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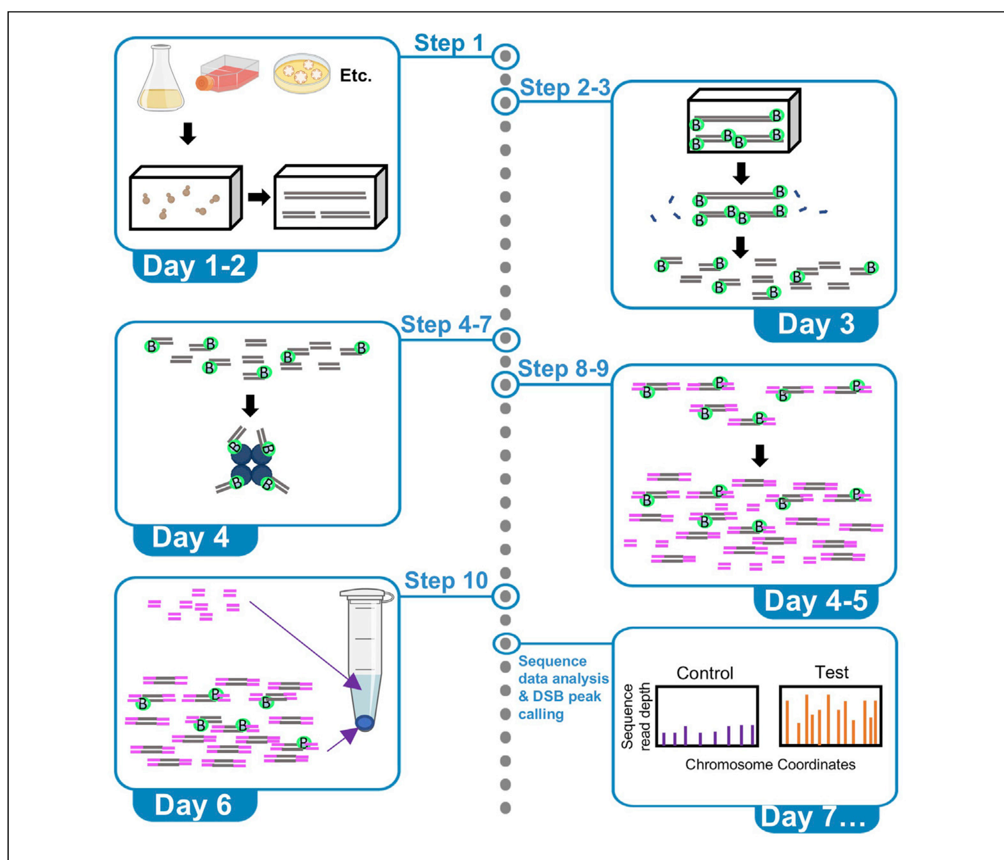
Genome-wide mapping of DNA double-strand breaks from eukaryotic cell cultures using Break-seq



We describe a genome-wide DNA double-strand break (DSB) mapping technique, Break-seq. In this protocol, we provide step-by-step instructions for cell embedment in agarose, in-gel DSB labeling and subsequent capture, followed by standard Illumina library construction and sequencing. We also provide the framework for sequence data processing and DSB peak identification. Finally, we present a custom designed 3D-printed device for processing agarose-embedded DNA samples. The protocol is applicable to *Saccharomyces cerevisiae*, as well as mammalian suspension, adherent, and 3D organoid cell cultures.

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Highlights

Genome-wide nucleotide resolution mapping of DNA DSBs

Break-seq shows high sensitivity and high specificity of DSB identification

Custom-designed device for processing agarose plugs, washing, and DSB labeling

Universally adaptable to any cell culture system

Joshi et al., STAR Protocols *2*, 100554
June 18, 2021 © 2021 The Author(s).
https://doi.org/10.1016/j.xpro.2021.100554

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Genome-wide mapping of DNA double-strand breaks from eukaryotic cell cultures using Break-seq

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https://doi.org/10.1016/j.xpro.2021.100554

SUMMARY

We describe a genome-wide DNA double-strand break (DSB) mapping technique, Break-seq. In this protocol, we provide step-by-step instructions for cell embedment in agarose, in-gel DSB labeling and subsequent capture, followed by standard Illumina library construction and sequencing. We also provide the framework for sequence data processing and DSB peak identification. Finally, we present a custom-designed 3D-printed device for processing agarose-embedded DNA samples. The protocol is applicable to *Saccharomyces cerevisiae*, as well as mammalian suspension, adherent, and 3D organoid cell cultures.

For complete details on the use and execution of this protocol, please refer to Hoffman et al. (2015) and Chakraborty et al. (2020).

BEFORE YOU BEGIN

Overview

Break-seq is a powerful method for genome-wide identification of DNA DSBs in eukaryotic organisms. It was initially developed for the model organism *Saccharomyces cerevisiae* (Hoffman et al. 2015), and had since been adapted to the mammalian cell cultures including suspension cells (Chakraborty et al. 2020), adherent cells, and 3-D organoids (unpublished). The method hinges on embedding cells in low-melting agarose prior to any manipulation of the DNA so as to minimize *in vitro* shearing of the DNA. It then involves T4 DNA polymerase-mediated end-repair labeling of DSB ends with biotinylated dATP, followed by sonication and size reduction of the chromosomal DNA released from agarose, capturing biotinylated DNA fragments with streptavidin beads, and finally, preparing a library of the captured DNA using standard Illumina adapters and sequencing platforms. This protocol provides detailed instructions for the ten steps beginning from cell culture growth to Break-seq library construction, as illustrated in the Graphical Abstract and detailed in "step-by-step method details".

1. **Cell culturing and agarose plug preparation** – We parse this step into 1.a and 1.b, for cell type-specific procedures using yeast and mammalian cells, respectively. We discuss the requirements of cell number for these cell types and present examples of cell culturing conditions for mapping spontaneous and replication stress-induced DSBs. After cells are harvested they are mixed with molten agarose to form "agarose plugs", followed by cell lysis in-gel, resulting in chromosomal DNA embedded in agarose. Hereafter all subsequent steps are identical for yeast and mammalian DNA samples.

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2. **In-gel labeling of DSBs by end-repair** – Following cell lysis the chromosomal DNA is exposed within the agarose matrix. The DNA ends are labeled by biotinylated dATP using T4 polymerase-mediated end-repair reaction.
3. **Agarose digestion and release of chromosomal DNA** – The end-repaired chromosomal DNA is released from the agarose plugs by β-Agarase digestion.
4. **Fragmentation of chromosomal DNA by sonication** – The size of chromosomal DNA is reduced to 300–500 bp fragments by sonication.
5. **End-repair of fragmented DNA** – Broken DNA ends are blunted by end-repair.
6. **A-tailing** – dATP is incorporated on the 3′-end of blunted DNA ends.
7. **Capturing labeled DNA by streptavidin beads** – Biotinylated DNA fragments are pulled down by incubation with streptavidin beads.
8. **Adapter ligation to (streptavidin-)BOUND DNA** – Standard Illumina adapters are ligated onto DNA ends.
9. **PCR amplification of labeled DNA** – Adapter-ligated DNA fragments are amplified using multiplexed Illumina primers.
10. **Free adapter removal by AMPure purification** – Adapter-ligated DNA is captured by, then eluted from, AMPure beads, thus removing un-ligated free adapters.

The constructed Break-seq libraries are subjected to Illumina sequencing. Sequence reads are aligned to the reference genome and DSB peaks are identified by peak calling algorithms. We also present essential commands using Bowtie2 and MACS2 for sequence alignment and peak calling, respectively, as an example (quantification and statistical analysis).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
1 X PBS	Corning	Cat#21-040-CV
Acetic Acid, Glacial ACS, 500 mL	Fisher	Cas 64-19-7
Alpha factor	Thermo Fisher Scientific	Custom Peptide: [NH2]WHWLQLKPGQPMY[COOH]
Ammonium sulfate	Fisher Bioreagents	Cas 7783-20-2
AMPure	Beckman Coulter	Ref A63880
Aphidicolin	AG Scientific	Cat#A-1026-1mg
β-Agarase I	NEB	Cat#M0392L
β-Mercaptoethnanol	Sigma-Aldrich	Cas 60-24-2
Bacto Peptone	Becton, Dickinson and Company	Ref 211677
Bacto Yeast Extract	Becton, Dickinson and Company	Ref 212750
BenchMark FBS, heat inactivated	Gemini Bioproducts	Cat#100-106
Biotin-14-dATP	Invitrogen	Cat#19524016
Dextrose anhydrous	Fisher Bioreagents	Cas 50-997
Difco Yeast Nitrogen Base w/o Amino Acids & Ammonium Sulfate	Becton, Dickinson and Company	Ref 233520
dATP	NEB	Cat#N0440S
dCTP	NEB	Cat#N0441S
dGTP	NEB	Cat#N0442S
dTTP	NEB	Cat#N0443S
DTT	Fisher Bioreagents	Cat# BP172-5
Dynabeads M-270 Streptavidin	Invitrogen	Cat#65305
EDTA	Sigma-Aldrich	Cas 6381-92-6
Fisherbrand Low-Retention Microcentrifuge Tubes	Fisher Scientific	Cat#02-681-320

