**Expression, purification and characterization of the GpC methyltransferase M.CviPI**

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Methylation footprinting can be used to map protein-DNA contacts at the resolution of individual DNA molecules[1]. Enzymes with various nucleotide specificities have been successfully used to footprint genomes including GpC, CpG and A methyl-transfereases[2–7]. Among these M.CviPI methylate DNA in GpC context, that is distinct from CpGs that are endogenously methylated in mammals. This feature has been leveraged to profile nucleosome occupancy[8,9]; the binding of General Transcription Factors and RNA Pol II[6]; the co-occupancy of Transcription Factors (TFs)[10] and the relation between TF binding and endogenous DNA methylation[11]. Here, we present a protocol for the production and purification of M.CviPI in *E. coli*. Our protocol routinely yields milligrams of protein at a quality and a concentration compatible with DNA footprinting applications. We characterize the purity and the activity of the purified enzyme, providing a benchmark for future production.

## I. Protein expression

The expression plasmid encoding M.CviPI (pBAD_HisMBP3C-M.CviPI; see Annex 1) is available upon request to the corresponding authors.

Transformation

- Add 1 µl of pBAD_HisMBP3C-M.CviPI plasmid (concentration ~ 100 ng/µl) to 50 µl of chemically competent NEBExpress® $I^q$ *E. coli* cells (NEB cat# C3037I)
- Incubate the mixture on ice for 30 min
- Heat-shock the mixture at 42°C for 20 sec
- Incubate the mixture on ice for 5 min
- Add 950 µl of sterile SOC medium (at room temperature) to the mixture
- Shake for 1 h at 37°C
- After the recovery time, plate out 50-100 µl of the mixture on an LB-agar plate supplemented with 100 µg/ml Ampicillin and incubate the plate overnight at 37°C
- Inoculate the rest of the mixture in 100 ml LB medium supplemented with 100 µg/ml Carbenicillin and 0.2% glucose in a 500 ml culture flask and shake overnight at 37°C. This will function as the preculture.

Expression

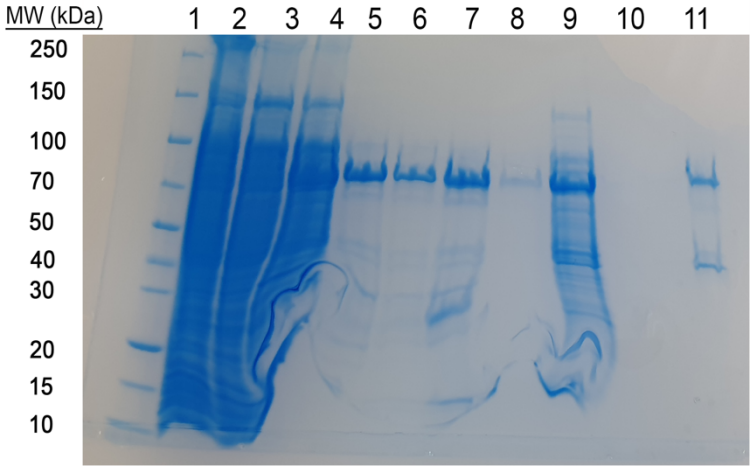
- Add 1 l LB medium supplemented with 0.2% glucose and 100 µg/ml Carbenicillin to a 5-liter culturing flask
  Note: we generally prepare 6 liters of *E. coli* culture for one large scale expression and purification experiment
- Add 10 ml of the preculture to each of the 5-liter shaking flasks
- Grow the cells at 37°C, 190 rpm until the $OD_{600}$ ~ 0.8
- Reduce the temperature to 18°C and induce protein expression by adding 0.02% L-arabinose (sterile) to each culture
- Grow the cells overnight at 37°C, 190 rpm. The expected end $OD_{600}$ is usually ~ 5.0
- Harvest the cells by centrifugation (4600 x *g*, 20 min, 4°C)
- Flash-freeze the cell pellets and store at -20°C until further usage

## II. Protein purification

Step 1: Combined MBPTrap and Ni-NTA affinity chromatography

| **MBPTrap** | |
|---|---|
| Column | 1 ml MBPTrap HP (Cytiva cat# 29048641) |
| Lysis buffer | 50 mM Tris pH 8.0 (@4°C), 1 M NaCl, 10% glycerol, 0.01 mg/ml DNase, 2 mM $MgCl_2$, Roche cOmplete™ EDTA-free protease inhibitor cocktail (Merck cat# 11873580001) |
| Wash buffer | 50 mM Tris pH 8.0 (@4°C), 1 M NaCl, 10% glycerol |
| Elution buffer | 50 mM Tris pH 8.0 (@4°C), 1 M NaCl, 10% glycerol, 10 mM maltose |
| **Ni-NTA** | |
| Column | 1 ml Protino Ni-NTA (Macherey-Nagel cat# 745410.5) |
| Lysis buffer | 50 mM Tris pH 8.0 (@4°C), 1 M NaCl, 10% glycerol, 0.01 mg/ml DNase, 2 mM $MgCl_2$, Roche cOmplete™ EDTA-free protease inhibitor cocktail (Merck cat# 11873580001) |
| Wash buffer | 50 mM Tris pH 8.0 (@4°C), 1 M NaCl, 10% glycerol, 20 mM imidazole |
| Elution buffer | 50 mM Tris pH 8.0 (@4°C), 1 M NaCl, 10% glycerol, 250 mM imidazole |
| **Dialysis** | |
| Dialysis buffer | 50 mM Tris pH 8.0 (@4°C), 50 mM NaCl, 10% glycerol |

