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Modified DAP-seq protocol using a high-yield wheat germ expression system for Cannabis sativa transcription factors

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We use this protocol and it's working

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Abstract

This protocol describes a modified DNA affinity purification sequencing (DAP-seq) workflow adapted from the Bartlett and O'Malley DAP-seq protocols. The main modification is the use of an engineered pIX-HALO-derived vector, **pIX-HALO-ΔccdB-SX**, in which the original **ccdB** cassette was replaced with **SacI** and **XhoI** restriction sites to enable direct cloning of synthesised transcription factor coding sequences. The protocol also uses a **high-yield wheat germ in vitro expression system** for Halo-tagged transcription factor production. Affinity purification conditions were further modified by using **0.05% NP-40** to increase wash stringency and **salmon sperm DNA** as a competitive blocker to reduce non-specific DNA binding. These modifications provide a streamlined DAP-seq workflow for plant transcription factor binding-site discovery, particularly in non-model species such as **Cannabis sativa**.



Guidelines

1. Use high-quality genomic DNA isolated from a tissue in which the transcription factors of interest are expected to be biologically active. For DAP-seq, this can be important because endogenous DNA methylation state may influence binding-site accessibility in the genomic DNA input library.
2. Prepare an adapter-ligated genomic DNA library with an insert size of approximately **150–200 bp**. This range provides a practical balance between read mappability and binding-site resolution. Fragmentation should be assessed for each sheared DNA tube individually, as shearing efficiency may vary between tubes.
3. For **pIX-HALO-ΔccdB-SX** plasmid preparation, aim for **9–10 μg plasmid DNA in 20 μL** for each wheat germ expression reaction. The **pIX-HALO-ΔccdB-SX** vector behaves as a low-copy plasmid; therefore, use the maximum recommended bacterial culture input for the chosen midi-prep kit.
4. HALO magnetic beads settle rapidly. Mix the bead suspension thoroughly before aliquoting and between aliquots to ensure equivalent bead input across samples.
5. This protocol uses **0.05% NP-40** during bead washing and affinity purification. This is a relatively stringent condition and may reduce non-specific background, but users should be aware that detergent stringency can affect recovery of weaker protein–DNA interactions.
6. Denatured salmon sperm DNA is included as a competitive blocker during DNA affinity purification to reduce non-specific DNA recovery.
7. Retain a small aliquot of each wheat germ expression reaction before bead capture for optional western blot analysis.

Materials

DNeasy Plant Mini Kit (Qiagen 69106)

Diagenode Bioruptor® UCD-200 (Diagenode)

NEBNext Ultra II DNA library prep kit (New England Biolabs inc. E7645S)

XhoI (New England Biolabs inc. R0146S)

T4 DNA ligase (New England Biolabs inc. M0202S)

Wheat germ TNT® high yield SP6 kit (Promega L3260)

Magne Halo Tag Beads (Promega G7281)

Salmon sperm DNA (Sigma Aldrich D9156)

Buffer EB (Qiagen 19086)

4150 Agilent Tapestation system

D1000 or D5000 screen tapes (Agilent)

Qubit™ 4 Fluorometer (Thermo Fisher Scientific)

Qubit™ dsDNA Quantitation Broad Range Assay (Thermo Fisher Scientific Q32850).

Safety warnings

1. Do not proceed with adapter ligation until the genomic DNA is sufficiently sheared. Poor or uneven shearing will affect library complexity, insert-size distribution and DAP-seq peak resolution.
2. Test each sheared genomic DNA tube individually. Do not assume that all tubes fragmented equally during Bioruptor treatment.
3. Do not over-dry plasmid DNA pellets during midi-prep. Over-dried plasmid DNA may be difficult to resuspend and may reduce the final concentration available for wheat germ expression.
4. Do not allow HALO magnetic beads to settle during aliquoting. Uneven bead distribution can cause large differences in protein capture and DNA recovery between samples.
5. During magnetic separation, avoid disturbing the bead pellet when removing supernatant. Bead loss will reduce recovery.
6. Do not skip the empty-vector HALO control. This control is important for estimating background DNA recovery.
7. Keep final DAP-seq libraries low-bind and avoid unnecessary freeze-thaw cycles.

