CHALMERS
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The Role of LigD in Non-Homologous End-Joining in *Mycobacterium tuberculosis*

Master's thesis in Biotechnology

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DIVISION OF CHEMICAL BIOLOGY

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Abstract

Repairing damaged DNA is necessary for the survival and accurate reproduction of organisms. To this end, numerous DNA damage repair pathways have evolved. Non-homologous end-joining is a repair pathway that repairs double-stranded breaks in DNA, without using a homologous sequence as a template. In bacteria, the two proteins necessary to facilitate non-homologous end joining are Ku and LigD, a DNA ligase. This project investigated how six different point mutations on LigD affected the DNA ligation activity. Both bulk and single molecule methods were utilized. For bulk assays, electrophoretic mobility assays, pulldown assays, and ligation assays were used to study how well Ku and LigD bind to and ligate DNA, and how the mutations affect this. The ligation activity was also investigated using nanofluidics, visualizing the DNA on a single molecule level. From these assays, it was shown that three of the point mutations (R198E, D162R, V194D) resulted in lowered ligation activity compared to wild type LigD. One of the mutations (K579E) increased the ligation activity. The mutation L580E caused DNA fragmentation, and for LigD D522R, the results from the assays were contradictory.

Keywords: *Mycobacterium tuberculosis*, DNA repair, NHEJ, LigD, Ku, protein-protein interactions, DNA-protein interactions, nanofluidics.

List of Acronyms

Below is a list of acronyms used in this thesis, listed in alphabetical order.

DDR – DNA damage response

DSB – Double Stranded Break

EMSA – Electrophoretic Mobility Shift Assay

HR – Homologous Recombination

LigD – Ligase D

NHEJ – Non-Homologous End-Joining

TB – Tuberculosis

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1. Background

1.1 Introduction

Non-homologous end-joining is a method to repair double-strand breaks in DNA that doesn't require a template. The damage repair pathway was first discovered in eukaryotes, but it has since been found in a diverse array of prokaryotes, including the bacterium *Mycobacterium tuberculosis*, the pathogen that causes tuberculosis in humans. The two proteins facilitating non-homologous end-joining in bacteria are Ligase D and Ku. Understanding how these proteins interact with each other and with DNA is central to understand how the bacteria maintains its genome, which could act as a target for drug development or shed further light on the mechanisms causing mutations in *Mycobacterium tuberculosis*.

1.2 Aim

The aim of this project was to study the effect of six different point mutations (predicted using Alphafold2 by collaborators from the Guerois Lab from Université Paris-Saclay) in two different domains on the protein Ligase D from *Mycobacterium tuberculosis*. The primary focus was on how each mutation affected the DNA ligation activity of the protein.

1.3 Theoretical Background

1.3.1 Tuberculosis

Mycobacterium tuberculosis is a pathogenic bacterium that can cause tuberculosis (TB) in humans. According to WHO, an estimated 10.6 million people were infected with tuberculosis in 2022, with an estimated 1.3 million people passing away from the disease [1]. As such, there is a great interest in furthering humanity's understanding of *M. tuberculosis*, and to use this knowledge to reduce the suffering caused by TB.

When droplets containing *M. tuberculosis* are breathed in, the bacteria is phagocytosed by alveolar macrophages. Through protein and lipid effectors, *M. tuberculosis* halts lysosomal trafficking pathways, continues to replicate in the cell, and induces necrosis. From there, *M. tuberculosis* infects other macrophages and neutrophils. It takes 2-8 weeks for the adaptive immune system to respond to the infection and help the macrophages form a granuloma, containing the infection. If the infection is successfully contained, the person will have latent tuberculosis. A person with latent TB doesn't show any symptoms, and can't infect other people, but still has these granulomas that were created to enclose *M. tuberculosis*. Circa 5-10% of the people infected with *M. tuberculosis* will develop active tuberculosis. This can be either from the primary infection not being successfully contained, or reactivation of latent TB (however, globally, reactivation seems to be the less common cause by far) [2].

1.3.2 DNA damage

DNA can be damaged by many different causes both internal and external, including reactive oxygen species, radiation, and spontaneous errors made during replication. Left as such, these damages will accumulate, leading to mutations and endangering the survival of the cell. Thus, it is imperative for an organism to have repair pathways that treat these damages. Organisms have evolved many different DNA damage responses (DDRs). Here, the focus will be on NHEJ (HR will also be mentioned).

A double stranded break (DSB) is one of the most severe forms of DNA damage. It occurs when the backbone of both DNA strands break in close proximity, thereby creating a complete break in the dsDNA [3].

Homologous recombination (HR) is one pathway to repair DSBs. It is error-free, but requires another copy of the damaged site nearby to act as template. *M. tuberculosis* is haploid, so there is only a sister chromatid to serve as template for HR during part of the cell life cycle (late S and G2 phases) [4].

1.3.3 NHEJ

Non-homologous end-joining (NHEJ) is a DSB repair pathway that does not require a homologous copy of the damaged DNA to be in close proximity. It can also repair DSBs where the ends have been damaged, or are noncomplementary. Compared to HR, NHEJ is more error-prone [5]. The pathway was first discovered in eukaryotes, but has since been discovered in many different archaea and bacteria. The pattern of which bacteria have NHEJ and which do not is sporadic, indicating that the machinery for NHEJ has been gained and lost several times across the domain [6].

In *M. tuberculosis*, the proteins facilitating NHEJ are Ku and Ligase D (LigD). Ku is a ring-shaped homodimeric protein that binds to the damaged ends of the DNA strands, protecting them from further damage, bridging the break, and recruiting LigD to the site. The protein consists of a ring-shaped core domain, from which a C-terminal domain arm extends. The core domain threads onto the DNA and can form a synapse across the DSB, but the C-terminal domain is required to stabilize this synapse and recruit LigD to ligate the break [7].

1.3.4 LigD

LigD is an ATP-dependent ligase. In *Mycobacterium tuberculosis* it consists of three catalytic domains with different activities: polymerase (POL), phosphoesterase (PE, but it's also called the nuclease domain), and ligase (LIG), listed from N- to C- terminal. The POL and PE domains process the ends of the DSB before LIG can seal the break. Different bacteria arrange the domains of LigD in different orders, or lack certain domains [8].

