May 16, 2025

Bar-Seq Library Preparation and Pooling: Primer Preparation

DOI
https://dx.doi.org/10.17504/protocols.io.n2bvj9n1nlk5/v1

Nina Alperovich[1], David Ross[1]

[1]NIST

Open Datasets Initiative
Align to Innovate

DOI: https://dx.doi.org/10.17504/protocols.io.n2bvj9n1nlk5/v1

Protocol Citation: Nina Alperovich, David Ross 2025. Bar-Seq Library Preparation and Pooling: Primer Preparation. **protocols.io** https://dx.doi.org/10.17504/protocols.io.n2bvj9n1nlk5/v1

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

Created: December 11, 2024

Last Modified: May 16, 2025

Protocol Integer ID: 115039

Keywords: primer preparation this protocol, 1st pcr in the barseq protocol, primer preparation, primers in the 2nd pcr, primers for the 1st pcr, 1st pcr primer, barseq protocol, tags to the barseq dna amplicon, barseq dna amplicon, similar naming strategy to the 1st pcr primer, seq automation instruction, aliquots for each primer, seq library preparation, sequencing flow cell adapter, reverse primer, pooling protocol, tube of primer, full set of primer, aliquots of primer, aliquot for the next primer, primer, next primer, forward primer, primer id, corresponding primer id, 1st pcr in row, seq, label as barseq-1, barseq-1, primer stock, barseq-1-f2, 1st pcr, 2nd pcr, pcr, preparation of aliquot, contamination, barseq-2-f, goal of this protocol, multiplexing tag, amplified pcr product, protocol, sequencing, automation instruction

Disclaimer

The protocol outlined in this document was created as a part of **Gro**wth-based **Q**uantitative **Seq**uencing-GROQ-Seq) Platform. The GROQ-Seq platform was created under **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

This protocol is a rigorous way to ensure no cross-contamination between primers used in the Bar-Seq Library Preparation and Pooling protocols (see Bar-Seq **Automation Instructions** & **User Instructions**). The goal of this protocol is to minimize the opportunities for cross-contamination between the primers for the 1st PCR (which attach the sample multiplexing tags to the BarSeq DNA amplicons) as well as the 2nd PCR (which attach sequencing flow cell adapters). To this end, this protocol is designed to make it physically impossible for cross-contamination to occur before the primers are loaded onto the automation system.

The main principle is “one at a time”: **only one primer stock should ever be opened at a time,** and only one primer’s aliquots should be prepared **or manipulated at a time** (except when they are loaded into the automation system).

- Each tube of primer, after being received from the vendor, should be opened only once, to prepare aliquots of primer + Master Mix and/or aliquots of primer + water for later use.
- The aliquots for each primer should be prepared one primer at a time.
- After preparation, primer + Master Mix aliquots should be stored at -20C until use.
- After preparation, primer + water aliquots should be stored at -80C until use.
- After preparation of aliquots for each primer, the user should clean the pipettors and the work area and then take a short break before preparing the aliquot for the next primer.
- For the full set of primers, this protocol could take as long as 3 days to complete. It should not be rushed, so plan accordingly.

Primers used in the 1st PCR are mutliplexing tags that cover the range of a 96 well plate.

- There are 8 forward primers that cover the 8 rows and 12 reverse primers that cover the 12 columns. This allows us to know exactly which well each sample comes from, which corresponds to the growth plate and condition the sample was produced by in the Pooled Growth-Based Assay.
- These are label as BarSeq_1 (for the 1st PCR in the BarSeq protocol) followed by they corresponding primer ID, which is a combination of either F for forward or R for reverse plus a number for the row or column, respectively. Example: BarSeq_1_F2 is a forward primer used in the 1st PCR in row 2.

Primers in the 2nd PCR are universal tags that enable attachment to the Illumina flow cell.

- There are only 2 of these primers, one forward and one reverse, because all of the amplified PCR product needs to attach to the flow cell in the same way for sequencing.
- These primers follow a similar naming strategy to the 1st PCR primers, but since there is only 1 forward and 1 reverse primer, they have no numbers. Example: BarSeq_2_F is the forward primer used in the 2nd PCR.