1) Resuspend the bacterial cell pellet (~ 45 ml of cell pellet from 6 l *E. coli* culture) in 150 ml lysis buffer
2) Lyse the cells by 4 passages through a Microfluidizer device
3) Centrifuge the lysate (30000 x *g*, 4°C, 45 min) (Soluble fraction)
4) Couple the 1 ml MBPTrap HP and Ni-NTA columns in tandem (MBPTrap first, Ni-NTA second) and load the cleared lysate with a flow rate of 1 ml/min using a peristaltic pump at 4°C (Flow through FT)
5) Uncouple both columns and wash and elute the MBPTrap and Ni-NTA columns separately
6) Wash the MBPTrap HP 2 x with 30 ml wash buffer (Wash W1, W2) at a flow rate of 1 ml/min using a peristaltic pump
7) Elute the MBPTrap with 6 ml elution buffer (Elution) at a flow rate of 1 ml/min using a peristaltic pump
8) Next, wash the Ni-NTA column with 15 ml lysis buffer and with 20 ml and 7.5 ml wash buffer, respectively (Wash W1, W2, W3) at a flow rate of 1 ml/min using a peristaltic pump
9) Elute the Ni-NTA column 2 x with 9 ml and 1.5 ml elution buffer (Elution E1, E2) at a flow rate of 1 ml/min using a peristaltic pump
10) Analyse all samples (indicated in green) via SDS-PAGE




1. Broad range MW marker
2. E. coli NEBExpress® $I^q$ pBAD_HisMBP3C-M.CviPI soluble fraction
3. Flow through MBPTrap – Ni-NTA
4. Wash 1 – Ni-NTA
5. Wash 2 – Ni-NTA
6. Wash 3 – Ni-NTA
7. Elution 1 – Ni-NTA
8. Elution 2 – Ni-NTA
9. Wash 1 – MBPTrap
10. Wash 2 – MBPTrap
11. Elution – MBPTrap

*Figure 1: SDS-PAGE of the MBPTrap and Ni-NTA affinity chromatography purification steps. Fractions indicated in blue were pooled and used for further purification steps.*

11) Pool the elution fraction of the MBPTrap with the Wash 2, Wash 3 and elution fractions of the Ni-NTA (indicated in blue in Figure 1)
12) Dialyse the pooled sample overnight ➪ 45 ml @ 0.55 mg/ml; $A_{260}/A_{280}$ ~ 0.55
13) As there was still quite some $His_6$-MBP-M.CviPI protein present in the flow through, this was run again on a Ni-NTA column. The flow through was loaded onto a 1 ml Ni-NTA column at a flow rate of 1 ml/min using a peristaltic pump
14) The Ni-NTA column was washed with 15 ml of lysis buffer and 15 ml of wash buffer at a flow rate of 1 ml/min using a peristaltic pump (Wash W1, W2)
15) The Ni-NTA column was eluted with 8 ml of elution buffer at a flow rate of 1 ml/min using a peristaltic pump (Elution E)
16) Pool the Wash 2 and elution fractions of the second Ni-NTA run
17) Dialyse the pooled sample overnight ➪ 23 ml @ 1.64 mg/ml; $A_{260}/A_{280}$ ~ 0.57

Step 2: HiTrap Heparin HP chromatography

| **HiTrap Heparin HP** | |
|---|---|
| Column | 1 ml HiTrap Heparin HP (Cytiva cat# 17040601) |
| Dialysis buffer | 50 mM Tris pH 8.0 (@4°C), 50 mM NaCl, 10% glycerol |
| Buffer A | 50 mM Tris pH 8.0 (@4°C), 100 mM NaCl, 10% glycerol |
| Buffer B | 50 mM Tris pH 8.0 (@4°C), 1 M NaCl, 10% glycerol |

1) Remove the samples from the dialysis tubing
2) Connect a 1 ml HiTrap Heparin HP column to a chromatography system (e.g. BioRad NGC, Åkta Pure, Åkta Purifier) and equilibrate the column with dialysis buffer at a flow rate of 1 ml/min

3) Load the dialysed samples onto the Heparin column at a flow rate of 1 ml/min ⇨ we loaded ~ 15 ml of sample per round
4) Wash the Heparin column with 5 ml of buffer A at a flow rate of 1 ml/min
5) Elute the Heparin column with a gradient going from 100 mM NaCl (buffer A) to 1 M NaCl (buffer) over a volume of 20 ml (20 column volumes)

