# The Functional and Structural Conservation of Kinesin-5 *CIN8* in *Saccharomyces Cerevisiae and Chaetomium Thermophilum*

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**Abstract**

Kinesin motor proteins, particularly those in the kinesin-5 family, are essential for regulating spindle dynamics and ensuring accurate and successful cell division in eukaryotes. In this study, we assess the functionality and structural conservation of Cin8 in two eukaryotic yeast species: *Saccharomyces cerevisiae*, a model organism for cell division studies, and *Chaetomium thermophilum*, a thermophilic filamentous fungus. Specifically, we investigate kinesin-5 *CIN8p*, an imperative motor protein involved in microtubule crosslinking, spindle assembly, and elongation during mitosis. Through a series of in vitro and in vivo experiments, we examined the structure-function relationship of Cin8p by analyzing ATPase activity, microtubule binding capacity, and cellular localization during mitotic spindle formation. Truncated constructs of *CtCin8* were compared with full-length constructs to determine the functional significance of specific domains in motor protein activity. All versions of CtCin8 exhibited robust ATPase activity and microtubule-binding capacity, confirming the structural/functional conservation of key motor domains. These results provide valuable insights into fundamental cellular processes and contribute to our understanding of mitotic spindle dynamics, with broader implications for integral research for cell division research.

## Background and Significance

*CIN8* is researched and analyzed in two different species: the budding yeast *Saccharomyces cerevisiae*(Sc) and the thermophilic filamentous fungus *Chaetonium thermophilum*(Ct). Cin8p is a member of the kinesin-5 family, a group of motor proteins that play an essential role in mitosis and meiosis by regulating microtubule dynamics[1]. Specifically, kinesin-5 motor proteins are critical for mitotic spindle assembly and spindle elongation during mitotic anaphase. During mitosis microtubules form a macromolecular structure known as the mitotic spindle that is responsible for the accurate segregation of chromosomes between the two daughter cells.[2].

In *S. cerevisiae*, two kinesin-5 motor proteins, Cin8p and Kip1p, are involved in mitotic spindle dynamics. The *CIN8* gene encodes Cin8p, while the *KIP1* gene encodes Kip1p. These proteins are functionally redundant, meaning that one can compensate for the other's dysfunction to maintain spindle formation[3]. However, the simultaneous loss of both proteins leads to a failure in spindle assembly, ultimately rendering the cell nonviable due to mitotic arrest. Although Cin8p has been studied extensively in *S. cerevisiae*, its conservation and functionality in other eukaryotes remain largely unexplored. To address this, we expanded our investigation to *Chaetonium thermophilum*, allowing us to assess the conservation of the *CIN8* gene across divergent species[4].

To analyze Cin8p's structure and function, full-length CtCIN8 and 3 truncated constructs containing only the motor domains were cloned from *Chaetonium thermophilum* and compared them to the budding yeast *Saccharomyces Cerevisiae*. These constructs were amplified via PCR and cloned into plasmids designed for expression in both bacterial and yeast systems. The experimental design focused on characterizing the kinetic properties of purified Cin8p to establish the relationship between its structure and function and utilizing two yeast strains: a wild-type strain expressing functional Cin8p one with mCherry-labeled tubulin to observe Cin8p localization, and the other modified genetically with a disrupted KIP1 gene and the ability to be able to degrade endogenous Cin8p in the presence of auxin[5]. These experiments provided insights into the specific cellular mechanisms underlying mitotic spindle function, helping us better understand how kinesin motor proteins ensure chromosome segregation.

The findings from this study not only contribute to our understanding of Cin8p's structure-function relationship but also offer broader implications for fields such as cell-replication fidelity. Previous studies have demonstrated that Cin8p and Kip1p play critical roles in maintaining chromosomal stability during cell division[6]. By exploring the underlying mechanisms, we hope to unlock new strategies for controlling motor protein function and applying this research to fields like cell replication which potentially allows us to control outcomes that weren't possible before.

**Preliminary Results**

Our preliminary results currently support data done for *Chaetonium thermophilum* specific constructs: CtCIN8 and 3 truncated constructs Ct-800, Ct-533, Ct-429 with bioinformatics being the exception. The approach is to complete our experimental data of CtCIN8 and aim for ScCin8 to undergo the same experiments which was noted in Future Directions. However, our Bioinformatic procedures were finalized with both CtCIN8 and ScCin8 data in direct comparison.

