bio-protocol

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Preparation of Cas9 Ribonucleoproteins for Genome Editing

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Abstract

Genome editing by the delivery of pre-assembled Cas9 ribonucleoproteins (Cas9 RNP) is an increasingly popular approach for cell types that are difficult to manipulate genetically by the conventional plasmid and viral methods. Cas9 RNP editing is robust, precise, capable of multiplexing, and free of genetic materials. Its transient presence in cells limits residual editing activity. This protocol describes the preparation of recombinant *Streptococcus pyogenes* Cas9 (SpCas9) protein by heterologous expression and purification from *Escherichia coli*, and the synthesis of CRISPR guide RNA by *in vitro* transcription and PAGE purification. SpCas9 is the first CRISPR Cas9 discovered (Jinek *et al*., 2012) and is also one of the most characterized Cas enzymes for genome editing applications. Using this formulation of Cas9 RNP, we have demonstrated highly efficient genome editing in primary human T and natural killer (NK) cells by electroporation, and in fungi and plants by polyethylene glycol-mediated transformation. Our protocol of Cas9 RNP preparation is consistent and straightforward to adopt for genome editing in other cell types and organisms.

Keywords: CRISPR, Cas9 RNP, sgRNA, *In vitro* transcription

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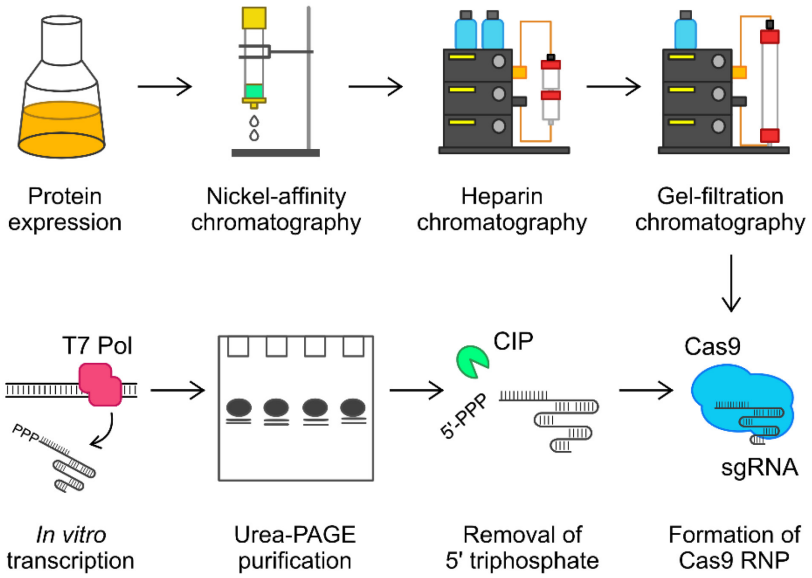
Graphical abstract:



Background

CRISPR-Cas9 genome editing is a revolutionary technology for research, biotechnology, agriculture, and medicine. Acting as molecular scissors, Cas9 nuclease induces a targeted DNA double-strand break at the site that is specified by the spacer sequence of guide RNA, creating a unique opportunity to modify genomic sequences through DNA repair pathways. The native form of Cas9 guide RNA consists of two RNA molecules: a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA) (Jinek *et al*., 2012). The crRNA and tracrRNA were engineered into a single guide RNA (sgRNA) fusion molecule to facilitate *in vitro* and *in vivo* applications (Jinek *et al*., 2012). Genome editing by the delivery of pre-assembled Cas9 RNP is robust and precise, and is advantageous over the standard plasmid and viral approaches when transient editing activity is desired. Cas9 protein and guide RNA can be synthesized and validated in advance, and stably stored in the -80°C freezer for genome editing on demand. These attributes are ideal for the manufacture of genome-engineered immune cells that complies with good manufacturing practice standards for research and clinical applications.

We have established robust Cas9 RNP genome-editing platforms for primary human T and NK cells, using our highly pure, stable, and non-toxic SpCas9 protein and synthetic guide RNA (Schumann *et al.*, 2015; Huang *et al.*, 2020, 2021). Cas9 RNP can be introduced to the immune cells, by either electroporation in a Lonza 4D Nucleofector system or a ThermoFisher Neon Transfection system, to achieve high gene-knockout efficiency and cell viability. Multiple Cas9 RNP of different specificities can be delivered in a single reaction to simultaneously target multiple genomic loci. Furthermore, the co-electroporation of synthetic DNA repair templates enables site-specific gene knock-in by Cas9-mediated homology-directed repair. We have also demonstrated that the same Cas9 RNP formulation worked efficiently in fungi and plants by other delivery methods (Lai *et al.*, 2017; Hsu *et al.*, 2021). We believe that this protocol would be of great value to the scientific communities for a wide range of genome editing applications in mammalian cells as well as other organisms.

In this protocol, we describe the procedures for the heterologous expression and purification of recombinant SpCas9 and the enzymatic synthesis of sgRNA. Our protocol focuses on high purity and low toxicity of Cas9 RNP for applications in primary immune cells, which are more sensitive to endotoxin and exogenous RNA. Our protocol offers several improvements to the protocols by Savić *et al.* (2019) and Lingeman *et al.* (2017), including an extended His tag to increase binding to the nickel-affinity column, use of a mild detergent to reduce endotoxin contamination, improved ion exchange and gel-filtration chromatography, and sterilization of protein for cell culture use. Our protocol for the enzymatic synthesis of sgRNA includes denaturing PAGE purification to extract the full-length sgRNA and remove the truncated products. We also perform a phosphatase treatment to remove the

immunogenic 5' triphosphate moiety that is highly toxic to human immune cells (Wienert *et al.*, 2018; Kim *et al.*, 2018; Huang *et al.*, 2021). We have shown that our guide RNA is of equally high quality to the commercial chemically synthesized guide RNA (Huang *et al.*, 2021), but for only a fraction of the cost to prepare. An overview of Cas9 and sgRNA preparation is illustrated in Figure 1.

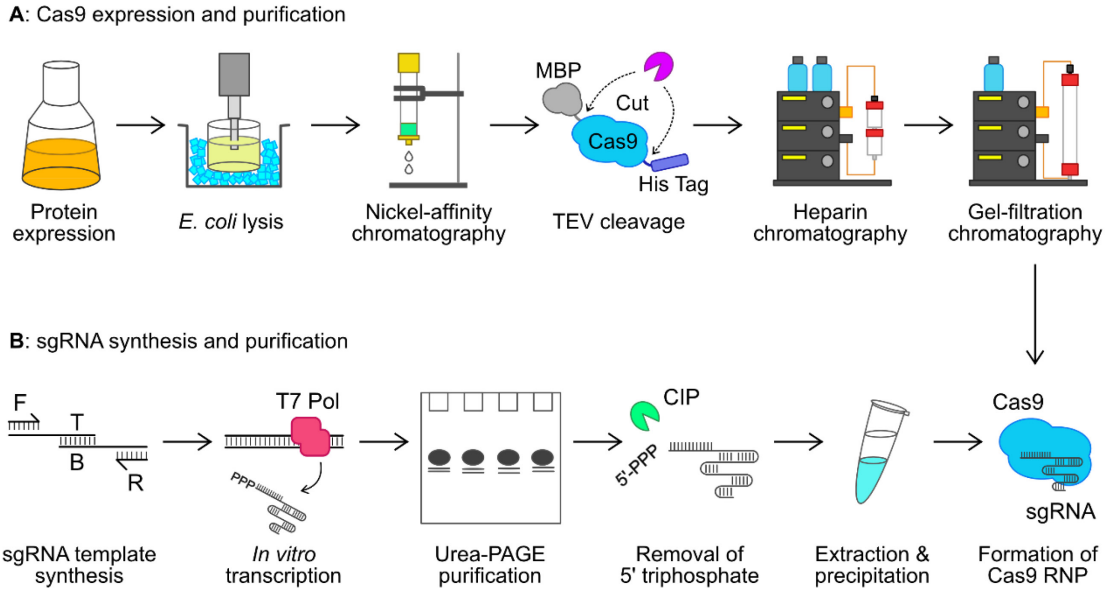


Figure 1. Overview of Cas9 and sgRNA synthesis and purification.
(A) Cas9 is expressed in *E. coli* and purified from *E. coli* lysate by nickel-affinity, ion-exchange (heparin column), and gel-filtration chromatography. (B) sgRNA synthesis begins with the assembly of sgRNA DNA template by overlapping PCR using four DNA oligonucleotides: top (T), bottom (B), forward (F), and reverse (R). The DNA template is then transcribed *in vitro* by T7 RNA polymerase (T7 Pol) to generate sgRNA. After sgRNA purification by urea-PAGE, the immunogenic 5' triphosphate group is removed by treatment with calf intestinal phosphatase (CIP). The resulting Cas9 and sgRNA are mixed to form Cas9 ribonucleoproteins (Cas9 RNP) for genome editing applications.

