Sequencing Library Preparation with Illumina DNA Prep Kit

For use with the Illumina DNA Prep kit (formerly Nextera Flex kit).

Note: Always centrifuge plates and tubes prior to opening to minimize the possibility of contamination.

Note: To avoid cross-contamination, change tips between each sample when adding or transferring samples or reagent master mixes.

Note: If you are using 8-strip tubes in the thermocycler, it is important to include at least 3 total strips of tubes to equilibrate lid pressure (empty “BALANCE” tubes are okay).

Note: When preparing PCR reactions, it is important to always work on ice (or cold blocks) to prevent heat-reactive components from activating prematurely. Additionally, keep in mind the time it takes your thermocycler lid to come up to reaction temperature, and pre-warm your thermocycler accordingly.

Note: Always allow SPRI beads to equilibrate to room temperature for 30 minutes before using.

Materials

Equipment:
→ P20 pipette (multichannel pipette optional)
→ P200 pipette (multichannel pipette optional)
→ Thermocycler
→ Tube centrifuge
→ Plate centrifuge
→ Vortex
→ 96-well plate magnet
→ Plate sealer
→ Timer
→ QuBit Fluorometer
→ Bioanalyzer

Consumables:
→ 96-well PCR plates
→ 96-well 0.8 mL Polypropylene Deepwell Storage Plate (midi plate) (2)
→ Microseal B adhesive plate seal (used to cover samples during reactions)
→ Microseal F foil seal (used for long-term storage)
→ 1.5mL microcentrifuge tubes
→ Capped 8-strip tubes
→ Pipette tips: P20, P200
→ Appropriate PPE and biosafety equipment

Reagents:
→ Illumina DNA Prep Kit
→ IDT by Illumina UD Indexes
→ Nuclease Free Water
→ Ethanol

Pre-Protocol Preparation

Preparing the index plate

1. Determine the index set to be used. IDT for Illumina UD Indexes can be used on any Illumina sequencing platform, while Illumina Nextera CD Indexes should only be used on the MiSeq and NextSeq platforms (for more information, see this link).

   Note: Please note that ***different*** wells of the ***same*** index plate should be used for samples in a sequencing batch (i.e., wells cannot be mixed and matched across index plates).

   Note: Each well on an index plate can be used only once (on a single sample, in a single batch). If you did not use all wells for an index plate, you can re-freeze the plate and use the unopened wells in a subsequent sequencing batch.

2. Using the appropriate table in Appendix A, write down the index(es) used for each sample (for UD indexes, write down the selected index; for CD indexes, write down both values from the appropriate cell). ***This is a crucial step in the process – if you forget to write down which indexes were used, your sequencing data cannot be processed in the bioinformatics pipeline.***

Tagment Genomic DNA

This step uses the Bead-Linked Transposomes to tagment DNA, which is a process that fragments and tags the DNA with adapter sequences.

Equipment:
→ P20 pipette (multichannel pipette optional)
→ P200 pipette
→ Thermocycler
→ Tube centrifuge
→ Vortex

Consumables:
→ 96-well PCR plates
→ Microseal B adhesive plate seal
→ 1.5mL microcentrifuge tubes
→ 8-tube strip
→ Pipette tips: P20, P200

Reagents:
→ Bead-Linked Transposomes (BLT) – *from Illumina DNA prep kit*
→ Tagmentation Buffer 1 (TB1) – *from Illumina DNA prep kit*
→ Nuclease Free Water

Protocol:

1. Bring Bead-Linked Transposomes (BLT) to room temperature. Vortex to mix. Do not centrifuge before pipetting. *Do not use BLT that has been stored at temperatures below 2C.*

2. Bring Tagmentation Buffer (TB1) to room temperature. Vortex to mix.

3. Prepare 20-23* DNA samples plus 1 negative control of nuclease-free water per library. Add 2-30µL of DNA to each well of a 96-well PCR plate so that the total input amount for each sample is 10-24ng**. Ensure that the same amount of DNA is added for each sample.

   Note: Number of DNA samples to multiplex depends on genome length, desired coverage, and sequencing technology to be used. This recommendation is for ~4M bacterial genomes, ~30x coverage, and a MiSeq run with the V2 reagent kit.

   Note: Input DNA can be as little as 1ng and as much as 500ng. The input amount should be recorded for each sample and the number of PCR cycles under "Amplify Tagmented DNA" adjusted accordingly. See Illumina DNA Prep Reference guide for adjustments if input DNA is <10ng or >24ng.

4. If the DNA volume for any sample is <30µL, add nuclease free water to the DNA sample to bring the total volume to 30µL.

