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NEBNext® Immune Sequencing Kit (Human) (E6320) V.1

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We use this protocol and it's working

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Abstract

This protocol details NEBNext® Immune Sequencing Kit (Human).

https://www.neb.com/-/media/nebus/files/manuals/manuale6320.pdf?rev=1dca310ed12a4e89b322063d31db030d&hash=D4D7E9FBED23CC7448447137DBFF8C0B

Guidelines

Overview

Immune repertoire sequencing is a powerful tool that can be used to analyze the immune response to diseases and pathogens from current and past exposures. Areas of particular interest include characterization of autoimmune diseases, oncology, discovery of neutralizing antibodies against infectious disease, or use as a diagnostic tool for residual disease detection.

In order to create the incredible genetic diversity required for antigen recognition by B and T Cells, lymphocytes construct unique antigen receptors via a process known as V(D)J recombination. In this process, each cell selects a single V, D, and J gene segment through genetic recombination, introducing additional non-germline-encoded nucleotides at the junctions. This process generates immune receptor diversity, the majority of which is encoded in the heavy chain complementarity determining region 3 (CDR3) (Figure 1).

Recent improvements in the read length and throughput of next-generation sequencing (NGS) platforms have resulted in a rise in the popularity of immune repertoire sequencing. The complex structure and sequence diversity of antibody-encoding genes have provided significant challenges to the development of a simple and reliable method to sequence immune receptor sequences.

The NEBNext Immune Sequencing Library Preparation Kit (Figure 2) provides all the components required for enrichment and sequencing of the B Cell Receptor (BCR) and T Cell Receptor (TCR) RNA transcripts present in a sample. NEBNext Immune Sequencing Kit has been developed and optimized to provide accurate sequencing of full-length immune transcript repertoires of B-cells and T-cells. This allows for exhaustive somatic mutation profiling across complete V, D and J segments, full isotype information analysis (IgM, IgD, IgG, IgA and IgE), and also allows for synthesis and expression of complete antibody chains for downstream immunological assays. In addition, this approach allows characterization of TRA and TRB chains. The method uses unique molecular identifier scheme specifically designed to barcode each mRNA molecule, allowing PCR copies derived from an individual mRNA to be collapsed into a single consensus sequence. This improves sequence accuracy by resolving PCR and sequencing errors. This also eliminates PCR bias, thus allowing for quantitative digital molecule counting.

Defining features of the Immune Sequencing method include:

a) Full-length variable sequences are generated (including isotype information), allowing downstream antibody synthesis and functional characterization not possible with approaches sequencing only the CDR3 region.

b) Variable region primers are not required, which reduces primer pool complexity and allows for the unbiased and simultaneous recovery of B Cell and T Cell receptor transcripts.

c) Unique molecular barcoding approach minimizes PCR biases and improves sequencing accuracy by allowing a consensus to be generated from duplicate sequencing reads originating from the same transcript. Moreover, unique barcoding enables accurate quantitation of each clone present in the sample.

d) High target capture efficiency allows for immune repertoire sequencing and analysis from sub-microgram quantities of total RNA.

Figure 1. Simplified representation of the structure of BCR or TCR showing the outcome of V(D)J recombination in mature lymphocytes.

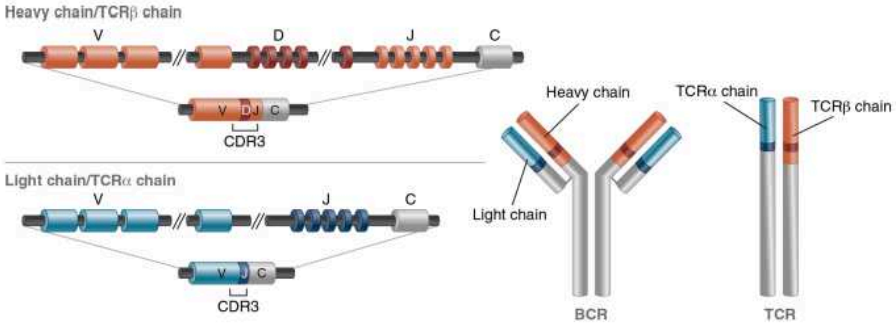
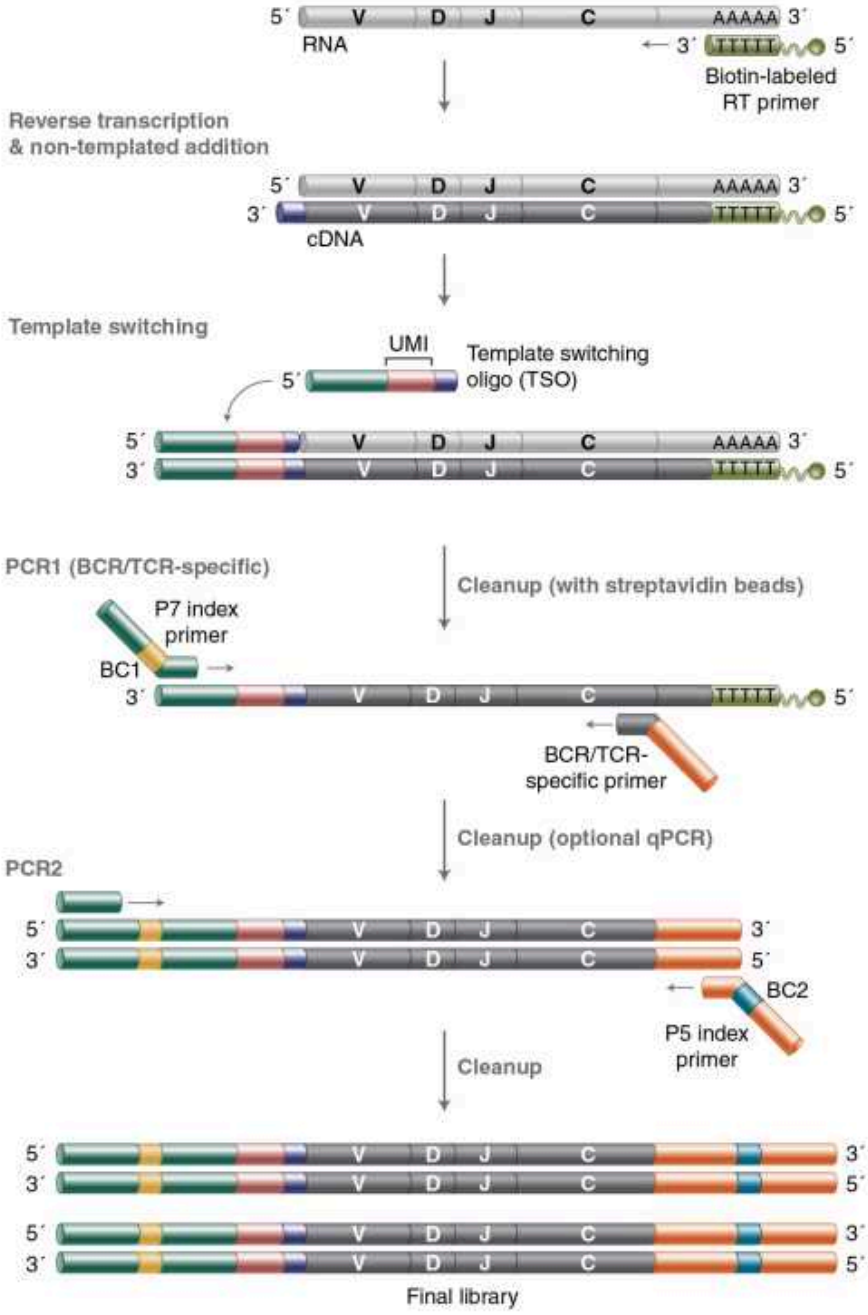