Materials and Reagents

Materials

1. Rainin pipette tips, 20-µL, 200-µL, and 1,000-µL (Mettler Toledo, catalog numbers: 30389291, 30389299 and 30389212)
2. Serological pipettes, 5-mL, 10-mL, and 25-mL (ThermoFisher, catalog numbers: 170355N, 170356N and 170357N)
3. 10-cm Petri dish (Fisher Scientific, catalog number: 08-757-105)
4. 200-µL PCR tube (Gunster Biotech, catalog number: MB-P08A)
5. Disposable plastic cuvette (BRAND, catalog number: 759115)
6. 1.5-mL microcentrifuge tube (Axygen, catalog number: MCT-150-C)
7. 15-mL conical tube (ThermoFisher, catalog number: 339650)
8. 50-mL conical tube (ThermoFisher, catalog number: 339652)
9. Nalgene Oak Ridge PPCO centrifuge tube, 42-mL (ThermoFisher, catalog number: 3119-0050)
10. 1-L polypropylene centrifuge bottle (Beckman Coulter, catalog number: A98813)
11. ColiRoller plating glass beads (Sigma-Aldrich, catalog number: 71013)

12. Polyethersulfone Membrane Filters, 0.45-µm pore size, 47 mm diameter (Sartorius, catalog number: 15406-47-N)
13. Millicup-FLEX filtration unit, 47 mm diameter (Millipore, catalog number: MCFLX4702)
14. Ni-NTA Superflow resin (Qiagen, catalog number: 30450)
15. Econo-column, 2.5 cm × 10 cm (Bio-Rad, catalog number: 7372512)
16. Econo-column funnels (Bio-Rad, catalog number: 7310003)
17. Clear 96-well microplate (PerkinElmer, catalog number: 6005640)
18. Amicon Ultra-15, 100-kDa MWCO (Millipore, catalog number: UFC910096)
19. 0.22-µm syringe filter, PES membrane, 33 mm diameter (Millipore, catalog number: SLGPR33RS)
20. 0.45-µm syringe filter, PES membrane, 33 mm diameter (Millipore, catalog number: SLHPR33RS)
21. HiTrap Heparin HP, 5-mL (Cytiva, catalog number: 17040703)
22. HiLoad Superdex 200 16/600 (Cytiva, catalog number: 28-9893-35)
23. Prefilter 6000 (GE Healthcare, catalog number: 18-4582-01)
24. Ultra-free MC centrifugal filter, 0.2-µm (Millipore, catalog number: UFC30GV08)
25. Mini-PROTEAN TGX precast gel (Bio-Rad, catalog number: 4561033)
26. Kimwipes disposable wipers (Sigma-Aldrich, catalog number: Z188956)
27. Large binder clip, 2” (Amazon, catalog number: B07V3VZLG1)
28. Disposable syringes, 3-mL and 10-mL (Terumo, catalog numbers: SS-03S and SS-10S)
29. 23G × 1 1/2" needle (Terumo, catalog number: NN-2338R)
30. 22G × 2” metal hub needle (Hamilton, catalog number: 91022)
31. Plastic food wrap (Amazon, catalog number: B00JPKW1RQ)
32. Green-fluorescing F_{254} silica thin-layer chromatography plate (Sigma-Aldrich, catalog number: 02554)
33. Disposable surgical blade (Feather Safety Razor, No. 10)

Reagents

1. SpCas9 expression plasmid (Addgene, catalog number: 182032)
2. Novagen *E. coli* Tuner (DE3) competent cells (Sigma-Aldrich, catalog number: 70623)
3. Difco LB Broth, Miller (Luria-Bertani) (BD, catalog number: 244610)
4. Terrific Broth (BD, catalog number: 243810)
5. Agar, bacteriology (VWR, catalog number: J637)
6. Glycerol (Fisher Scientific, catalog number: G/0650/17)
7. $Na_2HPO_4•7H_2O$ (Sigma-Aldrich, catalog number: S9390)
8. KH_2PO_4 (Sigma-Aldrich, catalog number: P0662)
9. NaCl (Sigma-Aldrich, catalog number: S9888)
10. NH_4Cl (Sigma-Aldrich, catalog number: 213330)
11. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (GoldBio, catalog number: I2481C100)
12. Kanamycin monosulfate (GoldBio, catalog number: K-120-50)
13. Difco Terrific broth (BD, catalog number: 243810)
14. HEPES (Sigma-Aldrich, catalog number: 54457)
15. KCl (Sigma-Aldrich, catalog number: P3911)
16. 2-mercaptoethanol (Sigma-Aldrich, catalog number: M3148)
17. Imidazole (Sigma-Aldrich, catalog number: I2399)
18. Triton X-100 (Sigma-Aldrich, catalog number: 93443)
19. Lysozyme (Sigma-Aldrich, catalog number: L4919)
20. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: 10837091001)
21. DNase I from bovine pancreas (Roche, catalog number: 11284932001)
22. Liquid nitrogen (local distributor)
23. NuPAGE LDS loading dye (ThermoFisher, catalog number: NP0007)
24. Bradford assay reagent (VWR, catalog number: E530)
25. TEV protease (Sigma-Aldrich, catalog number: T4455)
26. 10× Tris-glycine-SDS buffer (Sigma-Aldrich, catalog number: T7777)
27. ExcelBand protein marker (SMOBIO, catalog number: PM2500)
28. InstantBlue Coomassie Protein Stain (Abcam, catalog number: 119211)

29. DNA oligonucleotides for the synthesis of sgRNA template (IDT DNA)
30. Qiagen EB buffer (Qiagen, catalog number: 19086)
31. H_2O, molecular biology grade (Fisher Scientific, catalog number: BP2819)
32. KAPA HIFI PCR kit (Roche, catalog number: KK2103)
33. 6× Gel Loading Dye Purple (NEB, catalog number: B7024S)
34. Agarose I (VWR, catalog number: 0710)
35. 10× TAE buffer (VWR, catalog number: K915)
36. SYBR Safe DNA gel stain (ThermoFisher, catalog number: S33102)
37. Diethylpyrocarbonate (DEPC) (Sigma-Aldrich, catalog number: 40718)
38. Tris (VWR, catalog number: 0826)
39. $MgCl_2$ hexahydrate (Sigma-Aldrich, catalog number: M2670)
40. Spermidine (Sigma-Aldrich, catalog number: 85558)
41. Polyethylene glycol (PEG) 2000 (Sigma-Aldrich, catalog number: 8.21037)
42. Dithiothreitol (DTT) (Roche, catalog number: 10708984001)
43. ATP (Sigma-Aldrich, catalog number: A2383)
44. UTP (Sigma-Aldrich, catalog number: U6625)
45. CTP (Sigma-Aldrich, catalog number: C1506)
46. GTP (Sigma-Aldrich, catalog number: G8877)
47. T7 RNA polymerase (Roche, catalog number: 10881767001)
48. RQ1 RNase-free DNase I (Promega, catalog number: M6101)
49. Formamide (Sigma-Aldrich, catalog number: F9037)
50. Bromophenol blue (Sigma-Aldrich, catalog number: 114391)
51. Xylene cyanol (Sigma-Aldrich, catalog number: X4126)
52. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: E9884)
53. Urea (Sigma-Aldrich, catalog number: U5128)
54. 5× TBE buffer (VWR, catalog number: J885)
55. 40% polyacrylamide, 29:1 (ACROS, catalog number: 330215000)
56. Ammonium persulfate (APS) (Sigma-Aldrich, catalog number: A3678)
57. N,N,N',N' Tetramethylethylenediamine (TEMED) (Sigma-Aldrich, catalog number: T9281)
58. Scienceware Aquet liquid detergent (Sigma-Aldrich, catalog number: Z273260)
59. Sigmacote siliconizing reagent (Sigma-Aldrich, catalog number: SL2)
60. 3 M sodium acetate solution, pH 5 (Sigma-Aldrich, catalog number: S7899)
61. Glycogen (Abcam, catalog number: AB146544)
62. Isopropanol (EMD Millipore, catalog number: 1.09634.2500)
63. Ethanol (Katayama Chemical, catalog number: 9270)
64. Quick CIP, calf intestinal alkaline phosphatase (NEB, catalog number: M0525)
65. Phenol:chloroform mix, low pH (VWR, catalog number: 0966)
66. Chloroform (Sigma-Aldrich, catalog number: C2432)
67. ToxinSensor chromogenic lyophilized Amebocyte lysate (LAL) endotoxin assay kit (GeneScript, catalog number: L00350)
68. HCl (Fisher Scientific, catalog number: A508-P500)
69. LB+glycerol medium (see Recipes)
70. Terrific Broth (see Recipes)
71. 20× modified M9 buffer (see Recipes)
72. 1 M IPTG (see Recipes)
73. 50 mg/mL kanamycin (see Recipes)
74. LB agar plate with kanamycin (see Recipes)
75. Lysis buffer (see Recipes)
76. Wash buffer (see Recipes)
77. Elution buffer (see Recipes)
78. IEX A buffer (see Recipes)
79. IEX B buffer (see Recipes)
80. Gel-filtration buffer (see Recipes)
81. 100 mM PMSF (see Recipes)

82. 10 mg/mL DNase I (from bovine pancreas) (see Recipes)
83. 0.5 N NaOH + 1 mM EDTA solution (see Recipes)
84. DEPC-treated H_2O (see Recipes)
85. 10× IVT buffer (see Recipes)
86. 50% PEG 2000 (see Recipes)
87. 25 mM NTP mix (see Recipes)
88. 1 M DTT (see Recipes)
89. 0.5 M EDTA (see Recipes)
90. 2× STOP solution (see Recipes)
91. Urea-PAGE premix (see Recipes)
92. 20% APS (see Recipes)
93. 1 mg/mL glycogen (see Recipes)
94. RNA dissolving buffer (see Recipes)

Equipment

1. Rainin Pipet-Lite XLS single channel pipettes, 2-µL, 20-µL, 200-µL, and 1,000-µL (Mettler Toledo, catalog numbers: 17014393, 17014392, 17014391, and 17014382)
2. Thermo Scientific S1 pipet fillers (ThermoFisher, catalog number: 9501)
3. Autoclave (TOMY, catalog number: SX700)
4. Vortex mixer (Scientific Industries, catalog number: G560)
5. AKTA Purifier 10 FPLC system (GE Healthcare, catalog number: 28406264)
6. Mini-PROTEIN Tetra vertical electrophoresis cell (Bio-Rad, catalog number: 1658004)
7. SimpliAmp PCR thermal cycler (ThermoFisher, catalog number: A24811)
8. Mini gel DNA electrophoresis system (ThermoFisher, catalog number: B1)
9. Owl P10DS vertical electrophoresis system (ThermoFisher, catalog number: P10-DS-1)
10. Bio-Rad PowerPac HV power supply (Bio-Rad, catalog number: 1645056)
11. Gel imaging system (Azure Biosystems, catalog number: C300)
12. Compact UV lamp (UVP, catalog number: 95-0016-14)
13. Bacterial shaking incubator with refrigerating function (TKS, catalog number: OSI-500R eco)
14. Cell density meter (WPA, catalog number: C08000)
15. Sonicator system with 0.5”-diameter probe horn (Qsonica, catalog numbers: Q700 and 4420)
16. Peristaltic pump (EYELA, catalog number: MP-2000)
17. Fresco 21 microcentrifuge (ThermoFisher, catalog number: 75002555)
18. Allegra centrifuge (Beckman-Coulter, catalog number: X-30R)
19. Avanti centrifuge (Beckman-Coulter, catalog number: J-25)
20. Centrifuge rotor for 50-mL conical tube (Beckman-Coulter, catalog number: SX440)
21. Centrifuge rotor for Oak Ridge PPCO tube (Beckman-Coulter, catalog number: JA-25.50)
22. Centrifuge rotor for 1-L polypropylene bottle (Beckman-Coulter, catalog number: JLA-8.1)
23. Electronic scale (Mettle Toledo, catalog number: ML3002)
24. pH meter (Mettle Toledo, catalog number: 51302807)
25. NanoDrop Lite Spectrophotometer (ThermoFisher, catalog number: ND-LITE-PR)
26. MilliQ water purification system (Millipore, catalog number: MP0024)
27. Water bath, set to 37°C and 42°C (FIRSTEK, catalog number: B206)
28. Heat block, set to 95°C (Major Science, catalog number: MC01N)
29. Stir plate (Corning, catalog number: PC620D)
30. 250-mL Erlenmeyer flask (Pyrex, catalog number: 4980)
31. 2.5-L Ultra-Yield flasks (Thomson, catalog number: 931136-B)
32. Pyrex 100-mL beaker (DWK Life Sciences, catalog number: 1000/04D)
33. Pyrex 400-mL glass beaker (DWK Life Sciences, catalog number: 1000/14D)
34. Pyrex 500-mL glass bottle (DWK Life Sciences, catalog number: 1516/08D)
35. Pyrex 1-L glass bottle (DWK Life Sciences, catalog number: 1516/10D)

