

## NEBNext Direct<sup>®</sup> Genotyping Solution

NEB #E9500B-S – Sample Prep Kit

NEB #E9530B-S – Target Enrichment Kit

Version 2.0\_4/24

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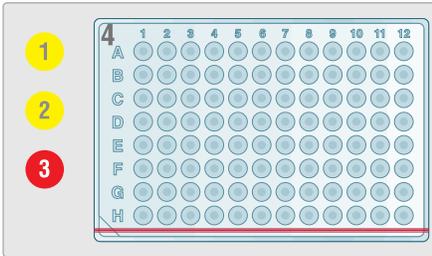
## The NEB #E9500B-S NEBNext Direct Genotyping Solution Sample Prep Kit Includes

NEB #E9500B-S contains NEB #E9500-1 (Box) and NEB #E9500-2 (Pouch), and is sufficient for preparation of up to 96 reactions.

### NEB #E9500-1

**Box: Store at -20°C.**

1. ○ (yellow) NEBNext Direct GS Fragmentation Buffer
2. ○ (yellow) NEBNext Direct GS Fragmentation Enzyme Mix
3. ● (red) NEBNext Direct GS 5' Adaptor Ligation Master Mix
4. ● (red) NEBNext Direct GS Indexed 5' Adaptor Plate



### NEB #E9500-2

**Pouch: Store at Room Temperature. Do not freeze.**

1. ● (brown) NEBNext Direct GS Stop Solution
2. ● (brown) NEBNext Direct GS Sample Purification Beads

## The NEB #E9530B-S NEBNext Direct Genotyping Solution Target Enrichment Kit Includes

NEB #E9530B-S contains NEB #E9530-1 (Box 1 of 2) and NEB #E9530-2 (Box 2 of 2), and is sufficient for preparation of up to 8 reactions.

### NEB #E9530-1

**Box 1 of 2: Store at -20°C.**

1. ○ (white) NEBNext Direct GS Hybridization Additive
  2. ● (green) NEBNext Direct GS 3' Blunting Enzyme
  3. ● (red) NEBNext Direct GS 3' Adaptor
  4. ● (red) NEBNext Direct GS Ligase
  5. ● (lilac) NEBNext Direct GS Specificity Enhancer Enzyme Mix
  6. ● (blue) NEBNext Direct GS Q5® Master Mix
- DGS01-DGS08 ● (dark blue) NEBNext Direct GS Index Primer Mix



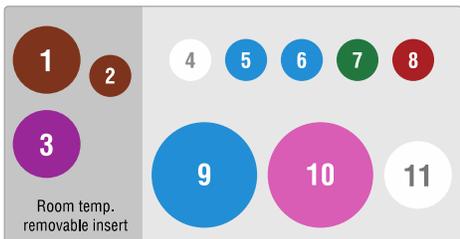
## NEB #E9530-2

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

1. ● (brown) NEBNext Direct GS Pre-Hyb Sample Purification Beads\*
2. ● (brown) NEBNext Direct GS Post-PCR Sample Purification Beads\*
3. ● (dark purple) NEBNext Direct GS Bead Wash 1 (BW1)\*\*
4. ○ (white) NEBNext Direct GS Hybridization Buffer
5. ● (blue) NEBNext Direct GS Streptavidin Beads
6. ● (blue) NEBNext Direct GS Bead Prep Buffer
7. ● (green) NEBNext Direct GS 3' Blunting Buffer
8. ● (red) NEBNext Direct GS 3' Adaptor Ligation Buffer
9. ● (blue) NEBNext Direct GS Hybridization Wash (HW)
10. ○ (light pink) NEBNext Direct GS Bead Wash 2 (BW2)
11. ○ (white) NEBNext Direct GS TE Buffer

\* This component should be stored at room temperature. Separate vials of Sample Purification Beads are supplied for protocol Step 1.5 (Pre-hybridization Bead Cleanup) and Step 1.13 (Purify and Size Select Amplified Fragments) for storage in pre-PCR and post-PCR spaces, if desired.

\*\* 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.



## Required Equipment

- Thermal cycler programmable to 100 µl with a programmable heated lid
- Agilent® TapeStation® or similar instrument
- Centrifuge for plates and tubes
- Fluorometric plate reader, Qubit® fluorometer, or other fluorometric device to quantify input DNA

## Required Materials

- E9500 – NEBNext Direct Genotyping Solution Sample Prep Kit
- E9530 – NEBNext Direct Genotyping Solution Target Enrichment Kit

E9500 and E9530 are both required for the NEBNext Direct GS workflow to convert molecules containing loci of interest into a sequence-ready library. Each kit can be purchased separately to enable the flexibility to pool anywhere from 4-96 samples from the Sample Prep Kit into a single reaction of the Target Enrichment Kit.

## Required Materials Not Included

- Molecular grade ethanol
- Sterile, nuclease-free water
- 0.2 ml PCR strip tubes and caps or 96/384 well PCR plates and seals
- 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 (optional 5 ml or 15 ml tube magnet)
- Microseal® 'B' PCR Plate Sealing Film, adhesive optical #msb1001 or similar PCR plate seal
- Agilent High Sensitivity D1000 ScreenTape and Reagents (Agilent, cat #5067-5584 and #5067-5585) or similar
- Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific, cat #P11496), Qubit dsDNA HS Assay Kit (ThermoFisher Scientific, cat #Q32854), or similar fluorometric assay to quantify input DNA

## Introduction

The NEBNext Direct Genotyping Solution provides scalable, cost-effective, sequence-based targeted genotyping for plant, animal and human applications. The approach is capable of supporting high-throughput processing of samples through high levels of sample multiplexing prior to hybridization-based enrichment of 100-5,000 marker loci (Figure 1).

### E9500 – NEBNext Direct Genotyping Solution Sample Prep Kit

Using the NEBNext Direct GS Sample Prep Kit, individual DNA samples are enzymatically fragmented and 5' index-tagged with an Illumina-compatible P5 adaptor. This adaptor incorporates two additional sequence elements: (1) an inline unique molecular identifier (UMI) to mark each unique DNA fragment within each sample followed by (2) an inline sample index to barcode each sample prior to pooling. After barcoding, up to 96 samples are subsequently pooled together into a single tube to be processed through the NEBNext Direct Genotyping Solution Target Enrichment Kit.

### E9530 – NEBNext Direct Genotyping Solution Target Enrichment Kit

Using the NEBNext Direct GS Target Enrichment Kit, biotinylated baits are hybridized to targets for 90 minutes, then target fragments are bound to streptavidin beads and trimmed to remove flanking, off-target sequence. Next, the 3' sequencing adaptor is ligated, and the captured fragments are PCR amplified to generate full length libraries.

The multiplexed libraries can be sequenced on any Illumina sequencing platform in a rapid sequencing strategy (Figure 2):

- 20 cycles of Read 1 sequences the inline UMI and sample barcode.
- 8 cycles of the i7 read (Index 1) sequences a second index added to all samples that were pooled and processed together through the NEBNext Direct GS Target Enrichment Kit. If a single target enrichment library pool is sequenced alone (only one i7 barcode is used), this read should be skipped.
- 75 cycles of Read 2 sequences the targeted regions containing loci of interest.