Figure 2. NEBNext Immune Sequencing Library Preparation Kit Workflow



Step I-Prepare target RNA

Immune Sequencing does not require mRNA isolation prior to library construction. Total RNA can be prepared from any sample or tissue known to contain B Cells or T Cells, such as peripheral blood mononuclear cells, bone

marrow, lymph nodes, or any other tissue in which infiltrating lymphocytes are present. Cells or tissues can be frozen, but intact, high quality RNA is required for optimal performance.

Step II-Perform molecular-tagging reverse transcription

During this 50-minute reaction full-length mRNA is reverse transcribed and the 3′ end of each cDNA is tagged with a DNA adaptor containing a unique molecular identifier (UMI) barcode.

Step III-Purify with Streptavidin Magnetic Beads

This 30-minute cleanup step removes all salts, reagents and unused cDNA adaptor oligonucleotides (TSO and RT Primers) allowing clean downstream amplification steps.

Step IV-Primary amplification of target fragments

This primary PCR step uses one primer which binds the universal cDNA adaptor and a primer mix targeting constant regions of the desired immune receptors. Products are then purified with Sample Purification Beads.

Step V-Secondary PCR cycle optimization with real-time PCR

This optional real-time PCR allows accurate assessment of target quantity leading to an optimized choice of PCR cycles for the final library PCR.

Step VI-Secondary PCR including Illumina® adaptor addition

This secondary PCR adds additional Illumina adaptor sequences to the target fragments (including Illumina multiplexing index sequences if desired). After Sample Purification Bead cleanup the libraries are ready for Bioanalyzer or TapeStation analysis, quantification and sequencing on the MiSeq platform.

Applications

The NEBNext Immune Sequencing Kit (Human) contains enzymes and buffers that are ideal to convert a small amount of human RNA input into indexed libraries for next-generation sequencing on the Illumina® MiSeq platform (Illumina, Inc). The workflow of NEBNext Immune Sequencing (Human) Kit is very user-friendly and fast with minimal hands-on time. Each of these components must pass rigorous quality control standards and are lot controlled, both individually and as a set of reagents.

For larger volume requirements, customized and bulk packaging is available by purchasing through the OEM/Bulks department at NEB. Please contact OEM@neb.com for further information.

Materials

The Kit Includes:

NEB #E6320S contains E2628S (Pouch with BCR and TCR primers), #E6325-1 (Box 1 of 2), and #E6325-2 (Box 2 of 2) and is sufficient for preparation of up to 24 reactions. NEB #E6320L contains E2628L (Pouch with BCR and TCR primers), #E6325-3 (Box 1 of 2), and #E6325-4 (Box 2 of 2) and is sufficient for preparation of up to 96 reactions. All reagents should be stored at t –20°C, 4°C or Room temperature as indicated below. Colored bullets represent the color of the cap of the tube containing the reagent.

NEB #E2628S/L, NEBNext Immune Sequencing Primers (Human)

Pouch: Store at -20°C.

- (blue) NEBNext IS BCR Primers (Human)
- (blue) NEBNext IS TCR Primers (Human)

NEB #E6325-1,

NEBNext Immune Sequencing Kit RT and PCR Reagents (24 reactions) **New England Biolabs Catalog #**E6320S

Box 1 of 2: Store at -20°C.

- (lilac) NEBNext IS RT Buffer (4X)
- (lilac) NEBNext IS RT Primer
- (lilac) NEBNext IS TS Oligos
- (lilac) NEBNext First Strand Synthesis Enzyme Mix
- (lilac) dNTP Solution Mix
- (lilac) NEBNext Cell Lysis Buffer
- (blue) Q5® Reaction Buffer (5X)
- (blue) Q5 Hot Start High-Fidelity DNA Polymerase
- (blue) NEBNext i701-i706 Primers
- (blue) NEBNext i501-i504 Primers
- (blue) NEBNext IS PCR2 Universal Primer
- (white) Nuclease-free Water
- (white) TE (0.1X)

NEB #E6325-3

NEBNext® Immune Sequencing Kit (Human) 96 reactions **New England Biolabs Catalog #**E6320L

Box 1 of 2: Store at -20°C.

