pA-Tn5

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3XFlag-pATn5 Protein Purification and MEDS-loading (5x scale, 2L volume)

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1 Works for me dx.doi.org/10.17504/protocols.io.8yrhxv6

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ABSTRACT

Here we describe the method used to purify and load pATn5 for CUT&Tag, https://www.protocols.io/view/bench-top-cut-amp-tag-z6hf9b6/abstract. This protocol was modeled after that in Picelli et al (Picelli et al., 2014) using the pTXB1-rbs_3XFlag-pATn5-FL plasmid available from Addgene: https://www.addgene.org/124601/. It yields, as written, **8.7 ml loaded pATn5** used at 1:200 dilution in CUT&Tag reactions, and **10.5 ml unloaded pATn5** for future use, both of which should be stored at -20C and can easily be scaled down.

EXTERNAL LINK

https://www.nature.com/articles/s41467-019-09982-5

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Kaya-Okur HS, Wu SJ, Codomo CA, Pledger ES, Bryson TD, Henikoff JG, Ahmad K, Henikoff S: CUT&Tag for efficient epigenomic profiling of small samples and single cells. Nature communications 2019, In press.

MATERIALS TEXT

Equipment

- RC5C Sorvall centrifuge or similar
- Branson Sonifier 250 fitted with the Branson Ultrasonics™ Sonifier™ Micro-tip
- Eppendorf 5804R, or similar

Reagents

- Ampicillin, 100mg/mL stock: Sigma-Aldrich A9518
- Carbenicillin, 100mg/mL stock: Disodium Salt Thermo Fisher Scientific cat# 10177012,
- LB: Miller's LB Broth, Corning Cat# 46050CM
- IPTG, 1M Stock: ISOPROPYL B-D-THIOGALACTOPYRANOSIDE, Sigma I6758-1G
- DTT, 1M stock: Sigma D0632
- Roche Complete EDTA free protease inhibitor (PI) tablets, Sigma 5056489001
- Chitin Resin (NEB S6651S)

Consumables:

- Econo-Pac® Chromatography Columns, (BIO-RAD 732-1010)
- 50 mL Conical Tubes, Fisher 14-959-49A
- 4x Millipore Centrifugal Filter Units 30K, Amicon Ultra-15 Cat# UFC903024
- Slide A Lyzer 10K MWCO Dialysis Cassettes, Fisher PI66830
- 18G needles
- foam buoy
- Zip Tie 1
- ¼ inch binder clip
- 2-inch stirbar

Buffers and Solutions

1L HEGX Buffer: add Protease Inhibitor Tablets just before use. Sigma #5056489001

1L HEGX Buffer:	Vol (mL)	Stock Solution
0.02 M HEPES-KOH at pH 7.2	20	1M
1 M NaCl	200	5M
0.001 M EDTA	2	0.5M
10% glycerol	100	100%
0.2% Triton X-100	2	100%
676 mL Water		

Tn5 Dialysis Buffer 5 L total (make 2x 2.5L in 4 L beakers)

2X Tn5 Dialysis Buffer: 1.6L total	Vol (mL)	Stock Solution
0.1M HEPES-KOH at pH 7.2	160	1M
0.2 M NaCl	64	5M
0.2 mM EDTA	0.64	0.5M
1.7 mM DTT	2.72	1M
0.2% Triton X-100	3.2	100%
20% glycerol	320	100%

1049.44 mL Water		

1M HEPES-KOH pH 7.2

- 400mL Water
- 119g HEPES
- ~7.12g KOH pellets to pH7.2
- Adjust water to 500mL
- Filter Sterilize
- Store at 4 °C

MEDS Assembly and In Vitro Assay

- Annealing Buffer (10mM Tris pH 8, 50mM NaCl, 1mM EDTA).
- MED Oligos: Eurofins, salt-free purification *(need reference for sequences here)*
- Glycerol, Sigma G5516
- 150ng HMW Lambda DNA (NEB# N3011)
- Proteinase K, Thermo Fisher Scientific 25530049
- SDS, Sigma L4509
- 1KB+ MW Marker, Fermentas SM1343
- HMW Lambda DNA, NEB# N301
- EtBr, Invitrogen 15585011
- Agarose, Thermo Fisher Scientific 16500500