The NEBNext Direct Genotyping Solution Sample Prep and Target Enrichment Kits can be flexibly combined to support variable amounts of sample pooling into the Target Enrichment Kit. Together, these two kits contain the oligonucleotides, enzymes, and buffers to enrich and convert molecules containing loci of interest into sequence-ready libraries.

**BED files:** BED files for the NEBNext Direct Genotyping Solutions 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 regions that are expected to be captured based on the baits that were designed (captured\_regions.bed), and 3) the subset of targeted regions that were unable to be designed for and will not be captured by the panel, typically due to high homology with other regions of the genome (targeted\_not\_captured\_regions.bed).

For human panels, BED files are provided in both hg19 and GRCh38/hg38 coordinates. For non-human panels, BED files are provided using the coordinates of the reference genome designated or supplied by the customer. If amplicon sequences are provided, a synthetic genome will be created from the amplicon sequences and the synthetic genome will be provided along with the corresponding BED files. 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 Customized Solutions Team at NEB. Please contact custom@neb.com for further information.

Figure 1. NEBNext Direct Genotyping Solution Workflow.

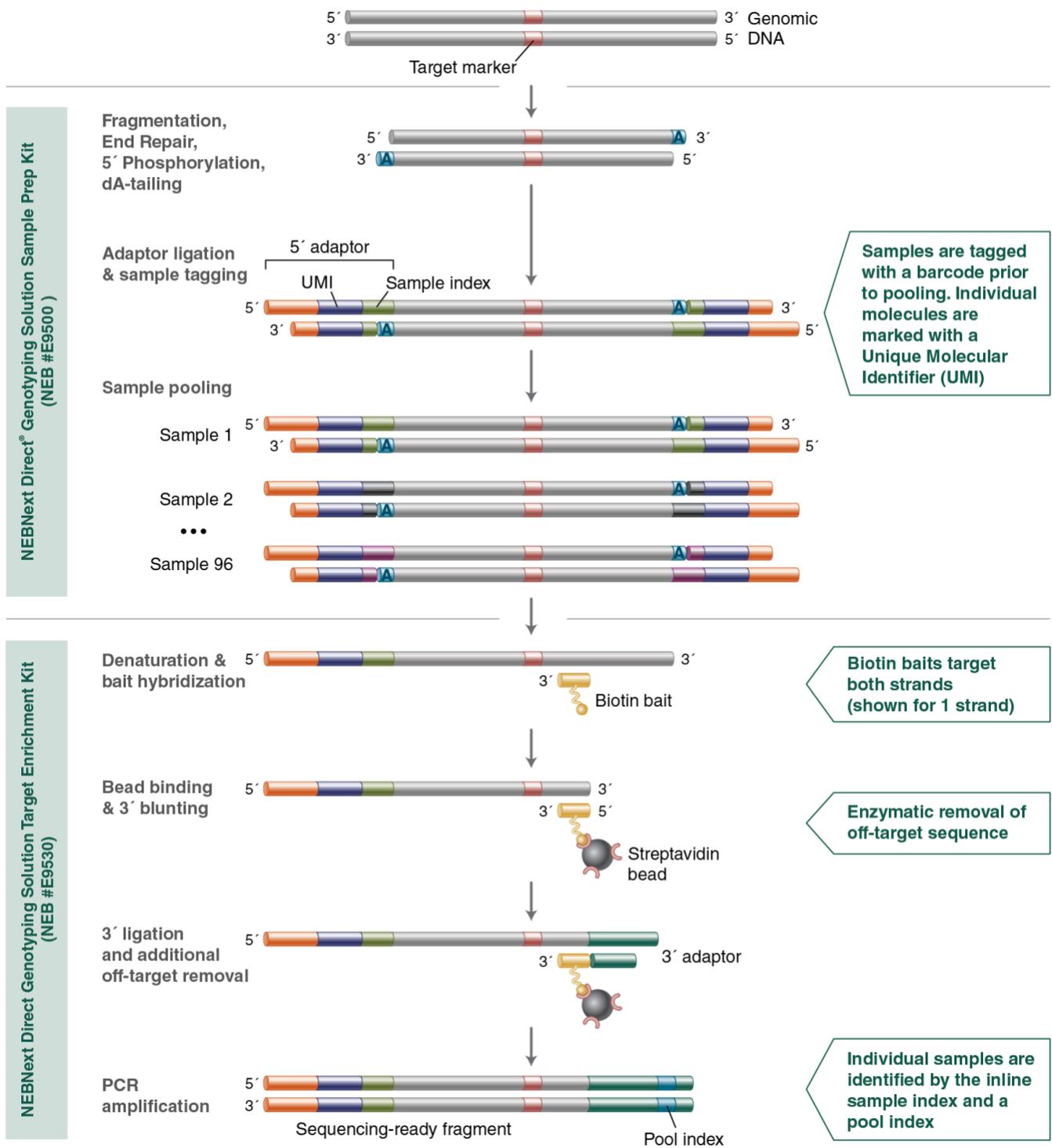
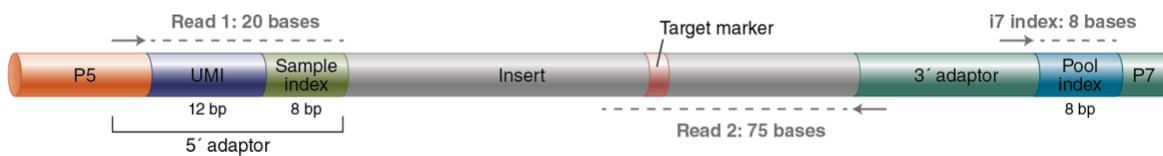


Figure 2. NEBNext Direct Genotyping Solution Sequencing Read Structure.



## Critical Guidelines

- Note that the captured molecules on streptavidin beads are unamplified, so any beads that are discarded during buffer exchanges or washes will result in fewer unique molecules in the final library and reduced overall yields. Take care to minimize bead loss during washes and buffer exchanges by checking the pipette tips for adhered beads prior to discarding.
- Perform all washes by pipetting up and down at least 10 times. Insufficient washing can lead to carryover of reagents, resulting in adaptor dimer formation and/or reduced yields. Foaming of the wash buffers may occur due to the presence of detergents. Moderate amounts of foaming will not impact performance.
- The 5' Ligation Master Mix, Hybridization Buffer, 3' Blunting Buffer, and 3' Ligation Buffer are viscous and should be pipetted slowly to ensure accurate volume delivery.
- **A thermal cycler programmable to 100 µl is required for this protocol.** A thermal cycler programmable to lower volumes is not compatible with the reaction volumes throughout the protocol and will negatively impact performance. The hybridization, bead binding, and wash volumes will exceed 100 µl, but a thermal cycler programmable to 100 µl is sufficient for these steps.
- NEBNext Direct Genotyping Solution panels and adaptors are designed to be sequenced on Illumina instruments with 20 cycles of Read 1 to sequence the 12 base UMI and 8 base sample index, 8 cycles of Index 1 to sequence the pool index, and 75 cycles of Read 2 to sequence the targets (Figure 2). **If a single target enrichment library pool is sequenced alone (only one i7 barcode is used), the Index 1 read should be excluded.** This protocol is incompatible with the Illumina Experiment Manager. See Section 4 and Section 5 for additional information.
- Reaction temperatures are carefully optimized for best target conversion. Strictly adhere to reaction temperatures and times throughout the protocol.

