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Purification and Activity Testing for Nanobody-Hia5 Fusions

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We use this protocol and it's working

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Abstract

This protocol is for the purification of nanobody-Hia5 fusions for use in DiMeLo-seq experiments. It also includes an assay for MTase activity in a non-targeted context, useful for validating individual preparations. The purification consists of a dual affinity sequence of Ni-NTA followed by amylose binding, leveraging the constructs' 6X Histidine and maltose binding protein (MBP) tags.

This protocol accompanies Sidhwani et al. (2025) "Histone H3 lysine methyltransferase activities control compartmentalization of human centromeres." and Gamarra et al. (2025) "DiMeLo-cito: a one-tube protocol for mapping protein-DNA interactions reveals CTCF bookmarking in mitosis" (DOI: 10.1101/2025.03.11.642717).



Materials

Buffers:

Lysis – 100 mL

- 50 mM Hepes pH 7.7
- 300 mM NaCl
- 0.5% Tx-100
- 5 mM β -mercaptoethanol (β -me)
- 10% glycerol

IMAC-X – 500 mL (20), 100 mL (300)

- 50 mM HEPES pH 7.7
- 300 mM NaCl
- 10% glycerol
- X mM imidazole
- 5 mM β -mercaptoethanol (BME)

Amylose wash buffer – 100 mL

- 50 mM HEPES pH 7.7
- 150 mM NaCl
- 10% glycerol
- 1 mM DTT

Introduction

- 1 This protocol covers the purification of Nanobody-MBP-Hia5 fusions (Addgene identifiers 239626 and 239627), as well as the validation of their in vitro methyltransferase activity. The workflow is a two-part, sequential affinity protocol consisting of a His/Ni-NTA affinity step followed by MBP/Amylose resin affinity finishing.

We find it easiest to perform resin washes in a 50mL conical tube and to elute manually off of a column support. However, any steps can be substituted with an HPLC equivalent if desired.

These constructs tolerate short periods (minutes to tens of minutes) at room temperature during later stages of purification, but activity is maximized when the sample is kept cold as much as possible, *especially after thawing the pellet but before the protein is washed out of the lysate.*

This protocol accompanies Sidhwani et al. (2025) "Histone H3 lysine methyltransferase activities control compartmentalization of human centromeres." and Gamarra et al. (2025) "DiMeLo-cito: a one-tube protocol for mapping protein-DNA interactions reveals CTCF bookmarking in mitosis" (DOI: 10.1101/2025.03.11.642717).

Reagents - Day 0

- 2 *Buffers can be made up to 1 week ahead of time and stored at 4C. Add reducing agents (BME/DTT) fresh on the day of use. HEPES pH anywhere from 7.5-7.8 is fine.*

For the purification

LYSIS - Make 50mL

50mM HEPES 7.7; 300 mM NaCl; 0.5% TritonX-100; 10% glycerol; 5mM BME

IMAC-20 - Make 500mL:

50mM HEPES 7.7; 300mM NaCl; 10% glycerol; 20mM imidazole; 5mM BME

IMAC-300 - Make 50mL:

50mM HEPES 7.7; 300mM NaCl; 10% glycerol; 300mM imidazole; 5mM BME

AMYLOSE WASH:

50mM HEPES 7.7; 150mM NaCl; 10% glycerol; 1mM DTT

Acquire or cast one polyacrylamide gel with at least 8 wells. Any percentage 10-15% works fine - we use 12.5%.