The POL domain adds nucleotides to the site of the break. If the DSB ends in an 5'-overhang, POL can catalyze fill-in of the templated nucleotides before ligation. The POL domain interacts strongly with 5' phosphate groups on DNA [9]. If the break is blunt, POL can insert non-templated nucleotides at the terminus. Both of these polymerase activities can give rise to mutations. For the fill-in, the POL domain is a relatively error prone polymerase, and the addition of a nucleotide to the site of the blunt DSB causes a frameshift mutation. Rarely, the POL domain can also add single nucleotides to the terminus of the ends that POL has already filled in [8]. *In vitro*, POL has been shown to have primase activity, preferring ribonucleotides over deoxynucleotides as a substrate. What role this preference for RNA plays in NHEJ repair *in vivo* has not been elucidated [9].

For *M. tuberculosis* LigD, the central domain PE has not been as well characterized as the other two domains. In *Pseudomonas aeruginosa*, which is another bacterium with NHEJ, the PE domain has shown 3'-phosphoesterase and 3' ribonuclease activity [8].

The LIG domain seals the two ends of the DSB together. If the LIG domain is inactivated *in vivo*, another DNA ligase (possibly Ligase C) can step in and ligate the break, though it reduces the efficiency of NHEJ [10]. The ligase domain has a high affinity for binding to DNA, and that affinity is enhanced by the presence of Ku [11].

1.3.5 Nanofluidics

Protein-DNA and protein-protein interactions can be studied through ensemble averaging or single molecule methods. With the resolution of single molecule methods, individual molecules and their behaviors can be distinguished. The nanofluidic chip used in this work is made out of fused silica (to keep DNA from sticking) with four loading wells. Wells one and two are connected by a U-shaped microchannel, as are wells three and four. The two microchannels are connected at their apexes by hundreds of nanochannels with the dimensions 100 x 150 nm, and a length of 500 μm. When confined in the nanochannels, the DNA is stretched due to the narrowness of the channels, while leaving the ends of the DNA free.

YOYO-1 is an DNA dye which becomes fluorescent when it binds to DNA. It is a homodimer that binds through intercalation, with either one or both of its monomers. When not bound to DNA YOYO-1 has a very low quantum yield, but it increases dramatically upon binding. It has a high affinity for DNA, and this in combination with the large difference in quantum yield makes YOYO-1 a popular choice when staining DNA. Due to the dye binding through intercalation, YOYO-1 binding will marginally extend the length of the DNA molecule and slightly reduce the rotation of the helix [12]. By staining DNA with YOYO-1, stretching it in nanochannels, and imaging it with fluorescence microscopy, individual DNA molecules can be visualized.

2. Methods

The proteins SNAP-Ku, Ku, LigD WT as well as the LigD mutants were expressed in *E. coli.* The purification was done according to the protocol established by Elin Persson [13]. To verify the concentrations of LigD WT and mutants they were analyzed on an SDS-Page gel (Invitrogen).

2.1 EMSA

EMSA titrations of were done of Ku on 50 bp DNA labeled 6-FAM. The DNA was blunt-ended and had a concentration of 5 nM, the Ku concentrations used were 0.1, 0.2, 0.5, 1, 2, 5, and 8 μM. The Ku and DNA was mixed in Buffer E (25mM Tris-HCl pH7.5, 100mM NaCl, 30mM KCl, 0.1mM EDTA, 0.05% Triton-X100, 500mg/mL BSA, 2% glycerol, 2mM DTT), incubated at room temperature for 30 min. The samples were loaded on a 1% agarose gel and ran at 80 V for 1 hour in 1XTAE buffer. The gel was imaged fluorescently using Chemidoc MP imaging systems (Bio-Rad)

WT LigD was titrated with and without Ku, on both blunt-ended DNA and DNA with a 4-bp overhang. Both types of DNA were 50 bp long and labelled with 6-FAM. LigD concentrations were 0.2, 1, 2.1, and 4.2 μM. The DNA, LigD, and Ku was added to Buffer E, and the samples were incubated at room temperature for 30 min at room temperature. The samples ran on an 1% agarose gel and were imaged the same way as for the EMSA Ku titration above.

2.2 Ligation assay

The concentrations used for LigD in the ligation assay were 0.2, 1, and 2 μM. For Ku, the concentration was 1μM. Ku was incubated with 300 μg of 1000 bp DNA (NoLimits, ThermoScientific) in Buffer L (50 mM Tris pH 7.5, 30 mM KCl, 30 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT) at 37ºC for 30 minutes. LigD was added at the indicated final concentration and incubated for 1 hour and 30 min. The reaction was stopped by the addition of the stop buffer

(5 mg/mL Proteinase K, 2% SDS, 0.1 M EDTA) and then incubated at room temperature for 30 min.

The samples were loaded on an 0.8% agarose gel and ran at 80V for 90 min in 1XTAE buffer. The gel was stained with the fluorescent dye SYBR gold nucleic acid stain (Invitrogen), and imaged in Chemidoc MP imaging systems (Bio-Rad). This assay was performed in triplicate.

2.3 Nanofluidics

1 μM of Ku and 0.5 μg/μL 10 000 bp DNA (NoLimits, ThermoScientific) were mixed with Buffer L, and incubated at 37 ºC for 30 minutes. Then, the LigD was added at a concentration of 0.2 μM, incubated again at 37 ºC for 90 minutes. 5 μL of 100 mM YOYO-1 from Invitrogen was added, corresponding to 1 dye molecule per 5 bp of DNA. Finally, it was incubated for ten minutes at 37 ºC.

Shortly before being loaded into the nanofluidic chip, the sample was diluted 20X times in Buffer S (10 mM Tris HCl pH 8, 10 mM KCl, 10 mM NaCl, 5 mM DTT, 0.06% SDS). 10 μL of the sample were loaded into one well, the other three wells were loaded with 10 μL Buffer S. The DNA was pushed into the nanochannels using pressure exerted by nitrogen gas. It was imaged with the help of the inverted fluorescent microscope Zeiss AxioObserver.Z1. For each LigD variant (and the controls), a minimum of 1000 DNA molecules were imaged. The molecules were counted and their lengths measured in ImageJ. The length of a monomer was defined as the median length of a molecule in the DNA-only control. The molecules were sorted into concatemers (defined as a molecule more than twice as long as the monomer) and monomers. By dividing the number of concatemers with the total number of molecules in each sample, the share of concatemers was calculated for each sample. The relative number of monomers for each DNA molecule was calculated by dividing the measured length by the median of the length of a monomer. This was done using Excel, and it was plotted in Origin 2023 and Excel.

