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MODELING A PARKINSON'S DISEASE LIKE STATE IN ZEBRAFISH LARVAE UTILIZING 6-HYDROXYDOPAMINE

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Abstract

Modeling A Parkinson's Disease-Like State In Zebrafish Larvae Utilizing 6-Hydroxydopamine

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Parkinsons' disease (PD) is a neurodegenerative disorder characterized by the loss of midbrain dopaminergic (mDA) neurons that currently has no cure. It is primarily characterized in patients by motor symptoms that include but are not limited to: tremor at rest, rigidity, akinesia, and postural instability affecting the quality of life for over 8.5 million people worldwide. The neurotoxin 6-hydroxydopamine (6-OHDA) has been used to induce a Parkinson's disease-like state in several animal models; however, assessment of 6-OHDA use in zebrafish demonstrated a lack of consensus on method and utility. We determined an optimized protocol utilizing 6-OHDA to produce a PD-like state as defined by a significant reduction of *tyrosine hydroxylase* (*th)*. Additionally, we found the mechanism of action for 6-OHDA toxicity to be conserved as defined by the upregulation

of super oxide dismutase *sod1* and *sod2*, and down regulation of mitochondrial dysfunction indicator *pink1*.

Chapter One

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that affects approximately 8.5 million people worldwide [1]. It is characterized by the loss of A-9 midbrain dopaminergic neurons (mDA) in the substantia nigra pars compacta. These neurons normally release Dopamine (DA), which modulates the sensitivity of movements by providing an implicit motivation for the motor action [2]; tuning activation and allowing for greater motor control of both gross and fine motor movements. The disorder is multifaceted including classic motor impairment and other symptoms like sleep abnormalities, depression and dysautonomia [3]. Additionally, PD has multiple hypothesizes and realized etiologies that make the development of treatment options difficult [4,5]. People who suffer from PD are diagnosed based on characteristic motor impairment symptoms that include resting tremors, rigidity, akinesia, and postural instability [1]. Diagnosis using motor impairment is problematic for clinicians, as it usually indicates advanced progression often diagnosis can be a decade after the initial symptoms obscuring the initial cause[6]. Following observation of the clinical phenotypic indicators, the PD diagnosis is confirmed with endpoint molecular markers including the aforementioned mDA neurodegeneration, which at this stage has passed a critical threshold of greater than 50 percent neuron loss [7].

Animals models have had to play a major role in Parkinson's research given these clinical challenges. The primary molecular marker assessed in research models is Tyrosine Hydroxylase (TH) [8], the rate limiting enzyme in the synthesis of dopamine [9] that converts Tyrosine to Levodopa (L-dopa). Other molecular markers for dopamine neurons, and by extension PD include Dopamine Transporter (DAT), responsible for the reuptake of extracellular dopamine into dopaminergic cells, and Vesicular Monoamine Transporter 2 (VMAT-2) involved in import of dopamine in synaptic vesicle axon terminal [10,11]. While these markers allow researchers to track mDA reduction, they do not account for the pathogenesis and progression of the disorder. I propose that this has hampered efforts to treat or cure the disorder, as evidenced by the lack of treatment options that stop the progression or reverse a Parkinsonian state and a plethora of options that mitigate symptoms for a limited amount of time [11]. Serious investment into research models that incorporate either well characterized or potential pathogenesis and progression markers into their observed outputs is required for the search of a long-term treatment option for PD. The reduction in the bioavailability of dopamine for signal transduction modulation is the key indicator and contributor for the phenotype of PD, but without models that actively incorporate pathogenesis and progression mechanisms of PD a suitable cure that can stop the mechanisms is very unlikely to be developed.

Current treatment options

Pharmacological dopamine-based avenues are the main focus in PD treatments for the alleviation and control of motor symptoms. L-dopa is a precursor for dopamine that can cross the blood brain barrier [12]. It is converted to dopamine in dopaminergic and noradrenergic neurons through decarboxylation [8]. Treatment of PD with L-dopa relies on

increasing the bioavailability of this critical DA precursor. Administration of L-dopa necessitates supplementary pharmaceuticals to enhance its efficacy and limit negative side effects, for example Carbidopa decreases the metabolism of L-dopa in the periphery of the body and allows for greater amounts of the compound to reach the mDa [1,12,13]. L-dopa has limited efficacy as the cellular processing of L-dopa to dopamine occurs in the cells that are dying as the disorder progresses [12,14]. L-dopa temporarily relieves symptoms masking the continued progression of the disorder.

Monoamine oxidase B (MAO-B) inhibitors are also utilized to treat PD [15]. MAO-B is a mitochondrial enzyme involved in the metabolism of dopamine and subsequent metabolites; therefore, inhibiting MAO-B increases bioavailability of dopamine [15]. Selegiline and Rasagiline are currently available FDA approved MAO-B inhibitors [13]. Like L-Dopa, the grim reality of PD is that those who are afflicted with the disorder have a small window of time after diagnosis where their symptoms are suppressed until the available treatments are rendered ineffective. A need for a different approach is required that emphasizes the analysis of PD etiology and explores non-dopaminergic pathways that are associated with PD.

Dopaminergic neurons

Dopaminergic neurons are located primarily in three major nuclei including the substantia nigra (A-9 group), ventral tegmental area (A10) and the retrobulbar field [16]. They are characterized by the production and release of dopamine for neuromodulation of pathways involved in cognitive function including motivation, reward, associations, and habit learning. PD is primarily a result of the loss of A9 neurons rather than the loss of any other mDA neuron type [17]. The presentation of PD brings into question as to why the

specific subclass of mDA neurons are being affected rather than all subclasses as they share significant morphology and functionality. Several ideas and hypothesis exist, but no single reason has been identified as to why. Identification of distinctions between mDA neurons and other neurons as well as single cell analysis of A-9 mDA neurons from one another is critical for understanding pathogenesis.

The identification and differentiation of mDA neurons from other cells begins early in development. The process of dopaminergic neuron formation can be broken down into four stages [18]. The first stage is marked by progenitors at the ventral ventricular mesencephalic surface giving rise to multiple neuronal cell types followed by the specification of the neuronal cell into dopaminergic neuronal precursors. The dopaminergic neuronal precursors then stop proliferation and begin displaying structural and early dopaminergic neuronal markers. The fourth and final stage is the maturation of the neurons and establishing connectivity. The last two stages are of significance due to the pathways involvement in the diagnosis of PD and the specification of dopaminergic neurons as medial or lateral [1,11].

The combined expression of *LMX1a* and *LMX1b*, LIM homeodomain transcription factors, is sufficient to specify mDA neurons in any part of the midbrain region following the establishment of the appropriate signaling network and transcriptional cascade [19,20,21]. The interactions between LMX1a and LMX1b are tangential to one another regulating mDA neuron proliferation and differentiation having overlapping and cross-regulatory functions [22]. LMX1a is key in the specification of the medial domain mDA, whereas LMX1b is essential for lateral mDA [21]. In addition, LMX1b signaling is required for isthmic development, which is key for long term maintenance of the mDA. Functionally, these

transcription factors have been shown to have a high binding affinity to transcription factor binding sites, and roles in repressing other transcription factors [22]. LMX1a and LMX1b are also important during the differentiation and formation of immature mDA neurons as they regulate the expression of *NEUROG2* and *MSX1* by upregulating them and repressing the expression of their negative regulators [20]. LMX1a and LMX1b repress *NEUROG2* which functions as a negative regulator *HES1*, and promotes MSX1 which inhibits lateral midbrain gene *NKX6-1*. MSX1 and *NEUROG2* are considered good indicators of immature dopaminergic neurons because in their absence neurons resemble hindbrain fates [23].

The maturation process of dopaminergic neurons is regulated by an auto regulatory loop of LMX1B and WNT1 signaling that regulates three key factors involved in mature mDA neuron formation in *NR4A2*, *EN1*, and *PITX3* [24]. Wingless and Int-1 (WNTs) are a secreted glycoprotein expressed in the midbrain, and signaling through its receptors is involved in the entire development of the midbrain and hindbrain region [25]. *NR4A2* is a transcription factor categorized in the nuclear receptor super family expressed in mDA neurons in the substantia nigra pars compacta and ventral tegmental regions of the brain in both developmental and adult stages [26]. The presence of *NR4A2* stipulates the expression of *TH*, DAT, and VMAT-2 [9,27]. NR4A2 facilitates transition from immature to mature mDA neruons, as such NR4A2 null organisms are completely devoid of TH [27]. *NR4A2* binds the response of element “NBRE” in the 5’ untranslated promoter regions of TH and DAT highlighting its direct role in their transcription [28]. PITX3 or paired-like homeobox protein is an additional indicator of mature differentiated mDA neurons [29]. PIX3 expression is only found in mature differentiated mDA neurons, and is similar to NR4A2

in that it is required for the preservation of a dopaminergic phenotype [30]. PITX3 presence is highly restricted and constitutive in the substantia nigra pars compacta and ventral tegmental mDA regions. The timing of expression is critical for mature cell differentiation between substantia nigra mDA neurons and ventral tegmental mDA neuons; where if PITX3 expression precedes TH expression that specifies mDA neurons that will occupy the substantia nigra and if TH expression precedes PITX3 expression the resulting mDA neuron is ventral tegmental [29]. The resulting temporal differences in expression at the end of maturation step results in the cell being involved in two of the three major subgroups of midbrain dopaminergic neurons having virtually identical cell fate maps until this point. Additionally, in order to better understand the dynamics of dopaminergic cells the incorporation of vesicle release protein (VMAT-2, *slc18a2*) and dopamine transporter (slc6a3, *slc6a3*) can be inferred [31,32].

Etiology

The causes of Parkinson's disease are categorized into two types: Genetic (30% of patients) or Idiopathic (70% of patients) [4,5]. Genome-wide association studies have identified loci highly associated with the disorder and specific genetic parameters for the disorder [33]. Interestingly, the genes/ single nucleotide polymorphisms found to be highly correlative with PD were associated with pathogenesis pathways thought to contribute to idiopathic forms of the disorder suggesting homology in the underlying mechanisms that contribute to a PD state [30,34].

α-synuclein misfolding and aggregation

The histopathological hallmark of PD is abnormal cytoplasmic deposits inside neuronal cell bodies of α-synuclein [35]. The abnormal α-synuclein aggregates form greater

order complexes called Lewy bodies [35]. The wild type or normal state of α-synuclein is as a mostly unfolded protein without tertiary structure, but it can exist as a stable tetramer that resist aggregation in aqueous solutions [36]. However, in a diseased state the α-synuclein confirmation becomes a β-sheet-rich amyloid-like structure that is prone to aggregation [37]. Current Hypotheses proposed for the conformational changes that lead to abnormal α-synuclein aggregation include phosphorylation of serine 129, ubiquitination, and truncation of the C-terminal [38]; however, the exact mechanisms that translate environmental stressors or events into the modifications of α-synuclein that result in an altered structure are not understood [39]. One hypothesis suggests the modification(s) alter the structure of α-synuclein at a chaperone protein binding site [40], resulting in the inability for the chaperone protein to properly fold α-synuclein potentially enabling the transition from a wild type 'healthy' state to a diseased one [40]. Genetic models are available that display the α-synuclein aggregation, but the models focus on the death of the mDA neurons and the clearing of the α-synuclein aggregates rather than the mechanistic action of the α-synuclein aggregation and investigation of the pathogenesis [41–43] Current α-synuclein models fail to account for the identified mechanisms of its aggregation and do not provide the ability to test pharmacological compounds that have the potential to prevent the phenotype from ever forming.

Mitochondrial Dysfunction

Neurons, including the mDA neurons, involved with PD have high energy requirements making their health and efficacy closely tied with the status of their mitochondria [44]. The mDA neurons have mechanisms to maintain healthy stocks of mitochondria that are disrupted for individuals who have PD [45]. Deregulation of normal

mitochondrial function or mitochondrial disfunction is heavily linked, and thought to contribute, to several neurodegenerative disorders' pathogenesis and progression, including PD [46]. In normal conditions, mitochondria are understood to be the bulk contributors of energy to the cell, they accomplish this by being the centers for oxidative phosphorylation subjecting them to high amounts of oxygen. That oxygen and subsequent possible oxidative free radicals drastically shift homeostatic equilibrium when the mDA neuron is being heavily utilized [46]. Additional sources of oxidative stress come from dopamine itself as the intermediates that exist in the catabolism of dopamine are reactive oxygen species (ROS) which are capable of producing free radicals. [14]. Additionally, the region the mDA neurons inhabit contains high amounts of neuromelanin which stabilize ROS when formed [47]. However, the presence of ROS is not the issue as the cell utilizes them as signaling molecules for post-transcriptional modification. The structure and efficiency of mitochondria are also regulated by ROS presence in a feedback loop [48].

Mitochondria exist in either a fused or divided state, the status of mitochondria dictating their efficacy and ability to interact with the rest of the cell [49]. Mitochondria that have undergone fusion, where two or more mitochondria are together as one, are more energetically efficient and are less prone to leak inner mitochondrial material or be targeted for destruction through mitophagy than those found divided having undergone fission [44]. The fusion of mitochondria is regulated by Optric atrophy 1 (OPA1) and mitofusins 1 and 2 (Mfn1 & Mfn2) [50]. Fission of mitochondria is regulated by activity in dynamin-related protein (Drp1). Targeted destruction of mitochondria or mitophagy is a very tightly regulated process initiated by the stabilization of mitochondrial surface protein

PTEN-induced putative kinase 1 (PINK1) when the surface membrane potential of the mitochondria is lost [51]. The stabilization of PINK1 results in a phosphorylation cascade that includes ubiquitin and Parkin [52]. The phosphorylated protein complex serves as a tag for the cell to destroy the mitochondria by the assembly of an autophagosome through ubiquitin signaling resulting in mitophagy. In a Parkinsonian state, mitochondrial dysfunction sets in when excess signaling from ROS shifts the population of mitochondria in a cell to undergo fission and divide making the mitochondria more prone to be cleared out by the cell [46]. Prolonged shifts can result in mDA neurons that are deprived of energy and signal for apoptotic death if enough mitochondria are indicated as damaged or if acute damage to mitochondria occurs and mitochondrial DNA or cytochrome C leaks out of the mitochondria [53]. Despite the significance and prevalence of mitochondrial dysfunction in neurodegenerative disorders like PD , it is treated more as an add on rather than an investigatory avenue and a potential pharmacological target [54].

The neurotoxin six-hydroxydopamine (6-OHDA) is a compound that is chemically similar to dopamine and enters dopaminergic cells through the same transporter as dopamine (DAT). The mechanism of 6-OHDA action is the formation of ROS producing catabolic intermediates that are unable to be cleared out by the cell [55]. 6-OHDA has been characterized to alter mitochondrial function and change the expression of genes related to mitochondrial dysfunction (Kulich M. et al., 2007). The 6-OHDA compound has been used since 1968 to model a PD-like state, but the animal and cell models that utilize the compound lack the integration of mitochondrial dysfunction as a contributor to the pathogenesis and progression of produced PD-like state especially in zebrafish [57]. The established models instead treat the intermediate genes and proteins as endpoint targets

rather than assess them in dose and exposure time dependent response curves. The lack of investigation in this area limits pharmacological targets as the use of neurotoxins like 6-OHDA in a single acute insult is a poor substitute for the years it takes for PD to set in [4,7]. Models need to be adapted or replaced in order to incorporate additional markers at more time points. Dosage and exposure time curves are performed in toxicological studies and provide the ability to observe the effects of compounds like 6-OHDA have on well characterized and understood pathways that are believed to contribute to pathogenesis. Investigating the effects of the compounds over time and across dosages that do not produce a molecular or behavioral phenotype of PD provide insights into the state of mDA neurons and the system before the PD-like state sets in. The study of protein-protein interactions and transcriptional modifications has revealed *NFE2L2* as a possible drug target for the recovery of Parkinsonian dysfunctional mitochondria [50,58]. The identified interactions of *NFE2L2* suggest a reversal of the molecular phenotype when the target is activated.

When pathogenic mechanisms are the forefront of a model interesting and otherwise unnoticed targets arise such as, TigarB inactivation [59]. The inactivation of TigarB leads to improved outcomes for PINK1$^{-/-}$ (knockout) *Danio rerio*. Studies that take into account the mechanisms when studying a Parkinsonian state provide a more complete molecular readout than those that don't and assist in the translation of information to new models or clinicians.

Mitochondrial dysfunction is understood to be an etiological contribution for disorders and conditions presenting varying symptoms [60]. This makes identifying a specific cause of Parkinson's disease difficult given the typical circumstances of symptom

presentation late in the progression of the disorder [7]. This fact brings up the question as to why mDA neurons in the substantia nigra and not other neurons or even other dopaminergic neurons in close proximity are not similarly affected in the onset of a Parkinsonian state [17]. As mentioned before the physical location of mDA neurons makes them more susceptible to mitochondrial dysfunction as they are in a neuromelanin rich area of the brain [16,47,61]. The neuromelanin rich region provides a microenvironment where reactive oxygen species can stabilize externally and cause damage [47]. Catecholaminergic neurons like mDA neurons are characterized by an age-related accumulation of neuromelanin giving the region a dark pigmentation [62]. The synthesis mechanism of melanin including neuromelanin is the oxidative polymerization of L-tyrosine [63]. Melanin synthesis is reliant on tyrosinase, an enzyme that catalyzes the oxidation of L-tyrosine. L-tyrosine can then undergo cyclization into 5-6-dihydroxyindole (DHI) and its carboxylated derivative 5,6-dihydroxyindole-2-carboxylic acid (DHICA) to become the main components of melanin [64]. The DHICA subunits are what give melanin the capacity to chelate metal ions and give them highly antioxidant properties [64]. Thus the presence of neuromelanin can be protective, but the complex formed in its accumulation transforms the microchemical environment shifting the ability for ROS to damage cells and tissues with age related degeneration of protein maintenance, stress and accumulation of neuromelanin [4,33,41].

An additional source of ROS comes from the use and recycling of dopamine in dopaminergic cells [14]. Intra-neuronal metabolism of dopamine produces toxic 3,4-dihydoxyphenylacetaldehyde (DOPAL) as the first byproduct DOPAL is hypothesized to destroy nigrostriatal dopamine terminals and contribute to dopamine deficiency that

characterizes PD [65]. There is a constant presence of dopamine in dopaminergic neurons due to the constant biosynthesis, vesicular leakage of dopamine in vesicles, and the reuptake of dopamine making the production and presence of DOPAL a constant in dopaminergic neurons. DOPAL toxicity has four hypothesized mechanisms including; protein cross-linking, oxidation to quinones, production of hydroxyl radicals, and the amplification of toxicity of other sources in the neuron [66–69]. Dopaminergic neurons operate in hostile environments that require highly structured and regulated processes for healthy operation; deterioration of the systems in place that help maintain operating conditions result in a gradual reduction in efficiency in the neurons and their eventual death.

Neuroinflammation and combined pathogenesis mechanisms

The role of neuroinflammation in PD stems from postmortem brain studies identifying the inflammation of T-lymphocytes, increased levels of pro-inflammatory cytokines in the substantia nigra pars compacta [70]. Neuroinflammation was originally thought to be a secondary associative occurrence in PD, but evidence suggest inflammatory responses contribute to the disorder's pathogenesis. Work in rodent models that utilize 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTPT) neurotoxins showed an inhibition of microglial activation with minocycline (tetracycline antibiotic) resulting in diminished lethality in mDA neurons in the substantia nigra suggesting that microglia-induced inflammation may contribute to the degeneration of these cells [71,72].

The pathogenesis of neurodegenerative disorders like PD is likely due to a combination of factors contributing to an eventual phenotype. The aggregation of α-

synuclein and mitochondrial dysfunction are thought to contribute to one another [73]. Where α-synuclein aggregates can interfere with mitochondrial function by disrupting the mitochondrial membrane causing mechanical tears. The production of ROS from dysfunctional mitochondria can change the free energy landscape of α-synuclein altering protein folding shifting towards forms that favor aggregation [74].

6-OHDA as a tool for Parkinson's disease modeling

The neurotoxin 6-OHDA is used to induce an idiopathic Parkinson-like state. It is chemically similar to Dopamine and utilizes the dopamine transporter (*slc6a3*) to enter dopaminergic neurons. Once inside the dopaminergic neuron 6-OHDA is partially metabolized and the reactants are redox cyclic oxidizers capable of producing ROS [75]. The neurotoxin 6-OHDA is autoxidizing in aqueous solutions and is capable of producing hydrogen peroxide, superoxide radical, and hydroxyl radicals. Mechanisms area available that provide protection against the ROS in the form of catalases and superoxide dismutase (SOD) [76]. Superoxide dismutase (SOD) is of particular interest as its role to turn radicals into much more manageable hydrogen peroxide makes it a possible target for pharmacological therapeutics [77].

Zebrafish

Danio rerio (Zebrafish) have been used as a model system for a parkinsonian state[59,78]. Zebrafish are teleost, the largest group of vertebrates, allowing for their use as tools to model human disease and disorders [79]. Zebrafish also have high structural and physiological conservation of key structures of the brain when compared to humans [80].

