Chapter 2

END-seq: An Unbiased, High-Resolution, and Genome-Wide Approach to Map DNA Double-Strand Breaks and Resection in Human Cells

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Abstract

DNA double-strand breaks (DSBs) represent the most toxic form of DNA damage and can arise in either physiological or pathological conditions. If left unrepaired, these DSBs can lead to genome instability which serves as a major driver to tumorigenesis and other pathologies. Consequently, localizing DSBs and understanding the dynamics of break formation and the repair process are of great interest for dissecting underlying mechanisms and in the development of targeted therapies. Here, we describe END-seq, a highly sensitive next-generation sequencing technique for quantitatively mapping DNA double-strand breaks (DSB) at nucleotide resolution across the genome in an unbiased manner. END-seq is based on the direct ligation of a sequencing adapter to the ends of DSBs and provides information about DNA processing (end resection) at DSBs, a critical determinant in the selection of repair pathways. The absence of cell fixation and the use of agarose for embedding cells and exonucleases for blunting the ends of DSBs are key advances that contribute to the technique's increased sensitivity and robustness over previously established methods. Overall, END-seq has provided a major technical advance for mapping DSBs and has also helped inform the biology of complex biological processes including genome organization, replication fork collapse and chromosome fragility, off-target identification of RAG recombinase and gene-editing nucleases, and DNA end resection at sites of DSBs.

Key words DNA double-strand breaks (DSBs), DNA damage, DNA repair, DSB mapping and quantification, Next-generation sequencing, Nucleotide resolution, End resection, Adapter ligation, Exonucleases, Agarose plugs

1 Introduction

Central to a cells ability to maintain genome stability are systems that monitor and repair DSBs. DSBs have been shown to occur in response to exogenous insults such as exposure to irradiation, chemotherapeutic and gene-editing agents, and from physiological processes such as replication, transcription, meiotic recombination in germ cells, and antigen receptor rearrangements in lymphocytes. If not rapidly and faithfully repaired, DSBs can serve as substrates

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for aberrant chromosomal rearrangements, which, in turn, can promote cancer and other pathologies.

1.1 Current and Historical Approaches to Map DSBs

A major limitation of current cytological and genomic approaches that assess DNA damage is their inability to map the chromosomal locations of DSBs and the resulting structures at breaks with precision and detail. Identifying the exact location of DSBs in the genome will greatly expand our understanding of the mechanism of their formation and will additionally allow for the monitoring of the kinetics of DNA processing and repair at DSBs. Precise DSB mapping will also allow for the integration of genome context (i.e., DNA composition, chromatin modifications, and processes like transcription and replication) in assessing their influence on the formation and repair of DSBs.

In recent years, the need for mapping and quantifying on- and off-target specificities of DSB-generating gene-editing nucleases in combination with the advances in next-generation sequencing has helped accelerate the development of genome-wide techniques to map DSBs. Many of these approaches are based on indirect readouts of DSB formation such as chromatin immunoprecipitation of DNA repair proteins (e.g., H2AX, SMC5, RPA, and other proteins [1–4]) or by detecting the outcome of improper DSB repair such as chromosomal translocations [5] or via the incorporation of an exogenously provided DNA repair sequence [6, 7]. Collectively, these outputs serve as surrogate marks for the site of a DSB. Each of these indirect approaches is not only unable to map the exact position of DSBs directly with sensitivity and robustness but also fails to provide features regarding the structure and processing of damaged DNA ends. These methodological limitations have prompted us to develop END-seq—a genome-wide, high-resolution, direct and quantitative readout for measuring DSBs in an unbiased manner that provides technological resolution to each of these major inadequacies. BLESS, and its recent modification BLISS, also map broken DNA ends in an unbiased genome-wide manner [8, 9]. These techniques are, however, associated with additional noise, primarily caused by the use of formaldehyde as a fixative. Importantly, neither BLESS/BLISS provides information about end structures at DSBs. END-seq, therefore, provides a major advance in the detection of double-strand breaks.

1.2 Overview of the END-seq Procedure

Briefly, in END-seq, native and unfixed human or mouse cells are embedded in agarose (Fig. 1). This critical step avoids the artificial generation of DSBs from mechanical processing and fixation of samples. After protein and RNA digestion, the DNA ends of DSBs are blunted with single-strand DNA-specific exonucleases, A-tailed (i.e., one “A” is added in a nontemplated manner to each blunted DNA end), and ligated within agarose plugs to a T-tailed DNA adapter which is labeled with biotin and compatible with

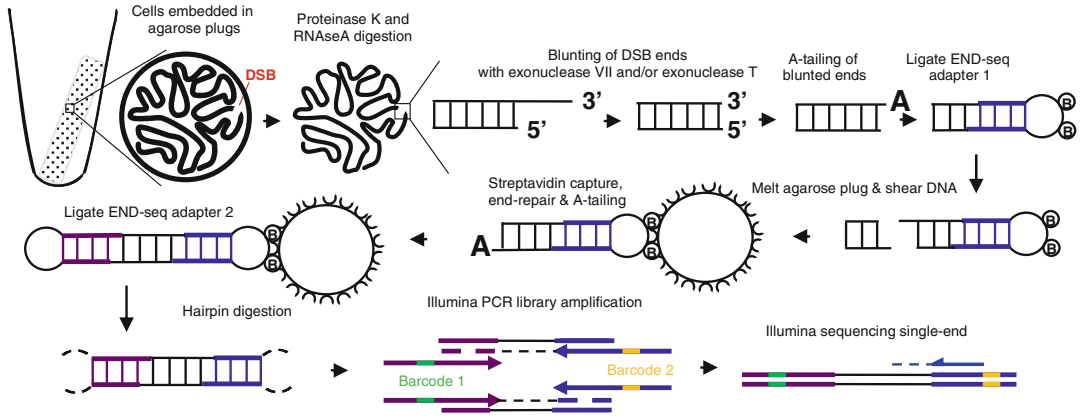

Fig. 1 Detailed schema of the END-seq methodology. Live cells are embedded in low-melting agarose following which agarose plugs are treated with proteinase K and RNase A to digest proteins and RNA, respectively. After blunting the single-strand DNA overhangs at DSBs with exonucleases (exonuclease VII and exonuclease T), A-tailing of the 3′ ends allows the ligation of a biotinylated hairpin adapter containing a 3′ T overhang and the Illumina's p5 adapter sequence ("END-seq adapter 1") within the plug. The agarose plug is then melted and DNA is extracted and sheared by sonication. Sheared fragments containing END-seq adapter 1 (and the DSB end) are captured with streptavidin-coated beads. The new ends created by sonication are end repaired and A-tailed, allowing ligation of a second hairpin adaptor containing Illumina's p7 sequence (named "END-seq adapter 2"). Adapter hairpins are digested away with the USER enzyme (a combination of an uracil DNA-glycosylase and endonuclease VIII) which digests DNA to create single-nucleotide gaps at uracil residues. PCR amplification using Illumina TruSeq primers with barcodes (denoted in yellow and green) result in a ready-to-use library in which the first base sequenced (read number 1) corresponds to the first base of the blunted DSB. (Figure has been adapted from [11])

Illumina sequencing. Next, high-molecular-weight DNA is extracted from the agarose plugs and fragmented by sonication. Subsequent purification of the biotinylated ends and ligation of a second adapter followed by PCR amplification result in a ready-to-sequence library in which the first base sequenced corresponds precisely to the first base of the blunted DSB. Furthermore, the number of reads mapping to the DSB is proportional to the frequency of DSBs in the cell population, which allows for the quantification of each DSB.

