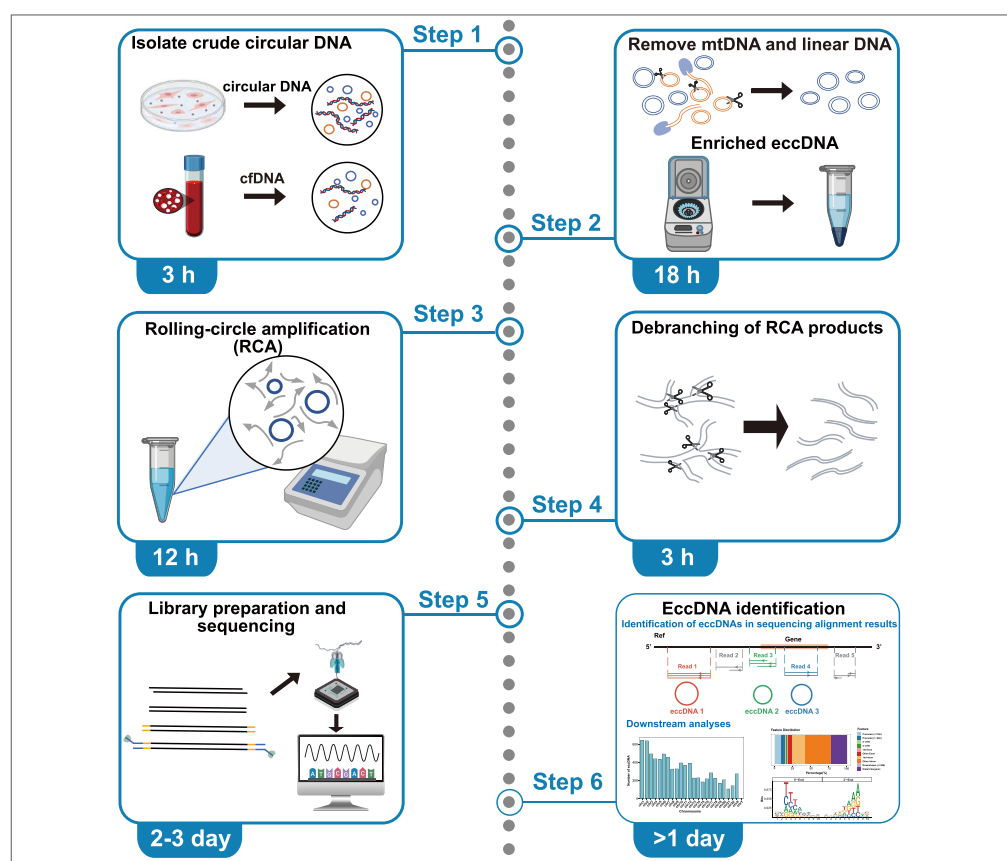


Protocol

Protocol for rolling circle amplification-based nanopore sequencing of eccDNA from cell and plasma samples



Extrachromosomal circular DNAs (eccDNAs) can drive copy number variation and genomic rearrangements and act as mobile regulators of gene expression. Here, we detail a protocol for eccDNA identification from cell and plasma samples. We describe steps for sample preprocessing with circular DNA enrichment, rolling-circle amplification and debranching, library preparation and sequencing, along with quality control tailored to the key steps.

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

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Highlights

Guidance on low-input circular DNA sample preparation

Steps for nanopore sequencing library preparation and sequencing workflow for eccDNA

Procedures for key-step quality control

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Protocol

Protocol for rolling circle amplification-based nanopore sequencing of eccDNA from cell and plasma samples

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SUMMARY

Extrachromosomal circular DNAs (eccDNAs) can drive copy number variation and genomic rearrangements and act as mobile regulators of gene expression. Here, we detail a protocol for eccDNA identification from cell and plasma samples. We describe steps for sample preprocessing with circular DNA enrichment, rolling-circle amplification and debranching, library preparation and sequencing, along with quality control tailored to the key steps.

For complete details on the use and execution of this protocol, please refer to Li et al.¹

BEFORE YOU BEGIN

Extrachromosomal circular DNA (eccDNA) is an independent genetic material widely found in eukaryotic organisms.² Emerging evidence underscores its critical roles in various biological processes, notably in cancer initiation, progression, and therapeutic resistance.^{3,4} Owing to its unique circular structure, which confers resistance to exonuclease degradation, eccDNA can be stably preserved in plasma and has emerged as a promising minimally invasive liquid biopsy biomarker.^{5,6} Adding to this complexity, eccDNA exhibits a broad size ranging from tens to millions of base pairs (bp). Current detection strategies generally fall into two categories based on this size variation⁷: those targeting large eccDNAs (>10 kb), which occur in over half of all human tumors,⁸ and those focusing on small eccDNAs (<10 kb), which are ubiquitously present across eukaryotes.⁹

In this protocol, we describe a standardized and optimized rolling-circle amplification (RCA)-based enrichment procedure and library preparation workflow tailored for small eccDNAs.^{10,11} The workflow incorporates comparative optimization of key steps based on existing methods, together with stepwise quality control measures, to improve experimental robustness and reproducibility.

Finally, while we use the human hepatocellular carcinoma cell line HepG2 and one pooled plasma sample as representative samples in this protocol, the protocol is broadly applicable to tumor cell lines and plasma samples from various eukaryotic species, with DNA extraction kits replaced as appropriate. Importantly, the number of linear DNA and mitochondrial DNA (mtDNA) digestion rounds and their duration should be adjusted based on sample type and characteristics, while the RCA reaction time should be adjusted according to the product concentration.



Innovation

The intrinsic low abundance and high dynamic range of eccDNA across diverse samples and biological conditions have historically made it challenging to achieve poor detection efficiency and low inter-assay reproducibility. By overcoming these fundamental bottlenecks, this protocol offers a flexible, robust covering sample processing through library preparation. Unlike previous methodologies, this protocol introduces strict quantitative standardization by precisely defining the optimal input amounts for critical steps. These steps are further refined through comparative analyses of existing methods to improve workflow performance. Rigorous quality control (QC) checkpoints are systematically embedded throughout the procedure. This step-by-step standardization coupled with continuous QC monitoring substantially reduces batch effects, improves reproducibility, and ensures reliable final sequencing results.

Preparation of cell samples

⌚ Timing: 1 h

High-quality circular DNA recovery strongly depends on using healthy, actively growing, morphologically normal cells that are free from contamination. Culture HepG2 cells (or the user's preferred cell line) in a humidified incubator at 37°C and 5% CO₂ in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin.

1. Harvest cells.
 - a. Remove the culture medium from the culture dish using a pipette.
 - b. Rinse the cells once with sterile PBS to remove residual serum.
 - c. Add enough trypsin-EDTA Solution to fully cover all cells.

Note: For a 10cm culture dish, add 2 mL trypsin-EDTA Solution.

 - d. Incubate in the incubator for 1–2 min. When the cells become rounded, add 2× volume of complete medium (4 mL) to stop the digestion.
 - e. Collect the cells.
 - i. Gently pipette up and down to disperse the cells.
 - ii. Transfer to a 15 mL centrifuge tube.
 - iii. Centrifuge at 200 ×g for 5 min at 23°C.
2. Use an appropriate method to count the cells, transfer the required number of cells ($1\text{--}5 \times 10^6$) to a collection tube, and then proceed to the next step.

Preparation of plasma samples

⌚ Timing: 1 h

For eccDNA library preparation from plasma samples, storage and handling are critical. Collect blood using EDTA-containing anticoagulant tubes, mix thoroughly immediately after collection, and store at 4°C. Centrifuge within 4 h to separate plasma and minimize genomic DNA (gDNA) contamination. If available, cell-free DNA (cfDNA) stabilization tubes can be used; aliquots may be stored at room temperature (approximately 23°C) for up to 1 week according to the manufacturer's instructions.

3. Centrifuge the blood collection tubes (6–10 mL) at 1,600 ×g for 10 min at 4°C to separate plasma.
4. Carefully transfer the plasma (approximately 2–5 mL) to a 1.5 mL centrifuge tube without disturbing the buffy coat, and avoid aspirating the cell layer.
5. Re-spin the 1.5 mL plasma tubes at 16,000 ×g for 10 min at 4°C.
6. Carefully transfer the supernatant into a new 1.5 mL centrifuge tube and store at –80°C.