Zebrafish are native to the eastern portion of the Ganges river and typically grow to a length of 2.5-4 cm in length [81]. Their major introduction as a genetics model was

brought about by George Streisinger due to their availability, high offspring volume, quick development, genetic malleability, and translucent embryo. [81]. For our purposes, zebrafish are ideal due to extensive work in the development of neuronal structure and physiology[82]. Zebrafish larvae in particular are of significant interest as their early development has been thoroughly investigated and characterized [83]. Zebrafish larvae are especially advantageous as the identification of neurotoxin based toxicological effects on single cells and whole organism is possible [84]. Their high permeability allows diffusion based delivery by soaking them in neurotoxin containing solution [80,85], and a lack of a blood brain barrier (BBB)/Blood – Cerebral Spinal Fluid Barrier until 7 days post fertilization (dpf) allows central nervous system access for otherwise impermeable compounds [86]. Lastly, zebrafish larvae have homologous mDA regions for Parkinson's disease modeling as early as 2 dpf [83,87]. This allows for a theoretical five-day window for the introduction of compounds that cannot enter the brain typically through soaking of the Zebrafish larvae in solution.

Expansion, development, and maturation of 6-OHDA use in Zebrafish larvae

In this study, we propose a repeatable identification of a PD-like state in zebrafish larvae when exposed to 6-OHDA through soaking. Additionally, we want to elucidate mechanisms of etiology identified in PD that are expected to occur within our model as described in other uses of 6-OHDA [56]. While the use of 6-OHDA in zebrafish larvae with the goal of producing a Parkinson's disease like state has been studied before [42,54,88], the results of these studies are varied and produce more questions than answers about the efficacy of 6-OHDA exposed zebrafish larvae to produce a Parkinson's disease-like state (See Table 1). Previously, no available protocol agreed on concentration of 6-OHDA,

supplemental reagents for stability, and timeline of exposure. We propose to generate a protocol that produces a clear PD-like state through qRT-PCR and behavior-based identification of a PD-like state. The identification of a PD-like state comes through the investigation of the presence of key reagents like L-ascorbic acid, methylene blue and HEPES buffer at differing concentrations (0-250 μM) as well as the timing and duration of 6-OHDA exposure. The primary factor for the identification of a PD-like state in zebrafish larvae is the reduction of the presence of tyrosine hydroxylase (*th*). We elected to utilize a qualitative approach through qRT-PCR utilizing *hprt1* as the endogenous control to quantify reductions of *th* as well as differential expression of other PD-related genes. Additionally, we wanted to incorporate markers that allow for greater understanding behind the etiology of PD by measurable outputs of *sod2* and *pink1* into the protocol. These markers will be used to identify pharmacological compounds that are able to modulate the neurotoxic effects of 6-OHDA affecting the ability of 6-OHDA to produce a PD-like state through ROS production derived mitochondrial dysfunction. We expected to produce a PD-like state and that was molecularly observed through 4 biological groups of AB strain zebrafish and parkinsonian behavior through 3 biological replicates.

Table 1

Compound/ protocol	Feng et al. 2014	Kalyn et al. 2019	Vaz et al. 2020
6-OHDA	10 µM – 250 µM	1 µM -100 µM	750 µM
L-Ascorbic acid	none	0.1 % - 0.01 %	0.2 %
Buffer(s) used	none	none	HEPES 10 mM
Abnormality	None described	Cardiac at 1 µM	None described
Treatment timeline	2-5 dpf	3 dpf -7 dpf	4 dpf - 5 dpf
Recording	5-7 dpf	7 dpf	6 dpf
Measured	Position in cuvette	Response to touch stimuli	DanioVision^{tm} movement

Table 1: Comparison of available 6-OHDA Zebrafish larvae protocols.
shows the differing currently available protocols and the variability they have in reagent selection, concentration, and timeline of exposure.

Chapter Two

Methods and Materials

Zebrafish husbandry care and selection

Zebrafish larvae were raised in-house or purchased from approved vendors. The wild type stains used were Segrest [Segrest farms, Florida], AB [ZIRC] and WIK [ZIRC]. In-house fish were maintained in a light (14-hour light / 10 hour dark schedule) and temperature-controlled room [water temperature of 82 °F (28 °C)]. Zebrafish adults were fed live brine shrimp twice daily and were kept in constant circulation systems. Adult health is assessed daily while feeding by looking at coloration and body morphology for deviations from a normal phenotype [89]. All Methodologies have been approved by the UT Tyler local *IACUC – protocol 705*.

Larval zebrafish embryos were collected from bulk mating in commercial mating tanks and placed into 100 x 15 mm petri dishes (maximum 80 staged embryos per plate) in embryo water solution (appendix). Plates were kept at 28 C in a glass door incubator. Larvae were dechorionated at 2 days post fertilization (dpf) and transferred to experimental solutions a 3 dpf. The embryo water solution was changed daily.

Exposing Zebrafish larvae to 6-Hydroxydopamine

The neurotoxin 6-hydroxydomaine hydrobromide [6-OHDA · HBr (Tocris) (6-OHDA)] used for experiments is highly unstable, and its use must be planned to the minute when prepared for administration. The instability of 6-OHDA necessitates L-ascorbic acid to increase stabilization. We performed dosage curves, different durations, and varied the time of application and duration for: 6-OHDA, L-ascorbic acid, Methylene

blue, and HEPES Buffer. Methylene blue is utilized almost ubiquitously in zebrafish husbandry as it acts like an antifungal compound and is useful to minimize incidental death associated with fungal spread in embryos. HEPES is used to stabilize pH in solution and is necessitated when high concentrations of L-ascorbic acid are utilized. This resulted in several workflows being attempted. Initial exposure used the maximum experimentally determined concentration of L-ascorbic acid (0.0013%) in embryo water [60 µg/mL Instant Ocean and 1 µL/ 100 mL of Methylene blue in RO water]. This solution was used to solvate the 6-OHDA and then expose the zebrafish to varying concentrations of 6-OHDA (0 µM- 250 µM). This protocol, which will be referred to as 0.0013% L-ascorbic acid.

A second methodology was developed utilizing quantitative Real-time reverse transcriptase polymerase chain reaction (qRT-PCR). The specifics of the qRT-PCR will be discussed later. The resulting workflow is referred to as 0.2% L-ascorbic acid, as we were able to increase the concentration of L-ascorbic acid using a HEPES pH buffered solution. The 0.2 % concentration of L-ascorbic acid is recommended for 6-OHDA solvation in an aqueous environment [90]. The high concertation of L-ascorbic acid requires the use of HEPES to reduce the effect pH effects of the L-ascorbic acid. The differences in reagent composition have been resulted in variability in in outcomes and had to be treated separately when behavior more molecular materials were harvested.

0.001% L-Ascorbic acid vehicle

The ascorbic acid solution was made by dissolving 13.3 mg of L-ascorbic acid into 100 µL of Embryo water to make 13.3% L-ascorbic acid solution. The 13.3% L-ascorbic acid solution was separated in 20 µL aliquots and frozen at -20 C° if not used that day. A 10 µL volume of the solution was added to 99.99 mL of embryo water to make a 0.001% solution. A mass of 2.345 mg of 6-OHDA was weighed into a 15 mL conical tube. The 15 mL conical tube was filled with 12.5 mL of the 0.001% L-ascorbic acid solution. The tube was mixed well resulting in a 750 µM 6-OHDA solution in 0.0013% L-ascorbic acid. The 750 µM solution was partitioned out and diluted with 0.001% L-ascorbic acid to make varying concentrations of 6-OHDA at final volumes of 12 mL. The dilutions were made in 15 mL conical tubes and the experimental concentrations of 6-OHDA were added to a 60 X 15 mm petri plate containing ~ 30 Zebrafish larvae. The larvae were moved to 0.001% L-ascorbic acid solution at 2 dpf and then either fresh 0.001% L-ascorbic acid solution or 0.001% L-ascorbic acid solution containing 6-OHDA. The solutions were replaced daily for a total of three days of exposure (ending on 5 dpf). Animals were assessed for behavior, euthanized, and molecular materials including either RNA or proteins were harvested.

0.2% L-Ascorbic acid vehicle

Zebrafish larvae were soaked in 6-OHDA at 3 dpf and moved into vehicle solution (0.2 % L-ascorbic acid in Embryo water buffered to pH 7.4 (actual pH 7.0) utilizing 40 mM HEPES) at 4 dpf. The fish were harvested at 6 dpf for molecular end points. The 6-OHDA solution was made from a 100 µM stock. The stock was prepared by weighing out 2.5 mg of 6-OHDA into a 15 mL conical tube. The tube was filled with

10 mL of the vehicle solution capped, mixed gently but thoroughly through inversion, and delicate shook. The solution was poured into a 250 mL screw top bottle. The process of adding 10 mL of solution mixing and pouring into the 250 mL screw top bottle was repeated three more time for a total of 40 mL of solution being added to the 250 mL screw top bottle. The volume was brought up to 100 mL for a final concentration of 100 μM of 6-OHDA.

The 100 μM concentration of 6-OHDA was used to make experimental solution of 6-OHDA. It was partitioned it out and diluted with more vehicle solution (ex. 30 μM 6-OHDA was made using 24 mL of the 100 μM 6-OHDA stock and 56 mL of the vehicle solution for a total of 80 mL of 30 μM 6-OHDA). The solution was added to 6 well plates with mesh inserts 12 mL per well with 12 larvae being in each well. Fish were kept in normal embryo water until 3dpf, when they were transferred into the 6-OHDA containing experimental solution by moving the filter cup into a new plate containing the 6-OHDA solution. The time from initially solvating the 6-OHDA to first contact with 6-OHDA was kept to a minimum. The solid 6-OHDA was kept at -20 C until absolutely needed. Everything was prepared and ready for 6-OHDA handling prior to removing the solid from the freezer. The larvae were left in the 6-OHDA solution for 24 hours, and then transferred into fresh vehicle solution daily until harvest at 6 dpf. We altered duration of exposure, concentration, and timing of exposure. Multi-day exposures were achieved in a similar manner, but the fish are plated into fresh 6-OHDA solution on additional days rather than being placed into vehicle solution (*figure 1*).

Figure 1

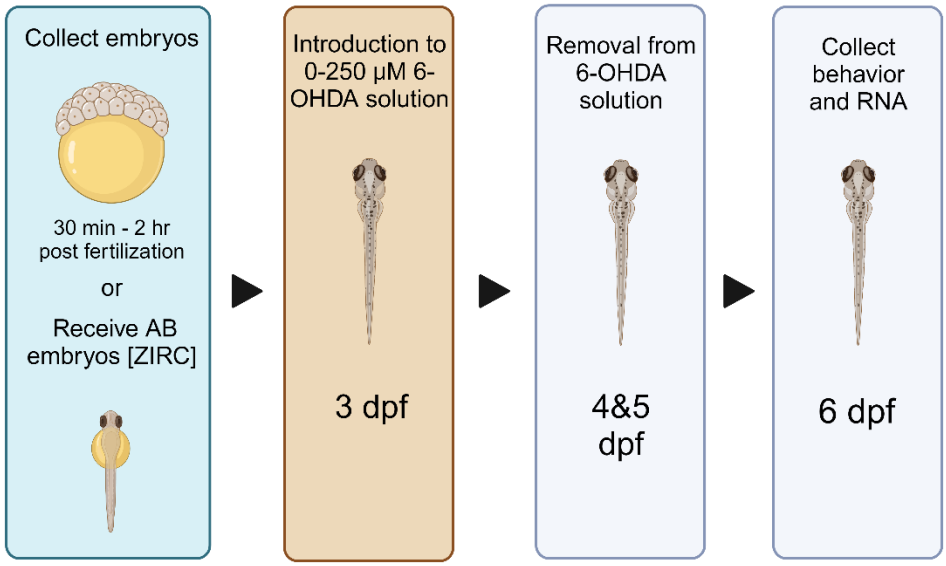

Figure 1: 6-OHDA exposure workflow. Final timeline for 6-OHDA exposure (Brown). Following removal from We kept the delivery vehicle (0.2 % L-ascorbic acid 40 mM HEPES pH 7.4 buffered Embryo water 24 ppm Methylene blue [Gray]) and collection date at 6 dpf constant but varied the concentration, duration and timing of 6-OHDA exposure Created with BioRender.com

Behavior

Zebrafish larvae behavior was measured through locomotion in set time intervals and conditions. Zebrafish behavior was gathered by placing the larvae inside a 96 well plate, one fish per well, that has the same solution the larvae were in at the last day of the experiment. Zebrafish larvae were recorded using a phantom video camera. The plate was placed onto a LED light plate that is off. The fish were acclimated for 30 minutes in the dark. The camera was set to record at 100 frames per second with 300 exposure and 10 seconds following that the LED light plate is turned on. The camera is allowed to record for 60 seconds in the light capturing burst activity. The light bar was left on, and the fish were allowed to acclimate an additional thirty minutes. After the light

acclimation period the camera was set to record for 10 minutes at 30 fps at 100 (light units).

The burst and activity video files were analyzed using the Daniovision settings for Ethovision v14. The fish were separated into arenas and analyzed as separate individuals. The data collected was average velocity, total distance moved, time spent moving, time spent inactive, and distance to first movement.

Manual behavior assessment was also done by scoring fish that moved in the 10- minute period and those who did not. Movement was categorized as having turned 180 and/or made is across the well in the 10-minute time periods. Manual assessment of behavior was done in a double-blind manner where the individual who assessed the fish generated the data and presented it to someone who was also blind to the experimental set up and only after generating trends and grouping the data was the second researcher unblinded. The first researcher that did the assessment remains blinded to the assessment.

RNA Extraction

RNA was extracted from at least 10 healthy fish in a single group (same 6-OHDA exposure). The ten fish threshold was experimentally determined based on RNA concentration and purity. Larvae were euthanized, placed into an acid-guanidinium-phenol based reagent (ambion TRIzol), and homogenized utilizing a single use polymer pestle. After thorough homogenization, ensuring that no tissue larger than a pen tip remained, the column purification was performed (Zymo research RNA extraction kit per manufacturer recommended protocol with the use of DNAase I).

Primer design and verification

Specific and efficient primers were required for the genes of interest and endogenous controls. The parameters for the genes were a 55 °C annealing temperature and a size range between 150 base pairs (bp) – 300 bp in length. Primers were designed utilizing Primer BLAST (National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were assessed with RNA from adult brains and 6 dpf larvae. First Strand synthesis of cDNA was performed utilizing the Sensifast cDNA synthesis kit (Meridian per manufacture's protocol). 400 ng of RNA was utilized in each 20 µL reaction; however, the cDNA was diluted 50 % to increase use per ng of RNA used. PCR was performed with the MyTaq Red PCR kit (Merdian per manufacturer's recommendations including thermal profile).

A 5 uL volume of the PCR product was loaded onto a 2.5% agarose gel for size verification and banding pattern analysis. Primers that produced a single band within 10 % error of the theoretical size were retained for further analyses. The remainder of the PCR reaction was cleaned up using the Monarch DNA Clean Up kit (New England Biolabs) or GeneJet PCR clean up kit (Fisher Scientific). Samples with sufficient concentrations were sent for Sanger sequencing (Eurofins SimplSeq kit).

Sequences were assessed using A Plasmid Editor (APE) [91] by peak quality and were BLASTed using blastn and megablast against the *Danio rerio* (taxid:7955) genome (GRCz11 release 106). Hits with the highest E- value and greater than 98.5% identity were required for a positive gene identification.

Primer concentrations were determined using a serial dilution of cDNA for the qRT-PCR reaction (Sensifast First Strand Synthesis, PowerTrack SYBR green w/ROX

reagent – Fisher Scientific). For all qRT-PCR experiments, we used half reactions compared to the manufacturer's protocol. We started with a 50 % dilution and performed a five-fold dilution series (50%, 25%, 12.5%, 6.25% 3.125%) No less than four concentrations were required per reaction.

The primer sets for the genes of interest and applicable endogenous control(s) were ran with five dilutions with each in triplicate. A non-template control for each primer set was ran on the same plate. The resulting Cq values were plotted against the natural log of the dilution factor ie. log base dilution factor (dilution) where in the 50% serial dilution a dilution of 25% would be -2. The slope of the resulting plot was transformed and inputted into: $2^{(-1/\text{slope})}$. The efficiency was determined by subtracting one from the result of the previous step. Efficiencies between 90 % and 110% were accepted in accordance to MIQE guidelines. An agarose gel was performed following the qRT-PCR to verify quality. The primer efficiency, amplification curves, and melt curves were analyzed in combination to determine the quality of run. We required single bands of the right size for verification. We accepted some primer dimers when amplification did not start until a high cycle number (35-40). Amplification curves were run in technical triplicate and all three curves were assessed for the same shape, size, and position. Outliers were eliminated from calculation, if all three amplifications were different the plots were compared to reactions of the same primer of a different sample(s) and the one that most closely resembled the size and shape of the rest was selected if sufficiency similar. Primer pairs were only considered if the non-template control had no curve present, or a very right shifted curve in the case for primer dimers. Primer dimers occasionally appeared on non-templates controls, but if the amplification Cq was less

than 30 on a non-template control the product was considered non-significant especially if what was an amplicon determined to have a different melting temperature and/or size based on melt curve and gel analysis. The melt curves had to be verified by a minimum of a single gel band for bp size determination.

qRT-PCR analysis

The qRT-PCRs were analysed with the ΔΔ CT method. This method requires an endogenous genetic control and a comparative sample control. The endogenous genetic control selected was *hprt1* and the comparative sample control was the 0 μM 6-OHDA group.

Protein harvesting

Protein was obtained from at least 50 zebrafish larvae from a single group. Larvae were euthanized and placed into either RIPA protein extraction buffer or Vesicle-Dell buffer (VDB) buffer.

RIPA

RIPA contains high amounts of detergent making it ideal for a quick quantitative analysis, but the resulting product cannot be analyzed for electrochemistry or binding/ affinity. Homogenization was performed with a single use pestle attached to a handheld homogenizer in a 1.5 mL Eppendorf tube.

VDB

The low detergent content of VDB buffer makes it ideal for quantification and electrophysiology of membrane/ vesicle bound proteins.

After euthanization, larvae were placed in a 1.5 mL centrifuge tube containing 750 µL of VDB buffer and homogenized with a polymer single use pestle attached to a tissue handheld homogenizer. After breaking down the larger chunks the sample was passed through a 1 mL syringe with a 21-gauge needle. The sample was placed into a 12 mL glass potter. The 1.5 mL centrifuge tube was filled with 750 µL VDB with protease inhibitor, mixed, and the solution was passed through the same needle before injecting into the 12 mL glass potter. The process of flushing the tube and syringe with fresh VDB was repeated two more times to minimize protein loss. The 12 mL glass potter was filled up to 12 mL with VDB containing protease inhibitor and homogenized thoroughly by performing at least ten strokes or until there are no visible tissue debris left in solution. The glass potter was emptied into a 50 mL conical tube. The glass potter was washed three times with12 mL of VDB buffer and emptied into the 50 mL conical tube to minimize protein loss. The 50 mL conical tube was spun at 1000 X g for 10 minutes at 4C producing a pellet (P1) and supernatant (S1). The supernatant is decanted into an appropriately sized conical tube and spun at 20,000 X g for 60 minutes at 4 C resulting in a second supernatant (S2) and pellet (P2). P2 contains the membrane extract and desired proteins. The supernatant removed; and the pellet was resuspended in VDB solution in the same tube 500 µL per 50 larval zebrafish.

Chapter Three

Results

Fish morphology, development, and mortality

The existing variability of 6-OHDA use in Zebrafish larvae necessitated a stepwise investigatory analysis of reagents, timing and duration of exposure to be done. Initially we focused on 6-OHDA concentration utilizing 0.001% L-ascorbic acid methodology; however, a transition occurred to utilizing 0.2 % L-ascorbic when the 0.001% L-ascorbic acid methodology proved to be inconsistent in the morphological and behavioral based results produced and could not sustain a significant reduction in behavioral locomotion.

0.001% L-Ascorbic acid vehicle

The 0.001% concentration was experimentally determined as the highest concentration of L-ascorbic acid that could be used with embryo water without buffering (*figure 2*). This concentration of L-ascorbic acid is on the lower end in terms of use in the realm of 6-OHDA experimentation.

Figure 2

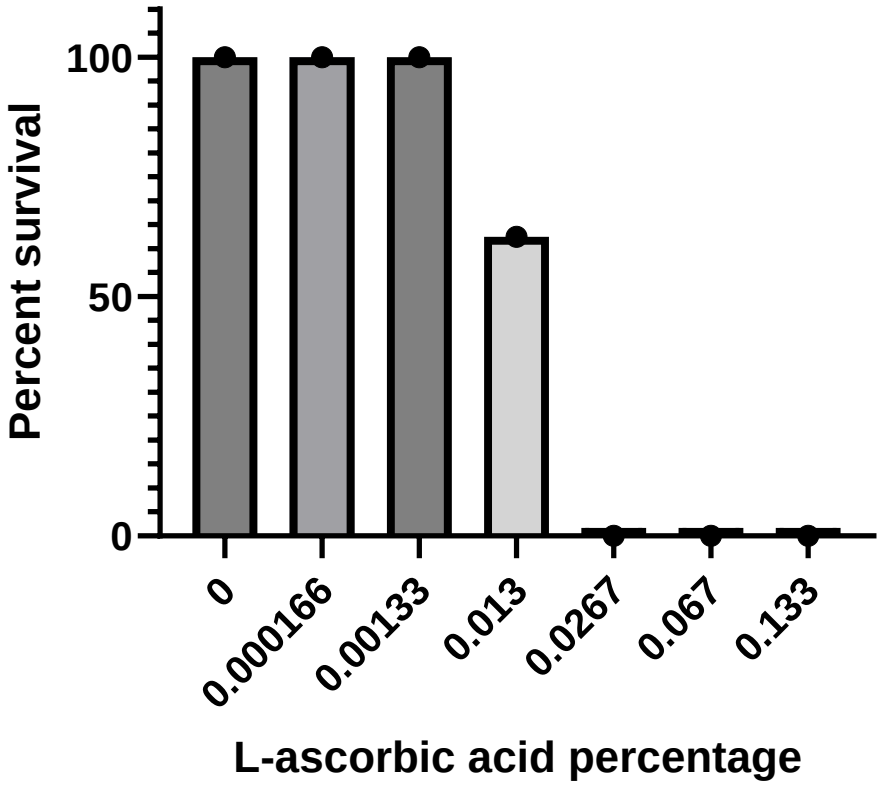

Figure 2: L-ascorbic acid mortality in embryo water no ph buffering: 0.001% l_asocrbic acid identified as highest concentration of L-ascorbic acid that can be utilized that has no noticeable effects of Zebrafish morphology and development. An n of 12 was utilized per group utilizing segrest fish.