One of the initial steps of DSB repair by homologous recombination (HR) is the generation of 3′ single-stranded DNA (ssDNA) via the 5′ to 3′ nucleolytic degradation of broken ends—a process termed DNA end resection [10]. DNA end resection inhibits an error-prone repair pathway called nonhomologous end joining and initiates repair using the high-fidelity homologous recombination (HR) pathway by recruiting the RAD51 recombinase that promotes strand invasion to complete HR. Since the blunting step in the END-seq protocol removes single-stranded overhangs and sequencing initiates at the first nucleotide in blunted double-stranded DNA, the resected interval can be calculated as the distance from the sequencing start site to the original position of the

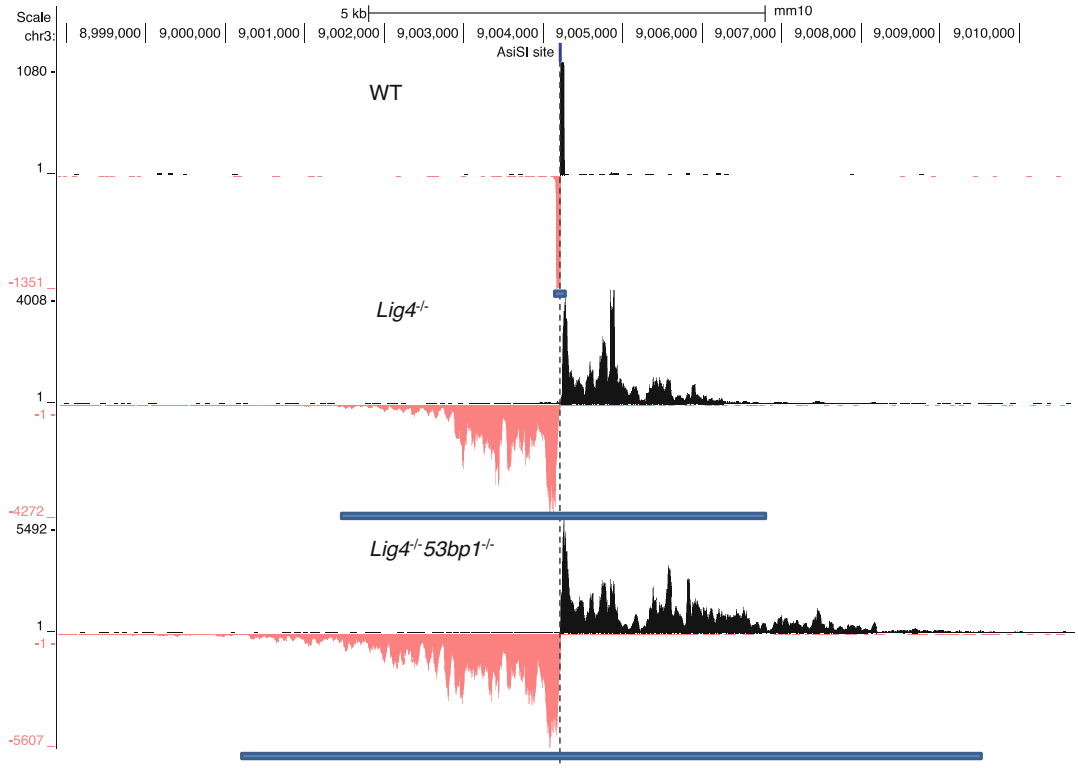

Fig. 2 Screen shot: a visual example of resection and DNA processing at a single DSB. A genome browser screen shot of one DSB in the murine genome located at chr3: chr3:9,004,213-9,004,220. The site of the break is highlighted with a vertical blue bar above the top track. The *y*-axis represents the total number of accumulated reads at the AsiSI site depicted in WT, *Lig4*$^{-/-}$ and *Lig4*$^{-/-}$*53bp1*$^{-/-}$ pre-B-cell lines. *Lig4*$^{-/-}$ are repair-deficient cells that allow for DSB persistence and resection while the combined ablation of LIG4 and the pro-NHEJ factor 53BP1 results in hyperresection. The accumulation of reads away from the DSB indicates end resection. Read coverage is colored in black or red for plus and minus strand alignments, respectively. Horizontal blue bars at the bottom of each track represent resected regions in the vicinity of the DSB

DSB. Given that each cell undergoes resection to differing extents, END-seq reveals a resection distribution profile of ends from the original site of the DSB (i.e., non resected) to several kilobases on each side of the break (i.e., resected) (Fig. 2).

1.3 Normalization Using Spike-Ins

Normalization of END-seq data is crucial to accurately compare the frequency of DSBs between samples. Although the same number of cells is used in all samples, normalization of DSBs by the total number of sequenced reads can lead to misleading interpretations. Simple read counts are unable to correct global changes in DSBs caused by specific experimental conditions (e.g., drug treatments, genotypes, time points) or even noise from apoptotic cells. Accurate comparison between samples, therefore, requires the use of an exogenous spike-in control. The spike-in control, used in the

END-seq procedure, are cells where a single, unique DSB can be induced by a zinc-finger nuclease in a repair-deficient cell line. This site has been shown to be broken in 100% of induced spike-in cells. Consequently, any differences in the total amount of reads at the spike-in DSB between samples can be used as a normalization control. In addition, the spike-in normalization allows for the estimation of the frequency of cells harboring any given DSB across the entire genome. For a 5% spike-in, the read counts at the unique zinc finger nuclease–induced DSB signifies a frequency of 5% of cells carrying a DSB in the total cellular population. Spike-in normalization, therefore, allows for the calculation of break frequency and enables quantitative comparisons between different samples in the experiment.

1.4 Applications and Uses of END-seq

END-seq has been successfully applied to detect and quantify DSBs produced by restriction endonucleases in vivo, genome editing nucleases, recombination-activating genes (RAGs) during VDJ recombination [11], activation-induced cytidine deaminase (AID) during class switch recombination, topoisomerase-2 induced breaks [12, 13], and at collapsed replication forks upon replicative stress [14]. DSB hotspots and resection during meiotic recombination may also be detected. END-seq has been effectively implemented in both murine and human cells across numerous genotypes and treatment conditions and represents a tool with great versatility and flexibility.

1.5 Sensitivity, Limitations, and Caveats

The ability to detect a DSB (i.e., sensitivity) using END-seq depends primarily on two factors: (1) the frequency of DSBs and (2) the spatial distribution at any given DSB in the pool of cells in a sample. Simply put, a higher signal (read count) will be detected by END-seq if more cells in the population have a DSB (i.e., high frequency) and the breaks at a specified location are proximal to each other between individual cells. We estimate that detection of a single DSB by END-seq necessitates that a break occurs at least in 1 in every 10,000 cells [11]. If DSBs are not coincident in their position between cells, they are not detected by END-seq as they will be indistinguishable from background. In other words, a major END-seq requirement for DSB detection is that the break sites be recurrent. Random DSBs, such as those generated after irradiation, cannot be detected by END-seq and their presence in the sample is reflected in increased noise which decreases the overall sensitivity of the method. It is worth stating that DNA repair–deficient genotypes increase END-seq sensitivity because it prolongs the time DSBs stay unrepaired.