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
GlutaMAX	Gibco	Cat#35050-061
Incert low melting agarose	Lonza	Cat#50123
KAPA HiFi HotStart ReadyMix	Roche	Ref 07958927001
Klenow 10× reaction buffer	NEB	E6055A
Klenow enzyme	NEB	E6054A
Lithium dodecyl sulfate	Sigma Chemical	Cat #L-4632
Magnesium acetate	Acros Organics	Cas 16624-78-5
N-Lauroylsarcosine sodium	Krackeler	45-L5125-500
Penicillin streptomycin solution	Corning	Cat#30-002-CI
Potassium acetate	J.T.Baker	Cas 127-08-2
Pronase protease	Millipore	Cat#53702-250KU
Propidium iodide	Fisher	Cat# AC440300250
Proteinase K	Fisher Bioreagents	Cat# BP1700-500
RPMI1640	Corning	Cat#15-040-CV
Sodium chloride	Fisher Bioreagents	Cat#BP358-212
Sodium hydroxide	Fisher Chemicals	Cat#AAA1603736
Sorbitol	Fisher Scientific	Cas 50-70-4
Succinic acid	Sigma-Aldrich	Ref 398055
SYTOX Green	Life Technologies	Cat#S7020
T4 DNA ligase	NEB	M0202S
Tris hydrochloride (Tris-HCl)	AMRESCO	Cas 1185-53-1
Tris-acetate	Fisher	Cat#T3294100G
Trizma® base (Tris-Base)	Sigma Life Science	Cas 77-86-1
Zymolyase 20T (Lyticase, Yeast lytic enzyme)	United States Biological	Cas 37340-57-1
Critical commercial assays		
End-It Kit Epicenter	Lucigen	Cat#ER81050
QIAquick PCR Purification Kit	QIAGEN	Cat#28106
Experimental models: Organisms/strains		
Human EBV transformed lymphoblastoid cell line: GM03200 (Fragile X)	Coriell Institute	GM03200; RRID:CVCL_AX76
Human EBV transformed lymphoblastoid cell line: GM06990 (control)	Coriell Institute	GM06990; RRID:CVCL_9587
S. cerevisiae	Standard laboratory strains	*MATa*, *BAR1*, or *bar1*
Oligonucleotides		
See Table 1 for sequences for Illumina primers/adapters	Integrated DNA Technologies (IDT)	N/A
Software and algorithms		
Bowtie 2	(Langmead and Salzberg 2012)	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Model-based Analysis for ChIP-seq (MACS, version 2.1.1)	(Zhang et al. 2008)	https://pypi.org/project/MACS2/
OpenSCAD parametric CAD software	OpenSCAD	www.openscad.org
Picard Mark Duplicates	Broad Institute/GitHub	http://broadinstitute.github.io/picard
Samtools	(Li et al. 2009)	http://samtools.sourceforge.net/
Other		
CHEF Mapper® XA System 50-well Plug Mold	Bio-Rad	Cat#1703713
M220 Focused-ultrasonicator	Covaris	SKU500295
Microtube AFA Fiber Pre-slit Snap-Cap 6×16 mm	Covaris	Cat#520045
NanoDrop™ 2000c Spectrophotometer	Thermo Fisher Scientific	Cat#ND-2000C

Table 1. DNA sequences for Break-seq adaptors and sequencing primers

Adaptor/primer name	Sequence
Solexa_1_top	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
Solexa_1_bottom	GATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
Solexa_2_top	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Solexa_2_bottom	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC
Universal Primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
Index Primer1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTC
Index Primer2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTC
Index Primer3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTC
Index Primer4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTC
Index Primer5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTC
Index Primer6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTC
Index Primer7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTC
Index Primer8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTC
Index Primer9	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTC
Index Primer10	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTC
Index Primer11	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTC
Index Primer12	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTC
Index Primer13	CAAGCAGAAGACGGCATACGAGATATCACGGTGACTGGAGTTC
Index Primer14	CAAGCAGAAGACGGCATACGAGATCGATGTGTGACTGGAGTTC
Index Primer15	CAAGCAGAAGACGGCATACGAGATTTAGGCGTGACTGGAGTTC
Index Primer16	CAAGCAGAAGACGGCATACGAGATTGACCAGTGACTGGAGTTC
Index Primer17	CAAGCAGAAGACGGCATACGAGATACAGTGGTGACTGGAGTTC
Index Primer18	CAAGCAGAAGACGGCATACGAGATGCCAATGTGACTGGAGTTC

Highlighted in red are the index sequences for each unique primer.

MATERIALS AND EQUIPMENT

Solutions for cell culture

Yeast cell culture media

YEPD – yeast extract peptone dextrose medium (1 l)

Reagent	Add
Yeast Extract	10 *g*
Bacto Peptone	20 *g*
20% w/v D-(+)-Glucose	100 mL

SC – synthetic complete medium (1 l)

Reagent	Add
Yeast Nitrogen Base	1.45 *g*
Ammonium Sulfate	5 *g*
Succinic Acid	10 *g*
Sodium Hydroxide (NaOH)	6 *g*
Amino Acid Powder	1.4 *g*
20% w/v D-(+)-Glucose	100 mL

Mammalian/lymphoblastoid cell culture media

RPMI medium (500 mL)		
Reagent	Final concentration	Add
RPMI1640	85% v/v	415 mL
Heat inactivated FBS	15% v/v	75 mL
GlutaMAX	1% v/v	5 mL
Penicillin streptomycin solution	1% v/v	5 mL

Solutions for agarose plug processing

Note: For all solutions described here on we have only used glass distilled (gD) H_2O, though we rationalize that other high grade purified water source can substitute for gD H_2O.

0.5 M EDTA, pH 8.0 (1 l)		
Reagent	Final concentration	Add
EDTA	0.5 M	186.21 *g*
NaOH pellets	To get to pH 8.0	15 *g*
12.5 N NaOH	To get to pH 8.0	~5 mL

- To 800 mL gD H2O add 186.21 g EDTA and 15 *g* NaOH pellets.
- Let it dissolve completely.
- Adjust the pH to 8.0 with 12.5 N NaOH.
- Bring the final volume to 1 l with gD H2O.
- Filter-sterilize and store at 22°C–26°C for up to a year.

0.2 M EDTA, pH 8.0 (1 l)		
Reagent	Final concentration	Add
EDTA	0.2 M	74.45 *g*
NaOH pellets	To get to pH 8.0	6 *g*
12.5 N NaOH	To get to pH 8.0	~5 mL

- To 800 mL gD H_2O add 74.45 *g* EDTA and 6 *g* NaOH pellets.
- Let it dissolve completely.
- Adjust the pH to 8.0 with 12.5 N NaOH.
- Bring the final volume to 1 l with gD H_2O.
- Filter-sterilize and store at 22°C–26°C for up to a year.

1% w/v Incert Agarose for Yeast Cells

- To 50 mL 50 mM EDTA add 0.5 *g* of Incert low melting agarose.
- Heat the mixture in the microwave for approximately 2 min, until the agarose is completely dissolved.
- The prepared mixture should be store in a 50°C water bath until ready for casting the agarose plugs.
- The mixture can be stored at 22°C–26°C for up to a year.

1% w/v Incert Agarose for Mammalian Cells

- To 50 mL 1× PBS add 0.5 *g* of Incert low melting agarose.
- Heat the mixture in the microwave for approximately 2 min, until the agarose is completely dissolved.
- The prepared mixture should be store in a 50°C water bath until ready for casting the agarose plugs.
- The mixture can be stored at 22°C–26°C for up to a year.

Spheroplasting Solution (42.04 mL)		
Reagent	Final concentration	Add
1 M Sorbitol	0.95 M	40 mL
0.5 M EDTA, pH 8.0	19 mM	1.6 mL
1 M Tris-HCl, pH 7.5	9.5 mM	0.4 mL
β-mercaptoethanol	13.6 mM	40 μL
Zymolyase 20-T	0.59 mg/mL	25 mg

⚠ CRITICAL: Prepare spheroplasting solution fresh right before adding it to the plugs. Stock solutions including 1 M Sorbitol, 0.5 M EDTA and 1 M Tris-HCl are shelf stable and can be prepared in advance.