Higher concentrations of L-ascorbic acid resulted in mortality, corpses of the fish were assessed, and the morphology of the dead fish indicated the cause to be due to the low pH from the L-ascorbic acid. The measured pH of 0.001% L ascorbic acid is 4.95.

Fish exposed to 6-OHDA utilizing 0.001% L-ascorbic acid embryo water have

variable mortality, developmental staging and mortality based on concentration and within cohorts (figure 3 and 4). The method produced variable outcomes outside of health and developmental trends regardless of fish strain used. Visual inspection of the larvae identified variability in body shape and shape along with differences in developmental staging across the three days of exposure.

Figure 3

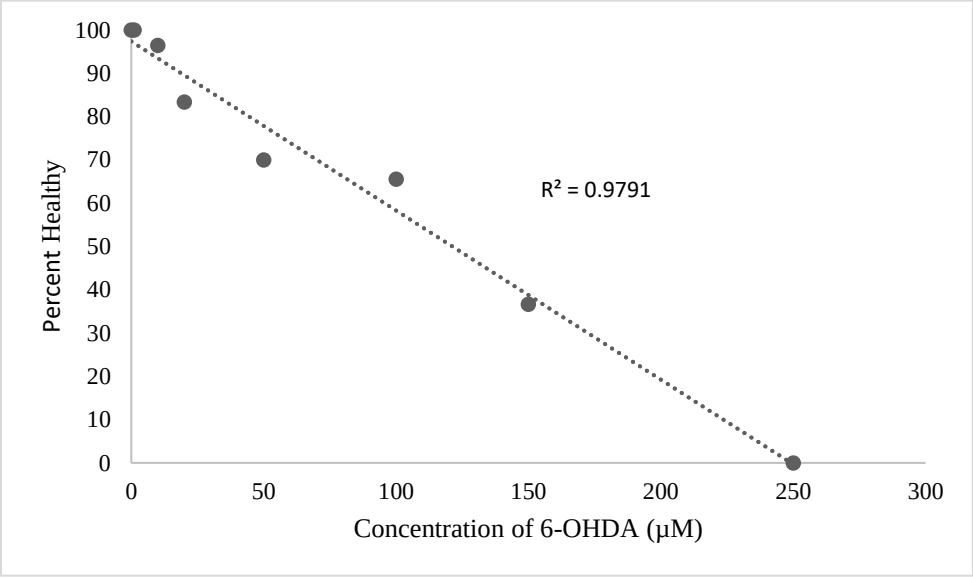

Figure 3: 6-OHDA effects on zebrafish larvae exposed for 3 days (2-5 dpf) utilizing 0.001%. Fish are Segrest strain n of 12 per concentration. Soaking began at 2 dpf and continued until 5 dpf where they were harvested, and behavior data collected.

Additionally, mortality utilizing unbuffered 0.001% L-ascorbic acid to deliver the 6-OHDA varied in within and in between biological replicate groups. Lethality when utilizing 6-OHDA in the aforementioned 0.001% manner was observed at 150 µM, and the fish are observed to have developmental delay relating to the timing of air bladder inflation

being shifted in chronological development by a day or more at concentrations above 70 µM (*figure 4*),

Figure 4

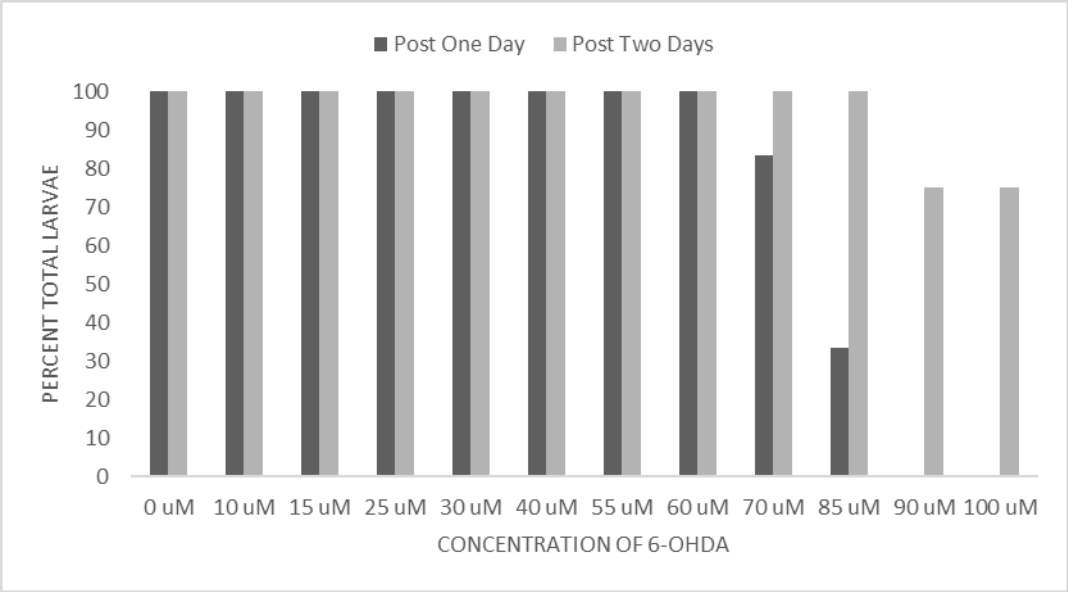

Figure 4: focused 6-OHDA effects over time on zebrafish larvae exposed for 3 days (2-5 dpf) utilizing 0.001% protocol utilized 12 fish per concentration and displays total number of healthy fish based on zebrafish morphological assessment.

Morphological observations when utilizing 6-OHDA in 0.001% L-ascorbic acid are deformations in the yolk sac, tail curvature, cardiac edema, and eye shape deformations. The morphological discrepancies appeared to have no patterns when comparing biological replicates, but within replicates patterns in specific phenotypes appeared but none of note when comparing the entire population of larvae exposed in this manner.

0.2% L-ascorbic Acid vehicle solution

When utilizing 0.2% L-ascorbic acid lethality increases with lower concentrations

of 6-OHDA (*figure 5*); however, no morphological or developmental differences were observed in the surviving fish (*figure 6*). we exclusively used the isogenic AB strain (ZIRC) for the 0.2% L-ascorbic acid solutions, as we were trying to reduce variability as much as possible. Initial probing of 6-OHDA concentrations utilizing viability then qRT-PCR assessment (see below) identified 30 μM as the optimal concentration capable of producing a PD-like state. Replicates of the 30 μM concentration were made; a total of 6 biological groups from 4 different cohorts were utilized in this manner (*figure 7*). Further exploration utilizing the 0.2% L-ascorbic acid methodology was done to identify timing and duration of 6-OHDA exposure (*figure 8*).

Figure 5

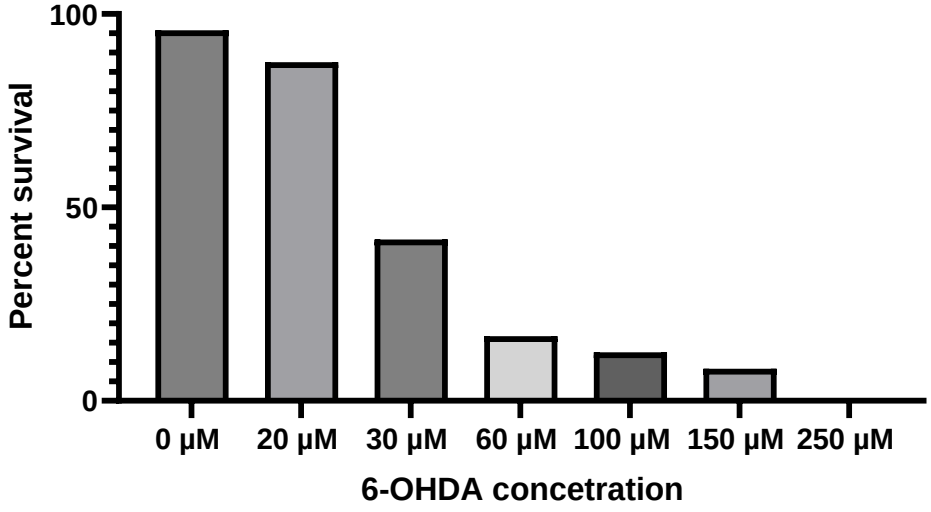

Figure 5: initial use of 6-OHDA in 0.2 % L-ascorbic acid shows survivorship in initial 6-OHDA use with 0.2 % L-ascorbic acid solution. Each concentration began with 24 zebrafish larvae of AB strain

The fish were all harvested at 6 dpf where total survivorship was assessed. Death in the fish primarily occurred during exposure (3-4 dpf). No morphological or developmental discrepancies were observed in the surviving fish when comparing to fish in the control (0 μM 6-OHDA) group (*figure 6*).

Figure 6

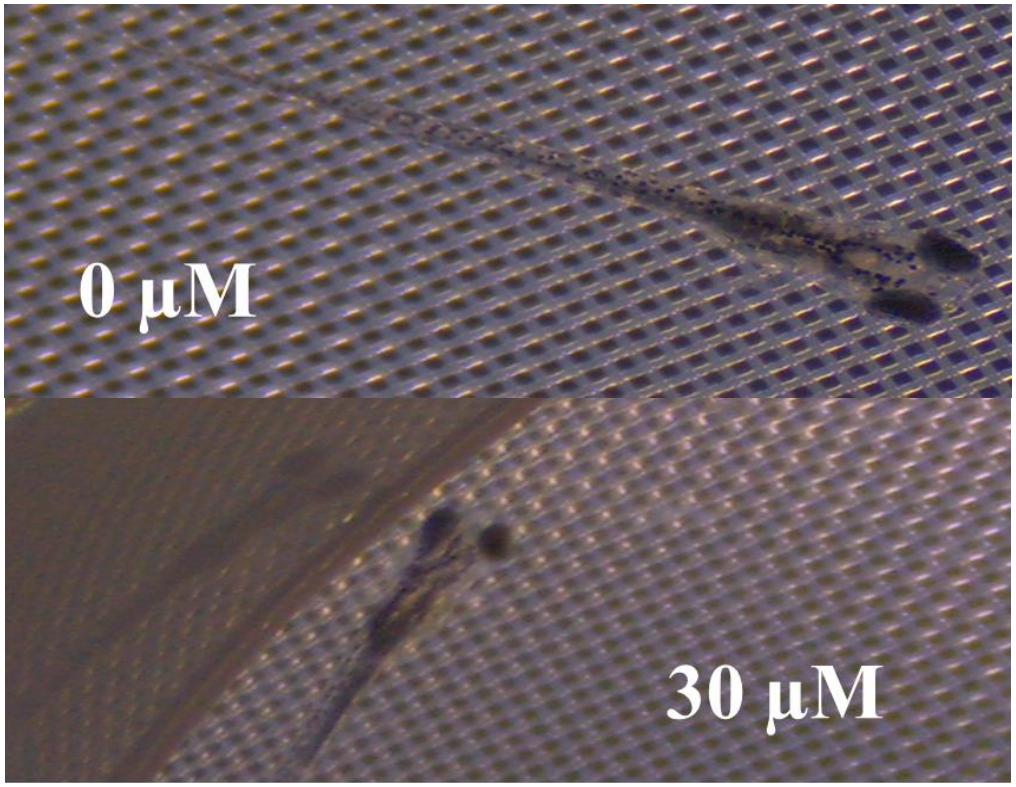

Figure 6: No morphological differences are seen in Zebrafish larvae at 4 dpf. Zebrafish larvae picture are 4 dpf after spending 24 hours in 0 μM (top) and 30 μM (bottom) 6-OHDA solution. No discernible morphological or developmental difference can be observed.

Figure 7

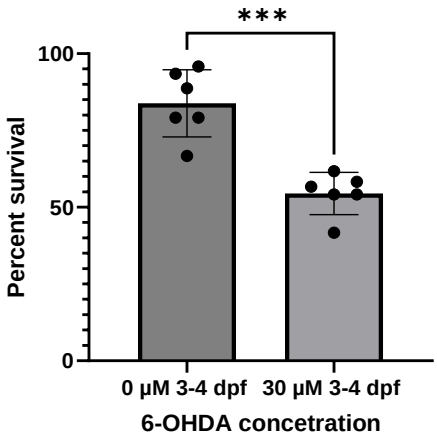

Figure 7: Biological Replicates of 6-OHDA exposure. A T-test was performed on the number of surviving fish between 30 µM 6-OHDA and the control 0 µM 6-OHDA. *** indicates a $p<0.001$ ($p=0.0002$) Test ran is a two tailed $t(10) = 5.5569$. The points represent different groups of fish n≥24 collected and assessed at 6 dpf

The six biological groups came from 4 different fish cohorts. The four fish cohorts were completely independent from each other. Their exposure dates ranged from Dec 2022 to August 2023 utilizing different stock bottles of 6-OHDA manufactured at different dates.

The lethality of 6-OHDA varies with its concentration, timing and duration of exposure (*figure 8*). Larvae exposed earlier (2 dpf) have morphological and developmental phenotypes similar to what was observed when utilizing 0.001% L-ascorbic acid. No morphological or developmental discrepancies were observed when exposure began on or after 3 dpf in surviving fish. Groups of fish exposed to lower concentrations of 6-OHDA for a longer period of time (20 µM 3-5 dpf) have higher survival than those exposed to higher concentrations of 6-OHDA for a single day (30 µM 3-4 dpf);

however, it did not change the molecular profile to indicate a PD-like state (see below).

Figure 8

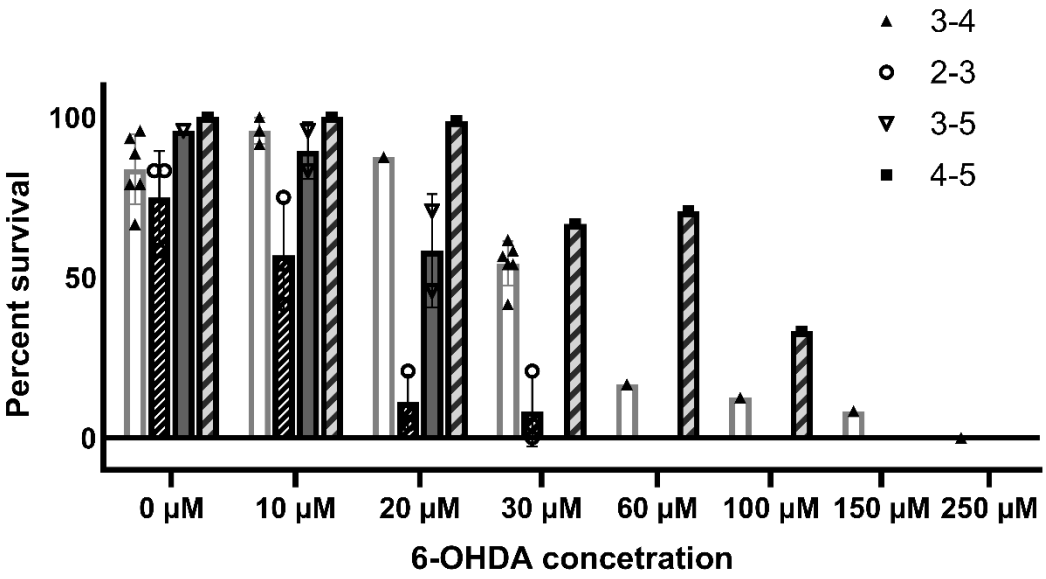

Figure 8: 6-OHDA based lethality is time and concentration dependent: shows the different mortality associated with 6-OHDA based on exposure concentration duration and timing of exposure. Each point began with 24 AB fish and surviving fish were indexed at 6

The higher lethality observed when utilizing higher percentages of L-ascorbic acid is particularly surprising as L-ascorbic acid is a known antioxidant and is expected to serve as a neuroprotective agent [92]. This indicates that the interactions of the compound kinetically favor the stabilization of 6-OHDA over neuroprotection based on observed lethality.

Methylene blue

Due to the understood role of methylene blue (MB) as a neuroprotective agent and its common inclusion in embryo mediums as an antifungal compound, its effect on 6-OHDA phenotypes was observed for a single cohort [89,93]. The presence of MB increased survivorship in fish exposed to 6-OHDA (*figure 9*). Given the impact, inclusion of methylene blue became standard for all further treatments.

Figure 9

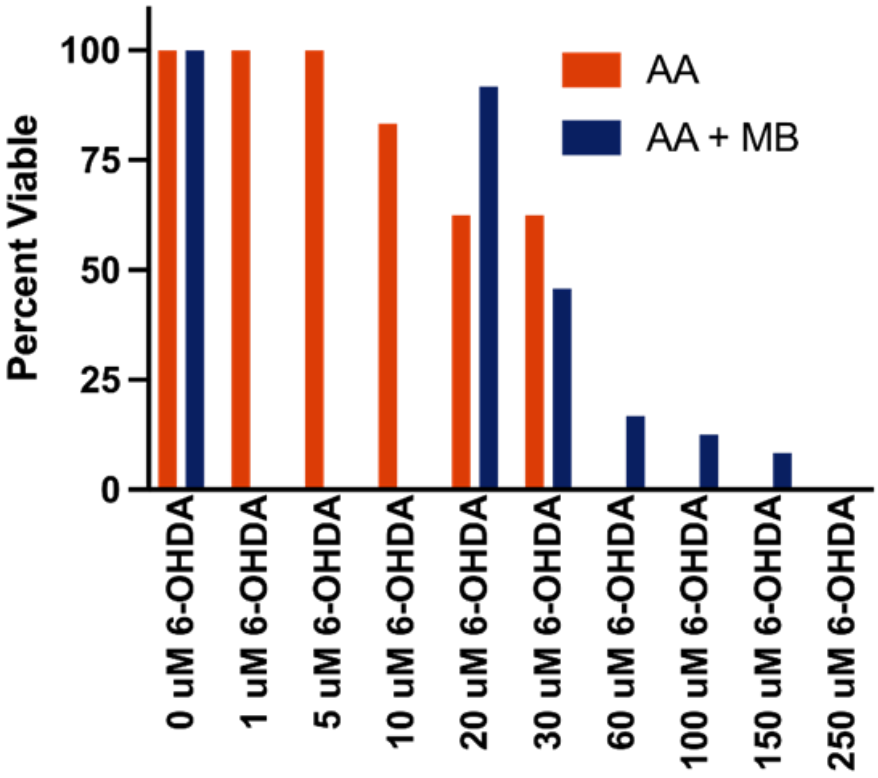

Figure 9: Methylene blue affects 6-OHDA associated mortality: depicts mortality in the presence and absence of MB. 24 fish were utilized per group. MB was utilized in a presence absence at a concentration of 1µ L/ 100 mL.

Future exploration into the efficacy of MB as an antiparkinsonian compound with the potential of affecting etiological pathways is planned.

Behavior

When utilizing the 0.001% protocol the behavior resulted in varied outcomes with only a single instance of significance occurring within a single fish cohort (*figure 10*). The fish used to achieve significance were Segrest. Attempts were made to reproduce the locomotive behavior, but it only occurred one time. Subsequent replications failed to yield the same result and the concern of the detection of a behavioral abnormality without the molecular identification of a PD-like state would result in the behavioral data being negative. This pivoted our scheme to utilize a molecular first approach through either protein or RNA as the primary output to identify a PD-like state in Zebrafish larvae utilizing 6-OHDA.

Figure 10

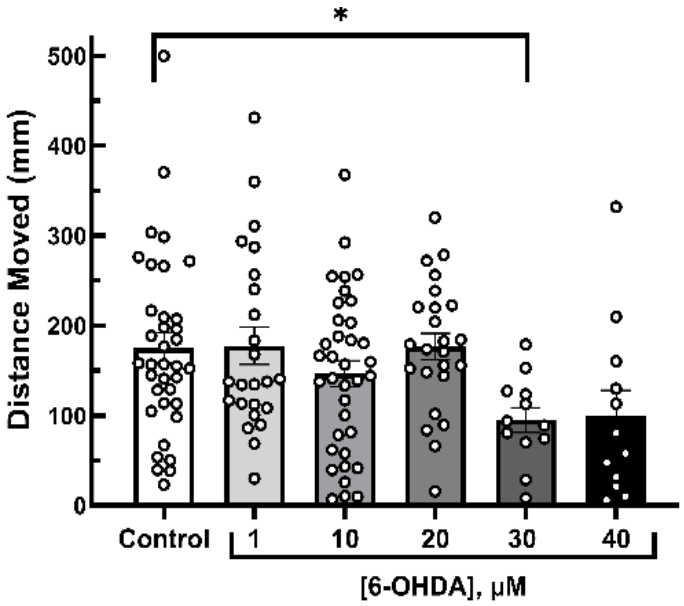

Figure 10: Locomotive behavior is reduced in 30 µM 6-OHDA fish: shows the behavioral outputs of Zebrafish larvae at 5 dpf after being exposed to a 6-OHDA. Each point represents the movement of a single fish in a single 96 well plate well measured over a period of 10 minutes.