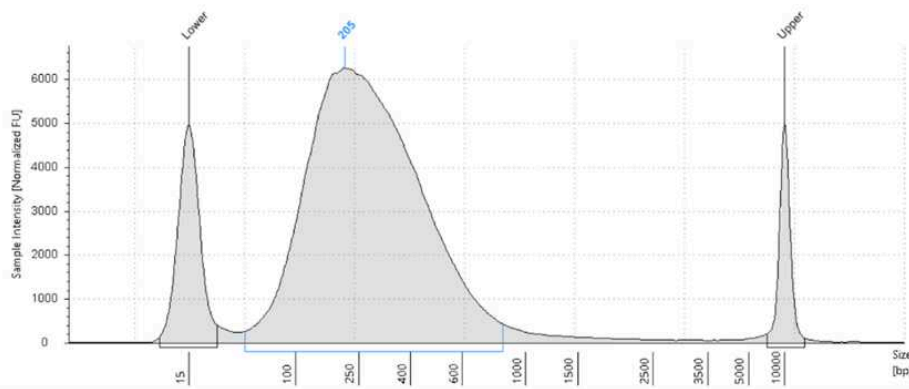


Generation of Adapter ligated DNA library with ~150-200bp insert size sufficient for at least 30 DAP-seq samples at 100ng library per sample

5h 15m 40s

- 1 Isolate high-quality genomic DNA from the tissue of interest. Where possible, use tissue in which the transcription factors being tested are expected to be biologically active, as relevant binding sites may be more likely to occur in an appropriate methylation context.
- 2 Isolate at least **30 µg genomic DNA**. Pool genomic DNA preparations if required and quantify the pooled DNA using Qubit. 2h
- 3 Switch on the cooling system for the Diagenode Bioruptor. Adjust the rear tap so that cooled water flows in and out of the Bioruptor at equilibrium. 30s
- 4 Allow the water in the cooling system to reach **4°C** before starting sonication.
- 5 Prepare **six low-bind microcentrifuge tubes**, each containing **100 µL genomic DNA at 50 ng/µL**. Keep tubes on ice. 5m
- 6 Shear genomic DNA using the Diagenode Bioruptor on the **high** setting with **30 s on / 30 s off** intervals for **20 min**. 20m
- 7 After 20 min, remove the tubes and place them immediately on ice. 10s
- 8 Assess DNA fragmentation for each tube individually using a **D1000 or D5000 ScreenTape**. 15m
- 9 Aim for a fragment distribution with a peak maximum of approximately **200 bp** and a relatively narrow distribution. An average insert size of **160–200 bp** is suitable for DAP-seq.

C1: 20 min sonication 50ng/ul 100 ul

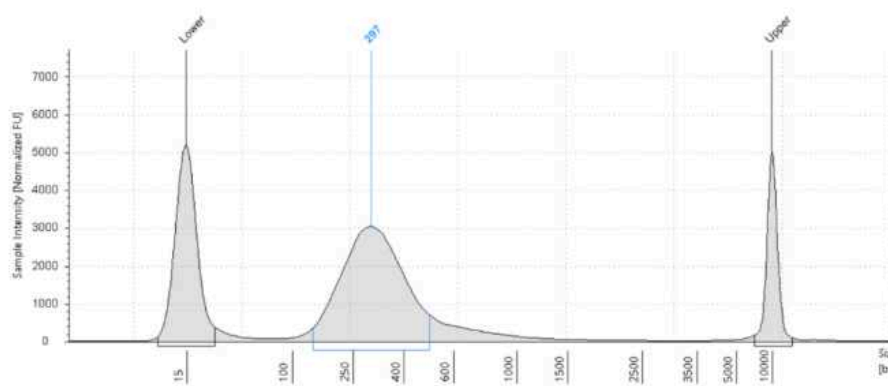


Fragmented DNA following a normal distribution of fragment sizes centred on 206 bp.