5. Vortex BLT (yellow cap) vigorously for 10 seconds to resuspend. Repeat as necessary.

6. Prepare the following reaction per sample:

Component	**Volume**
BLT	10 µL
TB1	10 µL
DNA	30 µL
Total Volume	**50 µL**

   Note: BLT and TB1 can be combined ahead of time into a master mix. Vortex tagmentation master mix thoroughly to resuspend and, *using a new pipette tip for each sample*, add 20µL master mix to each well of the plate containing a sample.

7. Pipette each sample 10 times to resuspend. Use fresh tips for each sample.

8. Seal the plate with Microseal B or other optically clear seal.

9. Run the following on a thermocycler:

Temperature	**Time**
55°C	15 min
10°C	∞

   Note: Choose the preheat lid option and set to 100°C. Set the reaction volume to 50µL.

Post Tagmentation Cleanup

This step washes the adapter-tagged DNA on the BLT before PCR amplification.

Equipment:
→ 96-well plate magnet
→ P20 pipette (multichannel pipette optional)
→ P200 pipette (multichannel pipette optional)
→ Thermocycler
→ Tube centrifuge
→ Vortex

Consumables:
→ Microseal B adhesive plate seal
→ Pipette tips: P20, P200

Reagents:
→ Tagment Stop Buffer (TSB) – *from Illumina DNA prep kit*
→ Tagment Wash Buffer (TWB) – *from Illumina DNA prep kit*

Protocol:

1. If precipitates are observed in Tagment Stop Buffer (TSB), heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved. Use at room temperature.
2. Add 10µL TSB to each sample well of the tagmentation reaction.
3. Slowly pipette each well 10 times to resuspend the beads.
4. Seal the plate with Microseal B.
5. Run the following on a thermocycler:

Temperature	Time
37°C	15 min
10°C	∞

   Note: Choose the preheat lid option and set to 100°C. Set the reaction volume to 60µL.

6. Place the plate on the magnetic stand and wait until the liquid is clear (~3 minutes).
7. Remove and discard supernatant, being careful not to disrupt beads.
8. Remove the sample plate from the magnetic stand and use a deliberately slow pipetting technique to add 100 µl TWB (at room temperature) directly onto the beads. A deliberately slow pipetting technique minimizes the potential of TWB foaming to avoid incorrect volume aspiration and incomplete mixing.
9. Pipette slowly until beads are fully resuspended.

10. Place the plate on the magnetic stand and wait until the liquid is clear (~3 minutes).
11. Using a multichannel pipette, remove and discard supernatant.
12. Repeat steps 8-11 for a total of two washes.
13. Remove the plate from the magnetic stand and use a deliberately slow pipetting technique to add 100µl TWB directly onto the beads.
14. Pipette each well slowly to resuspend the beads.
15. Seal the plate and place on the magnetic stand until the liquid is clear (~3 minutes). Keep on the magnetic stand until Step 4 of the Amplify Tagmented DNA section below. The TWB remains in the wells to prevent overdrying of the beads.

Amplify Tagmented DNA

This step amplifies the tagmented DNA using a limited-cycle PCR program. The PCR step adds adapters and sequences required for sequencing cluster generation.

Equipment:
→ 96-well plate magnet
→ P20 pipette (multichannel pipette optional)
→ P200 pipette (multichannel pipette optional)
→ Thermocycler
→ Tube centrifuge
→ Plate centrifuge
→ Vortex

Consumables:
→ Microseal B adhesive plate seal
→ 1.5mL microcentrifuge tubes
→ Pipette tips: P20, P200

Reagents:
→ Enhanced PCR Mix (EPM) – *from Illumina DNA prep kit*
→ Index adapters (tubes or plates)
→ Nuclease-free water

Protocol:

1. Thaw EPM on ice. Invert to mix, then briefly centrifuge.
2. Thaw Index Adapters at room temperature. Spin briefly before use.
3. Prepare the following master mix per sample:

Component	Volume
EPM	20 µL
Nuclease-free water	20 µL
Total Volume	**40 µL**

Note: Vortex master mix briefly and centrifuge at 280 x g for 10 seconds.

4. With the plate of tagmented DNA on the magnetic stand, use a 200µL pipette to remove and discard supernatant J (foam that remains on well walls does not adversely affect the library).

5. Remove plate from magnet and ***immediately*** add 40µL master mix directly on to the beads. Use a separate pipette tip for each sample well.

6. Immediately pipette to mix until beads are fully resuspended. Seal the sample plate and centrifuge at 280 x g for 3 seconds.

7. Prepare the selected index plate by centrifuging the selected index plate at 1000 x g for 1 minute to settle liquid away from the seal.