The behavioral data has several limitations and additional factors that can influence outputs such as time of day, light acclimation, and temperature. While the impact of the factors can and was reduced, limited man hours and scheduling made it impossible to perfectly reproduce exact collections requiring a significant effect of 6-OHDA locomotion to identify significance over background noise. Additionally, we attempted to cut variability by only utilizing very well characterized isogenic strains of Zebrafish (AB) instead of polygenic strains (Segrest) or more variable isogenic strains (WIK) [94–96]. The AB fish have low genetic coefficients as compared to other commonly available zebrafish strains enabling researchers to more easily detect behavioral differences and have single responses instead of multiphasic responses in populations [95].

When the 0.2 % delivery vehicle was used a repeatable behavioral output was observed though instances of akinesia-like behavior increasing in surviving Zebrafish larvae exposed to 30 μM 6-OHDA as compared to the control 0 μM fish (*figure 11*).

Figure 11

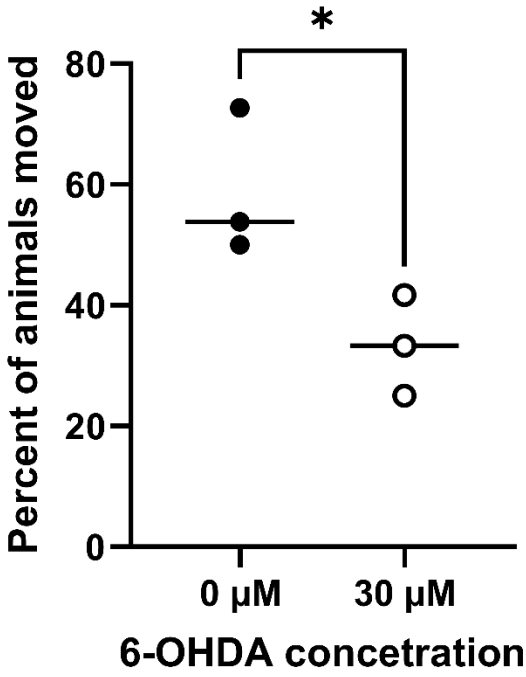

Figure 11: Instances of akinesia-like behavior increase in 30 µM 6-OHDA exposed fish. The points represent the percentage of larvae that moved in a ten-minute period. Each point represents the percentage of 10-12 larvae that moved in a 24 well plate where each plate has one well per fish with 12 control and 12 surviving one day 3-4 dpf 30 µM 6-OHDA exposed fish. A total of three plates were generated. The larvae are 6 dpf at time of

The akinesia-like behavior is indicative of a PD-like state and the identification of the behavioral output is both easy and inexpensive to perform. Unlike when utilizing the 0.001% L-ascorbic acid delivery of 6-OHDA, the 0.2% L-ascorbic acid resulted in a constant behavioral output.

Ethovision daniovision proved to be difficult to effectively apply with the aforementioned recording set up. The high camera framerate combined with the light plate having fluctuations / light pulsating (not visible to the human eye) as it got older made the software unable to maintain sufficient accuracy of fish detection and tracking to utilize the software or data generated from it.

Primer design

A total of 994 primers were designed utilizing NCBI primer design tool for the entire NIH 16 project of those, 122 were selected and tested for the application to PD modeling and the etiological mechanisms of PD (appendix). Some primers were taken from primers that were utilized in publications, but they did not translate well for our use[97,98]. Specifically, the primers for the dopaminergic targets *th* and *slc6a3* and the *sod1* and *sod2* ROS markers in the aforementioned articles did not function for our purposes and conditions. Of the 497 primer pairs assessed, 100 of them produced single bands and 9 of them have resulted in unique targets that have sufficiently good primer efficiency at standard conditions (*table 2*).

Table 2: qRT-PCR primers used to identify a PD-like state utilizing 6-OHDA exposed Zebrafish larvae

Gene target	**Forward primer sequence**	**Reverse primer sequnece**	**Efficency**
gapdh	tgaggttaaggcagaaggcg	aagtagcacctgcatcaccc	106.7
hprt1	ccgagagttgaggaccgttt	agacgttcagttcggtccat	98.38%
th	gacggaagatgatcggagaca	ccgccatgttccgatttct	92.66
slc6a3	gccaccttcaatcctcccaa	cgccagtttgtcacagaagc	102.39
slc18a2	tgacggtggtcgtgcctatt	gaggcaaacaacagacccac	92.33%
pink1	gggaaaggttgtaacgcagc	gaagcagaaagtcgaagcgg	91.17
sod1	tccacgtccatgcttttggt	tcagcggtcacattacccag	105.3
sod2	taggtgctgtgacctccaga	tcaatgcaggctgaagggag	97.77
gad1b	tgtgtccgatggcttgagtc	cacacggaggatggttcaca	91.71

Gene target	Primer orientation	Primer sequence	Tm	Efficency
gapdh	forwad	tgaggttaaggcagaaggcg	60	106.7
gapdh	reverse	aagtagcacctgcatcaccc	60	106.7
hprt1	forwad	ccgagagttgaggaccgttt	60	98.38%

hprt1	reverse	agacgttcagttcggtccat	60	98.38%
th	forwad	gacggaagatgatcggagaca	60	92.66
th	reverse	ccgccatgttccgatttct	60	92.66
slc6a3	forwad	gccaccttcaatcctcccaa	60	102.39
slc6a3	reverse	cgccagtttgtcacagaagc	60	102.39
slc18a2	forwad	tgacggtggtcgtgcctatt	60	92.33%
slc18a2	reverse	gaggcaaacaacagacccac	60	92.33%
pink1	forwad	gggaaaggttgtaacgcagc	60	91.17
pink1	reverse	gaagcagaaagtcgaagcgg	60	91.17
sod1	forwad	tccacgtccatgcttttggt	60	105.3
sod1	reverse	tcagcggtcacattacccag	60	105.3
sod2	forwad	taggtgctgtgacctccaga	60	97.77
sod2	reverse	tcaatgcaggctgaagggag	60	97.77
gad1b	forwad	tgtgtccgatggcttgagtc	60	91.71
gad1b	reverse	cacacggaggatggttcaca	60	91.711

All primers (*table 2*) produced amplicons that had sequence results that hit the desired target as their first NCBI Mega blast result with higher than 98.5% identity. Discrepancies came from sequence error where a ‘n’ was present rather than an erroneous base call. . Initially *gapdh* and *hprt1* were utilized as dual anchor endogenous controls but *gapdh* expression was reduced with 6-OHDA presence. While the effect was not significant, it created additional noise that masked the effects of 6-OHDA on the genes of interest (GOI). Additionally, *hprt1* Cq values were closer to the range of our GOI than *gapdh* making it a much more suitable as an endogenous control (*figure 12*, additional Cq figures in appendix)

Figure 12

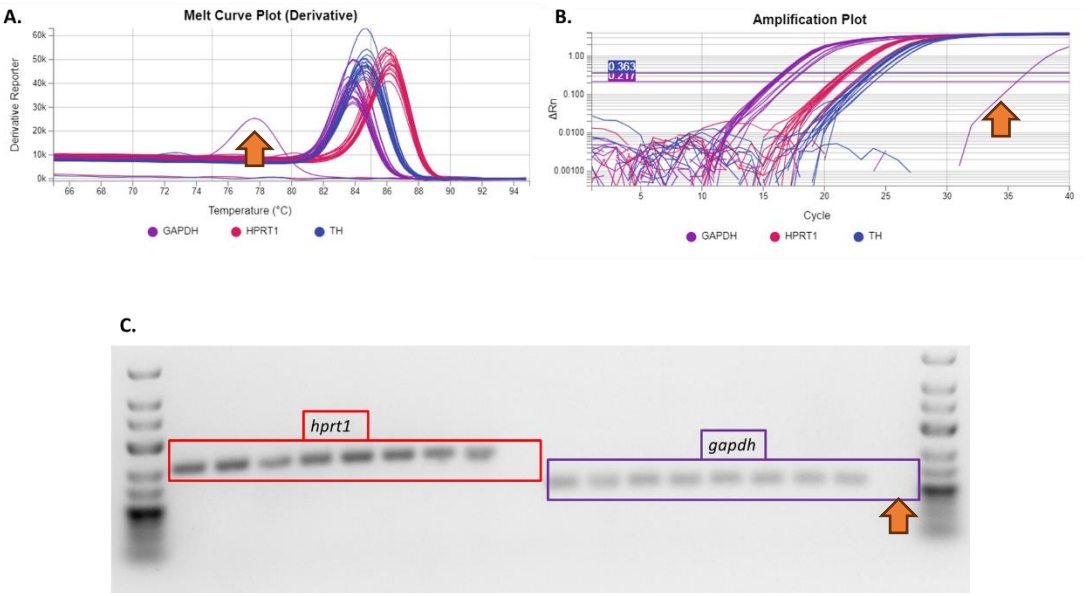

Figure 12: Representative Melt Curve, Amplification, and gel. A representative output per qPCR run. The arrows indicate a non-template control amplification that was detected in the melt curve and amplification but not on a gel. The amplification is determined as a primer dimer and the primer pair is deemed acceptable A. is the Melt curve plot of the products. B. is the real-time amplification plot , and C. is the gel of the products.

RNA extractions

A total of 40 RNA extractions were performed with 35 of them being successful. RNA was extracted at 6 dpf with concentration and purity being identified with a nanodrop (*table 3*).

Table 3

Sample name	Date of collection	Concetrtion	260/280	260/230
MB Control (3-4 dof)	12.20.2022	139.8	2.00	2.19
MB Vehicle (3-4 dpf)	12.20.2022	141.5	2.04	2.15
MB 20 µM (3-4 dpf)	12.20.2022	119.2	2.03	2.17
MB 30 µM (3-4 dpf)	12.20.2022	66.3	2.01	2.05
NMB Control (3-4 dpf)	12.20.2022	151.2	2.05	1.91
NMB Vehicle (3-4 dpf)	12.20.2022	100.5	2.01	2.06

NMB 1 µM (3-4 dpf)	12.20.2022	94.1	2.00	2.12
NMB 5 µM (3-4 dpf)	12.20.2022	145.1	2.02	2.18
NMB 10 µM (3-4 dpf)	12.20.2022	165	2.00	2.21
NMB 20 µM (3-4 dpf)	12.20.2022	119.4	2.01	2.11
NMB 30 µM (3-4 dpf)	12.20.2022	145.2	2.00	2.15
2-3dpf 0 µM A	3.13.2023	87.4	2.00	2.06
2-3dpf 0 µM B	3.13.2023	116.9	2.00	2.05
2-3dpf 0 µM C	3.13.2023	185.4	2.02	2.15
2-3dpf 10 µM A	3.13.2023	101.5	2.00	2.09
2-3dpf 10 µM B	***3.13.2023***	***111.6***	***1.87***	***1.36***
2-3dpf 10 µM C	3.13.2023	164.8	2.01	2.15
3-4 dpf 0 µM A	3.13.2023	112.2	1.98	2.14
3-4 dpf 0 µM B	***3.13.2023***	***104.6***	***1.97***	***1.63***
3-4 dpf 0 µM C	3.13.2023	148	2.02	2.12
3-4 dpf 10 µM A	3.13.2023	243.6	2.01	2.06
3-4 dpf 10 µM B	3.13.2023	238	2.00	2.18
3-4 dpf 10 µM C	3.13.2023	181.6	2.00	2.11
3-4 dpf 20 µM A	3.13.2023	80.1	1.96	2.01
3-4 dpf 20 µM B	3.13.2023	170.1	2.00	2.16
3-4 dpf 20 µM C	3.13.2023	75.5	1.96	1.9
3-4 dpf 30 µM A	3.13.2023	110.5	1.99	2.08
3-4 dpf 30 µM B	3.13.2023	92	1.96	2.01
3-4 dpf 30 µM C	***3.13.2023***	***59.2***	***1.9***	***1.42***
0 um 3-5 6ohda 4-10-23 1	4.10.2023	191.999	2.00	2.26
0 um 4-5 6ohda 4-10-23	4.10.2023	290.183	2.03	2.24
0 um 3-4 6ohda 4-10-23	4.10.2023	332.367	2.05	2.19
10 um 3-5 6ohda 4-10-23 1	4.10.2023	228.047	2.02	2.24
10 um 4-5 6ohda 4-10-23 1	***4.10.2023***	***325.069***	***2.02***	***1.65***
10 um 3-5b 6ohda	4.10.2023	125.56	2.04	2.06
20 um 3-5b 6ohda 4-10-23 2 2 1	4.10.2023	77.291	2.00	1.98
20 um 3-5a 6ohda 4-10-23 2 2 2 1	***4.10.2023***	***170.692***	***2.00***	***1.62***
20 um 4-5 6ohda 4-10-23 1	4.10.2023	148.648	2.01	2.01
30 um 4-5 6ohda 4-10-23	4.10.2023	118.545	1.98	2.28
30 um 3-4 6ohda 4-10-23	4.10.2023	86.618	2.00	2.15
60 um 4-5 6ohda 4-10-23	4.10.2023	171.65	2.00	2.15

qRT-PCR

Analysis of RNA products by qRT-PCR revealed a significant reduction of *th* indicating a reduction in Da neurons and a PD like state in 6 dpf larvae exposed to 30 μM 6-OHDA for 24 hours from 3 to 4 dpf (*figure 13*) [8]. No significant difference was observed for the other dopaminergic markers, *slc6a3 and slc18a2*. To assess the mechanism of PD, we analyzed other transcripts as well. A significant reduction of pink*1*, and a significant upregulation of *sod1* and *sod2* (*Figure 13*) are consistent with a lasting state of elevated ROS response and a state of mitochondrial dysfunction[56,77,99].

Figure 13

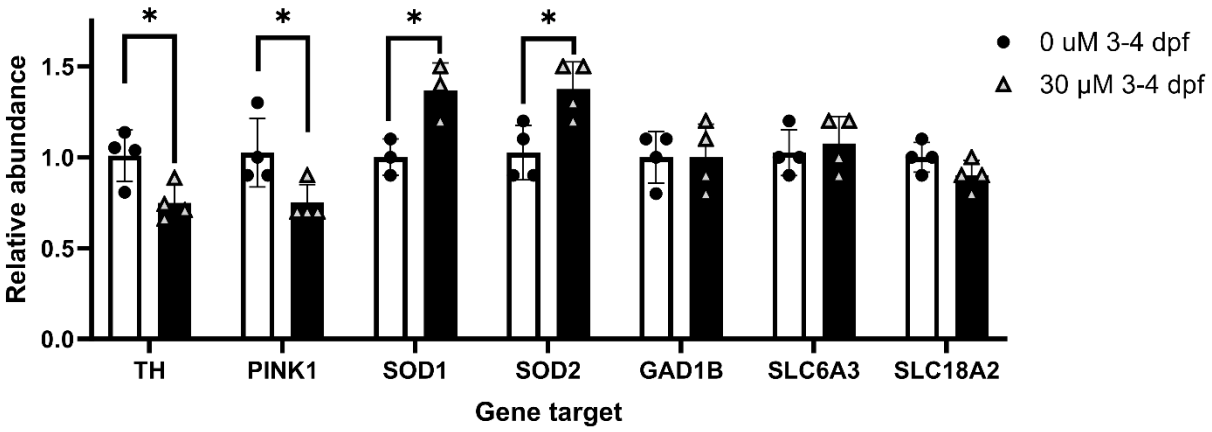

Figure 13: Relative abundance of gene of interest under acute exposure to 6-OHDA 3-4 dpf shows the relative abundance of the GOI compared to endogenous control *hprt1*. Each point represents RNA harvested from a group of at least 10 fish (each started with 24 but ~50% morality is observed using the methodology). Fish strain used is AB. The RNA came from a total of four biological groups from three different fish cohorts (except for *sod1*). * Indicates $p<0.05$.

The expression of GABAergic neuron marker *gad1b* remains unchanged suggesting specific neural degeneration (*figure 13*).

To assess if lower doses of 6-OHDA for longer durations would provide a better approach, larvae were soaked in 10 µM 6-OHDA for 48 hours from 3-5 dpf; however, this treatment showed no change in any of the genes of interest (*figure 14*).

Figure 14

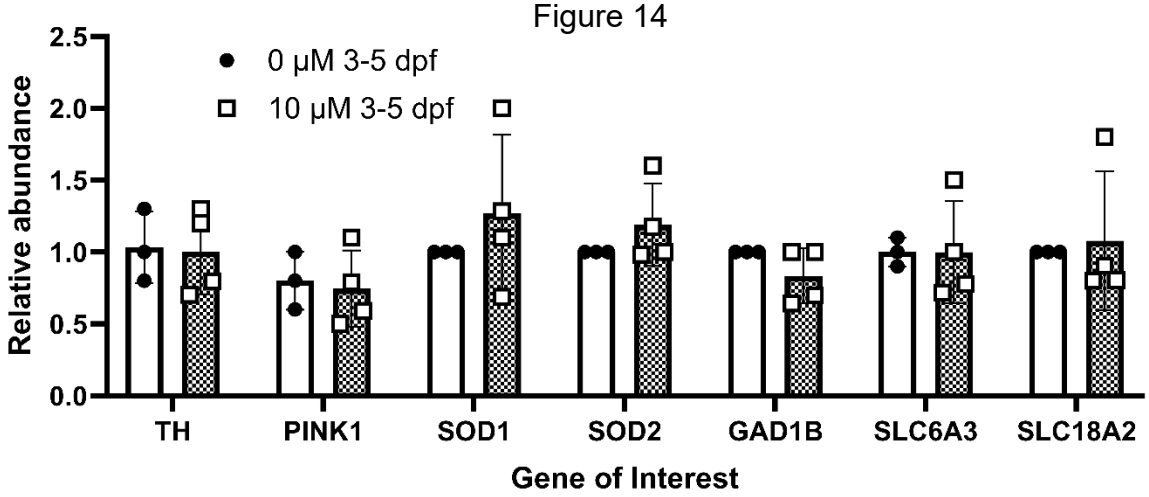

Figure 14: Relative abundance of Genes of Interest under extended exposure to 6-OHDA: No indication of a PD-like state when 6-OHDA is used fresh daily over 48 hours when starting at 3 dpf. The relative abundance of the GOI compared to endogenous control *hprt1*. Each point represents RNA harvested from a group of at least 15 fish (each started with 24 but morality is observed using the methodology). Fish strain is AB. The RNA came from three

The 10 µM concentration of 6-OHDA was selected as it was the highest concentration that did not show mortality following a single day of exposure starting at 3 dpf.

Additionally, to assess treatment window, we tried exposure starting later in development, specifically 4 dpf. No reduction in *th* was observed at the concentrations of 6-OHDA utilized acute exposure beginning at 4 dpf (*figure 15*). However, reductions in *pink1* expression are observed (expression difference is greater than 0.2) indicating that the animals were experiencing a state of mitochondrial dysfunction.

Figure 15

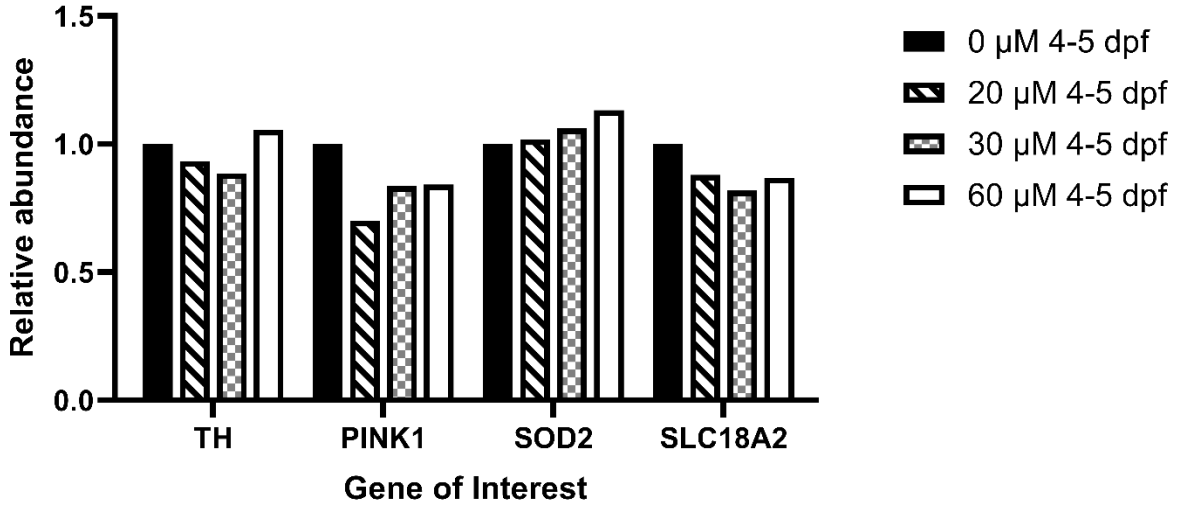

Figure 15: Relative abundance of genes of interest under acute 4-5 dpf 6-OHDA exposure: There are no indicators of a PD-like state when utilizing 6-OHDA in zebrafish starting at 4 dpf of exposure. Each bar represents at least 12 fish of RNA (starting n of 24)

The mitochondrial dysfunction appeared without the activation of the ROS detoxifying pathway as *sod2* is unchanged. It is possible we just assessed *sod2* expression too early as levels are trending upwards. Later collection time points would be required to see if this is the case.

