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USER MANUAL v1.1

DepleteX® Rare Transcript Boost Kit

NOVA-512860

For post-library depletion of Illumina NGS libraries

Contents

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Product Overview

The DepleteX® Rare Transcript Boost Kit removes the highest expressing transcripts from RNA-Seq libraries prior to sequencing, allowing the user to better detect less abundant transcripts and rare isoforms. The kit leverages the CRISPR Cas9 endonuclease and associated guide RNA to remove abundant protein-coding transcripts (~70% of total sequencing reads in next-generation sequencing (NGS) data) from poly(A)-selected RNA-Seq libraries prepared from fibroblasts and whole blood.

Description	
Total Assay Time	~18 hours (Includes 16 hours overnight incubation)
Hands-on Time	~1 hour
Samples Per Kit	24 samples
Sample Type	RNA-Seq Poly(A)-Selected DNA Library from Human Fibroblast or Whole Blood
DNA Library Input	10 ng
Average DNA Library Size	≥ 420 bp
Designed to Deplete	~4,450 high expressing transcripts (TPM >30) constituting ~70% of sequencing reads from RNA-Seq libraries
Method	CRISPR-Cas9 mediated depletion
Validated Library Prep	NEBNext® Ultra II Directional RNA libraries prepared with the Poly(A) mRNA Magnetic Isolation Module

Workflow

The protocol involves a 16-hour incubation for effective depletion. We recommend beginning the protocol at the end of the workday on Day 1 and continuing with the remaining portion of the protocol on the morning of Day 2.

1 **RNA Library Prep**

2 **DepleteX™ Rare Transcript Boost Kit (Post-Library Depletion)**

Step A: Depletion of Highly Expressed RNA

☐ Hands-on time: ~30 min | Total time: ~17 hours (including 16-hour incubation)

1. RNP Complex Formation & CRISPR Digestion
2. Bead Size Selection

Safe Stopping Point

Step B: PCR Amplification

☐ Hands-on time: ~30 min | Total time: ~1 hour

1. PCR Amplification
2. Bead Cleanup

Safe Stopping Point

3 **Sequencing**

Kit Contents and Storage

The DepleteX Rare Transcript Boost Kit contains enough material to deplete 24 RNA-Seq libraries. The kit contents and storage temperatures are indicated in the tables below.

ASY1064: Depletion Reagents (8 depletion reactions per box)

3 boxes
Storage at -20°C

Kit contents	Part number	Quantity per box
Cas9	REA1039	1 tube
10X Cas9 Buffer	REA1040	1 tube
RNase Inhibitor	REA1041	1 tube
Nuclease-Free Water	REA1042	1 tube
Tris Buffer	REA1043	1 tube
P5 Primer	REA1044	1 tube
P7 Primer	REA1045	1 tube
2X PCR Mix	REA1046	1 tube

ASY1070: Guide RNA

1 Bag
Storage at -80°C

Kit contents	Part number	Quantity per bag
Guide RNA (High Expressing RNA)	REA1047	1 tube

Required Materials and Equipment

Type	Item	Supplier
Plastic Consumables	0.5 mL, 1.5 mL DNA LoBind Tubes	Eppendorf (Cat# 022431021)
	0.2 mL thin wall PCR tubes	General Lab Supplier
	Low-Retention, Filtered, Sterile Tips (10 µL, 20 µL, 200 µL and 1000 µL)	General Lab Supplier
Reagents	AMPure XP Beads	Beckman Coulter (Cat# A63881)
	Absolute Ethanol, 200 Proof	General Lab Supplier
	Qubit dsDNA HS Assay Kit	ThermoFisher Scientific (Cat# Q32854)
Equipment	Single Channel Pipettes (10 µL, 20 µL, 200 µL, and 1000 µL)	General Lab Supplier
	Multichannel Pipettes (10 µL, 20 µL, and 200 µL)	General Lab Supplier
	Vortex Mixer	General Lab Supplier
	Microcentrifuge	General Lab Supplier
	PCR Magnetic Rack or Stand	General Lab Supplier
	Ice Bucket	General Lab Supplier
	PCR Thermal Cycler	General Lab Supplier
	Qubit Fluorometer	ThermoFisher Scientific (Cat# Q33238)
	Automated electrophoresis Instrument such as Agilent TapeStation or 2100 BioAnalyzer	General Lab Supplier