- 10 If a tube is insufficiently sheared and still contains excess large fragments, return it to the Bioruptor for an additional **10–20 min** using the same settings. 20m
- 11 During additional shearing, keep all Bioruptor positions occupied. If a sample tube is complete, replace it with a tube containing **100 µL water** to maintain balanced sonication conditions.
- 12 Repeat fragmentation assessment and additional shearing as needed until all six tubes show the desired fragment-size distribution. 15m
- 13 Proceed to adapter ligation using the NEBNext Ultra II DNA library prep kit (E7645S) using the maximum of 1 µg (20 µL) of sheered gDNA input per reaction. The 6 tubes of sheered DNA is sufficient for 30 reactions. Each adapter ligated library contains enough DNA for 1.6 100ng DAP-seq samples, therefore 30 reactions will provide enough adapter ligated library for 48 100ng DAP-seq samples. Follow the protocol as documented for ligation.
- 14 Proceed to adapter ligation using the **NEBNext Ultra II DNA Library Prep Kit**. 2h
- 15 For each ligation reaction, use the maximum recommended input of **1 µg sheared genomic DNA in 20 µL**.
- 16 Use the six sheared DNA tubes to prepare up to **30 adapter-ligation reactions**.

- 17 During double-sided size selection, use the kit conditions for either **150 bp** or **200 bp** insert size. In this protocol, the **150 bp insert-size condition** was used.
- 18 For the final elution of size-selected library from SPRIselect beads, elute in EB buffer for **10 min** rather than the minimum 2 min to improve DNA recovery.
- 19 Assess each size-selected, adapter-ligated library using a **D1000 or D5000 ScreenTape** to confirm successful ligation and size selection.
- 20 Pool the adapter-ligated libraries into a single low-bind microcentrifuge tube.
- 21 Run the pooled adapter-ligated library on a **D1000 or D5000 ScreenTape** for final reference.

B1: Pooled adapter lib



Pooled Adapter-ligated DNA library with an average insert size of ~150 bp (+150 bp adapter content).

- 22 Store the pooled adapter-ligated genomic DNA library at **-20°C** for up to **2 weeks**.

Plasmid preparation

- 23 Prepare pIX-HALO plasmid DNA for each transcription factor construct and the HALO empty-vector control using a midi-prep kit, such as the Qiagen Plasmid Midi Kit or an equivalent Promega kit.



- 24 Aim to recover **9–10 µg plasmid DNA in 20 µL (450–500 ng/µL)** for each wheat germ expression reaction.
- 25 Quantify plasmid DNA concentration.
- 26 Store plasmid DNA at **–20°C** until required.

Wheat germ expression and HALO bead capture

- 27 For each transcription factor or HALO empty-vector control, combine:
 - 20 µL pIX-HALO plasmid DNA, 450–500 ng/µL
 - 30 µL TNT Wheat Germ Master Mix
- 28 Gently mix by pipetting **5 times** using a P200 pipette set to approximately **40 µL**.
- 29 Incubate the wheat germ expression reaction at **25°C for 2 h**.
- 30 During the expression reaction, prepare HALO magnetic beads.
- 31 Transfer the required volume of HALO magnetic beads to a low-bind microcentrifuge tube.
- 32 Place the tube on a magnetic rack and allow the beads to collect.
- 33 Remove and discard the storage buffer.
- 34 Wash the beads with an equal volume of **PBS + 0.05% NP-40**.
- 35 Place the beads on the magnetic rack and remove the wash buffer.
- 36 Repeat the bead wash for a total of **three washes**.

- 37 After the final wash, resuspend the beads in approximately **85% of the original bead volume** using **PBS + 0.05% NP-40**.

For example, if **300 µL** beads were used, resuspend in approximately **250 µL PBS + 0.05% NP-40**.
- 38 Mix the bead suspension thoroughly.
- 39 Aliquot **20 µL bead suspension** into each PCR tube.
- 40 Between aliquots, close the bead stock tube and invert to maintain an even bead suspension.
- 41 Add **50 µL PBS + 0.05% NP-40** to each bead aliquot.
- 42 Add **40 µL** of the corresponding wheat germ expression reaction to each bead aliquot.
- 43 Store the remaining **10 µL** of each expression reaction at **-20°C** for optional western blot QC.
- 44 Cap the tubes and invert by hand **5-10 times** to mix the bead and protein solutions.
- 45 Rotate the tubes end-over-end for **1 h** at low-to-medium speed. The solution is viscous, so use a rotation speed that allows the liquid to travel fully between both ends of the tube.
- 46 Place the tubes on a magnetic rack and allow the beads to collect.
- 47 Remove the supernatant. The supernatant may be retained if assessment of unbound protein is desired.
- 48 Remove the tubes from the magnetic rack.



