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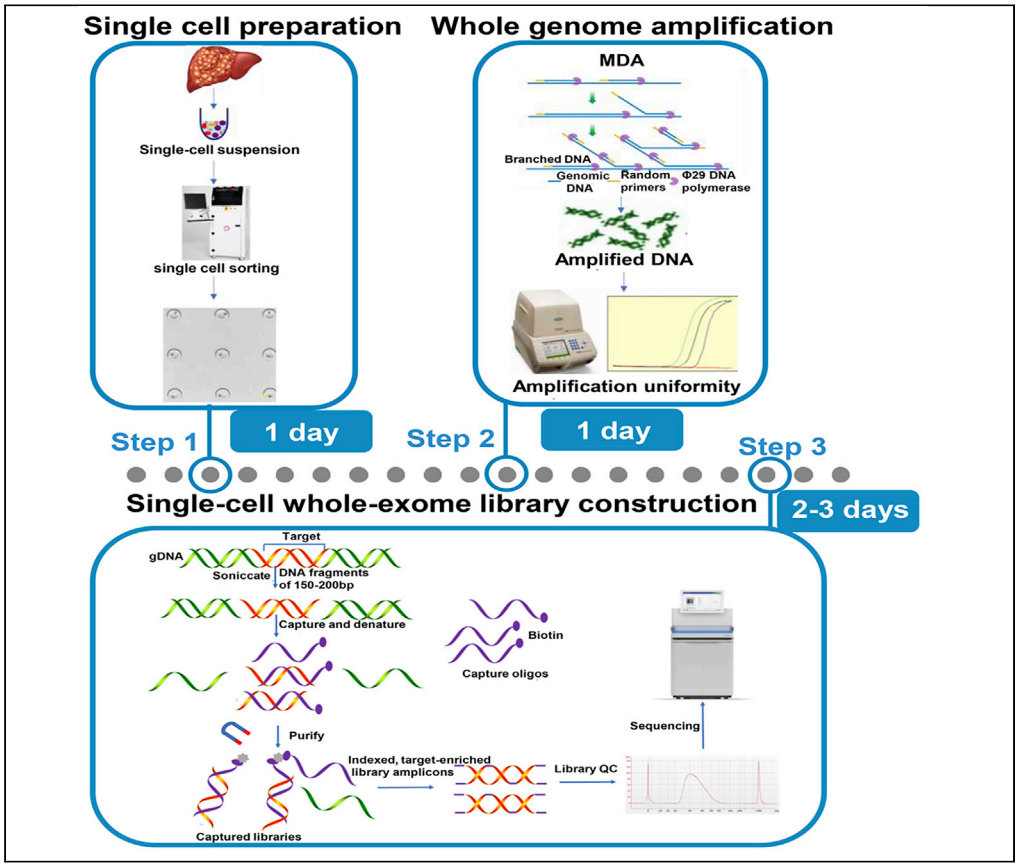
Protocol

Isolation of single cells from human hepatoblastoma tissues for whole-exome sequencing



By combining single-cell processing with whole-exome sequencing, we have developed single-cell whole-exome sequencing to investigate the mechanisms of hepatoblastoma development and to provide potential targets and therapeutic approaches for clinical treatment. In the following protocol, we outline the steps involved in single-cell sorting, whole-genome amplification, amplification uniformity estimation, and whole-exome library construction. In addition to the cells we use, this protocol is also suitable for other cell lines and cell types.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Single-cell exome sequencing of human hepatoblastomas

Protocol enables the detection of low-frequency variants

Protocol reveals tumor genetic heterogeneity and improves genome coverage

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Isolation of single cells from human hepatoblastoma tissues for whole-exome sequencing

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SUMMARY

By combining single-cell processing with whole-exome sequencing, we have developed single-cell whole-exome sequencing to investigate the mechanisms of hepatoblastoma development and to provide potential targets and therapeutic approaches for clinical treatment. In the following protocol, we outline the steps involved in single-cell sorting, whole-genome amplification, amplification uniformity estimation, and whole-exome library construction. In addition to the cells we use, this protocol is also suitable for other cell lines and cell types.
For complete details on the use and execution of this protocol, please refer to Jian et al. (2023).

BEFORE YOU BEGIN

Whole-genome sequencing (WGS) is a key tool for characterizing the dynamics changes of intracellular DNA, providing us with comprehensive biological information, but the sample preparation is complex and the large amount of sequencing data can mask low frequency mutations and be costly. However, human disease-associated mutations are largely concentrated in exon region.[1] Through whole-exome sequencing (WES), DNA can be captured and enriched in the exon region of the whole genome to identify variants in exome regions and to identify disease-causing mutations,[2] so WES has become an important tool for diagnosing rare genetic disorders in patients with complex clinical presentations. Hepatoblastoma, one of the most rapidly increasing cancers in children under 5 years of age [4], is poorly studied and has no suitable drug targets. Moreover, the existing adult drugs are not suitable for children, so we have a mission to discover new biomarkers. Here, we provide a facile protocol for single-cell whole-exome sequencing. It is helpful to study the mechanism of hepatoblastoma development and provide potential targets and treatment methods for clinical treatment.

Preparing the environment and materials

Timing: flexible

1. Prepare the work bench with ethanol, RNAseZAP (Rapid RNase remover), DNA-OFF (Surface DNA contamination remover), or similar before you begin.
2. Place AMPureXP beads at 20°C–25°C at least 30 min. The beads should not be frozen under any circumstances.
3. Since we use multiple 8-tube strips to form a 96-well plate to collect cells. Therefore, before cell sorting begins, 8-tube strips are taken and combined into a 96-well plate.

Note: We recommend preparing all reagents just before use to keep the solutions fresh and perform all steps on ice unless otherwise noted.

