NEBNext Direct® Custom Ready Panels

NEB #E6631S/L/X

8/24/96 reactions

Version 3.0_6/20

Table of Contents

The Kit Includes

NEB #E6631S contains #N6631S (Pouch with baits), #E6635-1 (Box 1 of 2), and #E6635-2 (Box 2 of 2) and is sufficient for preparation of up to 8 reactions.

NEB #N6631S

Pouch: Store at –20°C.

1. ○ (white) NEBNext Direct Custom Ready Baits

NEB #E6635-1

Box 1 of 2: Store at –20°C.

1. ● (brown) NEBNext Direct Stop Solution
2. ● (brown) NEBNext Direct DNA Nicking Enzyme
3. ● (brown) NEBNext Direct DNA Nicking Buffer
4. ○ (white) NEBNext Direct Hybridization Additive
5. ● (green) NEBNext Direct 3′ Blunting Enzyme Mix
6. ● (yellow) NEBNext Direct dA-Tailing Enzyme
7. ● (red) NEBNext Direct 3′ Adaptor
8. ● (red) NEBNext Direct Ligase
9. ● (orange) NEBNext Direct 5′ Blunting Enzyme Mix
10. ● (red) NEBNext Direct 5′ UMI Adaptor

11. (lilac) NEBNext Direct Cleaving Enzyme Mix
12. (blue) NEBNext Direct Q5® Master Mix

D01-D08 NEBNext Direct Index Primer Mix D01-D08

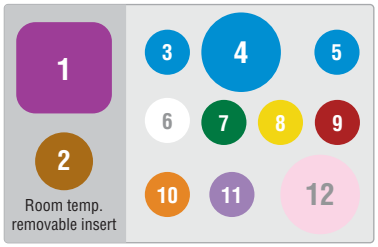
NEB #E6635-2 (Cont.)

Box 2 of 2: Store at 4°C. Do not freeze.

1. (dark purple) NEBNext Direct Bead Wash 1 (BW1)*
2. (brown) NEBNext Sample Purification Beads**
3. (blue) NEBNext Direct Streptavidin Beads
4. (blue) NEBNext Direct Hybridization Wash (HW)
5. (blue) NEBNext Direct Bead Prep Buffer
6. (white) NEBNext Direct Hybridization Buffer
7. (green) NEBNext Direct 3′ Blunting Buffer
8. (yellow) NEBNext Direct dA-Tailing Buffer
9. (red) NEBNext Direct Adaptor Ligation Buffer
10. (orange) NEBNext Direct 5′ Blunting Buffer
11. (lilac) NEBNext Direct Cleaving Buffer
12. (light pink) NEBNext Direct Bead Wash 2 (BW2)

* This component should be stored at room temperature. Upon arrival, you may notice a precipitate, which is normal. Once BW1 reaches room temperature, gently invert the bottle ~ 10 times to dissolve the precipitate. Do not use BW1 until all of the precipitate has dissolved.

** This component should be stored at room temperature.



NEB #E6631L contains #N6631L (Pouch with baits), #E6635-3 (Box 1 of 2), and #E6635-4 (Box 2 of 2) and is sufficient for preparation of up to 24 reactions.

NEB #N6631L

Pouch: Store at –20°C.

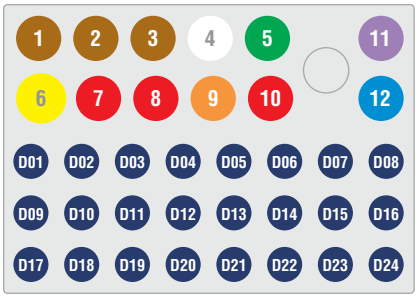
1. ○ (white) NEBNext Direct Custom Ready Baits

NEB #E6635-3

Box 1 of 2: Store at –20°C.

1. ● (brown) NEBNext Direct Stop Solution
2. ● (brown) NEBNext Direct DNA Nicking Enzyme
3. ● (brown) NEBNext Direct DNA Nicking Buffer
4. ○ (white) NEBNext Direct Hybridization Additive
5. ● (green) NEBNext Direct 3′ Blunting Enzyme Mix
6. ● (yellow) NEBNext Direct dA-Tailing Enzyme
7. ● (red) NEBNext Direct 3′ Adaptor
8. ● (red) NEBNext Direct Ligase
9. ● (orange) NEBNext Direct 5′ Blunting Enzyme Mix
10. ● (red) NEBNext Direct 5′ UMI Adaptor
11. ● (lilac) NEBNext Direct Cleaving Enzyme Mix
12. ● (blue) NEBNext Direct Q5® Master Mix

D01-D24 NEBNext Direct Index Primer Mix D01-D24



NEB #E6635-4 (Cont.)

Box 2 of 2: Store at 4°C. Do not freeze.

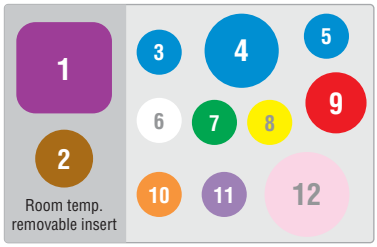
1. ● (dark purple) NEBNext Direct Bead Wash 1 (BW1)*
2. ● (brown) NEBNext Sample Purification Beads**
3. ● (blue) NEBNext Direct Streptavidin Beads
4. ● (blue) NEBNext Direct Hybridization Wash (HW)
5. ● (blue) NEBNext Direct Bead Prep Buffer
6. ○ (white) NEBNext Direct Hybridization Buffer
7. ● (green) NEBNext Direct 3′ Blunting Buffer
8. ● (yellow) NEBNext Direct dA-Tailing Buffer
9. ● (red) NEBNext Direct Adaptor Ligation Buffer
10. ● (orange) NEBNext Direct 5′ Blunting Buffer

11. (lilac) NEBNext Direct Cleaving Buffer

12. (light pink) NEBNext Direct Bead Wash 2 (BW2)

* This component should be stored at room temperature. Upon arrival, you may notice a precipitate, which is normal. Once BW1 reaches room temperature, gently invert the bottle ~ 10 times to dissolve the precipitate. Do not use BW1 until all of the precipitate has dissolved.

** This component should be stored at room temperature.



NEB #E6631X contains #N6631X (Pouch with baits), #E6635-5 (Box 1 of 2), and #E6635-6 (Box 2 of 2) and is sufficient for preparation of up to 96 reactions.

NEB #N6631X

Pouch: Store at –20°C.

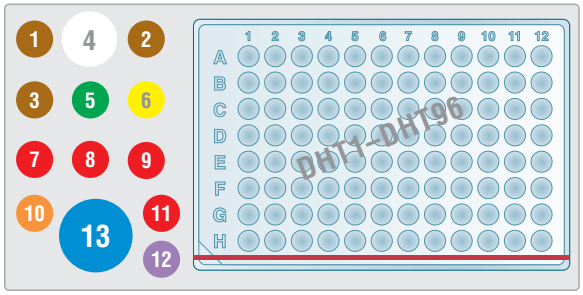
1. (white) NEBNext Direct Custom Ready Baits

NEB #E6635-5

Box 1 of 2: Store at –20°C.

1. (brown) NEBNext Direct Stop Solution
2. (brown) NEBNext Direct DNA Nicking Enzyme
3. (brown) NEBNext Direct DNA Nicking Buffer
4. (white) NEBNext Direct Hybridization Additive
5. (green) NEBNext Direct 3′ Blunting Enzyme Mix
6. (yellow) NEBNext Direct dA-Tailing Enzyme
7. (red) NEBNext Direct 3′ Adaptor
8. (red) NEBNext Direct Ligase
9. (red) NEBNext Direct Ligase
10. (orange) NEBNext Direct 5′ Blunting Enzyme Mix
11. (red) NEBNext Direct 5′ UMI Adaptor
12. (lilac) NEBNext Direct Cleaving Enzyme Mix
13. (blue) NEBNext Direct Q5® Master Mix

DHT1-DHT96 NEBNext Direct Index Primer Mix Plate



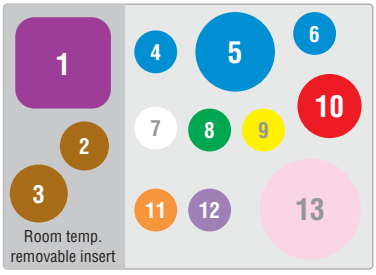
NEB #E6635-6 (Cont.)

Box 2 of 2: Store at 4°C. Do not freeze.