8. Add 10µL of the appropriate index adapter to each sample, being extremely careful to avoid contamination. **Change pipette tips between each addition and *ensure you have written down the index(es) added to each sample.***

9. Using a pipette set to 40 µL, pipette 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.

10. Seal the plate with Microseal B and centrifuge at 280 x g for 30 seconds.

11. Run the following on a thermocycler:

Temperature	Time
68°C	3 min
98°C	3 min
98°C	45 sec
62°C	30 sec
68°C	2 min
Repeat steps 3, 4 & 5 for a total of 8 cycles	
68°C	1 min
10°C	∞

Note: Choose the preheat lid option and set to 100°C. Set the reaction volume to 50µL. Wait until the machine comes to temperature before adding samples.

Note: Number of cycles assumes 10-24ng input DNA. Consult the Illumina DNA Prep Reference Guide for cycle recommendations for different input values.

12. At this point, DNA can be stored at 2-8°C for up to 3 days.

Clean Up Libraries

This step uses double-sided bead purification procedure to purify the amplified libraries.

Equipment:
→ Magnetic plate stand
→ P20 pipette (multichannel pipette optional)
→ P200 pipette (multichannel pipette optional)
→ Thermocycler
→ Tube centrifuge
→ Plate centrifuge
→ Vortex

Consumables:
→ 96-well 0.8 mL Polypropylene Deepwell Storage Plate (midi plate) (2)
→ 96-well PCR plate
→ Microseal B adhesive plate seal
→ Microseal F foil seal
→ 1.5mL microcentrifuge tubes
→ Pipette tips: P20, P200

Reagents:
→ Sample Purification Beads (SPB) – *from Illumina DNA prep kit*
→ Resuspension Buffer (RSB) – *from Illumina DNA prep kit*
→ Freshly prepared 80% ethanol
→ Nuclease-free water

Protocol:

1. Allow Sample Purification Beads to come to room temperature for 30 minutes before use. Vortex beads before ***each*** use and pipette slowly due to the viscosity of the solution.
2. Thaw RSB and bring to room temperature. Vortex to mix.
3. Centrifuge at 280 × g for 1 minute to collect contents at the bottom of the well.
4. Place the plate on the magnetic stand and wait until the liquid is clear (~5 minutes).
5. Transfer 45 µl supernatant from each well of the PCR plate to the corresponding well of a new midi plate.
6. Vortex and invert SPB multiple times to resuspend.
7. Add 40 µl nuclease-free water to each well containing supernatant.
8. Add 45 µl SPB to each well containing supernatant.
9. Pipette each well 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.

10. Seal the plate and incubate at room temperature for 5 minutes.
11. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
12. During incubation, thoroughly vortex the SPB (undiluted stock tube), and then add 15 µl to each well of a new midi plate.
13. Transfer 125 µl supernatant from each well of the first plate into the corresponding well of the second plate (containing 15 µl undiluted SPB).
14. Pipette each well in the second plate 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
15. Discard the first plate.
16. Incubate the sealed midi plate at room temperature for 5 minutes.
17. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
18. Without disturbing the beads, remove and discard supernatant.
19. With the plate on the magnetic stand, add 200 µl fresh 80% EtOH without mixing.
20. Incubate for 30 seconds.
21. Without disturbing the beads, remove and discard supernatant.
22. Repeat steps 19-21 for a total of two washes.
23. Use a 20 µl pipette to remove and discard residual EtOH.
24. Air-dry on the magnetic stand for 5 minutes. Avoid over-drying beads.
25. Remove from the magnetic stand.
26. Add 32 µl RSB to the beads.
27. Pipette to resuspend.
28. Incubate at room temperature for 2 minutes.
29. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
30. Transfer 30 µl supernatant to a new 96-well PCR plate.
31. At this point, libraries can be safely stored frozen (-25°C to 15°C) for up to 30 days.

Pool Libraries

Equipment:
→ P20 pipette (multichannel pipette optional)
→ P200 pipette (multichannel pipette optional)
→ Tube centrifuge
→ Vortex

Consumables:
→ 96-well PCR plate
→ 1.5mL microcentrifuge tubes
→ Pipette tips: P20, P200

Reagents:
→ Nuclease-free water

Protocol:

1. Quantify each library individually using a Qubit Fluorometer.
2. Calculate average fragment size and assess library quality using a TapeStation or BioAnalyzer.

   Note: Do not pool libraries that are primarily primer dimers. The average fragment size for this protocol is approximately 600bp. Primer dimers will show up on a BioAnalyzer or TapeStation as shorter fragments, approximately 150-200bp in length.
3. Dilute all libraries to 5nM in nuclease-free water.
4. Pool individual libraries in equimolar amounts by adding 5 µL of each 5nM library.