Institutional permissions

This project was permitted and approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Hepatoblastoma tissue	Shanghai Children's Medical Center	NA
Chemicals, peptides, and recombinant proteins		
RNAseZAP	Thermo Fisher	Cat# AM9780
DNA-OFF	Takara	Cat# 9036
AMPureXP beads	Beckmann Coulter	Cat# A63881
PBS	Thermo Fisher	Cat# 10010-049
DPBS, no calcium, no magnesium	Thermo Fisher	Cat# 14190250
FBS	Thermo Fisher	Cat# 26140079
DMEM	Thermo Fisher	Cat# 11965118
Ethanol 200 proof	Millipore Sigma	Cat# 7023-500ML
sciCLEAN8	Scienion	Cat# C-5283
Collagenase IV	Sigma-Aldrich	Cat# C5138-500MG
Nuclease-free water	Qiagen	Cat# 129114
Red blood cell lysis solution (10×)	Miltenyi Biotec	Cat# 130-094-183
Trypan blue stain (0.4%)	Thermo Fisher	Cat# T10282
KOH_8M	MACKLIN	Cat# P822103
DTT	Sangon Biotech	Cat# B645939-0001
EDTA	Thermo Fisher	Cat# R1021
HCl [2 N]	VWR	Cat# R008
Tris-HCl solution (1 M, pH 7.5)	Sangon Biotech	Cat# B548124-0500
Agarose	Sigma-Aldrich	Cat# A9539-50G
1 kb Plus DNA ladder	Thermo Fisher	Cat# 10787026
TBE	Sangon Biotech	Cat# B040124
Low TE buffer	Thermo Fisher	Cat# 12090-015
Dynabeads™ MyOne™ Streptavidin T1	Thermo Fisher	Cat# 65602
Critical commercial assays		
REPLI-g® Single Cell Kit	Qiagen	Cat# 157046333
Fast Sybr green master mix	Life Tech	Cat# 4385612
24 DNALabChipX-Mark	PerkinElmer	Cat# CLS145331
HT DNA NGS 3K Reagent Kit	PerkinElmer	Cat# CLS960013
SureSelectXT Reagent Kit, Illumina (ILM) platforms	Agilent	Cat# G9611B
Herculase II Fusion DNA Polymerase	Agilent	Cat# 600677
SureSelect XT Human All Exon V6	Agilent	Cat# 5190-8864
10× End Repair Buffer	Agilent	Cat# 5190-3609
T4 DNA Ligase	Agilent	Cat# 5190-3612
T4 DNA Polymerase	Agilent	Cat# 5190-3614
Klenow DNA Polymerase	Agilent	Cat# 5190-3613
T4 Polynucleotide Kinase	Agilent	Cat# 5190-3616
Agilent QPCR NGS Library Quantification Kit	Agilent	Cat# G4880A
QIAamp DNA Mini Kit	Qiagen	Cat# 51306
Qubit™ dsDNA HS Assay Kit	Thermo Fisher	Cat# Q32854

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Locus-specific primer (1 μM)	This paper	On Table S1# Sequence of locus-specific primer
Exo-resistant random primer- 5 ′ -N p N p N p N p N p ^{S}N p ^{S}N- 3 ′	Thermo Fisher	Cat# SO181
SureSelectXT Indexes	Agilent	On Table S2# Sequence of SureSelectXT indexes
Other		
CELLEN ONE	Scienion	Cat# cellenONE X1
SpeedVac Vacuum Concentrator	Thermo Fisher	Cat# SPD130DLX
Fluorescence image analysis system	Tanon	Cat# Tanon 4600SF
Automated cell counters	Invitrogen	Cat# AMQAX1000
Covaris M220 Focused-Ultrasonicator	Covaris	Cat# M220
MicroAmp Clear Adhesive Film	Thermo Fisher	Cat# 15036
Tween 20	Sigma-Aldrich	Cat# P9416-50ML
0.2 mL mMaxymum recovery flat cap	Axygen	Cat# PCR-02-L-C
Axygen0.2 mL PolypropylenePCRTubeStrips,8Tubes/Strip, Clear, Nonsterile	Axygen	Cat# PCR-0208-C
cellnVIALs (100 μL)	Scienion	Cat# CEV-5801-100
15 mL tubes	Labselect	Cat# CT-002-15
1.5 mL DNA LoBind Tubes	Eppendorf	Cat# 22431021
2 mL DNA LoBind Tubes	Eppendorf	Cat# 22431048
20 μL filter tips	Axygen	Cat# TF-20-R-S
1000 μL filter tips	Biosharp	Cat# BS-1000-TRS
200 μL filter tips	Biosharp	Cat# BS-200-TRS
10 μL filter tips	Biosharp	Cat# BS-10-TRS
Vacuum filter	SORFA	Cat# 610130
SmartStrainer 70 μm	Miltenyi Biotec	Cat# 130-098-462
Invitrogen Countess Cell Counting Chamber Slides	Thermo Fisher	Cat# C10228
microTUBE-50 AFA Fiber serew-cap	Covarious	Cat# 520166
Qubit assay tubes	Thermo Fisher	Cat# Q32856

MATERIALS AND EQUIPMENT

Lysis Buffer		
Reagent	Final concentration	Amount
KOH (8 M)	400 mM	50 μL
DTT (1 M)	100 mM	100 μL
EDTA (0.5 M)	10 mM	20 μL
ddH2O		830 μL
Total	**N/A**	**1 mL**

Store at 4°C for up to a month.

Stop Buffer		
Reagent	Final concentration	Amount
HCl (2 N)	400 mM	200 μL
Tris-HCl (1 M)	600 mM	600 μL
ddH2O	N/A	200 μL
Total	**N/A**	**1 mL**

Store at 4°C for up to a month.

⚠ CRITICAL: KOH and HCl have strong corrosivity. Avoid contact with skin and eyes and do not inhale or ingest these substances. Wear lab coat, goggles and gloves as required during use.

Alternatives: Common reagents can be replaced by reagents provided by other suppliers, as long as their quality and purity are the same as those used in the protocol; In addition to using Covaris M220 for DNA fragmentation, Covaris E220 or other models can also be used; Common consumables can also be provided by other suppliers.

The resources listed in the table are based on our protocol. Generally speaking, chemicals and resources can be purchased from any reliable commercial source, not limited to those listed in our table.

STEP-BY-STEP METHOD DETAILS

Single cell preparation

Timing: 1 day

During this step, we outline the steps of preparation for cellenONE X1 single-cell isolation procedure.

Preparation for cellenONE X1 single-cell isolation procedure

Timing: 30 min

1. Preparation of the filtered and degassed ddH_2O.
   a. Filter 600 mL ddH_2O into the bottle by using 0.22 μm vacuum-driven filter and degas the water in the ultrasonic cleaner for 20 min at 20°C–25°C.
   b. Place the bottle gently on the shelf.
2. Change the wash bottle with fresh doubly deionized water (with resistivity of 18 MΩ cm at 25°C) produced by deionized water purifier and then empty the waste bottle.
3. Place the recovery vial (100 μL microcentrifuge tube) and wash tray (a 2 cm × 1.5 cm × 5 cm accessory) in position. Add 20 μL 70% ethanol or 1 × PBS (reuse the dispensed cells) to the recovery vial.

   Note: Before starting the cellenONE X1 system, make sure there is nothing that could prevent the axis from moving freely in the enclosure.

4. Press the power button.
5. Open the chiller of the the cellenONE X1 system (2 switches behind and one button in front).
6. Open the cellenONE® software.
7. Select the "File" option – click on "Cooling unit control" to set the temperature of the target plate.
8. To Install the nozzle (Figure 1):
   a. Push nozzle 1 into the profiled rail (position 1, frontal view), so that most of its length is inserted.
      i. Carefully move the nozzle forward so that the glass capillary passes through the hole at the far end.
      ii. Push forward until it comes to an end stop.