36. GL45 Screw cap Twin Hose Connector for Pyrex bottles (Fisher Scientific, catalog number: 15193927)
37. Three-Prong extension clamp stand (Fisher Scientific, catalog number: 057697Q)
38. Rotary shaker (ARGOS, catalog number: R2200)
39. SpeedVac vacuum concentrator (EYELA, catalog number: CVE-2100)
40. Microplate reader (Tecan, catalog number: M1000Pro)
41. Vacuum pump (Rocker, catalog number: 400)

Software

1. UNICORN, for AKTA FPLC operation (version 5.31)
2. GraphPad Prism 9, for statistics and data visualization (version 9.3.1)
3. ImageJ, for image processing (version 1.53a)

Procedure

A. Expression and purification of SpCas9 protein

1. Overexpression of SpCas9 in *E. coli* Tuner (DE3)
   a. Preparation of *E. coli* growth media and stock solutions (see Recipes).
      i. Prepare 50 mL of Terrific Broth. Store at room temperature.
      ii. Prepare one flask of 100 mL of LB+glycerol media in a 250-mL Erlenmeyer flask for starter culture. Store at room temperature and use within a week.
      iii. Prepare three flasks of 1,000 mL of LB+glycerol media in 2.5-L Ultra-Yield flasks for large culture. Store at room temperature and use within a week.
      iv. Prepare the 20× modified M9 buffer. Store at room temp for up a year
      v. Prepare stock 1 M IPTG and 50 mg/mL kanamycin solutions. Store at -20°C for up to two years.
      vi. Prepare LB agar plate containing 50 µg/mL of kanamycin. Store at 4°C for up to a month.
      vii. Prepare Lysis buffer. Store at 4°C for up to a month.
   b. Transformation of *E. coli* (Day 1)
      i. Thaw chemically competent Tuner (DE3) cells on ice.
      ii. Add 1 ng of SpCas9 expression plasmid (Addgene) to 50 µL of competent cells.
      iii. Mix well by tapping, and incubate the tube on ice for 20 min.
      iv. Place the tube in a 42°C water bath for 60 s to heat shock *E. coli*.
      v. Incubate the tube back on ice for 2 min.
      vi. Add 500 µL of Terrific Broth to the *E. coli* and mix well by tapping.
      vii. Recover *E. coli* at 37°C for 1 hour with shaking.
      viii. Spread 100 µL of the cells on a 10-cm LB agar plate containing 50 µg/mL of kanamycin with sterile glass beads until the plate is fully dried.
      ix. Empty out the beads and incubate the plate at 37°C for 18–20 h for *E. coli* colonies to form.
      x. Store the plate at 4°C and use within two weeks.
      Note: Tuner (DE3) is not a stable strain to maintain the cas9 plasmid. We recommend fresh transformation for every batch of Cas9 expression.
   c. Set up of starter *E. coli* culture (Day 2)
      i. Add 2.5 mL of 20× modified M9 buffer and 102 µL of 50 mg/mL kanamycin solution to the 100-mL LB+glycerol medium to reconstitute the complete medium.
      ii. Use sterile pipette tips to pick 4–5 single colonies from the LB agar plate and inoculate in the complete medium.
      iii. Incubate the starter culture at 30°C for 20 h with 200 rpm shaking. Do not overgrow the culture at a higher template for a longer period of time.
   d. Expression of SpCas9 in large culture (Day 3)
      i. Split the 100-mL starter culture into two 50-mL conical tubes.

ii. Centrifuge at 4,000 × *g* and 25°C for 10 min to collect the *E. coli*, and decant the media.
iii. Resuspend the two tubes of *E. coli* pellets in a total of 15 mL of Terrific Broth by vortexing.
iv. Add 25 mL of 20× modified M9 buffer and 1,030 µL of 50 mg/mL kanamycin solution to the 1,000-mL LB+glycerol medium to reconstitute the complete medium.
v. Add 5 mL of *E. coli* suspension to each flask of the complete medium. Set up three flasks.
vi. Incubate the flasks at 37°C with 200 rpm shaking.
vii. Monitor the *E. coli* density by sampling 500 µL of the culture and determine the absorbance at 600 nm.
viii. When the absorbance at 600 nm reaches 1.5, remove the flasks from the incubator and incubate them in ice water for 10 min to lower the temperature. Swirl the flasks by hand every 2–3 min.
ix. Add IPTG to 500 µM final concentration.
x. Incubate the flasks at 16°C with 200 rpm shaking overnight for 18 h to induce Cas9 expression.

e. Harvest and storage of *E. coli* (Day 4)
i. Transfer the *E. coli* culture from the flasks to 1-L polypropylene centrifuge bottles.
ii. Balance the bottles before centrifugation on an electronic scale, and adjust the weight by deionized water.
iii. Centrifuge at 3,500 × *g* and 4°C for 15 min to collect the *E. coli*, and decant the media.
iv. Add 70 mL of ice-cold Lysis buffer per flask to resuspend the *E. coli* pellet.
v. Use a combination of vortexing and pipetting to break up the *E. coli* pellet. Keep the resuspended *E. coli* on ice.
vi. Combine the *E. coli* suspension and proceed immediately to Step 2b for *E. coli* lysis by sonication.
vii. Alternatively, freeze the *E. coli* suspension at -80°C by aliquoting the *E. coli* suspension into 50-mL conical tubes. Fill the tube to ~45 mL to avoid cracking the tube during freezing.
viii. Transfer the tubes to -80°C to freeze the cells. The freezing process partially disrupts the *E. coli* cell membrane and helps increase cell lysis. The frozen *E. coli* suspension is stable at -80°C for at least one year.

2. Purification of SpCas9
a. Preparation of purification buffers and stock solutions (see Recipes)
i. Prepare Lysis, Wash, and Elution buffers for nickel-affinity chromatography. Store at 4°C for up to a month.
ii. Prepare IEX A and IEX B buffers for heparin ion exchange chromatography. Store at 4°C for up to a month.
iii. Prepare Gel-filtration buffer. Store at 4°C for up to a month.
iv. Prepare 100 mM PMSF stock solution. Store at -20°C for up to a year.
v. Prepare 10 mg/mL DNase I (from bovine pancreas) solution. Snap freeze in 1-mL aliquots in liquid nitrogen and store at -80°C for up to two years.
vi. Prepare 0.5 N NaOH + 0.1 mM EDTA solution. Store at 4°C for up to a year.

b. Lysis *E. coli* by sonication (Day 5)
i. Thaw six tubes of the frozen *E. coli* suspension (~300 mL) in cold tap water.
ii. Mix the *E. coli* suspension by gently inverting the tubes every 5 min until the ice is completely melted.
iii. While the cell pellet is thawing, set up a 400-mL glass beaker containing a stir bar and a stir plate below the sonication probe.
iv. Dissolve 30 mg of lysozyme in 30 mL of Lysis buffer in the beaker at room temperature.
v. Pour the thawed *E. coli* suspension into the beaker.
Note: The thawed E. coli suspension should be viscous due to partial cell lysis induced by freezing.
vi. Place the beaker at the center of an ice bucket, and surround the beaker with ice to keep the temperature low during sonication.
vii. Place the ice bucket containing the beaker on the stir plate, and set to 100 rpm to homogenize the *E. coli* suspension.
viii. While stirring, slowly add 3 mL of 100 mM PMSF solution drop by drop into the *E. coli* suspension.
ix. While stirring, add 1 mL of 10 mg/mL DNase I (from bovine pancreas) into the *E. coli* suspension.

x. Submerge the sonication probe at ~1.5 inch deep into the *E. coli* suspension.
Note: The E. coli suspension should appear opaque and has a light-yellow color before sonication.
xi. Lyse the *E. coli* by sonication (power output 40, pulse-on 20 ss, pulse-off 20 ss, total process time 10 min) under constant stirring to homogenize the cell lysate and reduce the heat generated by the sonication probe.
Tip: Homogenization by stir bar may not be effective in the initial rounds of sonication due to high viscosity of the E. coli *lysate. We occasionally pause the sonication and use a spatula to stir the lysate a few times. Repeat this method as often as necessary to help homogenize the temperature and content. The lysate should gradually become less viscous and more translucent as sonication progresses.*
xii. Transfer ~37 mL of the *E. coli* lysate into an Oak Ridge PPCO tube. Use as many tubes as necessary. Do not overfill the tubes to avoid leaking during centrifugation.
xiii. Balance the tubes on an electronic scale, and adjust the weight by adding Lysis buffer.
xiv. Centrifuge at 20,000 × *g* and 4°C for 30 min. Combine the supernatant in a clean 500-mL glass bottle pre-chilled on ice.
xv. Filter the supernatant through a 0.45-µm filter to completely remove the cell debris. Collect the soluble *E. coli* lysate in a clean 500-mL bottle pre-chilled on ice.
xvi. Sample 5 µL of the soluble lysate, mix with 40 µL of 1× NuPAGE loading dye, and heat the mixture at 95°C for 5 min for SDS-PAGE analysis.