1. (dark purple) NEBNext Direct Bead Wash 1 (BW1)*
2. (brown) NEBNext Sample Purification Beads**
3. (brown) NEBNext Sample Purification Beads**
4. (blue) NEBNext Direct Streptavidin Beads
5. (blue) NEBNext Direct Hybridization Wash (HW)
6. (blue) NEBNext Direct Bead Prep Buffer
7. (white) NEBNext Direct Hybridization Buffer
8. (green) NEBNext Direct 3′ Blunting Buffer
9. (yellow) NEBNext Direct dA-Tailing Buffer
10. (red) NEBNext Direct Adaptor Ligation Buffer
11. (orange) NEBNext Direct 5′ Blunting Buffer
12. (lilac) NEBNext Direct Cleaving Buffer
13. (light pink) NEBNext Direct Bead Wash 2 (BW2)

* This component should be stored at room temperature. Upon arrival, you may notice a precipitate, which is normal. Once BW1 reaches room temperature, gently invert the bottle ~ 10 times to dissolve the precipitate. Do not use BW1 until all of the precipitate has dissolved.

** This component should be stored at room temperature.



Required Equipment

- Thermocycler programmable to 100 µl
- Agilent® Bioanalyzer® or similar instrument

Required Materials Not Included

- 1X TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Molecular grade ethanol
- Molecular grade water
- 0.2 ml PCR strip tubes or plates
- Eppendorf® DNA LoBind® 2 ml tubes (VWR, cat#: 80077-234)
- Additional microcentrifuge or conical tubes to prepare master mixes
- 96-well plate magnet or PCR tube magnet
- Microcentrifuge tube magnet
- Agilent High Sensitivity DNA Kit (Agilent, cat#: 5067-4626) or similar

Introduction

Target enrichment, coupled with next generation sequencing (NGS), enables high-throughput, deep sequencing of genomic regions of interest. NEBNext Direct is a novel, hybridization-based capture method offering significant advantages over traditional in-solution hybridization and multiplex PCR protocols.

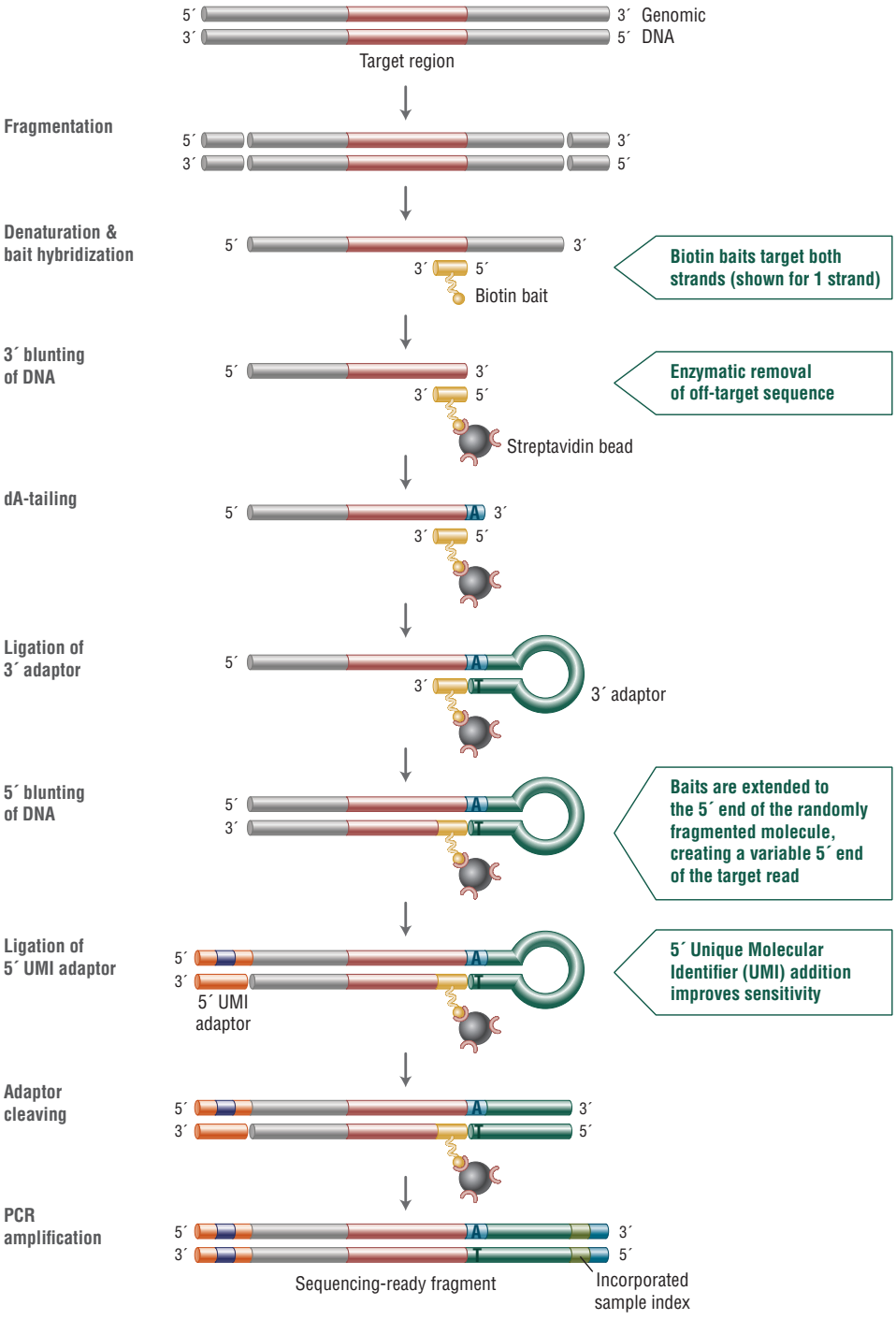
In the NEBNext Direct target enrichment approach (Figure 1), enzymatically nicked or Covaris® sheared DNA is hybridized to biotinylated oligonucleotide baits that capture both strands of the target DNA and define the 3′ ends of the regions of interest. After hybridization, the bait-target hybrids are bound to streptavidin beads and any 3′ off-target sequence is removed enzymatically. This combination of hybridization with enzymatic removal of 3′ off-target sequence enables greater sequencing specificity relative to conventional hybridization-based enrichment methods. The trimmed targets are then converted into Illumina-compatible libraries that include a 12 bp unique molecular identifier (UMI) in the Illumina i5 index location and an 8 bp sample barcode in the Illumina i7 index location. The NEBNext Direct enrichment method can be performed within one to two days and is compatible with most automated liquid handling instruments.

The NEBNext Direct Custom Ready Panels are designed to enrich for the complete exonic content of genes selected from the Custom Ready portfolio for next generation sequencing on the Illumina platform. This kit contains the oligonucleotides, beads, enzymes and buffers required to convert the desired fragments into a sequence-ready library. Custom Ready Panels are designed for PE150 sequencing.

BED files: BED files for the NEBNext Direct Custom Ready Panels are provided for review prior to purchase. Three files are provided: 1) The regions targeted in the design of the panel (targeted_regions.bed), 2) the actual regions where baits could be designed (captured_regions.bed), and 3) the subset of targeted regions not anticipated to be captured by the panel (targeted_not_captured_regions.bed). BED files are provided in GRCh37/hg19 and GRCh38/hg38 coordinates. Additional copies of the BED files can be requested via email to NEBNextDirect@neb.com

Lot Control and Functional Validation: Each kit component must pass rigorous quality control standards, and for each new lot the entire set of reagents is functionally validated together by construction and sequencing of an indexed library on the Illumina sequencing platform. For larger volume requirements, customized and bulk packaging is available by purchasing through the NEB Solutions department at NEB. Please contact nebsolutions@neb.com for further information.

Figure 1. NEBNext Direct Workflow.