   Note: If several nozzles are used, repeat the procedure for each one.

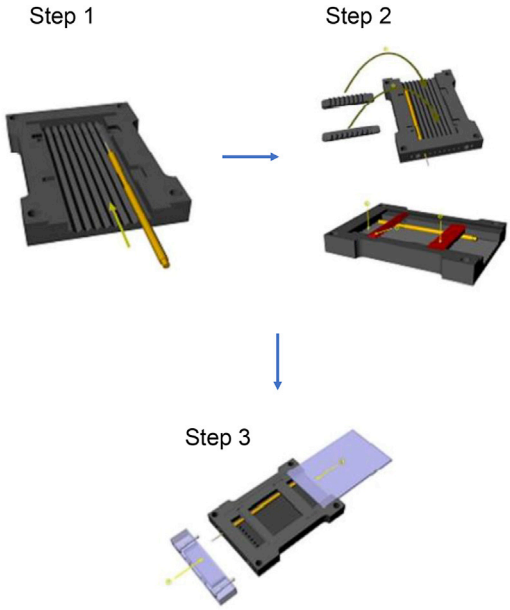

Figure 1. Installation diagram of sorting needle

b. Once the required nozzles have been inserted, as described in step 1, place the guide clip over the nozzles.
   i. Engage the locking clip at an angle of 10–15 degrees, pushing it against the action of the nozzle spring.

Note: When inserting the locking clip, ensure that all the nozzle springs are actuated.

c. Slide the sealing plate into its guide until it comes to a stop.
   i. The sealing plate retains the guide clip in position.
   ii. keep the nozzles clean.

⚠ CRITICAL: An additional protective cover can be attached which prevents breakage of the capillaries and protects the user from consequent risk of injury.

9. Click on the "Do task" drop-down box – execute the "Prime EtOH" task, to perform the alcohol cleaning process of the nozzle pipe.

Needed: 70% ethanol (about 200 mL), flush bottle, wash bottle.

In step 2, connect the PDC (piezo dispense capillary), the sorting needle for the cellenONE X1 system, to the dispenser head manifold after seeing a drop of water come down.

Note: Avoid over-tightening.

The deck and positions of cellenONE X1 are shown in (Figure 2).

10. Click on the "Do task" drop-down box – execute "Flush_PDC" task to wash the PDC.
11. Before starting an experiment, make sure that the nozzle is clean and free of any particles both inside and out. If the nozzle is not clean, try following procedures.

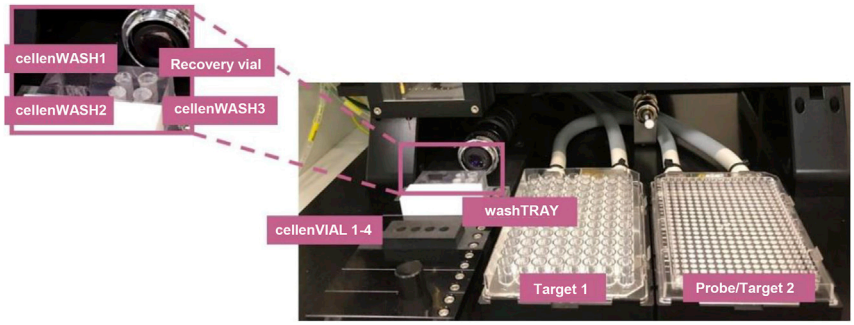

Figure 2. CellenONE X1 deck and positions

a. Prepare 2 mL 0.5% sciCLEAN8 (C-5283) liquid in the wash tray. Click on the "Do task" drop-down box - execute "sciClean" (clean inside) or "sciClean outside" (clean outside) to clean the nozzle.
b. Wipe the outside of the nozzle very gently with Kimwipe paper with 70% ethanol.
c. Wipe the tip very gently with sciClean paper with diluted sciClean liquid.
d. Airex (exhaust air).

12. Nozzle setup (Figure 3).

Click "camera" icon.

13. Align the center of the PDC's tip with the red cross using the x, y, z stepper. Adjust the voltage and pulse parameter to the recommended value.

Record the offset values by pressing "set nozzle parameters button"

14. Set the parameters for voltage and pulse you obtained for your nozzle into the according fields.
    a. Start the parameters by clicking the set nozzle parameter button.
    b. Start continuous dispensing and check the drop.
    c. Adjust the voltage and pulse until the drop distance is 450 $\pm$ 25 μm.
    d. Set nozzle parameter.
    e. Check the drop in the drop volume.

Note: Make the drop 3 times, the volume should be similar and the volume std deviation should <0.5%.

15. Check "Main" to ensure that the sorting and collection procedure selected is appropriate for our sorting model (Figure 4A).

Click "Target" – "Load field": CellenONE-slide (for slide) to set parameters for the collection.

Click "CellenONE"-"well (for 96-well-plate)" to select the program suitable for sorting into 96-well plates (Figure 4B).

Click "Target"- "field setup" to set the relevant parameters of the cell collection well plate. Click "erase all" to clear the previously selected collection wells, and then click on "set all "to reset all the desired collection wells. Then set No. of Drops = 1 (1 cell per well) to ensure that only one cell is sorted in each well (Figure 4C).