Chapter Four

Discussion

Protocol developed for 6-OHDA use in Zebrafish larvae

While it has been established that 6-OHDA administration causes the depletion of dopamine by disrupting mitochondrial functionality its effectiveness when used in Zebrafish larvae has been contested [42,54,55,88]. However, we have observed a reliable decrease in the dopaminergic marker *th* indicating 6-OHDA is capable of producing a Parkinson's disease-like state in zebrafish larvae (figure 12). A Parkinsonian behavior is also present in the zebrafish larvae with instances of akinesia increasing in surviving 30 µM 6-OHDA exposed fish (figure 10). Additionally, the 6-OHDA neurotoxin appears to induce a state of mitochondrial dysfunction through high ROS presence when utilized in an acute manner observed by the upregulation of mitochondrial gene *pink1* and oxidative radical makers *sod1* and *sod2*. When comparing the effects of 6-OHDA against the other dopaminergic markers the Dopamine Transporter, *slc6a3,* and Vesicle Monoamine Transporter Protein 2, *slc18a2,* no significant difference was observed indicating that these makers are not ideal for the identification of a Parkinsonian state when taking whole organism samples as their expression is not exclusively in dopaminergic neurons in zebrafish larvae [100].

The absence of morphological and developmental differences between surviving acute one day (3-4 dpf treatment) 30 µM 6-OHDA exposed fish and control fish in combination with the expression of *gad1b* not being affected by our protocol utilizing 6-OHDA indicates that this neurotoxin is having selective neurodegenerative effects on dopaminergic neurons. Future directions should include using other methodology to

validate the selectivity of 6-OHDA utilizing our methodology, for example immunohistochemical or TH binding/activity assays.

When we attempted to refine the approach and reduce the mortality while maintaining the dopaminergic response, we saw that neither lowering concentration, extending the time (figure 13), or starting the exposed when the fish are older and more resilient (figure 14), produced the molecular fingerprint of a PD-like state induced by 6-OHDA. The high lethality (~50 %) seen in our generated protocol is necessitated in order to reproducibly generate a PD-like state (figure 10 and 12).

Zebrafish health and morphology under different reagent conditions

The presence of L-ascorbic acid in the solution stabilizes 6-OHDA. We propose this allows more neurotoxin to enter the larvae and propagate damage [75]. This likely explains the occurrence of 6-OHDA being more lethal at the same concentrations in the presence of 0.2% L-ascorbic acid as compared to 0.001% L-ascorbic acid. The additional stability also resulted in increased consistency observed in lethality of using 0.2% L-ascorbic. The use of 0.2% L-ascorbic acid requires the use of a buffered vehicle solution. Based on other work, we utilized 40 mM HEPES pH 7.4 buffer in order to have neutral pH. Acidified water with no buffer have developmental and morphological effects in larvae that are observed in addition to the effect of 6-OHDA. In addition, lower levels of L-ascobic acid in combination with 6-OHDA can lead to morphological defects in surviving larvae [42,101]. This is problematic when assessing locomotive behavior and call into question the validity of movement effects attributed to the neurological effects of 6-OHDA rather than differences in body shape and size as a result of promiscuous 6-OHDA interactions.

Methylene blue (MB) is a commonly used antifungal in zebrafish larval experimentation. In our preparations, it has an effect on survivability on zebrafish larvae. We conducted a presence versus absence analysis of 1µL/ 100 mL of MB in vehicle, and observed a profound difference within the fish cohort was observed correlating with the previous results in mice exposed to 6-OHDA [93,102]. MB has neuroprotective properties pertaining to metabolic enhancement and its antioxidant characteristics, and is an effective treatment for carbon monoxide and cyanide poisoning due to its interactions with the Electron Transport Chain [103].. 6-OHDA was observed to increase ROS response in the form of upregulation of *sod1* and *sod2* therefore possibly explaining the reduced mortality observed in the larvae with MB present in their vehicle solution [55]. In addition, MB interacts with L-ascorbic acid to form derivative neuroprotective compounds through reduction of MB [104,105]. The interactions of MB and L-ascorbic acid requires careful consideration in the mechanism of action of any pharmacological compounds used as the neuroprotective/ neuromodulating effects could be altered in the presence of methylene blue. Further investigation should be made in the ability for MB to be utilized as an Anti-parkinsonian compound.

Initial variability in utilizing the 0.001% protocol was thought to be attributed to the use of polygenic fish [Segrest] instead of isogenic strains like AB [ZIRC] and WIK [ZIRC] [81,94]. General expectations from the use of polygenic verses isogenic strains is that behavioral and molecular outputs will be less varied because of lesser coefficient of variation seen in isogenic strains [95]. In addition, the sex determination strategies seen in isogenic, polygenic and true wild fish can vary [94]. This is problematic as the environmental condition of the fish can influence sex determination in certain strains of zebrafish that in

turn can influence behavior when assessing fish [96]. AB fish were selected as our target strain as they should have less variability in behavior and development with one another, and that choice seems to be reflected in the reproducibility and consistency of mortality, molecular, and behavioral results (figures 6, 10 and 12).

Timing of 6-OHDA exposure

The timing of Zebrafish larvae exposure to 6-OHDA has an immense effect on developmental and morphological outcomes. Zebrafish exposed to 6-OHDA at 2 dpf and earlier have developmental and morphological deficits that appear [101]. We observed a similar effect in our 2-3 dpf treatment cohort as toxic and developmental effects of 6-OHDA were present. This sets the 3 dpf mark as the ideal starting point for researchers that want to utilize 6-OHDA without morphological or developmental issues appearing. The absence of morphological or developmental factors provides higher levels of clarity when assessing behavior. Body size, shape and development are critical factors in locomotive ability in zebrafish and discrepancies between groups in these factors can result in shifting of observed outputs[84,89]. An extended use of 6-OHDA (20 μM 3-5 dpf) utilizing 0.2 % L-ascorbic acid 40 mM HEPES 7.4 Buffered embryo water resulted in a reduction in *th* and *pink1* expression without significant difference in *sod2* expression in a single cohort of fish; however, variability of phenotype led to no significant changes being observed over multiple cohorts. This indicates that the extended use results in 6-OHDA may be capable of inducing a Parkinsonian state through mitochondrial disruption without having a heightened whole organism response to reactive oxygen species ; however, more work would be needed to determine conditions that would reduce variability to make this an effect protocol. The elevated *sod2* expression is an understood

mechanism of neuroprotection cells and tissues can utilize when high levels of ROS are present. The dynamic response may be useful to investigate further as one protocol could be utilized to more closely emulate an acute event seen in some Parkinson's patients through injury or exposure to neurotoxins while the other used to emulate a more chronic condition seen in many idiopathic forms of PD [1,36,37].

Exposure on or before 2 dpf is problematic as development of immature dopaminergic neurons has not concluded diminishing signaling that occurs for normal development increasing the likelihood of secondary effects occurring [27,106]. The maturation process of dopaminergic neurons influences surrounding cells and tissues and its disruption can lead to development or morphological deficiency that can affect the collection of molecular and behavioral outputs [10]. However, this can be advantageous as Zebrafish larvae are great models for developmental work [82]. Developmental fate mapping has allowed researchers to adopt a directed differentiation tactic when taking pluripotent stem cells to mature dopaminergic neurons [107–109]. The information has uncovered targets for improving existing cells such as *nr4a2b,* a transcription factor categorized in the nuclear receptor super family expressed in mDA neurons in the substantia nigra and ventral tegmental area regions of the brain in both developmental and adult stages [26]. The presence of NR4A2 stipulates the expression of Tyrosine Hydroxylase [27]. Affecting early development in larvae in this manner can uncover effects seen in adulthood as NR4A2 expression is involved in the viability of mDA neurons long after differentiation [106]. The incorporation of developmental makers is crucial in understanding morphological discrepancies seen in Zebrafish larvae exposed to 6-OHDA during key time periods such as the maturation and formation of mDA neurons. A possible

future route of exposure is identifying the effects of 6-OHDA on immature dopaminergic neurons expressing NR4A2 in high quantities. The acuity of qRT-PCR is at its limit when trying to identify early developmental markers like NR4A2, but utilizing an RNA-seq approach, especially in a single cell format, for different developmental stages could provide insights into the effects that early neurological damage has on PD etiology [110]. 6-OHDA could be utilized in more specific timepoints where more acute effects can be observed and identified.

Exposure to 6-OHDA at 4 dpf does not appear to have Parkinsonian effects, as defined by the lack of *th* reduction at the same effective concentration from our 3 dpf protocol. This result makes 3 dpf a critical time point for 6-OHDA exposure to produce a PD-like state. The discrepancy between a 3dpf and 4 dpf treatment starting point for 6-OHDA exposure is possibly a result of blood brain barrier (BBB) development and its selectivity/ impermeability increasing with age [86]. The BBB becomes largely impermeable at 5 dpf and finished choroid plexus formation at 7 dpf securing the Blood-CSF barrier [87,111]. Previous work has shown that 6-OHDA does not cross the BBB as readily as other neurotoxins associated with PD. We propose that at this stage lower availability limits is effect, since we do observe lower relative expression of *pink1* in the 4 dpf treated fish (figure 12). This suggests that a state of mitochondrial dysfunction preceding measurable effects on dopaminergic neurons (reduced *th* expression) indicating that the mechanism of 6-OHDA toxicity is occurring, but lower 6-OHDA access is sparing the end result of Da neuron loss [55].

Future directions

The developed protocol will be utilized in novel classes of positive allosteric modulators that have the potential to modulate a parkinsonian state though non-dopamine based changes in nicotinic receptors [112]. Additionally, the molecular and physiological change of healthy dopaminergic neurons and animal into those that are PD-like cane be developed by takin intermediate time point sample [7]. This approached could give insights over more intermediate markers that can be translated into potential pharmacological targets for an ethological approach to PD treatment [11]. Additional confirmation of the reduction of *th* seen though the qRT-PCR is planned though the gather protein samples via radio labeled ligand for TH and immunohistochemical staining of TH [113].

Final conclusions

In summary, I have developed a method such that 6-OHDA can be utilized to induce a PD-like state based on qRT-PCR and behavioral analysis. The optimal dose and treatment time in our hands is 30 μM 6-OHDA for 24 hours between 3 and 4 dpf. Importantly, although there is a 50% mortality, analysis of the morphologically normal survivors demonstrates a significant reduction of *th*, a specific marker for DA neurons. This effect would be specific as we do not see significant differences in markers for other neural cell types, i.e. *gad1b*. In addition, we demonstrate the pathologic mechanism is at least partially through mitochondrial dysfunction as *pink1*, *sod1*, and *sod2* expression differences suggest a conserved response to 6-OHDA [114,115]. We propose that this protocol established a pathway to utilized 6-OHDA treated zebrafish as a tool for drug screens and a possible etiological information network.

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Appendix

Detailed protocols

Reverse transcriptase cDNA synthesis

cDNA synthesis is a critical step in the qPCR process. The reagent work up is based on the SensiFAST cDNA kit. The protocol can be broken up into four categories: (1) preparation of the area, (2) preparation and combination of reagents, (3) Thermal cycle synthesis, and (4) post synthesis processing.

Reagents needed

DO NOT PULL RNA or REVERSE TRANSCRIPTASE from freezer(S) until absolutely needed and place back in freezer IMMEDIATELY after use

95-70% ethanol

3% hydrogen peroxide

DEPC water

RNA

5x TransAMP Buffer

Reverse Transcriptase

Equipment needed

Pipettors (1 µL to 20 µL volume capable)

Vortex mixer

Table top centrifuge 1.5 mL

Thermal cycler

ICE bucket

Tube chiller for 1.5 mL tubes

Tube chiller for 0.2 mL tubes/ tube stips

0.2 mL tube or 0.2 mL tube strip (8)

Preparation of the area

Preparation of the working area is essential to avoid RNAase eating/ reacting with the RNA itself and avoid contamination.

1. The working area and instruments are sprayed with 95-70% ethanol and let evaporate.
2. The working area and instruments are cleaned with 3 % hydrogen peroxide by soaking a paper towel in the solution and wiping the area or pouring out the 3% hydrogen peroxide onto the bench top and wiping with paper towels.
3. Get an ice bucket and tube chillers
4. Get needed tube for reaction (one 0.2 mL tube per 20 µL reaction up to 50 µL per 0.2 mL tube)

Preparation and Combination of reagents

This section involved the preparation of reagents for use and their assembly for the reaction. A full thaw of reagents is required before manipulation though pipetting/ vortexing/ centrifugation. Once thawed all samples and reagents need to be vortexed and briefly centrifuged. When pipetting from stocks pipette mix by pipetting up and down slowly at least three times (This serves to pre-wet the tip as well and give more of an accurate volume) before transferring the desired volume of sample or reagent. Reaction tube should be place an kept on chiller inside of ice bucket as well as reagents and sample(s) until they are used and then immediately store after use.

1. Pull the RNA from the -80 C and acquire 400 ng of material (volume will depend on sample concentration) and place into a clean DNAase/RNAase free 0.2 mL tube (reaction tube)
2. Transfer 4 µL of 5x TransAMP Buffer to the same Reaction tube that contains the RNA
3. Transfer 1 µL of Reverse Transcriptase to the same reaction tube as before
4. Fill the reaction tube up to 20 µL total volume using DNase/RNase free-water or DEPC water

Table 1. List of available RNA samples and a quick volume guide.					
sample name	needed RNA (µL)	RT buffer	RT	Water	total
2dpf 0 µM A	4.58	4	1	10.42	20
2dpf 0 µM B	3.42	4	1	11.58	20
2dpf 0 µM C	2.16	4	1	12.84	20
2dpf 10 µM A	3.94	4	1	11.06	20
2dpf 10 µM B	3.58	4	1	11.42	20
2dpf 10 µM C	2.43	4	1	12.57	20
3dpf 0 µM A	3.57	4	1	11.43	20
3dpf 0 µM B	3.82	4	1	11.18	20
3dpf 0 µM C	2.70	4	1	12.30	20
3dpf 10 µM A	1.64	4	1	13.36	20
3dpf 10 µM B	1.68	4	1	13.32	20
3dpf 10 µM C	2.20	4	1	12.80	20
3dpf 20 µM A	4.99	4	1	10.01	20
3dpf 20 µM B	2.35	4	1	12.65	20
3dpf 20 µM C	5.30	4	1	9.70	20
3dpf 30 µM A	3.62	4	1	11.38	20
3dpf 30 µM B	4.35	4	1	10.65	20
3dpf 30 µM C	6.76	4	1	8.24	20
MB Control	2.86	4	1	12.14	20
MB Vehicle	2.83	4	1	12.17	20
MB 20 µM	3.36	4	1	11.64	20
MB 30 µM	6.03	4	1	8.97	20

Thermal cycle synthesis

This section is the actual reaction cDNA synthesis step. The thermal cycler is required in this section. Make sure to preprogram the cycler or maintain the reaction tube in ice/ chiller until ready for use.

1. After combing, gently vortex the tube and then centrifuge down in microcentrifuge or salad spinner.
2. Load the sample into the thermocycler and initiate the following thermal profile
    a. 25 °C for 10 min
    b. 42 °C for 15 min
    c. 48 °C for 15 min
    d. 85 °C for 5 min
    e. 4 °C hold (or chill on ice)

Post-Synthesis processing

The process is for the dilution of the cDNA and ensuring and even distribution of products.

1. Add equal (20 µL) volume of DEPC water to the cDNA product for a 50% dilution.
    a. When adding the DEPC water pipette: mix gently but thoroughly at least 10 times. The use of ice cold DEPC water is recommended to avoid temperature dependent degradation of cDNA.
2. After the cDNA has been diluted it is ready for use, for best results use immediately the same day or store at <-20 C for long term storage.
    a. Results from fresh and stored may vary with freeze/thaw cycles especially in genes with low expression.

qPCR set up and running

This step involves the assembly and use of cDNA from the previous protocol and the reagents for qPCR including the PowerTrack SYBR Green Master Mix, primer(s), and DEPC water. For Best results this process should be done immediately after the cDNA synthesis the same day to have best acuity in results. The protocol can be broken into three groups: (1) Master mix preparation, (2) Plate assembly, (3) qPCR thermal cycler operation

It is important to design your qPCR plate before attempting to do any of the following steps in order to have an idea of reagent amounts needed based on number of wells needed and the specific combination of Primer and sample in each well. Utilize the Design and Analysis tool app and utilize an Excel file to quickly generate volume totals.

Reagents needed

2X buffer PowerTrak Master mix

DEPC water

Primers

70% ethanol

Equipment needed

1.5 mL Eppendorf tubes

96 well plate

Plate sealer

Kimwipes

Soft plastic/ resin scraper

Plate cooler

Micro-centrifuge

qPCR thermocycler

USB with program installed.

Master mix preparation

Master mix preparation is critical as error(s) that occur here are very difficult to detect during analysis. It is important to practice very good pipetting skills and pipette mix before transferring from stocks and ensuring the tip of the pipette is 'pre-wet' with solution before transferring to ensure accuracy and precision in pipetting. Stock solutions should be fully thawed, and gently vortexed end spun down prior to pipetting.

1. Begin the preparation of master mix by pipetting out needed 2x mastermix for single sample use into a 1.5 mL clean DNAase and RNAase free eppendorf tube
    a. It it better to pipette all needed 2X master mix at this stage to prevent degradation of the reagents by quickly putting it back into the freezer after use
2. In to the same Eppendorf as before Add the needed cDNA (or just equivalent volume of water for non template control)
3. Into the same Eppendorf as before, add the needed amount of water for the whole sample
4. Dilute 20 µM of F+ R primers to 4 µM (volume can vary) in a separate clean and DNAase/ RNAase free tube
    a. 20 µM F+R primers is prepared by mixing 4 µL of a Forward primer 100 µM (stock) and 4 µL of a reverse primer 100 µM (stock) with 12 µL of water. This produces 20 µL of 20 µM F + R primer (scale to needed volume min 20 µL)

Final volumes needed can vary depending on the type of plate set up. However, one well will contain 5 µL of 2X master Mix, 2 µL of water, 1 µL of cDNA (sample) , and 2 µL of primer. When preparing the master mix you will add the needed amount of 2X master, water and cDNA (sample) based on the total number of wells for the sample. To avoid shortage from pipette loss multiply your tube number by 1.25. So in a plate set up that has 15 tubes that need a single cDNA amount you will prepare a master mix for 18.75 wells. The primer(s), while prepared separately, should be prepared in the same thought where you multiply the reagent needed for one well by the amount of reagent you would need for 1.25X wells that have the primer for your plate. You will need 2 µL of primer (4 µM F+R) for each well in the plate.

Plate assembly

This process should be performed immediately after the preparation of master mix and primer dilution. If the master mix/ primers need to be transported across campus place the 1.5 mL tubes in a tube chiller. The tube chiller should be placed on top of a Styrofoam ice box with ice. In this step you will need an applied biosystems 96 well plate with the appropriate plate sealer. In the end each well will have 10 µL of solution for 10 µL reaction.

1. Begin by adding appropriate primers to designated wells in 96 well plate. Two µL of 4 µM F+R primer into each well.
    a. When pipetting, pipette mixing before taking from primer tube as this step pre-wets the tip and helps mix the primers in such a way that the concertation is more uniform for across loaded samples. Use a single tip for each primer type.
    b. Pipetting the primer first allows for visual inspection of addition as the primer is the only contents in the well and is visible when placed in the bottom of the well.
2. Pipette in 8 µL of your sample specific master mix.
    a. Pipette mix the 1.5 mL Eppendorf tube containing the master mix prior to transferring; this process ensures that your pipette tip is pre-wet for more accurate transfers and that your sample specific master mix is a homogenous mixture increasing the probability of more uniform distribution.
    b. Use a separate tip for each well and pipette to the bottom of the well.
    c. When adding them add the contents by sample type and do not switch sample type until all wells for the specific sample type are filled.
3. Seal the plate with plate sealer.
    a. The plate sealer is a single piece of single side adhesive clear film with a backing. The seal has perforations on two sides dividing the seal into three sections. One large middle section and two evenly sizes smaller pieces on either side
    b. Place the plate on a clean surface or over fresh kimwipes and grab a soft plastic/ resin scraper (clean with ethanol and dry with fresh kimwipes)
    c. Remove the backing from the large middle piece and line up the middle piece over the plate as to where the edges of the plate go beyond the edge or perimeter well by >1mm on each side.
    d. Once seal has been placed and adequate coverage of whole plate is achieved go over the seal with cleaned plastic/ resin scraper to push out any air pockets starting from the middle and working to the edges.
    e. After pushing the seal flat against the plate rip off the smaller edge pieces (if the seal is place correctly the perforations should be close to the edge of the plate skirt to pushing and tearing with the skirt edge is possible)
    f. Take the plastic/ resin scraper and line the rows and edges of the plate wells to ensure an individual seal around each well.

4. Take the sealed plate and spin it down at 300 G for one minute in swing centrifuged (ensure correct inserts and balance plate) or microcentrifuge (salad spinner)
    a. Dry the swing centrifuge with Kimwipes before and after operation.
    b. When transporting the plate put the plate into plate chiller

qPCR thermal cycler operation

This process is quick if the program has been set/ prepared prior. Make sure program is set for 10 µL reactions with 55 °C annealing temperature thermal profile. Profile type is delta delta Comparative CT with melt curve.