2 Materials

2.1 Common Reagents and Instruments

1. Two water baths.
2. Eppendorf Thermomixer C fitted with 50 mL and 1.5 mL adapters or equivalent.
3. Wide-bore pipette filtered tips, 200 μL, VWR, Catalog # 46620-642 or equivalent.
4. Orbital platform shaker that can reach at least 180 rpm, FINEPCR shaker model SH30 or equivalent.

2.2 Embedding Cells in Agarose Plugs

1. Metal block from a dry bath or a thermoblock.
2. Puregene Proteinase K enzyme (Qiagen Catalog # 158920, 5 mL).
3. Plug mold, cell suspension buffer, and 2% agarose from Bio-Rad CHEF Mammalian Genomic DNA Plug Kit (Catalog # 170-3591). Prepare aliquots of the agarose included in the kit by melting the entire bottle in boiling water for 15 min and distributing the melted agarose into 500 μL aliquots in 1.5 mL tubes. Aliquots can be stored at 4 °C. Use one or more 500 μL aliquots, as needed, for each new procedure.
4. Lysis buffer (10 mM Tris–HCl pH 8, 50 mM EDTA, 150 mM NaCl, 1% SDS in nuclease free water. The lysis buffer should be stored at room temperature to avoid SDS precipitation.)

2.3 RNAse Treatment of Plugs

1. Plug wash buffer (10 mM Tris, pH 8.0, 50 mM EDTA, in nuclease free water).
2. Bio-Rad screened cap, Catalog # 1703711.
3. Puregene RNAse A solution, 5 mL, Qiagen, Catalog # 158924.
4. TE buffer (10 mM Tris, pH 8, 1 mM EDTA, in nuclease-free water).

2.4 Blunting of DSBs Ends, A-Tailing, and Ligation of END-Seq Adapter 1

1. EB buffer (10 mM Tris, pH 8.0, in nuclease-free water).
2. NEB Exonuclease VII and 5× Exonuclease VII buffer, Catalog # M0379L, 10,000 units/mL.
3. Homemade 5× Exonuclease VII buffer (250 mM Tris–HCl (pH 8.0), 250 mM sodium phosphate (pH 8.0), 50 mM 2-mercaptoethanol, 40 mM EDTA, pH 8 at 25 °C). Store in 1.8 mL aliquots at −20 °C.
4. NEB Exonuclease T, Catalog # M0265L, 5000 units/mL.
5. Rotatory mixer for 1.5 mL tubes, Labnet mini-labroller rotator H5500.

Table 1
Sequence of adapters and primers used in the END-seq protocol

Adapter or primer name	Sequence 5′–3′
END-seq adapter 1	Phosphate GATCGGAAGAGCGTCGTGTAGGGAAAGAGTGUU biotin-dTU biotin-dTUUACAC TCTTTCCCTACACGACGCTCTTCCGATC∗T
END-seq adapter 2	Phosphate GATCGGAAGAGCACACGTCUU UUUUUUAGACGTGTGCTCTTCCGATC∗T
TruSeq barcoded primer p5	AATGATACGGCGACCACCGAGATCTACACN NNNNNNNACACTCTTTCCCTACACGACGCT CTTCCGATC∗T
TruSeq barcoded primer p7	CAAGCAGAAGACGGCATACGAGANNNNN NNNGTGACTGGAGTTCAGACGTGTGCTCTTC CGATC∗T

See **Note 14** for adapter annealing
NNNNNNNN represents 8 nucleotide barcodes, following Illumina's recommendations (https://support.illumina.com/downloads/index-adapters-pooling-guide-1000000041074.html). "∗" denotes a phosphothiorate bond

6. NEBuffer 4, NEB Catalog # B7004S (also used in Subheading 2.6).
7. NEB Klenow Fragment (3′ → 5′ exo-), Catalog # M0212L, 5000 units/mL (also used in **step 2.6**).
8. NEBNext dA-Tailing Reaction Buffer, alternatively NEBuffer 2 supplemented with 0.2 mM of dATP (1×) (also used in Subheading 2.6).
9. NEB Quick Ligation Kit includes Quick Ligase (2000 U/μL) and 2× Quick Ligase Buffer, Catalog # M2200L (also used in Subheading 2.6).
10. END-seq adapter 1 (*see* Table 1 and note indicated for adapter annealing).
11. NEBuffer 2, Catalog # B7002S (also used in Subheading 2.6).

2.5 DNA Sonication and Shearing

1. Covaris S2 or S220 focused ultrasonicator or alternatively any sonicator (e.g., Bioruptor Plus, Diagenode, Catalog # B01020001) that gives a narrow size distribution of DNA fragments around 175 bp.
2. Covaris Holder microTUBE, Catalog # 500114.
3. NEB β-Agarase I, Catalog # M0392L, 1000 units/mL.
4. Dialysis membrane filter, MF, 0.1 μm, Millipore, Catalog # VCWP04700.
5. SDS 10% Solution, Ambion, Catalog # AM9823.
6. Covaris microTUBE AFA Fiber Pre-Slit Snap-Cap 6 × 16 mm, Catalog # 520045.
7. Sodium acetate (3 M), pH 5.5 Ambion, Catalog # AM9740.

8. Glycogen from mussels, 20 mg/mL, Roche, Catalog # 10901393001 or equivalent.
9. Nanodrop spectrophotometer or equivalent.

2.6 End-Repair, A-Tailing, and Ligation of END-Seq Adapter 2

1. ThermoFisher DynaMag-2 Magnet Catalog # 12321D or equivalent.
2. PCR machine, Bio-Rad T100 or equivalent.
3. END-seq adapter 2 (*see* Table 1 and Note indicated for adapter annealing).
4. Dynabeads MyOne Streptavidin C1 Catalog # 65002.
5. Dynabeads binding buffer 2× (10 mM Tris–HCl, 1 mM EDTA, 2 M NaCl, pH 7.5 in nuclease-free water).
6. Dynabeads wash buffer 1× (5 mM Tris–HCl, 0.5 mM EDTA, 1 M NaCl, pH 7.5 in nuclease-free water).
7. dNTP 100 mM Set Bioline Catalog # 39025 (make 10 mM mix combining dATP, dTTP, dCTP, dGTP).
8. NEB T4 ligase buffer 10×, Catalog # B0202S.
9. NEB T4 Polynucleotide Kinase, Catalog # M0201L, 10,000 units/mL.
10. NEB T4 DNA Polymerase, Catalog # M0203L, 3000 units/mL.
11. NEB DNA Polymerase I, Large (Klenow) Fragment, Catalog # M0210L, 5000 units/mL.
12. NEB USER Enzyme, Catalog # M5505L, 50 units, 1000 units/mL.
13. KAPA HiFi HotStart ReadyMix, Catalog # KK2601.
14. Illumina Truseq 8 nucleotide barcoded primer p5 (Table 1).
15. Illumina Truseq 8 nucleotide barcoded primer p7 (Table 1).