*Cloning (Exp 2)*

CtCIN8 and 3 truncated constructs: Ct-800, Ct-533, Ct-429, whose numbers correspond to the amino acid truncations, were amplified through a polymerase chain reaction or PCR. We decided to design our primers using a cloud based biotech platform Benchling and selected restriction enzyme sites HindIII and BamHi to cleave both PCR products and the plasmids/vectors. Restriction sites were engineered for bacterial overexpression vector (pET24b-6xHis), and yeast shuffle vectors (pGFP-C-FUS and pΔGFP) to allow the desired gene to be cloned. After the PCR products undergo column purification, they are digested by the restriction enzymes along with Calf intestinal alkaline phosphatase (CIAP) for vector religation inhibition and graded through DNA electrophoresis. The DNA fragments were run in 0.8% agarose gel in 1X TBE buffer at

120V and examined for preliminary results. The agarose gel imaged after the loading dye traveled more than ~75% downstream the gel **(Figure 1A)**. Ligation of CIN8 fragments into vectors with ligase buffer and ligase and then heat shocked to transform with E.coli DH5α. The isolated and inoculated clones from plasmid DNA then were tried and transformed in bacterial strain (BLR) for the pET24 constructs and the two yeast strains for the yeast shuttle vector constructs. More specifically we used a modified E. coli strain BLR, a derivative of BL21(DE3) with pGroRIL, that inducibly produces our protein for purification and subsequent enzymatic analysis. This BLR strain contains the plasmid pGroRIL which is imperative for assisting folding of proteins in a cell due to its chaperone genes and coupled with Cin8p expression allows to increase yield of the product. Utilizing electroporation, we used DDY904, which contains a mCherry-tubulin gene, and Y119, which lacks a functional KIP1 and has its CIN8 gene fused with an AID degron signal for yeast transformation **(Figure 1B)**.

*Bioinformatics (Exp 3)*

Bioinformatic tools were the foundation to assessing key details of the Cin8 gene along with the differences between the ScCin8 and CtCin8 sequences. Utilizing the sequence analysis tool for pairwise needle sequence alignment from EMBL-EBI, we found a Genome Identity percentage of 1653/3893 or 42.5% between the two protein sequences, ScCin8 and CtCin8, and the same percentage for the Genome Similarity Percentage at 1653/3893 or 42.5% **(Figure 2)**. We also found a Gap percentage of 920/3893 or 23.6% which seems to indicate that the two's genome structures have a high degree of similarity and low discrepancy. From this data, we also found the highest genome similarity amongst the bases of around 200 to 280. Additionally we found the Molecular Weight of CtCin8 and ScCin8 protein sequences utilizing ExPASy and found them to be 306 kDa and 254 kDa respectively. The similar molecular weights along with the percent identity reinforce the notion that the sequences are highly conserved amongst each other.

*Bradford Assay (Exp 1)*

Bradford Assay was the procedure for determining protein concentration for our targets. Coomassie Brilliant Blue or just Coomassie was the dye employed for our Bradford assay. Coomassie by itself has a peak absorption at 465 nm and is red-brown in color but once protein bound turns a blue color with peak absorption of 595 nm binding most commonly to basic amino acids, lysine, arginine and histidine. Most notably, the absorbance value increases accordingly to the quantitiy of bounded protein. After adding Coomassie to standard concentrations of bovine serum albumin (BSA) and to various volumes of your unknown protein, we measured the absorbance and plotted our data to create a standard curve **(Figure 3)**. The standard curve allowed us to find the protein construct molar concentration to be 9.31 mol/L, concentration of the unknown protein sample 7.4 µg/µL and concentration of our construct (Ct-429) 795 KDa.

*Malachite Green Assay (Exp 1)*

Malachite Green Assay was used to determine the ATPase activity of Cin8p by measuring the amount of free phosphate released from ATP hydrolyzed to ADP. The free phosphate complexes with molybdate ions and finally with the malachite green dye at low pH causing a movement in the max absorption of the dye. Two important statistics are calculated based on the Malachite Green Assay: Enzyme Activity (U) and Specific Activity(SA) [1 SA unit = μmol min-1mg-1]. Absorbance of our Assay was calculated at 650nm and plotted as a bar graph representing activity with MT or without MT, along with different tested conditions such as with ATP or without ATP **(Figure 4)**. Using this graph and the data found from this assay, we found the average extinction coefficient of the class for phosphate was 99.4 mM^-1cm^-1. Using SA as the dependent, we found that the SA without MT was 0.006024 μ/mg and with MT to be 0.012 μ/mg. Additionally, we found the extent of stimulation of ATPase activity with MT to be 2. Simply put, this means MT binding leads to around double ATPase Activity. This outcome is likely caused by Kinesin requiring ATPase activity as a power source when bonding to MT for inducing movement.

