"New" UROP Proposal

Title of Proposal: Optimizing the Sequencing Process for Hepatitis Delta Virus (HDV)

STATE THE PROBLEM/TOPIC

Hepatitis Delta Virus (HDV) is primarily associated with co-infection with Hepatitis B Virus (HBV). An infection with both HDV and HBV is the most fatal form of viral hepatitis with a 20% fatality rate. An estimated 254 million people worldwide are infected with HBV and approximately 15-40% will develop chronic hepatitis leading to cirrhosis, liver failure, and hepatocellular carcinoma **(Tong et al. 2006)**. It is estimated that at least 48 million people globally are infected with HDV **(Jumabayeva et al. 2022).**

The genome of HDV is a satellite RNA and the smallest of any known human virus. HDV encodes just one protein, the HDV antigen (HDAg) **(Huang and Lo 2014)**. HDAg has two isoforms, but neither form is sufficient to form a complete viral particle. It requires the envelope of another virus for virion assembly and release. HDV's reliance on a more competent "helper virus" for replication has primarily been associated with the HBV. However, in vitro and in vivo experiments have demonstrated HDV can utilize other enveloped viruses unrelated to HBV for this purpose **(Crobu et al. 2024) (Perez-Vargas et al. 2019).** This finding suggests a broader mechanism of action and potential avenues for infection. Establishing an optimized sequencing process will help identify the presence of HDV in these potential alternative sites of infection and transmission.

RNA sequencing has become increasingly common with the growing fields of transcriptomics and metatranscriptomics. RNA sequencing requires more precision than DNA sequencing to maintain RNA's chemical stability. Each additional step increases the risk of human error or contamination but also offers opportunities for optimization based on research goals **(Conesa et al. 2016).** The unique properties of HDV add further complications to sequencing techniques. Despite these challenges, there are several points in the process that can be optimized to meet the specific aims of sequencing HDV.

RELEVANT BACKGROUND/LITERATURE REVIEW

The chemical differences between RNA and DNA are small but significantly impact the behavior of the molecules. The double-stranded helix of DNA is famously stable and, under optimal conditions, can hold massive amounts of information for thousands of years **(Dabney, Meyer, and Pääbo 2013)**. The structure of RNA prevents the formation of the same stable double helix structure. RNA tends to form more complex secondary structures through intramolecular base pairing. For example, in HDV there is a secondary structure known as a "nested double pseudoknot" where, within 90 nucleotides, there are five separate regions of self complementation **(Wadkins et al. 1999).**

RNA is the second most abundant biomolecule in a cell after protein, constituting 20% of the cell's dry weight, compared to 55% for protein and 3% for DNA **(Alberts 2015).** The noise-to-signal ratio with isolated RNA is much higher, making it more challenging to target specific RNA molecules. This requires greater precision and additional processing steps that introduce more opportunities for contamination and degradation.

Scientific investigations involving RNA requires precision to maintain the RNA's chemical stability and avoid unintended RNase contamination. RNA is an inherently unstable molecule. The degradation of RNA is encouraged by many ribonucleases (RNases), proteins specifically designed to break it down. Despite consisting of a large portion of the cell's biomass, RNA has a median half-life of about 2 minutes **(Baudrimont et al. 2017).** RNA molecules are plentiful, but short-lived. As a comparison, the time between protein synthesis and protein degradation by proteases ranges from 4 to 14 hours **(Chen, Smeekens, and Wu 2016).** Although the diverse functions of RNA are still being discovered, the primary role of the biomolecule is in the synthesis of proteins. They are exported from the nucleus and rapidly degraded by RNases shortly after performing their function.

The ubiquity of RNases and short half-life of cytoplasmic RNA is protective, especially against viral infection. Cellular RNA originates in the nucleus, whereas viral RNA originates extracellularly. Rapid degradation of RNA in the cytoplasm can prevent viruses from establishing a successful infection. This defensive maneuver, however, makes it difficult to study RNA **(Farrell 2023).**

In HDV literature, it is widely assumed that HDV transmission relies exclusively on parasitizing the HBV surface antigen (HBsAg) for packaging and release from the host cell. This would mean that the significant presence of

the HDV particle requires HBV as the helper virus. However, recent studies have suggested that this may not be the only method of transport the HDV particle can utilize.