MEDS Analysis: 6% Acrylamide Gel for DNA- 1xTBE gel, run in 1xTBE buffer

- 1.35 mL 40% Acrylamide 19:1
- 90 uL 10% APS (freshly made)
- 13.5uL TEMED
- 0.9mL 10x TBE
- 6.65mL Water
- Alternatively, you can purchase a premade gel from Invitrogen: https://www.thermofisher.com/order/catalog/product/EC62652BOX?SID=srch-srp-EC62652BOX
- 6X Fermentas Orange Loading Dye, Thermo Fisher Scientific R0631
- 10X TBE, Fisher BP1333-1

5XTAPS-MgCl2-PEG8000

- 50 mM TAPS-NaOH pH8.5, Millipore Sigma T5130-25G (From 1M TAPS, pH to 8.5 with NaOH)
- 25 mM MgCl2
- 40% PEG 8000 (Fisher 202452)

Protein Analysis

- Novex; 4-20% Tris-Glycine Gel 1.0 mm, 12 well WEDGE, Thermo Fisher Scientific cat# XP04120PK2
- Pierce™ Bovine Serum Albumin Standard Pre-Diluted Set, Thermo Fisher Scientific cat# 23208
- PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa, Thermo Fisher Scientific cat 26620
- 2-Mercaptoethanol, BIO-RAD cat#1610710
- 1xTris Glycine Buffer: http://cshprotocols.cshlp.org/content/2006/1/pdb.rec9132.full?sid=7968c375-3662-40ea-9003-cde2e1e384a9

3x SDS sample buffer

http://cshprotocols.cshlp.org/content/2006/1/pdb.rec10075.full?sid=4f034b3d-e1db-4b81-b971-87014561be54

- 188 mM Tris-Cl (pH 6.8)
- 3% SDS
- 30% glycerol
- 0.01% bromophenol blue
- 15% β-mercaptoethanol

Coomassie brilliant blue R250 (0.1% w/v)

http://cshprotocols.cshlp.org/content/2007/2/pdb.rec10716.full?sid=92093ab7-2ae7-4b2e-90ab-f676ab3ba5de

- Coomassie brilliant blue R250
- Methanol
- Acetic acid

Prepare the Coomassie brilliant blue R250 in a 60/30/10 (v/v/v) mixture of H_2O, methanol, and acetic acid.

Destaining solution for Coomassie brilliant blue R250

http://cshprotocols.cshlp.org/content/2007/2/pdb.rec10717.full?sid=98f359ff-80c0-4da8-8c24-f6c4bfa5a66e

- Methanol
- Acetic acid

Mix H_2O, methanol, and acetic acid in a ratio of 50/40/10 (v/v/v).

Material for PAGE Analysis of pATn5 purification:

- • Novex; 4-20% Tris-Glycine Gel 1.0 mm, 12 well WEDGE, Thermo Fisher Scientific cat# XP04120PK2
- • Pierce™ Bovine Serum Albumin Standard Pre-Diluted Set, Thermo Fisher Scientific cat# 23208
- • 2-Mercaptoethanol, BIO-RAD cat#1610710
- • 1xTris Glycine Buffer, http://cshprotocols.cshlp.org/content/2006/1/pdb.rec9132.full?sid=7985b22c-dd10-4305-9221-83116986f47a

Reagents for testing transposase activity in vitro:

- loaded and unloaded pATn5 in 50% glycerol
- High molecular weight DNA (NEB #N3011 Lambda DNA) diluted 1:10 from 500ng/uL to 50ng/uL
- 5XTAPS-MgCl2-PEG8000
- 1% SDS
- Proteinase K
- 0.7% Agarose gel made with 1x TAE and running buffer

SAFETY WARNINGS

For safety warnings and hazard information, please refer to the SDS (Safety Data Sheet).