25% Sarkosyl

- In a hood, add 250 *g* of sarkosyl to 700 mL of autoclaved glass distilled (AgD) H_2O. Let it dissolve completely for ~ 16 h.
- Bring the final volume to 1 l with AgD H_2O.
- Aliquot and autoclave prior to storing at 22°C–26°C for up to five years.

10% Sarkosyl

- In a hood, add 100 *g* of sarkosyl to 700 mL of AgD H_2O. Let it dissolve completely for ~ 16 h.
- Bring the final volume to 1 l with AgD H_2O.
- Aliquot and autoclave prior to storing at 22°C–26°C for up to five years.
- Bring the final volume to 1 l with gD H_2O.
- Autoclave and store at 22°C–26°C for up to two years.

1× NDS solution (500 mL)		
Reagent	Final concentration	Add
10% Sarkosyl	1%	50 mL
EDTA	0.5 M	93 *g*
Tris-Base	10 mM	0.6 *g*
NaOH pellets	To get to pH >8.0	~10–15 *g*
4 N NaOH	To get to pH = 9.5	7–8 mL

- To 350 mL gD H_2O add 93 *g* EDTA and 0.6 *g* Tris-Base.
- Adjust the pH to greater than 8.0 with 100 to 200 pellets of solid NaOH.
- Add 50 mL of 10% Sarkosyl.
- Adjust pH to 9.5 with concentrated NaOH (4 N).
- Bring the final volume to 500 mL with gD H_2O.
- Filter-sterilize and store at 22°C–26°C for up to two years.

0.2× NDS Solution (1 l)

- To 500 mL gD H_2O add 200 mL of 1× NDS solution.
- Bring the final volume to 1 l with gD H_2O.
- Filter-sterilize and store at 22°C–26°C for up to two years.

LDS solution (1 l)		
Reagent	Final concentration	Add
Lithium dodecyl sulfate (LDS)	1%	10 *g*
0.5 M EDTA	100 mM	200 mL
1 M Tris-HCl, pH 8.0	10 mM	10 mL

- To 250 mL of gD H_2O, add 10 *g* LDS, 200 mL 0.5 M EDTA, pH 8.0, and 10 mL 1 M Tris-HCl, pH 8.0.
- Once dissolved, bring the volume to 1 l with gD H_2O.
- Filter-sterilize and store at 22°C–26°C for up to two years.

Lysis buffer (for mammalian cells, 50 mL)		
Reagent	Final concentration	Add
25% Sarkosyl	1%	2 mL
0.5 M EDTA	0.475 M	47.5 mL
Proteinase K	200 mg/mL	500 μL

- To 25 mL of 0.5 M EDTA add 2 mL of 25% Sarkosyl and 500 μL of 200 mg/mL Proteinase K.
- Bring the volume to 50 mL with 0.5 M EDTA.

⚠ CRITICAL: Prepare the lysis buffer right before adding it to the plugs. 25% Sarkosyl and 0.5 M EDTA are shelf stable and can be prepared in advance.

Solutions for DSB labeling and library construction

Biotinylated dNTP mix (1 mM of each dNTP, 294 μL)		
Reagent	Final concentration	Add
dTTP, dCTP, dGTP (100 mM each)	1 mM each	2.94 μl
100 mM dATP	0.83 mM	2.44 μl
0.4 mM Biotin-14-dATP	0.17 mM	125 μl

- Mix 125 μl of 0.4 mM Biotin-14-dATP, 2.44 μl of 100 mM dATP and 2.94 μl each of 100 mM dTTP, dCTP, dGTP respectively.
- Add 157.74 μl of AgD H_2O and mix properly.
- Store at −20°C for up to two years.

2× B&W buffer (200 mL)		
Reagent	Final concentration	Add
1 M Tris-HCl, pH 7.5	10 mM	2 mL
0.2 M EDTA, pH 8.0	1 mM	1 mL
5 M NaCl	2 M	80 mL

- Mix 2 mL 1 M Tris-HCl, pH 7.5, 1 mL of 200 mM EDTA, pH 8.0 and 80 mL 5 M NaCl.
- Bring the final volume to 200 mL with AgD H_2O.
- Filter-sterilize and store at 22°C–26°C for up to a year.

Making Solexa adapters

Solexa_1 (100 µL)		
Reagent	Final concentration	Add
100 µM Solexa_1_top	50 µM	50 µL
100 µM Solexa_1_bottom	50 µM	50 µL

Solexa_1 (100 µL)

- Prepare the two primers (Solexa_1_top and Solexa_1_bottom) at 100 µM in TE, pH 8.0 and store at 4°C.
- Mix equal volumes of Solexa_1_top and Solexa_1_bottom (50 µl each).
- Incubate at 95°C in a thermocycler for 5 min.
- Turn off machine at the end of the 5 min and let it cool to 22°C–26°C gradually (about 1.5 h) and store at –20°C for up to five years.

Solexa_2 (100 µL)		
Reagent	Final concentration	Add
100 µM Solexa_2_top	50 µM	50 µL
100 µM Solexa_2_bottom	50 µM	50 µL

- Prepare the two primers (Solexa_2_top and Solexa_2_bottom) at 100 µM in TE, pH 8.0 and store at 4°C.
- Mix equal volumes of Solexa_2_top and Solexa_2_bottom (50 µl each).
- Incubate at 95°C in a thermocycler for 5 min.
- Turn off machine at the end of the 5 min and let it cool to 22°C–26°C gradually (about 1.5 h) and store at –20°C for up to five years.

List of buffers that do not have special instructions-

- 50 mM EDTA
- 1 M Sorbitol
- 1 M Tris-HCl, pH 8.0
- 1 M Tris-HCl, pH 7.5
- TE pH 8.0
- TE 0.1
- 12.5 N NaOH
- 4 N NaOH
- 1 M Dithiothreitol (DTT)
- 1 M Tris Acetate, pH 7.8

3D-printed humidity chamber

A 3D-printed humidity chamber was designed to facilitate the in-gel labeling reaction and agarose plug washing while minimizing damage to the sample due to handling (Methods video S1). The chamber consists of a base with lip to control the height of the plug, a porous tray and a cap to minimize solution evaporation. A second design connects three chambers with channels to increase sample throughput and permit solution exchange via an external peristaltic pump (Methods video S2).

Table 2. Summary of 3D-printed humidity chamber components

Object	Description	Source and function call
Base	port-less chamber for humidity control and plug washing	breakseq.scad base()
Cap	top for base with handle cutout	breakseq.scad cap()
Mesh	square mesh grid for holding plug	breakseq.scad mesh()
Handle	tool for removing mesh from base	breakseq.scad handle()
Tray	alternative mesh design which incorporates handle	breakseq.scad multicell()
Multicell	three bases connected with channels for flow operations	breakseq-multi.scad multicell()
multicap	cap for multicell configuration	breakseq-multi.scad multicap()

All objects were designed using OpenSCAD parametric CAD software (www.openscad.org). The source code for the designs is provided in the supplemental information [or on github: https://www.github.com/bobthechemist/breakseq]. Objects in STL format are provided and can be printed with any 3D printer capable of printing PLA filament on a heated bed with at least a 130×130 mm^2 print surface. All objects were printed with a z-layer height of 0.2 mm without support material. A summary of the objects, their description, and the STL file link is shown in Table 2.

For the single-chamber design, a base (Figure 3A), a tray or a mesh with a handle (Figure 3B, showing the tray only), and a cap (Figure 3C) must be printed. For the multi-chamber setup, one each of the multicell and multicap (Figure 3D) are needed along with three copies of tray. Any peristaltic pump capable of incorporating 5 mm OD/3 mm ID silicone tubing can be used (Figure 3E). For tubing of different sizes, the variable *out_port_id* in *breakseq-multi.scad* should be modified and a new STL object generated. To minimize leaking, tubing should be permanently attached to 3D printed components via adhesive or epoxy. For a custom peristaltic pump controller based upon the 12 V pump from Adafruit (https://www.adafruit.com/product/1150), standard pulse width modulation circuitry using either integrated circuits or a microcontroller can be used. Priming the outflow tubing may be necessary in order to avoid overflow of the chambers.

⚠ CRITICAL: It is critical to monitor the water level in the chamber to ensure maintenance of humidity. Make sure that the end-repair reaction buffer nicely hovers over the plugs and that the lid is closed properly to maintain humidity.