Section 1
Protocol for NEBNext Direct Custom Ready Panels

Critical Guidelines

- Note that the captured molecules on streptavidin beads are unamplified, so any beads lost to pipette tips will result in fewer unique targets in the final library and reduced overall yields. Take care to minimize bead loss during washes.
- Perform all washes by pipetting up and down at least 10 times. Insufficient washing can lead to carryover of previous reagents, resulting in adaptor dimer formation and reduced yields. Foaming of the wash buffers may occur due to the presence of detergents.
- A thermocycler programmable to 100 µl is required for this protocol. A thermocycler programmable to only 50 µl is not compatible with the incubations. The hybridization, bead binding, and wash volumes will exceed 100 µl, but a thermocycler programmable to 100 µl is sufficient for these steps.
- Hybridizations can be performed for 90 minutes or longer. For larger panels (greater than ~70 kb), an overnight hybridization can significantly increase the number of unique molecules captured, resulting in higher coverage and fewer PCR duplicates with deep sequencing. Note that these benefits will not be apparent with low sequencing depth datasets. See Figure 1.2A & 1.2B, pages 17–18, for a comparison of 90 minute and 16 hour hybridization times across panel sizes and sequencing depths.
- Note that the Post-ligation Washes (Sections 1.8.7 and 1.10.7) contain different steps than the Post-reaction Washes (Sections 1.6.6, 1.7.6, 1.9.7 and 1.11.7).
- This protocol is incompatible with the Illumina Experiment Manager. For guidelines on how to run samples on an Illumina MiSeq, see Section 4.
- Reaction temperatures are carefully optimized for best target conversion. Strictly adhere to reaction temperatures and times throughout the protocol.

Data Processing

- For information on bioinformatic utilization of unique molecular identifiers (UMIs) please refer to Using Unique Molecular IDs with NEBNext Direct – Data Usage Guideline Page within the "Usage Guidelines & Tips" panel in the "Other Tools & Resources" tab at: www.neb.com/E6631.
- We have developed and optimized a pipeline for processing of data directly from FASTQ files using open-source bioinformatics tools. Details on this pipeline can be found at: https://github.com/DirectedGenomics/DemoPipeline.
- The i5 UMI index is recorded as a series of 12 Ns for the sequencing run. Please confirm that the BCL to FASTQ converter to be used tolerates Ns in the reported i5 index sequence prior to analysis.

Helpful Tips

- If you are preparing more than one sample, we recommend making master mixes using ~10% more volume than required per reaction.
- A multichannel pipette (200 µl) is recommended for washing and mixing steps if preparing multiple samples.
- To save time, buffers for all master mixes in steps 1.6–1.11 (3′ Blunting through Adaptor Cleaving) can be pre-aliquoted and kept at 4°C until needed. Enzyme can be added to the buffers during the previous incubation and kept on ice until needed.
- It is ideal to use a thermocycler on a bench with accessible workspace for this protocol.

Before You Begin

- Bring all wash solutions and beads (Box 2) to room temperature before use.
- Bead Wash 1 (BW1) may contain a precipitate due to shipping at 4°C. This is normal and should go back into solution once the buffer reaches room temperature (at least 4 hours followed by gently inverting the bottle ~10 times). Do not use until all precipitate has dissolved. If necessary, incubate the bottle in a 25°C or 37°C incubator or water bath to dissolve the precipitate.

Protocol for NEBNext Direct Custom Ready Panels

Symbols

Stopping points in the protocol.

This caution sign signifies a step in the protocol that has multiple paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

●

Colored bullets indicate the cap color or label stripe of the reagent to be added to a reaction.

1.1. Quantify DNA

1.1.1. Quantify input DNA using a Qubit™ dsDNA HS Assay Kit or a Quant-iT™ PicoGreen™ dsDNA Assay Kit. Do not use a UV-based assay for quantification.

1.2. DNA Nicking

If you are starting with cell-free DNA, skip DNA Nicking and go directly to Bait Hybridization in Section 1.3.

If you prefer to fragment with Covaris®, skip DNA Nicking and shear input DNA in 52 µl (total volume) of 1X TE. Follow Covaris recommendations for instrument and microtube setup using the 200 bp target size protocol for shearing in 50 µl. Transfer 50 µl of the sheared DNA to a PCR well, then proceed directly with bait hybridization in Section 1.3.

1.2.1. Take out the stop solution to thaw.

1.2.2. Set up the following reaction on ice. First, mix the DNA, buffer and water in a PCR tube or well. Add the enzyme last.

REAGENT	PER REACTION
DNA (10-1,000 ng)*	1-38 µl
● (brown) NEBNext Direct DNA Nicking Buffer	4 µl
● (brown) NEBNext Direct DNA Nicking Enzyme	3 µl
Ice-cold, nuclease-free H_2O	variable
Total Volume	45 µl

*For detection of somatic variants, we strongly recommend starting with a minimum of 100 ng DNA input.

1.2.3. Mix the reaction thoroughly by pipetting up and down 10 times. Incubate at 18°C for 15 minutes. Reactions may be incubated capped or uncapped.

1.2.4. Stop the reaction by moving the samples to ice and adding 5 µl of ● (brown) stop solution. Mix thoroughly by pipetting up and down 10 times.

1.2.5. Proceed directly to Bait Hybridization in Section 1.3.

Note that at this stage, the DNA is nicked, not fragmented. Therefore, these reactions cannot be analyzed for size as dsDNA on non-denaturing gels or on Agilent's TapeStation, Bioanalyzer or similar instruments.

1.3. Bait Hybridization

1.3.1. Make a hybridization master mix by adding the following components for the appropriate number of reactions. Vortex the hybridization buffer to mix well prior to pipetting.

REAGENT	PER REACTION	WITH 10% OVERAGE
○ (white) Hybridization Buffer	47 µl	51.7 µl
○ (white) Hybridization Additive	20 µl	22 µl
○ (white) NEBNext Direct Custom Ready Baits	5 µl	5.5 µl
Total	72 µl	79.2 µl

1.3.2. Mix the master mix well by vortexing for 3–5 seconds and centrifuge briefly.

1.3.3. To each sample of nicked or fragmented DNA from Section 1.2, add 72 µl of hybridization master mix for a final volume of 122 µl. Mix by pipetting up and down 10 times. Seal the PCR plate or cap tubes securely to avoid evaporation.

1.3.4. Run the following program with the heated lid set to 105°C and place the samples in the thermocycler after the block temperature reaches 95°C:

10 mins @ 95°C

90 mins or longer* @ 60°C

Hold @ 60°C

*See Figure 1.2A & 1.2B, page 17–18 for panel performance at 90 minutes and 16 hours.

1.3.5. While the samples are incubating, prepare ● (blue) Streptavidin beads (see Streptavidin Bead Preparation in Section 1.4).

1.3.6. After the incubation at 60°C and when Section 1.4 (Streptavidin Bead Preparation) is complete, unseal the tubes/wells, leave the samples on the thermocycler at 60°C with the lid open and proceed to Bead Binding in Section 1.5.

1.4. Streptavidin Bead Preparation

1.4.1. Warm Streptavidin beads to room temperature (~15 minutes).

1.4.2. Vortex the Streptavidin beads to resuspend.

1.4.3. For each reaction, 75 µl of beads are required (82.5 µl with 10% overage). In a 2 ml Eppendorf tube, add the appropriate volume of beads for the number of reactions performed.

Note: Use multiple 2 ml tubes if performing more than 12 reactions. It is not recommended to exceed 1 ml of beads per 2 ml Eppendorf tube.

1.4.4. Place the tube(s) on a magnet and wait for the solution to clear (~1 minute). Remove the supernatant, and then remove the tube(s) from the magnet.

1.4.5. Add 150 µl of ● (blue) Hybridization Wash (HW) per reaction (165 µl with 10% overage) to the beads and resuspend by vortexing or pipetting.

1.4.6. Place the tube(s) on a magnet and wait for the solution to clear (~1 minute). Remove the supernatant, and then remove the tube(s) from the magnet.

1.4.7. Repeat Steps 1.4.5–1.4.6 twice for a total of 3 washes.

1.4.8. Resuspend the beads in 30 µl of ● (blue) Bead Prep Buffer per reaction (33 µl with 10% overage).

1.4.9. Keep the beads at room temperature until bait hybridization (Section 1.3) is completed.

Note: For Sections 1.5–1.11, the thermocycler lid should be open and unheated. The PCR tubes can remain uncapped for ease of mixing. However, if it is preferred, tubes can be capped during incubations.

1.5. Bead Binding

1.5.1. Immediately before use, vortex the washed Streptavidin Beads (from Step 1.4.9) in Bead Prep Buffer to resuspend.

1.5.2. Add 30 µl of resuspended beads to each reaction (from Step 1.3.6) while the samples are on the thermocycler at 60°C, and then mix gently by pipetting up and down 10 times.

Note: If you have a large number of samples, the Streptavidin Beads may start to settle as you distribute the beads to each reaction. To prevent this, pipette up and down or vortex the beads frequently to keep them in suspension while pipetting into individual reactions.