Note: Avoid repeated freeze–thaw cycles; limit freeze–thaw to ≤ 3 times.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Pooled human plasma sample used for cfDNA extraction	This paper	N/A
Chemicals, peptides, and recombinant proteins		
FastDigest MspI enzyme	Thermo	Cat# FD1344
Exonuclease V	NEB	Cat# M0345
Adenosine 5'-Triphosphate (ATP) (10 mM)	NEB	Cat# P0756
Nuclease-free water (NFW)	Solarbio	Cat# R1600
DNA extraction solution (phenol-chloroform-isoamyl alcohol, 25:24:1)	Solarbio	Cat# P1012
Glycogen	Roche	Cat# 10901393001
3 M sodium acetate	Solarbio	Cat# A1070
TE Buffer	Solarbio	Cat# T1120
Phi29 MAX DNA Polymerase	Vazyme	Cat# EP0091
dNTPs (10 mM)	NEB	Cat# N0447
Recombinant Albumin (20mg/mL)	NEB	Cat# B9200
Pyrophosphatase, Inorganic	Thermo	Cat# EF0221
T7 Endonuclease I	NEB	Cat# M0302
T4 Ligase	NEB	Cat# E6056
Critical commercial assays		
2x ChamQ Universal SYBR qPCR Master Mix	Vazyme	Cat# Q711
E.Z.N.A.® Plasmid DNA Mini Kit I	Omega	Cat# D6943
Plasma Cell-Free DNA Extraction Kit	APOSTLE	Cat# A17622CN
Qubit™ 1x dsDNA HS Assay Kits	Thermo Fisher Scientific	Cat# Q33231
SPRI beads	Beckman	Cat# B23317
NEBNext FFPE DNA Repair Mix	NEB	Cat# E7360
Ultra II End-prep enzyme mix	NEB	Cat# E7546
NEB Blunt/TA Ligase Master Mix	NEB	Cat# M0367
Native Barcoding Kit 24 V14	ONT	Cat# SQK-NBD114.24
Flow Cell Wash Kit	ONT	Cat# EXP-WSH004
Deposited data		
Raw nanopore sequencing data for eccDNA profiling of Li et al. ¹	This paper	PRJCA052047
Raw nanopore sequencing data for eccDNA profiling of four Huh7 cell samples and one pooled human plasma sample	This paper	PRJCA069021
Experimental models: Cell lines		
Human: HepG2	Wuhan Procell Biotechnology Co., Ltd.	Cat# CL-0103
Human: Huh7	Shanghai Xunyi Biotechnology Co., Ltd.	Cat# MXC58
Oligonucleotides		
Exo-random primer (500 μM)	Thermo	Cat# SO181
Primer ND1 Forward: CCCTAAAACCCGCCACATCT Reverse: GAGCGATGGTGAGAGCTAAGGT	This paper	N/A
Primer H-GAPDH Forward: CTGGGCTACACTGAGCACC Reverse: AAGTGGTCGTTGAGGGCAATG	This paper	N/A
Primer MIR621-A Forward: GGATCATATTCCACGCTGACTAT Reverse: AGTCCCATTTCTATTTTACTTCG	This paper	N/A
Primer MIR621-B Forward: AATCACTGACCAACCAACCAA Reverse: TTTGGTCAGTGAATACATACATATGGC	This paper	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primer MIR4319-A Forward: CTGTACTAAGTCAGAGCCC Reverse: ATTTAGAGACATTCTTATGC	This paper	N/A
Primer MIR4319-B Forward: CTATACACAGAAGAGGAGGCAGTGC Reverse: CTGACCTCCTCATTCCACCTAC	This paper	N/A
Primer MIR148A-A Forward: CTGGAAGACCGGAATAGAG Reverse: CCCCTCTGGCCTTGCCCCCTCCC	This paper	N/A
Primer MIR148A-B Forward: CAGCCCCTTTGCTGTGACATTG Reverse: CAAGAATCGCACACTCTCCTT	This paper	N/A
Primer MIR1307-A Forward: CCAGTTTCCATAGTAATGACTCCCTGA Reverse: AACTGGTAAAACAGCCTGGCA	This paper	N/A
Primer MIR1307-B Forward: TCTGTGCCTGTTGTGTGTCG Reverse: TCTTTGTGTAAGTACATGGGAAGATGTC	This paper	N/A
Other		
Phase Lock Gel Heavy Tubes	TIANGEN	Cat# WM5-2302831
Qubit™ Fluorometer	Thermo Fisher Scientific	Cat# Q33226
Qubit™ Assay Tubes	Thermo Fisher Scientific	Cat# Q32856
Qsep100 Advance	Bioptric	https://bioptric.com.cn/ProductDetail/5186703.html
High Sensitivity Kilo Base Cartridge Kit	Bioptric	Cat# C105153
DynaMag™-2 magnetic rock	Thermo Fisher Scientific	Cat# 12321D
PromethION sequencing platforms	ONT	Cat# PRO-INT002
PromethION sequencing flow cells R10.4.1 (DNA)	ONT	Cat# FLO-PRO114M

STEP-BY-STEP METHOD DETAILS

Isolate crude circular DNA

⌚ Timing: 3 h

In this step, we describe the crude extraction of circular DNA from tumor cell lines and circular cfDNA from plasma samples. The protocol was adapted from the manufacturer's instructions for the [E.Z.N.A.® Plasmid DNA Mini Kit I](#) (for cell lines) and the [Plasma Cell-Free DNA Extraction Kit](#) (for plasma samples), with modifications.

1. For cell samples.

- Collect about $1-5 \times 10^6$ cells. Centrifuge at $200 \times g$ for 5 min at 23°C. Aspirate and discard the culture media.
- Wash with 500 μ L of PBS once and resuspend the cells. Centrifuge at $200 \times g$ for 5 min at 23°C. Aspirate and discard the PBS.
- Add 250 μ L of RNase A-supplemented Solution I (E.Z.N.A.® Plasmid DNA Mini Kit I), and thoroughly resuspend the cell pellet by vortexing or pipetting to maximize circular DNA yield.

Note: Add RNase A to Solution I before first use. Store RNase A-supplemented Solution I at 4°C.

- Add 250 μ L of Solution II (E.Z.N.A.® Plasmid DNA Mini Kit I) and mix by gently inverting the tube 4–6 times until the lysate becomes clear. If necessary, incubate at room temperature (approximately 23°C) for 2–3 min to ensure complete lysis.

⚠ CRITICAL: Avoid vigorous mixing to prevent shearing of chromosomal DNA, which lowers plasmid purity. Additionally, do not allow the lysis reaction to exceed 5 min.

Note: Store Solution II tightly capped when not in use to prevent acidification caused by atmospheric CO₂.

- e. Add 350 µL of Solution III (E.Z.N.A.® Plasmid DNA Mini Kit I) and immediately mix by inverting the tube 4–6 times until a white, flocculent precipitate forms.

△ CRITICAL: Immediate and thorough mixing upon the addition of Solution III is essential to prevent localized precipitation.

- f. Centrifuge at 20,000 ×g for 10 min to form a compact white pellet. Insert a HiBind® DNA Mini Column into a 2 mL collection tube.
- g. Carefully transfer the cleared supernatant from the previous step into the column.

Note: Avoid transferring cellular debris, as it can reduce DNA purity and clog the column.

- h. Centrifuge at 20,000 ×g for 1 min, discard the flow-through, and reuse the collection tube.
- i. Add 500 µL of HBC Buffer (E.Z.N.A.® Plasmid DNA Mini Kit I) to the column and incubate at room temperature (approximately 23°C) for 1 min.

Note: Ensure that the HBC Buffer has been diluted with 100% isopropanol before use, according to the manufacturer's instructions.

- j. Centrifuge at 20,000 ×g for 1 min. Discard the flow-through and reuse the collection tube.
- k. Add 700 µL of DNA Wash Buffer (E.Z.N.A.® Plasmid DNA Mini Kit I) to the column and incubate at 23°C for 1 min.