- lilac) NEBNext IS RT Buffer (4X)
- (lilac) NEBNext IS RT Primer
- (lilac) NEBNext IS TS Oligos
- (lilac) NEBNext First Strand Synthesis Enzyme Mix
- (lilac) dNTP Solution Mix
- (lilac) NEBNext Cell Lysis Buffer
- (blue) Q5 Reaction Buffer (5X)
- (blue) Q5 Hot Start High-Fidelity DNA Polymerase
- (blue) NEBNext i701-i712 Primers
- (blue) NEBNext i501-i508 Primers
- (blue) NEBNext IS PCR2 Universal Primer
- (white) Nuclease-free Water
- (white) TE (0.1X)

NEB #E6325-2/#E6325-4, NEBNext Immune Sequencing Kit cDNA Purification Reagents

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

- (white) Streptavidin Magnetic Beads
- (white) NEBNext IS Bind and Wash Buffer (2X)
- (white) 0.1% Tween
- (white) NEBNext Sample Purification Beads*

*This component should be stored at Room temperature.

Required Materials Not Included

SYBR® Green **Thermo Fisher Scientific Catalog #**S7563

• 80% Ethanol (freshly prepared)

DNA LoBind Tubes **Eppendorf Catalog #**#022431021

NEBNext® Magnetic Separation Rack **New England Biolabs Catalog #**S1515S or

96S Super Magnet **Alpaqua Catalog #**A001322

• Thermal cycler
• qPCR machine and associated 96 well plates
• Bioanalyzer®/TapeStation® and associated consumables
• MiSeq® and v3 600 cycle sequencing reagent
• Nuclease-free Water

- Tube rotating mixer

6-Tube Magnetic Separation Rack **New England Biolabs Catalog #**S1506S

- DNase RNase-free PCR strip tubes (USA Scientific 1402-1708)

Kit Components

NEB #E6320S Table of Components

	A	B	C
	NEB #	**PRODUCT**	**VOLUME**
	E6321A	NEBNext IS RT Buffer (4X)	0.120 ml
	E6322A	NEBNext Cell Lysis Buffer	0.012 ml
	E6323A	dNTP Solution Mix	0.108 ml
	E6324A	NEBNext IS RT Primer	0.024 ml
	E6326A	NEBNext IS TS Oligos	0.024 ml
	E7761A	NEBNext First Strand Synthesis Enzyme Mix	0.048 ml
	E6327A	Hot Start High-Fidelity DNA Polymerase	0.060 ml
	E6328A	Q5 Reaction Buffer (5X)	0.600 ml
	E2626A	NEBNext IS BCR Primers (Human)	0.048 ml
	E2627A	NEBNext IS TCR Primers (Human)	0.048 ml
	E6329A	NEBNext IS PCR2 Universal Primer	0.072 ml
	E6355A	Streptavidin Magnetic Beads	0.360 ml
	E6356A	NEBNext IS Bind and Wash Buffer (2X)	11.3 ml
	E6357A	0.1% Tween	3.0 ml
	E6358A	NEBNext Sample Purification Beads	2.8 ml
	E6331A	TE (0.1X)	2.3 ml
	E6332A	Nuclease-free Water	1.5 ml
	E6333A	NEBNext i701 Primer	0.008 ml
	E6334A	NEBNext i702 Primer	0.008 ml

	A	B	C
	E6335A	NEBNext i703 Primer	0.008 ml
	E6336A	NEBNext i704 Primer	0.008 ml
	E6337A	NEBNext i705 Primer	0.008 ml
	E6338A	NEBNext i706 Primer	0.008 ml
	E6339A	NEBNext i501 Primer	0.036 ml
	E6340A	NEBNext i502 Primer	0.036 ml
	E6341A	NEBNext i503 Primer	0.036 ml
	E6342A	NEBNext i504 Primer	0.036 ml

NEB #E6320L Table of Components

	A	B	C
	NEB #	**PRODUCT**	**VOLUME**
	E6321AA	NEBNext IS RT Buffer (4X)	0.480 ml
	E6322AA	NEBNext Cell Lysis Buffer	0.048 ml
	E6323AA	dNTP Solution Mix	0.432 ml
	E6324AA	NEBNext IS RT Primer	0.096 ml
	E6326AA	NEBNext IS TS Oligos	0.096 ml
	E7761AA	NEBNext First Strand Synthesis Enzyme Mix	0.192 ml
	E6327AA	Q5 Hot Start High-Fidelity DNA Polymerase	0.240 ml
	E6328AA	Q5 Reaction Buffer (5X)	2.400 ml
	E2626AA	NEBNext IS BCR Primers (Human)	0.192 ml
	E2627AA	NEBNext IS TCR Primers (Human)	0.192 ml
	E6329AA	NEBNext IS PCR2 Universal Primer	0.288 ml
	E6355AA	Streptavidin Magnetic Beads	1.440 ml
	E6356AA	NEBNext IS Bind and Wash Buffer (2X)	45.2 ml

	A	B	C
	E6357AA	0.1% Tween	11.8 ml
	E6358AA	NEBNext Sample Purification Beads	11.1 ml
	E6331AA	TE (0.1X)	9.2 ml
	E6332AA	Nuclease-free Water	5.8 ml
	E6333AA	NEBNext i701 Primer	0.032 ml
	E6334AA	NEBNext i702 Primer	0.032 ml
	E6335AA	NEBNext i703 Primer	0.032 ml
	E6336AA	NEBNext i704 Primer	0.032 ml
	E6337AA	NEBNext i705 Primer	0.032 ml
	E6338AA	NEBNext i706 Primer	0.032 ml
	E6347AA	NEBNext i707 Primer	0.032 ml
	E6348AA	NEBNext i708 Primer	0.032 ml
	E6349AA	NEBNext i709Primer	0.032 ml
	E6352AA	NEBNext i710 Primer	0.032 ml
	E6353AA	NEBNext i711 Primer	0.032 ml
	E6354AA	NEBNext i712 Primer	0.032 ml
	E6339AA	NEBNext i501 Primer	0.144 ml
	E6340AA	NEBNext i502 Primer	0.144 ml
	E6341AA	NEBNext i503 Primer	0.144 ml
	E6342AA	NEBNext i504 Primer	0.144 ml
	E6343AA	NEBNext i505 Primer	0.144 ml
	E6344AA	NEBNext i506 Primer	0.144 ml
	E6345AA	NEBNext i507 Primer	0.144 ml
	E6346AA	NEBNext i508 Primer	0.144 ml