1. Insert USB with run instructions into the computer next to the thermocycler.
    a. Open the program on the USB and ensure settings are correct for SYBR green chemistry with ROX indicator 10 µL reaction volume at 55 °C anneal temp based on comparative ΔΔ CT template.
2. Turn on the qPCR thermocycler (switch is on back) and open the tray by pressing the button on the top right of the screen (touch screen)
    a. The tray will open and ensure the plate is inserted with the A1 well on the top left corner of thermocycler insert.
3. Once the plate is inserted close the tray and return to the computer
4. Now navigate to the start run tab on the computer and press the drop down arrow on the right side of the bottom and select the machine
    a. **SELECTING THE MACHINE IN THE START THE RUN DROP DOWN WILL AUTOMATICALLY START THE RUN SO MAKE SURE THE PLATE IS INSERTED IN THE RIGHT ORENTATION PRIOR TO SELECTION**
5. Wait the run sequence and do periodic quality checks
    a. Quality checks is seeing background values after 1 or two cycles for all wells. Once some samples + primers have ‘taken off’ highlight technical replicates and check their application profiles they should be identical in shape and position if the primer and sample are the same. Primers should ‘take off’ all around the same cycle number (1-2 cycle range unless doing primer efficiency run) irrespective of sample.
6. At the end of the run an internal quality check will be ran based on the non-template control (negative control) melt curve and the ROX values. If the seal was poor or if the non-template control produced a melt curve similar to the template the program will indicate errors.
7. Run the sample (a single one per prime and sample type) on an 2.5 % agarose gel to characterize band patterns.

6-OHDA soaking in 0.0013% L-ascorbic acid

Introduction

Goal is to add 6-OHDA to larval Zebrafish in a concentration that has no discernable wholistic morphological differences between 6-OHDA fish and fish that have not been exposed to 6-OHDA. Treated fish can be then used in behavioral assessment or protein assessment using Tyrosine Hydroxylase (TH) as a biomarker.

Materials

- Appropriate Mask for working with 6-OHDA
- Double gloving when working with 6-OHDA
- Lab coat
- glass pasture pippetes (regular, cut, and narrow)
- 6-OHDA(s) 1.3 mg (3X)
- 200mL of Embryo water (3X)
- 13.5 mg of L-Ascorbic acid(s)
- Conical tube X 9 (x3)
- 250 mL container
- 100 mL container

Procedure

- Reagent work up
- Ascorbic acid stock
- L-ascorbic acid needs to be used in a super stock form to ensure a single batch can be used through the experiment. This solution is made at the beginning of the experiment when the Zebrafish are 2dpf. It is used fresh the first on the first treatment day and kept frozen until needed for subsequent treatment days.

1. Weigh 13.5 mg of L-ascorbic acid
2. Add 100 uL of embryo water
3. Mix thoroughly by vortexing until clear
4. Use the appropriate amount for the first treatment (see below)
5. Store at -20C in aliquots for the remaining days

- The solution is thawed, vortex mixed, and the spun down to ensure efficient and even solution concentration on each subsequent day.
- Ascorbic Acid working solution

6. 0.0013% L-ascorbic acid (Working solution) is made by diluting 10 uL 13 % L-ascorbic acid (super stock) in 100 mL of embryo water.
7. Add appropriate amount of 0.0013% ascorbic acid to conical tubes following table below.

Table 1: 6-OHDA treatment of Zebrafish Larvae at 2-4 dpf

	A	B	C	D
1	Concentration of 6OHDA	Volume of 100 uM 6OHDA (mL)	Volume of 0.0013% ascorbic acid (mL)	Number of Embryos
2	0 uM	0	12	12
3	20 uM	2.4	9.6	12
4	30 uM	3.6	8.4	12
5	40 uM	4.8	7.2	12

- Addition of L-Ascorbic acid working solution

8. Once the Conical tubes are ready begin either pulling zebrafish larvae from incubation and placing them in a 60X 15 mm plate (n of 12) ensuring minimal water/ solution is in them or extracting zebrafish larvae from a 6-OHDA containing solution in a 60 X 15 mm plate if on second or third day of treatment.
9. The use of wide pore pipettes is recommended to extract the larvae from solutions making sure to work up in concentrations (ie using the same tip by stating at low concentrations and working to higher concentrations of solutions).
10. Make sure to label plates with the concentration of 6-OHDA, Initial and if there are several plates label them alphabetically (ie 20 µM A, 20 µM B).

- 6-OHDA addition
- This process should take no longer than 30 minutes from initially hydrating the 6-OHDA with the initial 10 mL of working ascorbic acid solution until the last solution is added. This is because of the high reactivity of 6-OHDA(aq) and the desired effects are primarily observed in the first 90 min of the solution's life span.

11. Weigh 1.3 mg of 6-OHDA(s) in a 12 mL conical tube
12. add 10 mL of Working L-ascorbic acid solution into the 12mL conical tube containing the 6-OHDA

13. Mix thoroughly and then place into a container that can hold 100 mL and add an additional 40 mL to make 100 µM 6-OHDA solution.
14. Add 100 um 6-OHDA stock according to Table 1: 6-OHDA treatment of Zebrafish Larvae at 2-4 dpf to make desire experimental solution to conical tubes already containing needed working ascorbic acid volumes.
15. Ensuring the solutions are mixed and then adding to zebrafish larve (n of 12) in 60 X 15 mm plates.

Membrane sample prep from Zebrafish (optional head and tail sectioning)

Introduction

This protocol is for taking live zebrafish (larvae, juveniles, adults) processing them into membrane preps

Materials

- Initial euthanasia
    - ice bucket/ freezer
    - water
- separation and dissection
    - 1X Vesicle-dell buffer (VDB)+ protease inhibitor
    - heat block
    - sharp (something to cut scalpel/ razor blade works well for larger fish but the edge of a large gauge syringe is better
    - 2x ice bucket
    - tubes for samples
- initial sample processing
    - coffee frothier pestle
    - syringe
- secondary processing
    - VDB = protease inhibitor cocktail
    - glass potter
    - Hamilton syringe

- centrifuge capable of 20,000XG

Procedure

- Euthanization

1. Transfer fish from collection tanks to petri dish with minimal water to allow the to break for faster cooling and euthanization.
2. Leave them in Ice/ freezer until a minute after they do not move after stimulus
3. place the fish onto another petri dish that contains chilled 1X VDB with protease inhibitor that on the cold heat block inside an ice buckets.

- Sample collection

4. In the chilled VDB+ protease inhibitor make a transverse cut behind the gil plate in a diagonal cut where the cut starts along the more ventral side ventral side of the gil plate and ends just in front of the dorsal fin area.

- A single chop is possible in larger fish with a scalpel or razor blade but finesse and slicing with the tip of a syringe is required for small juveniles and larvae as the contents (including their brain) squeeze out with a forceful chop

5. place the Head and tail in sperate a labeled (contents, date , and initials) tube containing 750 uM of VDB+ protease inhibitor

- Pick up the head by the lip/ mouth to avoid squeezing out contents and the rest of the body (tail) by the actual tail fin to avoid squeezing out contents.
- For accurate and better results measure the mass of the tube with just the 1X VDB buffer with the protease inhibitor and measure after all samples have been collected. Measuring totals
- Initial sample processing

6. Insert a plastic pestle with the coffee frothier into the tube and homogenize

- This step is where significant loss can occur make sure to pulse homogenize and only homogenize at the bottom of the tube as to avoid spilling.

7. After breaking down the larger chunks pass the sample through a syringe 10 times
8. store in ice if further processing the same day or if processing later store at -20

- After total initial processing weigh the tube again to account for loss
- Secondary sample processing

9. take the tube with the sample and the 750 uM solution and dilute with more VDB with protease inhibitor (keep track or volume added)

- (this and subsequent steps are done at the pharmacy building the 1X VDB + protease inhibitor is made with 50 mL VDB + 20uL of aliquoted protease inhibitor cocktail)

10. using a adequate sized glass potter further homogenize the sample

- At least 10 strokes of homogenization, preferably until there are no visible particulates

11. after homogenization using a Hamilton syringe pass the entire solution into an appropriate sized container (50mL conical tube for anything 40-25 mL and a 15ml conical for solution samples 12-10 mL in volume)
12. Centrifuge the tube at 1000 xG for 10 min @ 4C resulting int P1 (pellet one) and S1 (supernatant 1).
13. Decant the S1 (supernatant) into an appropriate tube and spin at 20,000 XG for 60 min @ 4C - resulting in S2 (supernatant 1) and P2 (pellet 2)

- Keep track of the inner and outer portion of the tube preferably labeling the tubes position and make sure you are using the correct centrifuge inset in this step

14. P2 contains the membrane extract and desired proteins - resuspend p2 in VDB (100ul/ Adult brain) and discard S2 supernatant.

Protein quantification (from membrane preps) by assay

Introduction

This protocol is for protein assay which can be used to determine the protien concentration in an unknown membrane prep

Materials

- Assay prep
    - Plate reader
    - 96 well plater
    - 660 nM Protein Assay Reagent
    - Albumin 2 mg/mL (Standard protein)
    - 1.5mL tubes
    - positive pressure pipette
- Running the Spectrophotometer
    - Software setup

Procedure

- Preparing the Assay prep

1. if the prepared protein is very viscous and opaque do serial dilutions taking into account volumes added until consistency is similar to 1:1 dilution standard.

- Make sure all your samples are kept on ice.

2. Prepare standard protein (albumin 2000mg/ mL) solutions from stock. Prep should result in 0,20,40,60,80,100,200,400,600,800, and 1000 mg/mL standards. make sure to vortex and spin down when pipetting. Use a positive pressure/ air displacement pipette making sure to change tips assuring to not cross contaminate and getting a consistent equal volume for dilutions and seral dilutions. Need 300 uL of each standard make about 1000 uL of each as you will need to dilute from dilutions to get the smaller concentrations.
3. Based on the number of samples and dilutions using a positive pressure pipette load standards and samples. Make sure to switch tips to ensure no cross-contamination occurs and equal pipetting occurs. Load samples based on the lay out on the program reading on the plate reader and make sure to vortex samples and standards before pipetting from them.

- Helps if you print out your plate reader plate diagram or draw it out on your note book and cross it out as you've added your sample.
- You are loading in triplicate 100 uL per well

4. Add 150uL of 660 protein assay reagent. calculate how much you will need and pour out 110% into a multi channel pipette trough and use a multichannel pipette.

- Use a new tip box to make it easy to set tips into the multi channel pipette.

5. Let the well incubate at room temp for one minute. initial minute is all the reactivity that is going to happen.

- You will notice the color change especially in the more concentrated samples (darker)
- Running the spectrometer

6. Open the plate reader and orientate based on the markings on plate frame.

- Familiarize yourself with the software of the plate reader and put in the wells you need to get absorbance from .

7. Set the plate to read and run the plate
8. The reading will give you several excel files and data ask your advisor what they need and print them out.

Recording Zebrafish on a 96 well plate

Introduction

After experimental treatment of 6-OHDA on the zebrafish larvae, their behavior needs to be assessed. Behavior values come from general movement of the zebrafish larvae as well as movement after stimuli. The behavior can be characterized by total movement over time based on the different concentrations of the 6-OHDA in solution. As well as immediate movement after a stimulus like light or sound . This process should begin no later than 2 pm as it takes a while and best mobility results occur earlier in the day/ morning.

Materials

- 96 well plate
- Zebrafish to fill the plate
- cut pasture pipettes
- small pore pasture pipettes (optional)
- Liquid 6-OHDA waste container
- Solid 6-OHDA waste container
- fitted respirator/ n-96 or equivalent fitted mask
- Lab coat
- Gloves
    - Double gloving when handling 6-OHDA solutions and containers that contact the 6-OHDA solutions

Procedure

- Extracting the Zebrafish

1. Extract the Zebrafish from the 60 X 15mm plates

- This step can optimally done with wide pore pipettes as the zebrafish are 5 dpf as this point and are difficult to capture as they are mobile if there are no morphological differences.
- Optionally you can use the small pore pipettes and extract solution from the 60 X15 mm plates enough to limit the mobility of the fish larvae and make it easier isolate and extract and add the solution back into the 96 well plate up the fill level of each well. (400 uL)
-

2. Place the zebrafish into the 96 well plate.

- when placing them into the plate make sure to note which fish from set concentrations make sure each well has the same volume of solution. If at all possible 'blind' the set up to another researcher that will be doing data analysis.
- Transporting the 96-well plate

3. place the 96-well plate in a topped plastic Tupperware container that con comfortably contain it and then place the container in another larger container that has a dog diaper in it.
4. Walk to the camera room RBN 4--

- Walk slowly and carefully making sure not to tip the container and not to trip or fall.
- Make sure to take some gloves with you to the room as well as your respirator/ mask and lab coat
- Physical set up for recording.
- Make sure to not turn on the lights of the room and make slow and deliberate actions when moving the 96-well plate NEVER REACHING BEHIND YOU OR STREACHING TO GRAB/ MANIPULATE ITEMS.

5. Don the appropriate ppe and place the 96 well plate on the squared off section of the light plate below the phantom camera.
6. Plug in the power strip at the back of the table behind the camera and to the left of the computer.
7. turn the power strip on and make sure the phantom camera and the computer are getting power

- you'll need to turn on the computer and make sure its charging as its an older laptop that works on windows xp the keys on the computer specifically the number keys.
-

8. Open the phantom camera app

General recipes

Fish care

Embryo water – 1 L RO water + 1.5 mL stock salt solution + 10 uL Methylene Blue

Stock salt solution – 140 g Instant ocean salt in 1 L RO water

8% bicarb – 40 g bicarbonate/ 500 mL RO water

6-OHDA soaking

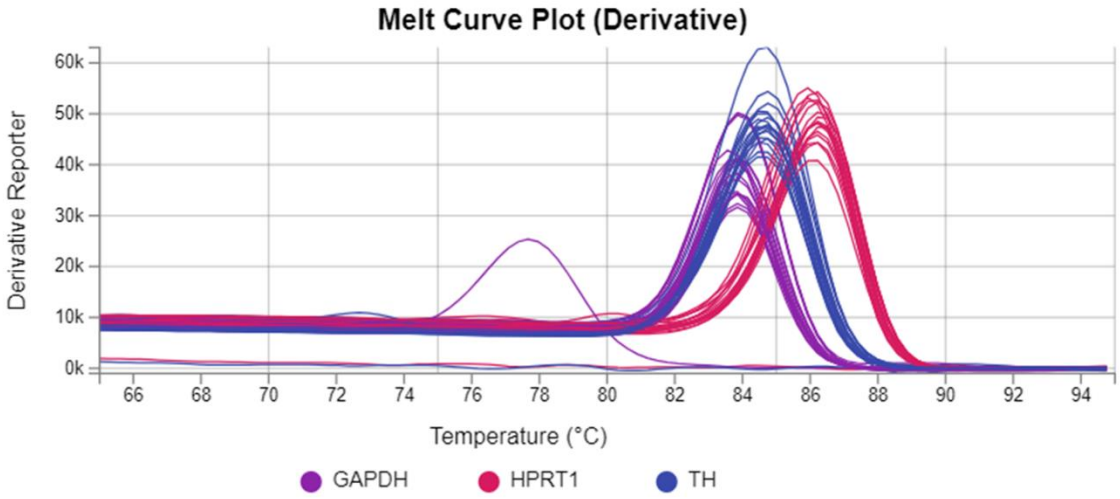
0.2 % L-ascorbic – 2 g L-ascorbic acid / 1L Embryo water or 1L 40 mM HEPES buffered Embryo water

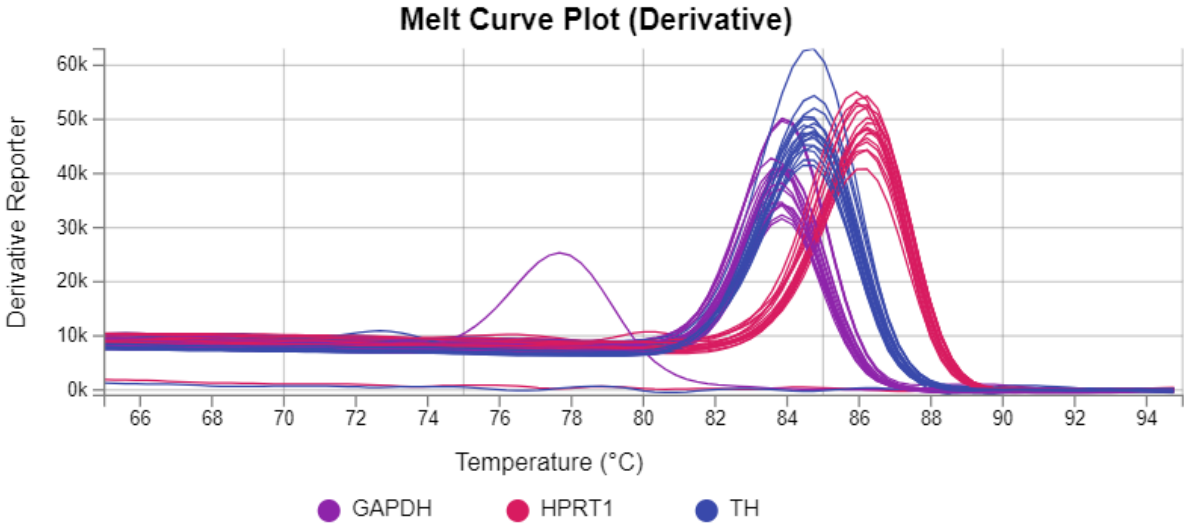
40 mM HEPES – 16.8878 mL 1M HEPES FA + 23.1015 mL HEPES NA + 960 mL Embryo water

