Purification and Quantification of protein-DNA interactions of Minichromosome Maintenance Protein (MCM) of *Pyrococcus abyssi*

Introduction:

MCM helicase proteins function as core components which unwinds parental DNA in eukarya and archaea [[1][2]]. These highly conserved, archaeal proteins are essential for uncovering important features of protein's functions as they provide a simplified model for understanding complex [2]. Practical objectives include purification of MCM protein, quantification of purified protein and of protein: DNA binding interactions.

Methods and Materials:

From SFP3 Practical manual, experimental details for purification of MCM protein were carried out with nickel affinity chromatography and Bradford assay procedures. SDS-PAGE gel was marked with molecular weight (M_w) markers to compare bands on gel and to indicate presence of MCM homo-hexamer in individual eluted fractions. Standard BSA curve was calibrated to calculate protein concentration for Electrophoretic Mobility Shift Assay (EMSA). Deviations included using 25 μl of solution D for FT. Sample $1_{diluted}$ was chosen to generate the volume fraction with MCM protein mole requirements in EMSA. With calculated protein concentrations (used in EMSA analysis), experimental details for EMSA were as followed from Practical Manual, where band images on polyacrylamide gel of MCM and dsDNA (with and without standard bp markers) were generated (Lane 1-10; with increasing MCM concentration in Lane 3-10). Fluorescently labelled bands (without standard bp markers) were visualized and quantified on software packages (ImageJ and Microsoft Excel) to mathematically estimate K_d values.

Results and Discussion:

Table 1:

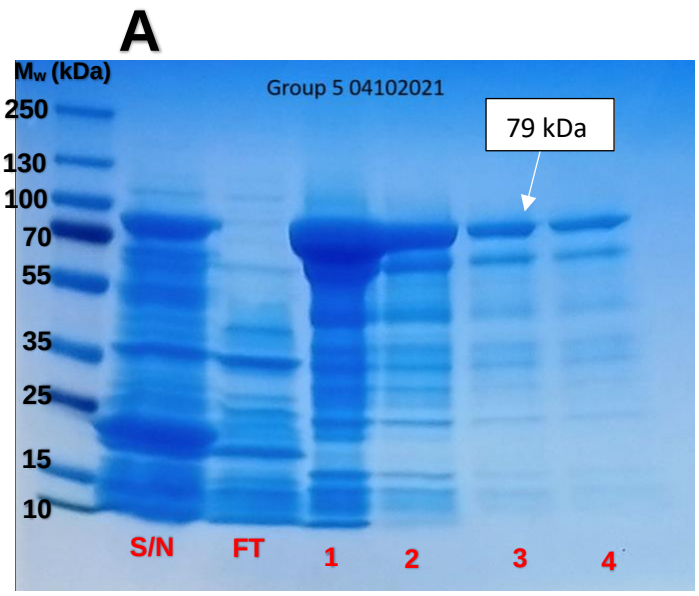
Standard Concentrations (mg/ml)	A_{595} (Reading 1)	A_{595} (Reading 2)	A_{595} (Reading 3)	A_{595} (Average)
0	0.000	0.000	0.000	**0.000**
0.1	0.0860	0.132	0.159	**0.126**
0.3	0.0330	0.362	0.307	**0.234**
0.5	0.372	0.531	0.587	**0.497**
0.7	0.586	0.620	0.567	**0.591**
0.9	0.623	0.603	0.784	**0.670**