c. Nickel-affinity chromatography (Day 6)
i. Wash 20 mL of Ni-NTA (50% (w/v) slurry in 20% (v/v) ethanol) with 20 mL of deionized water in a 50-mL conical tube. Mix by gently inverting the tube.
ii. Centrifuge at 3,000 × *g* and 4°C for 5 min, and discard the supernatant by pipetting.
iii. Resuspend the Ni-NTA resin in 40 mL of Lysis buffer. Mix by gently inverting the tube.
iv. Centrifuge at 3,000 × *g* and 4°C for 5 min, and discard the supernatant by pipetting.
v. Resuspend the Ni-NTA resin in 20 mL of Lysis buffer by pipetting.
vi. Transfer the Ni-NTA slurry to the 500-mL bottle containing the soluble *E. coli* lysate.
vii. Place a stir bar into the bottle, and incubate the bottle inside an ice bucket on top of a stir plate.
viii. Mix the Ni-NTA and *E. coli* lysate on ice by stirring at 100 rpm for 20 min to capture the His-tagged Cas9 protein.
ix. Connect a Bio-Rad Econo-column (2.5 cm × 15 cm) to an Econo-column funnel, and set up the column on a clamp stand. Perform the following steps at 4°C, and allow the lysate and buffers to flow through the column by gravity.
x. Load the Ni-NTA and lysate slurry into the column by pipetting. Discard the flow through.
xi. Wash the column with 400 mL of Lysis buffer.
Note: Load the buffers by pipetting slowly and try not to disturb the resin bed. Lysis buffer contains 0.5% (v/v) Triton X-100 to help wash off endotoxin from the column.
xii. Wash the column with 400 mL of Wash buffer.
Note: Wash buffer contains 1 M of KCl to help remove nucleic acid contaminants that bind to the positively-charged active site of Cas9.
xiii. Add Elution buffer to the column by pipetting, and collect a series of 5-mL fractions in 15-mL tubes.
xiv. Check each fraction by Bradford assay by sampling 5 µL of eluate from each fraction and mixing with 50 µL of 1× Bradford reagent in a 96-well plate.
Note: Bradford assay reagent is brown, but turns blue when mixed with proteins. The color change is visible to the naked eye.
xv. Combine the fractions with a substantial amount of protein in an Amicon Ultra tube (15-mL, 100-kDa MWCO).
xvi. Centrifuge at 4,000 × *g* and 4°C for 15 min. Refill the tube with more protein eluate, and mix the content by gently inverting the tube 4–5 times.
xvii. Repeat the centrifugation process to concentrate the eluate down to a final volume of ~2 mL.
xviii. Pipette mix the concentrated eluate gently.

xix. Sample 5 µL of the concentrated eluate, mix with 40 µL of 1× NuPAGE loading dye in a 1.5-mL microcentrifuge tube, and heat the mixture at 95°C for 5 min for SDS-PAGE analysis.
xx. Transfer the eluate to a new 50-mL conical tube.
xxi. Mix TEV protease to the concentrated eluate to cleave the maltose binding protein fusion and 12× His tag from the recombinant Cas9 protein. The recommended ratio is 1:100 (w/w) or 10,000 unit (1 mg) TEV protease to 100 mg of target protein measured by the absorbance at 280 nm.
xxii. Incubate the reaction at 4°C overnight for ~18 h.

d. Heparin ion exchange chromatography (Day 7)
i. Sample 5 µL of the TEV reaction mixture, mix with 40 µL of 1× NuPAGE loading dye in a 1.5-mL microcentrifuge tube, and heat the mixture at 95°C for 5 min for SDS-PAGE analysis.
ii. Add 10 mL of IEX A buffer to the TEV reaction mixture using a 10-mL serological pipette. Submerge the pipette into the mixture, gently swirl the solution and slowly release IEX A buffer.
Tip: Although the compositions of Elution and IEX A buffers are similar, we sometimes observe protein precipitation when IEX A buffer is added too quickly, resulting in loss of Cas9 protein. It is important to introduce the first few mL of IEX A buffer to the concentrated Cas9 and TEV mixture slowly.
iii. Slowly add another 20 mL of IEX A buffer by the same method.
iv. Pass the protein mixture through a 0.45-µm syringe filter to remove protein precipitation. *Note: When Step A-2-d-ii is performed correctly, there should be no visible protein precipitation. When substantial precipitation occurs, centrifuge the protein solution at 4,000 × g and 4°C for 15 min to remove the precipitant before filtration. Regardless of precipitation, filtration is recommended to remove particles and help prolong the life of columns.*
v. Connect two 5-mL HiTrap Heparin HP columns in tandem.
vi. Equilibrate the columns with 50 mL of IEX A buffer by a peristaltic pump at a flow rate of ~3 mL/min at 4°C.
vii. Load the protein mixture into the columns by a peristaltic pump at ~3 mL/min at 4°C. Collect the flow through fraction.
viii. Sample 5 µL of the flow through fraction, mix with 40 µL of 1× NuPAGE loading dye in a 1.5-mL microcentrifuge tube, and heat the mixture at 95°C for 5 min for SDS-PAGE analysis.
ix. Perform system wash on AKTA Purifier FPLC with IEX A (inlet A) and IEX B (inlet B) buffers, and then equilibrate the flow path with IEX A buffer.
Tip: The FPLC pump rinsing solution (20% ethanol) must be changed monthly to avoid microbial growth in the FPLC system and reduce the risk of endotoxin contamination in the protein sample.
x. Connect the Heparin columns to the FPLC, and wash the columns with 100 mL of IEX A buffer at 3 mL/min.
xi. Elute the protein with a linear gradient from 0% to 80% of IEX B buffer over 150 mL at 3 mL/min.
xii. Combine the peak fractions as shown in Figure 2.
xiii. Transfer the protein eluate to Amicon Ultra tube (15-mL, 100-kDa MWCO).
xiv. Centrifuge at 4,000 × *g* and 4°C for 15 min. Refill the tube with more protein eluate, and mix the content by gently inverting the tube 4–5 times.
xv. Repeat the centrifugation process to concentrate the eluate down to a final volume of ~1.5 mL. Store on ice.
xvi. Sample 5 µL of the concentrated Heparin eluate, mix with 40 µL of 1× NuPAGE loading dye in a 1.5-mL microcentrifuge tube, and heat the mixture at 95°C for 5 min for SDS-PAGE analysis.

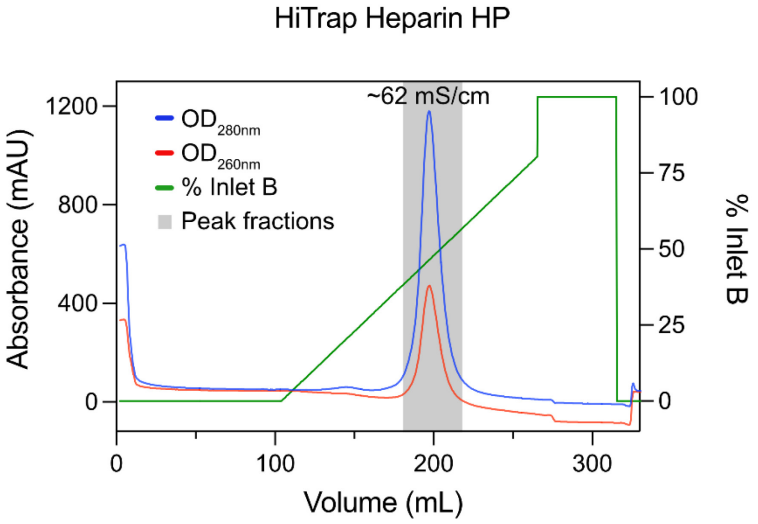


Figure 2. The elution profile of Heparin chromatography.
Cas9 is eluted at a conductivity of ~62 mS/cm in our buffer system.

e. Superdex 200 gel-filtration chromatography (Days 7 and 8)
   i. Starting on Day 7 after heparin chromatography, connect a 2-mL sample loop to the FPLC and perform a system wash with deionized water.
   ii. Connect a prefilter 6000 and a HiLoad Superdex 200 16/600 column to the FPLC, and set the pressure alarm and flow rate (0.8–1 mL/min) according to the manufacturer's protocol throughout the purification.
   iii. Clean the flow path (including the sample loop, prefilter, Superdex column, and fraction collector) with 250 mL of 0.5 N NaOH + 0.1 mM EDTA solution.
   iv. Rinse the flow path with 250 mL of deionized water.
   v. Equilibrate the flow path with 250 mL of Gel-filtration buffer.
   vi. On Day 8, transfer the concentrated protein into a 3-mL syringe connected to a 22G × 2" metal hub needle. Avoid bubbles.
   vii. Load the concentrated protein into a 2-mL sample loop on FPLC.
   viii. Inject the protein into the FPLC at 1 mL/min.
   ix. Combine the peak fractions at ~62 mL retention volume after injection, as shown in Figure 3.
   x. Transfer the combined Cas9 fractions to an Amicon Ultra tube (15-mL, 100-kDa MWCO).
   xi. Centrifuge at 4,000 × *g* and 4°C for 15 min. Refill the tube with more protein and mix the content by gently inverting the tube 4–5 times.
   xii. Repeat the centrifugation process to concentrate the Cas9 protein to ~2 mL.
   xiii. Sample 5 µL of the concentrated Cas9 protein, mix with 40 µL of 1× NuPAGE loading dye, and heat the mixture at 95°C for 5 min for SDS-PAGE analysis.
   xiv. Measure the absorbance of Cas9 protein at 280 nm using a NanoDrop Lite spectrophotometer.
   xv. Estimate the molar concentration of Cas9 by Beer's Law. The theoretical extinction coefficient of recombinant Cas9 is 126410 $M^{-1}cm^{-1}$.
   xvi. Continue to concentrate Cas9 to 40 µM. If over-concentrated, dilute Cas9 by Gel-filtration buffer.