1.5.3. Change the thermocycler temperature to 48°C and incubate the reactions for 10 minutes.

1.5.4. Remove the samples from the thermocycler and place on a magnet. Wait for the solution to clear (~15 seconds), remove the supernatant, and then remove the samples from the magnet.

1.5.5. Add 150 µl of HW to each sample. **Mix by pipetting up and down 10 times.** Place the samples on a thermocycler set at 62°C (lid open) and incubate for 5 minutes.

1.5.6. Remove the samples from the thermocycler and place on a magnet. Wait for the solution to clear (~15 seconds), remove the supernatant, and then remove the samples from the magnet.

1.5.7. Repeat Steps 1.5.5–1.5.6 for a total of 2 washes at 62°C.

1.5.8. Add 150 µl of ○ (light pink) Bead Wash Buffer 2 (BW2) to each sample. **Mix by pipetting up and down 10 times.**

Samples can be kept at room temperature in BW2 buffer for up to 30 minutes. If longer storage is required, place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet. Resuspend the beads in 100 µl of 1X TE. DNA-bound beads can be stored in 1X TE for up to 24 hours at 4°C. When ready to proceed with the protocol, place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet. Add 150 µl of BW2 to each reaction, mix gently by pipetting up and down 10 times, and then proceed directly to 3´ Blunting of DNA in Section 1.6.

1.6. 3´ Blunting of DNA

1.6.1. While the beads are suspended in BW2 buffer, make a 3´ Blunting master mix by adding the following components in a sterile nuclease-free tube for the appropriate number of reactions. Vortex the 3´ Blunting Buffer to mix well prior to pipetting.

REAGENT	PER REACTION	WITH 10% OVERAGE
● (green) 3´ Blunting Buffer	97 µl	106.7 µl
● (green) 3´ Blunting Enzyme Mix	3 µl	3.3 µl
Total	100 µl	110 µl

1.6.2. Mix the master mix well by vortexing for 3–5 seconds and centrifuge briefly.

1.6.3. Place the DNA-bound beads on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.6.4. Add 100 µl of 3´ Blunting master mix (from Step 1.6.1) to each sample. **Gently mix by pipetting up and down 10 times**. Incubate the samples at 37°C for 10 minutes on a thermocycler with the thermocycler lid open.

Note: For optimal performance, it is important that the incubation time for this step is closely followed.

1.6.5. Proceed immediately with the Post-reaction Wash (Section 1.6.6).

1.6.6. Post-reaction Wash

1.6.6.1. Place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.6.6.2. Add 150 µl of ● (dark purple) Bead Wash Buffer 1 (BW1) to each sample. **Mix by pipetting up and down 10 times**. Place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.6.6.3. Add 150 µl of BW2 to each sample. **Mix by pipetting up and down 10 times**.

Samples can be kept at room temperature in BW2 buffer for up to 30 minutes. If longer storage is required, place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet. Resuspend the beads in 100 µl of 1X TE. DNA – bound beads can be stored in 1X TE for up to 24 hours at 4°C. When ready to proceed with the protocol, place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet. Add 150 µl of BW2 to each reaction, mix gently by pipetting up and down 10 times, and then proceed directly to dA-Tailing in Section 1.7.

1.7. dA-Tailing

1.7.1. While the beads are suspended in BW2 buffer, make a dA-Tailing master mix by adding the following components in a sterile nuclease-free tube for the appropriate number of reactions. Vortex the dA-Tailing Buffer to mix well prior to pipetting.

REAGENT	PER REACTION	WITH 10% OVERAGE
○ (yellow) dA-Tailing Buffer	97 µl	106.7 µl
○ (yellow) dA-Tailing Enzyme	3 µl	3.3 µl
Total	100 µl	110 µl

1.7.2. Mix the master mix well by vortexing for 3–5 seconds and centrifuge briefly.

1.7.3. Place the DNA-bound beads on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.7.4. Add 100 µl of dA-Tailing master mix (from Step 1.7.1) to each sample. **Gently mix by pipetting up and down 10 times**. Incubate the reactions at 37°C for 10 minutes on a thermocycler with the thermocycler lid open.

1.7.5. Proceed immediately with the Post-reaction Wash (Section 1.7.6).

1.7.6. Post-reaction Wash

1.7.6.1. Place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.7.6.2. Add 150 µl of BW1 to each reaction and then **mix by pipetting up and down 10 times**. Place the samples on a magnet and wait for the solution to clear (~ 15 seconds). Remove the supernatant, and then remove the reactions from the magnet.

1.7.6.3. Add 150 µl of BW2 to each sample. **Mix by pipetting up and down 10 times**.

Samples can be kept at room temperature in BW2 buffer for up to 30 minutes. If longer storage is required, place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet. Resuspend the beads in 100 µl of 1X TE. DNA-bound beads can be stored in 1X TE for up to 24 hours at 4°C. When ready to proceed with the protocol, place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet. Add 150 µl of BW2 to each reaction, mix gently by pipetting up and down 10 times, and then proceed directly to 3′ Adaptor Ligation in Section 1.8.

1.8. 3′ Adaptor Ligation

1.8.1. While the beads are suspended in BW2 buffer, make a 3′ Adaptor Ligation master mix by adding the following components in a sterile nuclease-free tube for the appropriate number of reactions. Vortex the Adaptor Ligation Buffer to mix well prior to pipetting.

REAGENT	PER REACTION	WITH 10% OVERAGE
• (red) Adaptor Ligation Buffer	80 µl	88 µl
• (red) 3′ Adaptor	10 µl	11 µl
• (red) Ligase	10 µl	11 µl
Total	100 µl	110 µl

1.8.2. Mix the master mix well by vortexing for 3–5 seconds and centrifuge briefly.

1.8.3. Place the DNA-bound beads on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.8.4. Add 100 µl of 3′ Adaptor Ligation master mix (from Step 1.8.1) to each sample. **Gently mix by pipetting up and down 10 times**.

1.8.5. Incubate the samples at 20°C for 15 minutes on a thermocycler with the thermocycler lid open.

1.8.6. Proceed immediately with the Post-ligation Wash (Section 1.8.7).

1.8.7. Post-ligation Wash

Note: The following wash steps are different than the Post-reaction Washes.

1.8.7.1. Place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.8.7.2. Add 150 µl of BW1 to each sample. **Mix by pipetting up and down 10 times**. Place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.8.7.3. Repeat Step 1.8.7.2 for a total of **2 washes** in BW1.

1.8.7.4. Add 150 µl of BW2 to each sample. **Mix by pipetting up and down 10 times**.

Samples can be kept at room temperature in BW2 buffer for up to 30 minutes. If longer storage is required, place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet. Resuspend the beads in 100 µl of 1X TE. DNA- bound beads can be stored in 1X TE for up to 24 hours at 4°C. When ready to proceed with the protocol, place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet. Add 150 µl of BW2 to each reaction, mix gently by pipetting up and down 10 times, and then proceed directly to 5′ Blunting of DNA in Section 1.9.

1.9. 5′ Blunting of DNA

1.9.1. While the beads are suspended in BW2 buffer, make a 5′ Blunting master mix by adding the following components in a sterile nuclease-free tube for the appropriate number of reactions. Vortex the 5′ Blunting Buffer to mix well prior to pipetting.

REAGENT	PER REACTION	WITH 10% OVERAGE
• (orange) 5′ Blunting Buffer	97 µl	106.7 µl
• (orange) 5′ Blunting Enzyme Mix	3 µl	3.3 µl
Total	100 µl	110 µl

1.9.2. Mix the master mix well by vortexing for 3–5 seconds and centrifuge briefly.

1.9.3. Place the DNA-bound beads on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.9.4. Add 100 µl of 5′ Blunting master mix (from Step 1.9.1) to each sample. **Gently mix by pipetting up and down 10 times**.

1.9.5. Incubate the samples at 20°C for 10 minutes on a thermocycler with the thermocycler lid open.

1.9.6. Proceed immediately with the Post-reaction Wash (Section 1.9.7).

1.9.7. Post-reaction Wash

1.9.7.1. Place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.9.7.2. Add 150 µl of BW1 to each sample. **Mix by pipetting up and down 10 times**. Place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.9.7.3. Add 150 µl of BW2 to each sample. **Mix by pipetting up and down 10 times**.