NEBNext Immune Sequencing Reverse Transcription and cDNA Synthesis

1

Note

RNA Sample Recommendations: The RNA sample should be free of salts (e.g., Mg2+, or guanidine salts) or organics (e.g., phenol and ethanol). High quality RNA with a RIN > 7 is recommended for optimal performance.

Starting Material: 10 ng – 1 µg enriched B cell or T Cell or B cell and T Cell total RNA, or 10 ng – 1 µg total RNA from peripheral blood mononuclear cells (PBMCs). The maximum volume of RNA input is 9 µL.

Mix the following components in a sterile nuclease-free tube:

	A	B
	COMPONENT	**VOLUME**
	(lilac) NEBNext IS RT Buffer (4X)	5 µl
	(lilac) NEBNext IS RT Primer	1 µl
	(lilac) NEBNext IS TS Oligos	1 µl
	(lilac) dNTP Solution Mix	2 µl
	(lilac) NEBNext First Strand Synthesis Enzyme Mix	2 µl
	(lilac) NEBNext Cell Lysis Buffer	0.5 µl
	Total RNA	1-9 µl
	(white) Nuclease-free Water	Variable
	Total Volume	20.5 µl

2

Set a 100 µl or 20 µl pipette to 15 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Note

It is important to mix well. The presence of a small amount of bubbles will not interfere with the performance.

3 Place in a thermal cycler, with the heated lid set to ≥ 80 °C, and run the following program:

	A	B	C
	CYCLE STEP	TEMP	TIME
	Incubation	42°C	40 minutes
	Inactivation	70°C	10 minutes
	Hold	4°C	∞

Purify the cDNA with Streptavidin Magnetic Beads

4 Prepare Bind and Wash Buffer (1X) to be used in Step 14.

4.1 Dilute NEBNext IS Bind and Wash Buffer (2X) to 1X with an equal volume of nuclease-free water (not provided).

Note

For each sample, you will need 100 µL Bind and Wash Buffer (1X).

5 Vortex Streptavidin Magnetic Beads to resuspend. Aliquot the total amount of Streptavidin Magnetic Beads needed (15 µL of Streptavidin Magnetic Beads per sample) into a clean RNase-free 1.5 mL tube.

6 Place the tube on a magnetic rack at Room temperature. Once the solution is clear (~ 00:02:00) carefully remove and discard the supernatant without disturbing the

2m

bead pellet.

7 Remove the tube from the magnet and wash the beads by adding 200 µL of undiluted NEBNext IS Bind and Wash Buffer (2X). Vortex briefly to resuspend the beads and quickly spin the tube in a microcentrifuge to collect any sample on the sides of the tube.

8 Place the tube on a magnetic rack. Once the solution is clear, carefully remove and discard the supernatant without disturbing the bead pellet.

9 Remove the tube from the magnet and wash the beads by adding 200 µL of undiluted NEBNext IS Bind and Wash Buffer (2X). Vortex briefly to resuspend the beads and quickly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on a magnetic rack. Once the solution is clear, carefully remove and discard the supernatant without disturbing the bead pellet for a total of two washes.

10 Remove the tube from the magnetic rack. Resuspend the beads in 20 µL of undiluted NEBNext IS Bind and Wash Buffer (2X) for each sample (e.g., for 4 samples, add 4 × 20 = 80 µL NEBNext IS Bind and Wash Buffer (2X). Vortex briefly to resuspend the beads.

11 Add 20 µL beads to the cDNA products (20.5 uL) from Step 3 Vortex briefly to mix.

12 Place the tube on a rotator for 00:15:00 at Room temperature . 15m

13 Quickly spin down the tube to collect any sample on the sides of the tube and place the tube on a magnetic rack. Once the solution is clear, carefully remove and discard the supernatant without disturbing the bead pellet.

14 Remove the tube from the magnetic rack. Add 100 µL diluted Bind and Wash Buffer (1X) (from Step 4). Vortex briefly to resuspend the beads.

Note

Vortexing is better than flicking the tube, as it minimizes foaming.

15 Quickly spin down the tube to collect any sample on the sides and place the tube on a magnetic rack. Once the solution is clear, carefully remove and discard the supernatant without disturbing the bead pellet.

16 Remove the tube from the magnetic rack and add 100 µL of 0.1% Tween. Vortex briefly to resuspend the beads.

Note

This step is critical to remove all the salt from the beads before elution.

17 Quickly spin down the tube to collect any sample on the sides of the tube and place the tubes on a magnetic rack. Once the solution is clear, carefully remove and discard the supernatant without disturbing the bead pellet. With a 20 ul pipette tip, carefully remove any residual liquid left at the bottom of the tube without disturbing the beads.

18 Remove the tube from the magnet and add 23 µL of 0.1% Tween. Vortex briefly to resuspend the beads and quickly spin in a microcentrifuge to collect any sample on the sides of the tube.

19 Place the tube in a thermal cycler, with the heated lid set at 105 °C, and run the following program:

	A	B	C
	CYCLE STEP	**TEMP**	**TIME**
	Incubation	95°C	3 minutes
	Hold	25°C	∞

20 Remove the tube from the thermal cycler, vortex briefly, spin down the contents and place on the magnetic rack.

21 Once the solution is clear, transfer 21 µL of the supernatant into a new tube and discard the beads.

Note

Safe Stopping Point:

It is safe to store the cDNA at -20 °C Overnight.