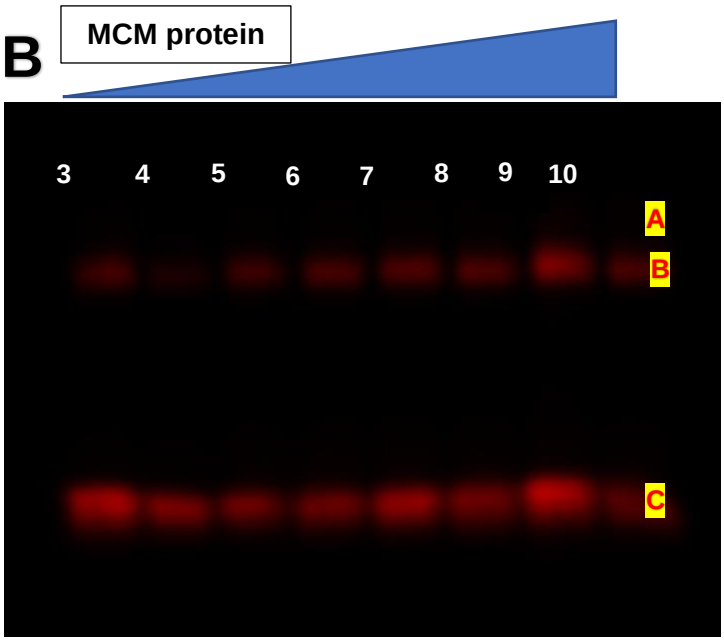
Table 2:

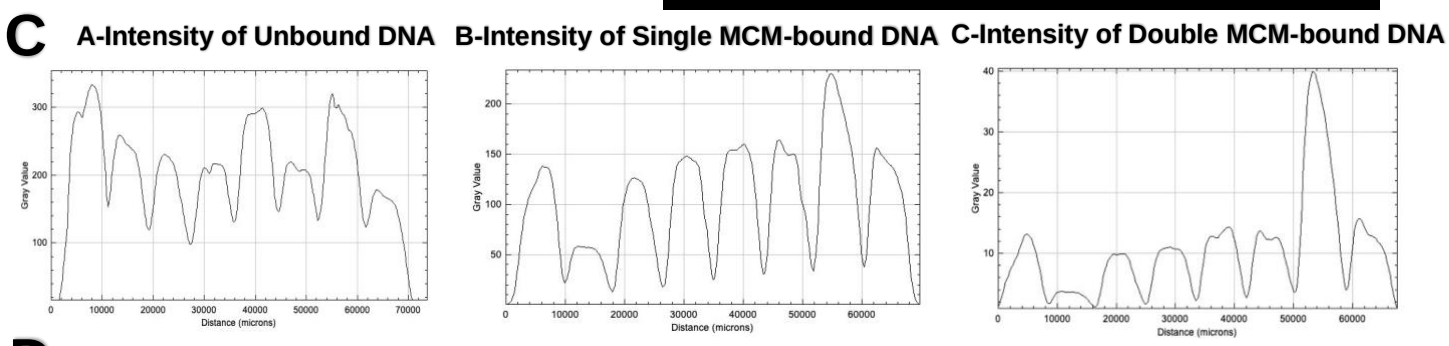
Eppendorf Tubes	Absorbance (A_{595})	Calculated MCM Protein Concentration (mg/ml)
S/N	1.127	1.428
FT	0.9600	1.209
1	1.971	2.531
$1_{diluted}$	0.9220	1.160
2	1.244	1.581
3	0.6170	0.7618
4	0.5610	0.6886

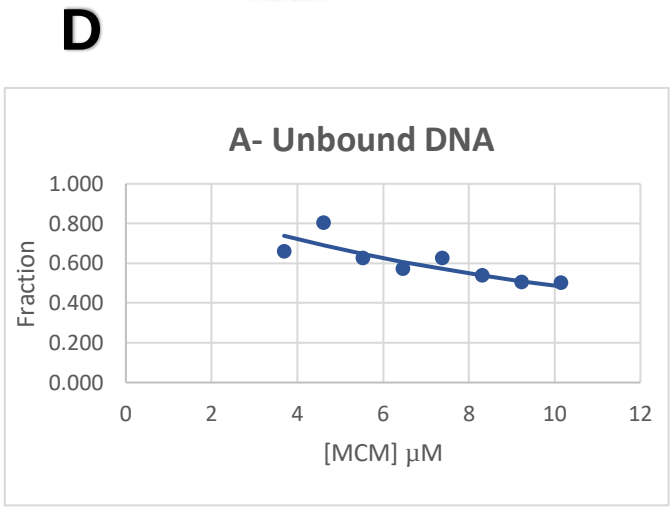
Table 1: Absorbance (A_{595}) of standard concentrations of MCM protein used to calibrate standard curve