2.7 PCR Product Cleanup, Gel Purification, qPCR, and Sequencing

1. ThermoFisher DynaMag-PCR Magnet Catalog # 492025 or equivalent.
2. DNA gel electrophoresis system.
3. Standard blue LED transilluminator.
4. Agencourt AMPure XP beads, Catalog # A63880 or equivalent.
5. QIAquick Gel Extraction Kit, Catalog # 28706.
6. KAPA Library Quantification Kit for Illumina platforms.
7. Bio-Rad CFX96 Touch Real-Time PCR detection system or equivalent.
8. NextSeq 550 Series or Illumina sequencer equivalent (HiSeq) and sequencing kits, Catalog # 20024906.

3 Methods

Each sample of END-seq corresponds to one agarose plug. The cell size limits the number of cells that can be used to make each plug (from two million of senescent MEFs (large cells) to 80 million of mouse thymocytes (small cells)). The volume of the cell pellet cannot exceed 12 μL. It is possible to make and process more than one plug per sample to increase the total number of cells and enhance the sensitivity of the method.

3.1 Day 1: Plug Making and Proteinase K Digestion

Prepare in advance two water baths or heat blocks, at 70 °C and 37 °C. Melt 500 μL aliquot(s) of 2% agarose (Bio-Rad kit) in a 70 °C water bath for 10 min until liquified and equilibrate at 37 °C water bath for at least 10 min before use.

1. Harvest cells, count and aliquot the desired number of cells. Depending on cell type, we recommend the following number of cells per plug: for stimulated B-cells, 20 million cells; T-cells,15 million; HCT116, seven million; HEK293T, seven million; HeLa, five million and MCF7, four million. As noted above, several plugs for the same sample can be made to increase sensitivity (usually to a total of 15 million) and processed in parallel until day 3 at which point the individual plugs can be combined (*see* **Note 1** for removing dead cells).
2. Spin cells and resuspend them in 1 mL of PBS and transfer to a 1.5 mL tube.
3. Add a fixed number of spike-in cells (2–5%) to each sample.
4. Spin down cells at 400 × *g* for 7 min at 4 °C.
5. Remove PBS from the 1.5 mL tube and leave approximately 50 μL covering the cell pellet. Slowly add 1 mL of PBS with a P1000 micropipette tip, do not disturb the pellet, and spin at 400 × *g* for 5 min at 4 °C (*see* **Note 2**).
6. Repeat **step 5**.
7. After the second PBS wash, remove PBS from the 1.5 mL tube and leave approximately 50 μL covering the cell pellet. Spin again for 10 s to remove any residual PBS droplets from the walls of the tube.
8. Use a 200 μL micropipette tip to remove the rest of the PBS and resuspend the pellet in 57 μL of Bio-Rad Cell Suspension Buffer. The cell pellet should occupy a volume of 5–13 μL and the mixture of cells and suspension buffer should be ~62 μL (60–70 μL range) (*see* **Note 3**).
9. Place one gel plug cast (10 plugs per cast, included in Bio-Rad CHEF Mammalian Genomic DNA Plug Kit) on an inverted metal block on ice. Set the smooth metal surface in contact

with the adhesive of the base of the gel plug cast and avoid contact with condensed water. This will allow the agarose to cool faster once the well is filled (*see* **Note 4**).

10. Equilibrate cells in a cell suspension buffer at room temperature (RT) for 5 min.
11. Prepare two pipettes, set one to 37.5 µL and the other one to 110 µL. Use a wide-bore p200 tip and take 37.5 µL of melted 2% agarose at 37 °C (pipet up and down twice in agarose) and add it to the 62.5 µL of cell suspension without mixing. Use the other P200 with a normal tip set at 110 µL and slowly mix ten times avoiding air bubble formation. Make sure the cell suspension and agarose are homogeneous. At this point, transfer the cell-agarose suspension to one well of the plug cast, creating a "dome" if needed. Repeat this step for each sample (Fig. 3a).
12. Place the plug cast on the inverted metal block in 4 °C refrigerator for 30 min to allow the agarose to polymerize. Avoid contact with condensed water.
13. For each sample prepare one 50 mL tube with proteinase K solution by adding 170 µL of Qiagen Proteinase K enzyme and 2.5 mL of lysis buffer (each tube can have up to four plugs from the same sample).
14. Transfer plugs into the 50 mL tube containing proteinase K solution by removing the tape from the bottom of the plug cast and ejecting plugs into a 50 mL tube using the plug mold plunger (included in the Bio-Rad kit) (Fig. 3a). Make sure all plugs are fully submerged in lysis buffer.
15. Cap the 50 mL tube and incubate in a Thermomixer C or water bath for 1 h at 50 °C with intermittent mixing (cycle 1: 15 s at 450 rpm followed by 15 min at 0 rpm) followed by 7 h at 37 °C (cycle 2: 15 s at 450 rpm followed by 15 min at 0 rpm) and finally hold at 23 °C (cycle 3: infinite 0 rpm). If using a water bath instead of a thermomixer, mix by hand every 10 min using a swirling motion for 1 h at 50 °C. Then, transfer the 50 mL tube to a water bath at 37 °C for 7 h to overnight. If plugs are immediately processed after 8 h of proteinase K treatment, then proceed until **step 3** in Subheading 3.2, and then keep plugs in plug wash buffer at room temperature overnight. Start with **step 4** in Subheading 3.2 the following day (*see* **Notes 5** and **6**).

3.2 Day 2: RNAseA Digestion

1. Prepare plug wash buffer (50 mM EDTA pH 8.0 and 10 mM TrisHCl pH 8.0) and TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) in molecular biology grade water. Mix thoroughly and leave at room temperature until use.