22

Note

- Use Option 22.1 for enriching B Cell Receptor (BCR) chains.
- Use Option 22.2 for enriching T Cell Receptor (TCR) chains.
- Use Option 22.3 for enriching both BCR chains and TCR chains.

STEP CASE

B Cell Receptor Chains PCR 1 36 steps

23 Add the following components to a sterile strip tube:

	A	B
	COMPONENT	**VOLUME**
	Purified cDNA (Step 21)	21 µl
	(blue) Q5 Reaction Buffer (5X)	10 µl
	(lilac) dNTP Solution Mix	1 µl
	(blue) Q5 Hot Start High-Fidelity DNA Polymerase	1 µl
	(blue) NEBNext IS BCR Primers (Human)	2 µl

	A	B
	(blue) NEBNext i7 Index Primer*	1 µl
	(white) Nuclease-free Water	14 µl
	Total Volume	50 µl

*Use only one i7 Primer per sample

24 Set a 100 ul or 200 ul pipette to 40 ul and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

25 Place the tube on a thermal cycler and perform PCR amplification using the following PCR cycling program:

	A	B	C	D
	CYCLE STEP	**TEMP**	**TIME**	**CYCLES**
	Initial Denaturation	98° C	60 seconds	1
	Denaturation	98° C	10 seconds	12
	Annealing	64° C	30 seconds	
	Extension	72° C	30 seconds	
	Final Extension	72° C	60 seconds	1
	Hold	4°C	∞	

Cleanup of PCR1 Amplification

41m

26 Vortex NEBNext Sample Purification Beads to resuspend. SPRIselect and AMPure® XP Beads can be used as well. If using AMPure XP Beads, please allow the beads to warm to Room temperature for at least 00:30:00 before use.

30m

27 Add 50 µL of resuspended NEBNext Sample Purification Beads to each 50 µl PCR reactions. Mix well by pipetting or brief vortexing.

28 Incubate for 00:05:00 at Room temperature . 5m

29 Quickly spin the tube in a microcentrifuge and place it on a magnetic rack. Once the solution is clear (~ 00:05:00) carefully remove and discard the supernatant without disturbing the bead pellet. 5m

Note

Caution: Do not discard beads.

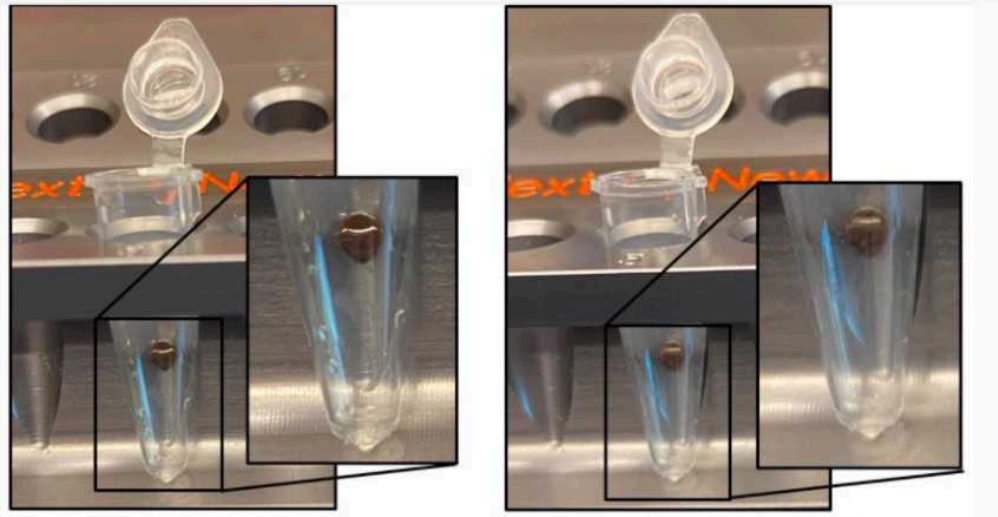
30 Wash beads by adding 200 µL of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at Room temperature for 00:00:30 , and then carefully remove and discard the supernatant. 30s

31 Wash beads by adding 200 µL of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at Room temperature for 00:00:30 , and then carefully remove and discard the supernatant once for a total of two wash steps. After supernatant is discarded, use a 20 µl pipette tip to carefully remove any residual liquid left at the bottom of the tube without disturbing the beads. 30s

32 Air dry the beads with more emphasis for up to 00:05:00 while the tube is on the magnetic stand with the lid open. 5m

Note

Caution: Do not over dry the beads. This may result in lower recovery of DNA target.



33 Elute DNA target from beads by adding 25 µL TE (0.1X) to beads. Mix well on a vortex mixer or by pipetting up and down at least 10 times. Incubate for at least 00:02:00 at Room temperature. Quickly spin the tube and place it on a magnetic rack to separate beads from supernatant. 2m

34 After the solution is clear (about 2 minutes), carefully transfer 23 µL supernatant to a new PCR tube.

Note

Safe Stopping Point: It is safe to store the PCR product overnight at -20 °C.

qPCR for PCR2 Cycle Optimization

35

Note

The qPCR step is optional; it is only used to identify the optimal final PCR cycling conditions, as each sample can contain varying amounts of amplifiable molecules. One can achieve a similar result by manually taking an aliquot of the PCR reaction every 5 cycles and visualizing each aliquot on Bioanalyzer or TapeStation to identify the optimal cycling number that yields the highest product with the lowest background. The number of cycles should be high enough to provide sufficient library fragments for a successful sequencing run, but low enough to avoid PCR artifacts and over-cycling (high molecular weight fragments on Bioanalyzer). Typically, most samples will amplify best in the range of 6–18 cycles.