Materials

Reagents and Consumables

Note: The volume of master mix and water, as well as the number of tubes required, is dependent on the amount of oligo received from the vendor for each of the primers listed in the table below. In the protocol you will use a provided excel sheet to calculate these values.

- Nuclease-free water - Thermo Scientific 4387936
- Master Mix - Thermo Scientific Phusion Flash High-Fidelity Cat. # F548
- 14 mL centrifuge tubes - Corning 352059
- 1.5 mL microcentrifuge tubes - ThermoFisher 68715

Primers used in 1st and 2nd PCRs

Note: Order all primers with PAGE purification.

- 1st PCR Primers should be ordered at 100nM (e.g. anything starting 'BarsSeq_1').
- 2nd PCR Primers should be ordered at 1µM (e.g., the two starting with 'BarSeq_2').

	A	B	C
	Primer name	Description	Sequence
	BarSeq_1_F1	forward barSeq PCR 1 primer, with sample multiplex tag: CGTGTATCTT, row A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNCGTGTATCTTGGCCTAGACGTGTGATAGACTCAGTCG
	BarSeq_1_F2	forward barSeq PCR 1 primer, with sample multiplex tag: ATTCATTGCA, row B	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNATTCATTGCAGGCCTAGACGTGTGATAGACTCAGTCG
	BarSeq_1_F3	forward barSeq PCR 1 primer, with sample multiplex tag: TCCTTCATAG, row C	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNTCCTTCATAGGGCCTAGACGTGTGATAGACTCAGTCG
	BarSeq_1_F4	forward barSeq PCR 1 primer, with sample multiplex tag: GAATGCACGA, row D	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNGAATGCACGAGGCCTAGACGTGTGATAGACTCAGTCG
	BarSeq_1_F5	forward barSeq PCR 1 primer, with sample multiplex tag: GGAATTGTTC, row E	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNGGAATTGTTCGGCCTAGACGTGTGATAGACTCAGTCG
	BarSeq_1_F6	forward barSeq PCR 1 primer, with sample multiplex tag: CCGGACCACA, row F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNCCGGACCACAGGCC

	A	B	C
			TAGACGTGTGATAGACTCA GTCG
	BarSeq_1_F7	forward barSeq PCR 1 primer, with sample multiplex tag: GACTTAGAAG, row G	ACACTCTTTCCCTACACGA CGCTCTTCCGATCTNNNN NNNNGACTTAGAAGGGCC TAGACGTGTGATAGACTCA GTCG
	BarSeq_1_F8	forward barSeq PCR 1 primer, with sample multiplex tag: TCTAGTCTTC, row H	ACACTCTTTCCCTACACGA CGCTCTTCCGATCTNNNN NNNNTCTAGTCTTCGGCC TAGACGTGTGATAGACTCA GTCG
	BarSeq_1_R1	reverse barSeq PCR 1 primer, with sample multiplex tag: TAGAGTTGGA, column 1	CTCGGCATTCCTGCTGAA CCGCTCTTCCGATCTNNN NNNNNTAGAGTTGGATCG CAGACGTTTTGCAGACTCC TG
	BarSeq_1_R2	reverse barSeq PCR 1 primer, with sample multiplex tag: AGAGCACTAG, column 2	CTCGGCATTCCTGCTGAA CCGCTCTTCCGATCTNNN NNNNNAGAGCACTAGTCG CAGACGTTTTGCAGACTCC TG
	BarSeq_1_R 3	reverse barSeq PCR 1 primer, with sample multiplex tag: ACTCTACAGG, column 3	CTCGGCATTCCTGCTGAA CCGCTCTTCCGATCTNNN NNNNNACTCTACAGGTCG CAGACGTTTTGCAGACTCC TG
	BarSeq_1_R 4	reverse barSeq PCR 1 primer, with sample multiplex tag: CGGTGACACC, column 4	CTCGGCATTCCTGCTGAA CCGCTCTTCCGATCTNNN NNNNNCGGTGACACCTCG CAGACGTTTTGCAGACTCC TG
	BarSeq_1_R5	reverse barSeq PCR 1 primer, with sample multiplex tag: GCGTTGGTAT, column 5	CTCGGCATTCCTGCTGAA CCGCTCTTCCGATCTNNN NNNNNGCGTTGGTATTCG CAGACGTTTTGCAGACTCC TG
	BarSeq_1_R 6	reverse barSeq PCR 1 primer, with sample multiplex tag: TGTGCTAACA, column 6	CTCGGCATTCCTGCTGAA CCGCTCTTCCGATCTNNN NNNNNTGTGCTAACATCG CAGACGTTTTGCAGACTCC TG
	BarSeq_1_R7	reverse barSeq PCR 1 primer, with sample multiplex tag: CCAGAAGTAA, column 7	CTCGGCATTCCTGCTGAA CCGCTCTTCCGATCTNNN NNNNNCCAGAAGTAATCG CAGACGTTTTGCAGACTCC TG
	BarSeq_1_R 8	reverse barSeq PCR 1 primer,	CTCGGCATTCCTGCTGAA CCGCTCTTCCGATCTNNN

