INSTRUCTION MANUAL

NEBNext® Immune Sequencing Kit (Human)

NEB #E6320S/L

24/96 reactions

Version 3.0_10/25

Table of Contents

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 –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.

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

NEB #E6325-1, NEBNext Immune Sequencing Kit RT and PCR Reagents (NEB #E6320S, 24 reactions)

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 RT and PCR Reagents (NEB #E6320L, 96 reactions)

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 (Life Technologies #S7563)
- 80% Ethanol (freshly prepared)
- DNA LoBind® Tubes (Eppendorf #022431021)
- Magnetic rack/stand (NEB #S1515S, Alpaqua®, cat. #A001322 or equivalent)
- 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
- Magnet stand or equivalent (for washing beads only) (NEB #S1506)
- DNase RNase-free PCR strip tubes (USA Scientific 1402-1708)

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 bias 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

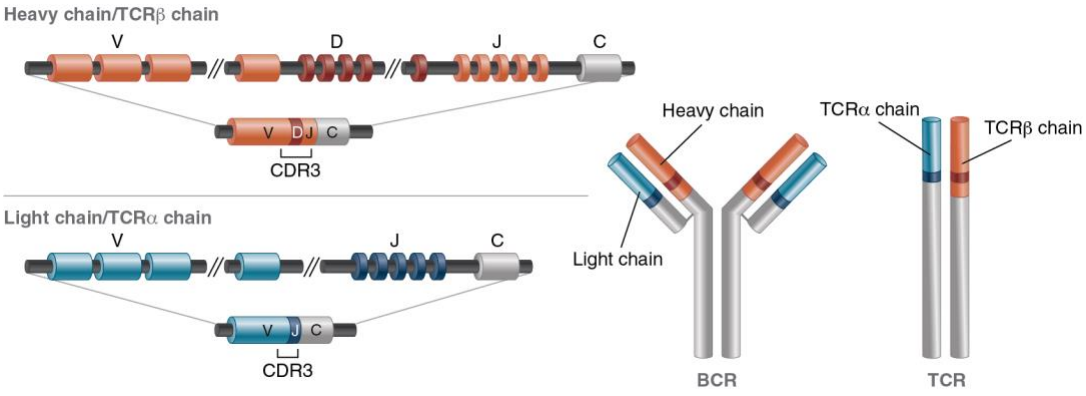
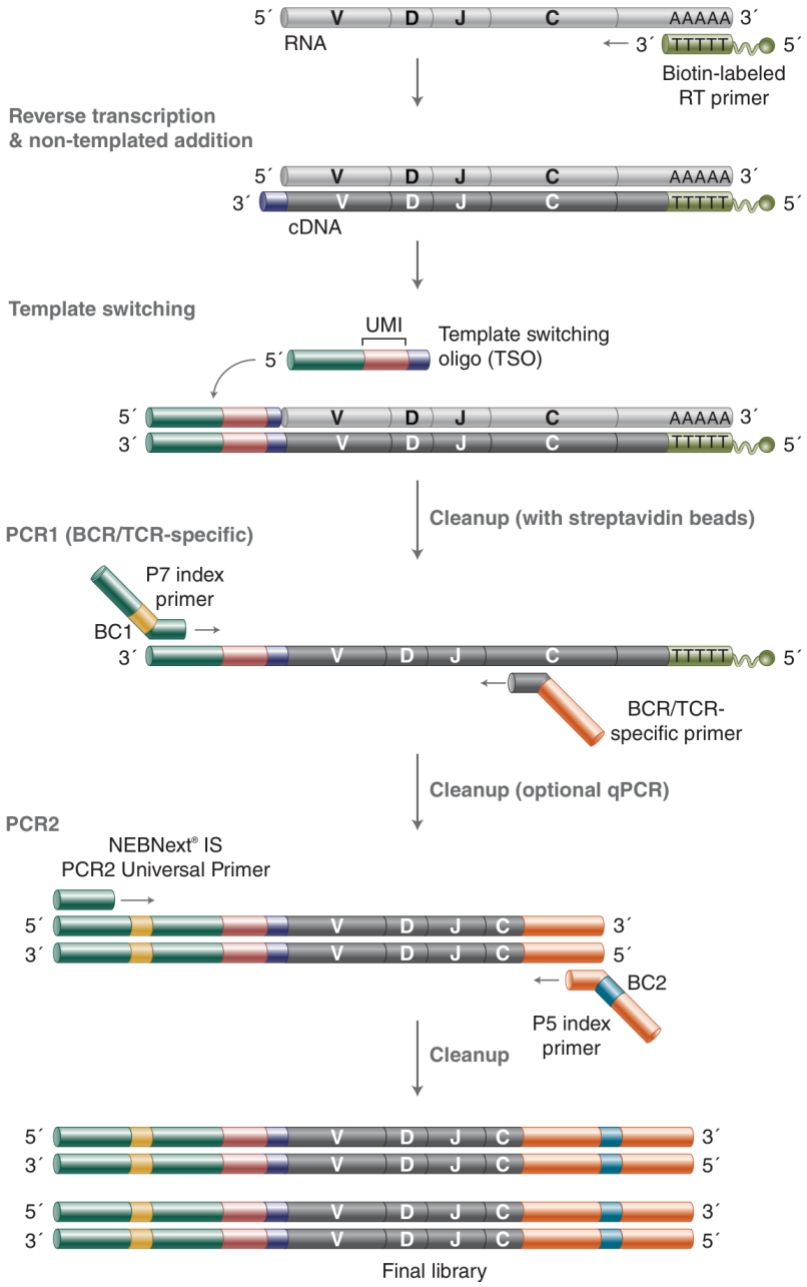