Alternatives: An alternative humidity chamber can be set up using any plastic container such as a 1-mL pipette tip box (Figure 3F). Remove the insert for tips, then replace it with a small perforated tray such as the insert of a 1-mL tip box). Add approximately 50 mL of AgD H_2O to submerge the insert legs halfway. Cut a piece of 5 cm × 5 cm parafilm and use gloved hands to place it on the tray. The parafilm-covered surface will be used for placing the agarose plugs for end-repair labeling reaction (Figure 3G). It is recommended that sample names be marked on the edges of the parafilm to prevent sample mix-up. We recommend using a Covaris sonicator for fragmentation of DNA. Alternatively, we have also obtained comparable results with a probe sonicator (VWR) with the following setting: output control 4.0, duty cycle 80%, 4 pulses first, cooling on ice for 1 min, followed by 4 more pulses.

STEP-BY-STEP METHOD DETAILS

Cell culture and agarose plug preparation

⏲ Timing: 2 days for step 1.a

⏲ Timing: ~3 days for step 1.b

1. Cell culture and agarose plug preparation for yeast cells and mammalian cells
   a. Cell culture and agarose plug preparation for yeast cells

Note: In our published study we had described the application of Break-Seq to map replication inhibitor (hydroxyurea, HU)-induced DSBs in wild type (WT) and a mec1 mutant strain in Saccharomyces cerevisiae (Hoffman et al. 2015). The experimental approach—briefly summarized—entailed synchronizing cells at the G1/S transition followed by releasing them into S phase in the presence of 200 mM HU. At the first level of comparison, i.e., within the same strain, we identified DSBs enriched in HU-treated sample compared to the G1 control sample using MACS2 peak calling in both samples simultaneously. Therefore, in this section we use this approach as an example. In addition to the current approach we have also compared DSBs in replication inhibitor-treated cells to those in vehicle-treated cells in asynchronous cell cultures (I.J. and W.F., unpublished data). Importantly, in this unpublished study we have also identified DSBs in vehicle-treated cells, i.e., spontaneously DSBs, by applying single-sample peak calling in MACS2. Therefore, our experimental approach can be extended to directly comparing different strains (WT or mutant) for spontaneous or drug-induced DSB formation. In all experiments we recommend embedding at least 10^8 cells in each agarose plug to ensure successful library production. That being said, we have observed that certain conditions such as prolonged (3 h) treatment with 50 mM HU reproducibly caused low success rate of library production despite embedding > 10^8 cells per plug. In those cases we have resorted to pooling more than two agarose plugs in a single DSB-labeling reaction or increasing the cell number per plug accordingly.

i. Grow cells in either YEPD or SC medium at 30°C unless temperature-sensitive strains are used. Varying experimental conditions (*e.g.*, with or without replication inhibitor treatment or cell cycle synchronization) can be applied. For each Break-seq sample approximately 100 mL of cell culture at OD_{660} of 0.6–0.8 is recommended, for it will yield approximately 4–5 agarose plugs each containing 10^8 cells.
ii. If using synchronized cell culture, log phase cells at OD_{660} of 0.3–0.4 are synchronized in G1 by the addition of alpha factor at 200 nM or 3 mM for *bar1* or *BAR1* strains, respectively, followed by incubation for approximately 1.5 generations, or until the unbudded cell population reaches at least 90%. The cell culture would reach an OD_{660} of 0.6–0.8 at this step. Replication inhibitors can be added at this step if desired. For instance, HU (an inhibitor of ribonucleotide reductase) is added at 200 mM.
iii. Immediately following the addition of replication inhibitor drugs cells are released from the G1 arrest into S phase by the addition of pronase at 0.02 mg/mL or 0.3 mg/mL for *bar1* or *BAR1* strains, respectively. Cells are harvested at discrete times during S phase.
iv. Add NaN_3 to the harvested cells to a final concentration of 0.1% (*e.g.*, add 2 mL of 10% NaN_3 to 200 mL cells). *Optional: Remove 1 mL for flow cytometry analysis using SYTOX Green to monitor S phase progression.* Transfer the cells to a Nalgene polypropylene bottle of the appropriate size and containing frozen 0.2 M EDTA (Figures 1A–1D, *e.g.*, 40 mL of 0.2 M EDTA for 200 mL of culture).

⚠ CRITICAL: The use of frozen 0.2 M EDTA ensures rapid cooling of the cells to arrest growth and minimize potential nucleolytic degradation.

v. Centrifuge at 3000 rpm (1966 *g*) for 5 min at 4°C.
vi. Wash the cell pellets twice by adding 5–10 mL of 50 mM EDTA then centrifuging as above, twice.
vii. Resuspend the cells in 50 mM EDTA to a final concentration of 2×10^9 cells/mL and warm the cell suspension in a 45°C water bath for 5 min.
viii. Add an equal volume of 1% low-melt InCert (or SeaPlaque) agarose in 50 mM EDTA, also prewarmed to 45°C.

⚠ CRITICAL: Make sure that agarose remains warm during the plug making process to prevent hardening. Process samples one at a time and keep the remaining samples in water bath. While mixing agarose with cells by pipetting, take precaution not to generate air bubbles before pipetting into plug molds. Perform this step carefully but swiftly as the

agarose will harden in epi tube within approximately 5 min.

ix. Mix the suspension thoroughly by vortexing, then pipette 100-µL aliquots into each CHEF agarose plug mold (~10^8 cells/plug). Agarose plugs are allowed to harden at 22°C–26°C for 15 min or 4°C for 5 min.

Note: This procedure will make 0.5% agarose, which is fairly fragile, but is crucial for in-gel enzymatic reactions such as end-repair.

x. Extrude the agarose plugs from the mold into the wells of a six-well cell culture plate (≤ 3 plugs per well).
xi. Add 6 mL of freshly prepared Spheroplasting Solution to each well. Incubate at 37°C for 4 h with constant but gentle shaking (abbreviated as "shaking" below).
xii. Aspirate the Spheroplasting Solution.
xii. Add 6 mL of LDS Solution. Incubate at 37°C with shaking for 15 min.
xiv. Aspirate the LDS Solution, then add 6 mL of fresh LDS Solution and incubate with shaking at 37°C for ~ 16 h.
xv. Next day, wash the agarose plugs twice by incubating with 6 mL of 0.2 X NDS with shaking at 22°C–26°C for 30 min each time.
xvi. Wash the agarose plugs thrice by incubating with 6 mL of TE pH 8.0 with shaking at 22°C–26°C for 30 min each time.

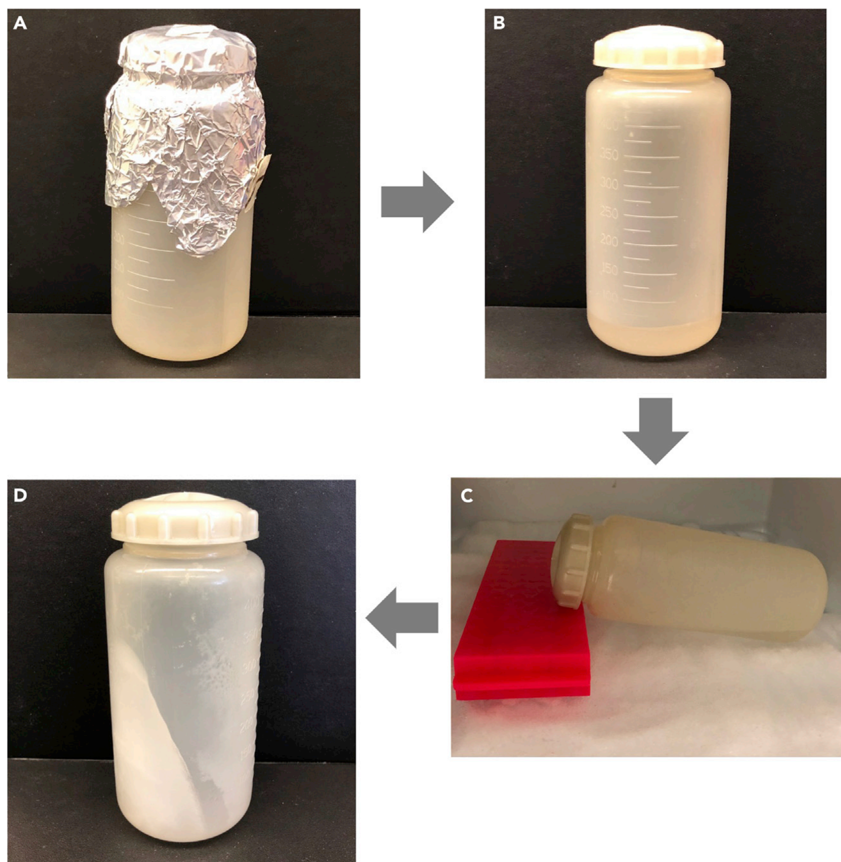

Figure 1. Preparation of frozen EDTA solution for harvesting yeast culture
An autoclaved 500-mL Nalgene bottle (A) is used to prepare 40 mL frozen 0.2 M EDTA by storage in a -20°C freezer (B–D).

xvii. Agarose plugs can be stored at 4°C in the six-well plate while submerged in TE pH 8.0 for up to 6 months. To prevent evaporation the plate is wrapped in plastic. During long term storage change the buffer TE pH 8.0 every month.