1 M HEPES FA – 12.4486 g HEPES FA / 50 mL embryo water

1 M HEPES Sodium salt 13.014g HEPES Na salt/ 50 mL embryo water

Melt and amplification curves





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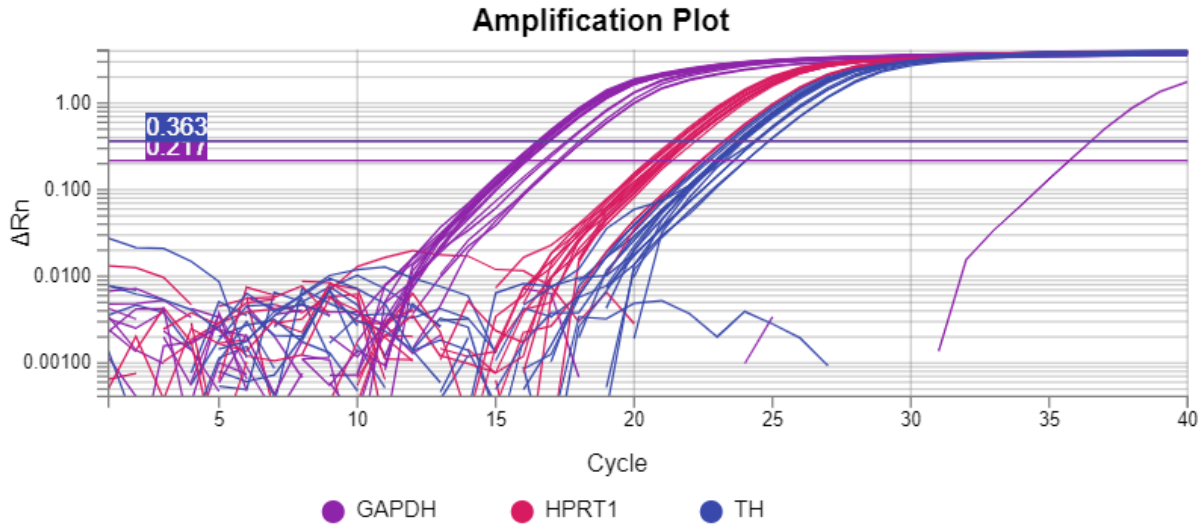
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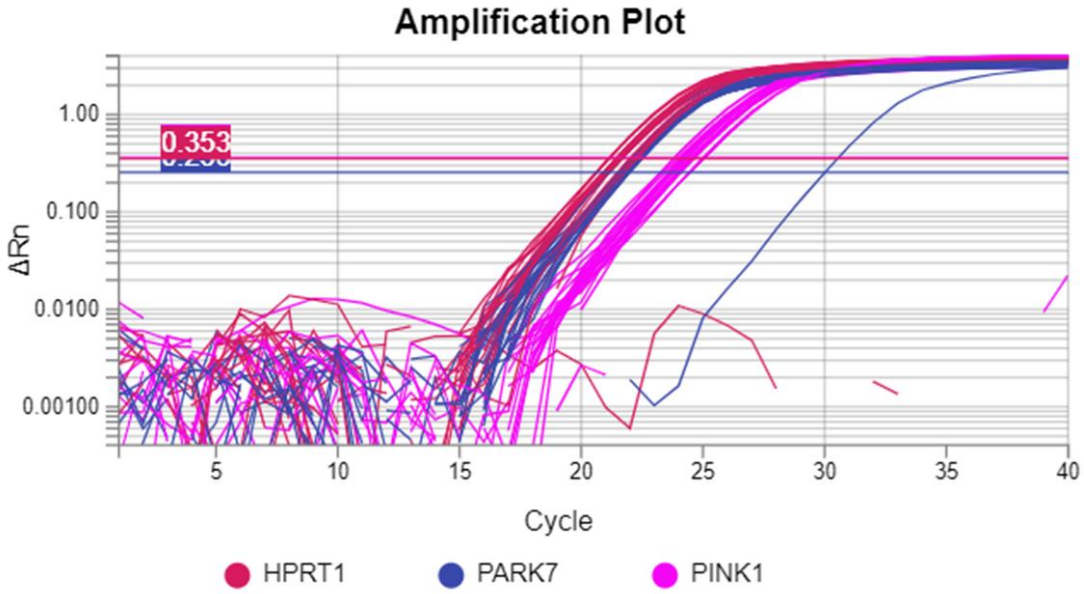
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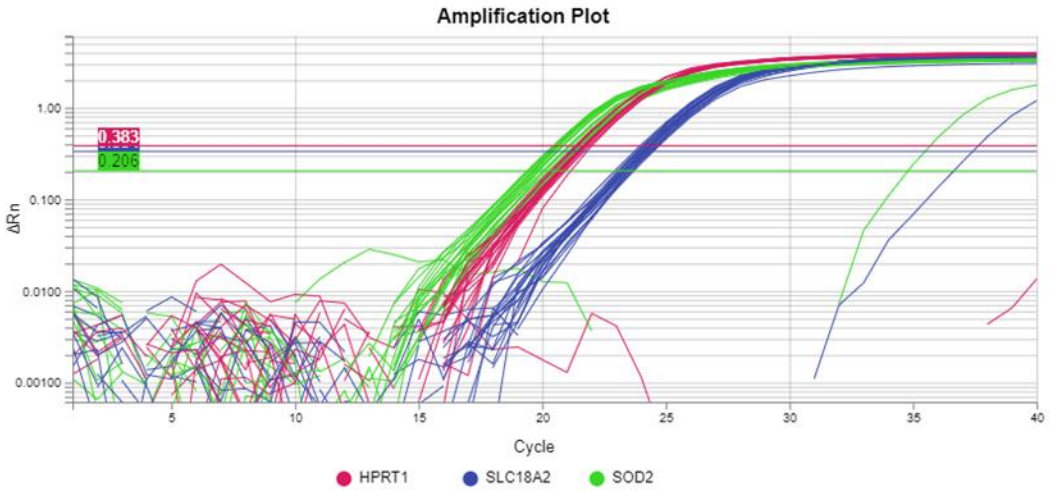
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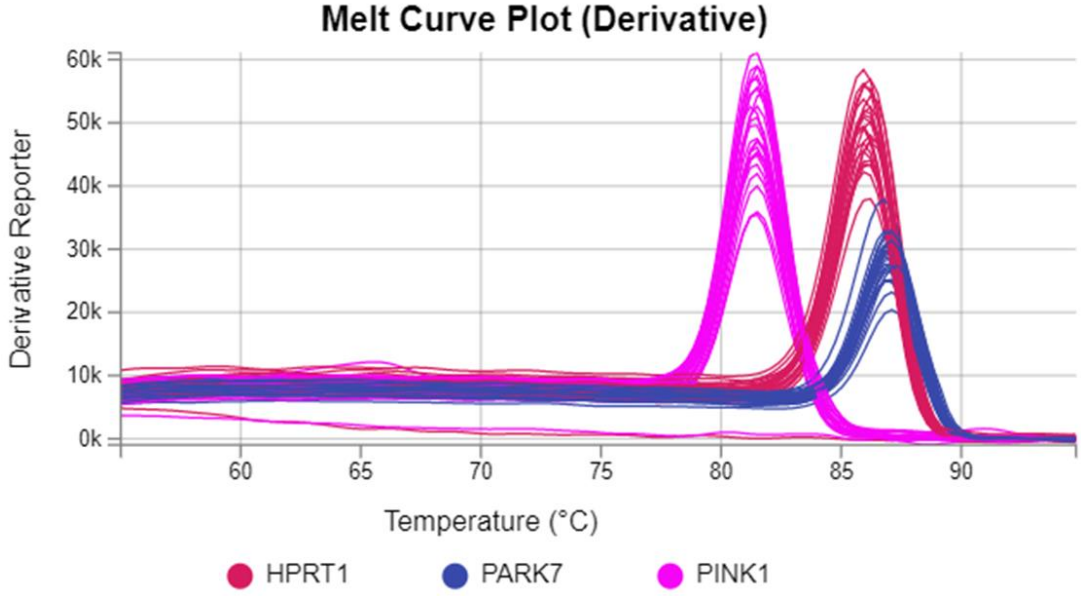
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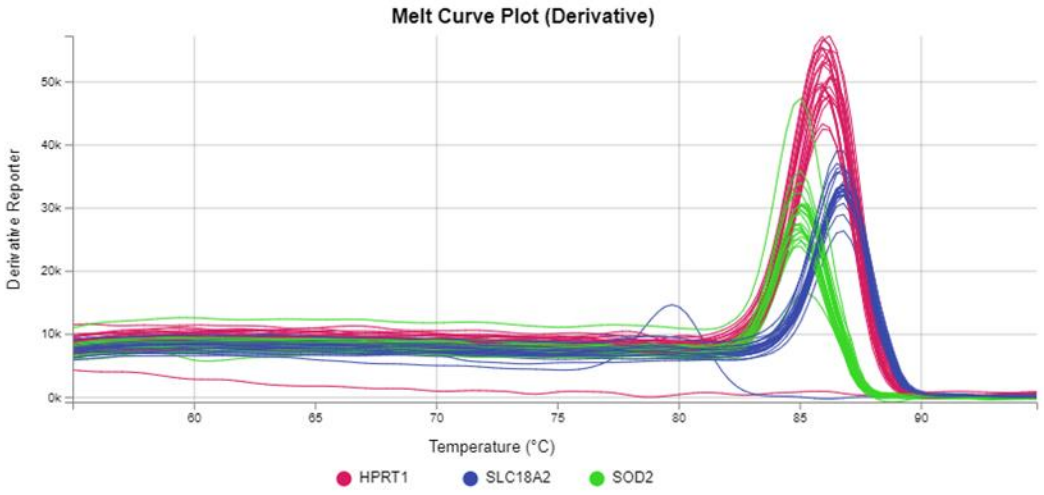
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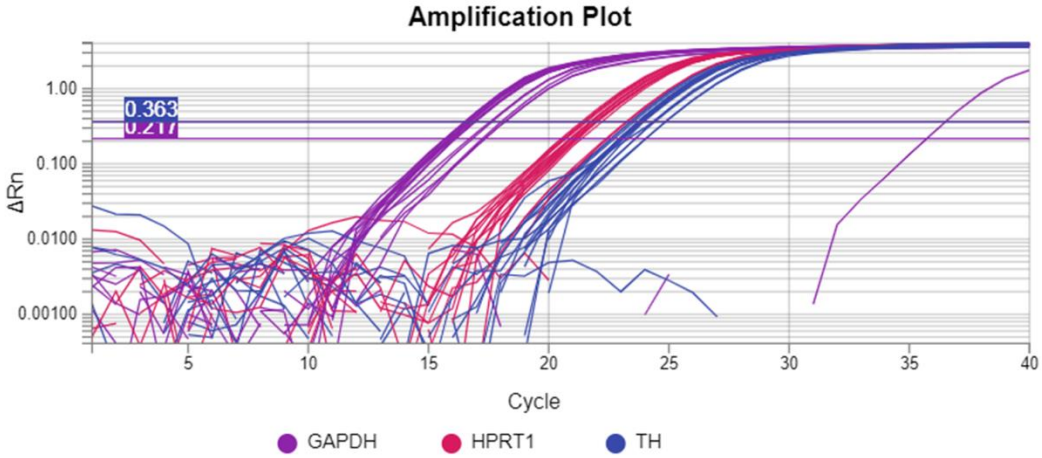
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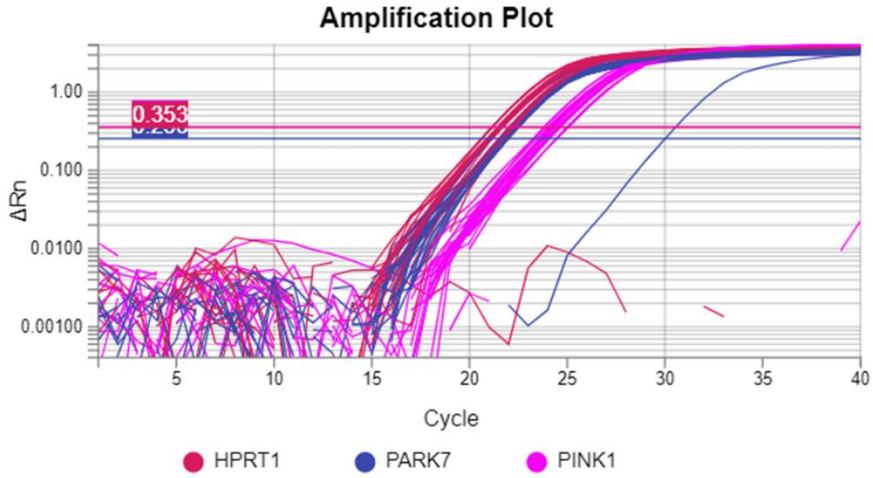
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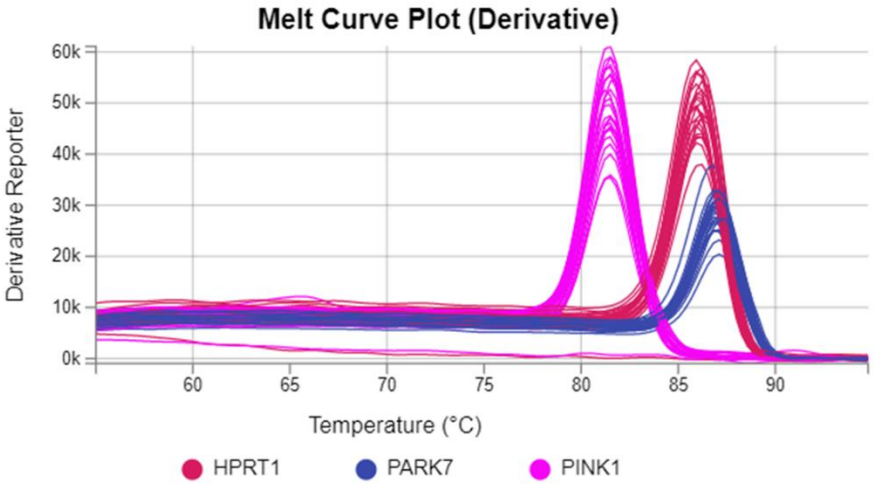
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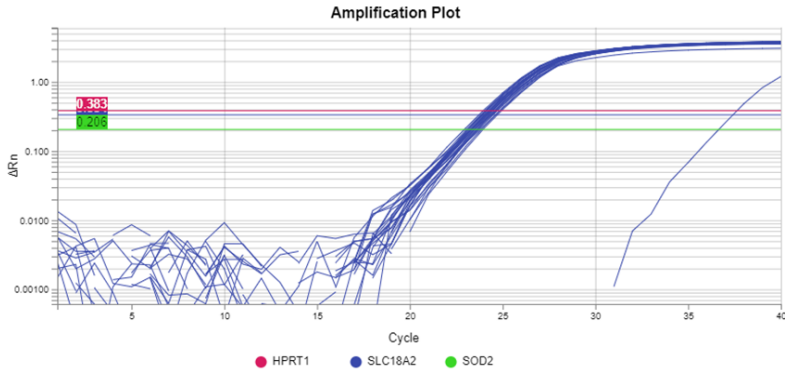
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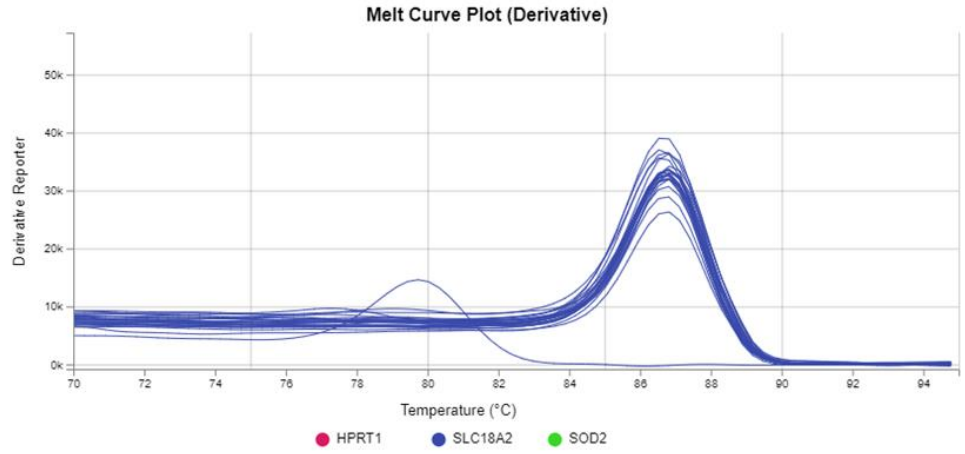
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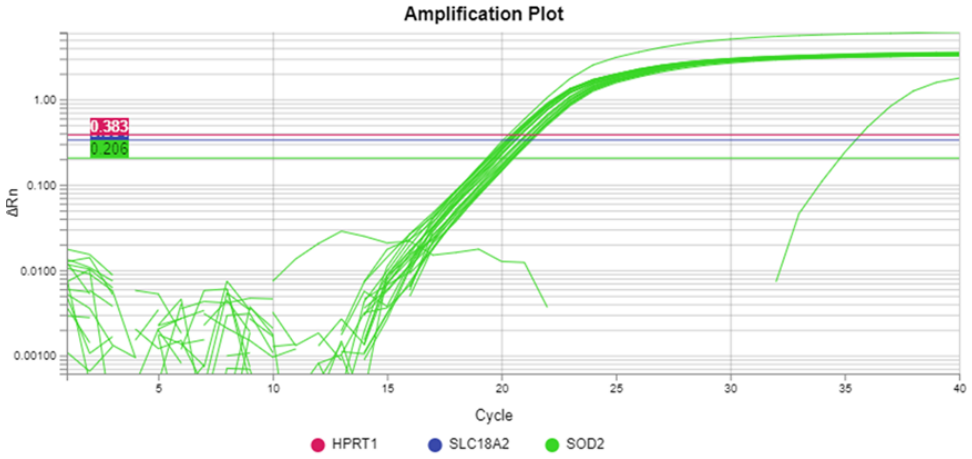
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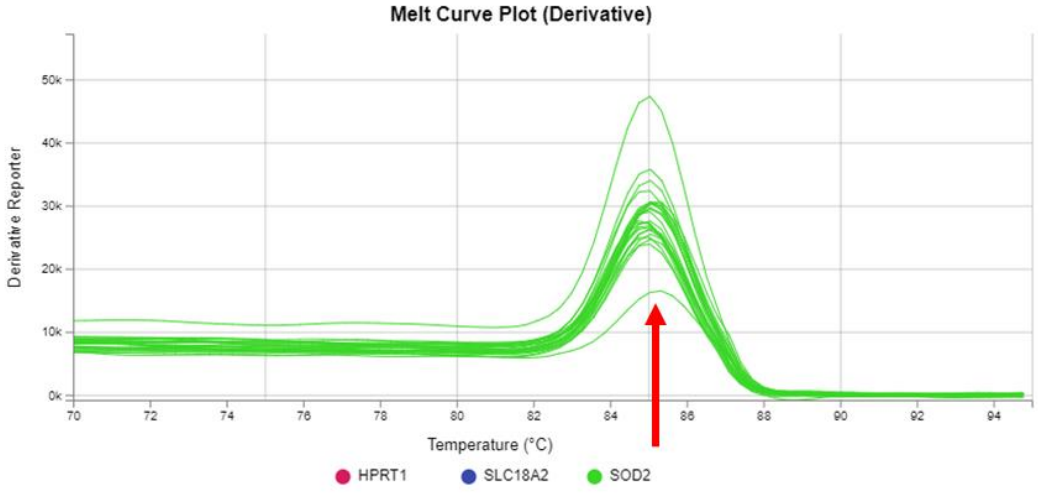
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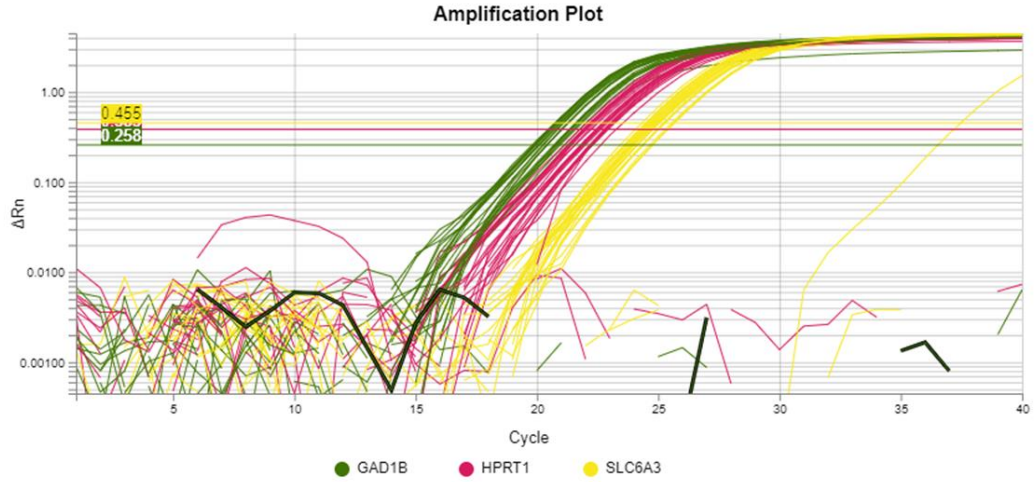
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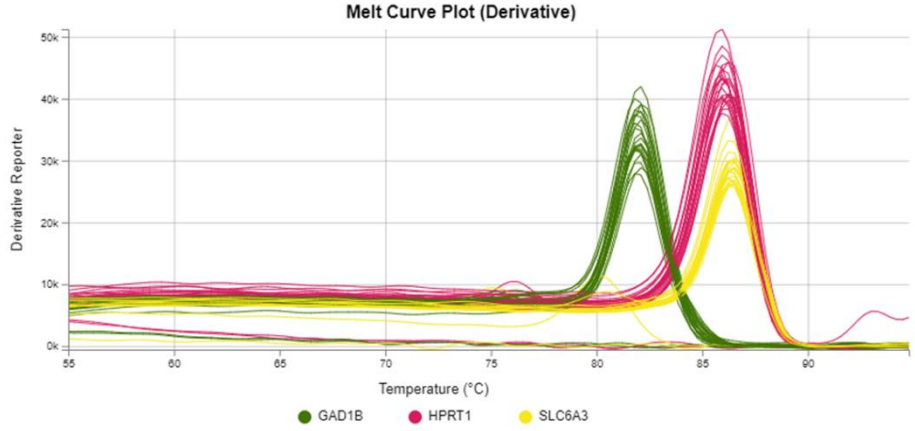
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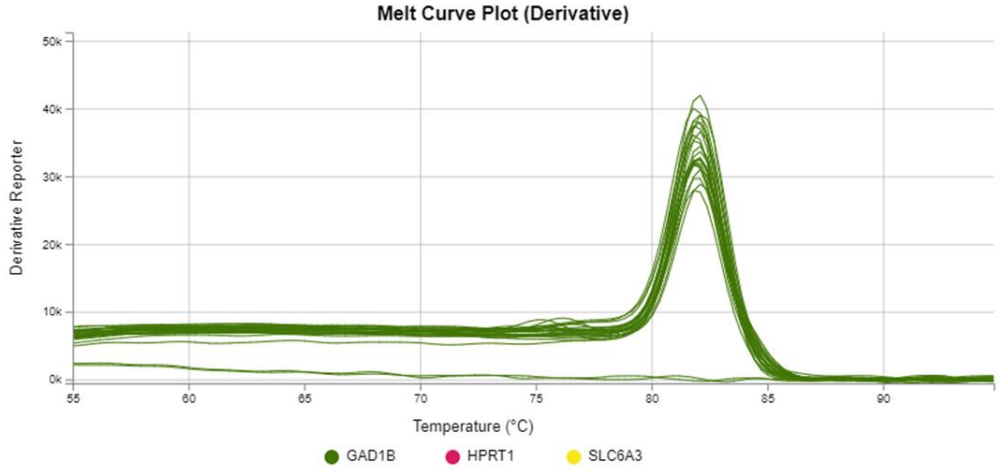
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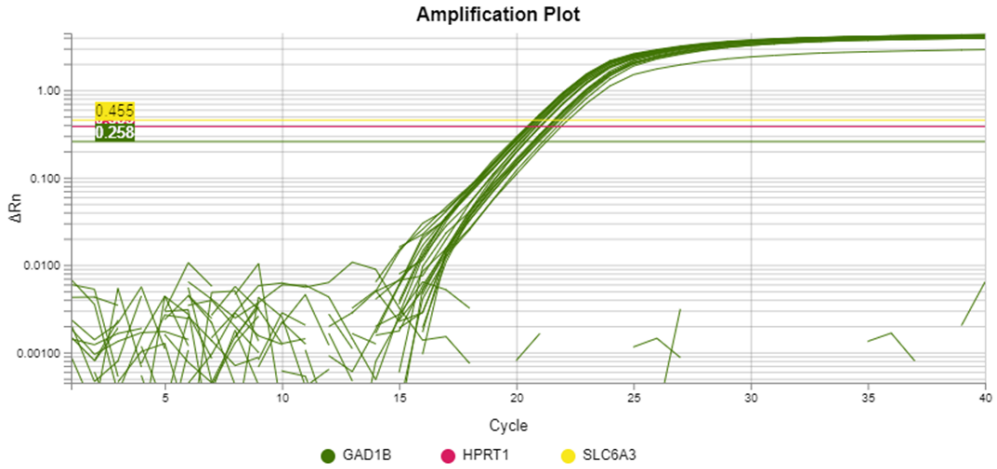
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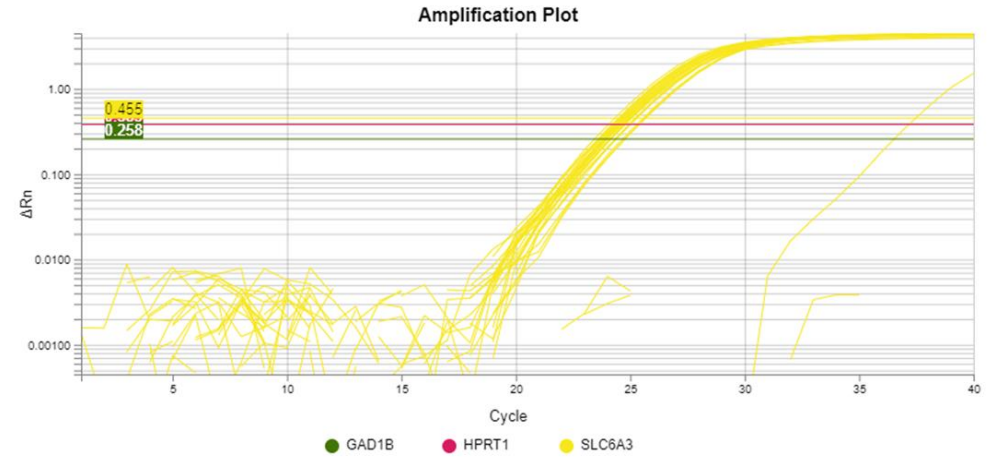
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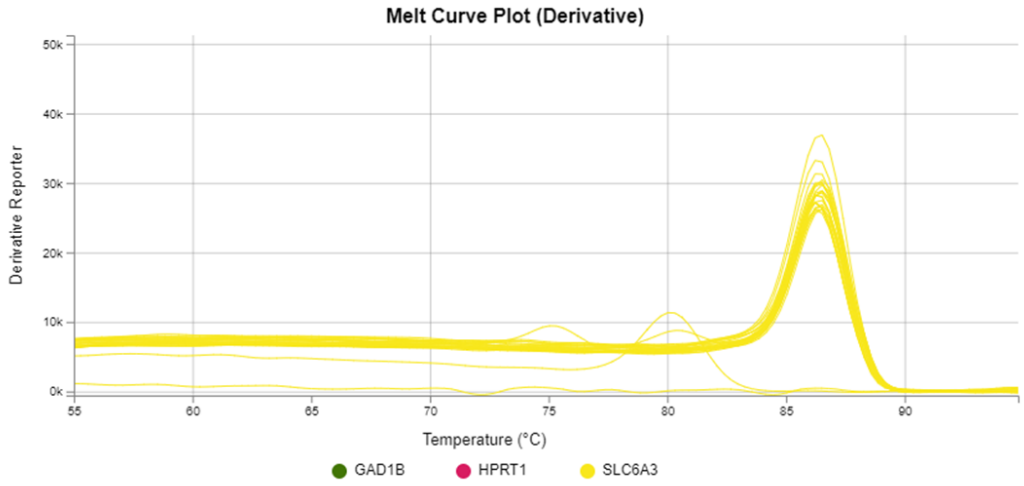
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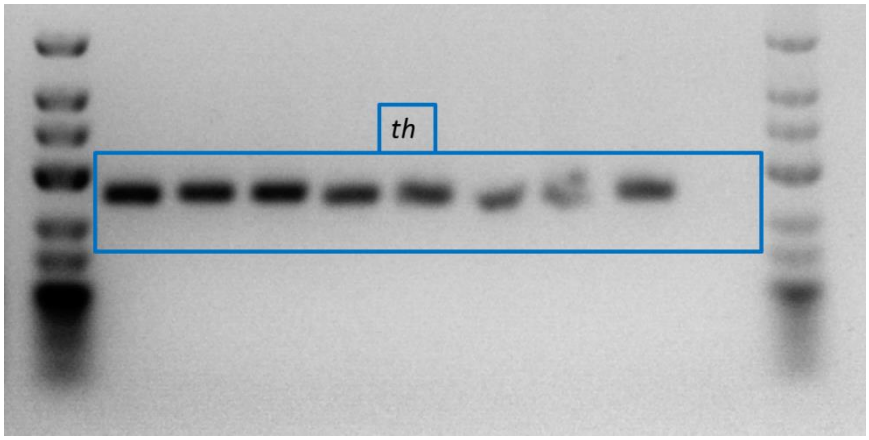
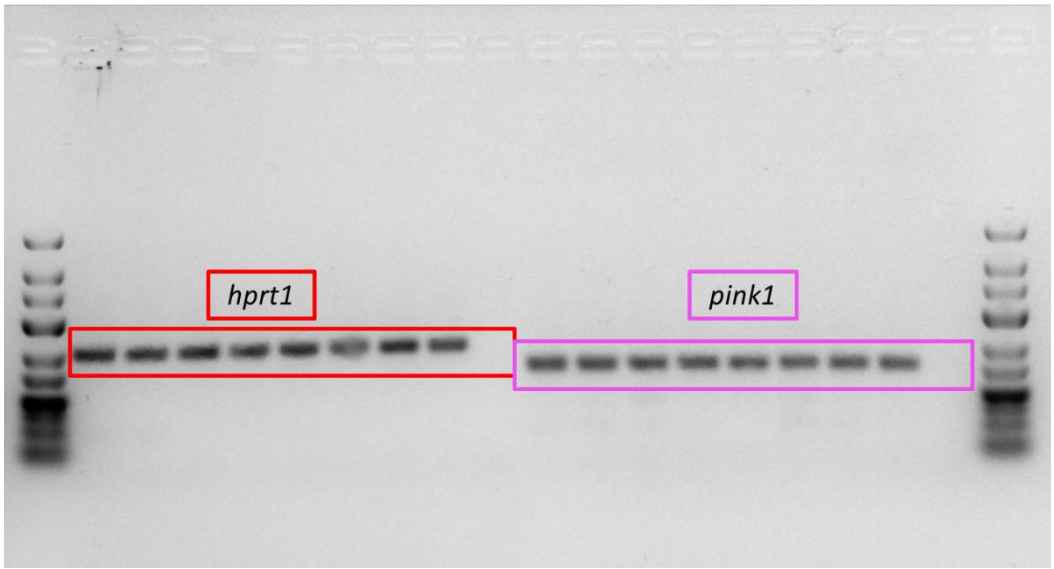
Temperature (°C)
GAD1B
HPRT1
SLC6A3