2.4 Pulldown assay

For each reaction, 3 μL magnetic beads (SNAP-Capture Magnetic Beads, New England Biolabs) were washed twice with 100 μL of Buffer L and resuspended in 100 μL of Buffer L. 10 μM SNAP-Ku or SNAP peptide (New England Biolabs) were added to the beads for immobilization and incubated in a thermo-shaker (LabTeamet) overnight at 4ºC, 600 RPM. The beads were quickly washed twice with 100 μL Buffer L and resuspended in 100 μL Buffer L with 10 μM LigD WT or LigD mutants, incubated for hour in a thermo-shaker at room temperature. The beads were then washed twice and resuspended in 10 μL NuPAGE™ LDS Sample Buffer (4X), and incubated at 95ºC for 15 minutes. The supernatant was loaded on a 4-12% Bis-Tris gel and ran at 200 V for 45 min. The gel was stained with the Pierce™ Silver Stain Kit according to the standard protocol, then imaged in the Chemidoc. This was done in triplicate.

3. Results and Discussion

The six different predicted LigD point mutations are D162R, V194D, R198E D522R, K579E, and L580E. The first three are in the POL domain, the latter three in the LIG domain, as illustrated by the schematic in Figure 1.

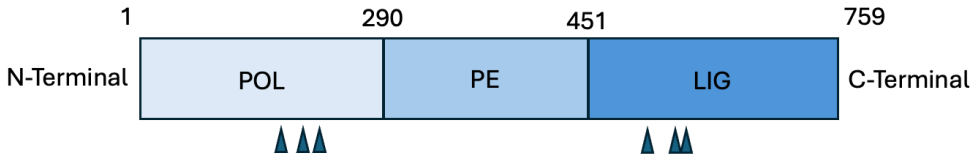

Figure 1: *Visualization of LigD domains, with approximate sites of the point mutations marked. The mutations in the POL domain are D162R, V194D, and R198E. The point mutations in the LIG domain are D522R, K579E, and L580E.*

The SDS-Page gel with purified LigD WT and LigD mutants can be seen in Appendix A. Electrophoretic mobility assays (EMSAs) were done to check how well Ku and LigD WT bind to DNA under different conditions. The EMSA gels are presented in Appendix B.

3.1 Ligation assay

To determine which concentration should be used in the main ligation assay, Ku was titrated (1-8 μM) on a constant concentration of WT LigD (0.5 μM) and DNA. Optimal binding of DNA was shown at 2 μM Ku, which corresponds to a Ku:LigD WT ratio of 4:1.
WT LigD was titrated (0.1, 0.2, 1, 2 μM) on constant Ku and DNA concentrations to confirm that this was an appropriate range of concentrations to compare LigD WT and LigD mutants. These two titrations can be seen in Appendix C.

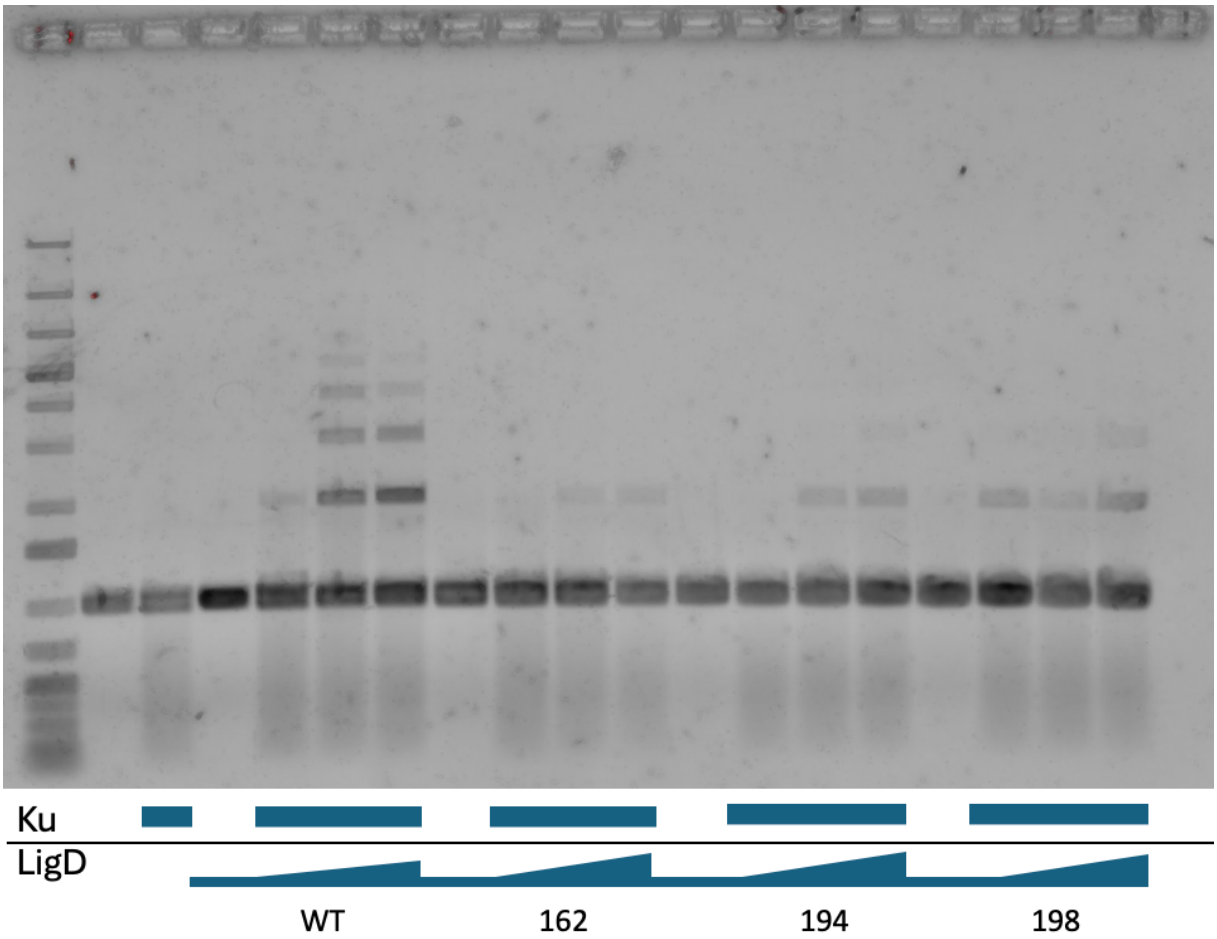

Figure 2: *Ligation assay of LigD WT and POL domain LigD mutants, using 1kb DNA fragments, with varying LigD concentrations (0.2, 1, 2 μM) and a constant Ku concentration (1 μM).*

The three mutants in the POL domain (R198E, D162R, V194D) all show lowered ligation activity compared to wild type LigD at the same concentrations. For R198E, it looks like the ligation activity is better at 0.2 μM than 1 μM. However, this might be because R198E was the LigD variant with the highest concentration of protein in stock, so that result may come from minor discrepancies in the actual volume aspirated when creating the dilution series for that mutant.