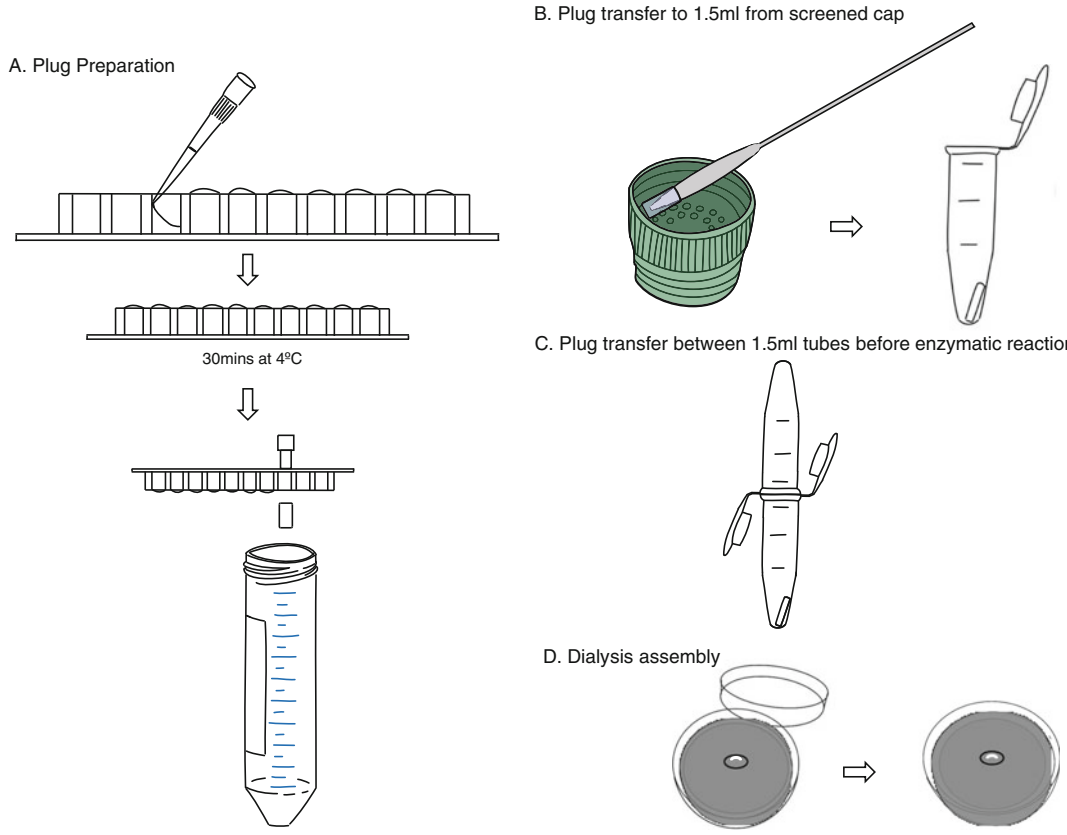

Fig. 3 Detailed depictions of individual steps in the END-seq protocol. (**a**) Plug preparation: cell suspension embedded in agarose is pipetted into a plug mold and allowed to solidify at 4 °C. Polymerized plugs are then transferred to a 50 mL conical tube containing lysis buffer with proteinase K. (**b**) Plug transfer from cap: Plugs are transferred after washes from a screened cap to a 1.5 mL tube using a disposable spatula. (**c**) Plug transfer between tubes: Transfer plug to a new tube for enzymatic reactions by superimposing the opening of two 1.5 mL tubes and tapping on a benchtop until the agarose plug slides down into the new tube. (**d**) Dialysis assembly: a dialysis membrane is floated on the surface of 15 mL TE in a 6 cm petri dish. Melted agarose sample is pipetted onto the center of the membrane. The dish is covered and samples are dialyzed for 1 h

2. Drain proteinase K solution through the screen cap fitted on the 50 mL tube and tap each tube on a benchtop to push plugs down to the bottom of the tube.
3. Rinse plugs three times with 15 mL of plug wash buffer by adding and draining plug wash buffer through the screened cap. Swirl tube and make sure plugs are fully immersed in wash buffer during each rinse (*see* **Note 7**). Plugs can be kept in plug wash buffer for several hours to overnight at room temperature.
4. Drain plug wash buffer through screened cap and tap the tube to ensure that the plugs moved down to the bottom of each tube.

5. Rinse plugs twice with 15 mL of TE buffer (as in **step 3**).
6. Wash plugs twice in 15 mL of TE with constant shaking for 15 min at RT (~180 rpm using an orbital platform shaker, or the minimum speed to see plugs moving). Make sure plugs are not settled at the bottom of the tube during washes.
7. Discard the last wash of TE through the screened cap. Remove the screened cap and add 2.5 mL of TE and 50 μL of Puregene RNAseA solution. Recap tubes, swirl to mix, make sure plugs are submerged, and incubate in a Thermomixer C for 1 h at 37 °C with intermittent mixing (cycle: 10 s at 450 rpm followed by 10 min at 0 rpm).
8. Drain RNase solution through the screened cap and tap plugs to the bottom of the 50 mL tube.
9. Rinse plugs three times with 15 mL of plug wash buffer. Swirl tube and discard buffer through the screened cap and tap plugs to the bottom of the tube (as in **Step 4**) before adding the next wash.
10. Wash plugs four times with 15 mL of plug wash buffer for 15 min at RT with constant agitation (~180 rpm using an orbital platform shaker). Do not discard final 15 mL of plug wash buffer.
11. Leave the plugs in plug wash buffer at 4 °C.

    This is a convenient stopping point. Plugs can be stored in plug wash buffer for up to 2 weeks at 4 °C.

3.3 Day 3: DSB End Blunting, A-Tailing, and Ligation of END-Seq Adapter 1

Please be aware that if there are more than two plugs per sample, perform the reactions and washes in 50 mL tubes instead of 1.5 mL tubes. Multiply the volume of reaction according to the number of plugs in each tube.

1. Discard plug wash buffer, rinse twice with EB buffer (10 mM Tris pH 8.0 in nuclease free water), and perform four washes with agitation (~180 rpm using an orbital platform shaker) for 15 min each at RT with EB buffer.
2. After discarding the last EB wash, invert the 50 mL tube and remove the screened cap. Use a disposable spatula to transfer the plugs from the interior of the screened cap to 1.5 mL tubes containing 1 mL of EB (Fig. 3b; *see* **Note 8**).

    Steps 3 and **4** are for treatment with exonuclease VII for resection and end structure studies. If this is not the aim of the experiment, proceed to **step 5**.
3. Remove EB buffer with a P1000 micropipette tip (*see* **Note 9**). Then, add 1 mL of 1× homemade exonuclease VII buffer and rotate for 15 min at RT in a rotatory mixer at 8 rpm. Repeat with 500 μL of 1× NEB Exonuclease VII buffer and rotate for 15 min at RT in a rotatory mixer at 8 rpm.

4. Remove the exonuclease VII buffer with a P1000 micropipette tip. Transfer plugs to a new 1.5 mL tube by inverting the opening of the tube over a new 1.5 mL tube (Fig. 3c). Tap on the bench until the plug moves down into the new tube. Remove any residual liquid from the wall and bottom of the Eppendorf tube with a P20 tip. Then, add 125 μL of exonuclease VII reaction mix (per plug):
   25.00 μL 5× NEB Exonuclease VII buffer.

   6.25 μL Exonuclease VII (10 U/μL).

   93.75 μL H_2O.

   Mix by tapping the tube with your fingers.

   Incubate at 37 °C for 1 h with mixing at 400 rpm (in the thermomixer, mix by tapping the tube with your fingers every 20 min).
5. Remove the exonuclease VII reaction mix with a P200 micropipette tip and wash twice with 1 mL of 1× NEBuffer 4 for 15 min at RT in a rotatory mixer at 8 rpm.
6. Remove NEBuffer 4 with a P1000 micropipette tip and transfer plug to a new 1.5 mL tube (Fig. 3c). Remove any residual liquid from the tube with a P20 tip. Then, add 125 μL per plug of ExoT reaction mix on the plug:
   12.5 μL 10× NEBuffer 4.

   6.25 μL exonuclease T (5 U/μL).

   106.25 μL H_2O.

   Mix by tapping the tube with your fingers.

   Incubate at 25 °C for 1 h with mixing at 400 rpm (in the thermomixer, mix by tapping the tube with your fingers every 20 min).
7. Remove the exonuclease T reaction mix and rinse with 1 mL of EB buffer. Decant and transfer plugs to a 50 mL tube containing 15 mL of EB and shake (~180 rpm using an orbital platform shaker) for 15 min at room temperature. Discard EB wash through the screened cap and tap plugs to the bottom of the tube before adding the next wash. Make sure plugs are submerged in EB buffer. Repeat the EB wash two additional times for a total of three washes.
8. After discarding the last EB wash, invert the 50 mL tube and remove the screened cap. Using a disposable spatula, scoop every plug from the interior of the screened cap to one 1.5 mL tube filled with 1 mL of 1× NEBNext dA-Tailing buffer and mix for 15 min at RT in a rotatory mixer at 8 rpm. Repeat the NEBNext dA-Tailing buffer wash one more time.

