

Jan 12, 2026

iSNS-seq protocol

DOI

<https://dx.doi.org/10.17504/protocols.io.kxygx4xo4l8j/v1>

Miiko Sokka¹, John M. Urban², nicola_neretti¹, Susan A. Gerbi¹

¹Brown University, Department of Molecular Biology, Cell Biology, and Biochemistry;

²Carnegie Institution for Science, Department of Embryology

iSNS-seq



Miiko Sokka

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Protocol Citation: Miiko Sokka, John M. Urban, nicola_neretti, Susan A. Gerbi 2026. iSNS-seq protocol. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.kxygx4xo4l8j/v1>

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Protocol status: Working

We use this protocol and it's working

Created: November 08, 2025

Last Modified: January 12, 2026

Protocol Integer ID: 231856

Keywords: SNS-seq, NS-seq, lambda-exonuclease, DNA replication origin mapping, seq protocol, dna replication origin detection, seq, protocol, improved short nascent strand, sequencing, dna

Funders Acknowledgements:

Susan Gerbi

Grant ID: GM121455

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Abstract

This is an improved short nascent strand sequencing (iSNS-seq) protocol used in the **Improved short nascent strand sequencing (iSNS-seq) enhances DNA replication origin detection and reduces non-origin biases** (pre-print <https://doi.org/10.64898/2025.12.22.696044>).

Materials

Three 500ml cultures of *Saccharomyces cerevisiae* yeast

YPAD (2% Peptone, 1% Yeast Extract, 2% Dextrose, 40ug/ml Adenine Sulfate)

EDTA-Glycerol (133mM EDTA-33% Glycerol)

10% Na-Azide

GSA bottles for centrifuging cells

SCE (1M Sorbitol, 100mM Na-Citrate pH7.2, 10mM EDTA)

Zymolyase 100T USBiologicals (Z1005)

SCE-Zymolyase master mix

20U/ul SUPERaseIn RNase Inhibitor Thermo AM2694

DNAzol (Invitrogen, 10503-027)

20 mg/ml proteinase K (Qiagen, 19131)

100% EtOH

80% EtOH

- TEN20 (50mM Tris pH8, 50mM EDTA, 20mM NaCl)
- phenol-chloroform
- 5M NaCl
- 2-propanol
- TE
- Qubit dsDNA BR
- TEN500 (50mM Tris pH8, 50mM EDTA, 500mM NaCl)
- 5%-30% Sucrose in TEN500
- SW41 polyclear tubes
- UPW
- 3M NaOAc
- PC-152
- AGE buffer
- SYBR Gold
- dsDNA 1kb ladder
- Amicon Ultra 10k filter
- NaOAc
- 100mM Tris pH8.0
- 1xNEBuffer2
- RecJF
- 10xPNKbuffer
- ATP
- T4 PNK
- 0.5M EDTA
- 2.5M NaOH
- 2.5M Acetic Acid
- AMPure beads

- 10xLMT
- Ilexo (NEB)
- Covaris S220
- NEBNext Ultra II End Prep Reaction Buffer
- NEBNext Ultra II End Prep Enzyme Mix
- USER Enzyme
- NEBNext Ultra II Ligation Master Mix
- NEBNext Ligation Enhancer
- NEBNext Adaptor for Illumina
- 10uM NEBNext Universal PCR Primer for Illumina
- 10uM NEBNext Index Primer for Illumina
- 2x SYBR Green Master Mix
- NEBNext Library Quant Kit for Illumina
- W303 yeast cells (from Joachim Lee)
- YPAD (2% Peptone, 1% Yeast Extract, 2% Dextrose, 40ug/ml Adenine Sulfate)
- EDTA-Glycerol (133mM EDTA-33% Glycerol)
- 10% Na-Azide
- Zymolyase 100T USBiologicals (Z1005)
- SCE (1M Sorbitol, 100mM Na-Citrate pH7.2, 10mM EDTA)
- SUPERaseIn RNase Inhibitor Thermo AM2694
- DNazol (Invitrogen, 10503-027)
- 20 mg/ml proteinase K (Qiagen, 19131)
- 1M Urea TE
- TEN500U (50mM Tris pH8, 50mM EDTA, 500mM NaCl, 1M Urea)
- 3M NaOAc
- NaOH
- Sodium Acetate
- ssRNA ladder (NEB N0362S)
- PC-152 Polyacryl Carrier (MRC)
- Amicon Ultra 10k 15ml (Millipore-Sigma, UFC901024)
- PN buffer for QIAquick spin purification of >17nt nucleic acids, DIY (40% (v/v) 5 M guanidinium chloride, 60% (v/v) isopropanol)
- 10U/ul T4 Polynucleotide Kinase (PNK), NEB [M0201]
- 10x PNK Buffer (700 mM Tris-HCl pH7.6, 100 mM MgCl₂, 50 mM DTT)
- 10mM ATP, NEB [P0756S]
- 5U/ul Lambda exonuclease, NEB M0262
- 10xLTM, Ilexo Tris-MgCl₂ Buffer (200mM Tris pH9.0, 10mM MgCl₂, 0.1% v/v Triton X-100)
- Klenow fragment(exo-) (NEB, M0212S)
- dNTPs (NEB N0447S)