Best Practices

General

- Periodically, optimizations and revisions are made to the kit components and user manual. Therefore, following the protocol included with or appropriate for the kit in question is important. This can be done by comparing the name and version number of the product to the name and version number of the protocol.
- This protocol describes the reagents, best practices, workflow, and method details for DepleteX Rare Transcript Boost kit.
- Use properly calibrated pipettes as library preparation is sensitive to pipetting error.
- Follow good laboratory practices throughout the protocol, including RNA-safe procedures when handling RNA. Ensure all pipette tips, microcentrifuge tubes, and other consumables are DNase- and RNase-free.
- When undertaking the protocol, always proceed immediately to the next step. If a stop is necessary, safe stopping points are available. Refer to the workflow schematic on page 3.

Reagent Handling

- Do not remove Cas9 and RNase Inhibitor from storage until before use. Maintain on ice during reaction setup. Return to -20°C immediately after use.
- Do not remove the guide RNA from storage until immediately before use. Maintain on ice during reaction setup. Return to -80°C immediately after use.
- We recommend a maximum of three freeze-thaw cycles for the Guide RNA. It is strongly recommended that multiple smaller aliquots of the Guide RNA be prepared when the reagent is first thawed if more than three freeze-thaw cycles are expected.
- Do not freeze AMPure® XP beads.
- Allow AMPure XP beads to come to room temperature before use. A thirty-minute incubation on the laboratory bench is usually sufficient.
- Vortex AMPure XP beads immediately before use. Ensure that the beads are in a uniform suspension before use.
- Use magnetic stands appropriate for PCR tubes.

Equipment Handling

- Thermal cycling should be performed with a heated lid, except where specified otherwise. Thermal cycler(s) must support uniform heating up to 100 µL sample volume.

Revision Log

Version	Date	Description
V1.0	December 2022	Launch
V1.1	April 2024	Edited for clarity and format. Updated reagent volume in Step A1.2.

Input Material

Please review and follow the guidelines below for preparing RNA-Seq libraries that are optimal for depletion:

NGS Library Prep Requirements

This product is designed for optimal performance with RNA-Seq libraries prepared from human fibroblast or whole blood. Ribosomal RNA depletion is not included with this product. We recommend the use of poly(A)-selected libraries to ensure that little or no rRNA remains in the library when depletion is performed.

The Jumpcode depletion protocol works most effectively with RNA-Seq libraries of ≥ 420 bp fragment sizes. For many library preparation methods involving RNA fragmentation by heat, this can be achieved with a combination of short RNA fragmentation times and dual SPRI bead size selection or agarose gel-based size selection. We recommend the highest practical RNA input quantity for the library preparation method of interest to ensure high molecular diversity in the prepared libraries. If the molecular diversity of the library is low, depletion is less likely to reveal previously unseen information and more likely to generate duplicates.

DepleteX Rare Transcript Boost Kit has been optimized with the NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760/E7765) with the Poly(A) mRNA Magnetic isolation Module (NEB #7490) to remove ribosomal RNA. Refer to the "NEBNext Ultra II RNA Library Prep Kit for Illumina" Instruction Manual (*Version 5.0_12/22, Section 1), for detailed information on the protocol and required reagents. The following conditions are recommended for RNA library preparation to ensure optimal depletion:

RNA-Seq Library Preparation:	Validated Input / Method:
Starting Material:	200 ng Total RNA from Human Fibroblast or Whole Blood
RNA Selection Method:	NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490) *Step 1
RNA Fragmentation Time:	5 minutes at 94°C (Target mRNA Size (nt): 400 – 1,000) *Step 1.2.37
Double-Sided Bead Size-Selection:	DNA Library AVG Size ≥ 420 bp (Target DNA Insert AVG Size: ≥ 300 bp) *Appendix A Step 6.2
PCR Enrichment:	~13 PCR Cycles *Step 1.9.3b

Before Proceeding to Step A.