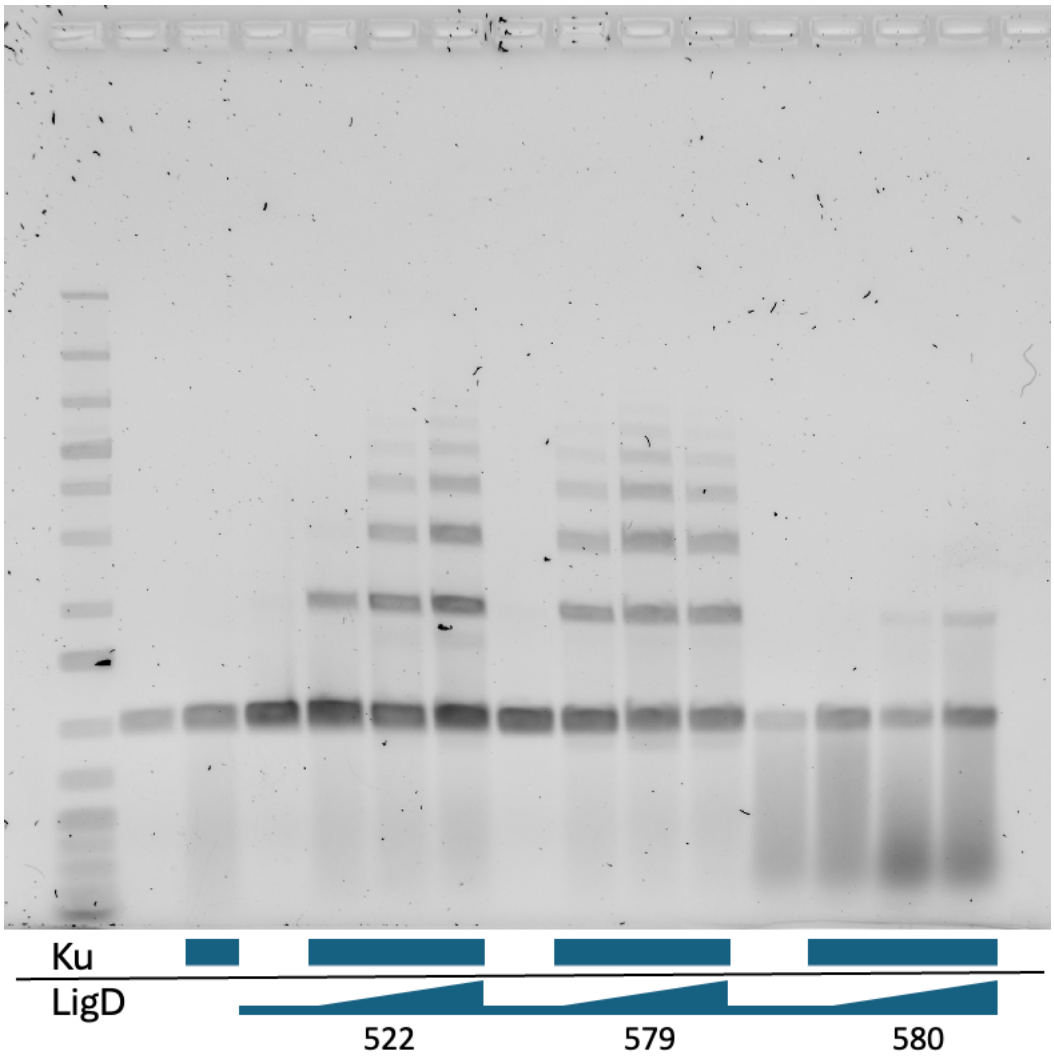

Figure 3: *Ligation assay of LIG domain LigD mutants using 1kb DNA fragments, with varying LigD concentrations (0.2, 1, 2 μM) and a constant Ku concentration (1 μM).*

D522R seems to have comparable ligation activity to WT LigD in Figure 1. K579E shows increased ligation activity compared to WT LigD, in particular at the lowest concentration of LigD (0.1 μM).

The LigD mutant L580E shows heavily decreased ligation activity, as well as an increased amount of smaller DNA fragments, as seen by the smear at the bottom of the gel. This could be attributed to the Ku-LigD580 complex not binding to the DNA ends and protecting them from degradation. However, since neither the DNA only or DNA plus Ku controls show the same degree of DNA fragmentation, and the smear darkens when the LigD concentration increases, the more probable explanation is that this nuclease activity is caused by the L580E mutation. Ku concentrations higher than the optimum Ku:LigD WT ratio caused similar smears, as can be seen in Figure C1.

3.2 Nanofluidics

Using nanofluidics for DNA allows for single-molecule resolution, without restricting the ends of the DNA molecule. For this reason, it was used to investigate ligation activity.