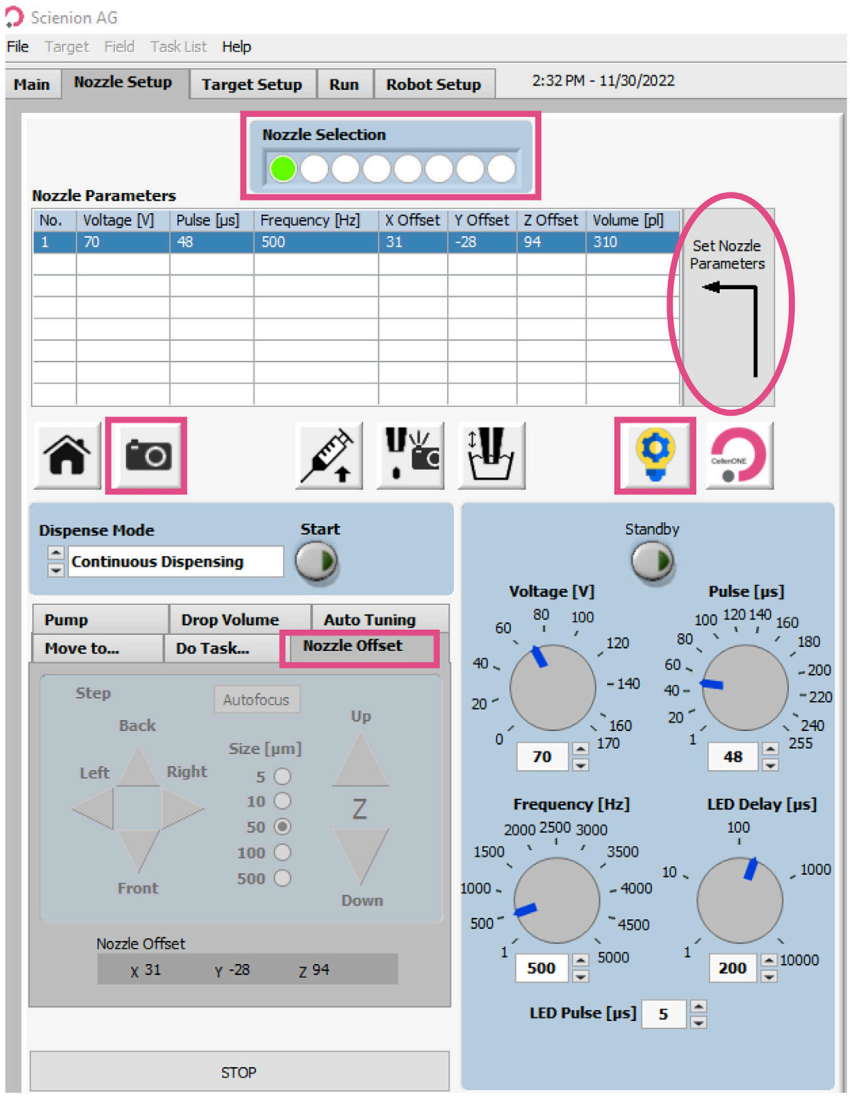

Figure 3. Nozzle Setup and Nozzle Offset tables for PDC alignment

Preparation for preprocessing of fresh or cryopreserved samples for single-cell droplet generation

Timing: 20 min

This section describes the process of preparing the cell suspension prior to single cell sorting.

⚠ CRITICAL: The process should be performed on ice to ensure cell viability, except for the enzymatic incubation process.

16. Shred and dissociate tissue.
    a. Rinse the tissue with pre-cooled dulbecco's phosphate buffered saline (DPBS) and remove the tissue into a 2 mL small centrifuge tube.
    b. Add 500 µL 0.05% collagenase IV and cut the tissue into pieces on ice, dissociate at 37°C for 8 min.
    c. Then 70 µm filter is used to filter the lysate and stop digestion with 10% fetal bovine serum (FBS)-containing dulbeccos modified eaglemedium (DMEM).

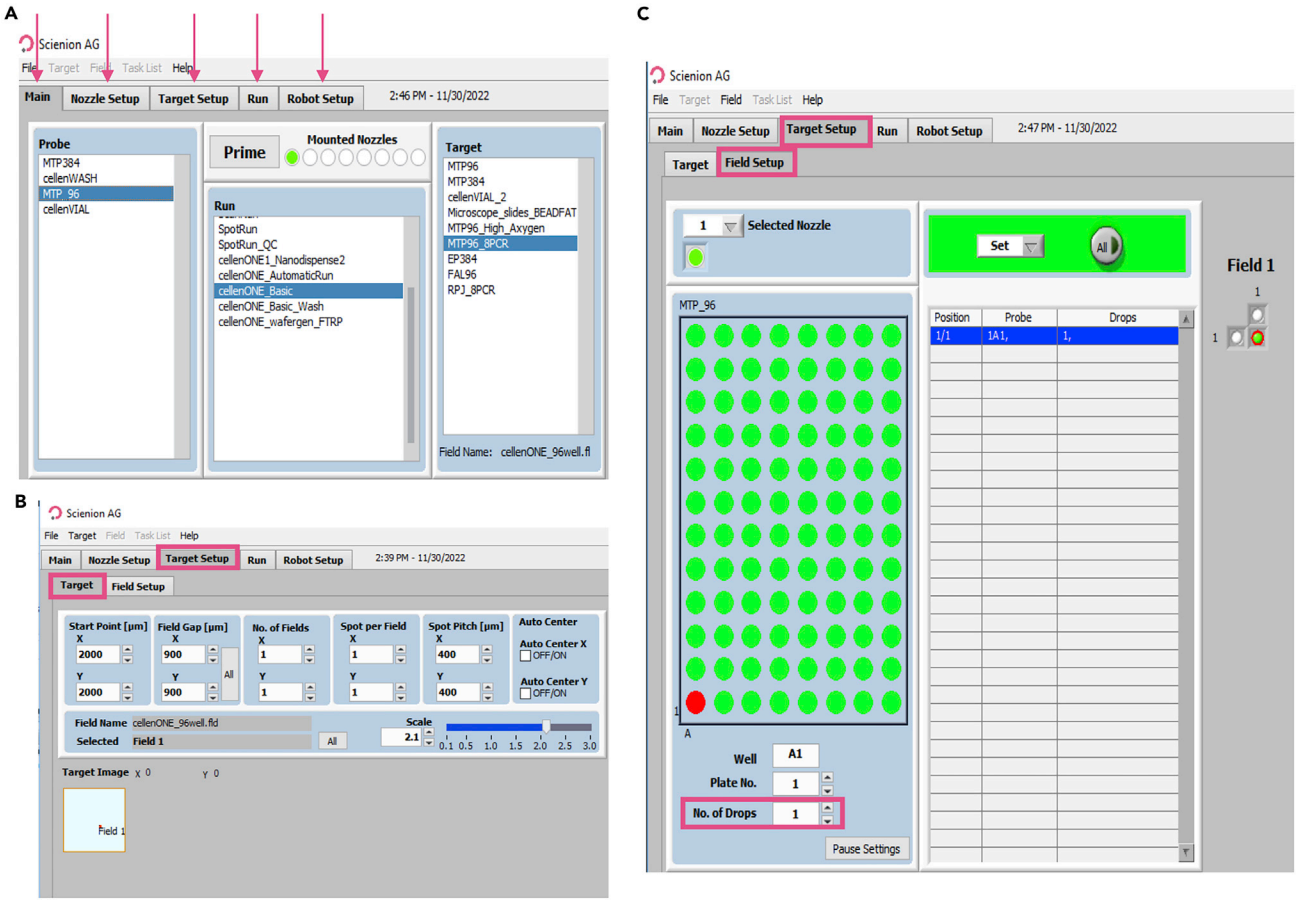

Figure 4. Sorting parameter setting

(A) Overview of setting options.

(B) "Target" to dispense single cells from well A1 of a probe 96 well plate into wells of a target 96 well plate; each well contains a single position and each well is set as a target.