Day 1 15m

1 Streak E. coli glycerol stock containing pTXB1-rbs_3XFlag-pATn5-FL plasmid onto an LB-Amp^{100} plate, incubate **Overnight** at **37 °C**

Day 2 2h

2 Select a single colony and make a patch plate for later, use within 1 week.

3 Make buffers and media.

See Materials section.

Day 3: Culture and Induce 8h

4 Early morning, with a sterile toothpick swipe through a bacterial patch to inoculate **30 ml** of LB broth+ carbenicillin 100ug/mL culture, let shake ~ **04:00:00** .

5 Put **1970 ml** of LB+ carbenicillin 100ug/mL in 6L flask at **37 °C** as well so it is already warm at the time of dilution.

Carbenicillin can be used in place of ampicillin. Carbenicillin is more stable, so it is potentially more effective at selecting only bacteria containing the plasmids of interest (for example, fewer satellite colonies will grow). However, it is also more expensive.

6 After **04:00:00** incubation use all 30 ml of starter culture to inoculate larger vessel.

7 Shake at **37 °C** , check OD600 periodically until it reaches O.D600 of 0.5-0.7. ~ **03:00:00**

8 Move to cold room to let cool ~ **01:00:00**

9 Induce culture with addition of fresh 1M IPTG to 0.25mM final and incubate on the **18 °C** shaker ~ **Overnight**

Day 4: Pellet and Freeze 2h

10 Pellet bacteria by centrifugation: Divide the culture between 6x **500 ml** bottles, centrifuge at 9500rcf, 4C for **01:00:00** 2h in a GS3 Rotor, RC5C Sorvall centrifuge.

11 Discard the supernatant.

12 Freeze bacterial pellets in dry ice and ethanol bath.

13 Store at -80 °C freezer until ready to process.

Day 5: Lyse, Chitin Binding 4h

14

Need 400 ml **HEGX+PI** today; make a total of 1L HEGX and add PI tablets as needed each day.

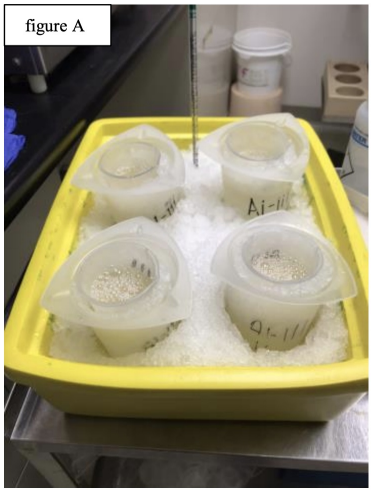
Remove pellets from freezer, thaw on ice and resuspend together in a combined total of 200 ml of chilled HEGX Buffer with Roche Complete EDTA free protease inhibitor (PI) tablets. Keep on ice while working.

15 Divide combined resuspension between 4x clean 100 ml plastic beakers nested in a larger plastic beaker filled with ice. See figure A.

16 While keeping in the ice nest, sonicate each sample 10-12 times, 00:00:45 each at 50% duty cycle and output level 7 on a Branson Sonifier 250 fitted with the Branson Ultrasonics™ Sonifier™ micro-tip. Between rounds of sonication keep in larger ice bucket (see figure A) and use a 2mL serological pipette to gently stir to facilitate cooling. Let each sample rest at least 00:00:45 between sonication rounds.

To prevent foaming keep the sonicator tip in the center of the sample. If excessive foaming occurs during sonication either wait for foam to settle or remove with a pipette and discard.

figure A



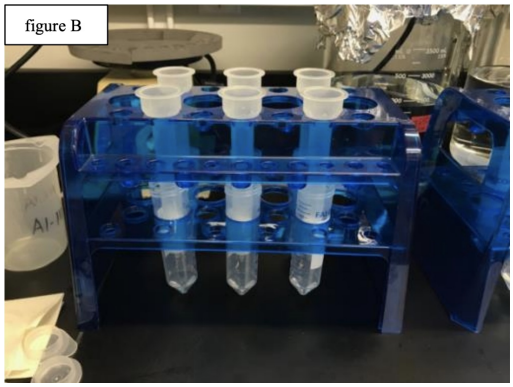
17 Transfer to fresh 50 mL conical tubes and pellet the lysate by centrifugation for 00:30:00 , 4 °C , at 16000 x g .