Please follow the guidelines below:

- Review the library preparation method(s). The libraries should have been prepared and stored under nuclease-free conditions.
- Ensure that the libraries are uniquely indexed.
- This protocol includes a 16-hour incubation for effective depletion. We recommend beginning the protocol at the end of the workday with overnight incubation, taking the protocol to completion the following day. Refer to the workflow schematic on page 3.

Protocol

Before attempting this protocol for the first time, it is highly recommended that the user read the "Input Material" section on page 7.

The protocol includes an overnight (16 hours) incubation step. We recommend starting the protocol at the end of the workday on Day 1 and complete the procedure on Day 2.

Step A: Depletion of Highly Expressed RNA

☐ Hands-on time: ~ 30 min | Total time: ~17 hrs

Reagent Preparation

Item	Storage	Handling
Poly(A)-Selected RNA-Seq Library ≥ 420bp (10 ng)	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
Cas9		
RNase Inhibitor		
Guide RNA (High Expressing RNA)	-80°C	
Nuclease-Free Water	-20°C	Thaw at room temperature. Vortex briefly and spin down. Keep on ice.
10X Cas9 Buffer		
AMPure XP Beads	4°C	Bring to room temperature. Vortex and invert mix.
80% Ethanol	Room Temperature	Prepare fresh.

A1: Ribonucleoprotein (RNP) Complex Formation & CRISPR Digestion

1. Dilute the RNA-Seq libraries you intend to deplete to a final concentration of 10 ng in a 9 µL volume using Nuclease-Free Water. Set aside for "CRISPR Digestion" reaction mix.
2. At room temperature, combine the following reagents in the order listed below in a 0.2 mL PCR tube:

RNP Complex Formation Reaction	Volume
Nuclease-Free Water	2.5 µL
10X Cas9 Buffer	1.0 µL
Cas9	2.0 µL
RNase Inhibitor	1.0 µL
Guide RNA (High Expressing RNA)	3.5 µL
Total Volume	10 µL

3. Mix gently by flicking the tube or pipetting up and down. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
4. Incubate the reaction mix at room temperature for 10 minutes. This constitutes the "Ribonucleoprotein (RNP) Complex."
5. To the tube containing the RNP Complex, add the following reagents in the order listed below at room temperature:

CRISPR Digestion Reaction	Volume
RNP Complex (Step A1.4)	10 µL
DNA Library (10 ng)	9 µL
10X Cas9 Buffer	1 µL

Total Volume	20 µL

6. Mix gently by flicking the tube or pipetting up and down slowly. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
7. Place the tube in a thermal cycler with a heated lid set to ≥80°C. Run the following program:

Temperature	Cycle Time
42°C	16 hours
65°C	5 min
4°C	HOLD

NOTE: CRISPR digestion for 16-hours is an overnight incubation step. The protocol is designed so that if digestion is begun in the evening day 1, the sample can be removed from the thermal cycler the next morning on day 2 and proceed with the remainder of the protocol. Use of a water bath is not recommended for this incubation step because of the tendency of liquid to evaporate and cool on the underside of the tube lid during overnight incubation at 42°C.

8. Following incubation, proceed to the next step on Day 2 (Bead Size Selection).

A2: Bead Size Selection

1. Add 30 µL of Nuclease-Free Water to the CRISPR digestion reaction from Step A1.7.
2. Add 30 µL (0.6X) of resuspended AMPure XP beads to the reaction. Mix well by pipetting up and down. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
3. Incubate at room temperature for 10 minutes. Mix the sample halfway through the incubation by pipetting up and down.
4. Place the tube on a magnetic stand until the solution is clear (3-5 minutes). Discard the supernatant without disturbing the beads.
5. Add 200 µL of freshly prepared 80% ethanol to the tube while still on the magnetic stand. Wait 30 seconds, then remove and discard the ethanol. Do not remove the tube from the magnetic stand while performing the ethanol wash.
6. Repeat the previous wash step.
7. Briefly spin the tube, place it back on the magnetic stand. Ensure that all residual ethanol is removed at this step.
8. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for up to 5 minutes.
9. Remove the tube from the magnetic stand and add 40 µL of Nuclease-Free Water to the beads. Mix well to resuspend the beads by pipetting up and down. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
10. Incubate at room temperature for 10 minutes. Mix the sample halfway through the incubation by pipetting up and down. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
11. Place the tube on the magnetic stand. Allow the solution to clear and transfer the supernatant containing the eluted DNA to a new 0.2 mL PCR tube.
12. Proceed to the next step (PCR Amplification).