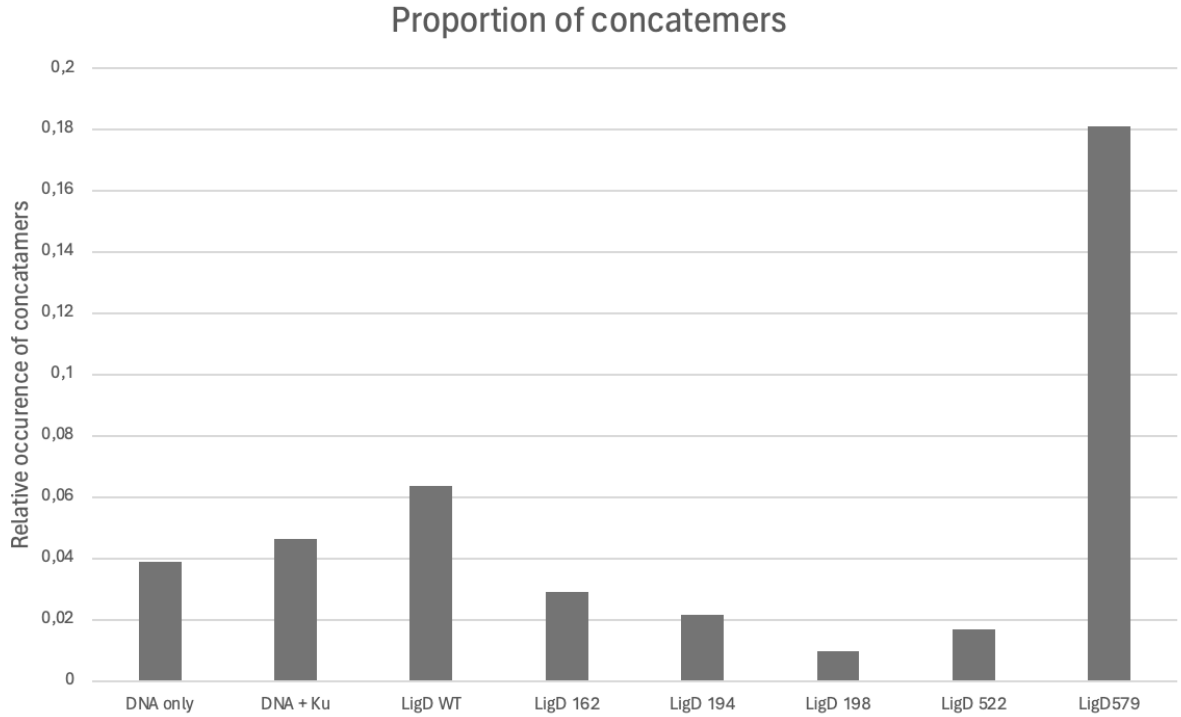

Figure 4: *Bar plot showing what share of the DNA molecules detected were concatemers for each LigD mutant, LigD WT and the controls.*

LigD K579E had the highest proportion of concatemers out of all the samples. This correlates well with the results seen in the ligation assay in Figure 3, where K579E showed an increased ligation activity compared to LigD WT. This increase was particularly noticeable at the LigD concentration of 0.2 µM, which is the same concentration used in this assay. The LigD mutants R198E, D162R, and V194D all had lower relative occurrence of concatemers than WT LigD, also in concurrence with the results in Figure 2. Of note is that D522R has the second lowest share of concatemers in Figure 4. This contradicts the results from the ligation assay presented in Figure 2, where it seemed to have a ligation activity comparable to LigD WT.

The DNA only and DNA plus Ku samples have a larger share of concatemers than all LigD mutants other than K579E. This could be due to impreciseness in measuring the molecules, as the data extraction method is still under development. Another factor that could have contributed is the potential that LigD has a condensing effect on DNA.

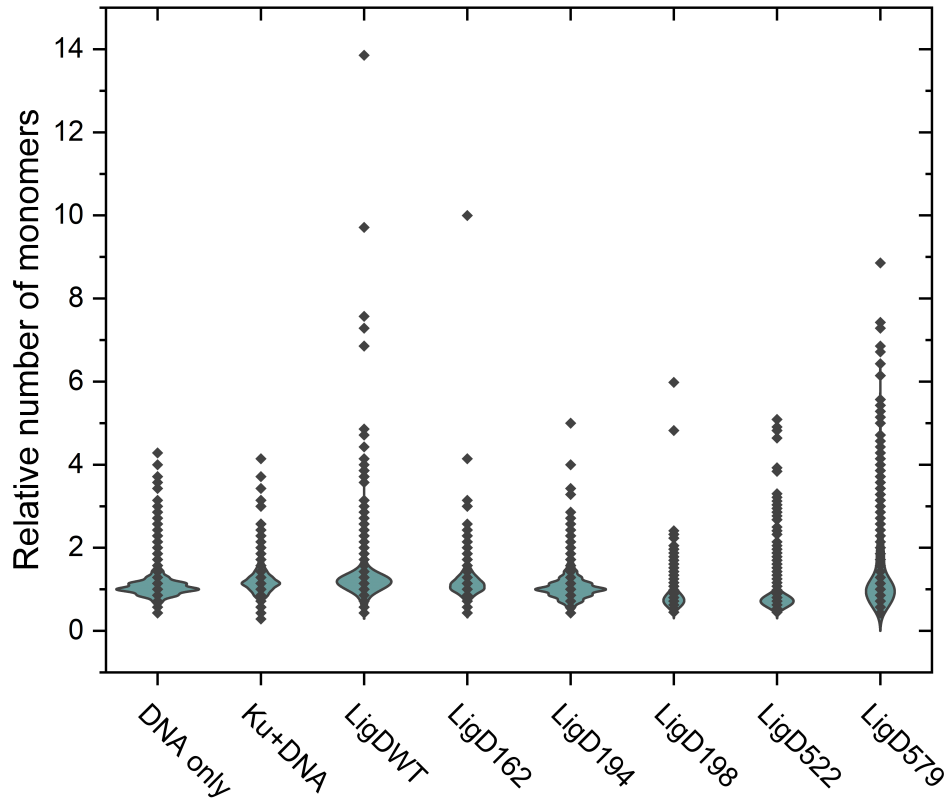

Figure 5: *Violin plot showing the distribution of the relative number of monomers per DNA molecule for LigD WT and LigD mutants, as well as the controls consisting of DNA with Ku, and only DNA.*

The median relative number of monomers for D522R and R198E are lower than the rest of the reactions. This indicates that the median recorded length of one monomer was shorter in these reactions, which could be why these mutants had such a low proportion of concatemers in Figure 4. That ligation assay indicated that D522R had a higher ligation activity than the POL-domain mutants, more similar to LigD WT.

LigD L580E is not presented in Figure 4 or 5. For L580E, DNA was only rarely seen in the microchannels, and was thus not able to be visualized in the nanochannels. Examples of images taken of LigD L580E in the microchannel can be seen in Appendix D. This fragmentation is consistent with the result from the ligation assay in Figure 3, indicating that the L580E mutation resulted in a LigD with very little ligation activity, instead showing increased nuclease activity.

The DNA molecules were counted after all the samples had been imaged. To capture over 1000 DNA molecules, certain samples required two imaging sessions. The molecules from these two biological replicates were pooled. The samples with two biological replicates are LigD WT, DNA only, V194D, D162R, and L580E (L580E was to verify the results discussed above). One possibility to take into account is that if D522R and R198E had also been repeated, the difference in median relative number of monomers might be changed or disappear.

3.3 Pulldown assay

To see how the mutations affected the protein-protein interactions, a pulldown assay was performed using SNAP-Ku.