HDV has also been detected in patients with Sjögren's disease, an autoimmune disorder of the salivary and lacrimal glands, without prior Hepatitis B infection **(Weller et al. 2016) (Hesterman 2023).** This suggests alternative HDV transmission methods without HBsAg. This possibility is further illustrated in experiments showing HDV using helper viruses unrelated to HBV, across several genera including vesiculovirus, flavivirus, and hepacivirus **(Perez-Vargas et al. 2019).** Creating a streamlined process that specifically targets HDV for sequencing will aid in further research into possible HDV infections with and without HBV co-infection.

Recent studies have discovered satellite RNA particles similar to HDV called HDV-like agents. These HDV-like RNA sequences have been found in meta-transcriptomic data from diverse hosts including birds, snakes, fish, amphibians, and invertebrates **(Chang et al. 2019).** Additionally, HDV-like sequences have been found in fungi and have been designated with the phylum Ambiviricota, characterized by circular RNA genomes "folding into a compact rod-like or branched secondary structure" with ribozymes similar to those found in HDV **(Kuhn et al. 2024).**

The discovery of diverse delta-like agents suggests this pathogen form is ubiquitous. However, sequencing HDV remains challenging, and delta-like agents face the same hurdles. An optimized protocol considering all unique challenges of HDV will be applicable to these delta-agents and essential in studying these new agents.

SPECIFIC ACTIVITIES AND TIMELINE

This project aims to refine the process in sequencing the HDV genome, which poses unique challenges due to its short length, high self-complementarity, and complex secondary structures. This work will aid in further research to understand HDV and related HDV-like viruses and their transmission mechanisms. The UROP funding will specifically help support the refinement of laboratory protocols for accurate and efficient HDV sequencing. We will research and conduct various methods of enzymatic ribosomal RNA depletion, RNA fragmentation, library preparation techniques for HDV sequencing. We will evaluate the outcomes of each step and determine the optimal process that is reproducible and accurate.

Research Activities

Literature Search: Conduct a comprehensive literature review of existing methods for rRNA depletion, RNA fragmentation, and library preparation. Identify and evaluate relevant studies and protocols. Hours: 10

Cell Culturing and Maintenance: Culture and maintain HEK293T cells under standard BSL-2 conditions, including media preparation, routine passaging every 3-4 days, monitoring cell conditions, and ensuring contamination-free conditions. Hours: 15

Cell Transfection: Transfect cultured cells with a plasmid using optimized transfection reagents and protocols. Monitor for successful transfection. Hours: 10

Method Selection and Reagent Procurement: Identify and select promising methods for rRNA depletion and RNA fragmentation. Procure necessary kits and reagents and determine campus labs capable of assisting with advanced steps. Hours: 10

rRNA Depletion Protocol Implementation: Implement and adjust selected rRNA depletion protocols to optimize efficiency. Test samples to assess RNA integrity. Hours: 20

RNA Fragmentation Protocol Testing and Optimization: Test and optimize RNA fragmentation protocols to achieve specific RNA fragment lengths suitable for sequencing. Hours: 20

Library Preparation Protocol Development: Develop RNA library preparation protocols, integrating optimized rRNA depletion and fragmentation methods. Hours: 20

Testing and Validation: Test and validate final products after sequencing. Adjust protocols to optimize reliability and reproducibility. Hours: 15

Total Hours: 120

Throughout the project Dr. XXX will provide direction on literature and methodology research and review proposed protocols. She will supervise the laboratory techniques and provide insight and feedback on

outcomes of both failed and successful experiments and ensure adherence to the timeline set in this proposal.

RELATIONSHIP OF WORK TO THE EXPERTISE OF THE MENTOR

Dr. XXX, my faculty mentor, has extensive research experience in virology, immunology, and molecular biology. Her work has focused on understanding viral mechanisms and host interactions from fascinating viruses like Marburgvirus and Ebola virus **(Kondratowicz et al. 2011)**, Cytomegalovirus **(Li et al. 2015)**, and Hepatitis Delta Virus **(Weller et al. 2016)** as well as looking at the link between viral infections and autoimmune diseases **(Gan et al. 2015)**. Her expertise in this field will be essential for addressing the unique challenges posed by HDV.

RELATIONSHIP OF THE WORK TO YOUR FUTURE GOALS

I have been assisting with research in Dr. XXX's lab for several months and her experience and mentorship have been invaluable. Her guidance has helped with past and current projects and shaped my future graduate school plans.

This project aligns with my educational and career goals in virology and molecular biology. I am applying to PhD programs this fall, and this research will prepare me for my time in graduate school. The time I've spent in Dr. XXX's lab has been incredibly valuable, and this project will further advance my experience in the field and prepare me for future research in the molecular biology of viruses.

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