Cloning of Thioredoxin Reductase-2 from *Drosophila Melanogaster for* Characterization of Catalytic Activity and Thermal Stability

Mustafa Dahbur, Adrian Ibarra, Emma Feeney, PhD
Biology Department
Loyola University of Chicago

Introduction

Thioredoxin (Trx) is a ubiquitous protein whose presence and function is highly conserved across species. Trx is a pleiotropic oxidoreductase (redox) enzyme that can combat oxidative stress in the cell by reducing disulfide bridges (R-S-S-R) to dithiols (R-SH-SH-R). It does so in an NADPH-dependent mechanism that transfers electrons to target proteins like an electron transport chain. Trx becomes inactive (oxidized) once it reduces other target proteins. To keep Trx in an active state (reduced) it must be regulated by another protein, Thioredoxin Reductase (TrxR) Thioredoxin Reductase 1 (TrxR-1) and 2 (TrxR-2) both function to reduce Thioredoxins disulfide active site. Thioredoxin Reductase 1 has been well characterized *in-vitro and in-vivo,* however Thioredoxin Reductase 2 has had little characterization both *in-vitro and in-vivo.*

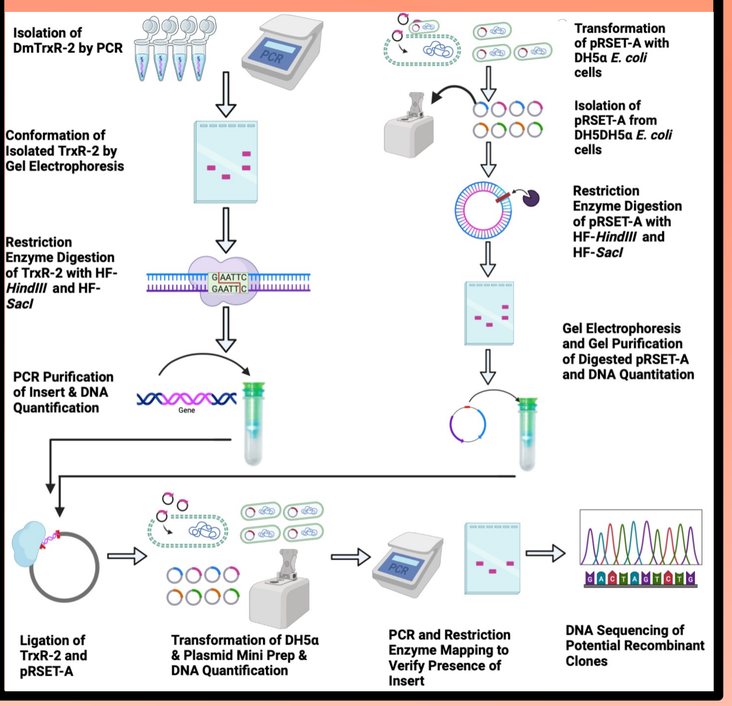
Hypothesis

- We hypothesize that TrxR-2 will biochemically function similarly toTrxR-1 based on the mechanism of catalysis the two proteins share.
- The goal of this project is to clone TrxR-2

Goals

- *To clone D. mel* gDNA into a pRSET-A vector using PCR- based cloning methods
- To establish a cloning protocol for future experiments and characterization of the protein
- Establish protein as a candidate for Databases like Design2Data (D2D). D2D aims to correct the predictive limitations, like accurately predicting structure and function given by protein modeling software by examining the functional parameters: catalytic efficiency and thermal stability of protein and protein variants.

Methods.



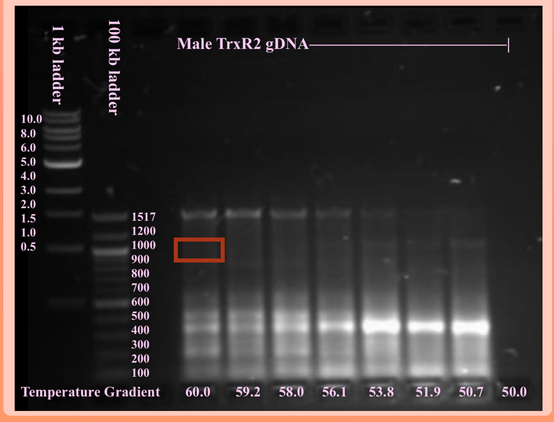


Figure 1 Temperature Gradient to Optimize Annealing Conditions for PCR. PCR product were run on a 1% Agarose TAE gel with 0.2 mg/mL of EtBr and observed under UV light. Lane 1 has NEB 1kb ladder and Lane 2 had NEB 100 bp ladder. Temperature gradient was set at the following temperatures: 60.0˚C, 59.2 ˚C, 58.0 ˚C, 56.1 ˚C, 53.8 ˚C, 51.9 ˚C, 50.7 ˚C, and 50.0 ˚C. Wells 4-10 have DmTrxR-2. The data shows that 60.0 ˚C (red box) is the most accurate annealing temperature.