(C) "Field Setup" to dispense single cells from well A1 of a probe 96 well plate into wells of a target 96 well plate; each well contains a single position and each well is set as a target.

d. Centrifugation at 500 × *g*, 4°C, 5 min, then discard the supernatant and resuspend with FBS-containing DMEM.

17. Lysis of red blood cells.

Lyse the red blood cells in suspension by adding an appropriate volume (approximately 3 times the volume of cell suspension) of red blood cell lysis buffer.

18. Quantification of cell number and viability.

Mix 10 µL cell suspension with an equal volume of 0.4% Trypan Blue and then load onto a cell counting chamber slides. Insert into the cell counter (Invitrogen, AMQAX1000, User guide) for cell number and viability assay.

Note: Keep the rest of the samples on ice or store at −80°C.

Single cell sorting

Timing: 10 min

The process mainly describes the sorting of prepared cell suspensions to obtain individual cells by the celleone single cell sorting system.

19. Resuspend the sample. Place it in the machine.
20. Aspirate 10 μL.
21. Check drop volume and set parameters.
22. Open CellenONE module.
23. Click "D" to take a reference background image. Click the Background button only when the nozzle is free of cells and/or particles and when PDC is in front of the camera.
24. Change the sample name to "particle size nozzle type volume"
25. Click "M" to do the mapping procedure.
26. Check the remaining volume.

⚠ CRITICAL: The isolation needs 4–5 μL. If it is not enough volume left, flush PDC and aspirate sample again.

Note: Change to "standby" mode when the sample was already aspirated but no movements were taken more than 30 s.

27. Click run to start the isolation and cells are sorted into these 8-tube strips (Open Z movement when using high 96-well plates).
28. Flush PDC immediately after isolation.

Whole genome amplification

Timing: 1 day

During this step, we outline the process of single cell multiple displacement amplification (MDA).

29. Single cell lysis.

The lysate was added to the 8-tube strips in which the sorted cells were collected for cell lysis.

Reagent	Final concentration	Amount
Single cell in microtube	N/A	~3 μL
Exo-resistant Random primer	0.5 μM	1 μL
Lysis buffer_MDA (400 mM KOH, 100 mM DTT, 10 mM EDTA) in-house made	N/A	3 μL
Flick, Cent, on ice 10 min		
Stop solution (400 mM HCl Tris-HCl and 600 mM Tris-HCl 1 M, pH7.5) in-house made	N/A	3 μL
Flick, Cent, on ice at least 2 min		

30. Make amplification mastermix.

Reagent	Final concentration	Amount
REPLI-g sc Reaction Buffer	N/A	30 μL
REPLI-g sc DNA Polymerase	N/A	2 μL
Mix and take 32 μL		
Total reaction		~42 μL

31. Amplification.

Steps	Temperature	Time	Cycles
Heatlid	75℃		
Amplification	30°C	90 min	1
Annealing	65°C	3 min	1
Hold	4°C	forever	

32. Amplified product purification.
    a. Add AMPureXP beads 75.6 μL into each 42 μL amplification product (1.8×), incubate at 20°C–25°C for 5 min.
    b. Put in the magnetic stand, when the solution is clear (~5 min), remove the supernatant.
    c. Resuspend with 150 μL 80% ethanol, incubate at 20°C–25°C for 5 min.
    d. Put in the magnetic stand, when the solution is clear (~5 min), remove the supernatant.
    e. Repeat steps c and d for total twice wash. Open the tube lid to air-dry (~5 min).
    f. Take the tube off the magnetic stand, add 32 μL nuclease-free (NF) water to elute beads, pipette several times, put back to the magnetic separator, wait until the solution is clear (~5 min).
    g. Transfer 30 μL supernatant to a new tube.

Note: Store the purified product at 4°C for short-term storage or −20°C for long-term storage.

33. Measure concertation using qubit.

Reagent	Amount
Qubit™ dsDNA HS Buffer	199 μL
Qubit™ dsDNA HS Reagent	1 μL
Total	**200 μL**

Purified product yields 10–12 μg.

34. Agarose gel electrophoresis.
    a. Weigh 0.5 g agarose and add 50 mL of 1× TBE solution to prepare 1% agarose gel.
    b. Then add 10 ng sample into each loading hole and run for 40 min under 100 V voltage.
    c. After electrophoresis, take out the gel and take photos with the gel imaging system. In the gel picture, a bright band over 10 kb (with a little smear) is desirable (Figure 5).

35. Amplification uniformity estimation.

Reagent	Final concentration	Amount
HCl (2 N) Fast Sybr green master mix (2×)	1×	5 μL
locus-specific primers forward (1 μM)	0.1 μM	1 μL
locus-specific primers reversed (1 μM)	0.1 μM	1 μL
NF water		2 μL
Aliquot 8 μL in to each well/tube		
diluted MDA product (1 ng/μL)	0.2 ng/μL	2 μL
Neg control (H2O)		2 μL
Unamplified genomic DNA as positive control (1 ng/μL)	0.2 ng/μL	2 μL

    a. Prepare real-time PCR mixture.
    b. Seal the plate and centrifuge briefly.

Steps	Temperature	Time	Cycles
Denaturation	95°C	20 s	1
Amplification	95°C	3 s	40
	60°C	30 s	
Hold	4°C	forever	

c. Incubate using the qPCR program below:
d. The relative uniformity values (RUVs) for 8 loci are calculated as follows:
Relative uniformity value (RUV) = 2 -(Cti-Ct0).
Cti denotes the Ct value of the locus in sample *i*, and Ct0 denotes the Ct value of the same locus in the unamplified genomic DNA.
RUV of unamplified genomic DNA should be 1.
An RUV closer to 1 indicates a more uniform amplification of the locus.

⚠ CRITICAL: Samples with RUV values between 0.25 and 4 for at least 6 of the 8 loci are selected for library construction.[3]

Single-cell whole-exome library construction

Timing: 2–3 days

This section describes sample preparation for paired- end multiplexed exome library preparation.

Sample preparation

36. Genomic DNA fragment.
a. Shear the DNA. 65 μL DNA was sheared to 150–200 bp by Covaris M220.

⚠ CRITICAL: The parameters are set as follows: peak power: 75, duty factor: 20%, cycles: 200, time: 250 s, and temperature: 18°C–22°C.