Samples can be kept at room temperature in BW2 buffer for up to 30 minutes. If longer storage is required, place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet. Resuspend the beads in 100 µl of 1X TE. DNA- bound beads can be stored in 1X TE for up to 24 hours at 4°C. When ready to proceed with the protocol, place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet. Add 150 µl of BW2 to each reaction, mix gently by pipetting up and down 10 times, and then proceed directly to 5′ Adaptor Ligation in Section 1.10.

1.10. 5′ Adaptor Ligation

1.10.1. While the beads are suspended in BW2 buffer, make a 5′ Adaptor Ligation master mix by adding the following components in a sterile nuclease-free tube for the appropriate number of reactions. Vortex the Adaptor Ligation Buffer to mix well prior to pipetting.

REAGENT	PER REACTION	WITH 10% OVERAGE
• (red) Adaptor Ligation Buffer	80 µl	88 µl
• (red) 5′ UMI Adaptor	10 µl	11 µl
• (red) Ligase	10 µl	11 µl
Total	100 µl	110 µl

1.10.2. Mix the master mix well by vortexing for 3–5 seconds and centrifuge briefly.

1.10.3. Place the DNA-bound beads on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.10.4. Add 100 µl of 5′ Adaptor Ligation master mix (from Step 1.10.1) to each sample. **Gently mix by pipetting up and down 10 times**.

1.10.5. Incubate the samples at 20°C for 20 minutes on a thermocycler with the thermocycler lid open.

1.10.6. Proceed immediately with the Post-ligation Wash (Section 1.10.7).

1.10.7. Post-ligation Wash

Note: The following wash steps are different than the Post-reaction washes.

1.10.7.1. Place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.10.7.2. Add 150 µl of BW1 to each sample. **Mix by pipetting up and down 10 times**. Place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.10.7.3. Repeat Step 1.10.7.2. for a total of **2 washes** in BW1.

1.10.7.4. Add 150 µl of BW2 to each sample. Mix by pipetting up and down 10 times.

Samples can be kept at room temperature in BW2 buffer for up to 30 minutes. If longer storage is required, place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet. Resuspend the beads in 100 µl of 1X TE. DNA- bound beads can be stored in 1X TE for up to 24 hours at 4°C. When ready to proceed with the protocol, place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet. Add 150 µl of BW2 to each reaction, mix gently by pipetting up and down 10 times, and then proceed directly to Adaptor Cleaving in Section 1.11.

1.11. Adaptor Cleaving

1.11.1. While the beads are suspended in BW2 buffer, make a Cleaving master mix by adding the following components in a sterile nuclease-free tube for the appropriate number of reactions. Vortex the Cleaving Buffer to mix well prior to pipetting.

REAGENT	PER REACTION	WITH 10% OVERAGE
• (lilac) Cleaving Buffer	95 µl	104.5 µl
• (lilac) Cleaving Enzyme Mix	5 µl	5.5 µl
Total	100 µl	110 µl

1.11.2. Mix the master mix well by vortexing for 3–5 seconds and centrifuge briefly.

1.11.3. Place the DNA-bound beads on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.11.4. Add 100 µl of Cleaving master mix (from Step 1.11.1) to each sample. **Gently mix by pipetting up and down 10 times**.

1.11.5. Incubate the samples at 37°C for 15 minutes on a thermocycler with the thermocycler lid open.

1.11.6. Proceed immediately with the Post-reaction Wash (Section 1.11.7).

1.11.7. Post-reaction Wash

1.11.7.1. Place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.11.7.2. Add 150 µl of BW1 to each sample. **Mix by pipetting up and down 10 times**. Place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet.

1.11.7.3. Add 150 µl of BW2 to each sample. **Mix by pipetting up and down 10 times**.

Samples can be kept at room temperature in BW2 buffer for up to 30 minutes. If longer storage is required, place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet. Resuspend the beads in 100 µl of 1X TE. DNA- bound beads can be stored in 1X TE for up to 24 hours at 4°C. When ready to proceed with the protocol, place the samples on a magnet and wait for the solution to clear (~15 seconds). Remove the supernatant, and then remove the samples from the magnet. Add 150 µl of BW2 to each reaction, mix gently by pipetting up and down 10 times, and then proceed directly to Library Amplification in Section 1.12.

1.12. Library Amplification

Note: Refer to Section 2 if using the NEBNext Direct Index Primer Mix Plate provided in NEB #E6631X. Refer to Section 3 for guidelines on index pooling.

1.12.1. Place the reactions on a magnet, wait for the solution to clear (~15 seconds), remove the supernatant then remove the reactions from the magnet.

1.12.2. Add 45 µl of nuclease-free, molecular grade water to each reaction. Mix gently by pipetting up and down 10 times to completely resuspend the beads.

1.12.3. Add the following components to a sterile strip tube/well in a PCR plate:

REAGENT	PER REACTION
• (blue) Q5 Master Mix	50 µl
• (blue) Index Primer Mix	5 µl
Resuspended beads (from Step 1.12.2)	45 µl
Total	100 µl

1.12.4. Gently mix by pipetting up and down 10 times. Seal the PCR plate or cap tubes.

1.12.5. Run the following program with the heated lid set to 105°C and place the samples in the thermocycler when the block temperature reaches 98°C:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	20-27*
Annealing	62°C	15 seconds	
Extension	72°C	20 seconds	
Final Extension	72°C	5 minutes	1
Hold	4°C	∞	

*Follow the PCR cycle number recommendations listed in Table 1.12.1.

Table 1.12.1: PCR Cycle Number Recommendations.

INPUT DNA	RECOMMENDED NUMBER OF PCR CYCLES < 10 kb PANEL SIZE	RECOMMENDED NUMBER OF PCR CYCLES > 10 kb PANEL SIZE
1,000 ng	22	20
500 ng	23	21
100 ng	25	23
10 ng	27	25

1.12.6. Proceed to Purify and Size Select Amplified Fragments in Section 1.13.

PCR reactions with beads can be stored for up to 24 hours at 4°C.

1.13. Purify and Size Select Amplified Fragments

1.13.1. If you detect significant evaporation from the PCR reaction, bring the volume up to 100 µl with molecular grade water.

1.13.2. Vortex the ● (brown) Sample Purification Beads to resuspend.

1.13.3. Add 85 µl of Sample Purification Beads to the PCR reactions. Mix well by pipetting up and down at least 10 times.

1.13.4. Incubate for 10 minutes uncapped at room temperature.

1.13.5. Place the tubes/PCR plate on a magnet. After the solution is clear (about 2 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets **(Caution: do not discard beads)**.

1.13.6. Add 200 µl of freshly prepared (same day) 80% EtOH while the tubes/plate are on the magnet. Incubate at room temperature for 30 seconds and then carefully remove and discard the supernatant.

1.13.7. Repeat Step 1.13.6 once for a total of **2 washes** in 80% EtOH, ensuring that all of the supernatant is removed from each reaction.

1.13.8. Incubate the samples, uncapped (or unsealed), at 37°C for 5 minutes on a thermocycler with the thermocycler lid open to dry the beads.

1.13.9. Remove the tubes/plate from the thermocycler and resuspend the dry beads in 102 µl of water. Incubate for 2 minutes at room temperature.

1.13.10. Place the tubes/plate on a magnet and allow the solution to clear (about 2 minutes).

1.13.11. Transfer 100 µl of the eluted library to fresh tubes/plate and add 85 µl of Sample Purification Beads. Mix well by pipetting up and down at least 10 times.

1.13.12. Incubate for 10 minutes at room temperature.

1.13.13. Place the tubes/plate on a magnet. After the solution is clear (about 2 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets **(Caution: do not discard beads)**.

1.13.14. Add 200 µl of freshly prepared (same day) 80% EtOH while the tubes/plate are on the magnet. Incubate at room temperature for 30 seconds and then carefully remove and discard the supernatant.

1.13.15. Repeat Step 1.13.14 once for a total of **2 washes** in 80% EtOH, ensuring that all of the supernatant is removed from each well.

1.13.16. Incubate the samples, uncapped (or unsealed), at 37°C for 2 minutes on a thermocycler with the thermocycler lid open to dry the beads.

1.13.17. Remove the tubes/plate from the thermocycler and resuspend the dry beads in 30 µl of 1X TE by gently pipetting (or gently vortex capped tubes/sealed plate, followed by a quick spin). Incubate for 2 minutes at room temperature.

1.13.18. Place the tubes on a magnet and allow the solution to clear (about 2 minutes).

1.13.19. Transfer 28 µl of the eluted library to a fresh tube.

1.13.20. Evaluate 1 µl of the eluted library on a High Sensitivity Bioanalyzer Chip or with a similar assay.

Figure 1.1: Example of libraries prepared with human DNA (NA19240).