Note: We have constructed Break-Seq libraries with success from samples stored for up to a year. Results were consistent with technical and biological replicates prepared a year prior.

b. Cell culture and agarose plug preparation for mammalian cells

Note: To date, we have published DSB mapping in human lymphoblastoid cells with and without replication stress induced by aphidicolin (a DNA polymerase inhibitor) dissolved in dimethylsulfoxide (DMSO) (Chakraborty et al. 2020). Below we describe this approach in details as an example. In addition, we have successfully mapped spontaneous DSBs (no drug treatment) in breast epithelial cell lines (MCF-7 and MCF-10A) as well as 3D organoid cultures of primary cells derived from patients with aggressive prostate cancer (W.F. and N. Wagaman, unpublished data). Therefore, we believe Break-Seq is widely compatible with different cell types as well as treatment conditions. Regardless the cell type we aim to produce at least 10^6 cells per agarose plug. Adjust the starting cell numbers erring for up to 20% loss during processing.

i. Grow the lymphoblastoid cells to exponential phase at a concentration of 5×10^5 cells/mL. For adherent cells grow to up to 80% confluence. After the aphidicolin incubation step (see below) the cell concentration would be ~10^6 cells/mL. We aim for 10^6 cells per agarose plug. Therefore, for each sample we require at least 5 mL of cell culture, which will produce 2 agarose plugs erring for loss during washes.
ii. Remove 1 mL of cells for flow cytometry analysis.

Note: This step is similar to the one described above with yeast cells except that mammalian cells are stained with propidium iodide.

iii. Aliquot the remaining cells. To experimental samples add aphidicolin to a final concentration of 0.2–0.6 μM. To a mock control sample add equal volume of DMSO, the solvent for aphidicolin stock solution.
iv. Harvest cells after 24 h incubation by centrifugation at 800 rpm (140 *g*) for 8 min at 22°C–26°C . Remove 1 mL before centrifugation for flow cytometry analysis.
v. Wash each cell pellet from 5 mL of cell culture with 2 mL of ice-cold 1× PBS buffer. Scale up if more cells are used. Spin down at 800 rpm (140 *g*) for 8 min at 22°C–26°C .
vi. Resuspend the cells in 500 μL ice-cold 1× PBS and transfer cell suspension to an epi tube.
vii. Spin down at 2000 rpm (874 g) in a table-top microcentrifuge at 4°C.
viii. Resuspend the cells in 100 μL of ice-cold 1× PBS to a final concentration of ~3×10^7 cells/mL.
ix. Make 1% low melting agarose in 1× PBS on hot plate. Equilibrate the solution in a 43°C water bath. Equilibrate the cells at 43°C as well for 5 min.
x. Add equal volume of 1% agarose to cells, mix and cast in a CHEF agarose plug mold. Cool the agarose plugs on ice for 10–15 min.

Note: Agarose plugs containing mammalian cells are translucent and are slicker and more fragile than those containing yeast cells (Figure 2). Plugs with yeast cells will become translucent after cell lysis.

⚠ CRITICAL: Make sure that agarose remains warm during the plug making process to prevent hardening. Process samples one at a time and keep the remaining samples in water bath. While mixing agarose with cells by pipetting, take precaution not to generate air bubbles before pipetting into plug molds (Figure 2).

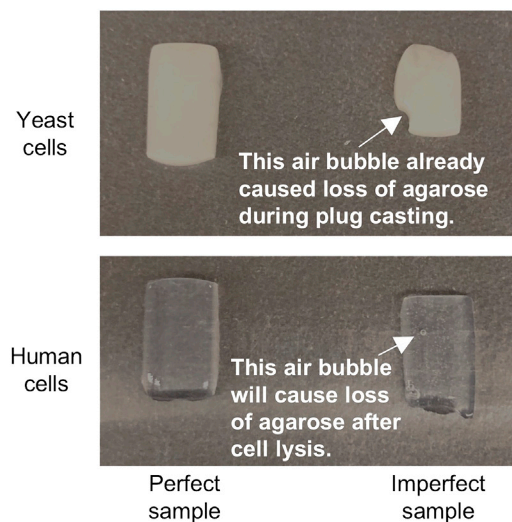

Figure 2. Comparison of agarose plugs prepared from yeast versus human cells

Demonstration of air bubbles trapped in agarose causing structural damage to the agarose plugs during and after plug casting, respectively.

xi. Extrude the plugs into 6 mL of lysis buffer in a 50 mL falcon tube. Incubate at 50°C for ~ 16 h.

xii. Change the lysis buffer by removing plugs from the old solution and transferring them into a new falcon tube with fresh lysis buffer. Incubate at 50°C for ~ 16 h again.

Note: Handle with care as agarose plugs will become transparent and might fall out with buffer if poured.

xiii. Wash the agarose plugs by incubating in 6 mL of TE pH 8.0 at 22°C–26°C for 1 h.

xiv. Repeat step 1.b.xiii (above) twice. Store agarose plugs in 6 mL of TE pH 8.0 at 4°C for ~ 16 h. Change for fresh TE pH 8.0 every month during long term storage.

DSB labeling and library construction

Timing: 5 h for step 2

Timing: 2 h for step 3

Timing: 1 h for step 4

Timing: 50 min for step 5

Timing: 40 min for step 6

Timing: 1 h for step 7

Timing: 16–18 h for step 8

Timing: 1 h for step 9

Timing: ~16 h for step 10

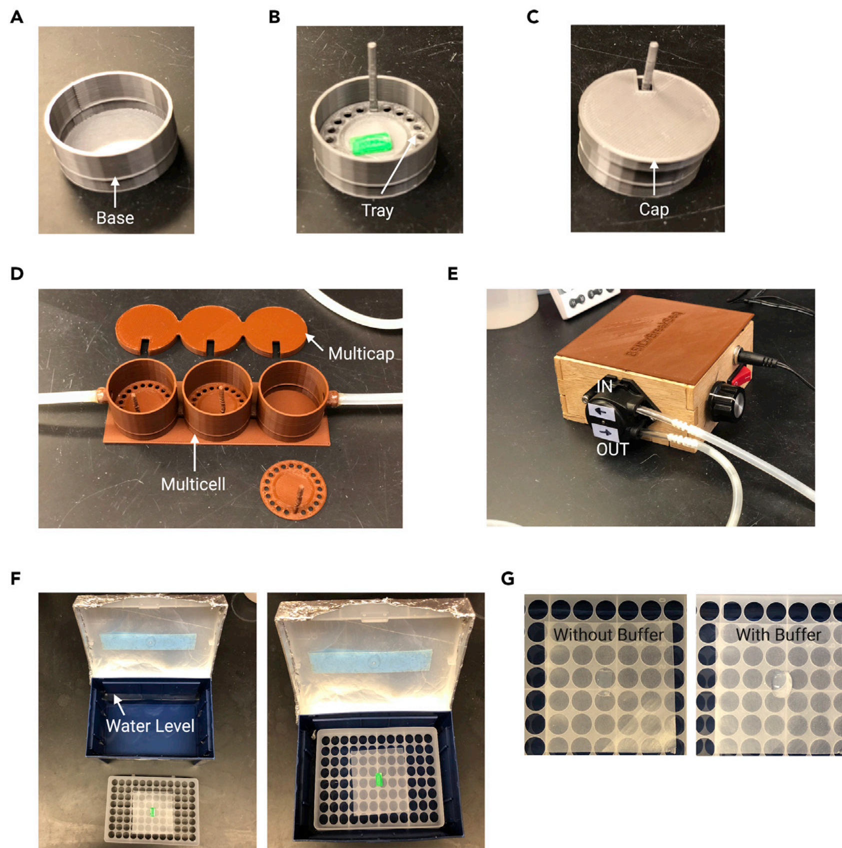

Figure 3. Humidity chamber(s) used for processing agarose plugs during labeling reactions and subsequent washing
(A–F) The figure shows a 3D printed design (A–E) and a jerry-rigged design if a 3D printer is not available (F). The components of the 3D printed humidity chamber include: a base which holds the buffer (A), a porous tray with a handle where the agarose plug (3D printed to life size 0.5 cm × 1 cm × 0.1 cm in green color) is placed, which fits into the base (B), a cap for the base and tray for maintaining humidity (C). (D) A multi-chamber design which contains three bases fitted with three individual trays and caps and connected to a pump. (E) A peristaltic pump which circulates solution through the multi-chambers. The directions of influx and efflux, are marked as "in" and "out", respectively. (F) A jerry-rigged humidity chamber using a 1-mL tip box as the base and the insert from a 1-mL tip box as the tray.
(G) A real agarose plug placed in the center of the parafilm laid on the platform of the jerry-rigged humidity chamber, without (left) and with (right) 100 μL End-repair reaction buffer. Note that the buffer surrounds the agarose plug and stays "hovered" with surface tension.