Safety warnings

 This protocol has been tested in *Saccharomyces cerevisiae*.

Yeast Cell Culture and Lysis

- 1 Inoculate three 60ml overnight cultures of budding yeast cells in **YPAD** at 30°C.
- 2 Autoclave three bottles of 500ml of **YPA(D)** for the next day.
- 3 Prepare **EDTA-Glycerol** (20ml 0.5M EDTA pH8, 30ml sterilized ddH₂O, 31g 100% Glycerol) divided in two GSA centrifuge bottles (volume of each bottle about 40ml), and freeze o/n at -70°C.
- 4 The next day, prepare three 500 ml logarithmic growth cultures by taking 18 ml of each saturated o/n culture per 500ml of **YPAD**. Grow for 3-5h until OD₆₀₀ is between 1.0–1.2 (~10×10⁸ cells). Expected yield is about 130µg gDNA per 500ml culture.
- 5 Spin cells down 3 minutes at 3,000 RPM, discard supernatant and pool cells each in a 250 ml RT sterilized water.
- 6 Add 2.5ml of **10% Na-Azide** with the cells and immediately aliquot in two frozen GSA bottles with EDTA-Glycerol and mix until thawed, keep on ice after that.
- 7 Spin cells at +4°C and wash once with cold water. Check how much volume the cells take up.
- 8 Soften cells with 19ml water supplemented with 1ml 1M Tris pH8.0 and 125µl BME for 10 min on ice, with occasional mixing.
- 9 Wash the cells once with cold water and aliquot in 8×2ml tubes (about ~0.5ml pellet size per tube).
- 10 Prepare (8.1x) SCE-Zymolyase master mix by combining 7.29ml SCE (1x=900µl), 54.3ul BME (6.7µl), 40.5µl SUPERaseIn (5µl) and 810ul Zymolyase (100µl).
- 11 Suspend each pellet in 1ml of cold SCE-Zymolyase master mix and incubate at 30 min at 37°C on rotation.
- 12 Spin spheroplasts 3 min at full speed and wash them 3x with cold 1ml SCE. Pool two pellets in final wash for a total of four tubes.



- 13 Lyse each spheroplast pellet in 1ml of DNAzol at RT by pipetting until homogenous.
- 14 Add 200µl 20mg/ml proteinase K (final 200 µg/ml) and incubate o/n at RT on gentle rotation.

DNA Extraction and Purification

- 15 Pellet the debris from crude lysate by centrifuging 10 min at 10,000g and save the supernatant into a new tube.
- 16 Add 0.5ml 100% EtOH and mix for 5 min on slow rotation, then spin 2 min at 4,000g.
- 17 Wash the pellets twice with 1ml 80% EtOH and suspend in 400ul TEN20S by gentle vortexing at RT until dissolved (about 30 min).
- 18 Spin the samples 15 min at full speed and discard the pellets that contain mainly polysaccharides and debris.
- 19 Pool samples for a total 28×800µl and remove residual polysaccharides and proteins with phenol-chloroform (in regular tube) and chloroform (in phase-lock tube).
- 20 Transfer the two upper phases in 2ml tubes, add 350ul of 5M NaCl and precipitate the nucleic acids by adding ~1x vol of 2-propanol, spin 15 min at RT and wash twice with 80% EtOH (save the sample in 2nd EtOH wash at -20°C until all three replicates are ready to proceed simultaneously).
- 21 Elute each pellet DNA in 0.5ml (4 × 0.5ml) of TE by end-over-end rotation for o/n at +4°C.

Denaturation and Sucrose Gradient Fractionations

- 22 Pool the DNA samples and check the dsDNA concentration by Qubit dsDNA BR and save 1µl of dsDNA to be run later in AGE.
- 23 Save 1.0ug of the nucleic acids sample for gDNA and two λ-exo controls.