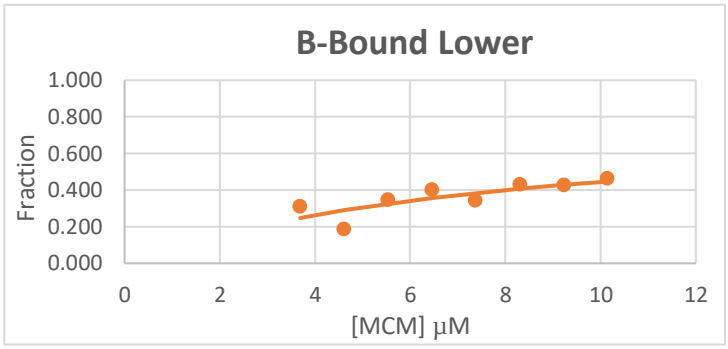
Table 2: Absorbance (A_{595}) of eluted samples and Calculation of MCM Protein Concentration with calibration curve of Bradford Assay (BSA) from Table 1 readings.











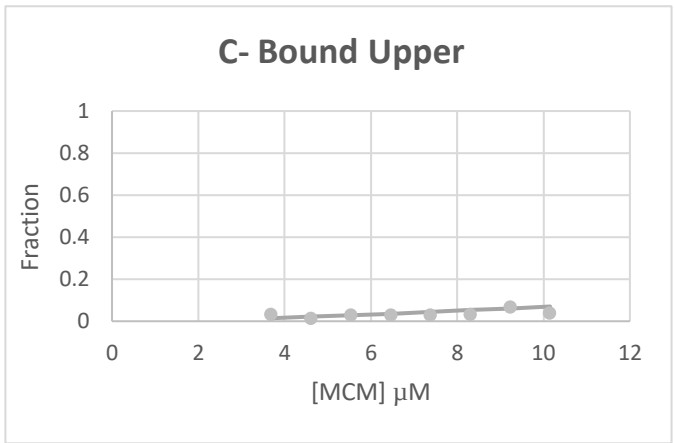


Figure A: Coomassie-stained SDS-PAGE gel of samples eluted from MCM purification via Nickel Affinity Chromatography. Molecular weight markers indicated. Large band (79 kDa), indicates presence of MCM helicase.
Figure B: Li-Cor scanner image of fluorescently labelled bands on gel electrophoresis with increasing concentrations of MCM protein (Lane 3 to 10). DNA tagged with 5IRD700; Band A, B (lower band) and C (upper band) show unbound DNA, single MCM bound DNA, double MCM-bound DNA respectively.
Figure C: Fluorescence Intensity readings generated from ImageJ software package, from rows A, B and C respectively from Li-Cor scanner image
Figure D: Fractions of Fluorescence Intensity against increasing MCM concentration. Line of best fit used to derive estimates of KD1 and KD2 values.

