JUMPCODE GENOMICS

USER MANUAL

Post-library depletion of Poly A selected RNA-Seq libraries

KIT1020, KIT1024, KIT1025, KIT1022, KIT1027

Contents

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Product overview

RNA samples typically have a small number of highly expressed, well-characterized transcripts that outcompete low expressing transcripts for sequencing coverage. Resequencing these transcripts adds little biologically relevant information. The relative lack of sequencing coverage, coupled with significant noise from high expressing transcripts, limits detection of low expressing genes, rare variants and unique fusion events that may be disease drivers.

CRISPRclean leverages Cas9 and CRISPR guide RNA to remove highly expressed reads from NGS libraries before sequencing with the goal to reduce wasted sequencing reads and increase the sensitivity for rare genomic or transcriptomic content.

Jumpcode compatible products for use in this protocol

Compatible Jumpcode products for this user manual	Compatible Jumpcode products for this user manual
KIT1020 High Expressing RNA Depletion Kit	~4,450 high expressing transcripts in fibroblast and whole blood
KIT1024 RNA Depletion Panel (Globin)	Human globin genes: HBA1, HBA2, HBB, HBD
KIT1025 RNA Depletion Panel (Insulin)	Human insulin gene: INS
KIT1022 RNA Depletion Panel (Liver)	94 most abundant genes in human liver tissue
KIT1027 RNA Depletion Panel (Ribo, Mito Genes)	90 human ribosomal and 10 mitochondrial protein-coding genes

Post-library depletion for poly A selected libraries with KIT1020 High Expressing RNA Depletion Kit

Description	
Assay time	1.5 day including 16 hours overnight
Hands-on time	~1 hour
Sample types	Fibroblast and whole blood
Input	Prepared poly A selected libraries
Jumpcode compatible products for use with this protocol	KIT1020 High Expressing RNA Depletion Kit by itself or used in combination with a second RNA Depletion Panel from the below list: • KIT1022 RNA Depletion Panel (Liver) • KIT1025 RNA Depletion Panel (Insulin) • KIT1027 RNA Depletion Panel (Ribo, Mito Genes)

Post-library depletion for poly A selected or ribodepleted libraries

Description	
Assay time	3 hours and 45 minutes
Hands-on time	1 hour and 15 minutes
Input	Prepared poly A selected libraries or ribodepleted libraries
Jumpcode compatible products for use with this protocol	• KIT1022 RNA Depletion Panel (Liver) • KIT1024 RNA Depletion Panel (Globin) • KIT1025 RNA Depletion Panel (Insulin) • KIT1027 RNA Depletion Panel (Ribo, Mito Genes)

Workflow for Post-library depletion for poly A libraries with KIT1020 High Expressing RNA Depletion Kit

The protocol involves a 16-hour (overnight) 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 (~3 hours) on the morning of Day 2.

1 Prepare Poly A selected or ribodepleted RNA-Seq libraries

Ribodepletion

Day 1: (end of workday)

Hands-on time: 10 min | Total time: 16 hrs, including overnight incubation

A. Ribonucleoprotein complex formation

B. CRISPR Digestion

Safe stopping Point

2

Day 2: (morning)

Hands-on time: <1 hour | Total time: ~3 hrs

C. Size Selection

D. Amplification

E. Size Selectio

Safe stopping Point

3 Sequencing

Workflow for post-library depletion for poly A selected or ribodepleted libraries

The protocol can be completed in under 4 hours.

1. Prepare Poly A selected or ribodepleted RNA-Seq libraries

2. **CRISPRclean depletion**

   Hands-on time: 1 hr 15 min | Total time: 3 hrs 45 min hrs

   A. Ribonucleoprotein formation
   B. CRISPR digestion
   C. Size selection
   D. Amplification
   E. Size selection

   Safe stopping Point

3. Sequencing

Kit contents and storage

Each kit contains enough material to deplete 24 RNA libraries. The kit contents and storage temperatures are indicated in the tables below.