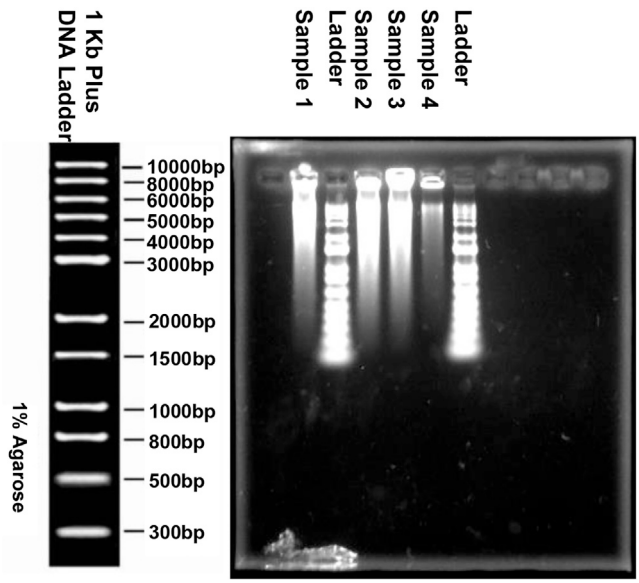

Figure 5. Picture of the MDA products gel run

b. Purification.
   i. Mix AMPure XP beads suspension thoroughly.
   ii. For each sheared DNA sample (approximately 65 μL), add 90 μL AMPure XP beads. Mix with a pipette ten times.
   iii. Incubate samples at 20°C–25°C for 5 min.
   iv. Put the tube on a magnetic separator. Wait for the solution to clear (3–5 min). Discard the supernatant carefully.
   v. Add 200 μL of 70% ethanol to each tube while keeping the tube in the magnetic stand. Remove the ethanol after 1 min. Repeat the wash procedure.
   vi. Seal the wells, spin, return to the magnetic stand and remove all ethanol. Airdry the samples (3–5 min).
   vii. Add 25 μL NF water to each tube, mix thoroughly, and incubate for 2 min at 20°C–25°C.
   viii. Put the tube on the magnetic stand, wait until the solution is clear. Transfer the supernatant (~ 24 μL) to a new tube.

c. Assess quality (optional).
   Refer to the reagent kit guide of 2100 Bioanalyzer instrument to perform the quality control process. The DNA fragments peak at 150–200 bp in size.

d. Repair the ends.
   i. Prepare End Repair master mix on ice, mix thoroughly.

Reagent	Final concentration	Volume for 1 reaction (for multiple reactions, prepare 10% excess)
NF water	N/A	17.6 μL
10× End Repair Buffer	1×	5 μL
dNTP Mix	N/A	0.8 μL
T4 DNA Polymerase	N/A	0.5 μL
Klenow DNA Polymerase	N/A	1 μL
T4 Polynucleotide Kinase	N/A	1.1 μL
Total	**N/A**	**26 μL**

   ii. Add 26 μL of the prepared mix to each tube. Mix well by pipet.
   iii. Incubate by using the following program without a heated lid.

Steps	Temperature	Time	Cycles
End-Repair	20°C	30 min	1
Hold	4°C	forever	

e. Purification.
   i. Mix AMPure XP beads suspension thoroughly.
   ii. For each 50 μL end-repaired DNA sample, add 90 μL of homogeneous AMPure XP beads. Mix with a pipette ten times.
   iii. Incubate samples at 20°C–25°C for 5 min.
   iv. Put the tube on a magnetic separator. Wait for the solution to clear (3–5 min). Discard the supernatant carefully.
   v. Add 200 μL of 70% ethanol to each tube while keeping the tube in the magnetic stand. Remove the ethanol after 1 min. Repeat the wash procedure.
   vi. Seal the wells, spin, return to the magnetic stand and remove all residual ethanol. Airdry the samples (3–5 min).
   vii. Add 16 μL NF water to each tube, mix thoroughly, and incubate for 2 min at 20°C–25°C.
   viii. Put the plate on the magnetic stand, wait until the solution is clear. Transfer the supernatant (~ 15 μL) to a new tube.

Pause Point: If the experiment does not proceed, the sample should be stored at −20°C.

f. dA-tail the 3′ end.
   i. Prepare dA-Tailing master mix on ice, mix well.

Reagent	Final concentration	Volume for 1 reaction (for multiple reactions, prepare 10% excess)
NF water	N/A	5.5 μL
10× Klenow Polymerase Buffer	1×	2.5 μL
dATP	N/A	0.5 μL
Exo(-) Klenow	N/A	1.5 μL
Total	**N/A**	**10 μL**

   ii. For each sample (~ 15 μL) add 10 μL of the prepared master mix, pipet to mix thoroughly.
   iii. Incubate by using the following program without a heated lid.

Steps	Temperature	Time	Cycles
dA-Tailing	37°C	30 min	1
Hold	4°C	forever	

g. Purification.
   i. Mix AMPure XP beads suspension thoroughly.
   ii. For each 25 μL dA- tailed DNA sample, add 45 μL of homogeneous AMPure XP beads. Mix with a pipette ten times.
   iii. Incubate samples at 20°C–25°C for 5 min.
   iv. Put the tube on a magnetic separator. Wait for the solution to clear (3–5 min). Discard the supernatant carefully.
   v. Add 200 μL of 70% ethanol into each tube while keeping the tube in the magnetic stand. Remove the ethanol after 1 min. Repeat the wash procedure.
   vi. Seal the tubes, spin, return to the magnetic stand and remove all residual ethanol. Airdry the samples (3–5 min).
   vii. Add 7.5 μL NF water to each tube, mix thoroughly, and incubate for 2 min at 20°C–25°C.
   viii. Put the plate on the magnetic stand, when the solution is clear, transfer the supernatant (~ 6.5 μL) to a new tube. Go to next step immediately.

h. Ligate the paired-end adapter.
   i. Prepare Ligation master mix on ice. Mix well.

Reagent	Final concentration	Volume for 1 reaction (for multiple reactions, prepare 10% excess)
NF water	N/A	7.75 μL
5× T4 DNA Ligase Buffer	1×	5 μL
SureSelect Adaptor Oligo Mix	N/A	5 μL
T4 DNA Ligase	N/A	0.75 μL
Total	**N/A**	**18.5 μL**

   ii. For each sample (6.5 μL), add 18.5 μL of the prepared master mix, pipet to mix well.
   iii. Incubate by using the following program without a heated lid.

Steps	Temperature	Time	Cycles
Ligation	20°C	15 min	1
Hold	4°C	forever	

Pause Point: If the experiment does not proceed, store the sample at −20°C.

i. Purification.
   i. Mix AMPure XP beads suspension thoroughly.

ii. For each 25 μL adapter- ligated DNA sample, add 45 μL of homogeneous AMPure XP beads. Mix with a pipette ten times.
iii. Incubate samples at 20°C–25°C for 5 min.
iv. Put the tube on a magnetic separator. Wait for the solution to clear (3–5 min). Discard the supernatant carefully.
v. Add 200 μL of 70% ethanol into each tube while keeping the tube in the magnetic stand. Remove the ethanol after 1 min. Repeat the wash procedure.
vi. Seal the tubes, spin, return to the magnetic stand and remove all residual ethanol. Airdry the samples (3–5 min).
vii. Add 16 μL NF water to each tube, mix thoroughly, and incubate at 20°C–25°C for 2 min.
viii. Put the tube on the magnetic stand, when the solution is clear transfer the supernatant (~16 μL) to a new tube.