- 24 Aliquot the nucleic acids sample in 4×0.5ml in 1.5ml tubes and denature by heating for 10 min at 95°C. Cool immediately on slushy ice. Save 1µl for input ssDNA.
- 25 Measure DNA concentration from the denatured ssDNA (to confirm denaturation) using Qubit dsDNA BR (1µl sample). The concentration of ssDNA should be roughly 1/10th of the dsDNA.
- 26 Run equal amounts of dsDNA | ssDNA samples on 1% AGE.
- 27 Prepare two 11ml 5%-30% Sucrose in TEN500 in SW41 polyclear tubes: 5.5ml of 30% sucrose TEN500 in the chamber closest to the valve (with magnet stirrer) and 5.5ml of 5% sucrose TEN500 in the chamber next to it (valves closed). Open the valve carefully and let the 30% sucrose flow for few seconds as slowly as possible. Then fully open the connecting valve and briefly press the top of the left (5%) chamber with a palm to force the flow. Cool down the gradient for about 2h at +4°C.
- 28 Run maximum of ~200µg dsDNA per 5-30% sucrose gradient in TEN500 for 11h, 35,000rpm at 4°C using SW-41 rotor.
- 29 Collect 0.5ml fractions (~23) from the bottom of the centrifuge tube.
- 30 Prepare precipitation mix: 1.36ml UPW, 170µl 3M NaoAc, 17µl PC-152.
- 31 Take 20µl samples from 11 fractions* (from each gradient) and add 91µl of the precipitation mix and 300µl etOH (3x vol).
- 32 Prepare re-elution mix: 136µl UPW, 34µl 6x AGE buffer, 17µl 100x SYBR Gold.
- 33 Wash the pellets once with 100ul 80% etOH, briefly vacuum-dry and redissolve in 11µl of re-elution mix by mixing 30 min at 30°C covered from light.
- 34 Run the fractions in two 1% AGE gels each with 12 wells, using dsDNA 1kb ladder as a size marker (0.5-1.0kb dsDNA corresponds to 0.5-2.0kb ssDNA).
- 35 Based on AGE, select 0.5–1.0kb fractions (500–2000nt of ssDNA) and pool them together.



- 36 Concentrate the nucleic acids using Amicon Ultra 10k filter (5,000 RCF, for 30 min to 1h at 4°C). Repeat the centrifugation by washing twice with 5ml of UPW.
- 37 Once the sample is concentrated enough (~400µl), precipitate the ssDNA with NaoAc and etOH in a 2ml tube and wash 2x with 80% etOH (15 min at full speed spin), and resuspend the precipitated nucleic acids in 81µl UPW.
- 38 Aliquot the sample (2×40ul) for Traditional and Improved SNS-seq protocols.

RecJ digestion

- 39 Add 5µl of 100mM Tris pH8.0 to the 45µl nucleic acids sample in UPW.
- 40 Heat the sample for 3 min at 95°C and then cool 1 min on slushy ice.
- 41 Add ice-cold 21µl of 10xNEBuffer2, 1µl of SUPERaseIn and 10µl of 30U/µl RecJF, incubate 16h at 37°C and inactivate the enzyme for 15 min at 75°C.
- 42 Purify the nucleic acids by adding 100µl of AMPure beads (1x), elute with 39µl of UPW. Leave the beads in solution.

PNK, NaOH & λ-exo digestion

- 43 Add 10µl 10xPNKbuffer, 5µl ATP, 1µl T4 PNK and incubate 30 min at 37°C.
- 44 Add 1ul 0.5M EDTA and inactivate the enzyme for 20 min at 65°C.
- 45 Purify the nucleic acids by adding 50µl of AMPure beads buffer (1x), elute with 40µl UPW. Discard beads.
- 46 Add 5µl 2.5M NaOH and hydrolyze the RNA for 15 min at 50°C.
- 47 Neutralize by adding 50µl of 2.5M Acetic Acid.

- 48 Purify the DNA by adding 50µl of AMPure beads (1x), elute with 86µl of UPW. Leave the beads in solution.
- 49 Add 10µl 10xLTM and 40µl of 50U/µl Ilexo (NEB) and incubate 16h at 37°C followed by inactivation of the enzyme for 15 min at 75°C.
- 50 Purify the DNA by adding 100ul of AMPure beads buffer (1x), elute with 20µl of UPW.