## Helpful Tips

- For Step 1.2 (Fragmentation and End Prep), we recommend making a master mix using 20% more volume than required per reaction. For Steps 1.6 (Bait Hybridization), 1.9 (3' Blunting of DNA), 1.10 (3' Adaptor Ligation), and 1.12 (Library Amplification), we recommend making master mixes using 10% more volume than required per reaction. Overage is included for each component to accommodate excess for master mixes.
- Multichannel pipettes (10 µl and 200 µl) are recommended for washing and mixing steps.
- To save time, master mixes for steps 1.6 (Bait Hybridization), 1.9 (3' Blunting), 1.10 (3' Adaptor Ligation) and 1.12 (Library Amplification) can be prepared during the previous incubation and kept on ice until needed.
- It is recommended to use a thermal cycler on a bench with accessible workspace for this protocol.

## Before You Begin

- Pre-chill sterile, nuclease-free water on ice or at 4°C for the Fragmentation and End Prep (Step 1.2).
- Bring all wash solutions from Box 2 of the NEBNext Direct GS Target Enrichment Kit (NEB #E9530-2) to room temperature before use.
- Bring the Sample Purification Beads from the NEBNext Direct GS Sample Prep Kit Pouch (NEB #E9500-2) and from Box 2 of the NEBNext Direct GS Target Enrichment Kit (NEB #E9530-2) 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 (~ 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/water bath to dissolve the precipitate.

## Section 1

### Protocol for use with NEBNext Direct Genotyping Solution

#### 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 number of samples being processed.



Stopping points in the protocol.



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

### NEBNext Direct Genotyping Solution Sample Prep Kit (NEB #E9500B-S)

#### 1.1. Quantify DNA

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

#### 1.2. Fragmentation and End Prep

- 1.2.1. Vortex the Fragmentation and End Prep Enzyme Mix thoroughly for 5–8 seconds prior to use.

**Note: It is important to vortex the enzyme mix prior to use for optimal performance.**

- 1.2.2. Set up the following reaction on ice, in either a 384-well plate or a 96-well plate. DNA should be diluted in pre-chilled, sterile, nuclease-free water to a total volume of **6 µl**. Aliquot DNA and water first, then add **2 µl** of master-mixed buffer and enzyme to samples.

REAGENT	PER REACTION	WITH 20% OVERAGE
DNA (10–100 ng)*	6 µl	N/A
• (yellow) Fragmentation Buffer	1.5 µl	1.8 µl
• (yellow) Fragmentation Enzyme Mix	0.5 µl	0.6 µl
<b>Total Volume</b>	<b>8 µl</b>	<b>N/A</b>

\* See Table 1.2.1 for guidelines on DNA input amounts.

**Table 1.2.1: Recommended DNA Input Amounts Based on Haploid Genome Size and Panel Size.**

HAPLOID GENOME SIZE	RECOMMENDED DNA INPUT AMOUNT FOR 100–500 SNP PANELS	RECOMMENDED DNA INPUT AMOUNT FOR 501–5,000 SNP PANELS
< 1 Gb	10–25 ng	25–50 ng
> 1 Gb	25–50 ng	50–100 ng

- 1.2.3. Seal the plate, briefly vortex the samples to mix, and quickly spin down the plate prior to incubation. Alternatively, samples may be mixed by pipetting up and down at least 10 times, then covered with a plate seal and quickly spun down for incubation.
- 1.2.4. Run the following program in a thermal cycler with the heated lid set to 75°C:

15 mins @ 37°C  
30 mins @ 65°C  
Hold @ 4°C



**If necessary, samples can be stored at -20°C for up to 16 hours; however, we recommend continuing with 5' Adaptor Ligation before stopping.**

### 1.3. 5' Adaptor Ligation with Sample Indexing

- 1.3.1. Determine the number of samples (between 4 to 96) that will be pooled together for a single hybridization.
- 1.3.2. Ensure that you choose a valid combination of barcoded adaptors following guidelines in Section 3, Table 3.1.
- 1.3.3. Refer to Section 2 on for guidelines on using the single-use ● (red) NEBNext Direct GS Indexed 5' Adaptor Plate.
- 1.3.4. Briefly spin down the sample plate from Step 1.2.4 to collect samples to the bottom of the wells.
- 1.3.5. Remove the plate seal and to each sample well add 5 µl of indexed 5' adaptor from the ● (red) NEBNext Direct GS Indexed 5' Adaptor Plate. The 5' Adaptor incorporates a unique sample index into each sample prior to pooling. **Use one unique indexed adaptor per sample** that will be subsequently pooled together.
- 1.3.6. Add 10 µl of ● (red) 5' Adaptor Ligation Master Mix to each sample.
- 1.3.7. Mix the samples by sealing the plate and vortexing thoroughly, followed by a quick spin, or by pipetting up and down at least 10 times.
- 1.3.8. Incubate the sample in a thermal cycler for 15 minutes at 20°C with the lid unheated.
- 1.3.9. Remove the plate from the thermal cycler and stop the ligation by adding 2.5 µl of ● (brown) Stop Solution. Mix well by sealing the plate and vortexing thoroughly, followed by a quick spin, or by pipetting up and down at least 10 times.

### 1.4. Sample Pooling and Bead Cleanup

- 1.4.1. Combine 24 µl from each uniquely indexed sample into an appropriately sized fresh, sterile, nuclease-free tube(s) based on the total volume specified in Step 1.4.3.

Note: Each pool of samples should contain all uniquely indexed samples intended for a single hybridization reaction.

- 1.4.2. Immediately before use, vortex the ● (brown) Sample Purification Beads thoroughly to resuspend.
- 1.4.3. Add ● (brown) Sample Purification Beads to each pool of samples for a 0.8X bead cleanup. See below for example volumes of Sample Purification Beads to add based on the number of samples pooled:

# OF POOLED SAMPLES	VOLUME OF POOLED SAMPLES	VOLUME OF SAMPLE PURIFICATION BEADS	TOTAL VOLUME
24	576 µl	461 µl	1,037 µl
47	1,128 µl	902 µl	2,030 µl
96	2,304 µl	1,843 µl	4,147 µl

- 1.4.4. Mix well by vortexing thoroughly or by pipetting up and down at least 10 times.
- 1.4.5. Incubate the samples for 5 minutes at room temperature.
- 1.4.6.  **For sample pools ≤ 46 samples:** Place the samples on a magnet and wait for the solution to clear.  
**For sample pools greater > 46 samples:** Either a 5 ml or 15 ml tube with the pooled samples and beads can be placed on an appropriate magnet, or the pooled samples and beads can be distributed into multiple 1.5 ml or 2 ml tubes and placed on a tube magnet.
- 1.4.7. 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 fragments (**Caution: do not discard the beads**).
- 1.4.8. While the tubes are on the magnet, add enough 80% EtOH (freshly prepared) to cover the bead pellet. For example, if the samples are in a 1.5 ml tube, ~1 ml of 80% EtOH is sufficient to cover the bead pellet; if the samples are in a 15 ml falcon tube, ~10 ml of EtOH is required to cover the bead pellet depending on the model of the magnet used.
- 1.4.9. Incubate the samples at room temperature for 30 seconds, then carefully remove and discard the 80% EtOH. Be careful not to disturb the beads that contain the DNA fragments.
- 1.4.10. Repeat steps 1.4.8–1.4.9 once for a total of **2 washes** in 80% EtOH. Ensure that all visible liquid is removed after the second wash.