**Future Direction**

While much of the research has been conducted under CtCIN8 and 3 truncated constructs: Ct-800, Ct-533, Ct-429, the future direction of this research is to commence formalized experiments to expand our preliminary results with ScCin8 and its truncated constructs. Additionally commencing more forms of experimentation can allow a more meticulous perspective of *CIN8* and the structural and functional conservation across both *Saccharomyces Cerevisiae* and *Chaetomium thermophilum.*

*Localization labeled with GFP*

CtCIN8 and 3 truncated constructs can be visualized through fluorescent microscopy by tagging the constructs with Green Fluorescent Protein(GFP) in addition with ScCin8 and its truncated constructs which allows specific cellular structures to be localized by taking advantage of a fluorescent signal. In addition, we can take advantage of the mCherry-labeled tubulin, a red fluorescent protein, that allows visualization of the mitotic spindle. This experiment is possible since genetic code is highly conserved which allows the transcriptional and translational machineries of yeast to recognize our desired sequences. We will determine the localization of *CIN8* construct in wild type cells (strain DDY904) instead of Y119 since the former contains a different additional fusion protein TUB1 gene with mCherry into the genome. Running this experiment can allow us to see if there is consistency amongst the GFP and Cherry results where the Cin8 protein localizes on the mitotic spindle. If a similar localization is the result, this would

demonstrate conservation of *CIN8* functions across the two species.

*Biochemistry in vitro ATPase activity/kinetics/MT binding*

In order to purify our protein of interest, and use the purified protein for biochemical assays, we look to utilize Affinity Chromatography using the 6xHis tag engineered on the C-terminus. Ni-NTA resin, agarose beads bound to nitrilotriacetic acid (NTA) chelates nickel (Ni2+), would be used to allow His-tagged proteins to bind within the eluting column. Imidazole would also be used to bind competitively with the nickel ion in order to elute our target protein since it is structurally similar to histidine. Protein concentration of each resulting fraction would be measured through Bradford Assay.

Additionally, a Microtubule Binding Assay will be commenced to test the ability of *CIN8* constructs to bind to MTs. By discovering if the presence of MTs shifts the proportion of *CIN8* from the soluble to the pellet fraction following high speed centrifugation, this assay allows us to quantifiably evaluate Cin8p's ability to interact with MT's in the presence of ATP.

Lastly, we aim to undergo NADH-coupled ATPase assay to assess ATPase activity of our *CIN8* constructs and compare the results to our prior ATPase experiment or the Malachite Green Assay. This assay is based on two reactions, part of the glycolysis and anaerobic lactic acid fermentation pathways to regenerate ATP hydrolyzed by the protein ATPase. The assay will monitor the loss over time of NADH at 340 nm, which is proportional to the hydrolysis of ATP. This data will allow us to compare the kinetics between ScCin8 and CtCin8 to quantifiably assess the conservation of structural and functional conservation of *CIN8* function.

*Phenotypic Analysis*

An obvious tactic is to visually affirm possible structural and functional conservation is phenotypic analysis of Cin8p constructs. Essentially, we would look for any change during our experiments in order to see if our ScCin8 and CtCin8 constructs have similar or different features. Confirmation of Auxin treatment reducing expression of the AID-tagged endogenous *CIN8* and that it does not affect expression of the GFP-tagged *CIN8* constructs that you have introduced on a plasmid is imperative for the basis of this procedure. The genomic copy of *KIP1* is nonfunctional in the Y119 strain but *CIN8* has a fused AID sequence which allows it to stay functional in the absence of auxin. Based on our bioinformatic analysis of CtCin8 and ScCin8 and current knowledge, we would expect both proteins to be nonfunctional and thus phenotypically qualifiable.