For the activity test

MTase ACTIVATION BUFFER - Make 10mL (This is the same as the DiMeLo-seq activation buffer. If planning DiMeLo-seq experiments, make 50mL and store at 4C for up to a month):

15mM Tris pH8; 15mM Na-Glutamate; 60mM K-Glutamate; 1mM EDTA pH8; 0.5mM EGTA pH8; 0.05mM spermidine; 0.1% BSA (*add BSA fresh day of use*)

Other materials

(Equivalent products may also be available through other suppliers for some items)

- *c3010 chemically competent cells*: **NEB**, Cat. No. **C3010I**
- *L-Rhamnose*: **Millipore-Sigma**, Cat. No. **R3875** {Sterilize through 0.22um or smaller filter before use}
- *cComplete EDTA-free protease inhibitor tablet (Must be the EDTA-free version, and NOT the mini version)*: **Roche**
- *Ni-NTA resin*: **Qiagen**, Cat. No. **30210**
- *Amylose resin*: **NEB**, Cat. No. **E8021**
- *Column Support: re-usable empty column support, if available, or single-use product such as*: **Bio-Rad**, Cat. No. **7321010**
- *D-(+)-Maltose monohydrate*: **ThermoFisher**, Cat. No. **A16266.22**
- *DpnI (for activity test)*: **NEB**, Cat. No. **R0176**
- *Lambda DNA (for activity test)*: **Oxford Nanopore Tech.**, part **LMD** in kit **EXP-CTL001**
- *S-Adenosyl methionine (SAM)*: **NEB**, Cat. No. **B9003S**
- *rCutSmart is included with most NEB restriction enzymes, including DpnI. The discontinued formulation (CutSmart) is also compatible with the activity test in this protocol.*

Methods - Day 1

- 3 Transform plasmid containing Nb-Hia5 fusion into NEB c3010 chemically competent cells (T7 Express *lysY* chemically competent *E. coli* cells). Inoculate directly into 100mL of autoclaved LB with 50ug/mL of filter-sterilized kanamycin and grow overnight 37C.

Prepare 2-4L of autoclaved LB, 1L per flask.

Methods - Day 2

- 4 Inoculate the prepared LB with 10mL of overnight culture per liter. Add 50ug/mL kanamycin and incubate at 37°C until OD600 reaches ~0.6.

Induce with 0.04% (2.5 mM) sterile rhamnose and grow at 24°C for 4 hours, or 18C overnight. Collect and combine pellets in a 50mL conical tube, wash once in 1X PBS, then spin down and freeze pellet at -80C.

Methods - Day 3

- 5 Thaw pellets and re-suspend in 40 mL lysis buffer with fresh BME and 1 fully-dissolved cOmplete protease inhibitor tablet.
- 6 Lyse with 3, 30-second rounds of sonication. *We use a Branson Sonifier 250 with power setting 7 and duty cycle 50%.* Stir sample gently between rounds and keep on ice.

NOTE: Cell lysates contain free proteases! Keep sample cold at all times after lysis and before purification. An ice bucket is the safest place for your sample when not in a chilled centrifuge rotor or cold room. Avoid unnecessary delays or pauses to the purification while the protein is still in the lysate.

OPTIONAL: For further protection against proteolysis, add 1 mM PMSF post-lysis, if available.

- 7 Centrifuge at 35k RPM for 45 mins, at 4°C. *We use a Beckman Optima L-100K centrifuge with a type 55.2Ti rotor.*

Meanwhile, wash 2 mL Ni-NTA resin 3X in IMAC-20. We do this by pelleting the resin in a bench top micro centrifuge and resuspending in buffer.

After centrifugation, transfer the lysate to a 50mL conical tube, then add the washed resin directly to the clarified lysate.

- 8 Incubate on an inverting rotator (5-25rpm) for 1 hr at 4C to promote protein binding to the resin.
- 9 Wash in bulk using 30-40 mL of IMAC-20 per wash. WASH SEQUENCE: 1 short wash, 2 10 min washes, 2 short washes.

For short washes, simply add the wash buffer to the conical tube containing the resin and invert the tube 5-10 times by hand before spinning down to pellet the resin. For long washes, place on an inverting rotor (5-25rpm) at 4C.

- 10 Re-suspend resin and place into an empty column support. Drain the wash buffer and elute with 8-10 mL of IMAC-300, into a 15mL conical tube. *When draining wash buffer, do not allow resin to dry for more than a few minutes.*

While the elution is dripping through, wash 1 mL of amylose resin 3X in amylose wash buffer.

Save an 18uL sample of the Ni-NTA eluate on ice for later SDS-PAGE analysis.