Pause Point: If the experiment does not proceed, the sample should be stored at −20°C.

j. Amplify the adapter-ligated library.
i. Prepare pre- capture PCR reaction mix on ice, mix well.

Reagent	Final concentration	Volume for 1 reaction (for multiple reactions, prepare 10% excess)
NF water	N/A	10.5 μL
SureSelect Primer	N/A	0.625 μL
SureSelect ILM Indexing Pre-Capture PCR Reverse Primer	N/A	0.625 μL
5× Herculase II Reaction Buffer	1×	5 μL
100 mM dNTP Mix	1 mM	0.25 μL
Herculase II Fusion DNA Polymerase	N/A	0.5 μL
Total	**N/A**	**17.5 μL**

ii. For each sample (7.5 μL), add 17.5 μL PCR reaction mixture, mix well by pipet.
iii. Incubate by using the following program.

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	2 min	1
Denaturation	98°C	30 s	4–6
Annealing	65°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	10 min	1
Hold	4°C	forever	

k. Purification.
i. Mix AMPure XP beads suspension thoroughly.
ii. For each 25 μL amplified DNA sample, add 45 μL of homogeneous AMPure XP beads. Mix with a pipette ten times.
iii. Incubate samples at 20°C–25°C for 5 min.
iv. Put the tube on a magnetic separator. Wait for the solution to clear (3–5 min). Discard the supernatant carefully.
v. Add 200 μL of 70% ethanol into each tube while keeping the tube in the magnetic stand. Remove the ethanol after 1 min. Repeat the wash procedure.
vi. Seal the tubes, spin, return to the magnetic stand and remove all residual ethanol. Airdry the samples (3–5 min).
vii. Add 15 μL NF water to each tube, mix thoroughly, and incubate for 2 min at 20°C–25°C.
viii. Put the tube on the magnetic stand, wait until the solution is clear. Transfer the supernatant (~15 μL) to a new tube.

Pause Point: If the experiment does not proceed, store the sample at −20°C.

l. Assess quality and quantity.
The quality is assessed as shown in c of step 36; while the quantity is assessed as shown in step 33. The size of DNA fragment peak here is approximately 225–275 bp.

Hybridization and capture

Timing: 16 or 24 h

This section describes the steps to hybridize prepared gDNA libraries with a specific probe capture library. After hybridization, the target molecule is captured on a streptavidin bead.

37. Hybridize DNA samples to the probe.
    a. Prepare 1.7 μL of a 221 ng/μL dilution of each library if the DNA concentration is higher than 221 ng/μL.
    b. For libraries concentration below 221 ng/μL, concentrate the samples using a vacuum concentrator as described in the user guide.
    c. Transfer each 1.7 μL gDNA library (375 ng) to a separate tube, sealed and keep on ice.
    d. Prepare the Hybridization Buffer at 20°C–25°C, Warm the buffer for 5 min at 65°C if a precipitation forms. Until used, keep it at 20°C–25°C.

Reagent	Final concentration	Volume for 1 reaction (for multiple reactions, prepare 10% excess)
SureSelect Hyb 1	N/A	3.315 μL
SureSelect Hyb 2	N/A	0.135 μL
SureSelect Hyb 3	N/A	1.325 μL
SureSelect Hyb 4	N/A	1.725 μL
Total	**N/A**	**6.5 μL**

    e. Prepare the SureSelect Block Mix. Until used, keep the mixture on ice.

Reagent	Final concentration	Volume for 1 reaction (for multiple reactions, prepare 10% excess)
SureSelect Indexing Block 1	N/A	1.25 μL
SureSelect Block 2	N/A	1.25 μL
SureSelect ILM Indexing Block 3	N/A	0.3 μL
Total	**N/A**	**2.8 μL**

    f. To each gDNA library prepared in step c, add 2.8 μL the prepared Mix. Mix well by pipette.
    g. Incubate by using the program below with a heated lid at 105°C.

Steps	Temperature	Time	Cycles
Blocking	95°C	5 min	1
Hold	65°C	≥ 5 min	

    h. Dilute SureSelect RNase Block according to the probe size, and keep the mixture on ice until use.

Probe size	RNase block dilution (parts RNase Block: parts water)	Volume of dilute RNase block required per hybridization reaction
≥ 3.0 Mb	25%(1:3)	2 μL
<3.0 Mb	10%(1:9)	5 μL

i. Prepare Capture Library Hybridization, Mix based on design size of the probe.

Reagent	Final concentration	Volume for 1 reaction (for multiple reactions, prepare 10% excess)
Hybridization Buffer mixture from step d	N/A	6.5 μL
25% RNase Block solution from step h	N/A	1 μL
Probe (with design ≥3 Mb)	N/A	2.5 μL
Total	**N/A**	**10 μL**

j. Add 10 μL of the prepared mix to each sample while keeping the sample tube at 65°C. Mix well by pipet.

k. All wells should be entirely sealed. Incubate 16 or 24 h at 65°C, with a 105°C-heated lid.

38. Prepare streptavidin-coated magnetic beads.
    a. Prepare SureSelect Wash Buffer 2 by warming it to 65°C until it is used in step 39.
    b. Resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads thoroughly.
    c. Add 25 μL beads to a fresh tube for each sample.
    d. Wash the beads: Add 200 μL SureSelect Binding Buffer. Mix thoroughly by pipet. Put the tube on a magnetic stand. When the solution is clear, remove the supernatant. Repeat this process for total 3 washes.
    e. Resuspend the beads in 100 μL of SureSelect Binding Buffer.
39. Capture the hybridized DNA using streptavidin-coated beads.
    a. Maintain the hybridization tube at 65°C, transferring each hybridization mixture to the tube containing 100 μL of washed streptavidin beads. Mix thoroughly by pipet.
    b. Incubate the capture tube at 20°C–25°C for 30 min at 1,400–1,800 rpm.
    c. Spin the tube briefly.
    d. Put the tube on the magnetic separator. Remove the supernatant when the solution is clear.
    e. Using 100 μL SureSelect Wash Buffer 1 to resuspend the beads and mix thoroughly by pipet.
    f. Incubate the samples at 20°C–25°C for 15 min and spin briefly.
    g. Put the tube on the magnetic separator. Remove the supernatant when the solution is clear.
    h. To wash the beads, add 100 μL Wash Buffer 2 (65°C prewarmed), pipette to mix well. Incubate at 65°C for 10 min. Put the tube on the magnetic separator, remove the supernatant when the solution is clear. Repeat the step for total 3 washes.