2. In-gel labeling of DSBs by end-repair

Note: The 3D printed humidity chamber will be used in the subsequent steps for preparing/washing the agarose plugs as well as for end-repair incubation in step 2.g. While using it for preparing/washing plugs, the plugs rest directly on the mesh and are submerged in the buffer. While using it for end-repair labeling the agarose plugs are placed on a piece of 2-cm diameter parafilm and the water level is just below the mesh as shown (Video 1). If not using the 3D printed device the agarose plugs can be washed in a six-well plate and the humidity chamber can be set up as described in Materials and Equipment, under "Alternatives".

a. Make 10× stock reaction buffer by adding the following reagents to 20 mL of AgD H_2O:

Reagent	Final concentration	Add
1 M Tris-Acetate pH 7.8	33 mM	16.5 mL
Potassium Acetate	66 mM	3.24 *g*
Magnesium Acetate	10 mM	1.07 *g*
1 M DTT	0.5 mM	250 μL

b. Bring the volume to 50 mL with AgD H_2O.
c. Transfer agarose plugs to a new six-well plate, one plug per well or per humidity chamber.

Note: If using the multi-chamber design substitute the washing steps below by connecting the multi-chamber base to the peristaltic pump and flow 50-mL of the respective buffer through the chambers. Adjust the pump speed to exhaust the 50-mL buffer in approximately 5 min.

d. Wash each plug twice with 5 mL TE 0.1 with rocking at 22°C–26°C for 15 min each.
e. Make 1× reaction buffer from 10× stock reaction buffer. Wash each plug twice with 5 mL 1× reaction buffer with rocking at 22°C–26°C for 30 min each.
f. During the last wash, prepare labeling reaction mix:

Reagent	Volume (μL)
End-repair buffer (10×)	10
Biotinylated dNTP mix	10
10 mM ATP	10
End-It enzyme mix	3
AgD H_2O	67

Note: We estimate that the maximal volume of each agarose plug is 100 μL. Thus, each 100 μL labeling reaction is intended for a single agarose plug. Scale up accordingly with multiple agarose plugs. For recipe of dNTP mix containing Biotin-14-dATP, refer to the beginning of the protocol. All other reagents herein are provided by the End-It kit.

g. Set up the labeling reaction by adding 100 μL of labeling mix to each plug in the humidity chamber. Add the reaction mixture dropwise on top of each plug and let it hover all over the plug. Close the lid on the humidity chamber.

Note: Before adding the labeling reaction mix to the plugs, dab-dry the plugs with the corner of a Kimwipe to remove any residual buffer from the previous step.

h. Incubate the humidity chamber containing agarose plugs in a 25°C incubator for at least 1.5 h.

Note: Longer incubation time up to 2.5 h is preferred.

i. Wash twice with 5 mL TE 0.1 with rocking at 22°C–26°C for 15 min each.
j. Wash/Equilibrate twice with 200 μL 1× β-Agarase buffer on ice for 30 min each.

Note: Set up ice bath in a large container such as a 20-liter sterilization pan. Place the lid of the six-well plate on ice as the tray to hold the plugs. Line the lid with parafilm and place plugs on the parafilm. The β-Agarase buffer would hover over the plug. Use a glass plate as a lid for protection.

3. Agarose digestion and release of chromosomal DNA
   a. Transfer each plug to an epi tube.

b. Melt the agarose by incubating in a 70°C water bath for 10 min and mix occasionally by flicking the tube.
c. Transfer the epi tube to a 42°C water bath and incubate for 5 min.
d. Add 3 μL β-Agarase to each plug and incubate at 42°C for 1 h.
e. Centrifuge at 13500 rpm (39803 *g*) for 30–60 sec to pellet the undigested agarose. Transfer the supernatant (containing DNA) to a new epi tube.

⚠ CRITICAL: It is important to ensure that all the agarose is digested/separated from the DNA before proceeding further as the leftover DNA tends to interfere with sonication.

Note: (Optional) Add 100 μL β-Agarase buffer to the pelleted agarose, repeat steps 3.d–3.e to increase yield.

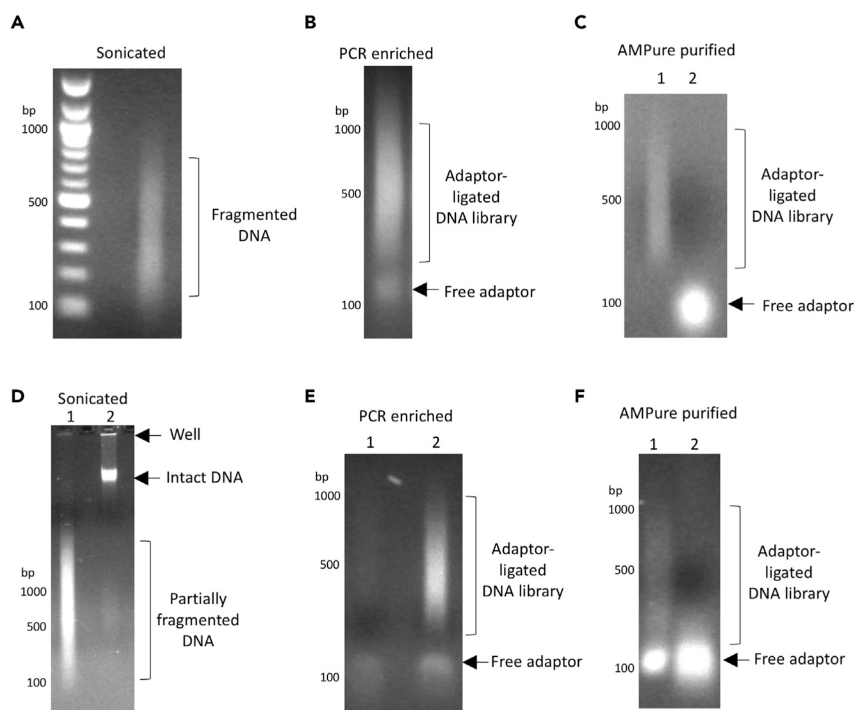

Figure 4. Exemplary agarose gel images of DNA resulted from step 4.h, step 9.d, and step 10.p for the same sample and examples for problems described in Problem 2

(A) Fragmented DNA to ~400 bp average size after sonication. The 100-bp DNA ladder is shown in (A), and only marked for other panels for clarity.

(B) PCR amplified DNA after adapter ligation (note the shift of average size of DNA to 500–600 bp) with noticeable level of free adapters after PCR.

(C) AMPure purified DNA library (lane 1) and flow-through containing free adapters (lane 2). Examples for problems described in Problem 2 are also presented (D-F).

(D) Unfragmented DNA after sonication. Lane 1, a sample with partially fragmented DNA; lane 2, a sample with no fragmentation at all.

(E) Low level of PCR product. Lane 1, a sample with low level of amplification of DNA. Lane 2, a sample with normal level of amplification of DNA as comparison.

(F) Retention of free adapters in the DNA library after AMPure purification . Lane 1, a sample after AMPure purification. Lane 2, flow-through of the sample in lane 1 after AMPure purification.

4. Fragmentation of chromosomal DNA by sonication (using Covaris M220)
   a. Transfer 100 μL of the β-Agarase-digested DNA sample from step 3.e above into a Covaris snap-cap tube (microtube AFA Fiber Pre-slit Snap-Cap 6×16 mm, Covaris #520045). Typically, there is excess volume of DNA sample from step 3.e, then transfer the excess volume into a second Covaris sanp-cap tube. Sonicate using the preloaded protocol from Covaris named "DNA_300_bp_130_μL_Snap_Cap_microtube".
   b. Transfer sonicated DNA to a new epi tube.
   c. Pool DNA from the same sample from multiple tubes in step 4.a. Transfer any remaining β-Agarase-digested DNA from step 3 to the same snap-cap tube above.
   d. Repeat sonication using the same preloaded protocol above.
   e. Pool the sonicated DNA from the same sample into the epi tube above. Repeat for all samples.
   f. Analyze 5 μL of DNA by electrophoresis on a 1.2% agarose gel to confirm the shearing of DNA to an average size of < 500 bp (Figure 4A).

   Note: Typically, we observe 400 bp average size. Please refer to the troubleshooting section for potential problems encountered with DNA sonication.
   g. Use Qiagen PCR cleanup kit to purify the DNA by following manufacturer's instructions. Elute each DNA sample with 50 μL of Elution Buffer (EB) provided by the kit.