1.4.11. Incubate the samples on the magnet at room temperature with the tube caps open for up to 5 minutes to dry the beads.

**Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.**



1.4.12. **For sample pools ≤ 46 samples:** Remove the tubes from the magnet and resuspend the beads in 200 µl of sterile, nuclease-free water to elute the samples from the beads.

**For sample pools > 46 samples:** Remove the tubes from the magnet. If the samples and beads were distributed across multiple 1.5 ml or 2 ml tubes for ethanol washes, divide 200 µl of sterile, nuclease-free water across the number of tubes used to resuspend each bead pellet and elute the samples from the beads. If the sample pool and beads were kept in a 5 ml or 15 ml tube, resuspend the beads in 200 µl of sterile, nuclease-free water to elute the samples from the beads.

1.4.13. Incubate for 2 minutes at room temperature.

1.4.14. Place the samples on a magnet and wait for the solution to clear.



1.4.15. **For sample pools ≤ 46 samples:** Transfer 198 µl of the eluted sample to a fresh tube.

**For sample pools > 46 samples:** If the samples and beads were distributed across multiple 1.5 ml or 2 ml tubes for ethanol washes, combine the eluates from the multiple tubes to transfer a combined total of 198 µl of elution into a fresh tube.

1.4.16. Proceed immediately with Step 1.5 using the NEBNext Direct Genotyping Solution Target Enrichment Kit (NEB #E9530), or store samples at -20°C for up to 2 weeks.



**Purified DNA sample pools can be stored for up to 2 weeks at -20°C.**

## NEBNext Direct Genotyping Solution Target Enrichment Kit (NEB #E9530B-S)

### 1.5. Pre-hybridization Bead Cleanup

1.5.1. Immediately before use, vortex the ● (brown) Pre-Hyb Sample Purification Beads thoroughly to resuspend.

1.5.2. Add 360 µl of ● (brown) Pre-Hyb Sample Purification Beads to the pooled samples from Step 1.4.15.

1.5.3. Mix well by vortexing thoroughly or by pipetting up and down 10 times with the pipette set to 500 µl.

1.5.4. Incubate the samples for 5 minutes at room temperature.

1.5.5. Place the samples on a magnet and wait for the solution to clear.

1.5.6. 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 fragments (**Caution: do not discard the beads**).

1.5.7. While the tubes are on the magnet, add 1 ml of freshly prepared (same day) 80% EtOH to each tube.

1.5.8. Incubate the samples at room temperature for 30 seconds, then carefully remove and discard the 80% EtOH. Be careful not to disturb the beads that contain the DNA fragments.

1.5.9. Repeat Steps 1.5.7.–1.5.8. once for a total of **2 washes** in 80% EtOH. Ensure that all visible liquid is removed after the second wash.

1.5.10. Incubate the samples on the magnet at room temperature with the tube caps open for up to 5 minutes to dry the beads.

**Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.**

1.5.11. Resuspend the dry beads in 62 µl of sterile, nuclease-free water to elute the samples from the beads.

1.5.12. Incubate for 2 minutes at room temperature.

1.5.13. Place the samples on a magnet and wait for the solution to clear.

- 1.5.14. Transfer 60  $\mu\text{l}$  of the eluted sample into a fresh PCR strip tube or plate. Take care not to transfer Sample Purification Beads with the eluted sample.

**Note: Any Sample Purification Beads carried over into the hybridization will increase the off-target molecules that are converted into the final library.**

## 1.6. Bait Hybridization

- 1.6.1. Make a Hybridization master mix by combining the following components for the appropriate number of reactions. Vortex the Hybridization Buffer to mix well prior to pipetting and pipette the buffer slowly to ensure accurate volume delivery.

REAGENT	PER REACTION	WITH 10% OVERAGE
◦ (white) Hybridization Buffer	40 $\mu\text{l}$	44 $\mu\text{l}$
◦ (white) Hybridization Additive	15 $\mu\text{l}$	16.5 $\mu\text{l}$
◦ (white) NEBNext Direct Genotyping Solution Baits	5 $\mu\text{l}$	5.5 $\mu\text{l}$
<b>Total Volume</b>	<b>60 <math>\mu\text{l}</math></b>	<b>66 <math>\mu\text{l}</math></b>

- 1.6.2. Mix the master mix well by vortexing for 3–5 seconds and centrifuge briefly.
- 1.6.3. To each pooled sample from Step 1.5.14, add 60  $\mu\text{l}$  of Hybridization master mix for a final volume of 120  $\mu\text{l}$ .
- 1.6.4. Seal the PCR plate or cap tubes securely to avoid evaporation. Mix well by briefly vortexing, followed by a quick spin. Alternatively, samples may be mixed by pipetting up and down 10 times prior to capping the tubes/sealing the plate for incubation.
- 1.6.5. Run the following program with the heated lid set to 105°C and place the samples in the thermal cycler after the block temperature reaches 95°C. Proceed to Section 1.7 during this incubation.
- 10 mins @ 95°C  
90 mins @ 60°C  
Hold @ 60°C
- 1.6.6. While the samples are incubating, prepare ● (blue) Streptavidin beads (see Streptavidin Bead Preparation in Section 1.7).
- 1.6.7. After the 90 min incubation at 60°C and when Section 1.7 (Streptavidin Bead Preparation) is complete, unseal the tubes/wells, leave the samples on the thermal cycler at 60°C with the lid open and proceed to Bead Binding in Section 1.8.

## 1.7. Streptavidin Bead Preparation

- 1.7.1. Equilibrate the ● (blue) Streptavidin Beads to room temperature (~15 minutes).
- 1.7.2. Immediately before use, vortex the ● (blue) Streptavidin Beads thoroughly to resuspend.
- 1.7.3. Multiply the number of hybridizations performed in Step 1.6 by 82.5  $\mu\text{l}$ , and transfer that volume of ● (blue) Streptavidin Beads into a sterile, nuclease-free tube. See table (below) for example volumes.

# OF HYBRIDIZATION REACTIONS	VOLUME OF BEADS	VOLUME OF HYBRIDIZATION WASH (HW)	VOLUME OF BEAD PREP BUFFER
2	165 $\mu\text{l}$	330 $\mu\text{l}$	66 $\mu\text{l}$
4	330 $\mu\text{l}$	660 $\mu\text{l}$	132 $\mu\text{l}$
8	660 $\mu\text{l}$	1320 $\mu\text{l}$	264 $\mu\text{l}$

- 1.7.4. Place the tube on a magnet and wait for the solution to clear (~1 minute). Remove the supernatant, and then remove the tube from the magnet.
- 1.7.5. Multiply the number of hybridizations performed in Step 1.6 by 165  $\mu\text{l}$  and transfer that volume of ● (blue) Hybridization Wash (HW) to the beads. See table in Step 1.7.3 for example volumes. Resuspend the beads by vortexing thoroughly followed by a quick spin or by pipetting up and down 10 times.
- 1.7.6. Place the tube on a magnet and wait for the solution to clear (~1 minute). Remove the supernatant, and then remove the tube from the magnet.