18

Chitin Binding: The remainder of the protocol is carried out at 4 °C or on ice. All buffers should be chilled on ice prior to use.

In 4 °C **room** prepare 10x Econo-Pac® Chromatography Columns (BIO-RAD 732-1010), each holds 20 ml Set-up columns (we use a rack intended for Qiagen Maxi-preps, figure B). Gently invert Chitin Resin (NEB S6651S) to fully suspend and add 2.5mL to each column. Wash each with 20 ml of HEGX Buffer, drain immediately, don't collect anything. Do not let resin dry out prior to addition of sample.

figure B



19 Add yellow cap to bottom of column.

20 Add supernatant from lysate spin to Chitin Resin slowly to prevent bubbles.

21 Cap column tightly and incubate Overnight at 4 °C on nutator. Check for leakage after 00:15:00 and 00:30:00 , wrap both ends in parafilm if necessary to prevent leakage.

Day 6: Wash and Cleavage Intein with DTT. 1h

22 Return columns to rack: remove yellow cap gently, hang rack over collection tube and uncap column. Drain unbound fraction into 50 mL tubes (save on ice or at 4 °C in the event troubleshooting is required later).

23 Wash column 2x with 20 ml HEGX collecting first wash into 50 mL tubes (save on ice or at 4 °C in the event troubleshooting is required later), the second wash can be wasted.

24 Add yellow cap to bottom of column.

25 Make 65 ml HEGX +PI with 100mM DTT and slowly add 6 ml to each sample.

26 Cap column tightly and nutate in cold room ~ 48:00:00

Day 7: Elute & Dialyze 4h

27 Return columns to rack: remove yellow cap gently, hang in rack over collection tube and uncap column. Drain unbound fraction into 15 mL conical tubes, combine at end. Cover resin with additional 2 ml of buffer and save at 4 °C until purification is verified.

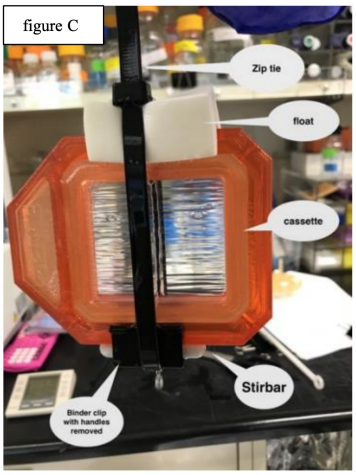
28 Record elution volume. ~ 50 ml

29 Dialyze the protein as described in the following substeps.

29.1 Assemble 2 Slide-A-Lyzer 10K MWCO Dialysis Cassettes

- Use a medium (1 ¼ inch) binder clip to secure a 2 inch stir bar and remove the clip handles gently. Use an 18-inch zip tie to secure a foam float to the cassette. See figure C.
- Hydrate the cassette by submerging and use a binder clip to anchor the cassette at the proper depth by clipping the zip tie tail to the lip of the dialysis beaker. Let hydrate according to product instructions

figure C



29.2 Using an 18 gauge needle load the Slide-A-Lyzer Cassettes with **25 ml** each. Follow product literature on how to properly load cassettes.

29.3 In a 4 L beaker, add both cassettes and dialyze for **01:00:00** - **02:00:00** in **2.5 L** of Tn5 dialysis buffer with DTT added to 1.7mM just before use.

29.4 Move samples to fresh Tn5 dialysis buffer, also **2.5 L** with DTT added to 1.7mM just before use, let dialyze **Overnight** at **4 °C** .

29.5 Recover sample from cassette and combine into a fresh 50mL conical tube. Hold on ice or at **4 °C** until next steps. Overnight storage is ok if this falls into a weekend.