Note: During the final wash, ensure that all the wash buffer has been removed.

i. Add 15 μL NF water to each tube, mix well by pipette. Until used, keep the samples on ice.

Indexing and sample processing for multiplexed sequencing

Timing: 1 h

This section describes the steps for adding index tags by amplification, as well as purifying and assessing the quality and quantity of the capture libraries.

Note: Only one of the 96 possible index primers is added in each reaction well.

40. Amplify the captured libraries.
    a. Assign each sample to the appropriate index.
    b. Prepare PCR reaction mix on ice, mix well by vortexing.

Reagent	Final concentration	Volume for 1 reaction (for multiple reactions, prepare 10% excess)
NF water	N/A	9.25 μL
5× Herculase II Reaction Buffer	1×	5 μL
Herculase II Fusion DNA Polymerase	N/A	0.5 μL
100 mM dNTP Mix	1 mM	0.25 μL
SureSelect ILM Indexing Post-Capture Forward PCR Primer	N/A	0.5 μL
Total	**N/A**	**15.5 μL**

c. Add 15.5 μL PCR reaction mix to a fresh PCR tube for each sample.
d. Put 2.5 μL indexing primer into each sample.
e. Transfer 7 μL bead-bound target-enriched DNA sample to the tube containing PCR reaction mix and indexing primer; Pipet to mix well.

Note: In case of future use, the remaining library-bound beads can be stored at –20°C.

f. Run the following PCR amplification program:

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	2 min	1
Denaturation	98°C	30 s	12
Annealing	57°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	10 min	1
Hold	4°C	forever	

g. Once the PCR amplification has been completed, spin the tube briefly.

41. Purification.
    a. Detailed steps are as follows:
        i. Mix AMPure XP beads suspension thoroughly.
        ii. For each 25 μL amplified DNA sample, add 45 μL AMPure XP beads. Mix with a pipette ten times.
        iii. Incubate samples at 20°C–25°C for 5 min.
        iv. Put the tube on a magnetic separator. Wait for the solution to clear (3–5 min). Discard the supernatant carefully.
        v. Dispense 200 μL of 70% ethanol into each tube while keeping the tube in the magnetic stand. Remove the ethanol after 1 min. Repeat the wash procedure.
        vi. Seal the tubes, spin, return to the magnetic stand and remove all residual ethanol. Airdry the samples (3–5 min).
        vii. Add 15 μL NF water to each tube, mix thoroughly, and incubate for 2 min at 20°C–25°C.
        viii. Put the tube on the magnetic stand, wait until the solution is clear. Transfer the supernatant (~15 μL) to a new tube.

Pause Point: If the experiment does not proceed, store the sample at −20°C.

42. Assess the quantity and quality of the libraries.

Refer to the reagent kit guide of 2100 Bioanalyzer instrument to perform the quality control process. The peak of DNA fragment size should be 250–350 bp. Qubit 4.0 was used to measure each library's concentration.

Stopping Point: If the experiment does not proceed, store the libraries at −20°C.

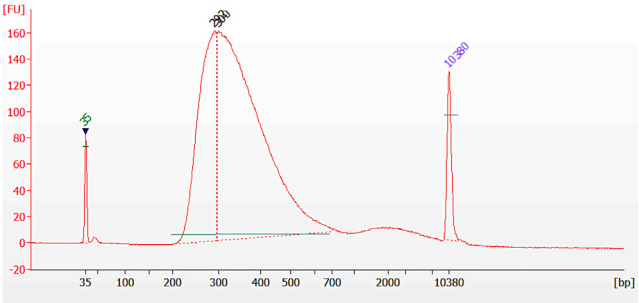

Figure 6. Post-capture analysis of amplified indexed library DNA using the 2100 Bioanalyzer and a High Sensitivity DNA Assay

43. Sequencing.

The constructed exome libraries can be sent to a commercial facility for sequencing.

EXPECTED OUTCOMES

Using our single-cell whole-exome library construction method, the average yield of six single cell exome libraries was more than 100 ng (T14:150 ng, T15:178.5 ng, T20:124.5 ng, T24:73.05 ng, N16:181.5 ng, N24:118.5 ng). And the peak of DNA fragment size located between 250–350 bp were obtained (Figure 6). By using Illumina NovaSeq platform, 150 bp paired-end sequencing is performed, yielding about 10 Gb of data.

LIMITATIONS

Although the selection can be made by the sorting photos retained by the machine, there is still a certain probability of bias. Therefore, the process of CellenONE X1 sorting cannot guarantee that there is only one cell in each well. So, the selection process of identifying cells that can be used for subsequent experiments is relatively long. For this problem, staining of sorted cells with dyes that do not affect subsequent experiments can be selected; In addition, the process of exon library construction is laborious and time-consuming, so corresponding automation scripts can be designed and automated workstations can be used to save labor costs.[4,5]

TROUBLESHOOTING

Problem 1

There are many empty wells after cell sorting by CellenONE X1, there are no cells in these wells (Step "single cell preparation", 27).

Potential solution

The ejection zone is too big, move the ejection zone boundary to reduce its size.

Problem 2

There are many doublets after cell sorting by CellenOne X1, this means that there are two cells in the same one well (Step "single cell preparation", 27).

Potential solution

Some cells are not detected, widen detection parameters (diameters, elongation, and circularity).

Problem 3

Low cell viability (Step "whole genome amplification", 29, 30, 31, 32, 33).

Potential solution

Set the chiller to 4°C and upload the cell sample from the 384 well source plate.

Problem 4

The homogeneity of amplification of most single-cell MDA products is poor (Step "whole genome amplification", 35).

Potential solution

Using corresponding methods to select MDA products with good amplification homogeneity for subsequent experiments. In addition to the method for detecting the homogeneity of amplification products provided in this protocol, reference can also be made to the amplification homogeneity detection method published by He et al.[6]

Problem 5

DNA fragments do not conform to expectations after shearing (Step "sample preparation", 36: a, b, c).

Potential solution

Prepare and shear the sample according to the maximum volume of the microtube.

Problem 6

There will be a small hill starting from 700 bp when too many cycles were used to amplify the sample (Step "hybridization and capture", 40: f).

Potential solution

Amplify the sample with less cycles.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Hui Wang (huiwang@shsmu.edu.cn).

Materials availability

No new material was generated using this protocol.

Data and code availability

The datasets supporting the current study have not been deposited in a public repository because further research is ongoing but are available from the corresponding author on request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102052.

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AUTHOR CONTRIBUTIONS

Methodology, J.H., M.M.; Reviewing and Editing, J.H., M.M., R.G., X.Z.; Supervision, H.W.; Conceptualization, J.H.; Writing - Original Draft, J.H., M.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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