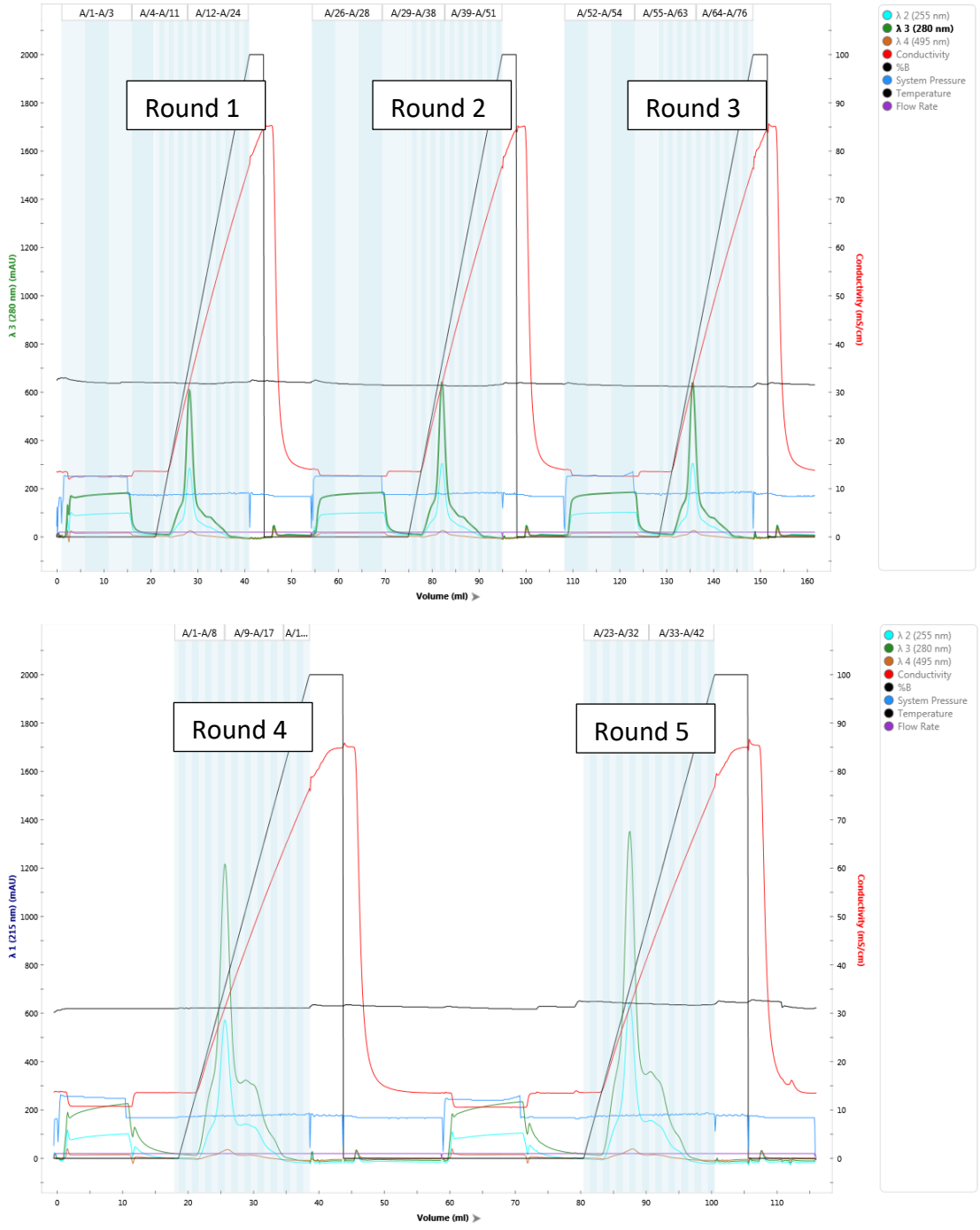
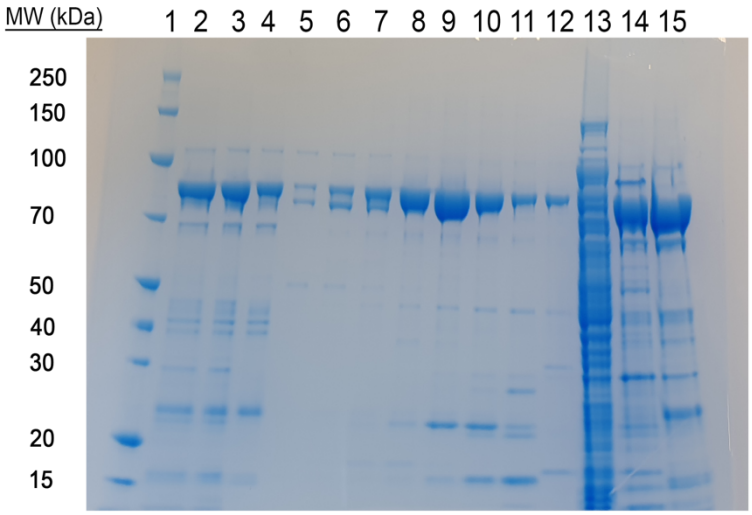



*Figure 2: Chromatograms of the HiTrap Heparin HP purification steps*




1. Broad range MW marker
2. $His_6$-MBP-M.CviPI sample before dialysis
3. $His_6$-MBP-M.CviPI sample after dialysis
4. Hep. round 1 – fraction A2
5. Hep. round 1 – fraction A8
6. Hep. round 1 – fraction A9
7. Hep. round 1 – fraction A10
8. Hep. round 1 – fraction A11
9. Hep. round 1 – fraction A12
10. Hep. round 1 – fraction A13
11. Hep. round 1 – fraction A14
12. Hep. round 1 – fraction A17
13. Wash 1 – 2nd Ni-NTA
14. Wash 2 – 2nd Ni-NTA
15. Elution – 2nd Ni-NTA