   Note: If the DNA quantity is much greater than 20 μg then multiple columns would be required. The maximum load we have tried is 15 μg per column without significant loss. The DNA can also be ethanol-precipitated instead of column purification. For ethanol precipitation add 10% volume of 3M Sodium Acetate (NaAc) pH 5.0 followed by two volumes of ice cold 100% ethanol to one volume of sonicated DNA. For 200 μL of sonicated DNA add 20 μL of NaAc and 400 μL of 100% ethanol. Incubate at −80°C for ~ 16 h and centrifuge to collect DNA on the next day.
   h. Measure DNA concentration using double stranded DNA setting on a NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific). Estimate potential loss rate during purification for future reference.

   Pause point: The samples can be stored for ~ 16 h at 4°C at this point.

5. End-repair of fragmented DNA
   a. Mix the sonicated/sheared DNA with the following reagents:

Reagents	Volume (μL)
Sheared DNA (8–11 μg of DNA)	67
10 X End-repair Buffer	10
2.5 mM dNTP mix	10
10 mM ATP	10
End-It enzymes	3

   b. Mix well and incubate at in a 25°C incubator for 45 min.
   c. Purify through a Qiagen PCR purification column. Elute DNA in 43 μL of EB. Measure DNA concentration using up to 1 μL of eluted DNA on a NanoDrop and use the remaining 42 μL for A-tailing.

6. A-tailing
   a. Mix the end-repaired DNA from the previous step with the following:

Reagents	Volume (μL)
DNA (end-repaired)	42
10× Klenow reaction buffer	5
Klenow enzyme	3

b. Mix well and incubate at 37°C for 30 min.
c. Purify through a Qiagen PCR purification column. Elute DNA in 52 μL EB.
d. Measure DNA concentration using up to 2 μL of eluted DNA on a NanoDrop. Calculate the amount of INPUT DNA from the remaining 50 μL prior to streptavidin purification below.

Note: We typically obtain 2.5 - 5 μg of INPUT DNA per sample. When the DNA amount falls below 1 μg it may result in too little bound DNA and prevent successful DNA library construction.

7. Capturing labeled DNA by Streptavidin beads
   a. Add 50 μL EB to each DNA sample above to make up the final volume of 100 μL.
   b. Mix the M270 Dynabeads suspension (10 mg/mL) well by swirling the bottle. Pipette 50 μL of Dynabeads suspension per sample into an epi tube situated on a magnet holder and let it sit for 2 min at 22°C–26°C before removing the supernatant.

⚠ CRITICAL: Use a low-retention microcentrifuge tube to minimize sticking of magnetic Dynabeads to the plastic.

Note: The binding capacity of the Dynabeads for dsDNA is ~10 μg/mg beads. Assuming each agarose plug contains 5 μg of DNA with 10% biotinylated DNA (see below), one needs 5 μL of Dynabeads. Thus, using 50 μL of beads per agarose plug exceeds the capacity by approximately ten-fold.

   c. Add 100 μL 1× B&W buffer, mix by pipetting 12 times.
   d. Place the epi tube on magnet and let it sit for 2 min at 22°C–26°C before removing the supernatant.
   e. Repeat steps 7.c and 7.d twice.
   f. Add 100 μL (twice the original volume, final concentration of Dynabeads 5 mg/mL) 2× B&W buffer, mix by pipetting 12 times.
   g. Add equal volume (100 μL) of DNA, mix by pipetting 12 times.
   h. Incubate at 22°C–26°C on a rotator for 30 min.
   i. Place the epi tube on magnet and let sit at 22°C–26°C for 2 min.
   j. Transfer the supernatant to a new epi tube and measure the concentration on a NanoDrop to calculate the amount of FLOWTHROUGH DNA.
   k. Calculate the amount of BOUND DNA and thus, biotinylated DNA, as follows:

BOUND DNA = INPUT DNA (step 6.d) – FLOWTHROUGH DNA (step 7.j)

Typically, we obtain 1–1.5 mg BOUND DNA, approximately 10% of INPUT DNA.
   l. Wash the Dynabeads twice with 100 μL 1× B&W buffer.
   m. Wash the Dynabeads twice with 100 μL EB.
   n. Resuspend Dynabeads in ligation mix (see the next step).

8. Adapter ligation to (streptavidin-)BOUND DNA
   a. Mix the following reagents and add to the Dynabeads from the previous step.

Reagents	Volume (μL)
50 μM Solexa_1	3
50 μM Solexa_2	3
10× T4 ligase buffer	5
T4 DNA ligase	3
AgD H_2O	36

b. Mix well and incubate at 22°C–26°C for ~ 16 h on a rotator.
c. Next day, wash twice with 200 µL 1× B&W buffer.
d. Wash twice with 200 µL EB.
e. Resuspend in 44 µL EB.

9. PCR amplification of labeled DNA
   a. Take 22 µL of the above DNA/Dynabead mix for each PCR using the KAPA HiFi HotStart ReadyMix.

   Note: As described above we typically obtain 1 – 1.5 µg of BOUND DNA on the Dynabeads. However, in certain conditions it could be as low as 200 ng. When the BOUND DNA is less than 500 ng, use the entire 44 µL of DNA/Dynabead mix for each PCR.
   b. PCR Reaction Mix (50 µL):

Reagents	Volume (µL)
DNA/bead mix	22
2× KAPA HiFi HotStart Ready Mix	25
Universal Primer (10 µM)	1.5
Unique Index Primer (10 µM)	1.5

   c. PCR amplify the DNA using the following PCR conditions:

Reaction conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	5 min	1
Denaturation	98°C	20 sec	18
Annealing	65°C	15 sec	
Extension	72°C	1 min	
Final Extension	72°C	5 min	
Hold	4°C	Forever	

   d. Transfer the PCR product into an epi tube, place the tube on the magnet and let sit for 2 min at 22°C–26°C . Analyze 4 µL of the supernatant by electrophoresis on a 1.5% agarose gel (Figure 4B). Pipette the rest of the supernatant into a fresh epi tube.

   Pause point: The samples can be stored at 4°C for ~ 16 h at this point.

10. Free adapter removal by AMPure purification
   a. Swirl the AMPure bottle to resuspend the magnetic particles.
   b. Use 1.8 volumes of AMPure beads per 1 volume of DNA solution (81 µL for each 50 µL PCR reaction). Transfer beads to PCR product in a low retention epi tube.
   c. Mix the AMPure beads and PCR product thoroughly by pipette mixing 10 times and vortexing for 10 sec.
   d. Incubate at 22°C–26°C for 10 min, with frequent mixing by vortexing.

   ⚠ CRITICAL: Frequent vortexing of the bead mix is important to avoid settling of the AMPure beads to the bottom of the tube.

   Note: This step is important to ensure sufficient binding of DNA to the beads. Do not put the mix on the magnet right away.
   e. Place the epi tube on the magnet and let sit for 5 min.

f. Transfer the supernatant to a fresh epi tube to save as flow-through for later analysis.
g. Ethanol-precipitate the flow-through as described above.
h. Resuspend the ethanol-precipitated DNA pellet in 10 μL of TE 0.1.
i. Without removing the tube from the magnet, add 200 μL of 70% ethanol onto the beads and incubate at 22°C–26°C for 30 sec. Discard the supernatant.

Note: Slowly drip the 70% ethanol onto the magnetic beads to wash them sufficiently.

j. Repeat step 10.i.
k. Leave the epi tube cap open to air dry for 20 min.

⚠ CRITICAL: This duration has been empirically determined in our lab environment. We recommend setting a timer for 20 min and then check the sample every 5 min. Once the beads appear dry we also double check by sniffing to ensure complete removal of ethanol. In most cases, 20 min is sufficient to allow ethanol to dissipate completely. Occasionally, we had to lengthen the drying period to up to 25 min.

l. Elute DNA by adding 40 μL of AgD H_2O or TE 0.1 and pipette mix 10 times and vortex for 10 sec.
m. Incubate at 22°C–26°C for 10 min with frequent mixing by vortexing.

Note: Lengthen the incubation to ~16 h at 4°C to increase binding of free adaptors to the beads.

n. Place the epi tube on magnet and let sit for 5 min.
o. Transfer the supernatant to a new epi tube. This resulting Break-seq DNA library should be stored at −20°C.
p. Analyze 1 μL of the DNA library as well as all of (10 μL) the adapter flow-through from step 10.h by electrophoresis on a 1.0% agarose gel (Figure 4C).

⚠ CRITICAL: Repeat steps 10.a–10.p if the free adaptors in the DNA library persist. Excessive free adaptors present in the final library might interfere with the sequencing reaction.