	A	B	C
		with sample multiplex tag: CTTATACCTG, column 8	NNNNNCTTATACCTGTCG CAGACGTTTTGCAGACTCC TG
	BarSeq_1_R 9	reverse barSeq PCR 1 primer, with sample multiplex tag: ACTAGAACTT, column 9	CTCGGCATTCCTGCTGAA CCGCTCTTCCGATCTNNN NNNNNACTAGAACTTTCG CAGACGTTTTGCAGACTCC TG
	BarSeq_1_R1 0	reverse barSeq PCR 1 primer, with sample multiplex tag: TTAGGCTTAC, column 10	CTCGGCATTCCTGCTGAA CCGCTCTTCCGATCTNNN NNNNNTTAGGCTTACTCG CAGACGTTTTGCAGACTCC TG
	BarSeq_1_R1 1	reverse barSeq PCR 1 primer, with sample multiplex tag: TATCATGAGA, column 11	CTCGGCATTCCTGCTGAA CCGCTCTTCCGATCTNNN NNNNNTATCATGAGATCGC AGACGTTTTGCAGACTCCT G
	BarSeq_1_R1 2	reverse barSeq PCR 1 primer, with sample multiplex tag: CTCACACAAG, column 12	CTCGGCATTCCTGCTGAA CCGCTCTTCCGATCTNNN NNNNNCTCACACAAGTCG CAGACGTTTTGCAGACTCC TG
	BarSeq_2_F	forward barSeq PCR 2 primer	AATGATACGGCGACCACC GAGATCTACACTCTTTCCC TACACGACGCTCTTCCGAT CT
	BarSeq_2_R	reverse barSeq PCR 2 primer	CAAGCAGAAGACGGCATA CGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTC CGATCT

High-level overview

1 **Note:** All steps in this protocol should be done in a biosafety hood to avoid contamination of primers. Clean the pipettors, items, and workspace (inside a biosafety cabinet or other clean hood space).

In this protocol you will make primer aliquots used in the 1st and 2nd PCRs of the BarSeq Preparation and Pooling protocol.

The primers from the 1st PCR and 2nd PCR are not prepared in the same way, so their creation is split into two distinct sections in this protocol.

1. First you will use an excel sheet template to calculate required reagents and consumables needed for all primers.
2. Then you will follow step-by-step instructions for creating the aliquots for primer used in the 1st PCR one-by-one. Primers used in the 1st PCR are first resuspended in water to achieve a primer solution of 20 µmol/L, then further diluted 10x into Master Mix, and finally aliquoted and stored at-20C until use.
3. Finally, you will create the aliquots for primers used in the 2nd PCR one-by-one. Primers used in the 2nd PCR are resuspended in water to achieve a primer solution of 100 µmol/L, further diluted in water to a concentration of 20 µmol/L, and then are aliquoted and stored at-80C until use.