*Figure 3: SDS-PAGE of the HiTrap Heparin HP purification steps. Hep.: HiTrap Heparin HP. Fractions indicated in blue were pooled and used for further purification steps.*

6) Pool fractions A11-A17 (Heparin round 1), A37-A43 (Heparin round 2) and A62-A68 (Heparin round 3) (indicated in blue in figure 3) ⇨ 20 ml @ 0.44 mg/ml; $A_{260}/A_{280}$ ~ 0.55
7) Pool fractions A8-A14 (Heparin round 4) and A29-A35 (Heparin round 5) ⇨ 13 ml @ 0.86 mg/ml; $A_{260}/A_{280}$ ~ 0.55

Step 3: Anion exchange chromatography

| **HiTrap Q HP** | |
|---|---|
| Column | 1 ml HiTrap Q HP (Cytiva cat# 17115301) |
| Dilution buffer | 50 mM Tris pH 8.0 (@4°C), 10% glycerol |
| Dialysis buffer | 50 mM Tris pH 8.0 (@4°C), 50 mM NaCl, 10% glycerol |
| Buffer A | 50 mM Tris pH 8.0 (@4°C), 100 mM NaCl, 10% glycerol |
| Buffer B | 50 mM Tris pH 8.0 (@4°C), 1 M NaCl, 10% glycerol |

1) The elution fractions from the HiTrap Heparin HP column that contain the $His_6$-MBP-M.CviPI protein have a NaCl concentration ~ 600 mM. These are diluted 12-fold with dilution buffer to obtain a final NaCl concentration ~ 50 mM.
2) Connect a 1 ml HiTrap Q HP column to a chromatography system (e.g. BioRad NGC, Åkta Pure, Åkta Purifier) and equilibrate the column with dialysis buffer at a flow rate of 1 ml/min
3) Concentrate the diluted samples to ~ 2 ml and load them onto the HiTrap Q HP column at a flow rate of 1 ml/min

4) Wash the HiTrap Q HP column with 10 ml of buffer A at a flow rate of 1 ml/min
5) Elute the HiTrap Q HP column with a gradient going from 100 mM NaCl (buffer A) to 1 M NaCl (buffer) over a volume of 20 ml (20 column volumes)

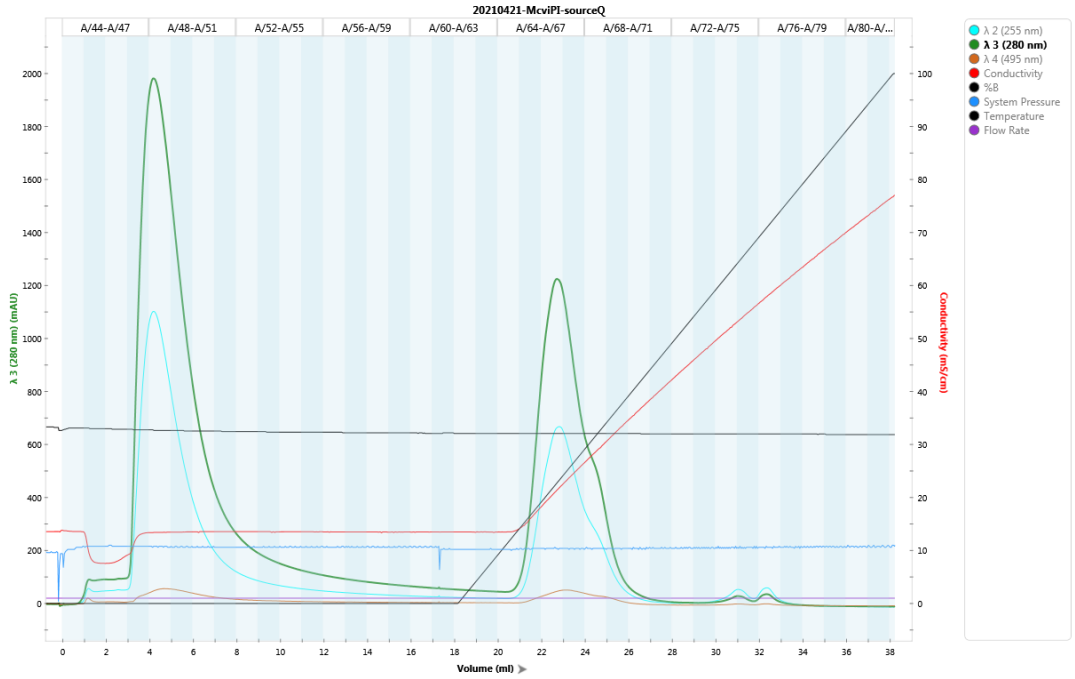
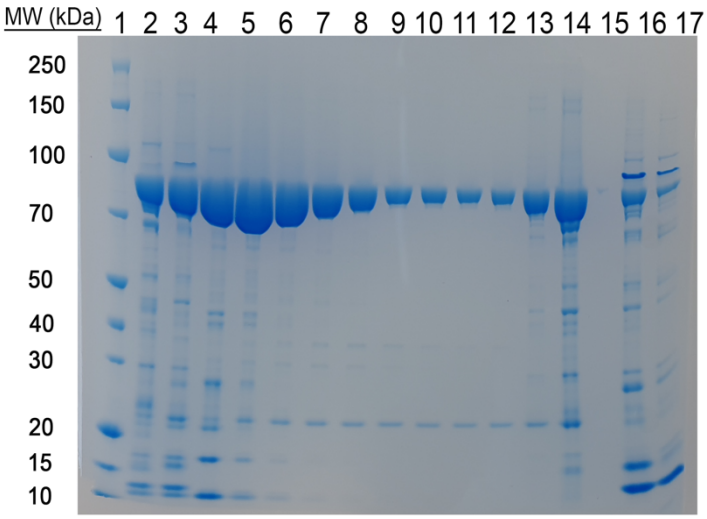