EXPECTED OUTCOMES

The amount of DNA extracted from each agarose plug post β-Agarase digestion, sonication and Qiagen PCR column purification (step 4.h) is 100–200 ng/μL. The expected concentration of DNA per sample post AMPure step (step 10.p) is in the range of 10 ng/μL to 40 ng/μL. We have obtained as low as 6 ng/μL and as high as 150 ng/μL. For HiSeq experiment the recommended minimal amount of DNA for sequencing is 50 ng per sample. For each Break-seq library we aim to obtain at least 20 million reads for yeast cells and 50 million for human cells.

QUANTIFICATION AND STATISTICAL ANALYSIS

Break-seq libraries were sequenced on Illumina HiSeq2500 (paired-end 150 bp) in multiplexed format. They can also be sequenced on the NovaSeq 6000 and NextSeq 1000/2000 platforms. The obtained fastq files were processed as follows:

1. Sequence reads from the fastq files were aligned to the reference genome using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml).

Command: bowtie2 -q -p 8 –local –fr -x REFERENCEGENOME −1 File_R1.fastq -2 File_R2.fastq -S File.sam

2. SAM files were then converted to BAM files using SAMtools (http://samtools.sourceforge.net/).

Command: Samtools view -Sb File.sam > File.bam

3. SAM files were then further processed using Picard tools (https://broadinstitute.github.io/picard/) to specifically select for unique and non-redundant reads and eliminate PCR duplicates. This was done using options -F 1024 and -f 2 in the Picard toolbox. The generated files containing unique and non-redundant reads bear the extension "pp.non-redundant.bam", which will be used in the subsequent steps.

Commands:

```
java -Xmx2g -jar picard.jar SortSam INPUT=File.bam OUTPUT=File.Sorted.bam SORT _ORDER=coordinate CREATE_INDEX=true VALIDATION_STRINGENCY=SILENT

java -Xmx2g -jar picard.jar MarkDuplicates INPUT=File.Sorted.bam OUTPUT=File.mskDup.bam METRICS_FILE=bam.log VALIDATION_STRINGENCY=SILENT ASSUME_SORTED=true

samtools view -bh -F 1024 File.mskDup.bam > File.mskDup.nr.bam

samtools view -bh -F 1024 -f 2 File.mskDup.nr.bam > File.pp.non-redundant.bam
```

4. The BAM files obtained after selecting for unique reads and removing redundant reads was then used to identify DSBs using Model-based Analysis for ChIP-seq (MACS2 version 2.2.6, https://github.com/macs3-project/MACS). See below for details. At this stage, it is recommended that the Break-Seq library complexity be calculated from the BAM files by preseq (Daley and Smith, 2013). We have rejected any library that has < 70% properly-paired reads from all sequence reads.

5. DSB hotspots could be identified within the Break-seq library from a test (*e.g.*, replication inhibitor-treated) sample alone, or as enriched DSBs in the Break-seq library from an experimental sample normalized to a control. The control can be a Break-seq library from an untreated sample, or a whole genome DNA-seq library from the same test sample. The latter provides a DNA copy number control for Break-seq signals. MACS2 commands for these different analyses are issued as follows with the yeast samples as an example:
   a. Break-seq from test sample alone
      Command: macs2 callpeak -t TEST.pp.non-redundant.bam –outdir PATHNAMEFOROUTDIRECTORY -f "BAMPE" -g REFERENCEGENOMESIZE –seed 1 -B –keep-dup all
   b. Break-seq from test sample normalized to Break-seq from control sample
      Command: macs2 callpeak -t TEST.pp.non-redundant.bam -c CONTROL.pp.non-redundant.bam –outdir PATHNAMEFOROUTDIRECTORY -f "BAMPE" -g REFERENCEGENOMESIZE –seed 1 B –keep-dup all
   c. Break-seq from test sample normalized to DNA-seq from test sample
      Command: macs2 callpeak -t TEST.pp.non-redundant.bam -c DNASEQTEST.pp.non-redundant.bam –outdir PATHNAMEFOROUTDIRECTORY -f "BAMPE" -g REFERENCEGENOMESIZE –seed 1 -B –keep-dup all
   d. Copy-number normalized Break-seq from test sample compared to copy-number normalized Break-seq from control sample

   Note: This step uses the pileup files generated from step 5.c above wherein we have normalized each Break-seq file to the DNA-seq file from the same sample to adjust for copy number variation.

Command: macs2 bdgdiff –t1 TEST.Breakseq.pileup.bdg –c1 TEST.DNAseq.lambda.bdg –t2 CONTROL.Break-seq.pileup.bdg –c2 CONTROL.DNAseq.lambda.bdg –d1 EffectiveSequencingDepthForTEST –d2 EffectiveSequencingDepthForCONTROL -g 60 -l 120 –o-prefix diff

6. For each sample we perform at least three biological replicate experiments, i.e., derived from three independent cell cultures. Within each cell culture, a given sample is processed for multiple agarose plugs, which then serve as technical replicate samples. Break-Seq DNA libraries derived from these replicates are processed identically through the previous 5 steps in this section, deriving a DSB location file (in bed or xlsx format by MACS2) for each replicate. These DSB peak files are then analyzed by DiffBind (Ross-Innes et al. 2012) for consensus DSB peak identification. Consensus DSB peaks are defined as those that appear in multiple replicate experiments, using criteria set by the user depending on the total number of replicates, *e.g.*, at least 2 out of 3 replicates, at least 3 out of 4 replicates, etc.

LIMITATIONS

We have tested Break-seq in multiple model systems including yeast, human lymphoblasts and fibroblasts, and human cultured organoids with success. In all cases we require >10^6 cells and we have not attempted constructing Break-seq libraries with fewer cells.

TROUBLESHOOTING

Problem 1

The concentration of the eluted DNA after β-Agarase digestion of agarose plug is very low.

Potential solution

The overall DNA quantity can be improved by increasing the number of cells embedded in agarose. The optimal number of cells should be determined empirically based on the cell type/strain. We have also pooled DNA from multiple agarose plugs (technical replicates from the same sample) after end-repair for a single Break-seq library successfully.

Problem 2

The DNA did not fragment well after sonication (Figure 4D, lanes 1&2).

Potential solution

This problem could arise as a result of inefficient β-Agarase digestion of the agarose. The undigested agarose might interfere with the sonication process. In this case we recommend a pulse spin at the highest speed in a tabletop centrifuge to rid the DNA of residual agarose, followed by another round of sonication.

Problem 3

The amount of DNA retained on beads (BOUND DNA from step 8.e) is low.

Potential solution

We typically obtain 1 – 1.5 μg of BOUND DNA on the Dynabeads. However, in certain conditions it could be as low as 200 ng. When the BOUND DNA is less than 500 ng, use the entire 44 μL of DNA/Dynabead mix for PCR by splitting it into two PCR reactions with the same index primer. The two reactions will then be combined at the AMPure purification (step 10.b). We do not recommend a secondary PCR using the primary PCR product to increase yield, which might impact the complexity of the Break-Seq library.

Problem 4

The amount of DNA after PCR is low (Figure 4E, lane 1).

Potential solution

This problem is often due to deficiency of the template BOUND DNA in the reaction, possibly as a result of erroneous quantification of the FLOWTHROUGH DNA. Increase the amount of template DNA in a new PCR.

Problem 5

High level of free adapters in the final library.

Potential solution

We have observed persistent retention of free adapters from the PCR product even after a second round of adapter removal by AMPure beads (Figure 4F, lane 1). This problem could be ameliorated by increasing the incubation time of the PCR enriched DNA with the AMPure beads from 10 min to for ~ 16 h at 4°C. If the problem persists, we recommend repeating the PCR by using increased amount of template DNA (bound to Dynabeads).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wenyi Feng (fengw@upstate.edu).

Materials availability

3D printed device used for agarose plug processing in this study is made by Robert LeSuer at SUNY Brockport. The source code for the designs is provided in the supplemental information [or on github: https://www.github.com/bobthechemist/breakseq].

Data and code availability

Select Break-seq datasets generated by the protocol have been published previously (Hoffman et al. 2015, Chakraborty et al. 2020). Additional datasets will be published elsewhere.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100554.

ACKNOWLEDGMENTS

We would like to thank members of the Feng Lab for support and helpful discussions. We thank V. Van Steenkist for systems support and P. Jenjaroenpun for sharing shell scripts for Break-seq analysis. This work was supported by the National Institutes of Health grants 5R00GM08137805 and 5R01-GM118799 and the Department of Defense CDMRP Discovery Award W81XWH-15-1-0204 to W.F.

AUTHOR CONTRIBUTIONS

W.F. conceptualized and designed the method. I.J. performed the experiments and standardized the method. R.L., J.D., and M.P. designed and produced the 3D-printed device.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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