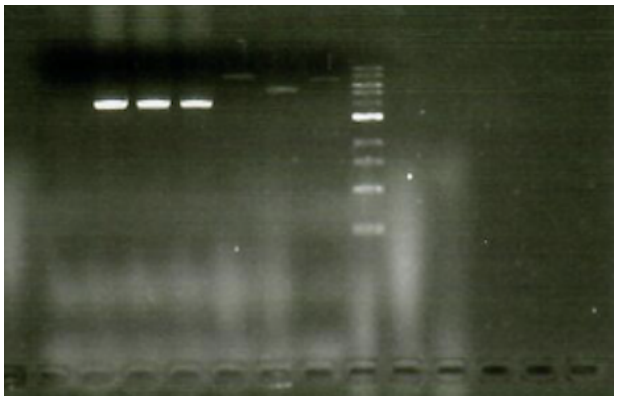
*Western Blotting*

While our protein analysis after SDS commenced under Coomassie, we believe that Western Blotting can be exercised to solidify results for conservation of *CIN8* functions across the two species. Coomasie allowed us to image all proteins in the sample making it useful for generalized protein visualization however Western Blotting allows us to specifically detect and identify target proteins from the sample since it uses antibodies that bind to the protein of interest. Following the same SDS procedure done for Coomassie, proteins are electrophoretically transferred to be bound to a solid support membrane, in this case polyvinylidene difluoride (PVDF). Histidine is our chosen primary antibody that would recognize specific antigen. Done successfully, we expect that there would be substantial evidence that Cin8p is expressed like the BLR constructs made in previous experiments.

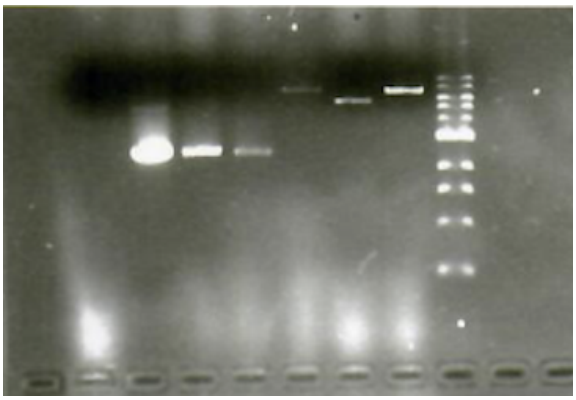
**Figures and Tables**

**1A)**

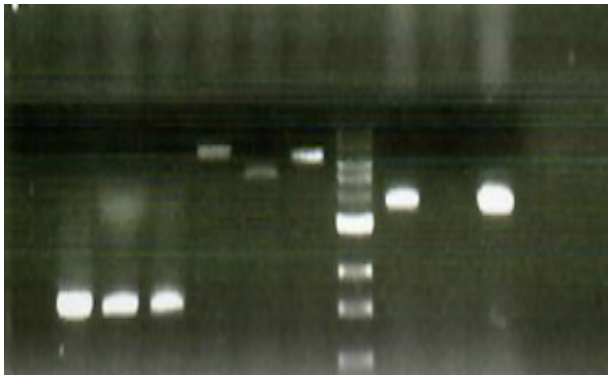
**i)**



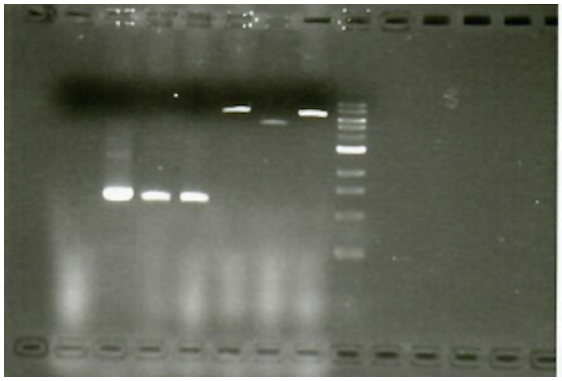
**ii)**



**iii)**



**iv)**



**v)**

**MW Std** (Cat# NEB N3232)

| Size (bp) | Mass (ng) |
|---|---|
| 10,002 | 42 |
| 8,001 | 42 |
| 6,001 | 50 |
| 5,001 | 42 |
| 4,001 | 33 |
| **3,001** | 125-bright band |
| 2,000 | 48 |
| 1,500 | 36 |
| 1,000 | 42 |
| 517 | 42 |