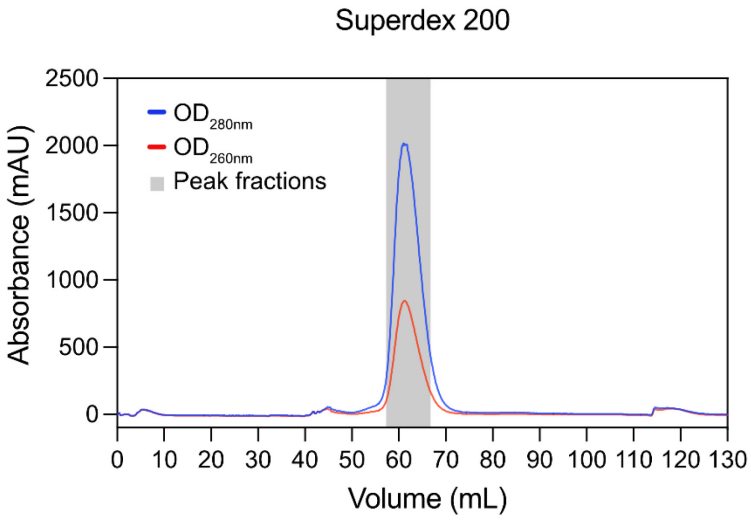


Figure 3. The elution profile of Superdex 200 chromatography.
The retention time of Cas9 is ~62 mL under our condition.

f. Sterilization, freezing, and analysis of Cas9 (Day 8)
   i. Pre-wet Ultra-free MC centrifugal filters (0.2-μm) with 400 μL of Gel-filtration buffer.
   ii. Centrifuge at 12,000 × *g* and 4°C for 1 min. Discard the flow through.
   iii. Transfer Cas9 protein to the centrifugal filters at 400 μL per tube.
   iv. Centrifuge at 12,000 × *g* and for 4°C 1 min. Discard the filter tube.
   v. Aliquot Cas9 in 200-μL PCR tubes at 11 μL per tube. Immediately snap-freeze the tube in liquid nitrogen after each pipetting.
   vi. Store the Cas9 aliquots at -80°C freezer. Cas9 protein is stable for at least a year. Avoid repeated freezing and thawing.
   vii. Dilute 10× Tris-glycine-SDS buffer to 1× in ddH_2O.
   viii. Run a Mini-PROTEAN TGX precast gel (10% PAGE) in Mini-PROTEIN Tetra vertical electrophoresis cell in 1× Tris-glycine-SDS running buffer to analyze the samples collected during the purification. Stain the PAGE with Coomassie protein stain (Figure 4A).
   Note: The final Cas9 protein carries at C-terminal a 3XFLAG affinity tag for detection and a 3× nuclear localization sequence for nuclear import (Figure 4B).

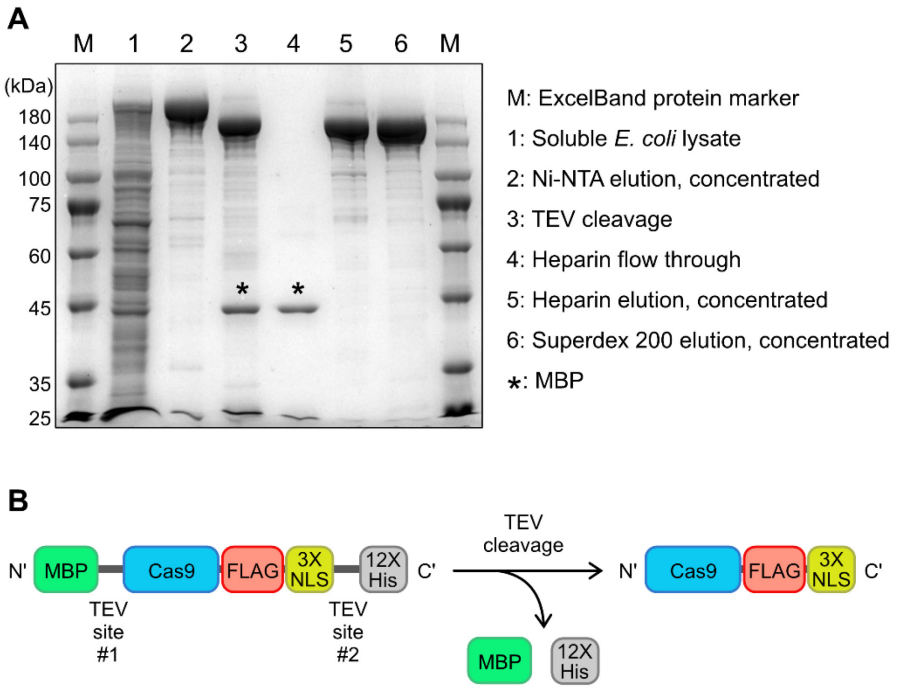


Figure 4. Purity of recombinant Cas9 protein.
(A) SDS-PAGE analysis of Cas9 protein sampled at different stages of purification. The gel image was taken by an Azure imaging system. (B) Recombinant Cas9 is expressed as a fusion protein carrying a maltose binding protein (MBP) at the N terminus, and a 3X FLAG tag, a 3X nuclear localization sequence (NLS), and a 12X His tag at the C terminus. There are two TEV sites to allow the separation of MBP and 12X His tag by TEV cleavage.

B. Synthesis and purification of sgRNA

1. Enzymatic synthesis of sgRNA
   a. Synthesis of sgRNA transcription template by overlapping PCR (Day 1)
      i. Prepare a 1 mM premix of Top and Bottom oligonucleotides in Qiagen EB buffer (sequences below). Store at -20°C for up to a year.

Top oligonucleotide
5'-*TAATACGACTCACTATA*G-guide sequence-GTTTTAGAGCTATGCTGGAAACAG CATAGCAAGTTAA-3'
Bottom oligonucleotide
5'-GCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAA CTTGCTATGCTGTTTCCAGCAT-3'

Note: The italicized sequence (TAATACGACTCACTATA) is the T7 promoter, where the T7 RNA polymerase binds and initiates RNA synthesis at the first guanosine (G, highlighted in grey) immediately downstream of the promoter sequence. Ideally, the spacer sequence should begin with a 5′ G to enable in vitro transcription (IVT). For targets without a G at the 5′ end of the spacer, it is essential to append an extra G to the full 20-nt spacer. The resulting sgRNA will carry the extra G at the 5′ end of sgRNA. Alternatively, the Ribozyme-gRNA-Ribozyme strategy can be used to preserve the full spacer sequence and provide flexibility at the 5′ end of the sgRNA (Rouet et al., 2018; Zhang et al., 2017).

      ii. Prepare a 10 mM premix of Forward and Reverse primers in Qiagen EB buffer (sequences below). Store at -20°C for up to a year.

Forward primer
5'-TAATACGACTCACTATAG-3'
Reverse primer
5'-GCACCGACTCGGTGCCACTTTTTCAAG-3'

Note: The annealing and extension of the Top and Bottom oligonucleotides is technically sufficient to generate a double-stranded DNA template for IVT. However, adding the Forward and Reverse primers to the PCR reaction significantly increases the yield of DNA template. The principle of overlapping PCR is illustrated in Figure 5A.

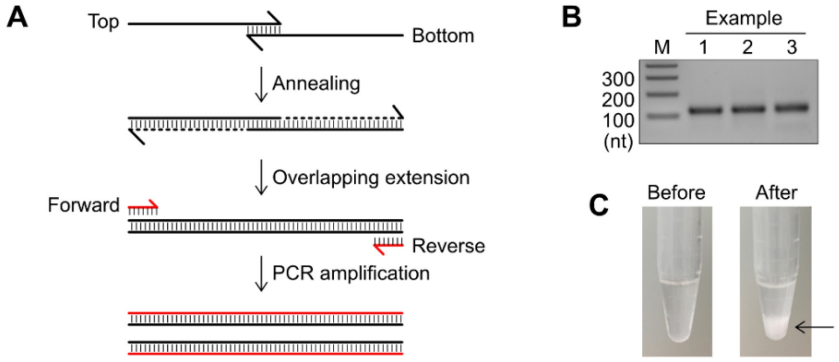


Figure 5. Synthesis of sgRNA DNA template by overlapping PCR.
(A) Schematic representation of overlapping PCR using four DNA oligonucleotides. (B) Visualization of the DNA template by DNA gel electrophoresis and SYBR Safe staining. Three examples of DNA templates are shown. (C) The white precipitation from IVT reaction is pyrophosphate, which is insoluble in water (marked by arrow).

iii. Set up a 30-µL PCR reaction using the KAPA HIFI PCR kit as indicated below:

Component	Volume (µL)	Final concentration
H_2O, molecular biology grade	18	n/a
5× KAPA HIFI buffer	6	1×
1 mM Top and Bottom premix	1.5	50 µM
10 mM Forward and Reverse premix	3	1 mM
KAPA dNTP	0.9	2 mM
KAPA HIFI polymerase	0.6	0.6 U

Note: Other brands of DNA polymerase and PCR kits can also be used to generate the DNA template. Refer to the manufacturer's protocols to optimize the PCR conditions.

iv. Run PCR in the following thermal cycler setting:

Temperature (°C)	Time (sec)	Cycle number
95	60	1
98	10	30
59	10	
72	10	
72	120	1
4	Hold	1

v. Mix 2 µL of PCR product with 5 µL of 1× Gel Loading Dye Purple.
vi. Run DNA gel electrophoresis in a 2% (w/v) agarose gel in 1× TAE buffer at 100 V in a Mini Gel electrophoresis system.
vii. Stain the gel with SYBR Safe, according to the manufacturer's instruction, to confirm the size of sgRNA template (Figure 5B).
viii. Proceed to IVT reaction without PCR cleanup. Alternatively, store at -20°C for up to a year.