Add the following components to a sterile strip tube:

	A	B
	COMPONENT	**VOLUME**
	Purified PCR1 DNA (Step 34)	5 µl
	(blue) Q5 Reaction Buffer (5X)	5 µl
	(lilac) dNTP Solution Mix	0.5 µl
	(blue) Q5 Hot Start High-Fidelity DNA Polymerase	0.5 µl
	(blue) NEBNext IS PCR2 Universal Primer	1 µl
	(blue) NEBNext i5 Primer*	1 µl
	SYBR Green (20X)**	0.25 µl
	(white) Nuclease-free Water	11.75 µl
	Total Volume	25 µl

* Use one i5 Primer per PCR reaction
** 20X working stock of SYBR Green can be made by diluting 1 µl (10,000X) SYBR Green (Life Technologies #S7563) into 399 µl DMSO and stored at 4°C for up to a month. You only need 0.2 x SYBR final in the qPCR reaction.

36 Set a 100 µl or 20 µl pipette to 20 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

37 Place the tube on a qPCR instrument and run the following PCR cycling program:

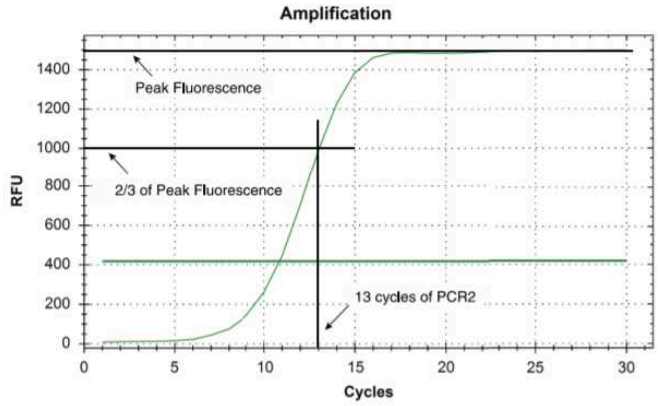
	A	B	C	D
	CYCLE STEP	**TEMP**	**TIME**	**CYCLES**

	A	B	C	D
	Initial Denaturation	98°C	60 seconds	1
	Denaturation	98°C	10 seconds	30
	Annealing and Extension	72°C	30 seconds (fluorescence on*)	
	Hold	4°C	∞	

* Select the SYBR channel.

38 The amplification curve for each sample is used to determine the optimal number of PCR cycles to use during PCR2 (Section: PCR2 for Final Library). The PCR cycle number is approximated by determining the cycle number (round down to the nearest whole number) corresponding to 2/3 of the peak fluorescence (see example in Figure 3; in this example 13 PCR cycles will be used in Section: PCR2 for Final Library).

Figure 3. Determining cycle number for PCR2 using amplification curve of sample from a qPCR run.



PCR2 for Final Library

39 Add the following components to a sterile strip tube:

	A	B
	COMPONENT	**VOLUME**
	Purified PCR1 DNA (Step 34)	10 µl

	A	B
	(blue) Q5 Reaction Buffer (5X)	10 µl
	(lilac) dNTP Solution Mix	1 µl
	(blue) Q5 Hot Start High-Fidelity DNA Polymerase	1 µl
	(blue) NEBNext IS PCR2 Universal Primer	2 µl
	(blue) NEBNext i5 Index Primer*	2 µl
	(white) Nuclease-free Water	24 µl
	Total Volume	50 µl

* Use one i5 Primer per PCR reaction

40 Set a 100 µl pipette to 40 µl then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

41 Place the tube on a thermal cycler and run the following PCR cycling program:

	A	B	C	D
	CYCLE STEP	**TEMP**	**TIME**	**CYCLES**
	Initial Denaturation	98°C	60 seconds	1
	Denaturation	98°C	10 seconds	X*
	Annealing and Extension	72°C	30 seconds	
	Hold	4°C	∞	

* Use number of cycles determined from Step 38. If qPCR was not done, see Table 1 for recommended number of PCR cycles. In this case the number of PCR cycles should be chosen based on input amount and sample type. The number of cycles should be high enough to provide sufficient library fragments for a successful sequencing run, but low enough to avoid PCR artifacts and over-cycling (high molecular weight fragments on Bioanalyzer).

Table 1. For enriched B Cell or T Cell RNA input, PCR2 cycle number is recommended as in the table:

	A	B	C
	TOTAL RNA INPUT	**PCR1 ASSAY PCR2**	**CYCLE NUMBER**
	B Cell RNA 1,000 ng	BCR	6-7
	B Cell RNA 100 ng	BCR	9-10
	B Cell RNA 10 ng	BCR	13-14
	T Cell RNA 1,000 ng	TCR	10-11
	T Cell RNA 100 ng	TCR	12-13
	T Cell RNA 10 ng	TCR	17-18

Note

If B and T cells are combined, choose a PCR2 cycle number in between the recommendations for B cell and T cell RNA.

Cleanup of PCR2 Amplification to Obtain Final Libraries

46m

42 Vortex NEBNext Sample Purification Beads to resuspend. SPRIselect and AMPure XP Beads can be used as well. If using AMPure XP Beads, please allow the beads to warm to Room temperature for at least 00:30:00 before use.

30m

43 Add 50 µL of TE (0.1X) to each 50 µl PCR reaction to obtain 100 µl mixture, and then add 50 µL resuspended NEBNext Sample Purification Beads to the 100 µl mixture. Mix well by pipetting or brief vortexing.