   Note: Only pool libraries with concentration <5nM if the library is relatively clear of primer dimers. In this case, add 5 µL of the library without diluting.
5. Run BioAnalyzer again to get final pooled library concentration and fragment size.

Appendix A

1. ***If using Illumina UD Indexes,*** the plate layouts for Plates A-D are available here and in the tables below. The specific sequence associated with each index (needed to prepare a sample sheet) is available here. If pooling fewer than 8 samples, please read the "Two-Plex Through Eight-Plex Pooling Strategies" of this site before selecting which indexes to use.

IDT for Illumina UD Indexes Plate A/Set 1

The following table depicts the plate layout for IDT for Illumina UD Indexes Plate A/Set 1. Write down the unique index to be added to each sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDP0001	UDP0009	UDP0017	UDP0025	UDP0033	UDP0041	UDP0049	UDP0057	UDP0065	UDP0073	UDP0081	UDP0089
B	UDP0002	UDP0010	UDP0018	UDP0026	UDP0034	UDP0042	UDP0050	UDP0058	UDP0066	UDP0074	UDP0082	UDP0090
C	UDP0003	UDP0011	UDP0019	UDP0027	UDP0035	UDP0043	UDP0051	UDP0059	UDP0067	UDP0075	UDP0083	UDP0091
D	UDP0004	UDP0012	UDP0020	UDP0028	UDP0036	UDP0044	UDP0052	UDP0060	UDP0068	UDP0076	UDP0084	UDP0092
E	UDP0005	UDP0013	UDP0021	UDP0029	UDP0037	UDP0045	UDP0053	UDP0061	UDP0069	UDP0077	UDP0085	UDP0093
F	UDP0006	UDP0014	UDP0022	UDP0030	UDP0038	UDP0046	UDP0054	UDP0062	UDP0070	UDP0078	UDP0086	UDP0094
G	UDP0007	UDP0015	UDP0023	UDP0031	UDP0039	UDP0047	UDP0055	UDP0063	UDP0071	UDP0079	UDP0087	UDP0095
H	UDP0008	UDP0016	UDP0024	UDP0032	UDP0040	UDP0048	UDP0056	UDP0064	UDP0072	UDP0080	UDP0088	UDP0096

IDT for Illumina UD Indexes Plate B/Set 2

The following table depicts the plate layout for IDT for Illumina UD Indexes Plate B/Set 2. Write down the unique index to be added to each sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDP0097	UDP0105	UDP0113	UDP0121	UDP0129	UDP0137	UDP0145	UDP0153	UDP0161	UDP0169	UDP0177	UDP0185
B	UDP0098	UDP0106	UDP0114	UDP0122	UDP0130	UDP0138	UDP0146	UDP0154	UDP0162	UDP0170	UDP0178	UDP0186
C	UDP0099	UDP0107	UDP0115	UDP0123	UDP0131	UDP0139	UDP0147	UDP0155	UDP0163	UDP0171	UDP0179	UDP0187
D	UDP0100	UDP0108	UDP0116	UDP0124	UDP0132	UDP0140	UDP0148	UDP0156	UDP0164	UDP0172	UDP0180	UDP0188
E	UDP0101	UDP0109	UDP0117	UDP0125	UDP0133	UDP0141	UDP0149	UDP0157	UDP0165	UDP0173	UDP0181	UDP0189
F	UDP0102	UDP0110	UDP0118	UDP0126	UDP0134	UDP0142	UDP0150	UDP0158	UDP0166	UDP0174	UDP0182	UDP0190
G	UDP0103	UDP0111	UDP0119	UDP0127	UDP0135	UDP0143	UDP0151	UDP0159	UDP0167	UDP0175	UDP0183	UDP0191
H	UDP0104	UDP0112	UDP0120	UDP0128	UDP0136	UDP0144	UDP0152	UDP0160	UDP0168	UDP0176	UDP0184	UDP0192