- 49 Add **100 μ L PBS + 0.05% NP-40** to each tube.
- 50 Allow the beads to settle briefly, then place the tubes on the magnetic rack.
- 51 Remove and discard the wash buffer.
- 52 Repeat the protein/bead wash for a total of **four washes**.
- 53 After the final wash, resuspend the beads in **40 μ L PBS + 0.05% NP-40**.

DNA affinity purification

- 54 Denature sheared salmon sperm DNA at **98°C for 10 min**.
- 55 Immediately place denatured salmon sperm DNA on ice.
- 56 To each tube of transcription factor-bound HALO beads, add:
 - **5 μ L sheared salmon sperm DNA**, 2 μ g/ μ L
 - 100 ng adapter-ligated genomic DNA library
 - Qiagen EB buffer to a final volume of **100 μ L**
- 57 Cap the tubes and invert by hand **5–10 times** until the solution is homogeneous.
- 58 Rotate the tubes end-over-end for **1 h** at low-to-medium speed.
- 59 Place the tubes on a magnetic rack and allow the beads to collect.
- 60 Remove and discard the supernatant.

- 61 Remove the tubes from the magnetic rack.
- 62 Add **100 μ L PBS + 0.05% NP-40** to each tube.
- 63 Allow the beads to settle briefly.
- 64 Place the tubes on the magnetic rack and remove the wash buffer.
- 65 Repeat the DNA/protein/bead wash for a total of **five washes**.
- 66 During the final wash, transfer the full bead suspension to a fresh strip of PCR tubes.
- 67 Place the fresh tubes on the magnetic rack and remove the final wash buffer.
- 68 Remove the tubes from the magnetic rack.
- 69 Resuspend the beads in **16 μ L Qiagen EB buffer**.
- 70 Gently pipette up and down to fully resuspend the beads.
- 71 Cap the tubes and heat at **98°C for 10 min** to release bound DNA.
- 72 Immediately place the tubes on ice.
- 73 Place the tubes on a magnetic rack and allow the beads to collect.

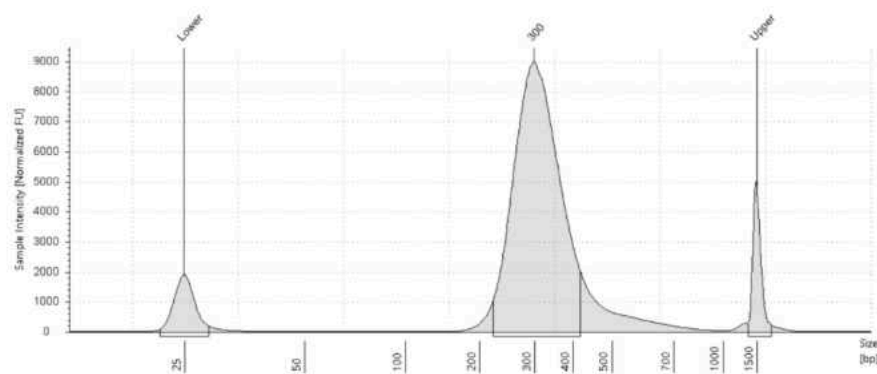


- 74 Transfer **15 µL supernatant** to a new PCR tube. This recovered DNA is used as input for indexing PCR.

Indexing and final DAP-seq library preparation

- 75 Continue the NEBNext Ultra II DNA Library Prep Kit protocol using **15 µL recovered DAP-seq DNA** as input for indexing PCR.
- 76 Follow the indexing PCR setup exactly as described in the NEBNext Ultra II protocol.
- 77 Amplify libraries using **14 PCR cycles**.
- 78 Perform the post-PCR cleanup according to the NEBNext Ultra II protocol.
- 79 For the final elution, elute purified DAP-seq libraries in **12 µL Qiagen EB buffer** instead of the larger elution volume recommended in the kit protocol.
- 80 Allow libraries to elute from the beads for **10 min** before recovering the eluate.
- 81 Assess final DAP-seq libraries using a **D1000 ScreenTape**.