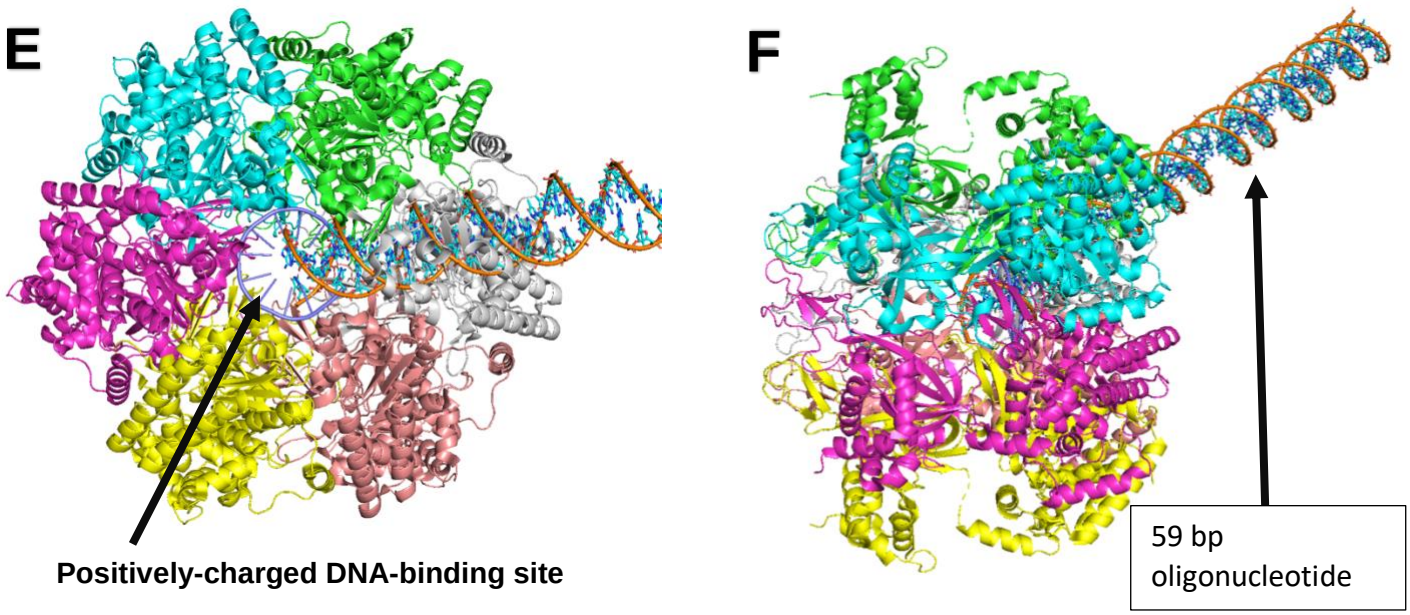


Figure E & F: PYMOL cartoon representation of homo-hexamer MCM helicase protein (PDB ID: 6MII) with 59 bp oligonucleotides (generated with PYMOL builder); top view orientation(E) and side view orientation (F) presented where each monomer (subunit) is shown in a different colour.

Coomassie-stained SDS-PAGE gel in Figure A presented clear bands of approximately 79 kDa in 5 fractions (S/N, 1, 2, 3, 4) which indicates the molecular weight of MCM monomer. This is true as eluted fractions (2, 3, 4) consistently have retained similar concentrations of protein at that band whilst other bands decreased. Whilst it was a successful purification, in Ni^{2+} Affinity Chromatography, once a protein is His-tagged, it can potentially form dimers/tetramers in presence of Ni^{2+}. This could lead a slightly inaccurate molecular mass readout which decreases reliability; a more reliable method would be ion exchange chromatography for higher adsorption specificity. Triplicate repeats (Table 1) from BSA also increased reliability of experiment.

From Figure B, there was human error as positive control (oligonucleotides only) and negative control (MCM helicase only) were not run on polyacrylamide gel, which is presented by absence of Lane 1 and Lane 2 in figure. As Lane 2 was supposed to be used as a control to avoid artefacts due to ethidium bromide staining in post-staining agarose gel, our experiment could not show if presence and degree of dissociation was due to electrophoretic method. This decreases accuracy of our collated data and hence, experiment reliability. Unexpectedly, Lane 4 showed relatively lesser fluorescent intensities in bands C and D which does not follow hypothetical trend of increasing MCM concentration; this is also observed in Figure C (all graphs) where there is significantly less intensity in Lane 4. This could also potentially be due to human error.