- 11 Add the washed amylose resin directly to the IMAC eluate and incubate on rotator for 90 mins, at 4C.

Meanwhile: Prepare 10mL of amylose elution buffer consisting of amylose wash buffer supplemented with 20mM maltose.

- 12 Spin down and save an 18uL sample of the unbound fraction (supernatant) from the amylose resin incubation on ice for later SDS-PAGE analysis.

Wash the resin in amylose wash buffer: 1X quick wash, 1X 10 min wash.

Meanwhile: wash the column support or; if single-use, ready a new one.

- 13 Place the resin in the clean column support, then elute into 3, 1.5 mL fractions in amylose elution buffer. Regular 2mL microcentrifuge ("Eppi") tubes work well for receiving the eluate. *Keep protein on ice as much as possible, but short periods at RT are acceptable at this stage.*

- 14 Run the samples of the Ni-NTA eluate, amylose resin supernatant, and amylose eluate fractions on the prepared acrylamide gel and quantify their concentrations. The first elution fraction should be quite pure and very concentrated - likely 1-2mg/mL. In 1.5mL total volume, this is enough for hundreds of DiMeLo-seq reactions. With this much yield, it is usually not worth concentrating the remaining fractions. If yield is lower, spin concentrate all fractions with appreciable protein, to at least 1mg/mL.

- 15 Add glycerol to a final concentration of 20%, distribute into 25-50 μ L aliquots, flash freeze in liquid nitrogen, and store at -80°C.

Protein should remain stable at this temperature for years, and individual aliquots can tolerate at least one freeze-thaw cycle. After thawing, spin aliquots down at max speed in a microcentrifuge for 10 minutes before use to clear aggregates, then re-quantify protein concentration before use. Re-freeze the unused portion of an aliquot by flash freezing in liquid nitrogen, and mark tubes to indicate that they have been thawed and re-frozen.

Activity Test

- 16 The nanobody-Hia5 constructs have two key functions: (1) methyltransferase activity and; (2) Nb-mediated target binding. This assay tests the former by checking for DpnI digestion of a DNA substrate after MTase treatment. DpnI digests lambda DNA only when

the adenine in its recognition sequence is methylated. The general workflow for the assay is:

1. Methylation of lambda DNA with purified Nb-Hia5 fusion
2. Treatment with DpnI
3. Agarose gel electrophoresis to assay digestion

Digestion of DNA indicates high MTase activity at the tested concentration. In the example at the end of this document, the "ASP4327" and "ASP4368" constructs are internal names for Hia5 with anti-mouse and anti-rabbit nanobodies, respectively.

Tip: The easiest way to do this assay is in PCR strip tubes incubated in a thermocycler or other small-tube incubator. The maximum volume per tube during this assay is 50uL.

- 17 Supplement MTase activation buffer with 160uM S-Adenosyl methionine (SAM). Treat lambda DNA in 20uL of this buffer for 1hr at 37C, using various concentrations of Hia5 fusion: 25nM, 50nM, 100nM. See example protocol for buffer specifications.

NOTE: We use 2uL/reaction of the lambda DNA available from Oxford Nanopore Technologies. Its concentration is 50ng/uL, and the full sequence is available on the ONT website: <https://nanoporetech.com/support/library-prep/kit-contents-and-composition/what-is-the-control-lambda-vial-lmd>

- 18 During MTase treatment, prepare the digestion buffer:

For each reaction, make 1X NEB CutSmart (or rCutSmart) with 1uL of DpnI. Make 1 additional reaction to ensure sufficient volume. For no digestion conditions, use (r)CutSmart alone.

- 19 Add 30 µL of digestion buffer (or (r)CutSmart), pipette to mix well, and incubate for 1hr at 37°C.
- 20 Run samples at 120V for 45 mins on a 1% agarose gel with EtBr (or equivalent). Run as much of the sample as possible, using larger wells if needed.

Example purification results

- 21  nB-Hia5_Fusions_Purification_Prot... 722.9KB