Note: Ensure that the DNA Wash Buffer has been diluted with 100% ethanol prior to use.

- l. Centrifuge at 20,000 ×g for 1 min. Discard the flow-through and reuse the collection tube.
- m. Repeat step k–l for a second wash with DNA Wash Buffer.
- n. Centrifuge the empty column at 20,000 ×g for 2 min to completely dry the column matrix.

△ CRITICAL: This dry-spin step is essential. Residual ethanol must be thoroughly removed prior to elution, as it can severely interfere with downstream applications.

- o. Transfer the column to a clean 1.5 mL microcentrifuge tube.

△ CRITICAL: To minimize DNA loss and ensure optimal recovery, it is strongly recommended to use low-binding/low-retention tubes and pipette tips for all sample handling throughout the protocol.

- p. Add 45 µL of Elution Buffer (E.Z.N.A.® Plasmid DNA Mini Kit I) or sterile deionized water directly onto the center of the column membrane.
- q. Incubate at room temperature (approximately 23°C) for 1 min, then centrifuge at 20,000 ×g for 1 min to elute the DNA.

Note: A single elution typically recovers approximately 70% of the bound DNA. An optional second elution can be performed to maximize DNA recovery. Specifically, the eluate from the first elution can be re-applied to the column, incubated for 1 min, and then centrifuged at 20,000 ×g for 1 min. This may help increase DNA recovery, albeit at a lower final concentration. Using 3 × 10⁶ cells, we typically obtain approximately 1 ng/µL (total yield: about 45 ng).

Table 1. qPCR reaction master mix

Reagent	Final concentration	Amount
DNA template	N/A	2 μ L
2 $\times$ ChamQ Universal SYBR qPCR Master Mix	1 $\times$	10 μ L
Forward primer (2 μ M)	0.2 μ M	2 μ L
Reverse primer (2 μ M)	0.2 μ M	2 μ L
NFW	N/A	4 μ L
Total	–	20 μ L

- r. Store recovered crude circular DNA at 4°C for short-term use. For long-term storage, store at –80°C.
- s. **QC:** Reserve a 2 μ L aliquot of the eluted DNA for quality control analysis by qPCR.
 - i. To prepare the sample for qPCR, dilute the 2 μ L crude circular DNA aliquot 1:100 with 198 μ L of nuclease-free water (NFW).
 - ii. Prepare the qPCR reaction master mix for two primer sets according to [Table 1](#).

Note: Use *ND1* primers to quantify mtDNA for estimating the extraction efficiency of circular DNA, and use *GAPDH* primers to quantify nuclear DNA for assessing the abundance of linear DNA.

- iii. Set up the qPCR cycling program according to [Table 2](#).

Note: Typical Ct values are approximately 15–20 for *ND1* and 25–30 for *GAPDH* (see [problem 1](#) and [potential solution](#) in the [troubleshooting](#) section).

2. For plasma samples.
 - a. Add 40 μ L of Proteinase K (Plasma Cell-Free DNA Extraction Kit) to a 15 mL tube, then add 1 mL of plasma, and finally add 100 μ L sample lysis buffer (Plasma Cell-Free DNA Extraction Kit).

Note: Avoid direct contact between Proteinase K and sample lysis buffer before adding the plasma.

Note: This section demonstrates the protocol using a 1 mL plasma sample. However, this kit can be used with 1–5 mL plasma samples. (For additional guidance, see [problem 2](#) and [potential solution](#) in the [troubleshooting](#) section.)

- b. Vortex to mix all components, then incubate at 60°C for 20 min.

Alternatives: For all incubation steps, use a water bath or a metal heat block as appropriate.

- c. After incubation, equilibrate the tube to room temperature (approximately 23°C). Place the tube in 23°C water to accelerate equilibration, if needed.
- d. Prepare the magnetic bead binding solution by combining 1.25 mL binding solution with 15 μ L magnetic beads (Plasma Cell-Free DNA Extraction Kit), and mix thoroughly to resuspend the beads.
- e. Add the prepared magnetic bead binding solution to the lysed sample. Briefly vortex or invert the tube more than 10 times to mix.

Note: Avoid excessive agitation to minimize foaming.

Table 2. qPCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	1
Denaturation	95°C	15 sec	40 cycles
Annealing	60°C	15 sec	
Extension	72°C	30 sec	

- f. Rock the tube on a rotator mixer at medium speed for 10 min to facilitate binding of cfDNA to the magnetic beads.

Alternatives: In the absence of a rotator mixer, whenever the protocol instructs “use a rotator mixer,” substitute it with “mix by gently inverting or flicking the tube once every minute.”

- g. Place the 15 mL tube on a magnetic rack for 2 min until the solution becomes clear and the beads collect along the tube wall.
- h. Carefully discard the supernatant without disturbing the beads.
- i. Wash step:
 - i. Remove the 15 mL tube from the magnetic rack. Add 500 μ L of Wash Buffer I (Plasma Cell-Free DNA Extraction Kit) and mix thoroughly to resuspend the magnetic beads.
 - ii. Spin down the contents of the tube at 1000 $\times$ g for 3 sec and transfer the suspension to a 1.5 mL low-binding microcentrifuge tube.
 - iii. Add another 500 μ L of Wash Buffer I to the 15 mL tube to rinse any residual magnetic beads from the tube wall. Transfer the rinse solution, including any remaining magnetic beads, to the same 1.5 mL low-binding microcentrifuge tube.

△ CRITICAL: Retain the 15 mL tube temporarily, as it will be rinsed again in the next step.

- iv. Place the 1.5 mL tube on a magnetic rack for 1 min until the solution becomes clear and the beads collect on the tube wall.
- v. Without disturbing the beads, use the supernatant from the 1.5 mL tube to rinse the inner wall and cap of the retained 15 mL tube. Transfer the rinse, including any residual beads, back to the 1.5 mL tube, then discard the 15 mL tube.
- vi. Place the 1.5 mL tube on the magnetic rack for 2 min until the solution becomes clear and the beads collect on the tube wall.
- vii. Carefully discard the supernatant.
- j. Remove the 1.5 mL tube from the magnetic rack. Add 1 mL of Wash Buffer I and vortex for 30 sec.
- k. Centrifuge the 1.5 mL tube at 1000 $\times$ g for 3 sec to bring down any liquid adhering to the cap or wall, then place it on the magnetic rack for 2 min until the solution becomes clear and the beads collect on the tube wall.
- l. Carefully discard the supernatant.
- m. Prepare the secondary wash working solution by mixing Wash Buffer II (Plasma Cell-Free DNA Extraction Kit) with absolute ethanol at a 1:4 ratio.

Note: Prepare at least 2 mL of working solution per sample (20% Wash Buffer II and 80% ethanol).

- n. Add 1 mL of the prepared secondary wash working solution to the 1.5 mL tube.
- o. Remove the tube from the magnetic rack and vortex for 30 sec.
- p. Centrifuge the 1.5 mL tube at 1000 $\times$ g for 3 sec, then place on the magnetic rack for 2 min until the solution becomes clear and the beads collect on the tube wall.
- q. Carefully discard the supernatant.

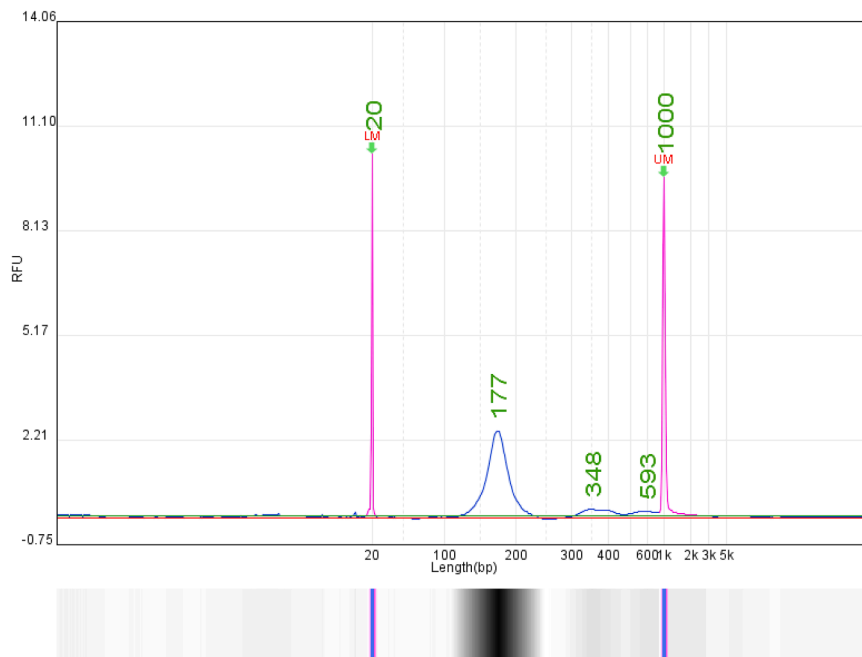


Figure 1. Plasma cfDNA fragment distribution
Representative fragment size profile of plasma cfDNA.