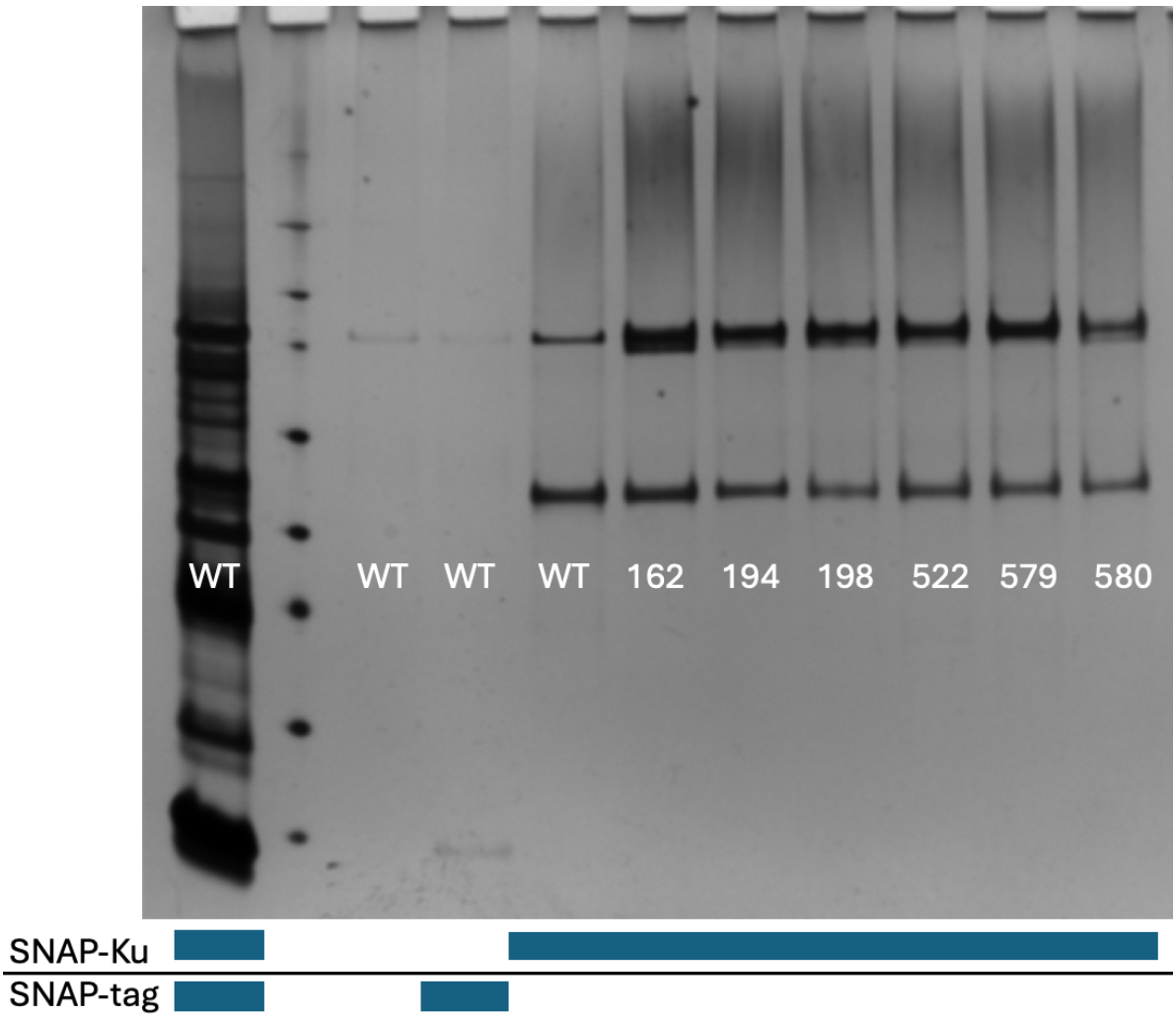

Figure 6: *Pulldown assay investigating the protein-protein interactions of Ku and LigD WT and mutants, using Ku .*

As seen in Figure 6, all LigD mutants were recovered by SNAP-Ku in the pulldown assay. This shows that the mutations did not significantly interfere with the Ku-LigD protein-protein affinity. However, this is done in the absence of any DNA. With DNA present, the Ku-LigD interaction might change. To continue investigating if the differences in ligation activities can be ascribed to the mutations disrupting the interaction between Ku and LigD, doing a pulldown assay with DNA present would be pertinent.

4. Conclusion

The ligation assay showed that the predicted LigD point mutations affected ligation activity in different ways. The mutations located in the POL domain (R198E, D162R, V194D) resulted in lowered ligation activity compared to wild type LigD at the same concentrations. For LigD K579E, the ligation activity increased, especially at low concentrations of LigD (0.2 μM). The mutation D522R showed no significant change in ligation activity compared to WT LigD in the bulk ligation assay, but in the nanofluidic ligation assay it seemed like the ligation activity was significantly lowered. This might be due to artefacts in data collecting or analysis, but would need to be examined further to verify. LigD L580E showed a decreased ligation activity. The mutation caused DNA fragmentation that could be clearly seen in the gel of the ligation assay. It also prevented the DNA incubated with LigD L580E from being analyzed using nanofluidics, since the DNA was so fragmented.

These assays don't show what the impact of these LigD point mutations would be in *M.tuberculosis*, or if increased or decreased ligation activity would have an effect on the survival of the organism. To examine these questions further, cell viability assays wherein the cells carrying these mutations are exposed to DSB-causing agents could be performed.

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Appendix A – SDS-Page gel of LigD WT and mutants