Figure 2. NEBNext Immune Sequencing Library Preparation Kit workflow



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 Customized Solutions team at NEB. Please contact custom@neb.com for further information.

Protocol for NEBNext Immune Sequencing Kit (Human)

Symbols

This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.

This caution sign signifies a step in the protocol that has two paths leading to the same end point.

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Colored bullets indicate the cap color of the reagent to be added.

RNA Sample Recommendations: The RNA sample should be free of salts (e.g., Mg^{2+}, or guanidine salts) or organics (e.g., phenol and ethanol). High quality RNA with a RIN > 7 is required 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). Note: The maximum volume of RNA input is 9 µl. PBMC's or other tissue sample types have variable amounts of immune cells and therefore optimization of the PCR cycle number may be necessary.

1. NEBNext Immune Sequencing Reverse Transcription and cDNA Synthesis

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

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**

A master mix of reaction components can be prepared before addition to the sample.

1.2. 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.

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

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

2. Purify the cDNA with Streptavidin Magnetic Beads

2.1. Prepare Bind and Wash Buffer (1X) to be used in Step 2.11.

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

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

COMPONENT	VOLUME FOR 1 REACTION	VOLUME FOR 10 REACTIONS*
NEBNext IS Bind and Wash Buffer (2X)	50 µl	550 µl
Nuclease Free Water	50 µl	550 µl
Final Volume of NEBNext IS Bind and Wash Buffer (1X)	100 µl	1100 µl

* We recommend preparing an excess of 10% when preparing Bind and Wash Buffer (1X)

2.2. Immediately before use, 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.

2.3. Place the tube on a magnetic rack (NEB #S1506 or equivalent) at room temperature. Once the solution is clear (~2 minutes) carefully remove and discard the supernatant without disturbing the bead pellet.

2.4. 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.

When processing 3 or more samples at the same time, it is possible to prepare beads in one 1.5 ml microcentrifuge tube.

a) Add the volume of beads that is required for the number of samples to be processed. Consider adding 10% overage when preparing reagents for more than 1 sample.