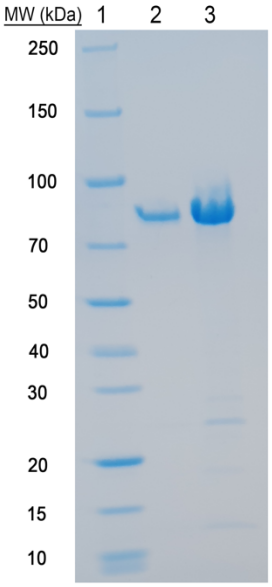

*Figure 4: Chromatogram of the HiTrap Q HP anion exchange purification*




1. Broad range MW marker
2. Load of round 4 & 5 of the HiTrap Heparin HP
3. Load of the HiTrap Q HP
4. HiTrap Q HP fraction A47
5. HiTrap Q HP fraction A48
6. HiTrap Q HP fraction A49
7. HiTrap Q HP fraction A50
8. HiTrap Q HP fraction A51
9. HiTrap Q HP fraction A52
10. HiTrap Q HP fraction A53
11. HiTrap Q HP fraction A54
12. HiTrap Q HP fraction A55
13. HiTrap Q HP fraction A65
14. HiTrap Q HP fraction A66
15. HiTrap Q HP fraction A67
16. HiTrap Q HP fraction A68
17. HiTrap Q HP fraction A69

*Figure 5: SDS-PAGE of the HiTrap Q HP anion exchange purification steps. Fractions indicated in blue were pooled and used for further purification steps.*

6) Pool fractions 47-55 of the anion exchange (indicated in blue in figure 5) ⇨ 8 ml @ 1.07 mg/ml; $A_{260}/A_{280}$ ~ 0.53
7) Concentrate the pooled elution fractions of the anion exchange chromatography to ~ 2 mg/ml and dilute with 90% glycerol to reach a final concentration of 50% glycerol
8) Final sample ⇨ 8.7 ml @ 1 mg/ml in 25 mM Tris pH 8.0 (@4°C), 50 mM NaCl, 50% glycerol
9) Aliquot the final sample (100 µl aliquots) and store at -20°C




1. Broad range MW marker
2. Final sample of purified $His_6$-MBP-M.CviPI (0.5 µl)
3. Final sample of purified $His_6$-MBP-M.CviPI (2.5 µl)

*Figure 6: SDS-PAGE of the final $His_6$-MBP-M.CviPI protein sample*

| **Final sample** | |
|---|---|
| Name | $His_6$-MBP-M.CviPI |
| Storage buffer | 25 mM Tris pH 8.0 (@4°C), 50 mM NaCl, 50% glycerol |
| Storage temperature | -20°C |
| Concentration | 1 mg/ml |
| Aliquot size | 100 µl |
| Total yield | 8.7 mg purified from 6 liter of *E. coli* expression culture |

**Comments**

- All protein purification steps are performed at 4°C
- Samples are kept on ice throughout the entire protein purification process
- The MBPTrap step is optional. It increases purity of the sample but reduces the yield.
- A 5 ml HiTrap Heparin HP can be used instead of a 1 ml column to avoid multiple rounds of Heparin column purification.

## III. Biophysical characterisation of the purified $His_6$-MBP-M.CviPI protein

Mass spectrometry analysis

To verify the identity of the purified $His_6$-MBP-M.CviPI sample, mass spectrometry analysis was performed at the EMBL Proteomics Core Facility.