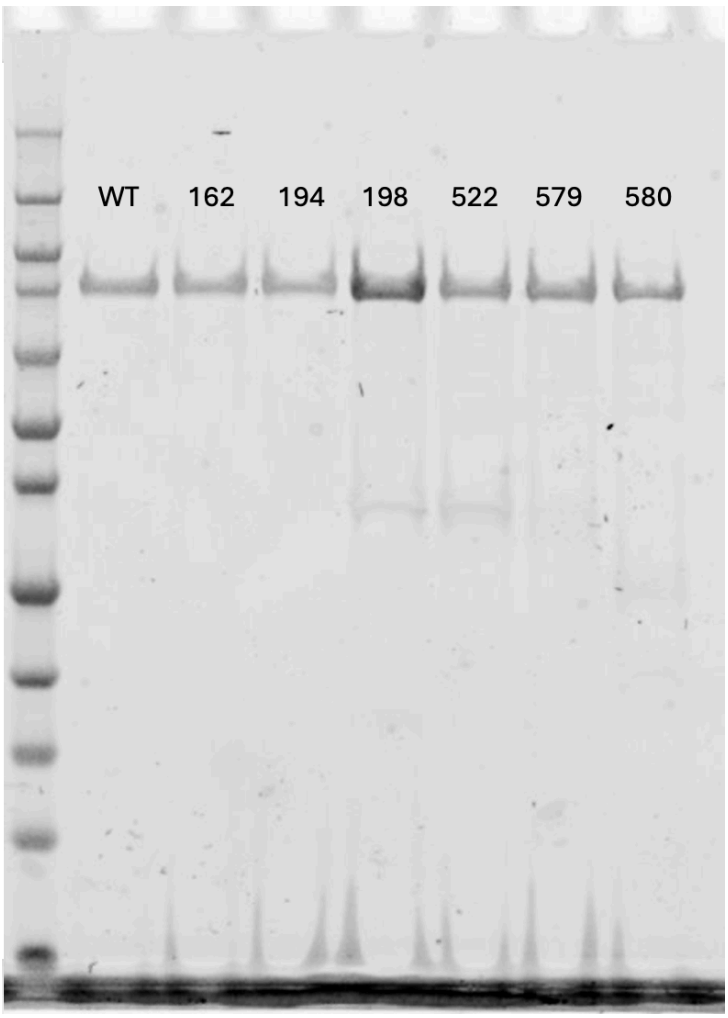

Figure A1: *SDS-Page gel of the LigD proteins (WT and six different point mutations) used in this project.*

Appendix B – EMSA gels

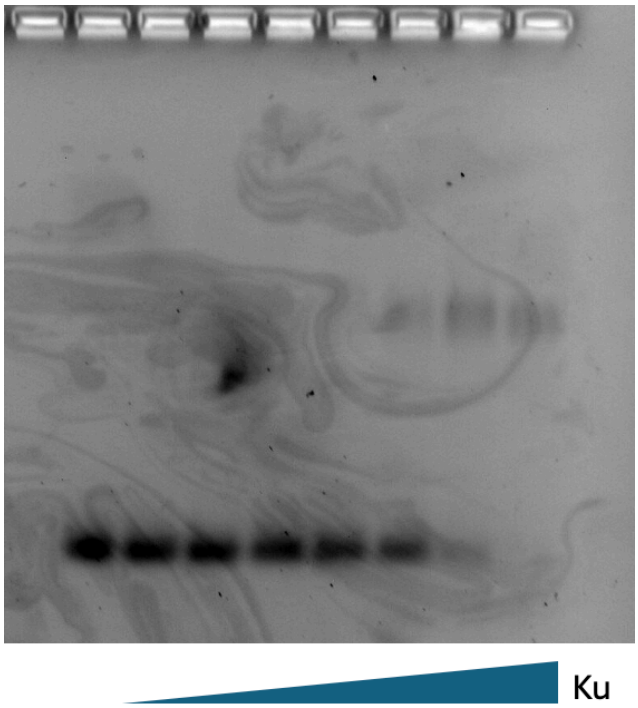

Figure B1: *Titration of Ku with a constant concentration of DNA. The concentrations of Ku are 0, 0.1, 0.2, 0.5, 1, 2, 5, 8 µM. The DNA concentration is 5 nM. Looking at the shift, 50% of the DNA should be bound to Ku at a concentration above 2 µM, but slightly less than 5 µM.*

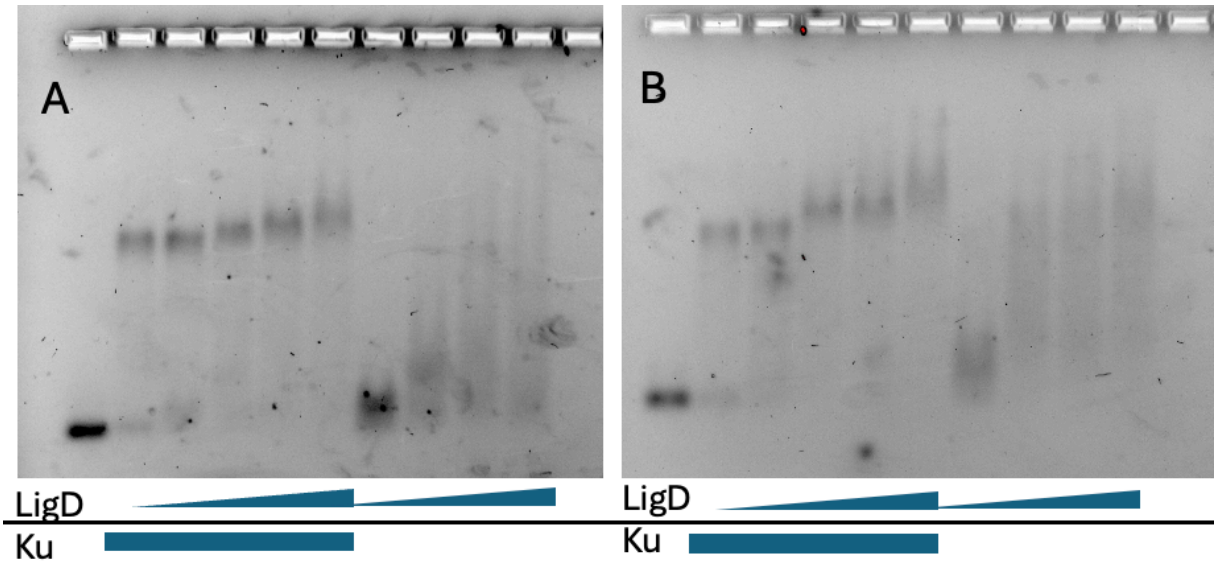

Figure B2: *EMSA, LigD titration with and without Ku, on DNA with sticky (A) and blunt ends (B). With Ku, there is no noticeable difference in LigD binding affinity to the two types of DNA. Without Ku, the bands are further smeared in both gels. The smeared bands for LigD on DNA with blunt ends without Ku have traveled less distance than the corresponding bands with overhang DNA. This indicates that in the absence of Ku WT LigD might bind to blunt DNA better than DNA with an overhang.*