NEBNext Ultra II Library Preparation

2h 30m

- 51 Take the SNS dsDNA sample and fill up the each volume to 130µl with TE.
- 52 Fragment the dsDNA to 300bp peak size using Covaris S220 and the following settings: Duty factor: 10%, Peak incident power (W): 140, Cycles per burst: 200, Time (seconds): 80, Volume (µl): 130
- 53 Purify the 300bp DNA by adding 13µl 3M NaOAc, 1µl PC-152 and 420µl 100% etOH, wash 2x w/ 80% etOH and elute in 50µl UPW.
- 54 Check the dsDNA concentration by Qubit HS. (One ng of 300bp dsDNA is 0.0051 pmol.)
- 55 End repair and dA tailing
- 56 In a PCR tube, mix 50µl of 300bp DNA, 7ul of NEBNext Ultra II End Prep Reaction Buffer and 3µl of NEBNext Ultra II End Prep Enzyme Mix and incubate in a PCR cycler (w/ heated lid on) 30 min at 20°C > 30 min at 65°C > hold at 4°C.
- 57 Determine if adaptor dilution is necessary. Aim for 5-fold molarity of adaptors:insert. [1:1 (no dilution, 15µM working concentration) for 100ng–1µg, 1:10 (1.5µM working concentration) for 5–99ng, 1:25 (0.6µM working concentration) for <5ng]
- 58 Ligation of NEBNext Adaptors
- 59 Add directly to the 60µl of End prepped DNA sample 30µl of NEBNext Ultra II Ligation Master Mix, 1µl of NEBNext Ligation Enhancer and 2.5µl of NEBNext Adaptor for Illumina. Mix well with a pipette.

- 60 Incubate at 15 min at 20°C in a thermocycler with the heated lid off.
- 61 Add 3µl of USER® Enzyme to the ligation mixture.
- 62 Mix well and incubate at 15 min at 37°C w/ heated lid on.
- 63 Samples can be stored overnight at –20°C.
- 64 Clean the adaptor-ligated DNA (96.5µl volume) by adding 87µl (0.9x) of AMPure beads, wash 2x with 80% etOH and elute with 10µl 0.1x TE and transfer 7.5µl to a PCR tube (discard beads).
- 65 Save 1.5µl (9%) of the remaining sample for qPCR amplification pre-check.
- 66 Samples can be stored overnight at –20°C before proceeding with library amplification step.
- 67 Library amplification: This section takes about 4h for qPCR and 1h 15 min – 1h 45 min for amplification and purification to complete (total time ≈ 5h).
- 68 Determination of the amount of cycles by qPCR
- 69 Required only once for each sample type: To check the correct number of cycles, mix with the 1.5µl sample 0.5µl of 10µM NEBNext Universal PCR Primer for Illumina, 0.5µl of any 10µM NEBNext Index Primer and 1.36 for Illumina and 2.5µl 2x SYBR Green Master Mix.
- 70 Run the samples in qPCR and determine the cycle number that corresponds to 50% of maximum amplification. Reduce 3 cycles (for using ~10% of starting material) from this number to get the final number of amplification cycles.
- 71 Library amplification by PCR
- 72 Add the following reagents to a PCR tube with the adaptor-ligated DNA to amplify the libraries



- 73 Perform the number of cycles determined by qPCR to amplify the library, using the following program: Usually 3–13 cycles in total, but can be higher for SNS samples.
- 74 Purify the amplified library by adding 45ul of AMPure beads (0.9x), elute with 50μl of 0.1x TE and repeat the purification by adding 45μl of AMPure beads buffer (0.9x). Finally elute the library in 16μl of 0.1x TE. Transfer 14μl to a new low-bind tube.
- 75 Samples can be stored indefinitely at –20°C.

QC (Fragment Analysis and Quantification)

- 76 Check the dsDNA concentration of the library from 1ul input volume using Qubit BR. If the concentration is below detection limit (0.2ng) it means the total amount of dsDNA in the 33μl sample is below 6.6ng. If the concentration is above the detection limit (100ng) the sample concentration is over 100ng/μl. In this latter case, measure the DNA concentration again from 1μl input using Qubit BR (detection limit 20–1000ng).
- 77 Perform fragment analysis with 2–4μl of the sample preferably at 0.5–5ng/μl concentration to see the library size distribution.
- 78 Library Quantification by qPCR Use NEBNext® Library Quant Kit for Illumina® (E7630).