- r. Repeat Steps v–w to perform a second wash.
- s. Keep the 1.5 mL tube on the magnetic rack, open the cap, and air-dry the beads at room temperature (approximately 23°C) for 3 min.
- t. Remove the 1.5 mL tube from the magnetic rack and add 25 μ L of elution buffer (Plasma Cell-Free DNA Extraction Kit).
- u. Vortex the tube thoroughly for 7 min to fully resuspend the beads and elute cfDNA from the magnetic beads.
- v. Place the tube on the magnetic rack for 1–2 min until the eluate becomes clear and the beads collect on the tube wall.
- w. Transfer the cfDNA-containing eluate to a new microcentrifuge tube using a pipette.
- x. Store recovered cfDNA at 4°C for short-term use. For long-term storage, store at -80°C .
- y. QC:
 - i. Measure the concentration of the recovered cfDNA using a Qubit fluorometer according to [the manufacturer's instructions](#).
 - ii. Assess the fragment size distribution using a Qsep100 Advance according to [the manufacturer's instructions](#).

Note: Samples with a total cfDNA yield of ≥ 25 ng and a typical cfDNA fragment size distribution are recommended for the next preparation step. See [Figure 1](#) as an example.

Remove mitochondrial DNA and linear DNA

⌚ Timing: 18 h

This step describes the enzymatic removal of mitochondrial and linear DNA from the crude circular DNA extract. This digestion is entirely optional and can be customized to selectively eliminate either one or both DNA fractions, depending on the specific research goals. For eccDNA investigations,

utilizing this depletion step effectively minimizes background signal interference and lowers sequencing expenses. Next, we present an optimized ethanol precipitation protocol for trace DNA purification. Although the abundance of eccDNA varies significantly across different samples and physiological states, this purification strategy maximizes recovery yield at a remarkably low cost. Nevertheless, exceptionally low initial sample input or suboptimal handling proficiency may still lead to diminished recovery.

3. The FastDigest MssI restriction enzyme recognizes and cleaves the GTTT⁺AAAC site on mtDNA, thereby linearizing it. Subsequently, Exonuclease V is used to digest both the residual linear DNA and the newly linearized mtDNA.
 - a. Assemble the reaction mixture as follows:

Reagent	Amount
DNA sample+NFW	22 μ L
NEBuffer 4 (10 $\times$)	3 μ L
ATPs (10 mM)	3 μ L
FastDigest MssI enzyme	1 μ L
Exonuclease V	1 μ L
Total	30 μ L

△ CRITICAL: Set aside a 2 μ L pre-digestion aliquot as a control before starting the the FastDigest MssI enzyme/Exonuclease V reaction for downstream QC.

- b. Mix gently by flicking the tube, and centrifuge at 1000 $\times g$ for 3 sec to collect the contents at the bottom.
 - c. **Cell samples:** incubate the reaction at 37°C for 15 h, followed by heat inactivation at 70°C for 30 min.
Plasma samples: incubate the reaction at 37°C for 10 h, followed by heat inactivation at 70°C for 30 min.
4. **QC:** Verify the digestion efficiency of mtDNA and linear DNA using qPCR.
 - a. Dilute the pre-digestion sample 30-fold by mixing 2 μ L of the sample with 58 μ L of NFW.
 - b. Dilute the post-digestion sample 10-fold by mixing 2 μ L of the sample with 18 μ L of NFW.
 - c. Prepare the qPCR reaction master mix for two sample groups with two primer sets according to [Table 1](#).

Note: Similarly, perform quality control using *ND1* and *GAPDH* primers.

- d. Set up the qPCR cycling program according to [Table 2](#).

Note: Effective digestion of linear and mitochondrial DNA is confirmed by a Ct value increase of at least 3–10 cycles post-digestion for both linear (*GAPDH*) and mitochondrial (*ND1*) targets.

Note: As an option, the digested sample can be purified again, followed by a second digestion step, to further enrich the target template, thereby reducing background and increasing the proportion of usable sequencing data in the final library (see [problem 3](#) and [potential solution](#) in the [troubleshooting](#) section).

5. Dilute the 28 μ L post-digestion sample with 172 μ L of NFW to a final volume of 200 μ L, and transfer the mixture into a clean 1.5 mL microcentrifuge tube.

6. Add 200 μ L of a 25:24:1 phenol-chloroform-isoamyl alcohol mixture to the sample and mix thoroughly by vortexing.

Note: Vortexing is essential to thoroughly mix the aqueous and organic phases, allowing phenol to fully interact with and denature the proteins present in the sample.

7. Transfer the mixture into a Phase Lock Gel (PLG) tube and centrifuge at 16,000 $\times g$ for 5 min at 23°C.

△ CRITICAL: Centrifuge the Phase Lock Gel tube at 16,000 $\times g$ for 30 sec prior to use.

8. Carefully transfer as much supernatant as possible (approximately 200 μ L) to a clean 1.5 mL microcentrifuge tube without disturbing the separation layer, and discard the PLG tube.

Note: The white gel inside the PLG tube distinctly separates the mixture into an upper aqueous phase and a lower organic phase.

△ CRITICAL: During the transfer, strictly ensure the pipette tip does not contact the white gel layer to prevent sample contamination.

9. Add 2 μ L of glycogen to the 1.5 mL microcentrifuge tube, mix by gently inverting the tube, centrifuge at 1000 $\times g$ for 3 sec, and incubate at room temperature (approximately 23°C) for 5 min.
10. Add 20 μ L of 3M sodium acetate to the sample, mix by gently inverting the tube, centrifuge at 1000 $\times g$ for 3 sec, and incubate at room temperature (approximately 23°C) for 5 min.
11. Add ice-cold 100% ethanol to bring the final volume to 1.2 mL. Incubate the mixture at -20°C for ≥ 10 h or -80°C for ≥ 30 min.

Note: Pre-chill the 100% ethanol at -20°C for at least 30 min prior to use.

12. Centrifuge the sample at 20,000 $\times g$ for 30 min at 4°C, then carefully aspirate the residual ethanol.

△ CRITICAL: During purification, eccDNA is often present at low concentrations. As a result, the precipitate is minimal and may be difficult to visualize, sometimes appearing as a thin line along the inner wall of the tube or as a small pellet adhering to the bottom. Care should be taken when discarding the ethanol to avoid disturbing or aspirating the precipitate.

Note: To maximize DNA recovery and prevent accidental loss of the pellet, it is acceptable to leave a small residual amount of ethanol at the bottom of the tube during this step.

Note: Prepare fresh 80% ethanol during the centrifugation step.

13. Wash the DNA pellet with 1 mL of fresh 80% ethanol. Centrifuge at 20,000 $\times g$ for 30 sec at 4°C, then carefully aspirate the supernatant using a pipette.

△ CRITICAL: Strictly ensure the DNA pellet remains in the tube, as it may dislodge from the tube wall during the 80% ethanol wash. Carefully aspirate as much residual ethanol as possible using a pipette tip without disturbing the pellet.

14. Allow the pellet to air-dry at room temperature (approximately 23°C) for 2 min by keeping the tube open.

△ **CRITICAL:** Air-dry the pellet just enough to evaporate the ethanol and resuspend it in TE buffer while still slightly moist, since an over-dried pellet significantly compromises DNA recovery efficiency.