IDT for Illumina UD Indexes Plate C/Set 3

The following table depicts the plate layout for IDT for Illumina UD Indexes Plate B/Set 2. Write down the unique index to be added to each sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDP0193	UDP0201	UDP0209	UDP0217	UDP0225	UDP0233	UDP0241	UDP0249	UDP0257	UDP0265	UDP0273	UDP0281
B	UDP0194	UDP0202	UDP0210	UDP0218	UDP0226	UDP0234	UDP0242	UDP0250	UDP0258 V2	UDP0266	UDP0274	UDP0282
C	UDP0195	UDP0203	UDP0211	UDP0219	UDP0227	UDP0235	UDP0243	UDP0251	UDP0259	UDP0267	UDP0275	UDP0283
D	UDP0196	UDP0204	UDP0212	UDP0220	UDP0228	UDP0236	UDP0244	UDP0252 V2	UDP0260	UDP0268	UDP0276	UDP02854
E	UDP0197	UDP0205	UDP0213	UDP0221	UDP0229	UDP0237	UDP0245	UDP0253	UDP0261	UDP0269	UDP0277	UDP0285
F	UDP0198	UDP0206	UDP0214	UDP0222	UDP0230	UDP0238	UDP0246	UDP0254	UDP0262	UDP0270	UDP0278	UDP0286
G	UDP0199	UDP0207	UDP0215	UDP0223	UDP0231	UDP0239	UDP0247	UDP0255	UDP0263	UDP0271	UDP0279	UDP0287
H	UDP0200	UDP0208	UDP0216	UDP0224	UDP0232	UDP0240	UDP0248	UDP0256	UDP0264	UDP0272	UDP0280	UDP0288

IDT for Illumina UD Indexes Plate D/Set 4

The following table depicts the plate layout for IDT for Illumina UD Indexes Plate B/Set 2. Write down the unique index to be added to each sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDP0289 V2	UDP0297	UDP0305	UDP0313	UDP0321	UDP0329	UDP0337	UDP0345	UDP0353	UDP0361	UDP0369	UDP0377
B	UDP0290 V2	UDP0298	UDP0306	UDP0314	UDP0322	UDP0330	UDP0338	UDP0346	UDP0354	UDP0362	UDP0370	UDP0378
C	UDP0291 V2	UDP0299	UDP0307	UDP0315	UDP0323	UDP0331	UDP0339	UDP0347	UDP0355	UDP0363	UDP0371	UDP0379
D	UDP0292	UDP0300	UDP0308	UDP0316	UDP0324	UDP0332	UDP0340	UDP0348	UDP0356	UDP0364	UDP0372	UDP0380
E	UDP0293	UDP0301 V2	UDP0309	UDP0317	UDP0325	UDP0333	UDP0341	UDP0349	UDP0357	UDP0365	UDP0373	UDP0381
F	UDP0294	UDP0302	UDP0310	UDP0318	UDP0326	UDP0334	UDP0342	UDP0350	UDP0358	UDP0366	UDP0374	UDP0382
G	UDP0295	UDP0303	UDP0311	UDP0319	UDP0327	UDP0335	UDP0343	UDP0351	UDP0359	UDP0367	UDP0375	UDP0383
H	UDP0296	UDP0304	UDP0312	UDP0320	UDP0328	UDP0336	UDP0344	UDP0352	UDP0360	UDP0368	UDP0376	UDP0384

2. ***If using Illumina CD Indexes,*** the plate layout for Nextera DNA CD Indexes is available here or in the table below. The specific sequence associated with each index (needed to prepare sample sheet) is available here. If pooling fewer than 8 samples, please read the "Two-Plex Through Eight-Plex Pooling Strategies" section of this site before selecting which indexes to use.

   Note: You should write down both the H7 and H5 number in the cell selected for each sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	H701 H505	H702 H506	H703 H517	H705 H505	H707 H506	H723 H517	H706 H505	H712 H506	H720 H517	H710 H505	H711 H506	H714 H517
B	H702 H517	H703 H505	H701 H506	H707 H517	H723 H505	H705 H506	H712 H517	H720 H505	H706 H506	H711 H517	H714 H505	H710 H506
C	H703 H506	H701 H517	H702 H505	H723 H506	H705 H517	H707 H505	H720 H506	H706 H517	H712 H505	H714 H506	H710 H517	H711 H505
D	H705 H503	H707 H503	H723 H503	H706 H503	H712 H503	H720 H503	H710 H503	H711 H503	H714 H503	H701 H503	H702 H503	H703 H503
E	H706 H516	H712 H516	H720 H516	H710 H516	H711 H516	H714 H516	H701 H516	H702 H516	H703 H516	H705 H516	H707 H516	H723 H516
F	H710 H522	H711 H510	H714 H513	H701 H522	H702 H510	H703 H513	H705 H522	H707 H510	H723 H513	H706 H522	H712 H510	H720 H513
G	H711 H513	H714 H522	H710 H510	H702 H513	H703 H522	H701 H510	H707 H513	H723 H522	H705 H510	H712 H513	H720 H522	H706 H510
H	H714 H510	H710 H513	H711 H522	H703 H510	H701 H513	H702 H522	H723 H510	H705 H513	H707 H522	H720 H510	H706 H513	H712 H522