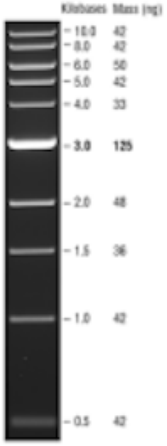
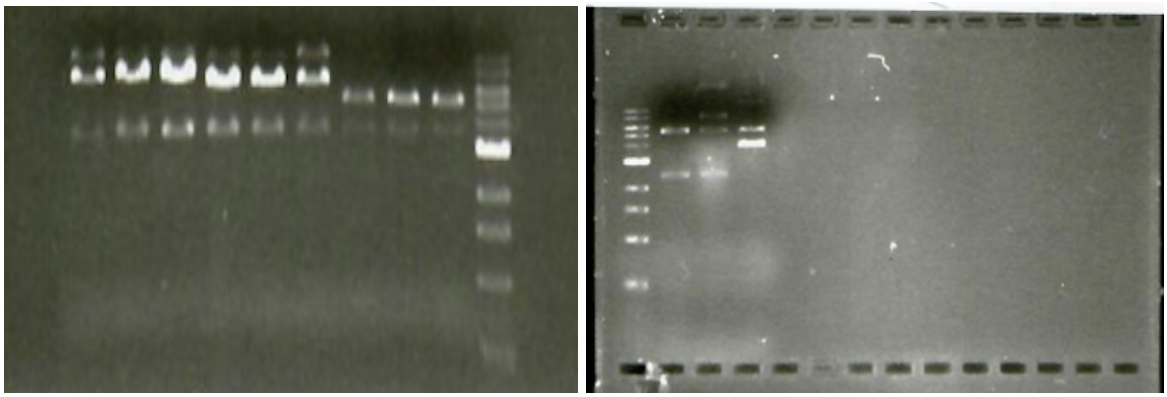


*Figure 1A*: Gel of Digested PCR products and Vectors from PCR. All images from i) to iv) visuals agarose gel where purified and digested DNA fragments were run to analyze PCR quality. The PCR product for the CtCin8 (i) and the three constructs Ct-800 (ii), Ct-533(iii) and Ct-429(iv) all produced serviceable results demonstrating the effectiveness of the PCR and the quality of the DNA chosen. Image v) represents the ladder that was run on the gel to be seen as a reference point for the DNA band size calculation.