15. Resuspend the DNA pellet in 30 µL of TE buffer just prior to complete drying.

Note: Because this section specifically enriches for low-abundance eccDNA, the resulting concentration is typically below the detection limit of standard fluorometric or spectrophotometric assays. Therefore, direct concentration measurement is not required at this step. Sample sufficiency for downstream experiments will be quality-controlled based on the yield and concentration of subsequent PCR amplification products.

16. Samples can be stored 1 day at 4°C or stored long term at –20°C or –80°C.

Rolling-circle amplification

⌚ **Timing:** 12 h

This section provides a comprehensive rolling-circle amplification protocol for eccDNA. This highly robust method yields sufficient library quantities for downstream sequencing even with a low DNA input. Furthermore, by rigorously controlling the amplification time, this protocol minimizes the amplification bias typically associated with extensive exponential amplification. Finally, remove residual primers and add fresh enzyme to further amplify the rolling-circle amplification products. This secondary amplification step ensures the enrichment of highly complete full-pass molecules.

17. Assemble the mix as follows:

Reagent	Amount
DNA sample+ NFW	13 µL
10× Phi29 MAX DNA Polymerase Reaction Buffer	2 µL
Exo-random primer	1 µL
Total	16 µL

18. Incubate the reaction mix at 95°C for 3 min in a thermal cycler, followed by immediate transfer to ice.

19. Assemble the reaction mixture as follows:

Reagent	Amount
dNTPs	2 µL
Recombinant Albumin	0.4 µL
Inorganic pyrophosphatase	0.6 µL
Phi29 MAX DNA Polymerase	1 µL
DNA sample from step 18	16 µL
Total	20 µL

Note: Supplement the 10× Phi29 buffer with DTT to a final concentration of 4 mM after it has undergone ten freeze-thaw cycles. Freshly opened buffer does not require additional DTT.

20. Mix the reaction gently by flicking the tube and centrifuge at 1000 ×g for 3 sec the 0.2 mL PCR tube to collect the contents.

For cell samples: Incubate the mixture at 30°C for 6 h for rolling-circle amplification of the sample.

For plasma samples: Incubate the mixture at 30°C for 10 h for rolling-circle amplification of the sample.

△ **CRITICAL:** QC: We recommend pausing the protocol after the 6- or 10-h amplification to quantify the DNA using a Qubit fluorometer. Heat inactivation should be performed only when the DNA concentration is ≥ 150 ng/ μ L for cell samples and ≥ 20 ng/ μ L for plasma samples, to ensure sufficient input material for library preparation (see [problem 4](#) and [potential solution in troubleshooting section](#)).

21. Incubate the reaction at 65°C for 10 min to heat-inactivate the Phi29 MAX DNA polymerase. Then cool the reaction to 4°C for storage or proceed to the next step.
22. Transfer the sample from the previous step into a 1.5 mL DNA tube, add 0.4 $\times$ (8 μ L) of SPRIselect beads, and incubate the mixture at room temperature (approximately 23°C) for 5 min on a rotator.

△ **CRITICAL:** To ensure the beads remain fully resuspended throughout the incubation, gently flick the tube every minute or place it on a rotator mixer.

23. Centrifuge the tube at 1000 $\times$ g for 3 sec to bring down any liquid adhering to the cap or wall, then place it on a magnetic rack until the eluate becomes completely clear and colorless.
24. Carefully discard the supernatant without disturbing the beads.
25. While maintaining the tube on the magnetic rack, wash the beads with 180 μ L of 85% ethanol.
26. Keep the beads undisturbed throughout; carefully aspirate and discard the ethanol.
27. Ensure complete removal of any residual ethanol. Remove the tube from the magnetic rack, open the lid, and air-dry the pellet for approximately 30 sec.

△ **CRITICAL:** Avoid over-drying the beads. Excessive drying will severely compromise the final DNA recovery efficiency.

28. Add 22 μ L of NFW to the tube and thoroughly resuspend the beads. Incubate the mixture at room temperature (approximately 23°C) for 10 min on a rotator.
29. Place the tube on a magnetic rack for at least 1 min until the eluate becomes completely clear and colorless. Carefully transfer of the clear eluate into a new 1.5 mL microcentrifuge tube.

Note: QC: Quantify the DNA concentration using a Qubit fluorometer. Prepare a 20-fold dilution of the sample with NFW. Subsequently, add 2 μ L of the diluted DNA to 198 μ L of the Qubit 1 $\times$ dsDNA HS working solution.

30. Prepare the reaction by combining the following components:

Reagent	Amount
10 $\times$ Phi29 MAX DNA Polymerase Reaction Buffer	3 μ L
dNTPs	5.5 μ L
Phi29 MAX DNA Polymerase	1.5 μ L
DNA sample	20 μ L
Total	30 μ L

31. Mix gently by flicking the tube and spin down at 1000 $\times$ g for 3 sec. Incubate the reaction at 30°C for 4 h to allow for DNA extension.

32. Quantify the amplified sample using a Qubit fluorometer. Dilute 2 μ L of the DNA sample 20-fold with NFW, and add 2 μ L of this dilution to 198 μ L of Qubit 1 $\times$ dsDNA HS working solution.

Note: QC: Ensure the DNA concentration reaches ≥ 100 ng/ μ L for cell samples and ≥ 20 ng/ μ L for plasma samples before proceeding to heat inactivation. If the concentration is lower, extend the amplification time at 30°C for an additional 2–6 h (see [problem 4](#) and [potential solution](#) in [troubleshooting](#) section).

33. Heat the reaction to 65°C for 5 min to inactivate the Phi29 MAX DNA polymerase. Cool the reaction to 4°C. Store the DNA sample at –20°C or proceed directly to downstream applications.

Debranching of RCA products

⌚ Timing: 3 h

In this section, we use T7 Endonuclease I to process 2 μ g of the complex, hyper-branched structures generated by rolling circle amplification. Although pore clogging may still occur during subsequent sequencing, this enzymatic treatment significantly reduces pore clogging and extends the effective sequencing time. If the debranched sample yield is insufficient for multiple library loadings, we recommend processing an additional 2 μ g of the RCA product.

34. Transfer 2 μ g of the DNA sample into a clean 0.2 mL PCR tube and adjust the total volume to 25 μ L with NFW.
35. Prepare the reaction by combining the following components:

Reagent	Amount
DNA sample+ NFW	25 μ L
NEBuffer 2	3 μ L
T7 Endonuclease I	2 μ L
Total	30 μ L

36. Mix the contents gently by flicking the tube, followed by a brief centrifugation. Incubate the reaction mixture at 37°C for 2 h to facilitate DNA debranching.

Note: In our latest experiments, we used the Huh7 cell line to compare T7 Endonuclease I digestion for 2 h versus 15 min. Although the 2 h T7 Endonuclease I digestion produced shorter read lengths in the resulting library, it generated higher sequencing output and a lower pore loss rate under the same sequencing time and flow-cell pore count. See [Figures 2A–2F](#) as an example. Furthermore, following eccDNAs identification with ECCFP, extending the digestion time to 2 h enabled detection of more unique eccDNAs than the 15 min condition, with improved reproducibility across replicates. See [Figures 3A and 3B](#) as an example. Notably, eccDNAs identified after 2 h digestion showed a relatively shorter length distribution. See [Figures 3C and 3D](#) as an example.

37. Transfer the DNA sample from the previous step into a 1.5 mL tube. Add 0.4 $\times$ (12 μ L) of SPRIselect beads and incubate for 5 min at room temperature (approximately 23°C) on a rotator.
38. Centrifuge the tube at 1000 $\times g$ for 3 sec to bring down any liquid adhering to the cap or wall, then place it on a magnetic rack until the supernatant becomes completely clear and colorless. Maintain the tube on the magnetic rack and carefully remove the clear supernatant.