44 Incubate for 00:05:00 at Room temperature .

5m

45 Quickly spin the tube in a microcentrifuge and place it on a magnetic rack. Once the solution is clear (~5 minutes) carefully transfer the 150 µL supernatant to a new tube without disturbing the bead pellet. **Save the supernatant and discard beads.**

46 Add 15 µL of resuspended NEBNext Sample Purification Beads to supernatant. Mix well by pipetting or brief vortexing.

47 Incubate for 00:05:00 at Room temperature . 5m

48 Quickly spin the tube in a microcentrifuge and place it on a magnetic rack. Once the solution is clear (~ 00:05:00) carefully remove and discard the supernatant without disturbing the bead pellet. **Do not discard beads.** 5m

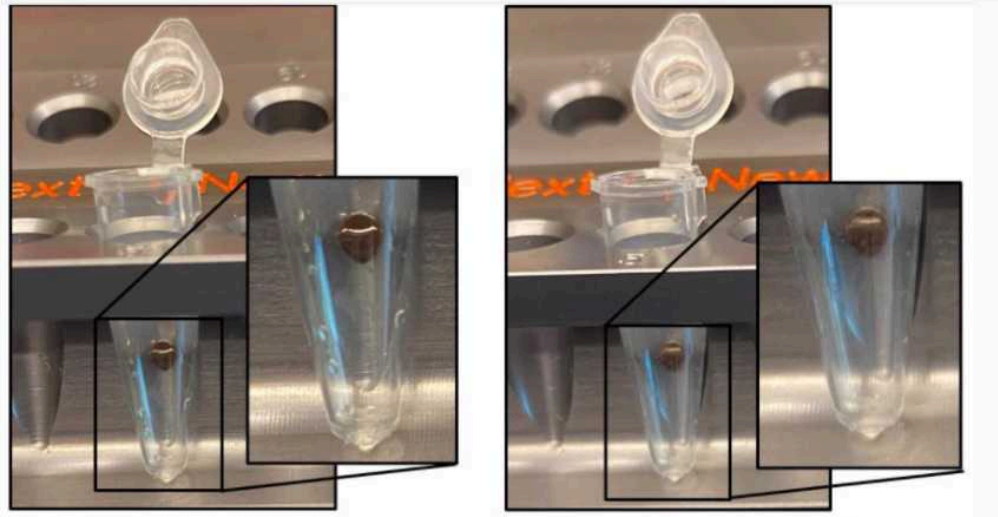
49 Wash beads by adding 200 µL of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at Room temperature for 00:00:30 , and then carefully remove and discard the supernatant. 30s

50 Wash beads by adding 200 µL of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at Room temperature for 00:00:30 , and then carefully remove and discard the supernatant once for a total of two wash steps. After supernatant is discarded, carefully remove any residual liquid left at the bottom of the tube without disturbing the beads with a 10 µl pipette tip. 30s

51 Air dry the beads for up to 00:05:00 while the tube is on the magnetic stand with the lid open. 5m

Note

Caution: Do not over dry the beads. This may result in lower recovery of DNA target.



52 Elute DNA target from beads by adding 20 µL TE (0.1X) to beads. Mix well on a vortex mixer or by pipetting up and down at least 10 times. Incubate for at least 00:02:00 at Room temperature . 2m

53 Quickly spin the tube and place it on a magnetic rack to separate beads from supernatant. After the solution is clear (about 2 minutes), carefully transfer 18 µL supernatant to a new PCR tube.

Note

Safe Stopping Point: It is safe to store the library overnight at -20 °C .

Assess Library Quality on a Bioanalyzer High Sensitivity Chip or TapeStation High Sensitivity D1000 ScreenTape®

54 Dilute library 5-fold in nuclease-free water.

55 Run 1 µL on a Bioanalyzer DNA High Sensitivity chip.

56 Check that the electropherogram shows the expected size distribution. B Cell Receptor chain libraries will have an average size of 647 bp (Figure 4). T Cell Receptor chain libraries will have an average size of 630 bp (Figure 5). BCR + TCR chain libraries will have an average size of 632 bp (Figure 6).

Figure 4. Example of B Cell Receptor library size distribution on a Bioanalyzer.

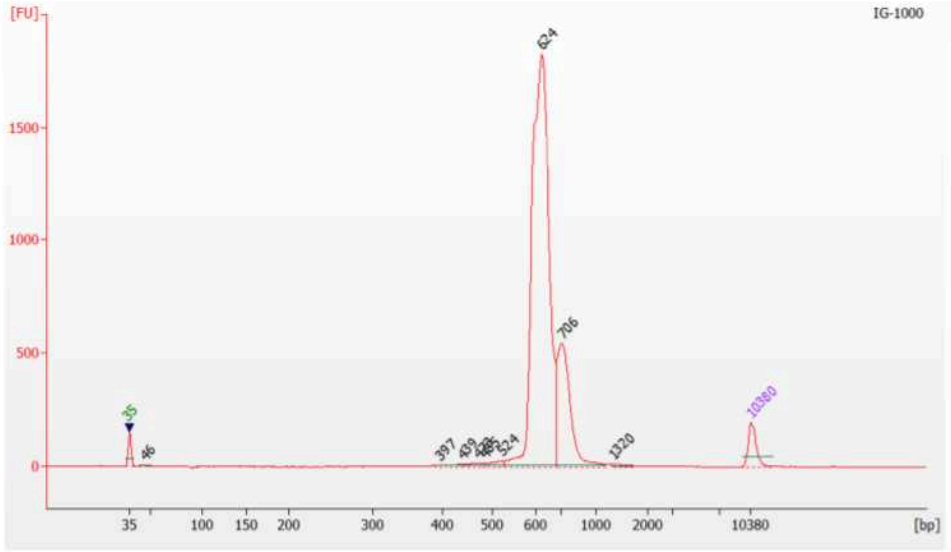


Figure 5. Example of T Cell Receptor library size distribution on a Bioanalyzer.