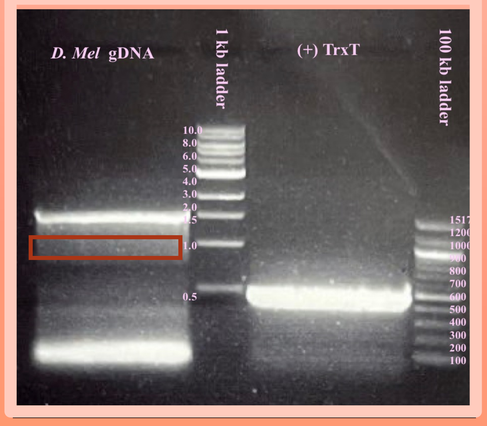


Figure 2 Successful Isolation of the DmTrxR-2 gene by PCR. PCR products were run on a 1% Agar TAE gel with 0.2 mg/mL of EtBr and observed under UV light. Lane 2 has NEB 1kb ladder and Lane 4 has NEB 100 bp ladder. Lane 1 shows our gene was isolated with an expected band size of ~1500 bp (red box) and Lane 3 shows the positive control, TrxT at ~500 bp.

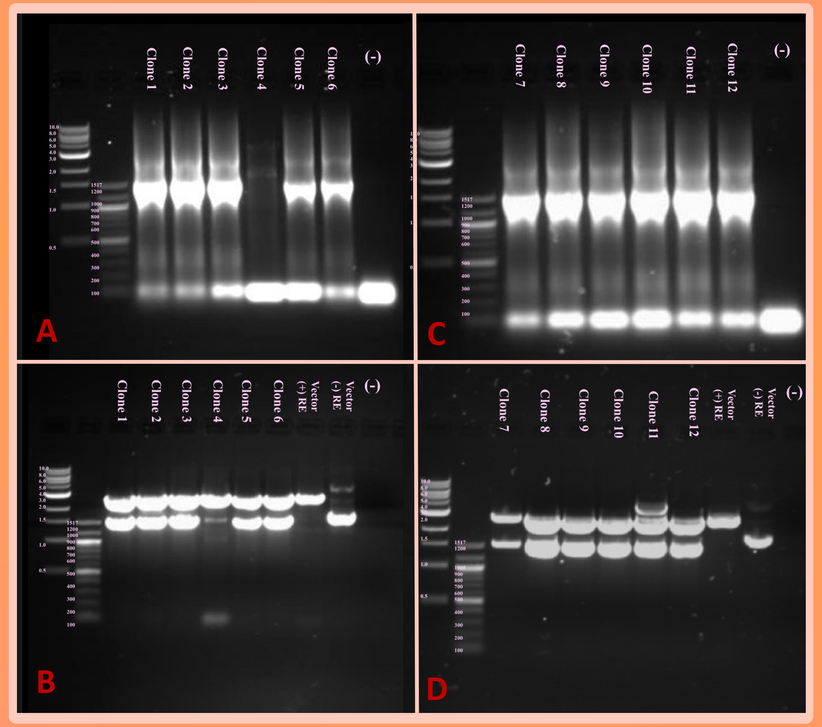


Figure 3 Verification of DmTrxR-2 within pRSET-A by PCR and Restriction Enzyme Mapping. All PCR products were run on a 1% Agar TAE gel with 0.2 mg/mL of EtBr and observed under UV light. All restriction enzyme digestions were done with HF-*SacI* and HF-*HindIII*. PCR was done using *D. mel* gDNA with upstream primer Sequence: 5'GAGCTCATGTCGACGATAAAGTTTCTGC-3' and the downstream primer sequence: 5'AAGCTTCTAACTGCAACAGGAGGCTG-3. All ladders used were NEB 1 kb ladder and NEB 100 bp ladder. **(A-C)** PCR of the expected recombinant clone was performed to verify the presence of the insert and that it was successfully ligated into the vector. Lanes 3-8 are potential clones. **(A)** Lanes 3, 4, 5, 7, and 8 contains potential clones with expected bands at ~1500 bp. Lane 6 does not show the expected band sizes. In **(C)** the insert was amplified in the vector in all lanes**. (B-D)** Restriction Enzyme double digest was done to confirm the identity of the recombinant plasmid. Lanes 3-8 had the digested clones. Lane 9 contained an empty vector with restriction enzymes and lane 10 contained empty vector with no restriction enzymes. Lane 11 was a negative control containing water. The insert is 1794 bp and the empty vector is 2.9 kb**. (B)** lanes 3, 4, 5, 7, and 8 all show the correct band sizes at ~1500 bp and ~3.0 bp. **(D)** The results for lanes 3, 4, 5, 6, and 8 show the expected band sizes at ~1500 bp and ~3.0 bp. Lane 7 has an extra indicating that it was cut twice.