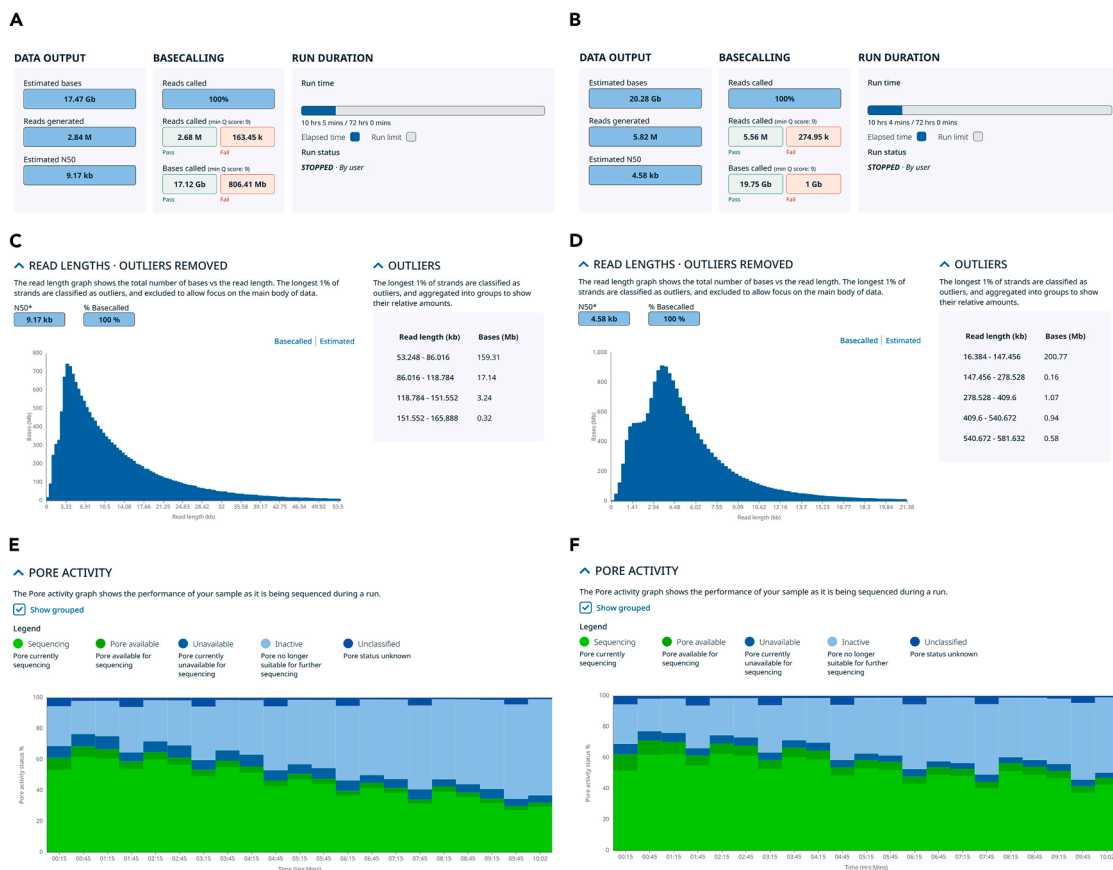


Figure 2. Comparison of sequencing performance by T7 digestion

Representative screenshots of ONT sequencing summary metrics from libraries prepared with 15 min T7 digestion (A, C, and E) or 2 h T7 digestion (B, D, and F), compared at the same sequencing time and flow-cell pore count.

(A and B) Sequencing output.

(C and D) Read length distribution.

(E and F) Pore loss rate.

39. While maintaining the tube on the magnetic rack, wash the beads with 180 μ L of 85% ethanol. Carefully aspirate and discard the ethanol.
40. Carefully aspirate any residual ethanol. Remove the tube from the magnetic rack, open the lid, and air-dry the pellet for approximately 30 sec.
41. Add 24 μ L of NFW to the tube and thoroughly resuspend the beads. Incubate the mixture for 10 min at room temperature (approximately 23°C) on a rotator.
42. Place the tube on a magnetic rack for at least 1 min until the eluate becomes completely clear and colorless. Carefully transfer the clear eluate into a new 1.5 mL microcentrifuge tube.
43. QC:
 - a. Quantify the DNA concentration using a Qubit fluorometer. Add 1 μ L of the DNA sample to 199 μ L of the Qubit 1 $\times$ dsDNA HS working solution.

CRITICAL: A minimum concentration of 25 ng/ μ L for the debranched DNA sample is highly recommended to enable dynamic adjustment of the library input.

- b. Assess the fragment size distribution of the T7-digested RCA product.

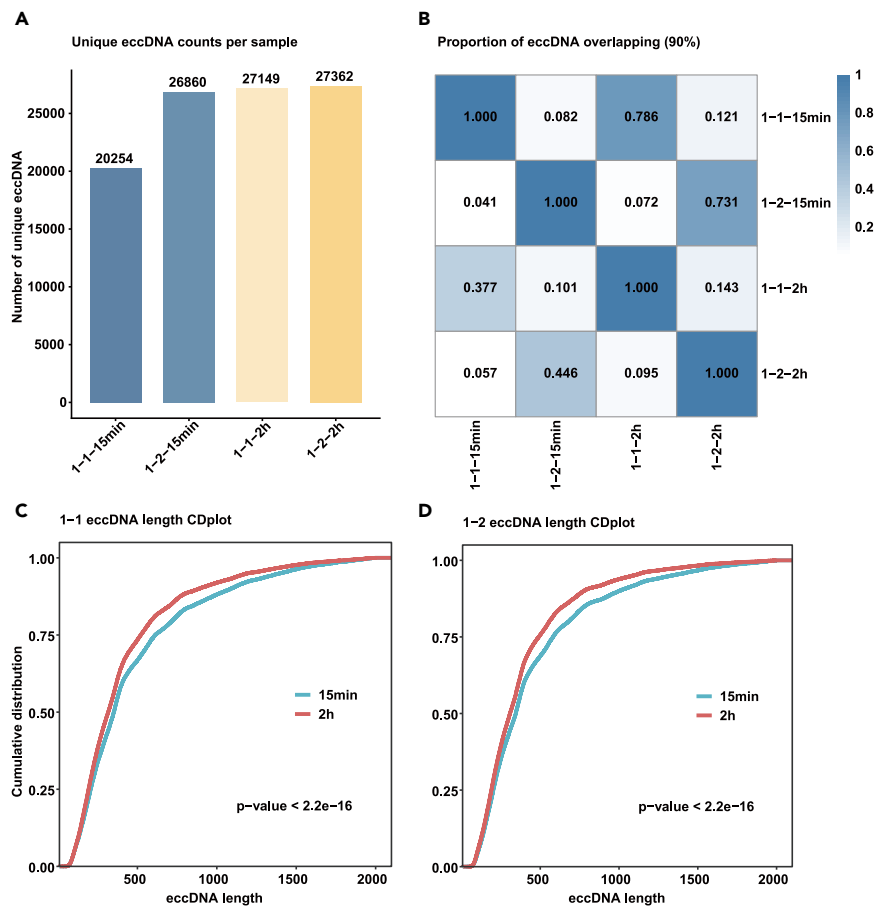


Figure 3. Effect of T7 digestion time on eccDNA identification

(A) Bar plot showing the number of unique eccDNAs identified after 15 min and 2 h T7 digestion.

(B) Heatmap showing the overlap of identified eccDNAs across replicates from the same sample under the two digestion conditions, using a 90% threshold.

(C and D) CD plots showing the length distribution of eccDNAs identified from the same sample after 15 min and 2 h T7 digestion.

⚠ **CRITICAL:** Sample fragment size typically ranges from 2.5 to 8 kb. While fragment length does not compromise library preparation and sequencing, an abundance of short fragments leads to a higher frequency of reads with full-pass ≤ 2 , ultimately decreasing the proportion of reads for eccDNA identification.

Nanopore sequencing library preparation and sequencing

⌚ **Timing:** 2–3 days

In this section, we based our library preparation approach on the official Oxford Nanopore Technologies (ONT) gDNA library preparation protocol to accommodate multiplexed eccDNA sequencing. We used the latest SQK-NBD114.24 kit in combination with PromethION sequencing flow cells R10.4.1. Alternative loading mass can also be used by adjusting the library loading amount accordingly.

44. Dilute 400 ng of DNA per sample to a final volume of 48 μ L with NFW, and proceed with DNA repair and end-preparation for each sample individually.