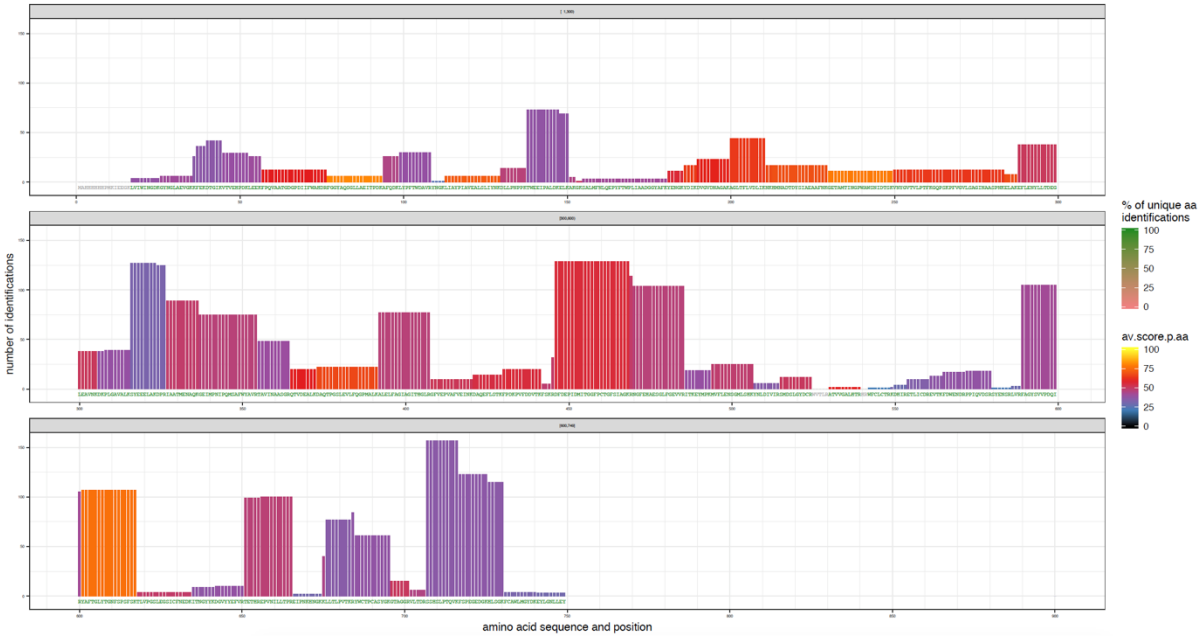



*Figure 7: Mass-spec confirms the identity and the integrity of the purified protein. 96.8% of the $His_6$-MBP-M.CviPI protein sequence is recovered by mass spectrometry.*

Nano-DSF analysis

Nano-differential scanning fluorimetry (Nano-DSF) was performed on a NanoTemper Prometheus NT.48 instrument to determine the melting temperature $T_m$ of the $His_6$-MBP-M.CviPI in the storage buffer. The measurement was performed in duplicate and indicates a reference melting temperature of 57.2°C.

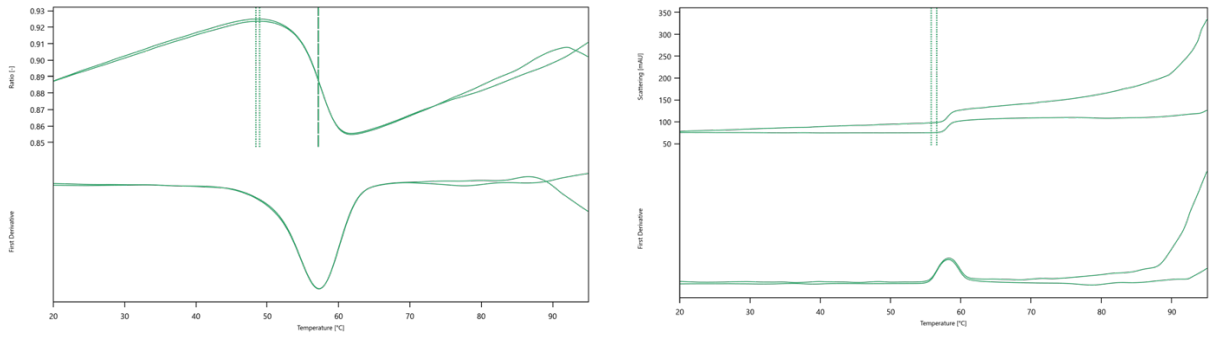



*Figure 8: Nano-DSF measurements of the $His_6$-MBP-M.CviPI protein sample. Theleft panel shows the ratio $F_{350nm}/F_{330nm}$ of the fluorescence emission intensities at 330 nm and 350 nm*

*and the first derivative hereof versus the temperature. The right panel shows the backscattering signal and the first derivative hereof versus the temperature.*

| Results of the Nano-DSF measurements | | | |
|---|---|---|---|
| **Capillary number** | **$T_{onset}$** | **$T_m$** | **$T_{turbidity}$** |
| 1 | 48.9°C | 57.2°C | 56.5°C |
| 2 | 48.4°C | 57.2°C | 55.8°C |

Mass photometry analysis

Mass photometry analysis was performed on a Refeyn Two$^{MP}$ instrument to assess the mass distribution present in the $His_6$-MBP-M.CviPI protein sample. The sample was diluted in PBS to a final concentration of 20 nM for the measurement. The main peak that is observed at 84 kDa corresponds very well to the calculated molecular weight of 83.7 kDa.