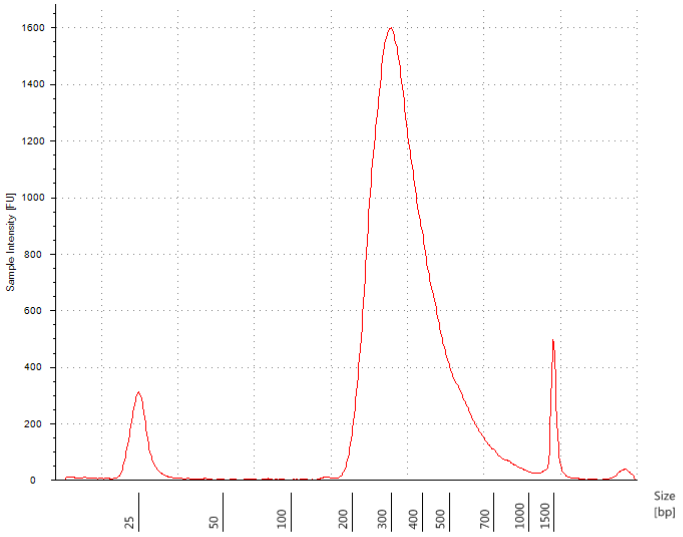


Figure 1.2A: Mean target depth achieved after PCR duplicate filtering with a 90 minute hybridization using 25–500 ng of DNA and panels containing 1–100 genes.

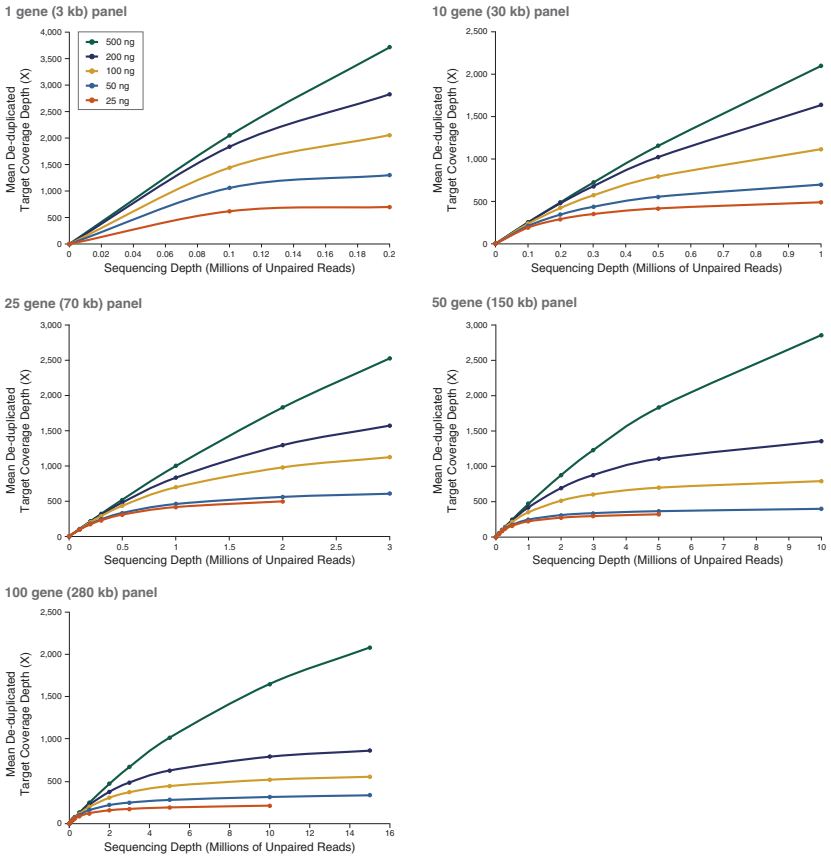
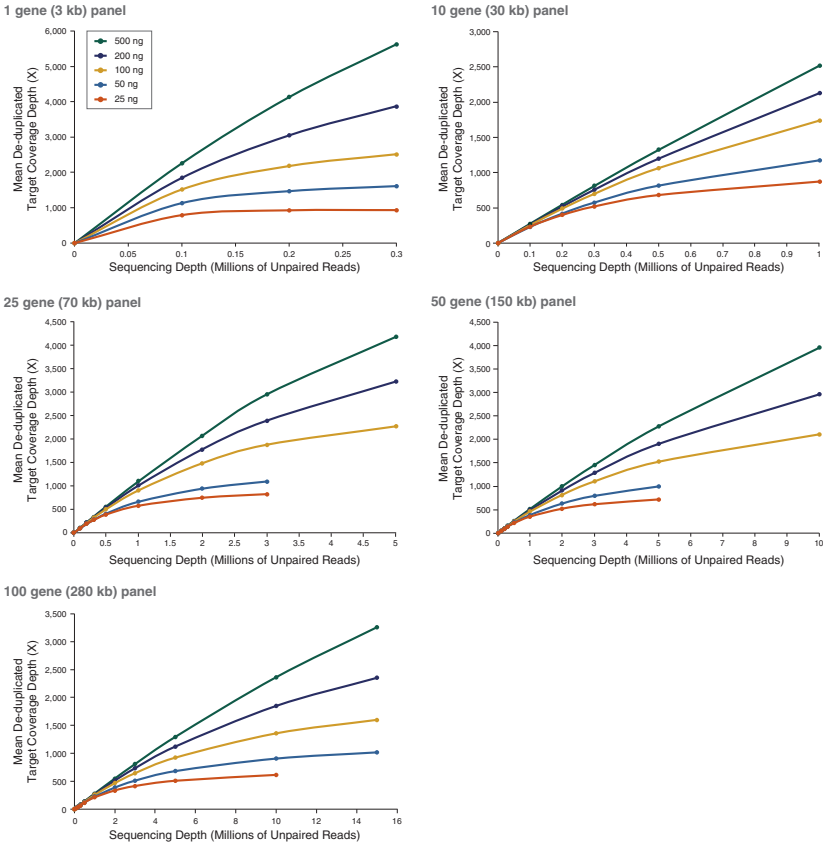


Figure 1.2B: Mean target depth achieved after PCR duplicate filtering with a 16 hour hybridization using 25–500 ng of DNA and panels containing 1–100 genes.



Section 2
Guidelines for Setting Up PCR Reactions (NEB #E6631X only)

Symbols

⚠

This caution sign signifies a step in the protocol that has multiple paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

2.1. PCR Amplification

⚠

For < 96 samples, follow the protocol in Section 2.1.1. For 96 samples, follow the protocol in Section 2.1.2.

2.1.1. Setting up the PCR reactions (< 96 samples)

2.1.1.1. Determine the number of libraries that will be amplified and pooled for subsequent sequencing.

2.1.1.2. Ensure that you choose a valid combination of barcode primers based on color balance guidelines (see Section 3).

2.1.1.3. Thaw the NEBNext Direct Index Primer Mix Plate for 10–15 minutes at room temperature.

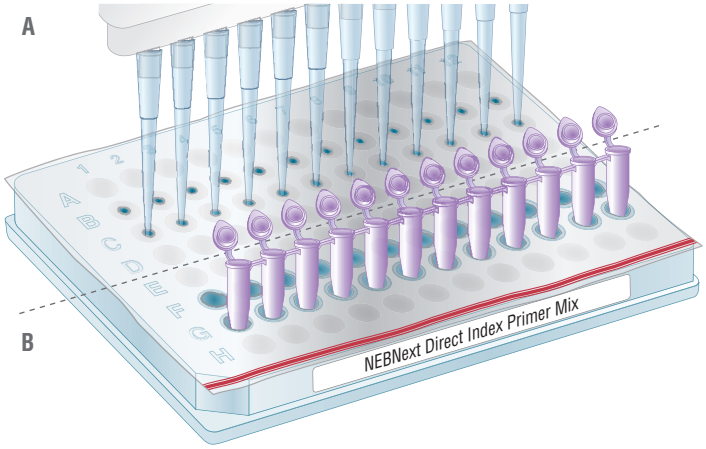
2.1.1.4. Remove the hard plastic plate cover. Briefly centrifuge the plate (280 × *g* for ~1 min) to collect all of the primer at the bottom of each well.