**1B)**

**i)** **ii)**



**iii)** **iv)**

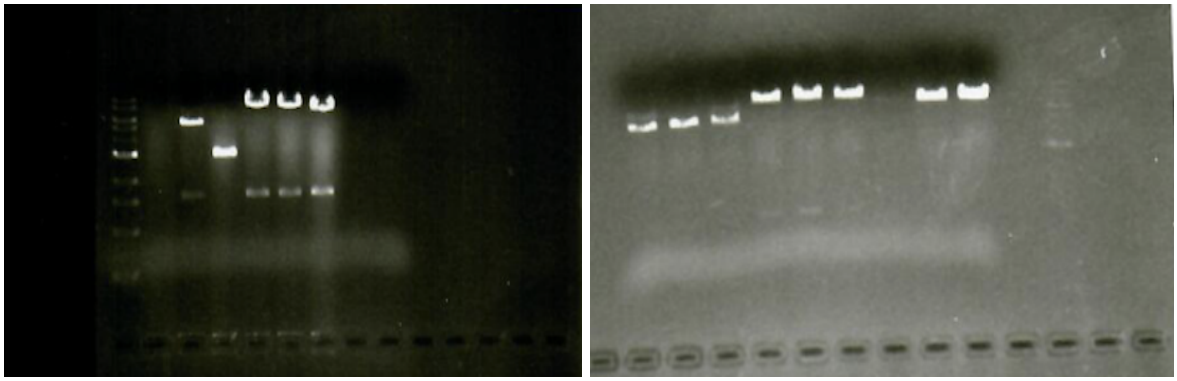


*Figure 1B*: Gel of Transformed Plasmids into Bacteria and Yeast. Successful clones for each truncation were put into the bacterial strain for pET24 constructs and the two yeast strains for the yeast shuttle vector constructs. The PCR product for the CtCin8 (i) and the three constructs Ct-800 (ii), Ct-533(iii) and Ct-429(iv) all produced serviceable results demonstrating the effectiveness of the PCR and the quality of the DNA chosen.

```
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# Program: needle
# Rundate: Sun 20 Oct 2024 02:23:28
# Commandline: needle
#    -auto
#    -stdout
#    -asequence emboss_needle-I20241020-022325-0816-34366872-p1m.asequence
#    -bsequence emboss_needle-I20241020-022325-0816-34366872-p1m.bsequence
#    -datafile EBLOSUM62
#    -gapopen 10.0
#    -gapextend 0.5
#    -endopen 10.0
#    -endextend 0.5
#    -aformat3 pair
#    -sprotein1
#    -sprotein2
# Align_format: pair
# Report_file: stdout
########################################

#=======================================
#
# Aligned_sequences: 2
# 1: EMBOSS_001
# 2: YEL061C_S288C
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 3893
# Identity:    1653/3893 (42.5%)
# Similarity:  1653/3893 (42.5%)
# Gaps:         920/3893 (23.6%)
# Score: 6840.5
#
#
#=======================================
```

emboss_needle-I20241020-022325-0816-34366872-p1m

*Figure 2*: Emboss Needle Percent Comparison between CtCin8 and ScCin8. Attached is the EMBL-EBI needle link to see the total genome sequence and its similarities. However in the data shown, a similarity score, identity score, gap score and total score are listed about the comparisons of CtCin8 and ScCin8. Overall, the data shows that the two genetically are closely aligned to each other.