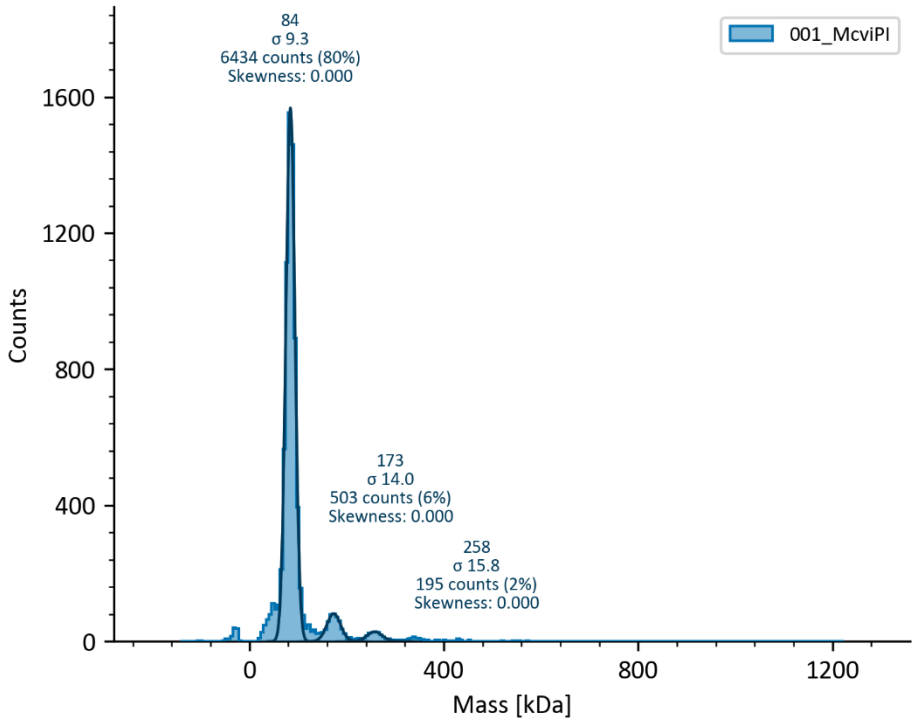



*Figure 9: Mass photometry analysis of the $His_6$-MBP-M.CviPI protein sample.*

## IV. Titration of M.CviPI activity *in vitro*

Titration of M.CviPI activity

Titration of the enzyme activity was performed using an *in vitro* DNA methylation assay. The tests were performed by methylation of lambda phage genomic DNA with increasing amounts of enzyme, followed by with a methyl-sensitive restriction digest. Reactions were performed

in a final volume of 20 µl. 1 µg of lambda DNA was incubated with a dilution series of M.CviPI. (120 to 15 ng/µl) for 60 min at 37°C. The enzymatic reaction was inactivated for 15 min at 65 °C. The resulting methylated DNA was digested with the methyl-sensitive restriction enzyme BbVI for 60 minutes at 37°C and ran on a 1% TBE gel (see figure 10).

1. Prepare a dilution series of M.CviPI (120, 105, 90, 75, 60, 45, 30 and 15ng/l) in water with a final volume of 10µl.
2. Prepare a master mix containing the lambda DNA, Methyltransferase reaction buffer, the S-adenosylmethionine and water.

| **Methylation reaction** | |
|---|---|
| Lambda DNA (500ng/ul) (N3011S) | 2 µl |
| 10x Methyltransferase reaction buffer | 2 µl |
| S-adenosylmethionine (SAM) (32mM) | 2 µL |
| M.CviPI protein (variable concentration) | 10 µL |
| Water | 4 µl |

3. Pipette reactions together, mix by pipetting and spin down quickly.
4. Incubate for 1 hr at 37 °C in a thermal cycler with the lid off.
5. Inactivate the reaction at 65 °C for 15min.
6. Prepare the digestion master mix containing the rCutsmart buffer, the BbVI restriction enzyme and water.

| **Digestion reaction** | |
|---|---|
| Methylation reaction (see above) | 15 µl |
| 10x rCutsmart buffer (B6004S) | 2 µl |
| BbVI restriction enzyme (R0173S) | 1 µl |
| Water | 2 µl |

7. Prepare a new set of PCR strip tubes and transfer 15 uL of the inactivated methylation reaction.
8. Add to each tube 5uL of the digestion master mix.
9. Mix by pipetting and spin down quickly.
10. Incubate for 1 hr at 37 °C in a thermal cycler with the lid off.
11. Prepare a 1% TBE gel.
12. Run the samples on a gel for 45min at 100V.