This protocol uses the following short-hand:

- nuclease-free water + primers solution = W + primers
- master mix + primers solution= MM + primers

Calculate all reagents and consumables required using the excel sheet

2 Use the Excel spreadsheet located in substep 2.1 to calculate the number of tubes and volume of reagents needed to prepare primers.

Note: The Excel spreadsheet has three sheet tabs:

1. 1st PCR primers
2. 2nd PCR primers
3. Print & Take to Lab

2.1 barseq_primer_prep.YYYY-MM-DD... 18.3KB

3 Save a copy of the spreadsheet as 'barseq-primer_prep.YYYY-MM-DD', replacing the “YYYY-MM-DD” in the file name with the date that the primers were received.

3.1 Enter the amount of each primer received (nmol) into the appropriate sheet tab.
- For primers used in the 1st PCR, enter information into the '1st PCR primers' sheet, under Column D labeled "amount of primer (nmol)" with the red background.
- For primers used in the 2nd PCR, enter information into the sheet '2nd PCR primers' sheet, under Column D labeled "amount of primer (nmol)" with the red background.

3.2 Save the spreadsheet again, and print out the 'Print & Take to Lab' sheet (i.e., the 3rd tab). You will use this printout as a reference for the rest of this protocol.

At the top of the print out you will see:
1. In the **Reagents** table: the total volume or nuclease-free water and Master Mix needed for the protocol to prepare primers for the 1st and 2nd PCR.
2. In the **Tubes** table: the total number of tubes needed for the protocol to prepare primers for the 1st and 2nd PCR.
3. In the **1st PCR Primers** table: all of the quantities (volumes and tubes) needed to prepare the 1st PCR primers - to be used in the *1st PCR primers* protocol sections.
4. In the **2nd PCR Primers** table: all of the quantities (volumes and tubes) needed to prepare the 2nd PCR primers - to be used in the *2nd PCR primers* protocol sections.

1st PCR primers: Gather and label tubes

4 Gather the tubes that will hold the final primer aliquots.
- Use sterile 1.5 mL micro-centrifuge tubes and keep them sealed at all times unless they are being manipulated.
- For MM + primer aliquots: The number of tubes for each primer is indicated in the column labeled “number of MM + primer aliquots.”
- For W + primer aliquots: The number of tubes for each primer is indicated in the column labeled “number of primer-only aliquots.” For some primers, this number may be zero.

4.1 Prepare labels and attach to aliquots.
- For MM + primer aliquots: Aliquot labels should include the primer name + "w/ Master Mix" + the date that the primers were received (matching the date in the spreadsheet file name) in the format YYYY-MM-DD. Example: "BarSeq_1_F1 w/ Master Mix 2024_12_17"
- For W + primer aliquots: Aliquot labels should include the primer name + "in water" + the date that the primers were received. Example: "BarSeq_1_F1 at in water 2024_12_17"
- Use printed labels if possible.

5 For each of the 1st PCR primers, prepare:

- one 1.5 mL reagent tube for nuclease-free water (20 tubes in total for a full set of 1st PCR primers)
- one 14 mL reagent tube for master mix (20 tubes for a full set of 1St PCR primers)

5.1 Prepare labels and attach to reagent tubes.
- Label each 1.5 mL water reagent tube with the name of one of the 1st PCR primers + "W reagent". Example: "BarSeq_1_F1 W reagent"
- Label each 14 mL master mix reagent tube with the name of one of the 1st PCR primers + "MM reagent". Example: "BarSeq_1_F1 MM reagent"
- Note: These tubes will be thrown away after use, so hand-written labels are ok.