45. In a 0.2 mL thin-walled PCR tube, mix the following reagents:

Reagent	Amount
DNA sample+ NFW	48 μ L
NEBNext FFPE DNA Repair Buffer	3.5 μ L
NEBNext FFPE DNA Repair Mix	2 μ L
Ultra II End-prep reaction Buffer	3.5 μ L
Ultra II End-prep enzyme mix	3 μ L
Total	60 μ L

46. Mix gently by flicking the tube and centrifuge at 1000 $\times$ g for 3 sec. Incubate the reaction in a thermal cycler at 20°C for 5 min, followed by 65°C for 5 min.
47. Transfer the DNA sample from the previous step into a 1.5 mL low-bind microcentrifuge tube. Add 1 $\times$ (60 μ L) of AMPure XP beads (Native Barcoding Kit 24 V14) and incubate for 5 min at room temperature.

Note: AMPure XP beads must be equilibrated to room temperature (approximately 23°C) for 30 min and vortexed thoroughly prior to use.

⚠ **CRITICAL:** To ensure the beads remain fully resuspended throughout the incubation, gently flick the tube every minute or place it on a rotator mixer.

48. Centrifuge the tube at 1000 $\times$ g for 3 sec to collect the liquid, then place it on a magnetic rack until the supernatant becomes completely clear and colorless. Maintain the tube on the magnetic rack and carefully remove the clear supernatant.

Note: Do not leave the sample on the magnetic rack for more than 2 min.

⚠ **CRITICAL:** Take extreme care to avoid disturbing the bead with the pipette tip during aspiration.

49. While maintaining the tube on the magnetic rack, wash the beads with 200 μ L of 80% ethanol. Carefully aspirate and discard the ethanol.
50. Repeat the previous step.
51. Carefully aspirate any residual ethanol. Remove the tube from the magnetic rack, open the lid, and air-dry the pellet for approximately 30 sec.

⚠ **CRITICAL:** Avoid over-drying the beads. The pellet should be dried just until its surface transitions from glossy to matte, strictly stopping before any cracks appear. Excessive drying will severely compromise the final DNA recovery efficiency.

Note: A brief centrifugation followed by reapplication to the magnet can be utilized to accelerate residual liquid removal. However, to prevent prolonged magnetic exposure that could magnetize the beads and hinder DNA elution, the primary air-drying step must be performed with the tube removed from the magnet.

52. Add 25 μ L of NFW to the tube and thoroughly resuspend the beads. Incubate the mixture for 10 min at room temperature (approximately 23°C).

⚠ **CRITICAL:** To ensure the beads remain fully resuspended throughout the incubation, gently flick the tube every minute or place it on a rotator mixer.

53. Place the tube on a magnetic rack for at least 1 min until the eluate becomes completely clear and colorless. Carefully transfer the clear eluate into a new 1.5 mL microcentrifuge tube.

54. **QC:** Quantify the DNA concentration using a Qubit fluorometer. Dilute 2 μL of the DNA sample 10-fold, and add 2 μL of the diluted sample to 198 μL of Qubit 1 $\times$ dsDNA HS working solution.
55. Select a unique barcode for each sample to be run together on the same flow cell. Add the following components to each sample according to the reaction setup below:

Reagent	Amount
DNA retained from purification	22.5 μL
Native Barcode	2.5 μL
Blunt/TA Ligase Master Mix	25 μL
Total	50 μL

56. Mix gently by flicking the tube, and spin down at 1000 $\times$ g for 3 sec. Incubate the reaction for 20 min at room temperature.

Note: It is recommended to perform this incubation in a thermal cycler set to 23°C.

57. Add 1 $\times$ (50 μL) of resuspended AMPure XP beads to the reaction and mix well by pipetting. Incubate for 5 min at room temperature (approximately 23°C).

△ CRITICAL: To ensure the beads remain fully resuspended throughout the incubation, gently flick the tube every minute or place it on a rotator mixer.

58. Centrifuge the tube at 1000 $\times$ g for 3 sec to collect the liquid, then place it on a magnetic rack until the supernatant becomes completely clear and colorless. Maintain the tube on the magnetic rack and carefully remove the clear supernatant.

Note: Do not leave the sample on the magnetic rack for more than 2 min.

△ CRITICAL: Take extreme care to avoid disturbing the bead with the pipette tip during aspiration.

59. While maintaining the tube on the magnetic rack, wash the beads with 200 μL of 80% ethanol.
60. Repeat the previous step.
61. Carefully aspirate any residual wash solution. Remove the tube from the magnetic rack, open the lid, and air-dry the pellet for approximately 30 sec.
62. Add 27 μL of NFW and thoroughly resuspend the beads. Incubate the mixture at 37°C for 10 min, gently flicking the tube every minute to ensure efficient elution.
63. Place the tube on a magnetic rack for at least 1 min until the eluate becomes completely clear and colorless. Carefully transfer the clear eluate into a new 1.5 mL microcentrifuge tube.
64. **QC:** Quantify the DNA concentration using a Qubit fluorometer. Dilute 2 μL of the DNA sample 10-fold, and add 2 μL of the diluted sample to 198 μL of Qubit 1 $\times$ dsDNA HS working solution.

Note: Depending on the number of samples to be sequenced, pool equimolar amounts of each barcoded sample into a 1.5 mL tube. For DNA fragments 3–10 kb in length, a total input of 400 ng is recommended; amounts ranging from approximately 75 to 150 fmol are completely viable. The final volume must remain under 65 μL . If the volume exceeds 65 μL , concentrate the mixture using a 2.5 $\times$ bead purification. Prepare one pooled library per sequencing run. For multiple sequencings, you can dynamically adjust the input amount of individual barcodes based on their respective sequencing yields (see [problem 5](#) and [potential solution](#) in the [troubleshooting](#) section).

65. Perform adapter ligation on the pooled barcoded sample as follows:

Reagent	Amount
The pooled barcoded sample	65 μ L
Native Adapter (NA)	5 μ L
NEBNext Quick Ligation Reaction Buffer (5 $\times$)	20 μ L
Quick T4 DNA Ligase	10 μ L
Total	100 μ L

Note: Pipette the Quick T4 DNA Ligase up and down only once before aspirating. Avoid excessive pipetting to prevent enzyme inactivation.

66. Mix gently by flicking the tube, and spin down at 1000 $\times$ g for 3 sec. Incubate the reaction for 20 min at room temperature.

Note: It is recommended to perform this incubation in a thermal cycler set to 23°C.

67. Add 0.5 $\times$ (50 μ L) AMPure XP beads to the reaction and mix well by pipetting. Incubate for 10 min at room temperature (approximately 23°C).

△ CRITICAL: To ensure the beads remain fully resuspended throughout the incubation, gently flick the tube every minute or place it on a rotator mixer.

68. Centrifuge the tube at 1000 $\times$ g for 3 sec to collect the liquid, then place it on a magnetic rack until the supernatant becomes completely clear and colorless. Maintain the tube on the magnetic rack and carefully remove the clear supernatant.

69. Add 250 μ L of Short Fragment Buffer (SFB) (Native Barcoding Kit 24 V14) to wash the AMPure XP beads. Resuspend by gently flicking the tube, followed by a quick spin down. Place the tube back onto the magnetic rack until the beads pellet, and carefully discard the clear supernatant.

70. Repeat the previous step.

71. Carefully aspirate any residual ethanol. Remove the tube from the magnetic rack, open the lid, and air-dry the pellet for approximately 30 sec. Avoid over-drying the AMPure XP beads.

72. Add 35 μ L Elution Buffer (EB) to the tube and thoroughly resuspend the beads. Incubate the mixture for 15 min.

△ CRITICAL: It is recommended to incubate the reaction at 37°C for 10 min using a heating block, gently flicking the tube every minute to mix.

73. Place the tube on a magnetic rack for at least 1 min until the eluate becomes completely clear and colorless. Carefully transfer the clear eluate into a new 1.5 mL microcentrifuge tube.

74. **QC:** Quantify the DNA concentration using a Qubit fluorometer. Add 1 μ L of the sample to 199 μ L of Qubit 1 $\times$ dsDNA HS working solution.

Note: The official protocol recommends a library input of 35 to 50 fmol. However, for our eccDNA libraries, an input mass between 150 and 300 ng provides sufficient sequencing throughput.