Compatible Jumpcode products for use in this protocol:

- KIT1020 High Expressing RNA Depletion Kit: ASY-1064, ASY-1070
- KIT1022 RNA Depletion Panel (Liver): ASY1064, ASY1065
- KIT1024 RNA Depletion Panel (Globin): ASY1064, ASY1071
- KIT1025 RNA Depletion Panel (Insulin): ASY1064, ASY1072
- KIT1027 RNA Depletion Panel (Ribo, Mito Genes): ASY1064, ASY1068

ASY1064 Depletion Reagents

8 samples per box
Stored 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

ASY1065 Guide RNA for Liver

24 samples per tube
Stored at -80°C

Kit contents	Part number	Quantity per box
Guide RNA for Liver	REA1048	1 tube

ASY1068 Guide RNA for Ribo, Mito Genes

24 samples per tube
Stored at -80°C

Kit contents	Part number	Quantity per box
Guide RNA for Ribo, Mito Genes	REA1051	1 tube

ASY1070 Guide RNA for High Expressing RNA

24 samples per tube
Stored at -80°C

Kit contents	Part number	Quantity per box
Guide RNA for High Expressing	REA1047	1 tube

ASY1071 Guide RNA for Globin

24 samples per tube
Stored at -80°C

Kit contents	Part number	Quantity per box
Guide RNA for Globin	REA1053	1 tube

ASY1072 Guide RNA for Insulin

24 samples per tube
Stored at -80°C

Kit contents	Part number	Quantity per box
Guide RNA for Insulin	REA1054	1 tube

Required materials and equipment provided by the user

Type	Item	Supplier
Plastics	0.5 mL, 1.5 mL DNA LoBind Tubes	Eppendorf 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	Poly(A)-selected or ribodepleted RNA-Seq libraries	General Lab Supplier
	AMPure XP Beads	Beckman Coulter A63881
	Absolute Ethanol, 200 Proof	General Lab Supplier
	Qubit dsDNA HS Assay Kit	ThermoFisher Scientific 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 for use with tubes	General Lab Supplier
	Ice Bucket	General Lab Supplier
	PCR Thermal Cycler	General Lab Supplier
	Qubit Fluorometer	ThermoFisher Scientific Q33238
	Automated electrophoresis such as TapeStation	General Lab Supplier
	DNA analysis instrument, such as the Agilent 2100 Bioanalyzer® System	General Lab Supplier

Best Practices

General

- Periodically, optimizations and revisions are made to the kit components and user manual. Therefore, it is important to follow the protocol included with or appropriate for the kit in question. This can be done by comparing the name and version number of the CRISPRclean product to the name and version number of the protocol. If you need further assistance in this regard, contact support@jumpcodegenomics.com.
- Workflow for Post-library depletion for poly A libraries with KIT1020 High Expressing RNA Depletion Kit describes the workflow and method details for CRISPRclean depletion with KIT1020 High Expressing RNA Depletion Kit as it applies to prepared poly A selected RNA-Seq libraries.
- Workflow for post-library depletion for poly A selected or ribodepleted libraries describes the workflow and method details for CRISPRclean depletion it applies to prepared poly A selected or ribodepleted RNA-Seq libraries.
- Use properly calibrated pipettes as library preparation is sensitive to pipetting error.
- Ensure that all pipette tips, microcentrifuge tubes, and other consumables are DNase- and RNase-free.
- Follow good laboratory practices throughout the protocol, including RNA-safe procedures when handling RNA.

For specific reagents

- Do not remove Cas9 and RNase Inhibitor from -20°C until before use. Return to -20°C immediately after use.
- Store the Guide RNA at -80°C. Do not remove the reagent from -80°C until time of use. Return it to -80°C immediately after use.
- We recommend a maximum of 3 freeze-thaw cycles for the Guide RNA. The Guide RNA tube contains material for 24 samples. It is strongly recommended that multiple smaller aliquots of the Guide RNA be prepared when the reagent is first thawed in order to reduce the number of freeze-thaw cycles affecting the Guide RNA.
- Do not freeze AMPure® XP beads.
- Allow AMPure XP beads to come to room temperature for 30 minutes before use.
- Vortex AMPure XP beads immediately before use. Ensure that they are in a uniform suspension before use.

Revision log

Version	Date	Description
V1.0	January 2023	Launch

Before starting protocol

- The kit produces best results with libraries size-selected for a relatively narrow fragment size range in which majority of fragments are >450 bp. 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.
- Libraries should be prepared and stored under nuclease-free conditions.
- Each depletion requires an input of 10 ng of library in a total volume no greater than 7 µL.