1st PCR primers: Fill reagent tubes for water and master mix

6 Clean the pipettors and workspace (inside a biosafety cabinet or other clean hood space).

7 Fill each water reagent tube with 650 µL of new, freshly opened nuclease-free water.

7.1 Cap each water reagent tube tightly and store at room temperature until use.

7.2 Close the source bottle of nuclease-free water and leave it closed at room temperature.

8 Fill each master mix reagent tube.

8.1 Thaw the necessary amount of new, freshly opened Master Mix stock tubes on ice.
- The amount of Master Mix needed for all primers is located in the **Reagents** table.
- If you are only preparing some of the primers, you can calculate the total volume of Master Mix needed by summing the corresponding primer entries in the **1st PCR Primers** table under the column labeled 'volume of master mix needed (µL)'.

8.2 Once thawed, vortex each Master Mix stock to completely re-dissolve any precipitates.

8.3 Spin each Master Mix stock to move all of the liquid to the bottom of the tubes.

8.4 For each primer, transfer the required volume of Master Mix into the corresponding Master Mix reagent tube.

- The volume required for each primer can be found in the column labeled “volume of master mix in MM reagent tube (µL)”

8.5 Securely cap each Master Mix reagent tube and store at 4C until use (or -20C if it will not be used the same day).

1st PCR primers: Resuspend primer

9 **Note:** It is crucial that you perform the following steps in this section for **only one primer at a time.**

10 Clean the pipettors and workspace (inside a biosafety cabinet or other clean hood space).

11 Spin the tube of primer received from the vendor to make sure all of the DNA is in the bottom of the tube.

12 Transfer the volume of nuclease-free water required to resuspend the primer from the water reagent tube to the vendor supplied tube containing the lyophilized primer.
- The volume is located in the column labeled “Volume of water required to resuspend primer (µL)”
- Discard the water reagent tube. Only the primer tube from the vendor will be used moving forward and will be referred to as the 'resuspended primer tube'.

13 Close the resuspended primer tube and allow the primer DNA to dissolve for 15 minutes at room temperature.

14 Vortex the tube briefly, at least three times to completely dissolve the primer DNA.

15 Spin the tube to make sure all of the primer DNA solution is in the bottom of the tube.

1st PCR primers: Add primer stock to master mix reagent tube

16 Transfer the required volume of resuspended primer solution to the Master Mix reagent tube for that primer.
- Volume located in the column labeled "Volume of primer stock to add to master mix (µL)"

17 Cap the Master Mix reagent tube tightly and vortex for 20 seconds, then spin the tube to move all of the Master Mix with primers to the bottom of the tube.

1st PCR primers: Create primer + master mix aliquots

18 Aliquot the Master Mix and primer solution into the pre-made "MM + primers" final aliquot tubes for that primer:
- For forward primers the volume is 450 uL.
- For reverse primers the volume is 350 uL.

19 Cap all of the aliquot tubes tightly and seal with ParaFilm.
- Throw away the Master Mix reagent tube.

1st PCR primers: Create primer + water aliquots

20 Use the remaining resuspended primer solution (if any is left in the vendor tube) to make the W + primer aliquots by pipetting the appropriate volume from the vendor tube to each of the premade "W +primer" final aliquot tubes
- For forward primers the volume is 45 uL
- For reverse primers the volume is 35 uL

20.1 Cap all of the aliquot tubes tightly, seal with ParaFilm.

20.2 Close the vendor's resuspended primer tube.
- If there is any remaining primer solution, store it at -80 C for PCR testing (e.g., one or two samples at a time).
- If no solution is left, throw away the tube.

21 Store all aliquots:
- The W + primer aliquot tubes should be stored at -80C.
- The MM + primer aliquot tubes should be stored at -20C.

1st PCR primers: Prepare for the next primer

22 Take at least a short break between each primer.

23 Clean the workspace and pipettors between each primer.

24 Repeat steps 9-21 for the remaining primers used in the 1st PCR.

Prepare the 2nd PCR primers

25 Primers BarSeq_2_F and BarSeq_2_R are used for the 2nd BarSeq PCR.
- They are typically ordered at a 10x higher scale than the 1st PCR primers, so the volumes are correspondingly higher.
- These primers are only stored in water. No master mix is used to prepare the aliquots of 2nd PCR primers.
- 2nd PCR primers are first resuspended to 100 µM in the vendor tube, further diluted down to 20µM in a dilution tube, before finally being aliquoted into aliquot tubes.
- All following steps refer to information found in the 'Preparing 2nd PCR Primers' table of the spreadsheet.