- 1.7.7. Repeat Steps 1.7.5–1.7.6 once for a total of 2 washes with ● (blue) Hybridization Wash (HW).
- 1.7.8. Multiply the number of hybridizations performed in Step 1.6 by 33  $\mu\text{l}$  and transfer that volume of ● (blue) Bead Prep Buffer to the beads. See table in Step 1.7.3 for example volumes. Vortex the beads in Bead Prep Buffer to resuspend.
- 1.7.9. Keep the beads in Bead Prep Buffer at room temperature until Bait Hybridization (Section 1.6) is completed.

**Note: For Sections 1.8–1.11 (Bead Binding through Off-Target Removal), the thermal cycler lid should be unheated. The lid can remain open and PCR tubes can remain uncapped for convenience. However, if it is preferred, tubes can be capped during incubations and the lid can be closed once the lid temperature has cooled down from hybridization.**

## 1.8. Bead Binding

- 1.8.1. Immediately before use, vortex the washed Streptavidin Beads (from Step 1.7.9) in Bead Prep Buffer to resuspend.
- 1.8.2. Add 30  $\mu\text{l}$  of resuspended beads to each hybridization reaction (from Step 1.6.7) while the samples are on the thermal cycler at 60°C and mix thoroughly by pipetting up and down 10 times.

**Note: If you have a large number of hybridizations, 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.**

- 1.8.3. Change the thermal cycler temperature to 48°C with the heated lid off and incubate the reactions for 10 minutes.
- 1.8.4. Remove the samples from the thermal cycler and place on a magnet. Wait for the solution to clear (~15 seconds), remove the supernatant, and then remove the samples from the magnet.
- 1.8.5. Add 150  $\mu\text{l}$  of ● (blue) Hybridization Wash (HW) to each sample. Mix by pipetting up and down 10 times. Place the samples on a thermal cycler set at 62°C and incubate for 5 minutes.
- 1.8.6. Repeat Steps 1.8.4–1.8.5 once for a total of 2 washes at 62°C.
- 1.8.7. Remove the samples from the thermal cycler and place on a magnet. Wait for the solution to clear (~15 seconds), remove the supernatant, and then remove the samples from the magnet.
- 1.8.8. Add 150  $\mu\text{l}$  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  $\mu\text{l}$  of 1X TE (provided). DNA-bound beads can be stored in 1X TE for up to 24 hours at 4°C (**Caution: do not freeze**). 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  $\mu\text{l}$  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 1.9.

## 1.9. 3' Blunting of DNA

- 1.9.1. 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 thoroughly to mix well prior to pipetting, and pipette the buffer slowly to ensure accurate volume delivery.

REAGENT	PER REACTION	WITH 10% OVERAGE
● (green) 3' Blunting Buffer	97 $\mu\text{l}$	106.7 $\mu\text{l}$
● (green) 3' Blunting Enzyme	3 $\mu\text{l}$	3.3 $\mu\text{l}$
<b>Total Volume</b>	<b>100 <math>\mu\text{l}</math></b>	<b>110 <math>\mu\text{l}</math></b>

- 1.9.2. Mix the master mix well by vortexing thoroughly 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 3′ Blunting master mix (from Step 1.9.1) to each sample. **Mix thoroughly by pipetting up and down 10 times.** Incubate the samples at 37°C for 10 minutes. Ensure that the thermal cycler lid has cooled to below 37°C, or keep the thermal cycler lid open.

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

- 1.9.5. Proceed immediately with the Post-reaction Wash (Section 1.9.6).
- 1.9.6. Post-reaction Wash
- 1.9.6.1. 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.
- 1.9.6.2. 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.
- 1.9.6.3. 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 (provided). DNA-bound beads can be stored in 1X TE for up to 24 hours at 4°C (**Caution: do not freeze**). 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 1.10.

### 1.10. 3′ Adaptor Ligation

- 1.10.1. 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 thoroughly to mix well prior to pipetting and pipette the buffer slowly to ensure accurate volume delivery.

REAGENT	PER REACTION	WITH 10% OVERAGE
● (red) 3′ Adaptor Ligation Buffer	80 µl	88 µl
● (red) 3′ Adaptor	10 µl	11 µl
● (red) Ligase	10 µl	11 µl
<b>Total Volume</b>	<b>100 µl</b>	<b>110 µl</b>

- 1.10.2. Mix the master mix by vortexing thoroughly 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 3′ Adaptor Ligation master mix (from Step 1.10.1) to each sample. **Mix thoroughly by pipetting up and down 10 times.**
- 1.10.5. Incubate the samples at 20°C for 15 minutes on a thermal cycler with the thermal cycler lid unheated.
- 1.10.6. When the 15 minute incubation is complete, proceed immediately to Off-Target Removal (Step 1.11). **Do not wash the beads in between 3′ Adaptor Ligation and Off-Target Removal.**
- 1.11. Off-Target Removal
- 1.11.1. To each sample from Step 1.10.6, add 5 µl of ● (lilac) Specificity Enhancer Enzyme Mix.
- 1.11.2. Mix thoroughly by pipetting up and down at least 10 times.

- 1.11.3. Change the thermal cycler temperature to 37°C with the thermal cycler lid unheated and incubate the samples for 15 minutes.
- 1.11.4. Proceed immediately with the Post-reaction Wash (Section 1.11.5).
- 1.11.5. Post-reaction Wash
- 1.11.5.1. 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.
- 1.11.5.2. 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.
- 1.11.5.3. 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 (provided). DNA-bound beads can be stored in 1X TE for up to 24 hours at 4°C (**Caution: do not freeze**). 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

Refer to Section 3, Table 3.2 for guidelines on index pooling.

- 1.12.1. For each hybridization, make a Library Amplification master mix by combining the following components in a sterile, nuclease-free tube for the appropriate number of reactions. Mix the Q5 Master Mix well prior to pipetting.

REAGENT	PER REACTION	WITH 10% OVERAGE
● (blue) Q5 Master Mix	50 µl	55 µl
Sterile, nuclease-free water	45 µl	49.5 µl
<b>Total Volume</b>	<b>95 µl</b>	<b>104.5 µl</b>

- 1.12.2. Place the samples on a magnet, wait for the solution to clear (~15 seconds), remove the supernatant then remove the samples from the magnet.
- 1.12.3. Move the samples to ice and add 95 µl of the Library Amplification master mix and 5 µl of ● (dark blue) indexed primer mix for a total volume of 100 µl for the PCR reaction. The indexed primer mix incorporates the pool index into each pool of samples that were processed through the NEBNext Direct GS Target Enrichment Kit as a single tube. **Use one unique indexed primer mix per reaction.**
- 1.12.4. Mix thoroughly by pipetting up and down 10 times, then cap the tubes or seal the plate.
- 1.12.5. Run the following program with the heated lid set to 105°C and place the samples in the thermal cycler when the block temperature reaches 98°C:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	12-21*
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

\* Follow the PCR cycle number recommendations listed in Table 1.12.1 and Table 1.12.2.