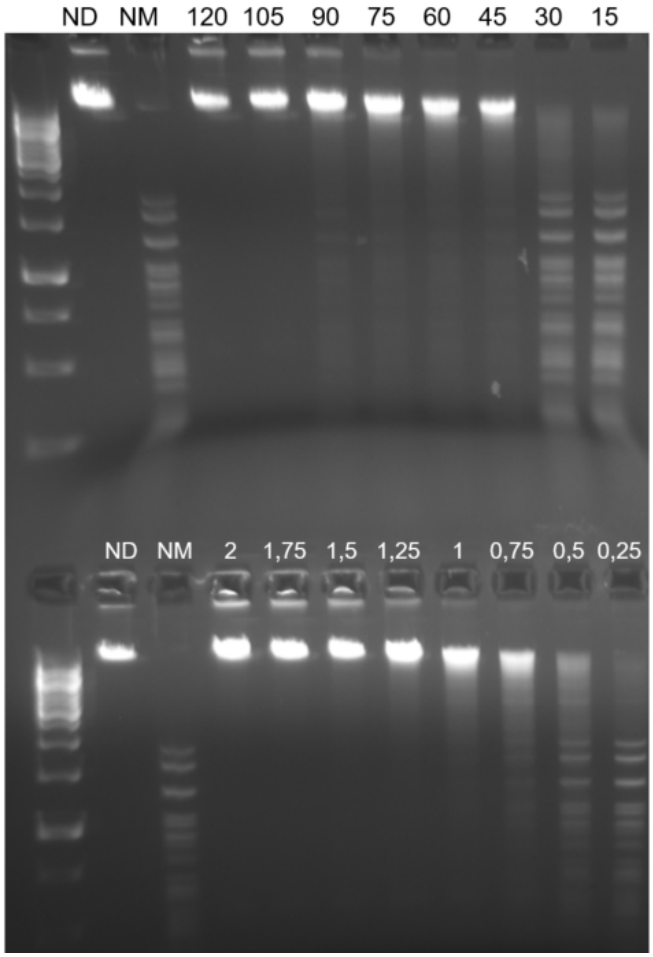



*Figure 10: In vitro activity assay to determine $His_6$-MBP-M.CviPI protein activity. The first 3 lanes in each panel consists of the 1kb ladder, no digestion control and the no methylation control. The upper panel shows the activity in a dilution series of the $His_6$-MBP-M.CviPI in ng/ µl. The bottom panel shows the activity a dilution series of the reference batch of M.CviPI in units/ µl.*

Determining the working concentration

The determination of the working concentration of His6-MBP-M.CviPI protein was done by comparing the activity to that of the reference batch. When looking at the bottom part of the gel in figure 10 a slight smear was seen in the 1 Unit sample. This amount was considered the golden standard for methylating 1 µg of DNA. Comparing this to the upper part of figure 10 this smearing was visible in the 90ng sample. Therefore, 90 ng/µl His6-MBP-M.CviPI protein appeared to be optimal amount to methylate 1 µg DNA.

## V. Quality controls

We controlled the activity of the purified enzyme in the SMF assay[1]. When performing SMF on S2 cells, we observed comparable levels of methylation to the reference datasets (Figure 11A), confirming the activity of the purified enzyme. Moreover, we tested the specificity of

the purified batch by methylating lambda DNA *in vitro*. We found that the purified M.CviPI methylates preferentially GpC contexts with similar unspecific activity in CCmG contexts (Figure 11B).

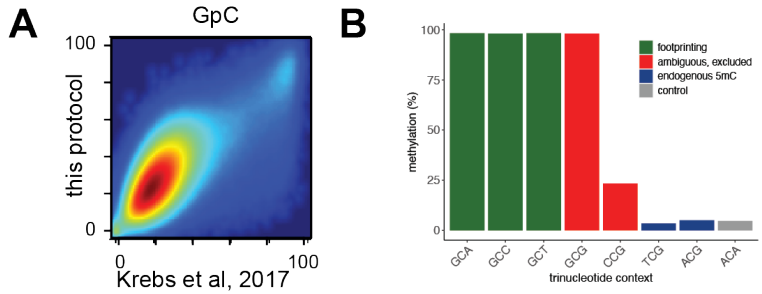



*Figure 11: (A) Smoothed scatterplot comparing the average methylation in GpC context in reference data (S2 cells- Krebs et al, Mol Cell 2017[6]) with data generated with M.CviPI purified with this protocol. (B) Nucleotide contexts methylated by M.CviPI purified with this protocol. Barplot representing the average methylation levels of lambda DNA in various trinucleotide contexts upon in vitro methylation with M.CviPI in saturating conditions. M.CviPI fully methylates GpCs regardless of the nucleotide context (green bars). It methylates CpGs in GCmG contexts and displays a low level of non-specific activity in CCmG context (<10%) (red bars); similar to the reference analysis in Kreibich et al, Mol Cell 2023[11].*

**Contributions:**

KL, KR: development of the protein purification strategy; RK: testing of the enzyme activity. ND: initial test of the purification procedure. KC: Data analysis. KL, RK, ARK, KR, writing of the manuscript. ARK review & editing. A.R.K, KR. Supervision. All authors discussed the results and commented on the manuscript.

## Annex 1: Characteristics of the pBAD_HisMBP3C-M.CviPI construct

Protein**: GpC methyltransferase M.CviPI originating from *Chlorella* virus**
Expression vector backbone: pBAD/His A (ThermoFischer cat# V43001)
Antibiotic resistance: Ampicillin
Fusion tags: N-terminal $His_6$-MBP-3C
Molecular weight: 83723.35 Da
Extinction coefficient: 117690 $M^{-1}$ $cm^{-1}$
pI: 6.14

Amino acid sequence:

MAHHHHHHPMKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPD
IIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTW
EEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLI
KNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGIN
AASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIPQMS
AFWYAVRTAVINAASGRQTVDEALKDAQTPGSLEVLFQ↓GPMALKALELFAGIAGITHGLRGFVEPVAF
VEINKDAQEFLSTKFPDKPVFDDVTKFSKRDFDEPIDMITGGFPCTGFSIAGKRNGFEHAESGLFGEVVRIT
KEYMPKMVFLENSGMLSHKYNLDIVIRSMDSLGYDCRWVTLRATVVGALHTRHRWFCLCTRKDHIRETL
ICDREVTKFDWENDRPPIQVDSRSYENSRLVRFAGYSVVPDQIRYAFTGLYTGNFSPSFSKTLVPGSLEGSI
CFNEDKITNGYYKDGVYYEFVRTETHREPVNILLTPREIPNKHNGKKLLTLPVTKRYWCTPCASYGKGTAG
GRVLTDRSSHSLPTQVKFSPEGEDGKHLSGKFCAWLMGYDKEYLGNLLEY*

$His_6$-tag
maltose binding protein(MBP)
HRV 3C protease cleavage site

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