For example, for 10 samples, add 165 µl beads [(10 x 15 µl) + 10%]

b) Add enough volume of Undiluted NEBNext IS Bind and Wash Buffer (2X) to fully resuspend the beads, at least 200 µl and up to the volume capacity of the 1.5 ml microcentrifuge tube.

For example, for 10 samples, add 1,500 µl Undiluted NEBNext IS Bind and Wash Buffer (2X)

COMPONENT	VOLUME 1 REACTION	VOLUME 10 REACTIONS*
Streptavidin Magnetic Beads (Step 2.2.)	15 µl	165 µl
Undiluted NEBNext IS Bind and Wash Buffer (2X) (Step 2.4.)	200 µl	1500 µl**

* We recommend preparing an excess of 10% when preparing Bind and Wash Buffer (1X)

** Maximum volume in a 1.5 ml tube.

2.5. Place the tube on a magnetic rack. Once the solution is clear (about 2 minutes), carefully remove and discard the supernatant without disturbing the bead pellet.

2.6. Repeat Steps 2.4.–2.5. once for a total of two washes.

2.7. 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 x 20 = 80 µl NEBNext IS Bind and Wash Buffer (2X)). Vortex briefly to resuspend the beads.

2.8. Add 20 µl beads to the cDNA products (20.5 µl) from Step 1.3. Vortex briefly to mix.

2.9. Place the tube on a rotating mixer for 15 minutes at room temperature. You can also use a Thermomix with no heat at 800 rpm for 15 minutes.

2.10. 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.

2.11. Remove the tube from the magnetic rack. Add 100 µl diluted Bind and Wash Buffer (1X) (from Step 2.1). Vortex briefly to resuspend the beads.

2.12. 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 (about 5 minutes), carefully remove and discard the supernatant without disturbing the bead pellet.

2.13. 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).

2.14. 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 (about 5 minutes), carefully remove and discard the supernatant without disturbing the bead pellet. With a 20 µl pipette tip, carefully remove any residual liquid left at the bottom of the tube without disturbing the beads.

2.15. 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.

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

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

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

2.18. Once the solution is clear (about 5 minutes), transfer 21 µl of the supernatant into a new tube and discard the beads.

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

3. PCR1 for VDJ Region Amplification and Index Barcode Incorporation

Use Option 3.1A for enriching B Cell Receptor (BCR) chains.
Use Option 3.1B for enriching T Cell Receptor (TCR) chains.
Use Option 3.1C for enriching both BCR chains and TCR chains.

3.1. Add the following components to a sterile strip tube:

3.1A. PCR1 for Enriching B Cell Receptor Chains

COMPONENT	VOLUME
Purified cDNA (Step 2.18.)	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
○ (white) NEBNext IS BCR Primers (Human)	2 µl
• (blue) NEBNext i7 Primer*	1 µl
○ (white) Nuclease-free Water	14 µl
Total Volume	**50 µl**

* Use only one i7 Primer per sample

For multiple reactions, a master mix of most reaction components can be made. Do not premix the i7 Primer with the other reaction components.

3.1B. PCR1 for Enriching T Cell Receptor Chains

COMPONENT	VOLUME
Purified cDNA (Step 2.18.)	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
○ (white) NEBNext IS TCR Primers (Human)	2 µl
• (blue) NEBNext i7 Primer*	1 µl
○ (white) Nuclease-free Water	14 µl
Total Volume	**50 µl**

* Use only one i7 Primer per sample

For multiple reactions, a master mix of most reaction components can be made. Do not premix the i7 Primer with the other reaction components.

3.1C. PCR1 for Enriching Both B Cell Receptor Chains and T Cell Receptor Chains

COMPONENT	VOLUME
Purified cDNA (Step 2.18.)	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
○ (white) NEBNext IS BCR Primers (Human)	2 µl
○ (white) NEBNext IS TCR Primers (Human)	2 µl
• (blue) NEBNext i7 Primer*	1 µl
○ (white) Nuclease-free Water	12 µl
Total Volume	**50 µl**

* Use only one i7 Primer per sample

For multiple reactions, a master mix of most reaction components can be made. Do not premix the i7 Primer with the other reaction components.