Note: We did not observe any difference in the yield of sgRNA using the purified DNA template (by Qiagen PCR cleanup kit) versus the unpurified template for IVT. The KAPA PCR buffer seems compatible with our IVT reaction. We have not tested other PCR kits for compatibility.

b. *In vitro* transcription by T7 RNA polymerase (Day 1)
   i. Prepare DEPC-treated H_2O. Store at room temperature for up to a year.
   ii. Prepare 10× IVT buffer, 50% (w/v) PEG 2000, 25 mM NTP mix, 1 M DTT and 2× Stop solution. Store the reagents at -20°C as 1-mL aliquots for up to a year (see Recipes).
   iii. Set up a 150-µL IVT reaction in a 1.5-mL tube as follows:

Component	Volume (µL)	Final concentration
DEPC-treated H_2O	52	n/a
10× IVT buffer	15	1×
50% PEG 2000	15	5%
1 M DTT	3	20 mM
sgRNA template	30	n/a
25 mM NTP mix	30	5 mM
T7 RNA polymerase	5	100 U

   iv. Incubate the reaction at 37°C for 2 h.
   Note: White precipitation of pyrophosphate should be visible after 30 min of incubation (Figure 5C). Longer incubation may not always increase sgRNA yield and is prone to sgRNA degradation. For sgRNA sequences that are difficult to transcribe, try to incubate the reaction at 42°C for 2 h.
   v. Add 1.5 µL of RNase-free DNase I and incubate at 37°C for 1 h to digest the sgRNA DNA template.
   vi. Mix in 150 µL of 2× Stop solution to terminate the reaction. Store the IVT reaction mixture at -80°C until PAGE purification. The IVT mixture is stable in the 1× Stop solution for at least 3 months.

2. PAGE purification of sgRNA
   a. Casting of denaturing urea-PAGE (Day 1)
      i. Prepare urea-PAGE premix and store at 4°C for up to a month.
      ii. Prepare 20% (w/v) APS. Store at 4°C for up to a week.
      iii. Thoroughly wash the glass plates, comb, and spacers with Aquet liquid detergent, and wipe them dry with paper towels. Other mild laboratory detergents are ok.
      iv. Lay the glass plates on top of a sheet of clean paper towel on the bench.
      v. Apply 100 µL of Sigmacote siliconizing reagent to the upper surface of each plate. Immediately wipe the solution evenly over the glass surface with Kimwipes. The solution should dry out quickly because of the solvent inside.
      vi. Use another piece of Kimwipes to remove excess residue. The glass surface should be clean and smooth, but not greasy.
      vii. Place the glass plates with the two Sigmacote-coated surfaces facing inward and the spacers sandwiched between to assemble the plate cassette.
      viii. Follow the manufacturer's instruction to install the urea-PAGE on Owl P10DS vertical electrophoresis system and seal the bottom of glass plates with 1% (w/v) agarose.
      Note: Other electrophoresis systems of similar gel sizes are also compatible with the protocol. We have obtained the same resolution using Hoefer SE 600 Series Vertical Electrophoresis System (Hoefer, catalog number: SE660-15-1.5).
      ix. Warm the urea-PAGE premix to room temperature. Cold premix will not polymerize properly.
      x. Mix the following components in a 100-mL beaker to make 75 mL of urea-PAGE.

Component	Volume	Final concentration
Urea-PAGE premix	75 mL	1×
20% APS	150 µL	0.04%
TEMED	75 µL	1/1,000×

xi. Stir the solution at room temperature for 2 min.
xii. Use a 25-mL serological pipette to transfer the solution into the plate cassette. Slowly pipette the solution down the side of plates to avoid bubbles.
xiii. Insert the comb between the plates and secure it with a large binder clip.
xiv. Allow the gel to polymerize at room temperature for at least 2 h before electrophoresis. The gel should start to polymerize within 10 min.
xv. For the best separation result, allow the gel to polymerize at room temperature overnight. Install the electrophoresis outer shield to prevent the gel from drying.
xvi. Alternatively, remove the polymerized gel from the electrophoresis system, store it in a plastic bag at 4°C, and use it within a week.

b. Electrophoresis of sgRNA on urea-PAGE (Day 2)
i. Bring the IVT reaction mixture (if stored at -80°C) and urea-PAGE (if stored at 4°C) to room temperature.
ii. Fill up the inner and outer buffer reservoirs with 0.5× TBE buffer.
iii. Gently flush the sample wells with 0.5× TBE buffer using a 10-mL syringe attached to a 23G needle.
iv. Load the IVT reaction mixture into the wells.
v. Connect the electrophoresis system to Bio-Rad PowerPac HV power supply. The final setup is illustrated in Figure 6A.
vi. Run the urea-PAGE at 300V for approximately 4 h until the bromophenol blue has run off the gel and xylene cyanol reaches the bottom of the PAGE (Figure 6B). The full-length sgRNA should be at the center of the PAGE. Lower voltage does not affect the gel resolution but takes more time to run.
vii. Disconnect the electrophoresis system from the power supply.
viii. Remove the urea-PAGE from the system and wipe off the excess buffer.
ix. Set the plates on a clean bench surface, and use a marker pen to label the front plate. Flip the plates over with the notched plate on top.

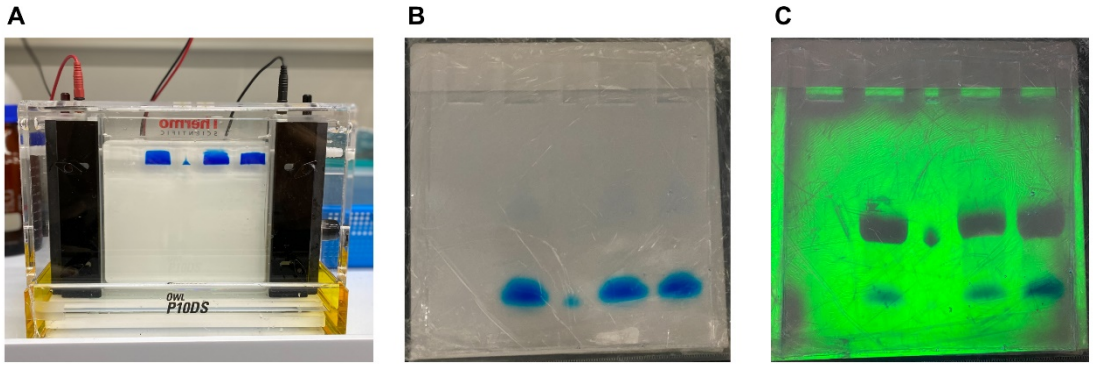


Figure 6. Visualization and extraction of sgRNA by gel electrophoresis.
(A) Setup of the urea-PAGE on Owl P10DS vertical electrophoresis system. The blue samples in the wells are sgRNA mixtures. The center well contains a purified sgRNA standard. (B) After electrophoresis, the urea-PAGE is separated from the glass plates, covered by plastic wrap, and placed on top of a fluorescent thin layer chromatography plate for imaging. The light blue color is xylene cyanol. Bromophenol blue has already run off. (C) Visualization of sgRNA bands in the gel by a compact UV lamp. The black and blue bands are sgRNA and xylene cyanol, respectively. Prolonged IVT reaction often leads to sgRNA degradation and the appearance of truncated, small fragments (not seen here). All images were captured with a smartphone camera.

c. Visualization of sgRNA on urea-PAGE (Day 2)
i. Use a clean and thin metal spatula to remove the spacers. The spacers should slide out easily.

ii. Use the spatula to gently pry off the notched glass plate at a corner. Sigmacote helps separate the glass plates from the gel.
iii. Cover the gel with a sheet of plastic food wrap, and carefully flip over the gel together with the front glass plate. The gel is now front-side up.
iv. Pry off the front glass plate with a spatula, and cover the gel surface with plastic wrap. To avoid confusion, use a marker pen to label the gel orientation on the plastic wrap.
v. Place the wrapped gel on a green-fluorescing F_{254} silica thin-layer chromatography plate (Figure 6B).
vi. Hold a UVP compact UV lamp on top of the gel. Turn on the UV to visualize the sgRNA bands, which appear black in the green background (Figure 6C).
Note: UV is hazardous. Always wear proper protective gear when working with UV. Point the UV lamp away from you.
vii. Use a marker pen to circle the sgRNA bands. Work quickly to avoid damaging sgRNA by long UV exposure.
viii. Turn off the UV lamp, and return the gel on a glass plate to the bench.
ix. Slice out the sgRNA band using a disposable surgical blade. Transfer the gel piece into a 15-mL conical tube using the surgical blade.

d. Extraction of sgRNA from PAGE (Days 2–4)
i. Prepare 70% (v/v) ethanol in ddH_2O. Store at room temperature.
ii. Prepare 1 mg/mL glycogen and store at -20°C.
iii. Insert a 5-mL serological pipette into the conical tube to crush the gel into fine pieces. Smaller the gel pieces lead to better diffusion of sgRNA from the gel.
iv. Add 1 mL of 3 M sodium acetate (pH 5) and 7 mL of DEPC-treated water into the tube. The final volume is approximately 9 mL.
v. Incubate the tube on a rotary shaker at 4°C overnight to allow sgRNA to diffuse out from the gel pieces into the sodium acetate buffer.
vi. Spin down the gel pieces at 2,500 × *g* and 4°C for 10 min.
vii. Transfer the solution to a new 15-mL conical tube by pipetting. The volume should be ~7–8 mL. It is ok to pick up some gel pieces.
viii. Pass the solution through a 0.45-μm syringe filter to completely remove the gel pieces.
ix. Add 20 μL of 1 mg/mL glycogen and mix well.
Note: Glycogen acts as a carrier to help precipitate sgRNA and must be mixed thoroughly before adding isopropanol.
x. Add 7 mL of 100% (v/v) isopropanol and mix well by inverting the tube.
xi. Incubate the tube at -20°C overnight to precipitate the sgRNA.
xii. Centrifuge at 4,000 × *g* and 4°C for 15 min to pellet the sgRNA. Discard the supernatant.
xiii. Add 1 mL of ice-cold 70% (v/v) ethanol to the tube, and use a 1-mL pipette tip to dislodge and break up the sgRNA pellet.
xiv. Transfer the mixture to a 1.5-mL tube, and centrifuge at 12,000 × *g* and 4°C for 2 min to spin down the sgRNA precipitation. Discard the liquid by pipetting.
xv. Add 1 mL of ice-cold 70% (v/v) ethanol to rinse the sgRNA pellet. Centrifuge at 12,000 × *g* and 4°C for 1 min, and discard the liquid.
xvi. Repeat the ethanol wash once.
xvii. Add 0.5 mL of absolute ethanol to rinse the sgRNA pellet. Centrifuge at 12,000 × *g* and 4°C for 1 min. Remove the liquid completely by pipetting.
Tip: Absolute ethanol absorbs trace amount of H_2O from the 70% ethanol wash and speeds up the drying process in a vacuum concentrator.
xviii. Dry the sgRNA pellet in a SpeedVac vacuum concentrator at room temperature for ~5 min. The sgRNA pellet should appear white when dried fully. The dried sgRNA pellet can be stably stored at -80°C for 2 years.