9. Remove the 1× NEB dA-Tailing buffer with a P1000 micropipette tip and transfer each plug to a new 1.5 mL tube (Fig. 3c). Remove any residual buffer droplets and add 125 μL of dA-Tailing reaction mix (per plug):
   12.5 μL NEB dA-Tailing buffer (10×).
   7 μL Klenow fragment (3′ → 5′ exo-) (5 U/μL).
   105.5 μL of H_2O.
   Mix by tapping the tube with your fingers.

   Incubate at 37 °C for 1 h with mixing at 400 rpm (in the thermomixer, mix by tapping the tube with your fingers every 20 min).
10. Remove the dA-Tailing reaction mix with a P200 micropipette tip, then add 1 mL of 1× NEBuffer 2 and mix for 15 min at RT in a rotatory mixer at 8 rpm.
11. Remove the 1× NEBuffer 2 with a P1000 micropipette tip and transfer plugs to a new 1.5 mL tube (Fig. 3c). Remove any residual buffer droplets and add 125 μL per plug of ice-cold Quick Ligation mix (prepare on ice) to the plug:
    112 μL 2× Quick Ligase Buffer (NEB).
    4 μL Annealed END-seq adapter 1 (10 μM).
    4 μL Quick Ligase (2000 U/μL NEB).
    5 μL H_2O.

    Incubate at 25 °C for 1 h with shaking at 400 rpm (in the thermomixer, mix by tapping the tube with the fingers every 20 min).
12. Remove the Quick Ligase Buffer mix with a P200 micropipette tip and rinse with 1 mL of plug wash buffer by pipetting up and down (be careful not to draw the plug into the pipette tip). Remove the plug wash buffer and add 1 mL of plug wash buffer and mix for 15 min at RT in a rotatory mixer at 8 rpm. Repeat this for a total of four washes.
13. Transfer plugs to a 50 mL tube with 45 mL of plug wash buffer by decanting and mix minimally for 8 h or overnight in a thermomixer C at 23 °C with intermittent mixing (cycle: 10 s at 450 rpm followed by 15 min at 0 rpm). This wash step helps eliminate any unligated and free END-seq adapter 1. The presence of the free adapter will increase the amount of primer-dimer formation in the PCR reaction.

3.4 Day 4: Plug Melting and DNA Shearing

Prepare in advance two water baths or heat blocks at 70 °C and 43 °C. Turn on the Covaris S220 sonicator and start the degassing and cooling procedure while performing drop dialysis (*see* **step 8** below).

1. Discard plug wash buffer from tubes in **step 13**, Subheading 3.3 above and rinse twice with TE buffer and perform four TE washes with agitation (~180 rpm on an orbital platform shaker) for 15 min at RT.
2. Discard TE Buffer through the screened cap and tap the tube to push plugs to the bottom of the tube.
3. Transfer one plug at a time with a disposable spatula to an empty 1.5 mL tube. Avoid any carryover of TE by touching the plug to the border of the screened cap to drain any excess liquid. If several plugs from the same sample are being processed simultaneously, pool them together in this step.
4. Pulse spin the microfuge tube for ~5 s at 2400 × *g* to spin the agarose plug to the bottom of the tube. This will facilitate efficient heat transfer and rapid melting of the agarose in the next step.
5. Melt the agarose plugs by incubating tubes in a water bath or heat block at 70 °C for 2 min. Incubate for 3 min if the volume is greater than 200 μL.
6. Immediately transfer tubes to a 43 °C water bath and incubate for 5 min.
7. In a separate 1.5 mL tube, place the total volume of beta-agarase enzyme needed for the melting step (1.5 μL × number of tubes) and leave at RT for at least 1 min before use. Add 1.5 μL of beta-agarase enzyme in the center of the melted plug in each tube. Slowly pipet up and down 3–5 times the entire volume of the melted plug with a P200 wide-bore tip and incubate at 43 °C for 45 min (a temperature difference of ±3 °C can inactivate the enzyme).
8. Setup drop dialysis as follows: pipet 15 mL of TE buffer into a 6 cm Petri dish for each sample and float a 0.1 μm dialysis membrane above the TE buffer (Fig. 3d). Place the lid back on the Petri dish and let the membrane hydrate for 10 min.
9. Pulse spin microfuge tube containing beta-agarase digested samples for 1 s at 2400 × *g* to spin down condensation.
10. Slowly pipet up and down the entire DNA solution with a P200 wide-bore tip and transfer it to the center of the dialysis membrane in a single drop (Fig. 3d). Do not touch the membrane with the tip as this could submerge the membrane resulting in sample loss. Place the lid on the petri dish and let samples dialyze for 1 h at room temperature (*see* **Note 10**).
11. Transfer DNA to a newly labelled 1.5 mL tube with a wide bore tip without touching the membrane. Add 1 μL of 10% SDS, 4 μL of proteinase K (20 mg/mL), vortex, and incubate for 15 min at 50 °C.

12. Spin down for 5 min at maximum speed in a microcentrifuge at RT to remove air bubbles.
13. Transfer DNA to a Covaris microTUBE AFA Fiber Pre-Slit Snap-Cap 6 × 16 mm using a P200 wide-bore tip. Pipet the entire DNA solution and slowly release it in the bottom of the Covaris tube to avoid making air bubbles. Fill with TE to the rim of the covaris tube (total tube volume is ~140 μL). If sample volume is more than 140 μL, add TE to have a total volume of 280 μL and distribute it between two Covaris tubes. Shear the DNA to a median size of 175 bp using the below cycle settings on a Covaris S220 series ultrasonicator.

    Cycle 1—duty 10%, intensity 175.0, cycles/burst 200, 240 s , 4–7 °C.
14. Transfer the sheared DNA to a new tube and add 80 μL of TE to a total volume of 200ul. To this add 1 μL of glycogen, 20 μL of 3 M sodium acetate pH 5.2, vortex well, and add 500 μL of 100% ethanol at RT. Vortex (do not pipette) and incubate tubes in dry ice for 15 min. In the case of multiple tubes of the same sample, combine the sheared DNA and adjust volumes accordingly.
15. Spin chilled samples at maximum speed for 20 min at 4 °C in a microcentrifuge. A white pellet should be visible.
16. Decant the supernatant, wash the pellet twice with 1 mL of ethanol 70% at RT, and spin at max speed for 5 min at 4 °C in a microcentrifuge.
17. Decant the supernatant, carefully remove all the ethanol droplets with a vacuum aspirator and a micropipette, and air dry the DNA pellet at RT.
18. After the pellets are dry, add 70 μL of TE to the pellet, vortex, and incubate at 50 °C for 5 min. Resuspend pellets with a micropipette P200 tip.
19. Spin at maximum speed for 5 min at RT in a microcentrifuge to pellet any insoluble material. Transfer solubilized DNA (supernatant) to a new tube and avoid taking any insoluble material or debris. Repeat this step as many times as is necessary until you have a clear DNA solution (*see* **Note 11**).
20. Measure DNA concentration with a nanodrop spectrometer (*see* **Note 12** on the expected recovered DNA amounts based on starting cell type and numbers).
21. Store DNA at −20 °C until ready to proceed to the next steps in Subheading 3.5.