The following guidelines should be used to determine the number of PCR cycles. To determine the total input in a hybridization, multiply the input amount per sample by the # of pooled samples per hybridization. For example, the total input in a hybridization of 96 pooled samples with 25 ng of input each is 2,400 ng and would require 15 PCR cycles for a 400 Mb genome and a 500 SNP panel.

The recommended cycle numbers listed should be used as a starting point to produce sufficient library concentrations for a sequencing run while avoiding PCR artifacts and over-cycling (high molecular-weight fragments on a Bioanalyzer or TapeStation). Some optimization may be required.

**Table 1.12.1: PCR Cycle Number Recommendations for 100-1,000 SNP Panels**

TOTAL INPUT IN THE HYBRIDIZATION	HAPLOID GENOME SIZE		
	400 Mb	3 Gb	15 Gb
240–800 ng	17	19	21
800–1,200 ng	16	18	20
1,200–2,400 ng	15	17	19
2,400–4,800 ng	14	16	18
4,800–9,600 ng	13	15	17

**Table 1.12.2: PCR Cycle Number Recommendations for 1,001-5,000 SNP Panels**

TOTAL INPUT IN THE HYBRIDIZATION	HAPLOID GENOME SIZE		
	400 Mb	3 Gb	15 Gb
240–800 ng	16	18	20
800–1,200 ng	15	17	19
1,200–2,400 ng	14	16	18
2,400–4,800 ng	13	15	17
4,800–9,600 ng	12	14	16

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



PCR products with beads can be stored for up to 24 hours at 4°C (**Caution: do not freeze**).

### 1.13. Purify and Size Select Amplified Fragments

- 1.13.1. If you detect significant evaporation in any of the PCR reactions, bring the volume up to 100 µl with sterile, nuclease-free water.
- 1.13.2. Immediately before use, vortex the ● (brown) Post-PCR Sample Purification Beads to resuspend.
- 1.13.3. Add 85 µl of Post-PCR Sample Purification Beads to each PCR reaction. Mix well by pipetting up and down at least 10 times.
- 1.13.4. Incubate for 5 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. While the samples are on the magnet, add 200 µl of freshly prepared (same day) 80% EtOH to each tube.
- 1.13.7. Incubate at room temperature for 30 seconds, and then carefully remove and discard the 80% EtOH. Be careful not to disturb the beads that contain the DNA fragments.

1.13.8. Repeat steps 1.13.6–1.13.7 once for a total of **2 washes** in 80% EtOH. Ensure that all visible liquid is removed after the second wash.

1.13.9. Incubate the samples on the magnet at room temperature uncapped or unsealed for up to 5 minutes to dry the beads.

**Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.**

1.13.10. Remove the tubes/plate from the magnet and resuspend the dry beads in 30  $\mu$ l of sterile, nuclease-free water or 1X TE by gently pipetting (or gently vortex capped tubes/sealed plate, followed by a quick spin).

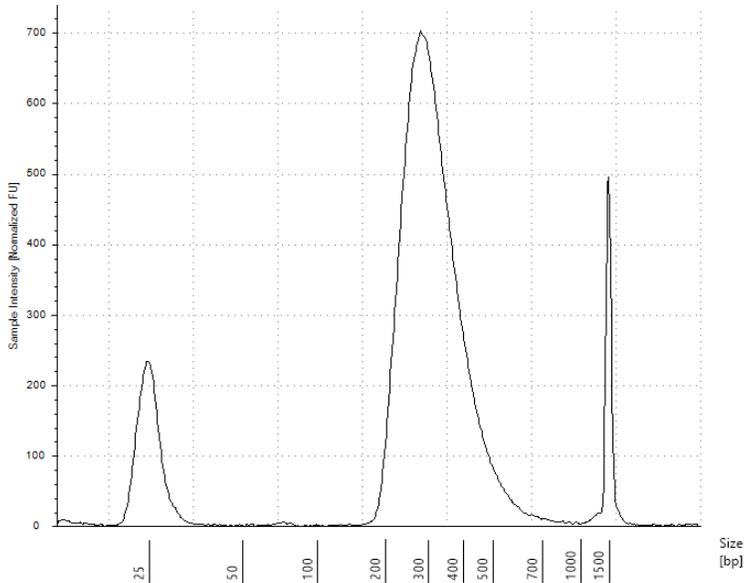
1.13.11. Incubate for 2 minutes at room temperature.

1.13.12. Place the tubes on a magnet and allow the solution to clear.

1.13.13. Transfer 28  $\mu$ l of the eluted library to a fresh tube.

1.13.14. Evaluate 1  $\mu$ l of the eluted library on an Agilent High Sensitivity D1000 ScreenTape or with a similar assay.

**Figure 1.1 Example of an Agilent TapeStation High Sensitivity D1000 trace of a library prepared from 96 maize samples (75 ng of DNA each) pooled and enriched with a 4,600 marker maize panel.**



## Section 2

### Guidelines for Using the Single-use, Indexed 5' Adaptor Plate

#### Symbols



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

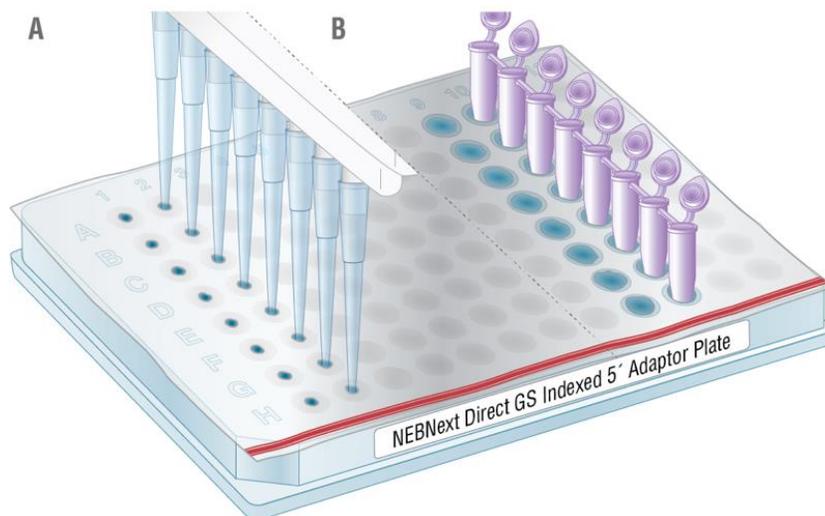
#### 2.1. Setting up the 5' Adaptor Ligation with Sample Indexing

- 2.1.1. Thaw the NEBNext Direct GS Indexed 5' Adaptor Plate for 10–15 minutes at room temperature.
- 2.1.2. Remove the hard plastic plate cover. Briefly centrifuge the plate (280 x g for ~1 min) to collect all of the adaptor at the bottom of each well.
- 2.1.3. Orient the ● (red) NEBNext Direct GS Indexed 5' Adaptor 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  $\mu$ l of the indexed adaptor to each sample. It is important to change pipette tips before piercing a new well to avoid cross contamination of indexed adaptors. Alternatively, the wells can be pierced using the bottom of clean PCR strip tubes (see Figure 2.1B) prior to pipetting the 5' adaptor. Use a new, clean strip tube for each new well to be pierced.

Note: There is enough adaptor in each well for one 5' ligation with Sample Indexing reaction. Do not reuse adaptor if the seal has been previously pierced to avoid contamination with other indexed adaptors.