3.2. Set a 100 µl or 200 µl pipette to 40 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.

3.3. Place the tube on a thermal cycler with the heated lid on or set at ≥ 105°C and perform PCR amplification using the following PCR cycling program:

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	∞	

* Depending on the content of target RNA of your samples, optimization of the PCR cycle number may be necessary.

4. Cleanup of PCR1 Amplification

4.1. 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 30 minutes before use.

4.2. Add 50 µl of resuspended NEBNext Sample Purification Beads to each 50 µl PCR reactions. Mix well by pipetting up and down at least 10 times or briefly vortexing.

4.3. Incubate for 5 minutes at room temperature.

4.4. Quickly spin the tube in a microcentrifuge and place it on a magnetic rack. Once the solution is clear (~5 minutes) carefully remove and discard the supernatant without disturbing the bead pellet. (**Caution: do not discard beads**).

4.5. Wash beads by adding 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

4.6. Repeat Step 4.5 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.

4.7. Air dry the beads for **up to** 5 minutes while the tube is on the magnetic stand with the lid open.

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

4.8. 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 2 minutes at room temperature. Quickly spin the tube and place it on a magnetic rack to separate beads from supernatant.

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

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

5. qPCR for PCR2 Cycle Optimization

The qPCR step is optional, but highly recommended while optimizing this kit for your specific samples; 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.

5.1. Add the following components to a sterile strip tube:

COMPONENT	VOLUME
Purified PCR1 DNA (Step 4.9.)	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 sample

** 20X working stock of SYBR Green can be made by diluting 1 µl (10,000X) SYBR Green (Life Technologies #S7563) into 499 µl DMSO and stored at 4°C for up to a month. You only need 0.2 x SYBR final in the qPCR reaction.

For multiple reactions, a master mix of most reaction components can be made. Do not premix the i5 Primer with the other reaction components.

5.2. 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.

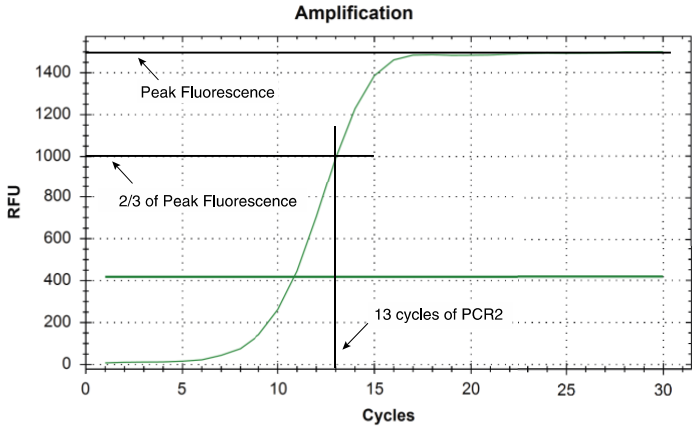
5.3. Place the tube on a qPCR instrument and run the following PCR cycling program:

CYCLE STEP	TEMP	TIME	CYCLES
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.

5.4. The amplification curve for each sample is used to determine the optimal number of PCR cycles to use during PCR2 (Section 6). 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 6).

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



6. PCR2 for Final Library

6.1. Add the following components to a sterile strip tube:

COMPONENT	VOLUME
Purified PCR1 DNA (Step 4.9)	10 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 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 sample

For multiple reactions, a master mix of most reaction components can be made. Do not premix the i5 Primer with the other reaction components.

6.2. 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.

6.3. Place the tube on a thermal cycler with the heated lid on or set at ≥ 105°C and run the following PCR cycling program:

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 5.4. 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:

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.

7. Cleanup of PCR2 Amplification to Obtain Final Libraries

7.1. 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 30 minutes before use.

7.2. 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 up and down at least 10 times, or briefly vortexing.

7.3. Incubate for 5 minutes at room temperature.

7.4. 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**.