NGS library prep requirements

High Expressing RNA Depletion is highly effective with NEBNext® Ultra II Directional RNA libraries prepared with the Poly(A) mRNA Magnetic isolation Module to remove ribosomal RNA. Refer to the Instruction Manual for NEBNext Ultra II RNA Library Prep Kit for Illumina (v3.1, Section 1) for detailed information on the protocol and required reagents. The following conditions are employed during preparation of these libraries to ensure optimal depletion:

1. At least 200 ng of total RNA input (into the poly(A) mRNA isolation procedure)
2. RNA fragmentation time of 5 minutes at 940C (refer to Step 1.2.37 of instruction manual)
3. Dual AMPure XP bead size selection to select fragments with an Agilent Bioanalyzer peak of 450 bp or greater (refer to Step 1.8.1 of instruction manual). The libraries under selection are referred to as libraries with 300 bp, 400 bp and 450 bp approximate insert sizes or 420 bp, 520 bp and 570 bp approximate final library sizes in Appendix A of the Instruction Manual for NEBNext Ultra II RNA Library Prep Kit for Illumina (v3.1, Section 1)
4. Thirteen PCR cycles (refer to Step 1.9.3b of instruction manual)

Protocol for post-library depletion for poly A libraries with KIT1020 High Expressing RNA Depletion Kit

The protocol involves a 16-hour (overnight) 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 (~3 hours) on the morning of Day 2.

Step A: Ribonucleoprotein complex formation

Hands-on time: 5 min | Total time: 15 min

Reagents preparation

Item	Storage	Handling
10X Cas9 Buffer	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
RNase Inhibitor		
Cas9		
Nuclease-Free Water		
Guide RNA	-80°C	

If using KIT1020 High Expressing RNA Depletion Kit by itself, then follow sub-section instructions for **Part 1**.

If combining two guide RNA sets together, then follow **Part 2** sub-section instructions:

- KIT1020 High Expressing RNA Depletion Kit in combination with KIT1025 RNA Depletion Panel (Insulin)
- KIT1020 High Expressing RNA Depletion Kit in combination with KIT1022 RNA Depletion (Liver)
- KIT1020 High Expressing RNA Depletion Kit in combination with KIT1027 RNA Depletion (Ribo, Mito Genes)

Part 1

1. If using KIT1020 High Expressing RNA Depletion Kit by itself, then add the reagents listed below in the given order to a nuclease-free microcentrifuge tube.

"RNP Complex Formation" Reaction Mix for using only KIT1020 High Expressing RNA Depletion Kit	Volume
Nuclease-Free Water	4.3 µL
10X Cas9 Buffer	1.2 µL
RNase Inhibitor	1.0 µL
Cas9	2.0 µL
High Expressing Human RNA Guide RNA	3.5 µL
Total Volume	**12 µL**

Part 2

1. If combining two guide RNA sets together, then add the reagents listed below in the given order to a nuclease-free microcentrifuge tube.

"RNP Complex Formation" Reaction Mix for using KIT1020 High Expressing RNA Depletion Kit with another RNA Depletion panel	High Expressing RNA Depletion with Insulin	High Expressing RNA Depletion with Liver or Ribo, Mito Genes
Nuclease-Free Water	2.0 µL	n/a
10X Cas9 Buffer	1.0 µL	1.0 µL
RNase Inhibitor	1.0 µL	1.0 µL
Cas9	2.9 µL	3.54 µL
High Expressing Human RNA Guide RNA	3.5 µL	3.5 µL
2nd Guide RNA set	1.6 µL (diluted 1:5 in Nuclease-free water)	2.65 µL
Total volume	**12 µL**	**~12 µL**

2. Mix the contents gently by carefully 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.
3. Leave the tube on the laboratory bench for 10 minutes at room temperature. This tube contains the **ribonucleoprotein complex (RNP)**.

Step B: CRISPR digestion

Hands-on time: 5 min | Total time: 16 hours

Reagents preparation

Item	Storage	Handling
10X Cas9 Buffer	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
Ribonucleoprotein complex (RNP) from Step A		
Poly A-selected RNA-Seq library you intend to deplete		
Nuclease-Free Water		
AMPure XP beads	4°C	Thaw the AMPure XP beads on the laboratory bench to bring them to room temperature. A minimum of 30 minutes at room temperature is recommended before use.

1. Obtain 10 ng of the library you intend to deplete. Add Nuclease-Free Water to bring the volume to 7 µL in a nuclease-free microcentrifuge tube. Mix gently.
2. To the tube containing the ribonucleoprotein complex (RNP) from Step A, add the following reagents at room temperature in the order given below.

"CRISPR Digestion" Reaction Mix	Volume
10 ng of NGS library	7.0 µL
10X Cas9 Buffer	1.0 µL
Ribonucleoprotein complex from Step A	12.0 µL
Total Volume	**20 µL**

3. Mix the contents gently by carefully 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 tube at 42°C for 16 hours in a thermal cycler with heated lid (>500C).