Volume recovered from Dialysis: ~ **42 ml**

Day 8: Concentrate Protein 1h 30m

30 Separate dialyzed protein between 4x Millipore Centrifugal Filter Units 30K, Amicon Ultra-15 Cat# UFC903024

31 Spin in Eppendorf 5804R at **2880 x g** and **4 °C** for **01:00:00** . Check at **00:20:00** intervals to assess progress, do not let volume drop below top of filters.

32 Recover concentrated sample from the upper filter portion of the tube, use an extra 500uL of buffer from lower catch tube to wash any extra protein off of each filter and add to collected protein.

Volume recovered from Concentrator: 7 mL + 2mL for rinsing filter = 9 mL

33 Add an equal volume of sterile 80% glycerol to the concentrated protein. Combined with the glycerol from dialysis the final glycerol concentration is ~50%.

34 Store at **-20 °C** .

PAGE Analysis of pATn5 purification

35

Material:
• Novex; 4-20% Tris-Glycine Gel 1.0 mm, 12 well WEDGE, Thermo Fisher Scientific cat# XP04120PK2
• Pierce™ Bovine Serum Albumin Standard Pre-Diluted Set, Thermo Fisher Scientific cat# 23208
• PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa, Thermo Fisher Scientific cat 26620
• 2-Mercaptoethanol, BIO-RAD cat#1610710
• 1xTris Glycine Buffer

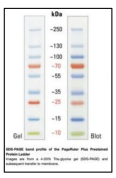
Run small volume of purified protein (and some from a previous batch if available) to compare yields. Include BSA standard range for quantification if desired. To the 3x Loading Dye from CSH Protocols, add 2-mercaptoethanol to 15% final before adding to the protein samples, heat to **100 °C** for **00:10:00** cool back to **Room temperature** prior to loading onto gel.

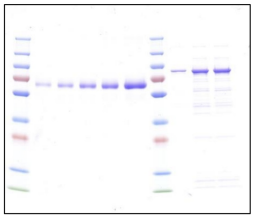
36 To make the BSA Standards dilute the 250 ng/uL BSA to 125 ng/uL and use increasing volumes on the gel.

37 Sample Prep and Gel Loading (figure D)

Lanes L→R	Volume of protein, uL	3xLoading Dye (+2-ME)	1xTE
Protein Marker	6	-	-
125ng BSA	1	3	10
250ng BSA	2	3	9
500ng BSA	4	3	8
750ng BSA	6	3	7
1.5ug BSA	12	3	-
Protein Marker	6	-	-
pATn5-Batch 2	1.5	3	10
pATn5-Batch 3	1.5	3	10
pATn5-Batch 3	3.0	3	8

figure D





Expected Sizes:
BSA: 66.5 kDa
pAtn5 before DTT: ~100kDA
pATn5 after DTT: ~73kDa

38 After loading samples run gel at 30mA for ~ **01:00:00** or until the loading dye leading edge runs to the bottom of the gel.

39 Transfer gel carefully to covered dish with Coomassie stain gently shake at least **01:00:00** to **Overnight** at **Room temperature** .

40 Replace stain with destain, add a rolled paper towel to dish to absorb excess Coomassie dye.

41 Replace destain with water, scan gel for notes.

Annealing of MEDS Oligos

42

Tube #	Seq Name	Seq 5' to 3'	OD	Vol. µl	nmol	µg	Len	MW	% GC Content	E260	Tm	Scale	Purif.	Vol in µl for 100 µM	Vol in µl for 200 µM
1	TN5MErev	[Phos]CTGTCTCTTATACACATCT	30	dry	178.8	1031.5	19	5768.8	36.8	167783	53.7	1.0 µmol	Salt-Free	1788	894
2	Tn5ME-A	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	161.5	dry	484.2	4959.6	33	10242.8	51.5	333456	70.9	1.0 µmol	Salt-Free	4842	1210.5
3	Tn5ME-B	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	188.3	dry	550.7	5839.5	34	10603	52.9	341956	71.9	1.0 µmol	Salt-Free	5507	2753.5

Data Sheet from Eurofins

43 Resuspend single-stranded salt-free oligos from Eurofins to 200uM in Annealing Buffer (10mM Tris pH8, 50mM NaCl, 1mM EDTA).