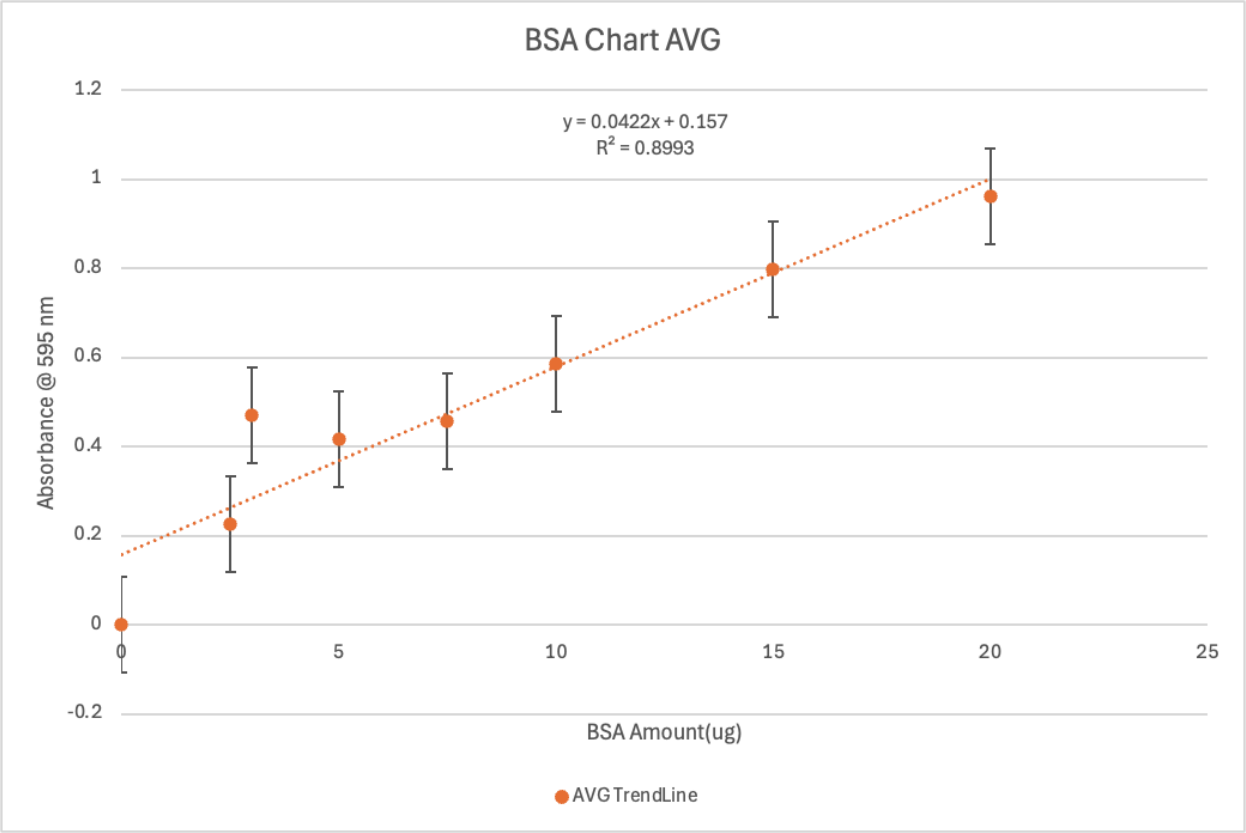


*Figure 3*: Bradford Assay Extinction Coefficient and Standard Curve Calibration: Average Absorbance at 595 nm plotted against the amount of BSA added in order to find a linear regression line. The regression line is shown as a line of best fit (y = 0.0422x + 0.157) and used to determine the protein concentration (ug). The High R^2 value of 0.8993 informs us that the data has a strong linear fit and can be reliably used as a means for predictions or other means.

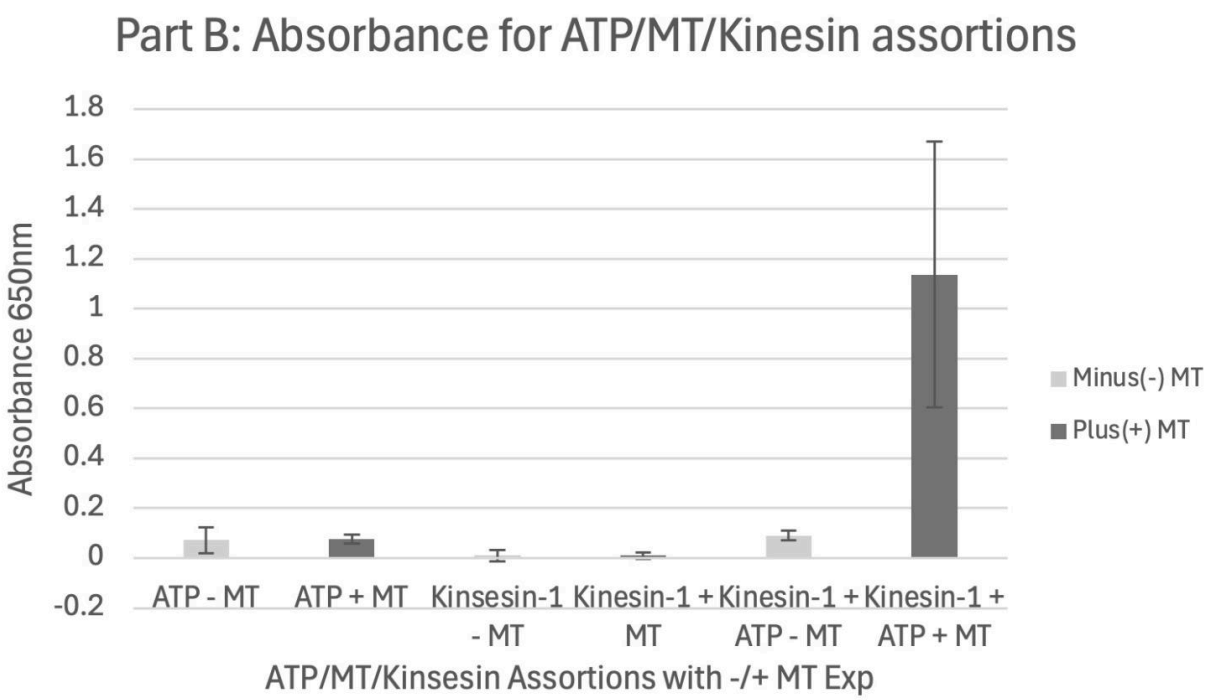


*Figure 4*: Specific Absorbance Activity of Human Kinesin-1 Protein in the Absence and Presence of MT: Average class absorbance value at 650 nm data under various conditions involving mainly presence/absence of MT. The bar chart demonstrates that while simply presence of Kinesin-1 along with MT as a dependent factor didn't influence much absorbance as well as ATP with MT as a dependent factor. However, Kinsen-1 along with MT and ATP had a tremendous output of absorbance while Kinsen-1 along with no MT and ATP didn't vary much in comparison to every bar. The error bounds represent the SD of the class data.

**References**


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