2.1.1.5. Orient the NEBNext Direct Index Primer Mix Plate as indicated in Figure 2.1 (red stripe towards the user). With a pipette tip, pierce the desired well(s) (Figure 2.1A) and transfer 5 µl of the primer mix required for the PCR reaction to the PCR plate/tubes. It is important to change pipette tips before piercing a new well to avoid cross contamination of indexed primers. Alternatively, the wells can be pierced using the bottom of clean PCR strip tubes (see Figure 2.1B) prior to pipetting the primer mix. Use a new, clean strip tube for each new well to be pierced.

Note: Each well contains the Universal Primer and the Index Primer. There is enough primer in each well for one PCR reaction. Do not reuse primer if the seal has been previously pierced to avoid contamination with other indexed primers.

2.1.1.6. Proceed with the PCR reaction according to Section 1, Section 1.12.

Figure 2.1: Orientation and piercing of the NEBNext Direct Index Primer Mix Plate.



2.1.2. Setting up the PCR Reactions (96 samples)

2.1.2.1. Thaw the NEBNext Direct Index Primer Mix Plate for 10–15 minutes at room temperature.

2.1.2.2. Remove the hard plastic plate cover. Briefly centrifuge the plate (280 × *g* for ~1 min) to collect all of the primer at the bottom of each well.

2.1.2.3. Orient the NEBNext Direct Index Primer Plate as indicated in Figure 2.1 (red stripe towards the user). With a pipette tip, pierce the wells (Figure 2.1A) and transfer 5 µl of the primer mix required for the PCR reaction to the PCR plate. It is important to change pipette tips before piercing a new well to avoid cross contamination of indexed primers. Alternatively, the wells can be pierced using the bottom of clean PCR strip tubes (see Figure 2.1B) prior to pipetting the primer mix. Use a new, clean strip tube for each new well to be pierced.

Note: Each well contains the Universal Primer and the Index Primer. There is enough primer in each well for one PCR reaction. Do not reuse primer if the seal has been previously pierced to avoid contamination with other indexed primers.

2.1.2.4. Proceed with the PCR reaction according to Section 1, Section 1.12.

Section 3
Index Pooling Guidelines

To download a sample sheet with the index sequences, visit the "Usage Guidelines & Tips" panel in the "Other Tools & Resources" tab on www.neb.com/E6631 – NEBNext Direct Custom Ready Panels (NEB #E6631).

For the HiSeq®/MiSeq, Illumina uses a red laser/LED to sequence bases A and C and a green laser/LED to sequence bases G and T. For each cycle, both the red and the green channel need to be read to ensure proper image registration (i.e., A or C must be in each cycle, and G or T must be in each cycle). If this color balance is not maintained, sequencing the index read could fail.

For the NovaSeq®/NextSeq®/MiniSeq® which utilize 2 color chemistry, valid index combinations must include some indices that do not start with GG in the first two cycles.

Tables 3.1, 3.2 and 3.3 list some valid combinations (up to 4-plex) that can be sequenced together. For combinations > 4 choose any 4-plex combination and add any other index as needed. For guidelines on how to choose combinations beyond what are listed below, visit the "Other Tools & Resources" tab on www.neb.com/E6631–NEBNext Direct Custom Ready Panels (NEB #E6631).

Table 3.1. Valid index primer combinations for NEB #E6631S.

PLEX	INDEX PRIMER
2	D01 and D02 D03 and D04 D05 and D06 D07 and D08
3	D01, D02 and D03 D02, D03 and D04 D03, D04 and D05 D04, D05 and D06 D05, D06 and D07 D06, D07 and D08
4	D01, D02, D03 and D04 D01, D04, D05 and D08 D02, D03, D04 and D05 D02, D04, D06 and D07 D03, D04, D05 and D06 D03, D05, D07 and D08 D04, D05, D06 and D07 D04, D05, D07 and D08 D05, D06, D07 and D08

Table 3.2. Valid index primer combinations for NEB #E6631L

PLEX	INDEX PRIMER
2	D01 and D02 D03 and D04 D05 and D06 D07 and D08 D09 and D10 D11 and D12 D17 and D23
3	D01, D02 and D03 D02, D05 and D16 D04, D05 and D06 D07, D08 and D09 D10, D11 and D12 D11, D12 and D14 D13, D17 and D22 D17, D23 and D24
4	D01, D02, D03 and D04 D02, D04, D06 and D07 D03, D04, D14 and D17 D05, D06, D07 and D08 D07, D08, D13 and D22 D08, D14, D21 and D22 D09, D10, D11 and D12 D13, D14, D16 and D17 D15, D17, D22 and D23 D16, D17, D20 and D24

Table 3.3. Valid index primer combinations for NEB #E6631X

PLEX	INDEX PRIMER (WELL POSITION)
2	DHT18 (B6) and DHT22 (B10) DHT37 (D1) and DHT42 (D6) DHT52 (E4) and DHT76 (G4) DHT68 (F8) and DHT95 (H11)
3	DHT4 (A4), DHT11 (A11) and DHT12 (A12) DHT15 (B3), DHT22 (B10) and DHT24 (B12) DHT25 (C1), DHT31 (C7) and DHT33 (C9) DHT37 (D1), DHT42 (D6) and DHT48 (D12) DHT49 (E1), DHT54 (E6) and DHT55 (E7) DHT64 (F4), DHT69 (F9) and DHT71 (F11) DHT76 (G4), DHT77 (G5) and DHT83 (G11) DHT87 (H3), DHT93 (H9) and DHT94 (H10)
4	DHT1 (A1), DHT2 (A2), DHT3 (A3) and DHT4 (A4) DHT5 (A5), DHT6 (A6), DHT8 (A8) and DHT10 (A10) DHT13 (B1), DHT14 (B2), DHT15 (B3) and DHT16 (B4) DHT17 (B5), DHT18 (B6), DHT19 (B7) and DHT20 (B8) DHT25 (C1), DHT26 (C2), DHT27 (C3) and DHT30 (C6) DHT28 (C4), DHT29 (C5), DHT32 (C8) and DHT35 (C11) DHT37 (D1), DHT38 (D2), DHT39 (D3) and DHT40 (D4) DHT45 (D9), DHT46 (D10), DHT47 (D11) and DHT48 (D12) DHT49 (E1), DHT50 (E2), DHT51 (E3) and DHT52 (E4) DHT56 (E8), DHT58 (E10), DHT59 (E11) and DHT60 (E12) DHT61 (F1), DHT62 (F2), DHT63 (F3) and DHT69 (F9) DHT64 (F4), DHT65 (F5), DHT66 (F6) and DHT67 (F7) DHT73 (G1), DHT74 (G2), DHT75 (G3) and DHT76 (G4) DHT80 (G8), DHT82 (G10), DHT83 (G11) and DHT84 (G12) DHT85 (H1), DHT86 (H2), DHT87 (H3) and DHT89 (H5) DHT91 (H7), DHT94 (H10), DHT95 (H11) and DHT96 (H12)

Section 4
Guidelines for Running Samples on the Illumina MiSeq

The NEBNext Direct protocol incorporates Illumina adaptor sequences; therefore, the libraries generated from this protocol may be sequenced on Illumina platforms including the MiSeq, NextSeq®, and HiSeq platforms. Here we describe the steps necessary to sequence NEBNext Direct libraries on the Illumina MiSeq.

If samples are run on the Illumina NextSeq, please note that while the i5 index is generated in the reverse complement orientation, no adjustments need to be made because the i5 UMI is a random sequence.

4.1. Reconfigure the MiSeq Reporter software to write both index reads to FASTQs.
By default, the Illumina MiSeq is set to not generate FASTQs for index reads. It is necessary to change this setting in order to make use of the 8 base sample barcode in the i7 position (index read 1) and the 12 base UMI in the i5 position (index read 2).

4.1.1. Open the MiSeq Reporter configuration file (C:\Illumina\ MiSeq Reporter\MiSeqReporter.exe.config).

4.1.2. Edit the "CreateFastqForIndexReads" setting in the MiSeq Reporter configuration file by adding the following line (or edit the existing line so the value is "1") between the <appSettings> tags:

<add key="CreateFastqForIndexReads" value="1"/>

4.1.3. Save the updated file on the MiSeq as C:\Illumina\MiSeq Reporter\MiSeqReporter.exe.config

4.2. Prepare a MiSeq sample sheet:

4.2.1. Download a MiSeq sample sheet from the "Other Tools & Resources" tab on the NEBNext Direct website at: https://www.neb.com/E6631 – NEBNext Direct Custom Ready Panels (NEB #E6631). Do not use the Illumina Experiment Manager to generate a sample sheet.