7.5. Add 15 µl of resuspended NEBNext Sample Purification Beads to supernatant. Mix well by pipetting up and down at least 10 times, or briefly vortexing.

7.6. Incubate for 5 minutes at room temperature.

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

7.8. Wash beads by adding 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

7.9. Repeat Step 7.8 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.

7.10. Air dry the beads for **up to** 5 minutes while the tube is on the magnetic stand with the lid open.

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

7.11. 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 2 minutes at room temperature.

7.12. 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.

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

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

8.1 Run 1 µl on a Bioanalyzer DNA High Sensitivity chip. A dilution may be necessary for running on a Bioanalyzer High Sensitivity DNA Chip.

8.2 Check that the electropherogram shows the expected size distribution. B Cell Receptor chain libraries will have an average size of 624 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

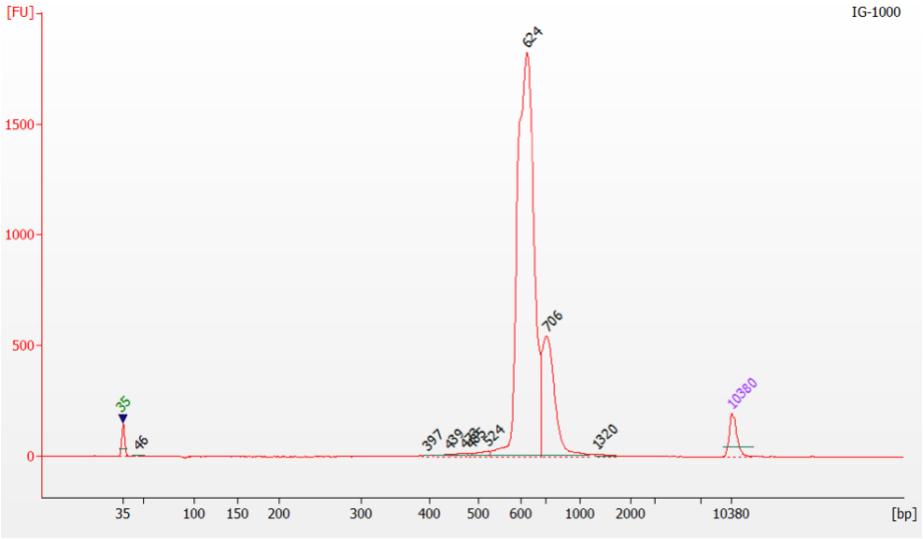


Figure 5. Example of T Cell Receptor library size distribution on a Bioanalyzer

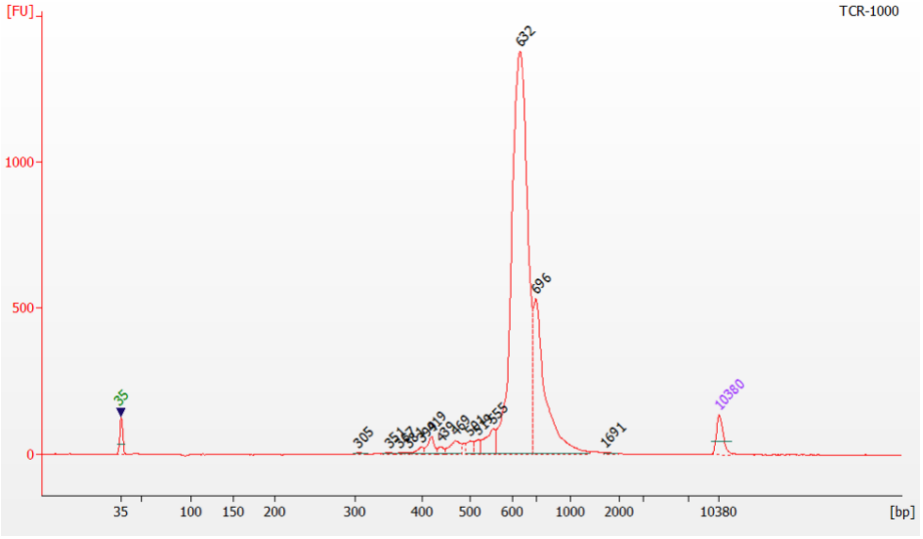
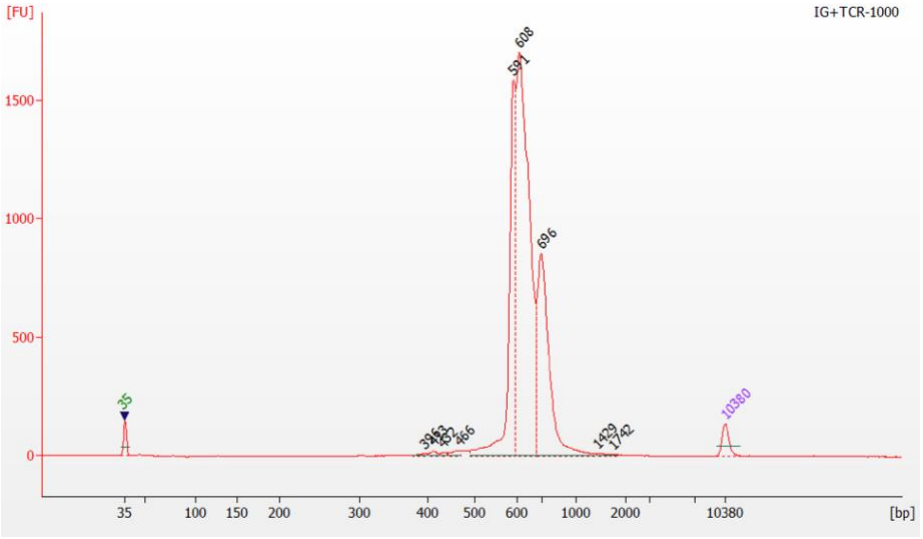


Figure 6. Example of B Cell Receptor chains and T Cell Receptor chains library size distribution on a Bioanalyzer



9. Sequencing and Data Analysis

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.

On the Miseq 7 – 8 pM. On the NextSeq 1000 / 2000 we typically load 650 pM – 1 nM. If the samples are monoclonal type or low diversity, 5-10% PhiX can be added to the pool