    This is a convenient stopping point. Samples can be stored at −20 °C for several weeks.

3.5 Day 5: Streptavidin Capture of Labeled DSB Ends, Blunting, A-Tailing Ligation of END-Seq Adapter 2, and PCR Library Amplification

Prepare in advance 9 mL of 1× bead wash and binding buffer (1× WB), 7 mL of EB per sample, and 10 mL of 2× bead binding buffer (2× BB) (*see* **Note 13**).

1. Add 35 μL of Dynabeads MyOne C1 in a 1.5 mL tube; wash beads with 1 mL of 1× WB. Place the tube on a DynaMag-2 Magnet for 1 min to ensure that all the beads are collected on the tube wall and remove the 1× WB with a P1000 tip. Repeat for a total of two washes.
2. Resuspend beads in 70 μL (twice the original volume of the beads) of 2× WB buffer; then add 70 μL of DNA sample (from **step 21**, Subheading 3.4), vortex, and mix (700 rpm) for 30 min at RT in a thermomixer. Vortex every 15 min to avoid beads settling to the bottom of the tube.
3. Place tubes in the magnet, remove supernatant, and wash beads three times with 1 mL of 1× WB, twice with 1 mL of EB, and once with 1 mL of 1× NEB T4 Ligase Buffer. Change tubes after the first EB wash. Beads can be kept on the magnet in 1× NEB T4 Ligase Buffer while preparing for the end repair reaction (steps below).
4. Remove the 1× NEB T4 Ligase buffer using a P1000 tip, resuspend beads in 50 μL of end repair reaction buffer, and incubate for 30 min at 24 °C with mixing (700 rpm) in the thermomixer. Vortex samples every 15 min.
   Prepare end-repair reaction buffer:
   5 μL 10× NEB T4 DNA Ligase buffer.
   2 μL 10 mM dNTPs.
   2 μL end repair enzyme mix (1:5:5 large Klenow fragment: T4PolI:T4PNK).
   41 μL H_2O to make final volume 50 μL.
   Repair enzyme mix:
   5 μL 3 U/μL T4 DNA Polymerase (NEB).
   1 μL 5 U/μL large Klenow fragment (NEB).
   5 μL 10 U/μL T4 DNA Polynucleotide Kinase (NEB).
5. Place tubes in the magnet, remove the END-repair reaction mix, and wash beads once with 1 mL 1× WB, twice with 1 mL of EB, and once with 1 mL of 1× NEBNext dA-Tailing Reaction Buffer. Change tubes after the first EB wash. Hold beads on the magnet in 1× NEBNext dA-Tailing Reaction Buffer while preparing the dA-Tailing reaction mix.
6. Remove 1× NEBNext dA-Tailing Reaction Buffer using a P1000 tip, resuspend beads in 50 μL of dA-tailing reaction mix, and incubate for 30 min at 37 °C with mixing (700 rpm) in the thermomixer. Vortex every 15 min.

Prepare dA-Tailing reaction mix:

5 μL 10× NEBNext dA-Tailing Reaction Buffer.

3 μL 5 U/μL NEB Klenow Fragment (3′ → 5′ exo-).

42 μL of H_2O.

7. Place tubes in the magnet, remove dA-Tailing reaction mix and wash beads once with 1 mL 1× NEBuffer 2. Hold beads on the magnet in 1× NEBuffer 2 while preparing the ligation reaction.
8. Remove 1× NEBuffer 2 using a P1000 tip and resuspend in 115 μL of ligation mix and incubate for 20 min at 25 °C with mixing (700 rpm) in the thermomixer. Vortex every 15 min.
   Prepare ligation mix: (prepare on ice).

   52.5 μL H_2O.

   57.5 μL 2× Quick Ligase Buffer (NEB).

   2 μL END-seq adapter 2 (0.50 μM).

   3 μL Quick Ligase (2000 U/μL NEB).
9. Inactivate the ligation reaction by adding 12 μL of EDTA 0.5 M, vortex, and place tubes in the magnet. Remove the ligation reaction using a P200 tip and wash beads three times with 1 mL 1× WB. Wash three times with 1 mL of EB. Change tubes after the first EB wash.
10. Remove the last EB wash and resuspend beads in 8 μL of EB. Add 10 μL of USER reaction, mix well, and incubate for 30 min at 37 °C with mixing (700 rpm) in the thermomixer. Vortex every 15 min.
    USER reaction:

    9 μL 2× Kapa HiFi HotStart Ready Mix.

    1 μL of USER enzyme 1 U/μL (NEB).
11. Place samples on ice and add 1 μL of Illumina Truseq 8 nt barcoded primer p5 (50 μM) and 1 μL of Illumina Truseq 8 nt barcoded primer p7 (50 μM). Then, add 38 μL of PCR master mix and transfer the total 60 μL of each sample to a PCR tube (PCR strip).
    PCR master mix:

    18 μL H_2O.

    20 μL 2× Kapa HiFi HotStart Ready Mix.
12. Perform the PCR using the following thermocycling parameters:
    (a) 45 s at 98 °C.
    (b) 16 cycles.
        15 s at 98 °C.
        30 s at 63 °C.

30 s at 72 °C.

(c) 5 min at 72 °C.

(d) Hold at 4 °C.

13. Place the PCR strip in a DynaMag-PCR Magnet and transfer the supernatant to a new 1.5 mL tube.
14. The PCR product is purified using 108 μL of AMPure XP beads following the manufacturer's protocol. Elute AMPure XP beads in 20 μL of EB.
15. Add loading buffer and run purified PCR product on a 2% agarose gel for 40 min at 90 V.
16. The PCR product should be between 200 bp and 400 bp and is excised from the gel. Avoid primer-dimer bands that are typically observed around 140 pb. (Fig. 4).
17. DNA is extracted from the gel slice using QIAquick Gel Extraction Kit following the manufacturer's protocol. The final column elution of DNA is done using 17 μL of prewarmed (55 °C) QIAquick EB.
18. Illumina libraries are quantified using the KAPA Library Quantification Kit for Illumina platforms following the manufacturer's protocol.
19. Typically 8–12 samples can be pooled per sequencing run with a NextSeq 500/550 High Output Kit v2.5 (75 Cycles) that yields between 35 and 50 million 75 bases single-end reads for each sample. We follow Illumina's NextSeq System Denature

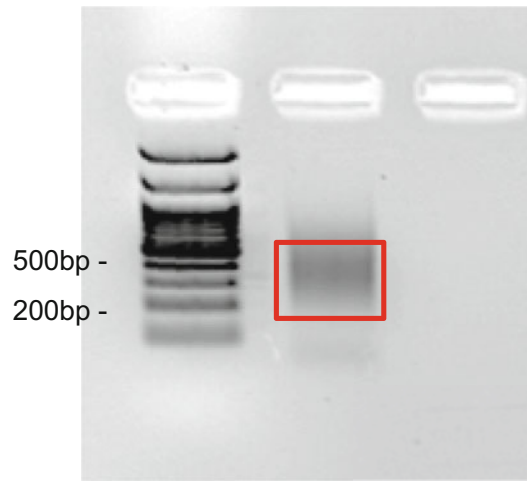

Fig. 4 Size distribution of sequencing-ready PCR product. The purified PCR library product is loaded on a 2% agarose gel and run for 40 min at 90 V. This results in a tight smear (red box) with a size distribution between 200 to 400 bp. Following gel purification and quantification, the DNA is ready to load onto an Illumina Next-Generation Sequencing Platform

and Dilute Libraries Guide and recommend barcode compatibility. Libraries are analyzed using a standard bioinformatics pipeline using Bowtie as an aligner and MACS as the peakcaller as previously described in [11–14].