4.2.2. Fill in the empty fields with your sample and barcode information.

4.2.3. Transfer the sample sheet file (*.csv) to the Miseq and save the file in D:\Illumina\Miseq Control Software\SampleSheets\

4.3. Pool, dilute, and denature samples* for an 8 pM final concentration following the MiSeq Denature and Dilute Libraries Guide and the Index Pooling Guidelines in Section 3, page 20.**

** The number of samples that can be pooled together will depend on the input amount, panel size, and number of reads required for each sample for the particular analysis being performed. For guidelines on the number of reads required for a desired target coverage, see Figure 1.2A & 1.2B, page 17–18.*

*** Based on quantification by a Qubit™ dsDNA HS Assay Kit, Agilent® High Sensitivity DNA Kit, or Agilent High Sensitivity D1000 ScreenTape. For other quantification methods, this concentration may need to be empirically determined for optimal cluster density.*

4.4. Follow the MiSeq System Guide to load your samples and run the MiSeq. When prompted, upload the sample sheet that was prepared in 4.1.2.

Kit Components

NEB # N6631S Table of Components

NEB #	PRODUCT	VOLUME
N6631S	NEBNext Direct Custom Ready Baits	0.04 ml

NEB #E6635S Table of Components

NEB #	PRODUCT	VOLUME
E7089-2	NEBNext Direct Stop Solution	0.04 ml
E7088-2	NEBNext Direct DNA Nicking Enzyme	0.024 ml
E7087-2	NEBNext Direct DNA Nicking Buffer	0.032 ml
E7050-2	NEBNext Direct Hybridization Additive	0.16 ml
E7084-2	NEBNext Direct 3′ Blunting Enzyme Mix	0.024 ml
E7015-2	NEBNext Direct dA-Tailing Enzyme	0.024 ml
E7011-2	NEBNext Direct 3′ Adaptor	0.08 ml
E7016-2	NEBNext Direct Ligase	0.16 ml
E7017-2	NEBNext Direct 5′ Blunting Enzyme Mix	0.024 ml
E7012-2	NEBNext Direct 5′ UMI Adaptor	0.08 ml
E7018-2	NEBNext Direct Cleaving Enzyme Mix	0.04 ml
E7019-2	NEBNext Direct Q5 Master Mix	0.4 ml
E7020-2 thru E7027-2	NEBNext Direct Index Primer Mix D01–D08	8 x 0.005 ml
E7009-2	NEBNext Direct Bead Wash 1	9.6 ml
E7048-2	NEBNext Sample Purification Beads	2 ml
E7049-2	NEBNext Direct Streptavidin Beads	0.6 ml
E7082-2	NEBNext Direct Hybridization Wash (HW)	6.0 ml
E7001-2	NEBNext Direct Bead Prep Buffer	0.4 ml
E7083-2	NEBNext Direct Hybridization Buffer	0.38 ml
E7080-2	NEBNext Direct 3′ Blunting Buffer	0.78 ml
E7004-2	NEBNext Direct dA-Tailing Buffer	0.78 ml
E7005-2	NEBNext Direct Adaptor Ligation Buffer	1.28 ml
E7081-2	NEBNext Direct 5′ Blunting Buffer	0.78 ml
E7007-2	NEBNext Direct Cleaving Buffer	0.76 ml
E7010-2	NEBNext Direct Bead Wash 2	10.8 ml

NEB #N6631L Table of Components

NEB #	PRODUCT	VOLUME
N6631L	NEBNext Direct Custom Ready Baits	0.12 ml

NEB #E6635L Table of Components

NEB #	PRODUCT	VOLUME
E7089-3	NEBNext Direct Stop Solution	0.12 ml
E7088-3	NEBNext Direct DNA Nicking Enzyme	0.072 ml
E7087-3	NEBNext Direct DNA Nicking Buffer	0.096 ml
E7050-3	NEBNext Direct Hybridization Additive	0.48 ml
E7084-3	NEBNext Direct 3′ Blunting Enzyme Mix	0.072 ml
E7015-3	NEBNext Direct dA-Tailing Enzyme	0.072 ml
E7011-3	NEBNext Direct 3′ Adaptor	0.24 ml

E7016-3	NEBNext Direct Ligase	0.48 ml
E7017-3	NEBNext Direct 5′ Blunting Enzyme Mix	0.072 ml
E7012-3	NEBNext Direct 5′ UMI Adaptor	0.24 ml
E7018-3	NEBNext Direct Cleaving Enzyme Mix	0.12 ml
E7019-3	NEBNext Direct Q5 Master Mix	2 x 0.6 ml
E7020-3 thru E7043-3	NEBNext Direct Index Primer Mix D01–D24	24 x 0.005 ml
E7009-3	NEBNext Direct Bead Wash 1	28.8 ml
E7048-3	NEBNext Sample Purification Beads	5 ml
E7049-3	NEBNext Direct Streptavidin Beads	1.8 ml
E7082-3	NEBNext Direct Hybridization Wash (HW)	18 ml
E7001-3	NEBNext Direct Bead Prep Buffer	1.2 ml
E7083-3	NEBNext Direct Hybridization Buffer	1.15 ml
E7080-3	NEBNext Direct 3′ Blunting Buffer	2.33 ml
E7004-3	NEBNext Direct dA-Tailing Buffer	2.33 ml
E7005-3	NEBNext Direct Adaptor Ligation Buffer	3.84 ml
E7081-3	NEBNext Direct 5′ Blunting Buffer	2.33 ml
E7007-3	NEBNext Direct Cleaving Buffer	2.28 ml
E7010-3	NEBNext Direct Bead Wash 2	32.4 ml

NEB #E6631X Table of Components

NEB #	PRODUCT	VOLUME
N6631X	NEBNext Direct Custom Ready Baits	0.48 ml

NEB #E6635X Table of Components

NEB #	PRODUCT	VOLUME
E7089-4	NEBNext Direct Stop Solution	0.48 ml
E7088-4	NEBNext Direct DNA Nicking Enzyme	0.288 ml
E7087-4	NEBNext Direct DNA Nicking Buffer	0.384 ml
E7050-4	NEBNext Direct Hybridization Additive	1.92 ml
E7084-4	NEBNext Direct 3′ Blunting Enzyme Mix	0.288 ml
E7015-4	NEBNext Direct dA-Tailing Enzyme	0.288 ml
E7011-4	NEBNext Direct 3′ Adaptor	0.96 ml
E7016-4	NEBNext Direct Ligase	2 x 0.96 ml
E7017-4	NEBNext Direct 5′ Blunting Enzyme Mix	0.288 ml
E7012-4	NEBNext Direct 5′ UMI Adaptor	0.96 ml
E7018-4	NEBNext Direct Cleaving Enzyme Mix	0.48 ml
E7019-4	NEBNext Direct Q5 Master Mix	4.8 ml
E7044-4	NEBNext Direct Index Primer Mix Plate	5 µl/well
E7009-4	NEBNext Direct Bead Wash 1	115.2 ml
E7048-4	NEBNext Sample Purification Beads	2 x 10 ml
E7049-4	NEBNext Direct Streptavidin Beads	7.2 ml
E7082-4	NEBNext Direct Hybridization Wash (HW)	72 ml
E7001-4	NEBNext Direct Bead Prep Buffer	4.8 ml
E7083-4	NEBNext Direct Hybridization Buffer	4.55 ml
E7080-4	NEBNext Direct 3′ Blunting Buffer	9.32 ml
E7004-4	NEBNext Direct dA-Tailing Buffer	9.32 ml

E7005-4	NEBNext Direct Adaptor Ligation Buffer	15.36 ml
E7081-4	NEBNext Direct 5′ Blunting Buffer	9.31 ml
E7007-4	NEBNext Direct Cleaving Buffer	9.12 ml
E7010-4	NEBNext Direct Bead Wash 2	130 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	4/18
2.0	Update Kit Includes with colored caps. Update Introduction text. Add Critical Guidelines, Helpful Tips and Before You Begin text. Adjust Step 1.2.4. Move Figures 2A and 2B to figures 1.2A and 1.2B. Update text on Section 3 to mention the Other Tools and Resources Tab instead of FAQs tab. Update Step 4.1.2 and 4.1.4 of Section 4. Add new Step 1.1 to Section 1 and update Section 1 text. Update title of Figure 2.1. Update text and remove tables in Section 4 Change kit component package numbers.	8/18
3.0	New format applied	6/20