In addition to the MiSeq 600 cycle kit we have also tested NEBNext Immune Sequencing libraries on the NextSeq 2000 600 cycle kit. The NextSeq 2000 produces a higher reads output that allows deeper sequencing for low abundance clonotype detection. NextSeq 2000 also produces a higher read quality and less sequencing failures. However, the best practice is to use unique dual index primers for libraries to load on patterned flow cell instruments like the NextSeq 2000. The current NEBNext Immune Sequencing Kit E6320 and E6330 do not supply UDI primers. Please contact NEB Technical support at info@neb.com to inquire about sequencing Immune-seq libraries on NextSeq 2000.

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: www.neb.com/E6320

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.

10. Index Pooling Guidelines

For more detailed indexing information please refer to the manual for NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1), NEB #E7600

To identify valid index combinations (up to 8-plex) for each set that can be sequenced together, please visit https://indexoligo.neb.com. For combinations > 8, choose any column and add any well combinations as needed.

Kit Components

NEB #E6320S Table of Components

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	Q5 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
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

NEB #	PRODUCT	VOLUME
E6321AA	NEBNext IS RT Buffer (4X)	0.480 ml
E6322AA	NEBNext IS 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
E6327AA	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
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 i709 Primer	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

Revision History

REVISION #	DESCRIPTION	DATE
1.0		N/A
2.0	Update protocol text.	12/21
3.0	Updated NEBNext Immune Sequencing Library Preparation Kit workflow (Figure 2), updated Sequencing and Data Analysis (Section 9) and Index Pooling Guidelines (Section 10), specified required input type, updated protocols.	10/25