> **Note:** This is an overnight incubation step. The protocol is designed so that if digestion is begun at 5 pm on Day 1, the sample can be removed from the thermal cycler at 9 am the next morning to be used in subsequent steps of this protocol.

> **Note:** We do not recommend a water bath for this incubation step because of the tendency of liquid to evaporate and cool on the underside of the tube lid during overnight incubations at 42°C.

5. After the 16-hour incubation at 42°C is complete, transfer the tube to ice for ~2 minutes.
6. Spin the tube briefly in the microcentrifuge. Transfer the tube to the laboratory bench. This tube contains the **CRISPR digested product**. Proceed immediately to the next step.

Step C: Size Selection

Hands-on time: 10 min | Total time: 50 min

Reagents preparation

Item	Storage	Handling
Nuclease-Free Water	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
CRISPR-digested product from Step B		
AMPure XP beads	4°C	Thaw the AMPure XP beads on the laboratory bench to bring them to room temperature. A minimum of 30 minutes at room temperature is recommended before use.
80% Ethanol	Room temperature	Freshly prepared

1. Add 30 µL of Nuclease-Free Water to the **CRISPR digested product** from Step B. Mix gently.
2. Add 30 µL (0.6 volumes) of well-resuspended, room-temperature AMPure XP beads, pipette several times to mix and incubate the tube at room temperature for 10 minutes. Halfway through the incubation, mix the sample by pipetting up and down a few times.
3. Place the tube on the magnetic stand. Allow the solution to clear (3 – 5 minutes) and discard the supernatant without disturbing the beads.
4. Add 200 µL of freshly prepared 80% ethanol to the tube. Wait 30 seconds, then remove and discard the ethanol. Do not remove the tube from the magnetic stand during this step.
5. Repeat the wash step with another 200 µL of 80% ethanol. Ensure that all residual ethanol is removed after this wash.
6. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for 5 minutes.
7. Add 40 µL of Nuclease-Free Water to the beads. Remove the tube from the magnetic stand. Mix the contents by pipetting up and down to fully resuspend the beads in the liquid. Allow the tube to sit at room temperature for 10 minutes. Halfway through the incubation, mix the sample by pipetting up and down a few times.
8. Place the tube on the magnetic stand. Allow the solution to clear and transfer the supernatant containing the eluted DNA to a thin-walled PCR tube. Place the tube on ice. This tube contains the **eluted DNA**. Proceed immediately to the next step.

Step D: Amplification

Hands-on time: 5 min | Total time: 65 min

Reagents preparation

Item	Storage	Handling
Nuclease-Free Water	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
P5 Primer		
P7 Primer		
2X PCR Mix		
AMPure XP beads	4°C	Thaw the AMPure XP beads on the laboratory bench to bring them to room temperature. A minimum of 30 minutes at room temperature is recommended before use.
80% Ethanol	Room temperature	Freshly prepared

1. Add the following components to the eluted DNA from Step 3h in the thin-walled PCR tube:

Component	Volume
Eluted DNA from Step C	~40 µL
P5 Primer	5 µL
P7 Primer	5 µL
2X PCR Mix	50 µL
Total volume	**100 µL**

2. Input the following parameters into a thermal cycler and perform a PCR:

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

Note: During this incubation period, remove the AMPure XP beads out of the refrigerator a minimum of 30 minutes prior to use to bring them to room temperature.

3. After the PCR is complete, remove the tube from the thermal cycler and briefly spin the PCR tube in a microcentrifuge before proceeding with the next step.

Step E: Size selection

Hands-on time: 10 min | Total time: 50 min

Reagents preparation

Item	Storage	Handling
Nuclease-Free Water	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
Tris Buffer		
AMPure XP beads	4°C	Thaw the AMPure XP beads on the laboratory bench to bring them to room temperature. A minimum of 30 minutes at room temperature is recommended before use.
80% Ethanol	Room temperature	Freshly prepared