4 Notes

1. If you suspect that there are dead cells, isolate live cells by Ficoll or Percoll. The following protocol is optimized for lymphocytes using Ficoll. For other cell types we recommend optimizing a Percoll gradient according to the density of the cell type.
   (a) In 15 or 50 mL tubes, add 5 mL of lymphocyte separation medium from Lonza (http://bio.lonza.com/uploads/tx_mwaxmarketingmaterial/Lonza_ManualsProductInstructions_Lymphocyte_Separation_Medium.pdf). The lymphocyte separation medium is a mixture of Ficoll and sodium diatrizoate (Hypaque) with density adjusted to 1.077 g/mL, and it can be used with human and mouse lymphocytes.
   (b) Centrifuge cells, remove media, resuspend pellet in 1 mL of cell growth media with 10% of serum, add 6 mL of PBS, and with a P1000 micropipette tip very slowly transfer the cell suspension on top of the lymphocyte separation media Ficoll solution. Be careful to avoid breaking the interphase between the cell suspension and the Ficoll. The interphase can be maintained if one inclines the tube and adds the cell suspension along the sides of the wall.
   (c) Spin at 450 × *g* for 25 min with the slowest acceleration and deceleration without any brakes.
   (d) Live cells fall in the interphase between Ficoll and PBS media while dead cells pellet in the Ficoll. Live cells are recovered using a P1000 micropipette tip and transferred to a new 50 mL tube with 40 mL complete media. Do not take more than 3 mL from the interphase.
   (e) Spin for 7 min at 450 × *g*. Discard media and resuspend the cell pellet in fresh complete media. Cells are counted and viability assessed with trypan blue staining.
   (f) Aliquot the number of cells necessary to make agarose plugs.
2. When washing the cells in PBS in preparation for embedding in agarose plugs, add PBS to wash the cells without disturbing the pellet after the first centrifugation. Small cells like lymphocytes can become very sticky. This washing step of the pellet without resuspension helps to remove any residual media components while minimizing cell loss.

3. The number of cells that fits in a plug depends on the cell size. The volume of cells and suspension buffer has to be between 62 and 70 μL, but the volume of suspension buffer added cannot be lower than 55 μL (meaning that the volume of the cellular pellet has to be between 7 and 15 μL). When adding 37.5 μL of 2% agarose to the cell suspension, the final agarose concentration should be between 0.75% and 0.8%. If the cell pellet size is too big, make two plugs. Lower volumes increase agarose concentrations and make the plugs so compacted that enzymes cannot enter, thereby dramatically lowering the efficiency of the entire END-seq process. Concentrations below 0.75% increase fragility of the plugs. Large numbers of cells in the plug increase mechanical resistance of the plugs in later steps while plugs with less than two million cells become very fragile (easily chipped) and necessitate special care.
4. When mixing agarose with the cells it is best to be quick to avoid solidification of the cell-agarose mixture before pipetting into the plug mold.
5. Monitor that plugs become clear during the first 30 min of the proteinase K reaction. After 10 min, you can observe the proteinase K advancing to the center of the plug and the borders of the plug become clearer. After 35–40 min the whole plug should be clear. If there are too many cells or the agarose concentration is too high, the center will remain undigested after 35–40 min. In this case, discard the plugs and start again. Although the effect of proteinase K during the first 30 min on plug clarity should be obvious, plugs from some cell types with a rich extracellular matrix can maintain cloudiness even after proteinase K treatment.
6. After the proteinase K reaction, plugs can be rinsed with wash buffer and stored until the next day at room temperature. Do not store them at 4 °C as the SDS in the lysis buffer remains in the plug. At 4 °C the SDS precipitates and will form crystals that could damage the DNA. If this happens, discard the plugs and start again.
7. Avoid plugs from being stranded in the screen cap or on the wall of the 50 mL tubes during reactions and washes. Always check how many plugs are in the buffer by stirring after every rinse and wash. If a plug is stranded, it will dry and enzymes will not be able to permeate the plug resulting in damaged DNA. In such a scenario, it is best to discard the plug and start again. We recommend discarding and adding washes in the same location on the screened cap. This will help prevent plugs from sticking to the cap after decanting. To facilitate the entrance of liquid through the screened cap, slightly tilt the 50 mL tube when pouring in fresh wash solution.

8. For transferring plugs from the screen cap to Eppendorf tubes, we recommend using a disposable spatula to avoid DNA contamination between different samples. Also avoid mixing plugs from different samples in the same proteinase K or RNAse reactions and/or washes. In the case of transferring plugs from one 1.5 mL tube to another, avoid using spatulas, simply transfer by connecting the opening of Eppendorf tubes, and decant the plug into the new tube (Fig. 3c).
9. When removing liquid from the 1.5 mL tubes with a plug inside, tilt the tube and introduce the tip of a 1000 mL micropipette to the bottom of the tube and aspirate. Be careful to not pull the plug into the tip as this will break the plug.
10. During the spot dialysis step, use forceps to grip the membrane by its edge and gently place on the TE surface while simultaneously maintaining the membrane in a horizontal position to prevent dipping or sinking of membrane.
11. Depending on the cell type, some white debris can appear after resuspending the DNA pellet in TE after ethanol precipitation. In this scenario, centrifuge the solution and transfer the clear supernatant to a new tube while avoiding the debris. Sometimes the debris does not precipitate and remains on the surface of the solution. In this case, remove the solution from the bottom of the tube.
12. Estimations of DNA concentration after Covaris sonication and DNA precipitation: for 20 million B-cells, around 65 micrograms of DNA should be recovered. For four million B-cells, approximately 14 micrograms are obtained.
13. Make fresh bead binding and wash solutions each time an experiment is performed. Mix beads well by vortexing and pipetting up and down before every use.
14. Order END-seq adapters 1 and 2 with HPLC purification. Reconstitute them by adding 1× NEB T4 Ligase Buffer to a concentration of 10 μM. Vortex the oligos and incubate at 50 °C for at least 5 min. Distribute the oligos in 500 μL aliquots in screwcap tubes and assemble them in a floater. Heat 1 L of water in a glass beaker in the microwave oven until it boils. Measure temperature with a thermometer and make sure it is higher than 95 °C. Put the floater with screwcap tubes containing oligos on the surface of the hot water and wait until it reaches room temperature, typically around 3–4 h. Finally, pool all aliquots and make 100 μL working aliquots. Aliquoted oligos can be stored at −20 °C indefinitely.

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