75. Perform nanopore long-read sequencing according to [the manufacturer instructions](#) using an appropriate flow cell, such as the PromethION sequencing flow cells R10.4.1 (DNA).

Identification of eccDNA

⌚ Timing: >1 day

In this section, we use EccDNA Caller based on Consecutive Full Pass (ECCFP), a novel bioinformatics pipeline developed by our group.¹ ECCFP is fully open-source and, compared with existing pipelines that identify small eccDNA molecules from long-read sequencing data based on RCA amplification, it combines the strengths of these approaches while being specifically optimized for RCA-derived data, thereby improving sensitivity, accuracy, and computational efficiency. For the detailed eccDNA bioinformatic analysis workflow, please refer to our previously published protocol.¹²

EXPECTED OUTCOMES

This workflow is designed to enable robust detection of eccDNA through standardized sample preparation and library construction procedures, and it is applicable to a variety of sample types. We used the human hepatocellular carcinoma cell line HepG2 and one pooled plasma sample for eccDNA detection and performed eccDNA identification using our published ECCFP identification pipeline. Across multiple independent experiments, we consistently generated high-quality libraries containing a sufficient number of ≥ 2 full-pass reads, which supports eccDNA identification and downstream analyses. For more specific expected results and performance metrics, please refer to the original text.¹ The results for the plasma samples are shown in [Figure 4](#).

LIMITATIONS

This workflow was validated using the HepG2 cell line and one plasma sample, and is generally applicable to most cell lines and plasma cfDNA samples. However, for cell lines and plasma cfDNA samples, performance may vary due to differences in starting material, including input amount, DNA quality, and fragment characteristics. Therefore, we recommend conducting preliminary experiments and optimizing conditions for key steps prior to application, especially by dynamically adjusting the QC and loading amount according to the starting material. In addition, eccDNA abundance changes dynamically across the cell cycle and in response to environmental stimuli; thus, inconsistent cellular states or stimulation backgrounds at the time of sample collection may reduce inter-sample reproducibility and comparability.

TROUBLESHOOTING

Problem 1

When applying this workflow to different sample types, differences in starting material often necessitate adjustments to the extraction strategy, as well as optimization of input amount and QC loading. In addition, eccDNA abundance dynamically changes across the cell cycle and in response to environmental stimuli. Therefore, even when all downstream steps are performed identically, inter-sample variability may still occur in terms of sequencing yield, background signal proportion, and the number of eccDNAs identified by ECCFP, ultimately affecting the robustness and reproducibility of the conclusions.

Potential solution

- We recommend performing small-scale pilot experiments prior to full runs for each sample category (cfDNA/cell line) and implementing dynamic standardization based on the amount and quality of starting DNA.
- Although this workflow is generally applicable to most cell lines, samples with extremely high or extremely low eccDNA abundance may require further tuning of the starting cell number or DNA input to achieve an optimal enrichment balance. During cell culture and early sample handling, gentle processing is also recommended to minimize unintended stimuli.

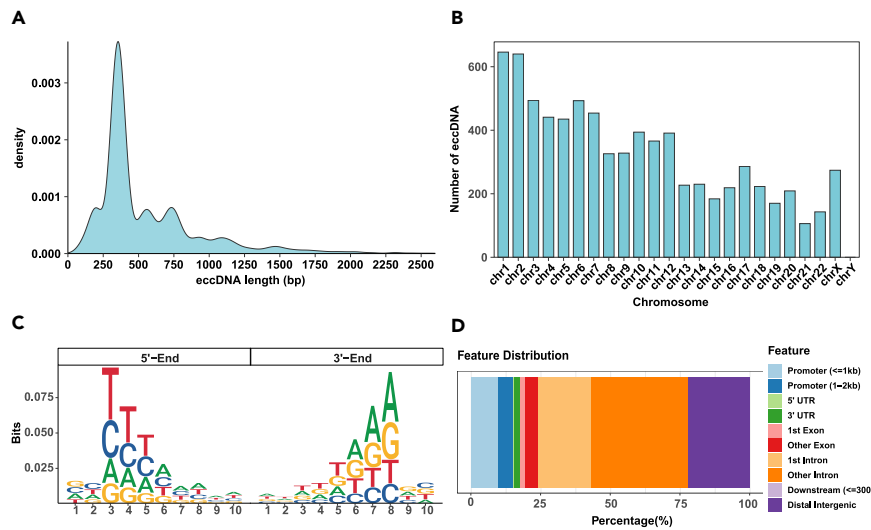


Figure 4. Characterization of eccDNA identified from plasma cfDNA

(A) Length distribution of identified eccDNAs.
(B) Chromosomal distribution of eccDNA counts.
(C) Sequence motif flanking eccDNA junction sites.
(D) Genomic feature annotation of eccDNAs.

- For cfDNA, we suggest optimizing using an initial input of 25 ng, setting the QC concentration to 20% of the corresponding cell-line level. When the input is limiting, the RCA incubation time can be extended to meet downstream library preparation requirements (RCA may be extended to 10–12 h).

Problem 2

This protocol demonstrates cfDNA extraction using a 1 mL plasma sample, but the kit is compatible with 1–5 mL plasma samples.

Potential solution

- When using plasma volumes other than 1 mL, the volumes of proteinase K, sample lysis buffer, binding buffer, magnetic beads, and elution buffer should be adjusted proportionally according to the plasma input volume.
- The wash steps remain unchanged
- Note that larger plasma inputs increase the total binding volume. Ensure that an appropriate tube size is used and that the magnetic beads are fully resuspended before binding and washing.

Problem 3

When using different sample types, the relative abundance of linear DNA and mtDNA may vary substantially. Therefore, a single round of digestion may not be sufficient to achieve efficient depletion across all samples.

Potential solution

If post-digestion qPCR QC indicates that linear DNA or mtDNA remains at a high level, we recommend performing 1–2 additional rounds of repeat digestion after purification to further improve depletion efficiency until the desired criteria are met. It is important to note that each additional purification/digestion cycle inevitably results in some DNA loss, which can reduce the final DNA yield and affect downstream library input. Accordingly, repeat-digestion decisions should be made in a QC-guided, stepwise manner to avoid unnecessary over-processing.

Problem 4

During RCA and Debranching, the DNA concentration may not reach the expected level.

Potential solution

- Because the amount of starting template can vary across different samples, amplification efficiency may also differ, leading to insufficient product accumulation. To avoid prematurely terminating the reaction, it is recommended not to inactivate the reaction during RCA. Instead, continue RCA until the product concentration reaches the predetermined target level, and only then perform inactivation.
- If the concentration has not reached the target, you can extend the RCA incubation by 2 h, followed by re-measuring the concentration, and repeat this step as needed until the desired concentration is achieved.

In some cases, however, extending amplification to 16 h may still not yield sufficient material for library construction. This may indicate that template material was lost during earlier extraction or purification, resulting in an insufficient amount of usable template. If this occurs, re-evaluate the input, purification recovery, and prior QC results, and adjust the sample-preparation workflow accordingly. Consider preparing a new sample.

Problem 5

Although the library input is distributed evenly across samples prior to loading based on the total concentration, uneven representation among different barcodes may still occur.

Potential solution

- After barcode ligation and purification, measure the concentration for each barcode/sample, and then pool the entire library to ensure as uniform a distribution as possible.
- If you plan to load the same sample multiple times to increase total data yield, you can further optimize the strategy by dynamically adjusting the loading amount for subsequent runs based on the barcode distribution observed in the first sequencing run.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be answered by the lead contact, Shaogui Wan (wansg@gmu.edu.cn).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Biyuan Miao (miaobiuyan666@163.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The sequencing data generated for this protocol, including four Huh7 cell line samples and one pooled plasma sample, have been deposited at the National Genomics Data Center under accession number PRJCA069021.
- The previously published HepG2 dataset used in this protocol is available at the National Genomics Data Center under accession number PRJCA052047.
- This protocol did not generate new code.

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AUTHOR CONTRIBUTIONS

B.M., P.X., and S.W. conceptualized the study; B.M., X.H., H.C., W.L., Q.Z., and H.L. conducted methodology and protocol optimization; B.M. drafted the original manuscript; S.W., J.G., and P.X. reviewed and edited the manuscript; all authors approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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