1. Add 60 µL (0.6 volumes) of well-resuspended, room-temperature AMPure XP beads to the supernatant from Step 4. Pipette several times to mix and incubate the tube at room temperature for 10 minutes. Halfway through the incubation, mix the sample by pipetting up and down a few times.
2. Place the tube on the magnetic stand. Allow the solution to clear (3 – 5 minutes) and discard the supernatant without disturbing the beads.
3. Add 200 µL of freshly prepared 80% ethanol to the tube. Wait 30 seconds, then remove and discard the ethanol. Do not remove the tube from the magnetic stand during this step.
4. Repeat the wash step with another 200 µL of 80% ethanol. Ensure that all residual ethanol is removed after this wash.
5. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for 10 minutes. Do not allow the beads to dry for longer than 10 minutes. Over-drying the beads could result in lower yields.
6. Add 30 µL of room-temperature Tris Buffer to the beads. Remove the tube from the magnetic stand. Mix the contents by pipetting up and down to fully resuspend the beads in the liquid. Allow the tube to sit at room temperature for 10 minutes.
7. Place the tube on the magnetic stand. Allow the solution to clear and transfer the supernatant containing the eluted DNA to a new nuclease-free microcentrifuge tube. Avoid transferring any beads along with the supernatant. The supernatant contains the fully prepared depleted library.

SAFE STOPPING POINT

Store the samples at -20°C

Protocol for post-library depletion for poly A selected libraries

Step A: Ribonucleoprotein complex formation

Hands-on time: 5 min | Total time: 15 min

Reagents preparation

Item	Storage	Handling
10X Cas9 Buffer	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
RNase Inhibitor		
Cas9		
Nuclease-Free Water		
Guide RNA	-80°C	

1. Follow the appropriate column according to which RNA Depletion Panel you intend to deplete. Combine the reagents listed below in the given order to a nuclease-free microcentrifuge tube.

"RNP Complex Formation" Reaction Mix	KIT1024 Globin or KIT1025 Insulin	KIT1022 Liver or KIT1027 Ribo, Mito Genes
Nuclease-Free Water	5.5 µL	5.5 µL
10X Cas9 Buffer	1.0 µL	1.0 µL
RNase Inhibitor	1.0 µL	1.0 µL
Cas9	0.9 µL	1.54 µL
Guide RNA	1.6 µL	2.65 µL
Total volume	**10 uL**	**~12 µL**

2. Mix the contents gently by carefully 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.
3. Leave the tube on the laboratory bench for 10 minutes at room temperature. This tube contains the **ribonucleoprotein complex (RNP)**.

Step B: CRISPR digestion

Hands-on time: 5 min | Total time: 65 min

Reagents preparation

Item	Storage	Handling
10X Cas9 Buffer	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
Ribonucleoprotein complex (RNP) from Step A		
Poly A-selected RNA-Seq library you intend to deplete		
Nuclease-Free Water		
AMPure XP beads	4°C	Thaw the AMPure XP beads on the laboratory bench to bring them to room temperature. A minimum of 30 minutes at room temperature is recommended before use.

1. Obtain 10 ng of the library you intend to deplete. Add Nuclease-Free Water according to the "CRISPR digestion" reaction mix table below to a nuclease-free microcentrifuge tube. Mix gently.
2. To the tube containing the ribonucleoprotein complex (RNP) from Step A, add the following reagents at room temperature in the order given below.

"CRISPR Digestion" Reaction Mix	KIT1024 Globin or KIT1025 Insulin	KIT1022 Liver or KIT1027 Ribo, Mito Genes
10 ng of NGS library	8 µL	7.31 µL
Nuclease-Free Water	1 µL	0 µL
10X Cas9 Buffer	1 µL	1 µL
Ribonucleoprotein complex from Step A	10 µL	11.69 µL
Total volume	**20.0 µL**	**20.0 µL**

3. Mix the contents gently by carefully 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 tube at 37°C for 60 minutes in a thermal cycler.

> **Note:** During incubation, the AMPure XP beads required for subsequent size selection steps may be removed from the refrigerator and placed on the laboratory bench to bring them to room temperature. A minimum of 20 minutes at room temperature is recommended before use.

5. After the incubation is complete, transfer the tube to ice for ~2 minutes.
6. Spin the tube briefly in the microcentrifuge. Transfer the tube to the laboratory bench. This tube contains the **CRISPR digested product**. Proceed immediately to Step C.