As Li-Cor image of fluorescent bands showed 2 retarded rows of bands in lower and upper bound with MCM addition, which implies that our 59 bp oligonucleotide can be bound by more than one MCM protein. Fluorescence intensity analyzed with ImageJ for bands A, B and C from figure

shown was graphed in Figure C. Data was collated for analysis in Excel packages where lines of best fit were created for respective 3 graphs (A, B and C) in Figure D, which estimated **KD1 and KD2** values (11 and 710 M respectively) from our experiment. This was a relatively reliable method which employed standard deviation and R value of 0.0435, which indicated a low positive value, expected from our experiment which quite a few human errors. Another limitation of SDS-polyacrylamide gel is that SDS resides in the deliberate denaturation of proteins prior to electrophoresis [3]. Enzymatic activity and protein-binding interactions generally cannot be determined which skews Kd values. Other methods to separate native proteins for helicase-function relationship should be employed.

From Figure B, this indicates that dsDNA can be bound by one or two MCM complexes at once. This could be due to the length of nucleotide sequence. From Figure E, potentially 2 hexameric units (2 MCM units) could pair up together when size of nucleotide is longer such as 59 nucleotide sequences as shown in experiment. From PYMOL, it is known that the central channel has a high positive charge which is indicative of neutralizing DNA charge which induces DNA unwinding. There is also a spiral-like structure in the central binding site which is quite similar to the helical structure of DNA; this potentially causes DNA to move into it easily and induce DNA unwinding. From Figure E and F, helicases could potentially bind DNA through central channel; as it has a ring-shaped topology, this likely aids in longer dsDNA sequence unwinding without dissociating from it. Furthermore, for longer dsDNA sequences, 2 hexameric helicases potentially could oligomerize/ pair together and induce a conformational change which changes Kd value as shown in our experiment. Conserved regions in Figure G are indicative that regions near binding site elements (spiral-like structure) play a significant role in DNA binding mechanism in MCM helicase.

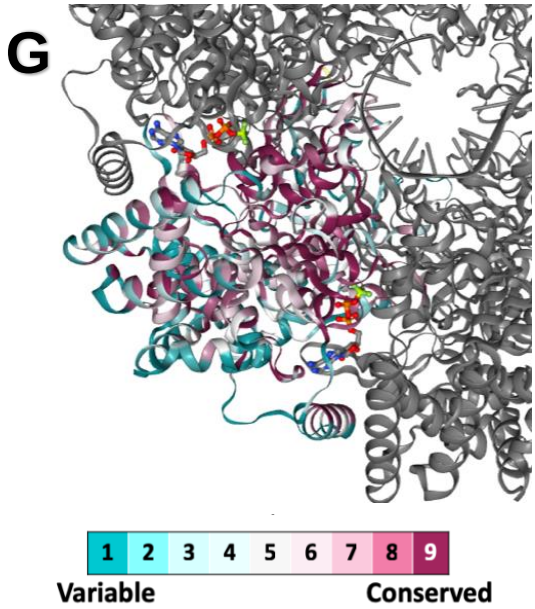


Figure G: CONSURF Analysis of one identical monomer subunit of MCM helicase (PDB ID: 6MII)

References:

1. Cannone, G., Visentin, S., Palud, A., Henneke, G. and Spagnolo, L., 2017. Structure of an octameric form of the minichromosome maintenance protein from the archaeon Pyrococcus abyssi. Scientific Reports, 7(1).
2. You, Z., De Falco, M., Kamada, K., Pisani, F. and Masai, H., 2013. The Mini-Chromosome Maintenance (Mcm) Complexes Interact with DNA Polymerase α-Primase and Stimulate Its Ability to Synthesize RNA Primers. *PLoS ONE*, 8(8), p.e72408.
3. Nowakowski, A., Wobig, W. and Petering, D., 2014. Native SDS-PAGE: high resolution electrophoretic separation of proteins with retention of native properties including bound metal ions. Metallomics, 6(5), pp.1068-1078.