- 2.1.4. Proceed with the 5' Ligation with Sample Indexing reaction according to Section 1.3.

**Figure 2.1: Orientation and piercing of the NEBNext Direct GS Indexed 5' Adaptor Plate.**



## Section 3

### Index Adaptor and Index Primer Pooling Guidelines

To download a sample sheet with the index sequences, refer to the Usage Guidelines located in the “Protocols, Manuals & Usage” tab on the product page for the NEBNext Direct Genotyping Solution Target Enrichment Kit (NEB #E9530), [www.neb.com/E9500](http://www.neb.com/E9500).

Tables 3.1 and 3.2 list valid barcode combinations that can be sequenced together based on color balance guidelines for Illumina sequencers. Low-complexity barcode combinations that do not maintain the recommended color balance could lead to failed sequencing of the sample indexes.

Table 3.1 lists some valid indexed 5' adaptor combinations (up to 8-plex) that can be sequenced together for the inline sample index. For combinations > 8, choose any column and add any additional combinations as needed.

Table 3.2 lists some valid indexed primer combinations (up to 4-plex) that can be sequenced together for the pool index in the Illumina i7 site. For combinations > 4 choose any 4-plex combination and add any other index as needed.

**Table 3.1: Valid index 5' adaptor combinations for NEB #E9500B-S.**

PLEX	WELL POSITION
< 4	Not recommended
4	A6, B6, C6, and D6 A12, B12, C12, and D12 B6, C6, D6, and E6 B12, C12, D12, and E12 C1, D1, E1, and F1 C7, D7, E7, and F7 E4, F4, G4, and H4 E10, F10, G10, H10
5	A1, B1, C1, D1, E1 A6, B6, C6, D6, E6 A7, B7, C7, D7, E7 A12, B12, C12, D12, E12 B1, C1, D1, E1, F1 B6, C6, D6, E6, F6 B7, C7, D7, E7, F7 B12, C12, D12, E12, F12 C1, D1, E1, F1, G1 C2, D2, E2, F2, G2 C4, D4, E4, F4, G4 C7, D7, E7, F7, G7 C8, D8, E8, F8, G8 C10, D10, E10, F10, G10 D4, E4, F4, G4, H4 D10, E10, F10, G10, H10
6–7	Any 5 plex plus 1-2 adjacent wells from the same column
8	Any column

**Table 3.2: Valid index primer combinations for NEB #E9530B-S.**

<b>PLEX</b>	<b>INDEX PRIMER</b>
2	DGS01 and DGS02 DGS03 and DGS04 DGS05 and DGS06 DGS07 and DGS08
3	DGS01, DGS02 and DGS03 DGS02, DGS03 and DGS04 DGS03, DGS04 and DGS05 DGS04, DGS05 and DGS06 DGS05, DGS06 and DGS07 DGS06, DGS07 and DGS08
4	DGS01, DGS02, DGS03 and DGS04 DGS01, DGS04, DGS05 and DGS08 DGS02, DGS03, DGS04 and DGS05 DGS02, DGS04, DGS06 and DGS07 DGS03, DGS04, DGS05 and DGS06 DGS03, DGS05, DGS07 and DGS08 DGS04, DGS05, DGS06 and DGS07 DGS04, DGS05, DGS07 and DGS08 DGS05, DGS06, DGS07 and DGS08

## Section 4

### Guidelines for Running Samples on the Illumina MiSeq

#### 4.1. 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 iSeq 100, MiSeq, NextSeq®, HiSeq® and Novaseq® platforms. Here we describe the steps necessary to sequence NEBNext Direct GS libraries on the Illumina MiSeq.

4.1.1. Prepare a MiSeq sample sheet:

4.1.1.1. Download a MiSeq sample sheet, NEBNext Direct GS Barcodes file, and refer to the “Sample Sheet Guidelines” located in the “Protocols, Manuals & Usage” tab on the NEBNext Direct GS Target Enrichment product page at [www.neb.com/E9500](http://www.neb.com/E9500). Do not use the Illumina Experiment Manager to generate a sample sheet.

4.1.1.2. Fill in the sample sheet with your sample and barcode information.

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

4.1.2. Pool, dilute, and denature samples for an 8–10 pM\* final concentration following the MiSeq Denature and Dilute Libraries Guide and the Index Pooling Guidelines in Section 3.

\* Based on quantification with an Agilent High Sensitivity DNA Kit or Agilent High Sensitivity D1000 ScreenTape using the region between 100–1200 bp. Some optimization may be required for optimal cluster density.

4.1.3. Follow the MiSeq System Guide to load your samples and run the MiSeq. When prompted, upload the sample sheet that was prepared in 4.1.1.

## Section 5

### Guidelines for Bioinformatic Processing of Sequencing Data

#### Introduction

NEBNext Direct GS libraries have a unique molecular identifier (UMI) followed by a sample barcode in the Read 1 position, with a second sample barcode in the Illumina *i7* site (Index 1) and the template read as Read 2 (Figure 2, page 5). The following sections describe how to partition the sequencing cycles into template reads, sample indexes, and UMIs for NEBNext Direct GS libraries using *bcl2fastq* or Picard tools.

#### 5.1. Guidelines for Demultiplexing NEBNext Direct GS Libraries Using *bcl2fastq*

##### 5.1.1. Prepare a sample sheet for *bcl2fastq*

The *bcl2fastq* software uses the sample barcodes to demultiplex in the order in which they are read by the sequencer. Therefore the “index” column in the sample sheet is required to contain the sample barcodes from the Read 1 site, while the “index2” column is required to contain the sample barcodes from the *i7* site. This sample sheet will differ from the one that is used to run the Illumina sequencer. To download a sample sheet with the barcode sequences for use with *bcl2fastq*, refer to the “Usage Guidelines” located in the “Protocols, Manuals & Usage” tab on the NEBNext Direct Genotyping Solution Target Enrichment Kit (NEB #E9530), [www.neb.com/E9500](http://www.neb.com/E9500).

##### 5.1.2. Run *bcl2fastq*

#### Options

The *bcl2fastq* software should be run with the following options:

```
--use-bases-mask y12i8,i8,y75  
--mask-short-adaptor-reads 10  
--barcode-mismatches 0
```

The first option identifies the 12 cycles of UMI and 8 cycles of the first sample barcode. The second option keeps the 12 bp UMI sequences from being identified as adaptor sequence and subsequently masked to “NNNNNNNNNNNN”. To prevent misassignment of a small number of reads (< 3%) to the wrong sample, we recommend allowing zero mismatches for barcode assignment by using the option “--barcode-mismatches 0”.

#### --use-bases-mask

The “--use-bases-mask” string specifies how to use each of the sequencing cycles:

- An “n” means ignore the cycle.
- A “Y” (or “y”) means the cycle is used as a template read.
- An “I” (or “i”) means the cycle is used as an index read.
- A number means that the previous character is repeated that many times.
- An asterisk “\*” means that the previous character is repeated until the end of this read or index (length according to the RunInfo.xml).

Each read mask is separated by a comma: “,”. NEBNext Direct GS libraries have three read masks. Note that as the sample index and UMI are combined in the same read mask, there is no comma separating them.

#### Output

Running *bcl2fastq* as described above will produce two fastq files: the *R1* fastq file will contain the UMI sequences and the *R2* fastq file will contain the Read 2 sequences.