Appendix C – Ku and WT titration for ligation assay

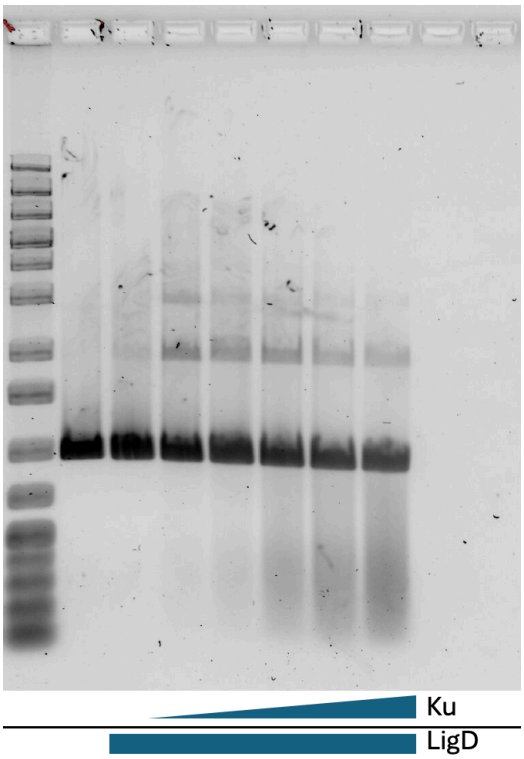

Figure C1: *Titration of Ku on DNA (300 µg). Maximum DNA binding is achieved at 2 µM of Ku.*

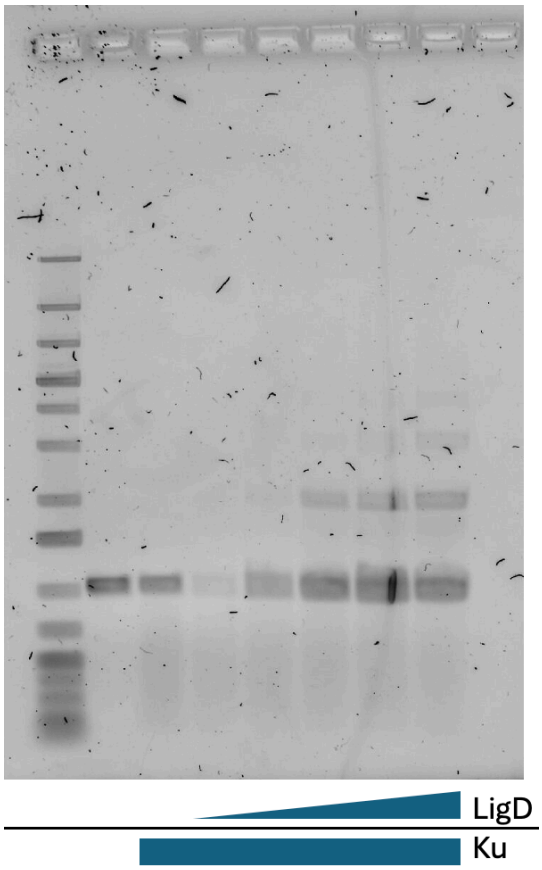

Figure C2: *Titration of LigD on Ku (0.1 µM) and DNA (300 µg). The LigD concentrations were 0.1, 0.2, 1, 2 µM.*

Appendix D – Imaging LigD 580 in microchannels

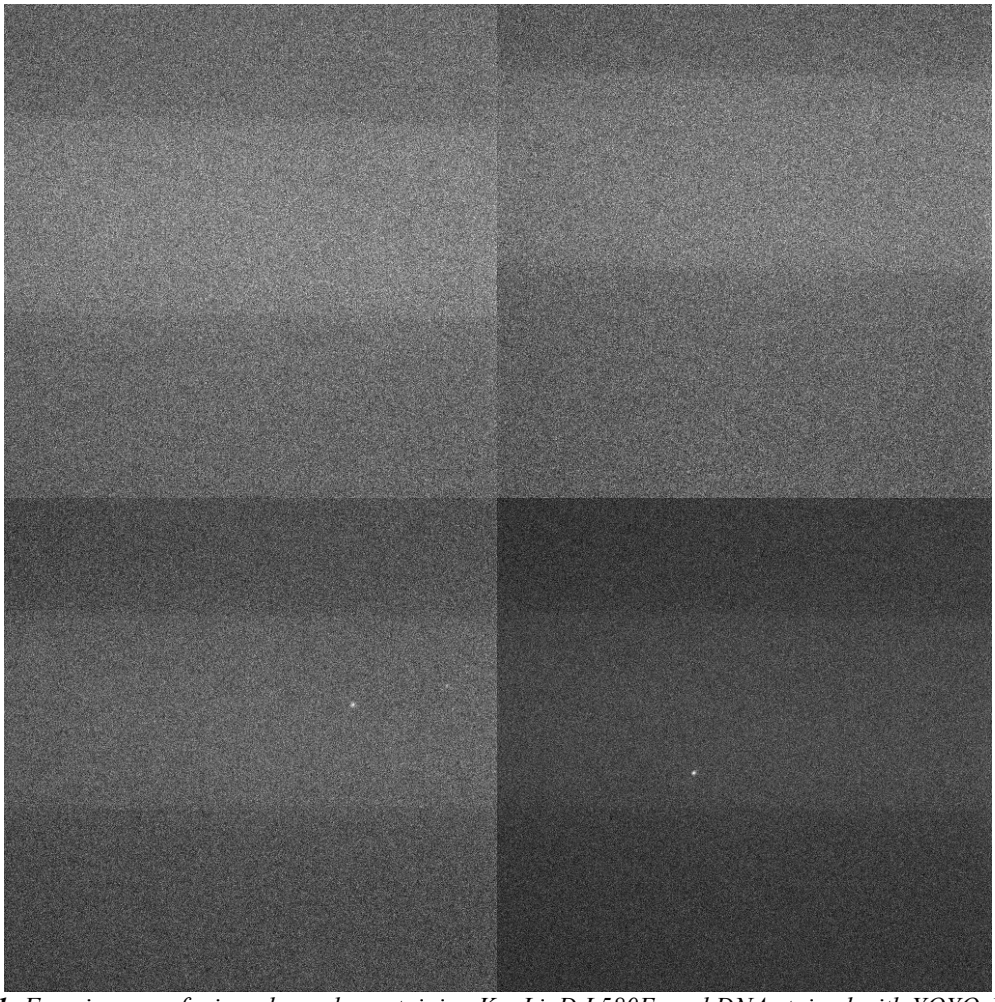

Figure D1: *Four images of microchannels containing Ku, LigD L580E, and DNA stained with YOYO-1. The bright spots in the lower two images are DNA molecules large enough to detect visually. The upper two images show no visible DNA molecules.*

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