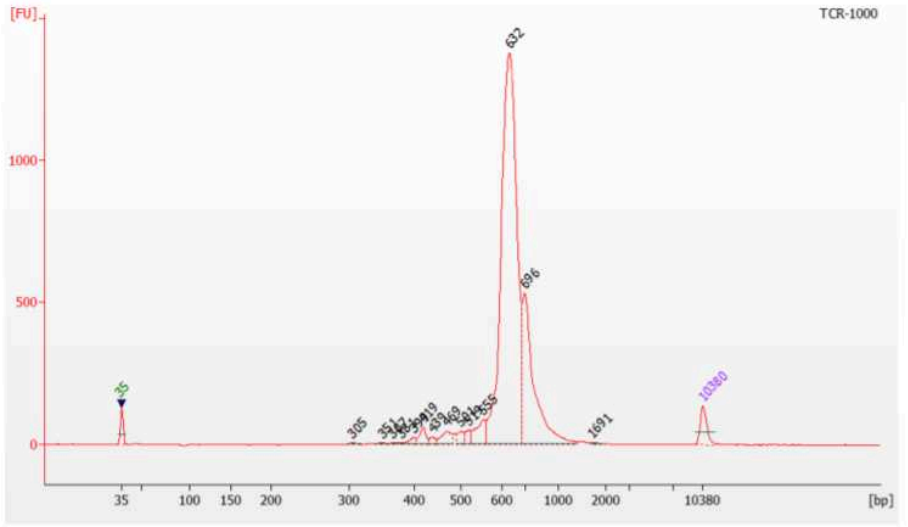
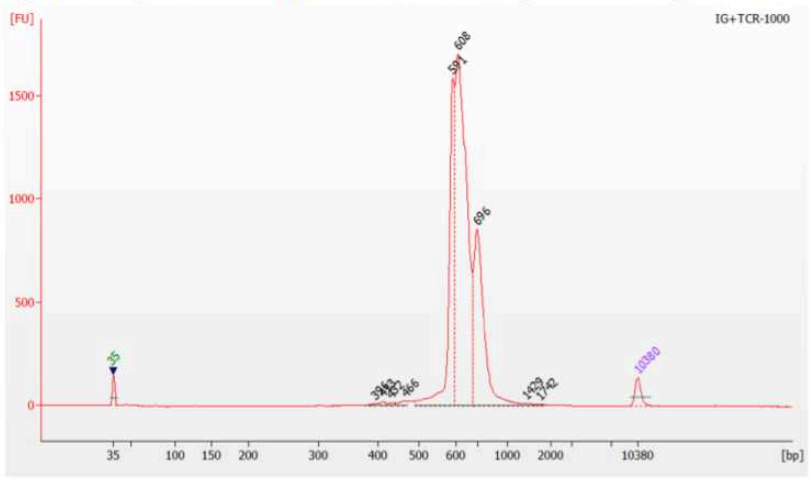


Figure 6. Example of B Cell Receptor chains and T Cell Receptor chains library size distribution on a Bioanalyzer.



Sequencing and Data Analysis

57 Sequencing should be performed on an Illumina MiSeq, using Illumina V3 sequencing chemistry. We recommend running 300 PE using a 600-cycle V3 MiSeq reagent kit with the index read between the first and second read.

Note

For information on data processing please refer to Data Usage Guideline Page within the "Usage Guidelines & Tips" panel in the "Other Tools & Resources" tab at: https://www.neb.com/products/e6320-nebnext-immune-sequencing-kit-human#Product%20Information

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://usegalaxy.org/u/bradlanghorst/w/presto-nebnext-immune-seq-workflow-v320.

Index Pooling Guidelines

58 For more detailed indexing information please refer to the manual for NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1), NEB #E7600. https://www.neb.com/-/media/nebus/files/manuals/manuale7600.pdf?rev=66702217f5614fae834024da17e8009b&hash=10C7820E5CB9543B5446A574B2DF2B31

For multiplexing samples, use Table 1 for some valid index combinations.

Table 1. Examples of valid index combinations.

PLEX	i7 PRIMERS	i5 PRIMERS
2	i701 and i702 i703 and i704 i705 and i706 i707 and i708 i709 and i710 i711 and i712	Any i5 primer
3	i701, i702 and i703 i703, i704 and i705 i705, i706 and i707 i707, i708 and i709 i709, i710 and i711	Any i5 primer
4	i701, i702, i703 and i704 i703, i704, i705 and i706 i705, i706, i707 and i708 i707, i708, i709 and i710 i709, i710, i711 and i712	Any i5 primer
5-12	Any valid i7 4-plex with any other i7 primers	Any i5 primer
7-12	Any 3 plex combination with and any other i7 primer (as needed)	i501, i502 and any other i5 primer (as needed) i503, i504 and any other i5 primer (as needed) i505, i506 and any other i5 primer (as needed) i507, i508 and any other i5 primer (as needed)
Greater than 12	Any 4 plex combination with any other i7 primer (as needed)	i501, i502 and any other i5 primer (as needed) i503, i504 and any other i5 primer (as needed) i505, i506 and any other i5 primer (as needed) i507, i508 and any other i5 primer (as needed)

Table 2. Index sequences of NEBNext i501 Primer – NEBNext i508 Primer:

PRODUCT	EXPECTED INDEX READ
	MiSeq
NEBNext i501 Primer	TATAGCCT
NEBNext i502 Primer	ATAGAGGC
NEBNext i503 Primer	CCTATCCT
NEBNext i504 Primer	GGCTCTGA
NEBNext i505 Primer	AGGCGAAG
NEBNext i506 Primer	TAATCTTA
NEBNext i507 Primer	CAGGACGT
NEBNext i508 Primer	GTACTGAC

Table 3. Index sequences of NEBNext i701 Primer – NEBNext i712 Primer:

PRODUCT	EXPECTED INDEX READ
NEBNext i701 Primer	ATTACTCG
NEBNext i702 Primer	TCCGGAGA
NEBNext i703 Primer	CGCTCATT
NEBNext i704 Primer	GAGATTCC
NEBNext i705 Primer	ATTCAGAA
NEBNext i706 Primer	GAATTCGT
NEBNext i707 Primer	CTGAAGCT
NEBNext i708 Primer	TAATGCGC
NEBNext i709 Primer	CGGCTATG
NEBNext i710 Primer	TCCGCGAA
NEBNext i711 Primer	TCTCGCGC
NEBNext i712 Primer	AGCGATAG