SAFE STOPPING POINT: If stopping, store the sample at -20°C.

Step B: PCR Amplification

☐ Hands-on time: ~30 min | Total time: ~1 hr

Reagent Preparation

Item	Storage	Handling
2X PCR Mix	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice.
P7 Primer	-20°C	Thaw at room temperature. Vortex briefly and spin down. Keep on ice.
P5 Primer		
Tris Buffer	-20°C	Bring to room temperature. Vortex and invert mix.
AMPure XP Beads	4°C	
80% Ethanol	Room Temperature	Prepare fresh.

B1: PCR Amplification

1. On ice, combine the following reagents in the order listed below in a 0.2 mL PCR tube:

Amplification Reaction	Volume
"Depleted DNA Library" (Previous Step A2.11)	40 µL
2X PCR Mix	50 µL
P7 Primer	5 µL
P5 Primer	5 µL
Total Volume	100 µL

2. Place the reaction in a thermal cycler with a heated lid set to 105°C. Run the following program:

Temperature	Cycle Time	Number of cycles
95°C	2 min	1 cycle
98°C	20 sec	10 cycles*
55°C	30 sec	
72°C	30 sec	
72°C	2 min	1 cycle
4°C	HOLD	

*The number of PCR cycles after depletion correlates inversely with DNA input. With the recommended input of 10 ng and 10 PCR cycles, a final total library yield of 100-300 ng is expected.

3. Proceed immediately to the next step (Bead Cleanup).

B2: Bead Cleanup

1. Add 60 µL (0.6X) of resuspended AMPure XP beads to the reaction. Mix well by pipetting up and down. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
2. Incubate the sample at room temperature for 10 minutes. Mix the sample halfway through the incubation by pipetting up and down.
3. Place sample tube on a magnetic stand until the solution is clear (3-5 minutes). Discard the supernatant without disturbing the beads.
4. Add 200 µL of freshly prepared 80% ethanol to the tube while still on the magnetic stand. Wait 30 seconds, then remove and discard the ethanol. Do not remove the tube from the magnetic stand while performing the ethanol wash.

5. Repeat the previous wash step.
6. Briefly spin the tube, place it back on the magnetic stand. Ensure that all residual ethanol is removed at this step.
7. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for up to 5 minutes.
8. Remove the tube from the magnetic stand and add 30 µL of Tris Buffer to the beads. Mix well to resuspend the beads by pipetting up and down. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
9. Incubate the sample at room temperature for 10 minutes. Mix the sample halfway through the incubation by pipetting up and down. Collect the contents at the bottom of the tube by briefly spinning the tube in a microcentrifuge.
10. Place the tube on the magnetic stand. Allow the solution to clear and transfer the supernatant containing the eluted DNA to a new DNA LoBind tube. This constitutes the depleted DNA library.

SAFE STOPPING POINT: If stopping, store the library at -20°C .

Subsequent Steps

Jumpcode Genomics recommends assessing the library yield using a dsDNA-specific fluorescence-based method (such as a Qubit fluorometer) and library size on an Agilent Bioanalyzer 2100 or equivalent instrument before sequencing for best results.

After the second (and final) bead cleanup, most DNA fragments in the depleted library should be larger than 420 bp. If so, the library can be loaded directly on an Illumina sequencer. If a large proportion of fragments are less than 420 bp, it is recommended that a gel-based size selection step be performed to isolate DNA between 420 bp and 800 bp in size. The DNA can be loaded on a sequencing instrument after gel purification.