BI: 1 Rep 3



Sample Table

Well	Conc. [ng/μl]	Sample Description	Alert	Observations
BI	66.3	1 Rep 3		

Representative D5000 TapeStation traces of final gene-expression DAP-sequencing library. Libraries showed the expected compact fragment-size distribution for a DAP-seq library suitable for sequencing.

- 82 Compare transcription factor DAP-seq libraries with the HALO empty-vector control.
- 83 Low DNA recovery in the HALO empty-vector control is expected and may indicate low background DNA recovery under these affinity purification conditions.
- 84 Store final indexed DAP-seq libraries at **-20°C** until pooling and sequencing.

Western Blot

- 85 Thaw the retained wheat germ expression reaction aliquot on ice.
- 86 For each sample, mix:
 - 1 μL wheat germ expression reaction
 - 1 μL 2× Laemmli buffer
- 87 Denature samples at **90°C for 5 min.**
- 88 Load **0.5 μL** of each denatured sample onto a **4–15% Criterion TGX Stain-Free Protein Gel.**

- 89 Load **4 μ L protein ladder**.
- 90 Run the gel at **100 V for 90 min**.
- 91 Prepare blocking solution by dissolving **2.5 g skim milk powder** in **50 mL 1 $\times$ TBS** to make **5% milk in TBS**.
- 92 Warm the blocking solution to **60°C** to dissolve the milk powder, then allow it to cool to room temperature before use.
- 93 Prepare Turbo transfer buffer.
- 94 Wet the transfer stacks and nitrocellulose membrane with Turbo transfer buffer.
- 95 Assemble the transfer stack in the Turbo transfer drawer in the following order:
 - wet transfer stack
 - nitrocellulose membrane
 - gel
 - wet transfer stack
- 96 Roll out bubbles carefully at each layer.
- 97 Close the transfer drawer and run the transfer using the default Turbo transfer program.
- 98 After transfer, confirm that the ladder has transferred to the membrane.
- 99 Place the membrane in a clean dish.
- 100 Add blocking solution and rock gently for **1 h** at room temperature. Do not use an orbital shaker.
- 101 Prepare primary antibody at **1:2,500** in **0.1% TBST**.

- 102 Discard the blocking solution.
- 103 Add primary antibody solution to the membrane.
- 104 Incubate overnight at **4°C** with gentle rocking. Do not use an orbital shaker.
- 105 Wash the membrane **3 × 10 min** with **0.1% TBST**.
- 106 Prepare secondary antibody at **1:20,000** in **0.1% TBST**.
- 107 Incubate the membrane with secondary antibody for **1 h** at room temperature with gentle rocking.
- 108 Wash the membrane **3 × 20 min** with **0.1% TBST**.
- 109 Prepare SuperSignal West Pico PLUS chemiluminescent substrate by mixing equal volumes of bottle A and bottle B. For one membrane, mix **2 mL bottle A + 2 mL bottle B**.
- 110 Place the membrane on clean plastic film or inside a cut plastic bag.
- 111 Apply substrate evenly across the membrane, ensuring the membrane is fully coated and free of bubbles.
- 112 Image using the ChemiDoc.
- 113 First acquire a colorimetric blot image for the ladder, using a short exposure such as **0.1 s**.
- 114 Then acquire a chemiluminescent image using the blot/chemi setting, starting with **0.1 s** exposure and adjusting if needed.



115 Merge the colorimetric and chemiluminescent images to show both ladder and signal.

Protocol references

Citation

O'Malley RC, Huang SC, Song L, Lewsey MG, Bartlett A, Nery JR, Galli M, Gallavotti A, Ecker JR (2016)
. Cistrome and Epicistrome Features Shape the Regulatory DNA Landscape. Cell.

<https://doi.org/10.1016/j.cell.2016.04.038>

LINK

Citation

Bartlett A, O'Malley RC, Huang SC, Galli M, Nery JR, Gallavotti A, Ecker JR (2017)
. Mapping genome-wide transcription-factor binding sites using DAP-seq. Nature protocols.

<https://doi.org/10.1038/nprot.2017.055>

LINK

Citations

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