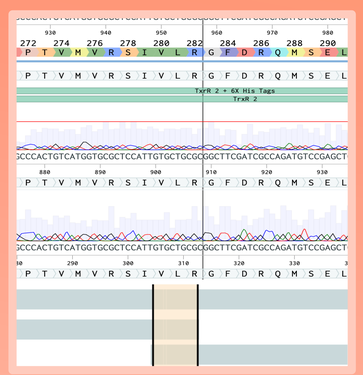


Figure 4 Sanger Sequencing of Clone 1 to Determine Identify of Clone
Clone 1 was selected for sequencing based on the Restriction Enzyme mapping and PCR data. Sequencing was done by GeneWiz and a BLAST was performed against FlyBase ID (FBgn0037170) on Benchling to confirm the identify of the insert and to identify any mutations induced by low-fidelity polymerase in PCR reaction. Highlighted box shows the forward and reverse sequence overlap indicating the entire gene was sequenced

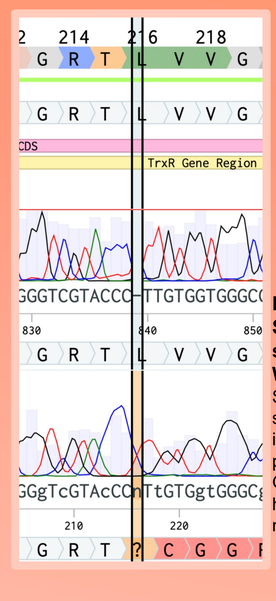


Figure 5 Sanger Sequencing Data showing Clone 1 may not be Wild
Sequencing data from clone 1 shows potential mutation in the insert. Black peaks are G, Red peaks are T, Blue peaks, and Green are A. The overlap in the highlighted box shows background noise in sequencing data.

Discussion

Summary of Results

- We successfully cloned DmTrxR-2 from *D. melanogaster* gDNA, however due to sequencing data background Nosie, true identify of insert can not be determined (Figure 5)
- Annealing temperature gradient showed the most optimal annealing temperature was 60.0˚C (Figure 1)
- Isolation of DmTrxR-2 by PCR with amplicon ~1500 bp, which strongly indicates we isolated the gene as the gene size is 1551 bp (Figure 2)
- Conformation that insert and vector were digested and subsequently ligated accurately as shown by Restriction Enzyme Mapping (Figure 3B-D) and PCR (Figure 3A-C). 10 out of 12 recombinant molecules were strongly shown to be potential clones. The insert size is 1551 bp and the vector size is 2897 bp. 10 out of the 12 clones showed the correct amplicons by PCR amplification at ~1500 bp.
- Sanger sequencing data showed that the entire gene was sequenced, and a BLAST was done to determine base pair identity and homology (Figure 4).

Discussion

- We were unable to characterize the protein due to protein induction troubleshooting and timing
- Thus, at this time, we can not contribute any biochemical data in respect to the enzyme.
- The SDS-PAGE gel (data not shown) showed that no fractions had proteins, indicating that we did not induce the enzyme
- We believe it to be either a low concentration of IPTG in the induction media or a shift in the reading frame due to addition of a base, resulting in a truncated protein (Figure 5)

Future Directions

- Future directions would be to establish protocols for efficient protein expression and purification
- Establish assays to characterize catalytic activity and thermal stability of protein
- Establish TrxR-2 enzyme as part of D2Ds protein engineering network.
- Use protein engineering to generate point mutation variants and collect functional biochemical data

References

Magnani F, Mattevi A. Structure and mechanisms of ROS generation by NADPH oxidases. Curr Opin Struct Biol. 2019 Dec;59:91-97. doi: 10.1016/j.sbi.2019.03.001. Epub 2019 Apr 30. PMID: 31051297.

Lu, J., & Holmgren, A. (2014). The thioredoxin antioxidant system. Free Radical Biology and Medicine, 66, 75–87. https://doi.org/10.1016/j.freeradbiomed.2013.07.036

Carlin DA, Hapig-Ward S, Chan BW, Damrau N, Riley M, Caster RW, et al. (2017) Thermal stability and kinetic constants for 129 variants of a family 1 glycoside hydrolase reveal that enzyme activity and stability can be separately designed. PLoS ONE 12(5): e0176255. https://doi.org/10.1371/journal.pone.0176255

Acknowledgements

We would like to thank our collaborators Dr Stefan Kanzok and his lab members. We would like to thank our P.I. Dr Emma Feeney for taking the time to guide us on this this project.