44 Mix equal volumes of both complementary oligos (at equimolar concentration) in a 1.5 ml microfuge tube, mix well and aliquot to in smaller tubes appropriate for thermal cycler.

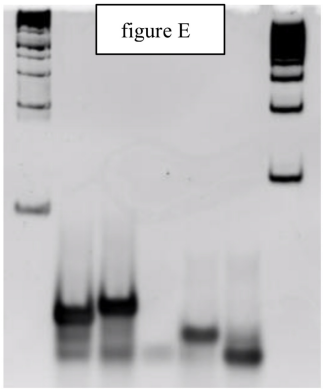
400 µl **Tn5MErev** + 400 µl **Tn5ME-A** = 800 µl → 4 tubes @ 200 µl
400 µl **Tn5MErev** + 400 µl **Tn5ME-B** = 800 µl → 4 tubes @ 200 µl

45 In MJ Cycler PTC-200 use ANNEALOL program which starts with a 95 °C 00:02:00 incubation and decreases temp in 5 °C increments incubating 00:05:00 each ending with a 00:05:00 incubation at 25 °C and holds at 8 °C afterwards. Store at -20 °C .

46 **Verify annealing:**
Run oligos, both ss and ds, on a 6% acrylamide 1xTBE (figure E) Lanes:

1. 1KB+ Marker 3uL
2. Rev+ ME-A 1uL +1uL 6X Fermentas Orange Loading Dye +6uL TE
3. Rev+ ME-B 1uL +1uL 6X Fermentas Orange Loading Dye +6uL TE
4. Rev 1uL +1uL 6X Fermentas Orange Loading Dye +6uL TE
5. ME-A 0.5uL +1uL 6X Fermentas Orange Loading Dye +6uL TE
6. ME-B 0.5uL +1uL 6X Fermentas Orange Loading Dye +6uL TE
7. 100 bp Marker 3uL

While annealing efficiency is not 100% it is robust, excess ssDNA will not load onto transposase and will wash away during CUT&Tag protocol.



Assembly of Tn5 with pre-annealed MEDS and in vitro assay to verify tagmentation activity

47 With oligo-mer:

- **600 µl 100 uM Tn5ME-rev/A oligo**
- **600 µl 100 uM Tn5ME-rev/B oligo**
- **7.5 ml 3xFlag-pATn5 (in 50% glycerol)**

The mixture is incubated for ~ **00:50:00** at **Room temperature** and then stored at **-20 °C** .

Testing transposase activity in vitro

48 In vitro activity assay is performed on 150 ng HMW Lambda DNA (NEB# N3011). Unloaded pATn5 or loaded pATN5 without $MgCl_2$ should have no impact on HMW DNA, loaded pATN5 in digestion buffer should digest HMW DNA to a smear. See figure F.

49 For each sample prepare the following reaction mix:

- **150 ng** HMW DNA
- **4 µl** 5XTAPS-$MgCl_2$-PEG8000 (water for no digestion buffer controls)
- **5 µl** pATn5-MEDS complex
- To 20ul Nuclease-Free H_2O

50 Incubate for **00:07:00** at **55 °C** .

51 To release and inactivate Tn5, add **2 µl** of 1%SDS (disrupt protein DNA interaction).

52 Incubate for **00:07:00** at **Room temperature** or **55 °C** .

53 Add **0.63 µl** proteinase K (20 mg/mL) to each reaction (degrade pATn5).

54 Incubate for **00:07:00** at **55 °C** .

55 Keep **On ice** or **4 °C** .

56 The samples can be analyzed by migration on 0.7% agarose gel in 1xTAE.

Gel Lanes: (figure F)

1. 150ng HMW lambda DNA
2. 1KB+ MW Marker
3. pATn5 (-) MEDS
4. pATn5 (+) MEDS, (-) digestion buffer
5. pATn5 (+) MEDS, (+) digestion buffer
6. pATn5[batch 2], (+) MEDS, (+) digestion buffer