2nd PCR primers: Gather and label tubes

26 Gather the tubes for the aliquots.
- Use sterile 1.5 mL micro-centrifuge tubes and keep them sealed at all times unless they are being manipulated.
- The number of tubes for each primer is indicated under the column labeled “number of primer + water aliquots”.

26.1 Prepare labels and attach to aliquots.
- Aliquot labels should include the primer name + "in water" (e.g., “BarSeq_1_F1 in water”), and the date that the primers were received in the YYYY-MM-DD format. e.g., BarSeq_1_F1 in water 2025-01-27
- Use printed labels if possible.

27 Gather 2 dilution tubes (one for each primer).
- Use sterile 14mL or 15 mL micro-centrifuge tubes and keep them sealed at all times unless they are being manipulated.

27.1 Label the dilution tubes.
- Dilution tube labels should include the primer name + "in water at 20µM" (e.g., “BarSeq_2_F in water at 20µM”)

28 Gather 2 reagent tubes (one for each primer).
- For both primers gather one 14 or 15 mL reagent tube.

28.1 Label each tube with its corresponding primer name + 'water reagent'. e.g., “BarSeq_2_F water reagent”

2nd PCR primers: Fill the water reagent tubes

29 Using the recently opened nuclease-free water stock from the preparation of the 1st PCR primers, distribute water to each of the water reagent tubes.
- Volume located in column labeled “total volume of water needed (µL)”

29.1 Cap each water reagent tube tightly and store at room temperature until use.

29.2 Close the bottle of nuclease-free water and leave it closed during the following steps.

2nd PCR primers: Resuspend primer

30 Note: It is crucial that you perform the following steps for only one primer at a time.

31 Clean the pipettors and workspace (inside a biosafety cabinet or other clean hood space).

32 Spin the tube of lyophilized primer received from the vendor to make sure all of the DNA is in the bottom of the tube.

33 Add the required amount of nuclease-free water from the water reagent tube to the vendor tube of the primer to prepare a 100 µM primer solution.
- The volume is found in the column labeled 'volume to resuspend primer to 100 µM (µL)'

34 Close the primer tube and allow the primer DNA to dissolve for 15 minutes (at room temperature)

35 Vortex the tube briefly, at least three times, to completely dissolve the primer DNA.

36 Spin the tube to make sure all of the primer DNA solution is in the bottom of the tube.

2nd PCR primers: Add water to dilution tube

37 Transfer the required amount of water from the water reagent tube to the dilution tube.
- The volume of nuclease-free water required for each primer is listed in the column labeled 'volume to dilute primer to 20 µM (µL)'

2nd PCR primers: Add primer stock to dilution tube

38 Transfer the appropriate amount of resuspended primer solution from the vendor-supplied tube to the dilution tube.
- The volume of resuspended primer solution required is in the column labeled 'volume of primer stock to add to dilution tube (µL)'

39 Vortex the dilution tube briefly, at least three times, to completely mix the primer DNA.

40 Spin the dilution tube to make sure all of the primer DNA solution is in the bottom of the tube.

2nd PCR primers: Create primer + water aliquots

41 Distribute the 20 umol/L primer + water aliquots into the corresponding labeled primer + water aliquot tubes:
- The volume/aliquot is 320 uL

42 Cap all of the aliquot tubes tightly and seal with ParaFilm.

43 Store the aliquots:
- the W + primer aliquot tubes should be stored at -80C.

44 If there is any remaining 20 umol/L primer solution, store it at -80C for testing.

2nd PCR primers: Prepare for final primer

45 Take at least a short break between each primer.

46 Clean the workspace and pipettors between each primer.

47 Repeat steps 32-46 for the second primer used in the 2nd PCR.

Acknowledgements

This protocol was formatted for Protocols.io by Dana Cortade (Align to Innovate) and David Ross (NIST).