69 The amount of amplified DNA should also be measured for each sample at each time point using Qubit or an equivalent DNA quantitative measurement.
- Expected yields are between 10 ng/uL and 30 ng/uL (in 45 uL), though some samples could have as little as 1 ng/uL (e.g., samples grown with the highest antibiotic concentration at the later time points).

Within each time point's samples, calculate the samples' final pooling volumes (Vf)

70 **Within each time point's samples (i.e., the 24 samples coming from each growth plate),** identify the the four lowest-concentration samples.

71 Calculate the mean of their concentrations in ng/µL, and define this concentration as the low concentration limit (C_{low}) for that time point.

72 For each sample, use the DNA concentration in ng/µL (C_n) and the following equation to calculate the pooling volume (V_n) for each sample:

- $V_n = C_{low}/(C_n + 0.1\ ng/uL)*15\ µL$

73 For each sample, set the final pooling volume (V_f) based on the following conditions:

- If $V_n < 4$ µL, set $V_f = 4$ µL
- If $V_n > 15$ µL, set $V_f = 15$ µL.

Within each time point, pool all samples and measure the pooled-DNA concentration

74 **Within each time point's samples (i.e., the 24 samples coming each growth plate),** pool together all samples into one container according to their final pooling volumes (V_f's) and store the remaining un-pooled samples at -20C.

- Note: This will give one pooled sample for each time-point (four pooled samples in total), each at a concentration of approximately 20 ng/uL and with a volume of 100 to 300 uL. This is much more DNA than is needed for sequencing.

Run a gel for each time point

75 Run a gel for each time point, using the pooled product DNA for each, and compare the results with those obtained during BarSeq method development.

- The gels should not have any visible bands that are shorter than the expected amplicon size (315 bp)
- Shorter bands indicate incomplete PCR cleanup that could result in a very low sequence count from Illumina sequencing.
- If the gels results look correct, proceed to the next step

Within each time point, pool all samples and measure the pooled-DNA concentration

76 For each of the four pooled samples, re-measure the DNA concentration and then store them at -20C until the next step can be accomplished.

Dilute each time point's sample and pool all samples for sequencing

77 To prepare samples for the sequencing provider, follow their instructions for **pre-made Illumina libraries.**

- Note: They usually give the sample requirements in molar concentration, typically 5 nmol/L, which is about 1 ng/uL for the BarSeq libraries. They usually require a minimum of 20 uL to 30 uL per lane of sequencing.

- As an example, using the numbers above, a sample protocol might be to dilute 5 µL of each pooled sample into nuclease-free water to a concentration of 1 ng/uL. This will give much more than the minimum required volume, but it avoids the extra variability from pipetting smaller volumes.

78 Combine the diluted time point samples to be run on the sequencer. In the following manner:

- For the first run: start with a single lane of NovaSeq, with all four time points combined in equal proportions. Then, after analyzing that data, if you need more data, order a second lane and send a second sample with only time points 2-4.
- For subsequent runs: order a single lane of NovaSeq with the combine the time points with the following ratios: ⅓:1:1:1 for time points 1-4 (or ratios determined from first run).

Protocol references

This protocol is based on a similar protocol first described by Tack et al., Mol Syst Biol (2021), https://doi.org/10.15252/msb.202010179