#### 5.2. Guidelines for Demultiplexing NEBNext Direct GS Libraries Using Picard

Demultiplexing NEBNext Direct GS libraries with Picard tools requires two processing steps. The first step (Step 5.2.2) is to assign each cluster on the sequencer to the appropriate barcode, and the second step (Step 5.2.3) is demultiplexing into fastq or unmapped BAM files.

#### Read Structure

Steps 5.2.2 and 5.2.3 require a [Read Structure](#), which is a string that defines the mapping of sequencing cycles into sequencing reads, sample indexes, and UMIs. The linked page provides a good description of the concept as well as a number of examples. All read lengths need to be specified explicitly, as Picard does not support variable length operators.

## The Read Structure for a NEBNext Direct GS library: 12M8B8B75T

### 5.2.1. Prepare a sample\_metadata.txt file.

Steps 5.2.2 and 5.2.3 require sample metadata in a format that differs from an Illumina sample sheet. Each step has its own requirements for identifying the metadata, so there are some columns with redundant information. The sample barcodes are defined in the order in which they are read by the sequencer. For NEBNext Direct GS libraries, the inline barcode is defined as barcode1 and the i7 barcode is defined as barcode2.

To download a sample\_metadata.txt file for use with the Picard tools in this section, refer to the “Usage Guidelines” located in the “Protocols, Manuals & Usage” tab on the NEBNext Direct Genotyping Solution Target Enrichment Kit (NEB #E9530), [www.neb.com/E9500](http://www.neb.com/E9500). This file can be edited in Excel and saved as tab delimited text file (.txt).

### 5.2.2. Assign clusters to barcodes.

Assignment of clusters to barcodes is performed by the Picard tool [ExtractIlluminaBarcodes](#). An example call is shown below. This will write the barcode files to the BASECALLS\_DIR, compress the output barcode files and use sixteen processors. To use all available processors on a system, set NUM\_PROCESSORS=0. Note that the heap size specified (the “-Xmx48g” in the command) should be 2-4G per processor to be used. To prevent misassignment of a small number of reads (< 3%) to the wrong sample, we recommend allowing zero mismatches for barcode assignment by using the option “MAX\_MISMATCHES=0”.

```
Java -Xmx48g -jar \  
  picard.jar ExtractIlluminaBarcodes \  
  VALIDATION_STRINGENCY=SILENT \  
  BASECALLS_DIR=Data/Intensities/BaseCalls \  
  OUTPUT_DIR=Data/Intensities/BaseCalls \  
  LANE=1 \  
  READ_STRUCTURE=12M8B8B75T \  
  BARCODE_FILE=sample_metadata.txt \  
  METRICS_FILE=Data/Intensities/BaseCalls/barcode_counts.lane-1.metrics.txt \  
  COMPRESS_OUTPUTS=true \  
  NUM_PROCESSORS=16 \  
  MAX_MISMATCHES=0
```

Note that the “BARCODE\_FILE” argument points to the sample\_metadata.txt file described in step 5.2.1. The “METRICS\_FILE” is an output file that has summary statistics about the assignment of clusters to barcodes.

### 5.2.3. Demultiplex samples.

Demultiplexing can be performed by either the Picard tool [IlluminaBasecallsToFastq](#), if you prefer the output to be a fastq file, or [IlluminaBasecallsToSam](#) if you prefer an unmapped SAM/BAM file as output. An example call that will write to a fastq file is shown below. There are small differences between the arguments of these two tools. Please see the linked pages for a full description of the available options.

```
java -Xmx36g -jar \  
  picard.jar IlluminaBasecallsToFastq \  
  VALIDATION_STRINGENCY=SILENT \  
  BASECALLS_DIR=Data/Intensities/BaseCalls \  
  LANE=1 \  
  RUN_BARCODE=000000000-BHRLY \  
  NUM_PROCESSORS=12 \  
  READ_STRUCTURE=12M8B8B75T \  
  MULTIPLEX_PARAMS=sample_metadata.txt \  
  READ_NAME_FORMAT=ILLUMINA \  
  INCLUDE_NON_PF_READS=false
```

Note that the “MULTIPLEX\_PARAMS” argument points to the sample\_metadata.txt file described above.

## Kit Components

NEB #E9500B-S Table of Components

NEB #	PRODUCT	VOLUME
E9501-2	NEBNext Direct GS Fragmentation Buffer	0.144 ml
E9502-2	NEBNext Direct GS Fragmentation Enzyme Mix	0.048 ml
E9503-2	NEBNext Direct GS Indexed 5' Adaptor Plate	0.005 ml/well
E9504-2	NEBNext Direct GS 5' Adaptor Ligation Master Mix	0.960 ml
E9505-2	NEBNext Direct GS Stop Solution	0.24 ml
E9506-2	NEBNext Direct GS Sample Purification Beads	2.0 ml

NEB #E9530B-S Table of Components

NEB #	PRODUCT	VOLUME
E9531-2	NEBNext Direct GS Hybridization Additive	0.16 ml
E9532-2	NEBNext Direct GS 3' Blunting Enzyme	0.024 ml
E9533-2	NEBNext Direct GS 3' Adaptor	0.08 ml
E9534-2	NEBNext Direct GS Ligase	0.08 ml
E9535-2	NEBNext Direct GS Specificity Enhancer Enzyme Mix	0.04 ml
E9536-2	NEBNext Direct GS Q5 Master Mix	0.4 ml
E9537-2	NEBNext Direct GS Index Primer Mix DGS01	0.005 ml
E9538-2	NEBNext Direct GS Index Primer Mix DGS02	0.005 ml
E9539-2	NEBNext Direct GS Index Primer Mix DGS03	0.005 ml
E9540-2	NEBNext Direct GS Index Primer Mix DGS04	0.005 ml
E9541-2	NEBNext Direct GS Index Primer Mix DGS05	0.005 ml
E9542-2	NEBNext Direct GS Index Primer Mix DGS06	0.005 ml
E9543-2	NEBNext Direct GS Index Primer Mix DGS07	0.005 ml
E9544-2	NEBNext Direct GS Index Primer Mix DGS08	0.005 ml
E9545-2	NEBNext Direct GS Pre-Hyb Sample Purification Beads	2.88 ml
E9546-2	NEBNext Direct GS Bead Wash 1 (BW1)	2.4 ml
E9547-2	NEBNext Direct GS Post-PCR Sample Purification Beads	0.68 ml
E9548-2	NEBNext Direct GS TE Buffer	2.4 ml
E9549-2	NEBNext Direct GS Hybridization Buffer	0.32 ml
E9550-2	NEBNext Direct GS Streptavidin Beads	0.6 ml
E9551-2	NEBNext Direct GS Bead Prep Buffer	0.24 ml
E9554-2	NEBNext Direct GS 3' Blunting Buffer	0.776 ml
E9555-2	NEBNext Direct GS 3' Adaptor Ligation Buffer	0.64 ml
E9552-2	NEBNext Direct G5 Hybridization Wash (HW)	6.0 ml
E9553-2	NEBNext Direct G5 Bead Wash 2 (BW2)	3.6 ml

## Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	9/19
2.0	Updated header and footer, legal footer and references to discontinued components.	4/24

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