3. Removal of 5' triphosphate moiety
a. Calf intestine phosphatase (CIP) reaction (Day 4)
i. Dissolve the sgRNA pellet in 50 μL of RNA dissolving buffer by incubating at room temperature for 5 min. Gently flick the tube to help dissolve sgRNA.

Note: Do not use a pipette to resuspend the sgRNA pellet because the pellet tends to stick to the inner wall of pipette tip.

ii. Dilute 1 µL of the sgRNA solution in 9 µL of RNA dissolving buffer.
Tip: The stock sgRNA is too concentrated and must be diluted for accurate absorbance measurement.
iii. Measure the absorbance of the diluted sgRNA at 260 nm on NanoDrop Lite spectrophotometer.
iv. Determine the molar concentration of sgRNA using the theoretical molecular weight of 34,900 g/mol. Multiply the tenfold dilution factor.
Note: The IVT reaction described in Step B-1-b yields 4–5 nmol of sgRNA, which is equivalent to 150 µg. Some sgRNA sequences are more difficult to transcribe due to the nature of the guide sequences, decreasing the yield of sgRNA.
v. Set up the CIP reaction in a 1.5-mL tube by mixing 1,000 pmol of sgRNA and 40 U of Quick CIP in a total of 60 mL in RNA dissolving buffer.
vi. Incubate the reaction at 37°C for 3 h to remove the immunogenic 5' phosphate moiety. Proceed immediately to the next step.

b. Extraction and precipitation of sgRNA (Days 4–5).
i. Prepare 0.3 M sodium acetate buffer (pH 5) by diluting from the 3 M stock solution in DEPC-treated water.
ii. Add 440 µL of 0.3 M sodium acetate buffer (pH 5) to the CIP reaction mixture to increase the total volume to 500 µL.
iii. Add 500 µL of phenol:chloroform mix (low pH, for RNA extraction).
iv. Mix well by vortexing for 20 s, and centrifuge at 12,000 × *g* at room temperature for 5 min.
v. Transfer the upper aqueous phase to a 1.5-mL tube.
vi. Add 500 µL of chloroform, mix well by vortexing for 20 s, and centrifuge at 12,000 × *g* at room temperature for 5 min.
Tip: Chloroform extraction ensures complete removal of phenol, which damages the Cas9 protein and is toxic to cells.
vii. Transfer the upper aqueous phase to a 1.5-mL tube.
viii. Add 10 µL of 1 mg/mL of glycogen and mix well to help precipitate sgRNA.
ix. Add 1 mL of 100% isopropanol, mix well, and incubate at -20°C overnight to precipitate the sgRNA.
x. Centrifuge at 12,000 × *g* and 4°C for 10 min to spin down the sgRNA pellet.
xi. Wash the sgRNA pellet with 1 mL of ice-cold 70% (v/v) ethanol three times.
xii. Wash the sgRNA pellet with 0.5 mL of absolute ethanol once.
xiii. Completely remove the liquid by pipetting.
xiv. Dry the sgRNA pellet in a SpeedVac vacuum concentrator at room temperature for ~5 min. The dried sgRNA pellet can be stably stored at -80°C for 2 years.

4. Refolding of sgRNA
a. Dissolve the sgRNA pellet in 30 µL of RNA dissolving buffer and determine the molar concentration as described in Step B-3-a-i.
b. Adjust the sgRNA concentration to 48 µM using RNA dissolving buffer.
c. Incubate the sgRNA at 65°C for 5 min to partially unfold the sgRNA and cool gradually to room temperature to refold the sgRNA.
d. Snap freeze the sgRNA in 10-µL aliquots in liquid nitrogen and store at -80°C. The sgRNA is stable for 2 years. Avoid repeated freezing and thawing.

C. Assembly of Cas9 RNP

1. Thaw Cas9 protein (40 µM) and sgRNA (48 µM) to room temperature.
2. Set up 20 µM of Cas9 RNP by mixing equal volumes of Cas9 protein and sgRNA.
Note: The molar ratio of Cas9 to sgRNA is 1 to 1.2. It is crucial to add Cas9 protein to sgRNA, but not in the reverse order, which is prone to trigger Cas9 aggregation and reduce the efficiency of Cas9 RNP. The RNP concentration is calculated based on the concentration of the protein, since it is the limiting component.

3. Swirl the mixture with the pipette tip while slowly releasing Cas9 protein into sgRNA.
4. Pipette mix the solution 4–5 times, and incubate the Cas9 RNP at 37°C for 5 min for the formation of ribonucleoprotein complexes.
5. Keep the Cas9 RNP at room temperature and use it within 2 h.

D. Endotoxin detection assay

1. Quantitate the level of endotoxin contamination in Cas9 and sgRNA using ToxinSensor chromogenic lyophilized Amebocyte lysate (LAL) endotoxin assay kit. Follow the manufacturer's protocol to reconstitute the assay reagents, and use the certified endotoxin-free tips and vials included in the kit.
2. Reconstitute LAL in 1.7 mL of LAL Reagent water.
3. Reconstitute Chromogenic substrate in 1.7 mL of LAL Reagent water.
4. Reconstitute Stop solution in 10 mL of 0.46 M HCl diluted in LAL Reagent water.
5. Reconstitute Color-stabilizer number 2 and number 3 solutions each in 10 mL of LAL Reagent water.
6. Reconstitute Endotoxin standard in 2 mL of LAL Reagent water to obtain a stock solution of 10 EU/mL.
7. Dilute the stock endotoxin standard in LAL Reagent water to 0.1 EU/mL, 0.05 EU/mL and 0.025 EU/mL and 0.01 EU/mL.
8. Dilute 10 µL of Gel-filtration buffer tenfold in 90 µL of LAL Reagent water. Set up three replicates.
   Note: Gel-filtration buffer is the Cas9 protein storage buffer and also is the base of RNA dissolving buffer.
9. Dilute 10 µL of stock Cas9 (40 µM) tenfold in 90 µL of LAL Reagent water. Set up triplicates.
10. Dilute 10 µL of stock sgRNA (48 µM) tenfold in 90 µL of LAL Reagent water. Set up triplicates.
11. Dispense 100 µL of LAL Reagent water (as blank), the diluted endotoxin standards, and the diluted Cas9 and sgRNA samples into different endotoxin-free vials. Set up triplicates.
12. Add 100 µL of reconstitute LAL to each vial. Mix well by swirling the vials gently to avoid foaming.
13. Incubate the vials at 37°C for 5 min.
14. Add 100 µL of reconstituted Chromogenic substrate solution to each vial. Mix well by swirling the vials gently to avoid foaming.
15. Add 500 µL of reconstituted Stop solution to each vial. Mix well by swirling the vials gently to avoid foaming.
16. Add 500 µL of Color-stabilizer number 2 solution to each vial. Mix well by swirling the vials gently to avoid foaming.
17. Add 500 µL of Color-stabilizer number 3 solution to each vial. Mix well by swirling the vials gently to avoid foaming.
18. Transfer 200 µL of the final solution into a 96-well plate.
19. Measure the absorbance at 545 nm by Tecan microplate reader.

Data analysis

1. Subtract the absorbance of the LAL Reagent water control (as blank) from the absorbance of the endotoxin standards, Gel-filtration buffer, Cas9, and sgRNA samples.
2. Plot the absorbance of endotoxin standards against the endotoxin concentrations in Prism 9 software.
3. Determine the best-fit straight line among the four standard concentrations to obtain the standard curve and equation (Figure 7A).
4. Calculate the concentration of endotoxin in the Gel-filtration buffer, Cas9, and sgRNA samples using the absorbance at 545 nm and standard equation (Figure 7B).

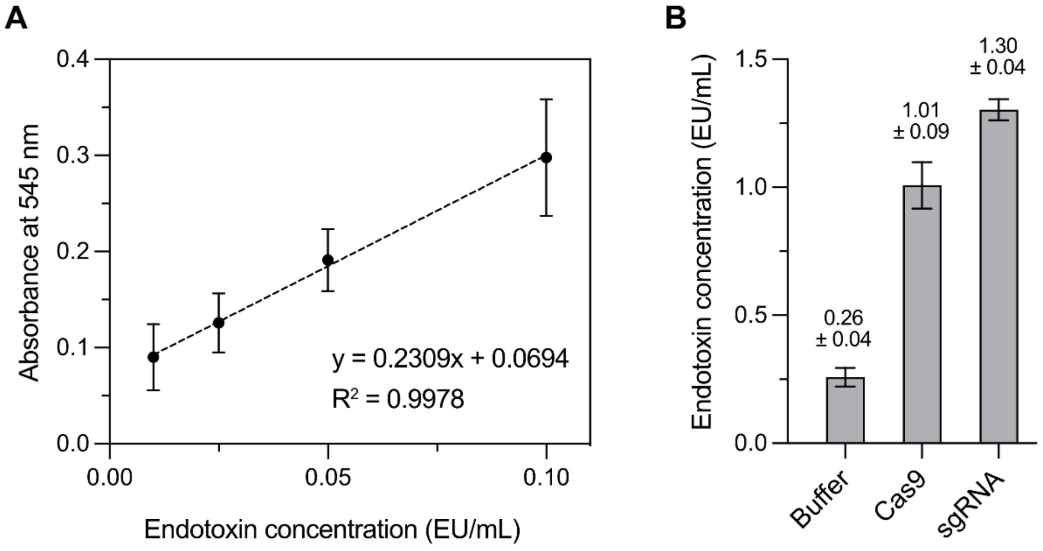


Figure 7. Analysis of Cas9 and sgRNA for endotoxin contamination.
(A) Endotoxin standard curve and conversion equation. (B) Average levels of endotoxin in the Gel-filtration buffer, Cas9, and sgRNA from three replicates.

Recipes

1. LB+glycerol medium

Reagent	Final concentration	Amount
Difco LB Broth	25 g/L	25 g
Glycerol	0.4% (v/v)	4 mL
ddH_2O	n/a	~980 mL
Total	n/a	1,000 mL

Autoclave at 121°C for 15 min. Store at room temperature.