Step C: Size Selection

Hands-on time: 30 min | Total time: 40 min

Item	Storage	Handling
Nuclease-Free Water	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
CRISPR-digested product from Step B		
AMPure XP beads	4°C	Thaw the AMPure XP beads on the laboratory bench to bring them to room temperature. A minimum of 30 minutes at room temperature is recommended before use.
80% Ethanol	Room temperature	Freshly prepared

1. Add 30 µL of Nuclease-Free Water to the **CRISPR digested product** from Step B. Mix gently.
2. Add 30 µL (0.6 volumes) of well-resuspended, room-temperature AMPure XP beads, pipette several times to mix and incubate the tube at room temperature for 10 minutes. Halfway through the incubation, mix the sample by pipetting up and down a few times.
3. Place the tube on the magnetic stand. Allow the solution to clear (3 – 5 minutes) and discard the supernatant without disturbing the beads.
4. Add 200 µL of freshly prepared 80% ethanol to the tube. Wait 30 seconds, then remove and discard the ethanol. Do not remove the tube from the magnetic stand during this step.
5. Repeat the wash step with another 200 µL of 80% ethanol. Ensure that all residual ethanol is removed after this wash.
6. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for 5 minutes.
7. Add 40 µL of Nuclease-Free Water to the beads. Remove the tube from the magnetic stand. Mix the contents by pipetting up and down to fully resuspend the beads in the liquid. Allow the tube to sit at room temperature for 10 minutes. Halfway through the incubation, mix the sample by pipetting up and down a few times.
8. Place the tube on the magnetic stand. Allow the solution to clear and transfer the supernatant containing the eluted DNA to a thin-walled PCR tube. Place the tube on ice. This tube contains the **eluted DNA**. Proceed immediately to Step D.

Step D: Amplification

Hands-on time: 5 min | Total time: 65 min

Reagents preparation

Item	Storage	Handling
Nuclease-Free Water	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
P5 Primer		
P7 Primer		
2X PCR Mix		
AMPure XP beads	4°C	Thaw the AMPure XP beads on the laboratory bench to bring them to room temperature. A minimum of 30 minutes at room temperature is recommended before use.
80% Ethanol	Room temperature	Freshly prepared

1. Add the following components to the eluted DNA from Step 3h in the thin-walled PCR tube:

Component	Volume
Eluted DNA from Step C	~40 µL
P5 Primer	5 µL
P7 Primer	5 µL
2X PCR Mix	50 µL
Total Volume	**100 µL**

2. Input the following parameters into a thermal cycler and perform a PCR:

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

Note: During this incubation period, remove the AMPure XP beads out of the refrigerator a minimum of 30 minutes prior to use to bring them to room temperature.

3. After the PCR is complete, remove the tube from the thermal cycler and briefly spin the PCR tube in a microcentrifuge before proceeding with the next step.

Step E: Size selection

Hands-on time: 30 min | Total time: 40 min

Reagents preparation

Item	Storage	Handling
Nuclease-Free Water	-20°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
Tris Buffer		
AMPure XP beads	4°C	Thaw the AMPure XP beads on the laboratory bench to bring them to room temperature. A minimum of 30 minutes at room temperature is recommended before use.
80% Ethanol	Room temperature	Freshly prepared

1. Add 60 µL (0.6 volumes) of well-resuspended, room-temperature AMPure XP beads to the supernatant from Step 4. Pipette several times to mix and incubate the tube at room temperature for 10 minutes. Halfway through the incubation, mix the sample by pipetting up and down a few times.
2. Place the tube on the magnetic stand. Allow the solution to clear (3 – 5 minutes) and discard the supernatant without disturbing the beads.
3. Add 200 µL of freshly prepared 80% ethanol to the tube. Wait 30 seconds, then remove and discard the ethanol. Do not remove the tube from the magnetic stand during this step.
4. Repeat the wash step with another 200 µL of 80% ethanol. Ensure that all residual ethanol is removed after this wash.
5. Open the cap of the tube while it is on the magnetic stand and allow the beads to air dry for 10 minutes. Do not allow the beads to dry for longer than 10 minutes. Over-drying the beads could result in lower yields.
6. Add 30 µL of room-temperature Tris Buffer to the beads. Remove the tube from the magnetic stand. Mix the contents by pipetting up and down to fully resuspend the beads in the liquid. Allow the tube to sit at room temperature for 10 minutes.
7. Place the tube on the magnetic stand. Allow the solution to clear and transfer the supernatant containing the eluted DNA to a new nuclease-free microcentrifuge tube. Avoid transferring any beads along with the supernatant. The supernatant contains the fully prepared depleted library.

SAFE STOPPING POINT

Store the samples at -20°C

Subsequent steps

It is recommended that the library be quantitated on an Agilent Bioanalyzer or TapeStation instrument (or other similar analytical instrument) prior to sequencing. The expected final yield should be in the range of 100 - 200 ng. After the second (and final) size selection, most DNA fragments in the CRISPRclean-treated library should be larger than 450 bp.