2. Terrific Broth

Reagent	Final concentration	Amount
Difco Terrific Broth	47.6 g/L	2.38 g
Glycerol	0.4% (v/v)	0.2 mL
ddH_2O	n/a	~48 mL
Total	n/a	50 mL

Autoclave at 121°C for 15 min. Store at room temperature.

3. 20× modified M9 buffer

Reagent	Final concentration	Amount
$Na_2HPO_4•7H_2O$	561 mM	150.4 g
KH_2PO_4	441 mM	60 g
NH_4Cl	187 mM	10 g
ddH_2O	n/a	~800 mL
Total	n/a	1,000 mL

Autoclave at 121°C for 15 min. Store at room temperature.

4. 1 M IPTG

Reagent	Final concentration	Amount
IPTG	1 M	2.38 g
ddH_2O	n/a	10 mL
Total	n/a	10 mL

Sterilize the solution with a 0.22-μm syringe filter. Store at -20°C.

5. 50 mg/mL kanamycin

Reagent	Final concentration	Amount
Kanamycin	50 mg/mL	500 mg
ddH_2O	n/a	10 mL
Total	n/a	10 mL

Sterilize the solution with a 0.22-μm syringe filter. Store at 4°C.

6. LB agar plate with kanamycin

Reagent	Final concentration	Amount
Difco LB Broth	25 g/L	25 g
Agar	15 g/L	15 g
50 mg/mL Kanamycin	50 μg/mL	1 mL
ddH_2O	n/a	~970 mL
Total	n/a	1,000 mL

a. Dissolve LB broth in ~980 mL of ddH_2O. Transfer the medium to a 1-L glass bottle.
b. Add 15 g of agar, drop in a stir bar, and autoclave at 121°C for 15 min.
c. Stir the medium on a stir plate, and allow to cool to 30–40°C.
d. Add 1 mL of 50 mg/mL kanamycin solution. Stir well.
e. Transfer 25 mL of the solution to 10-cm Petri dishes by a serological pipette.
f. Allow the agar plates to cool and dry in a laminar flow hood for 30 min.
g. Store the plates at 4°C in a plastic bag.

7. Lysis buffer

Reagent	Final concentration	Amount
HEPES	20 mM	4.77 g
KCl	500 mM	37.28 g
Glycerol	10% (v/v)	100 mL
2-mercaptoethanol	10 mM	700 µL
Imidazole	70 mM	4.76 g
Triton X-100	0.5% (v/v)	5 mL
ddH_2O	n/a	~860 mL
Total	n/a	1,000 mL

a. Adjust the pH to 7.5 with KOH, and pass the buffer through a 0.45-µm filter in a Millicup-FLEX filtration unit.
b. To degas the buffer, drop a stir bar into the bottle, attach the GL45 screw cap, and place the bottle on a stir plate.
c. Seal one of the hose connectors on the GL45 cap with parafilm. Attach the other connector to a vacuum pump via a hose.
d. Turn on the vacuum pump and the stir plate. Air bubbles will start to emerge under constant stirring.
e. Turn off the vacuum and stir plate after 30 min or when air bubbles are no longer visible in the buffer.
f. Remove the stir bar and replace the GL45 cap with a standard bottle cap.
g. Store the buffer at 4°C for up to a month.

8. Wash buffer

Reagent	Final concentration	Amount
HEPES	20 mM	4.77 g
KCl	1000 mM	74.55 g
Glycerol	10% (v/v)	100 mL
2-mercaptoethanol	10 mM	700 µL
Imidazole	100 mM	6.8 g
ddH_2O	n/a	~820 mL
Total	n/a	1,000 mL

Adjust the pH to 7.5 with HCl. Filter and degas the buffer as described in Lysis buffer. Store the buffer at 4°C for up to a month.

9. Elution buffer

Reagent	Final concentration	Amount
HEPES	20 mM	4.77 g
KCl	300 mM	22.37 g
Glycerol	10% (v/v)	100 mL
2-mercaptoethanol	10 mM	700 µL
Imidazole	400 mM	27.2 g
ddH_2O	n/a	~880 mL
Total	n/a	1,000 mL

Adjust the pH to 7.5 with HCl. Filter and degas the buffer as described in Lysis buffer. Store the buffer at 4°C for up to a month.

10. IEX A buffer

Reagent	Final concentration	Amount
HEPES	20 mM	4.77 g
KCl	300 mM	22.37 g
Glycerol	10% (v/v)	100 mL
2-mercaptoethanol	5 mM	350 µL
ddH_2O	n/a	~880 mL
Total	n/a	1,000 mL

Adjust the pH to 7.5 with KOH. Filter and degas the buffer as described in Lysis buffer. Store the buffer at 4°C for up to a month.

11. IEX B buffer

Reagent	Final concentration	Amount
HEPES	20 mM	4.77 g
KCl	1000 mM	74.55 g
Glycerol	10% (v/v)	100 mL
2-mercaptoethanol	5 mM	350 µL
ddH_2O	n/a	~820 mL
Total	n/a	1,000 mL

Adjust the pH to 7.5 with KOH. Filter and degas the buffer as described in Lysis buffer. Store the buffer at 4°C for up to a month.

12. Gel-filtration buffer

Reagent	Final concentration	Amount
HEPES	20 mM	4.77 g
KCl	150 mM	11.18 g
Glycerol	10% (v/v)	100 mL
2-mercaptoethanol	1 mM	70 µL
ddH_2O	n/a	~890 mL
Total	n/a	1,000 mL

Adjust the pH to 7.5 with KOH. Filter and degas the buffer as described in Lysis buffer. Store the buffer at 4°C for up to a month.

13. 100 mM PMSF

Reagent	Final concentration	Amount
PMSF	100 mM	0.17 g
Absolute ethanol	99%	10 mL
Total	n/a	10 mL

Store at -20°C for up to a year.

14. 10 mg/mL DNase I (from bovine pancreas)

Reagent	Final concentration	Amount
DNase I from bovine pancreas	10 mg/mL	10 mg
Lysis buffer	1×	10 mL
Total	n/a	10 mL

Snap freeze in 1-mL aliquots in liquid nitrogen and store at -80°C for up to two years.

15. 0.5 N NaOH + 1 mM EDTA solution

Reagent	Final concentration	Amount
NaOH	0.5 N	20 g
EDTA	1 mM	0.29 g
ddH_2O	n/a	~1,000 mL
Total	n/a	1000 mL

Filter and degas the buffer as described in Lysis buffer. Store the buffer at 4°C for up to a year.

16. DEPC-treated H_2O

Reagent	Final concentration	Amount
0.1% DEPC	0.0001% (v/v)	1 mL
ddH_2O	n/a	999 mL
Total	n/a	1,000 mL

a. Stir the solution at room temperature for 1 h.
b. Autoclave for 15 min.
c. Store the solution at room temperature.

17. 10× IVT buffer

Reagent	Final concentration	Amount
Tris	500 mM	3.05 g
$MgCl_2$ hexahydrate	300 mM	3.05 g
Triton X-100	0.1%	50 µL
Spermidine	20 mM	145 mg
DEPC-treated H_2O	n/a	~45 mL
Total	n/a	50 mL

a. Clean the stir bar, glass beaker and pH meter probe with Aquet liquid detergent. Rinse well with ddH_2O and then DEPC-treated H_2O.
b. Adjust the pH to 8 with HCl. Sterilize the solution with a 0.22-µm syringe filter. Store at -80°C as 1-mL aliquots for up to two years.

18. 50% PEG 2000

Reagent	Final concentration	Amount
PEG 2000	50% (w/v)	5 g
DEPC-treated H_2O	n/a	~6 mL
Total	n/a	10 mL

Sterilize the solution with a 0.22-µm syringe filter. Store at -80°C as 1-mL aliquots for up to two years.

19. 25 mM NTP mix

Reagent	Final concentration	Amount
Tris	20 mM	0.12 g
ATP	25 mM	0.69 g
UTP	25 mM	0.69 g
CTP	25 mM	0.66 g
GTP	25 mM	0.66 g
DEPC-treated H_2O	n/a	~48 mL
Total	n/a	50 mL

a. Clean the stir bar, glass beaker, and pH meter probe with Aquet liquid detergent. Rinse well with ddH_2O and then DEPC-treated H_2O.
b. Adjust the pH to 7 with HCl. Sterilize the solution with a 0.22-μm syringe filter. Store at -80°C as 1-mL aliquots for up to two years.

20. 1 M DTT

Reagent	Final concentration	Amount
DTT	1 M	1.54 g
DEPC-treated H_2O	n/a	10 mL
Total	n/a	10 mL

Sterilize the solution with a 0.22-μm syringe filter. Store at -80°C as 1-mL aliquots for up to two years.

21. 0.5 M EDTA

Reagent	Final concentration	Amount
EDTA	0.5 M	14.6 g
ddH_2O	n/a	100 mL
Total	n/a	100 mL

Adjust the pH to 8.5 with NaOH. Sterilize the solution with a 0.22-μm syringe filter. Store at room temperature.

22. 2× STOP solution

Reagent	Final concentration	Amount
Formamide	95% (v/v)	38 mL
Bromophenol blue	0.05% (w/v)	0.02 g
Xylene cyanol	0.05% (w/v)	0.02 g
0.5 M EDTA	20 mM	1.6 mL
Total	n/a	40 mL

Store at -20°C as 1-mL aliquots for up to two years.

23. Urea-PAGE premix

Reagent	Final concentration	Amount
Urea	7 M	105 g
40% Polyacrylamide	10% (v/v)	62.5 mL
5× TBE	0.5	25 mL
ddH_2O	n/a	82.5 mL
Total	n/a	250 mL

Store at 4°C for up to a month.

24. 20% APS

Reagent	Final concentration	Amount
APS	20%	0.2 g
ddH_2O	n/a	1 mL
Total	n/a	1 mL

Store at 4°C for up to a week.

25. 1 mg/mL glycogen

Reagent	Final concentration	Amount
Glycogen	1 mg/mL	1 mg
DEPC-treated H_2O	n/a	1 mL
Total	n/a	1 mL

Store at -20°C as 1-mL aliquots for up to two years.

26. RNA dissolving buffer

Reagent	Final concentration	Amount
Gel-filtration buffer	1×	10 mL
$MgCl_2$ hexahydrate	10 mM	20 mg
Total	n/a	10 mL

Store the buffer at -20°C for up to a year.

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Competing interests

The authors declare no competing interests.

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