Gels

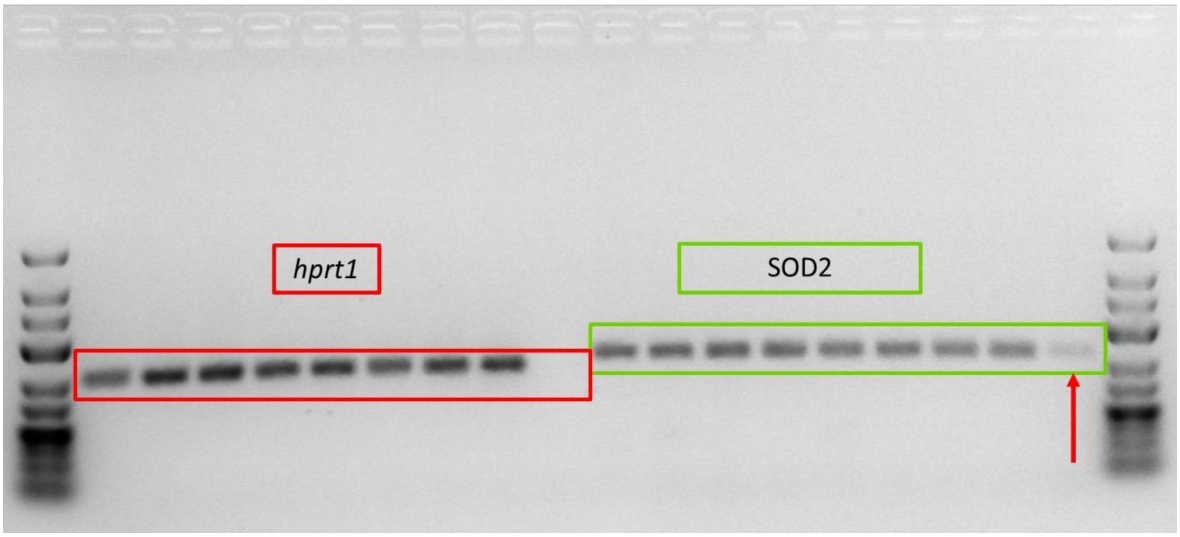
Each gene target has 9 samples, the first 4 left to righ are the different biological groups 0 uM (A through D) the next 4 are 30 uM (A through D) and the last one is the non-template control



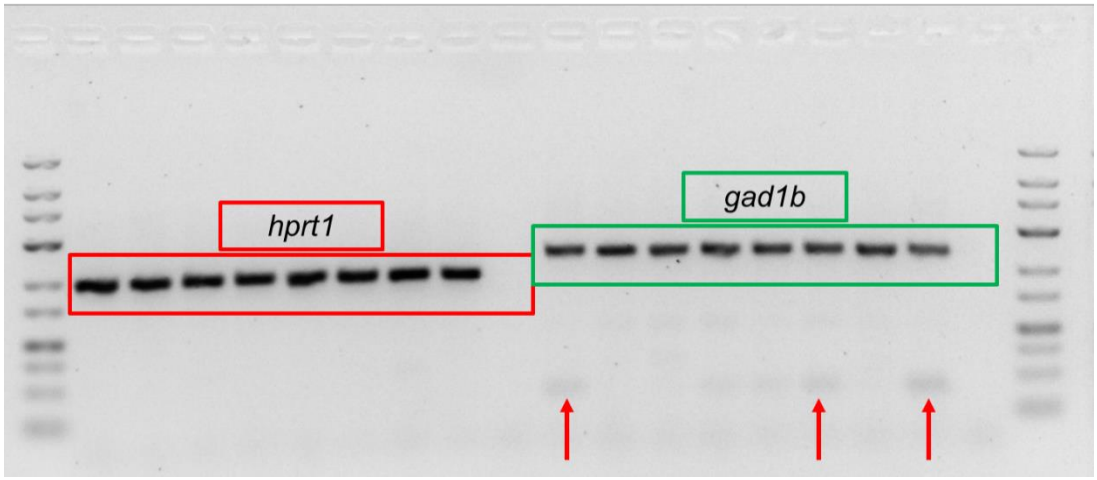
Each gene target has 9 samples, the first 4 left to righ are the different biological groups 0 uM (A through D) the next 4 are 30 uM (A through D) and the last one is the non-template control

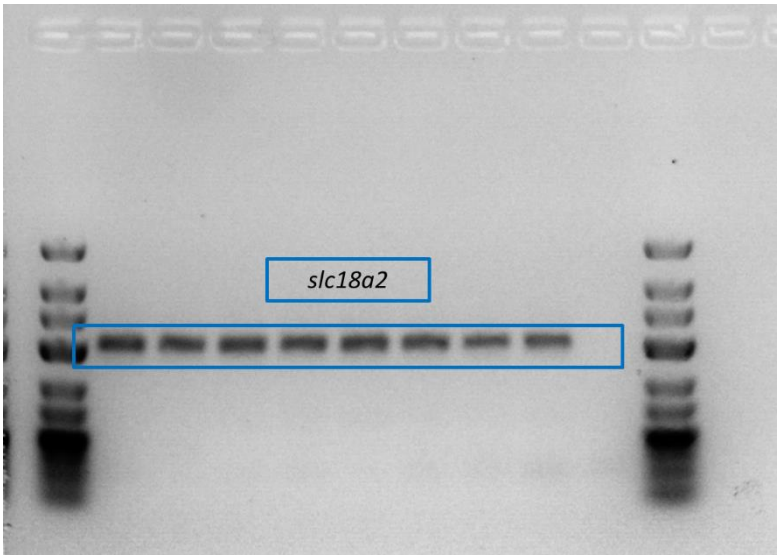


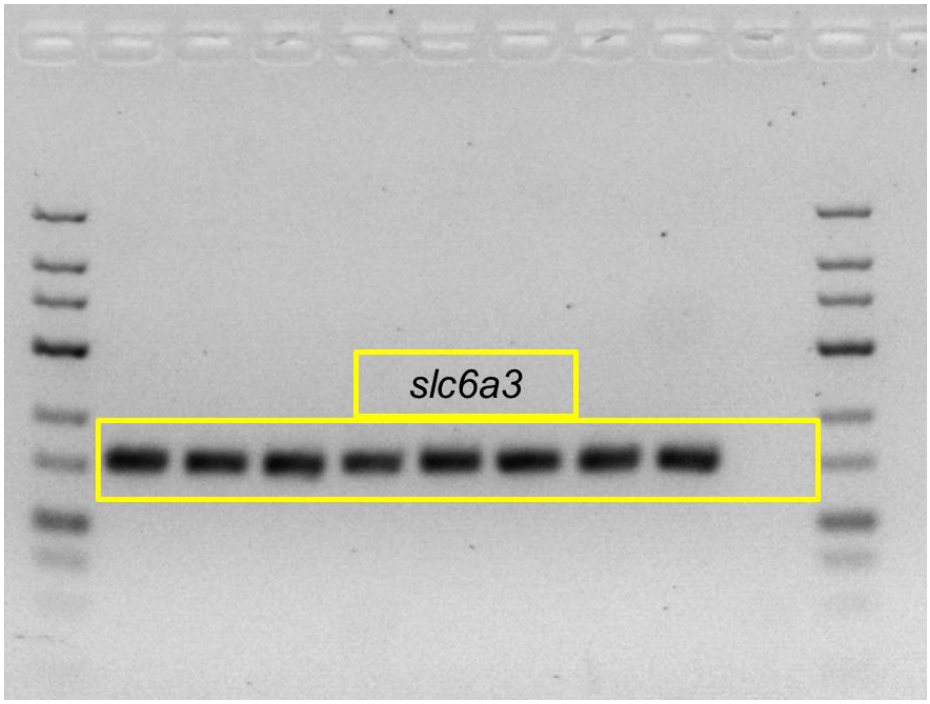
Each gene target has 9 samples, the first 4 left to righ are the different biological groups 0 uM (A through D) the next 4 are 30 uM (A through D) and the last one is the non-template control



Each gene target has 9 samples, the first 4 left to righ are the different biological groups 0 uM (1 through 4) the next 4 are 30 uM (1 through 4) and the last one is the non-template control







6-OHDA soaking trials

Trial	L-ascorbic acid concetration	Methylene blue	HEPES	6-OHDA µM	exposure time	exposure start	collection age	Number of fish	Percent healthy	percent alive	notation
A-1	0.001%	P	0	0	3 days	2dpf	5 dpf	29	100	100	none
A-1	0.001%	P	0	1	3 days	2dpf	5 dpf	30	100	100	none
A-1	0.001%	P	0	10	3 days	2dpf	5 dpf	28	96.42	100	none
A-1	0.001%	P	0	20	3 days	2dpf	5 dpf	30	83.33	100	none
A-1	0.001%	P	0	50	3 days	2dpf	5 dpf	30	70	100	dev delay
A-1	0.001%	P	0	100	3 days	2dpf	5 dpf	29	65.51	100	dev delay
A-1	0.001%	P	0	150	3 days	2dpf	5 dpf	30	36.6	80	dev delay

A-1	0.001%	P	0	250	3 days	2dpf	5 dpf	30	0	0	dev delay
A-2	0.001%	P	0	0	3 days	2dpf	5 dpf	12	100	100	none
A-2	0.001%	P	0	10	3 days	2dpf	5 dpf	12	100	100	none
A-2	0.001%	P	0	15	3 days	2dpf	5 dpf	12	100	100	none
A-2	0.001%	P	0	25	3 days	2dpf	5 dpf	12	100	100	none
A-2	0.001%	P	0	30	3 days	2dpf	5 dpf	12	100	100	none
A-2	0.001%	P	0	40	3 days	2dpf	5 dpf	12	100	100	none
A-2	0.001%	P	0	55	3 days	2dpf	5 dpf	12	100	100	none
A-2	0.001%	P	0	60	3 days	2dpf	5 dpf	12	100	100	none
A-2	0.001%	P	0	70	3 days	2dpf	5 dpf	12	100	100	dev delay
A-2	0.001%	P	0	85	3 days	2dpf	5 dpf	12	100	100	dev delay
A-2	0.001%	P	0	90	3 days	2dpf	5 dpf	12	75	100	dev delay
A-2	0.001%	P	0	100	3 days	2dpf	5 dpf	12	75	100	dev delay
A-3	0.001%	P	0	0	3 days	2dpf	5 dpf	12	100	100	none
A-3	0.001%	P	0	20	3 days	2dpf	5 dpf	12	100	100	none
A-3	0.001%	P	0	30	3 days	2dpf	5 dpf	12	100	100	none
A-3	0.001%	P	0	40	3 days	2dpf	5 dpf	12	100	100	none
B-1	0.20%	A	40 mM	0	1 day	3 dpf	6 dpf	24	95.8	95.8	none
B-1	0.20%	A	40 mM	20	1 day	3 dpf	6 dpf	24	87.5	87.5	none
B-1	0.20%	A	40 mM	30	1 day	3 dpf	6 dpf	24	41.7	41.7	none
B-1	0.20%	A	40 mM	60	1 day	3 dpf	6 dpf	24	16.7	16.7	none
B-1	0.20%	A	40 mM	100	1 day	3 dpf	6 dpf	24	12.5	12.5	none
B-1	0.20%	A	40 mM	150	1 day	3 dpf	6 dpf	24	8.3	8.3	none
B-1	0.20%	A	40 mM	250	1 day	3 dpf	6 dpf	24	0	0	none
B-2	0.20%	P	40 mM	0	1 day	3 dpf	6 dpf	24	95.8	95.8	none
B-2	0.20%	P	40 mM	20	1 day	3 dpf	6 dpf	24	87.5	87.5	none
B-2	0.20%	P	40 mM	30	1 day	3 dpf	6 dpf	24	41.7	41.7	none
B-2	0.20%	P	40 mM	60	1 day	3 dpf	6 dpf	24	16.7	16.7	none
B-2	0.20%	P	40 mM	100	1 day	3 dpf	6 dpf	24	12.5	12.5	none
B-2	0.20%	P	40 mM	150	1 day	3 dpf	6 dpf	24	8.3	8.3	none
B-2	0.20%	P	40 mM	250	1 day	3 dpf	6 dpf	24	0	0	none
B-2	0.20%	A	40 mM	0	1 day	3 dpf	6 dpf	24	100	100	none
B-2	0.20%	A	40 mM	1	1 day	3 dpf	6 dpf	24	100	100	none
B-2	0.20%	A	40 mM	5	1 day	3 dpf	6 dpf	24	95.83333333	95.8333333	none
B-2	0.20%	A	40 mM	10	1 day	3 dpf	6 dpf	24	79.16666667	79.1666667	none
B-2	0.20%	A	40 mM	20	1 day	3 dpf	6 dpf	24	62.5	62.5	none
B-2	0.20%	A	40 mM	30	1 day	3 dpf	6 dpf	24	58.33333333	58.3333333	none
B-2	0.20%	A	40 mM	60	1 day	3 dpf	6 dpf	24	0	0	none
B-3	0.20%	P	40 mM	0	1 day	3 dpf	6 dpf	24	66.66666667	66.6666667	none
B-3	0.20%	P	40 mM	0	1 day	3 dpf	6 dpf	24	79.16666667	79.1666667	none
B-3	0.20%	P	40 mM	0	1 day	3 dpf	6 dpf	24	79.16666667	79.1666667	none

B-3	0.20%	P	40 mM	10	1 day	3 dpf	6 dpf	24	95.83333333	95.8333333	none
B-3	0.20%	P	40 mM	10	1 day	3 dpf	6 dpf	24	91.66666667	91.6666667	none
B-3	0.20%	P	40 mM	10	1 day	3 dpf	6 dpf	24	100	100	none
B-3	0.20%	P	40 mM	20	1 day	3 dpf	6 dpf	24	54.16666667	54.1666667	none
B-3	0.20%	P	40 mM	20	1 day	3 dpf	6 dpf	24	54.16666667	54.1666667	none
B-3	0.20%	P	40 mM	20	1 day	3 dpf	6 dpf	24	58.33333333	58.3333333	none
B-3	0.20%	P	40 mM	30	1 day	3 dpf	6 dpf	24	54.16666667	54.1666667	none
B-3	0.20%	P	40 mM	30	1 day	3 dpf	6 dpf	24	54.16666667	54.1666667	none
B-3	0.20%	P	40 mM	30	1 day	3 dpf	6 dpf	24	58.33333333	58.3333333	none
B-3	0.20%	P	40 mM	0	1 day	2 dpf	6 dpf	24	58.33333333	58.3333333	none
B-3	0.20%	P	40 mM	0	1 day	2 dpf	6 dpf	24	83.33333333	83.3333333	none
B-3	0.20%	P	40 mM	0	1 day	2 dpf	6 dpf	24	83.33333333	83.3333333	none
B-3	0.20%	P	40 mM	10	1 day	2 dpf	6 dpf	24	41.66666667	41.6666667	dev delay
B-3	0.20%	P	40 mM	10	1 day	2 dpf	6 dpf	24	54.16666667	54.1666667	dev delay
B-3	0.20%	P	40 mM	10	1 day	2 dpf	6 dpf	24	75	75	dev delay
B-3	0.20%	P	40 mM	20	1 day	2 dpf	6 dpf	24	4.166666667	4.16666667	dev delay
B-3	0.20%	P	40 mM	20	1 day	2 dpf	6 dpf	24	8.333333333	8.33333333	dev delay
B-3	0.20%	P	40 mM	20	1 day	2 dpf	6 dpf	24	20.83333333	20.8333333	dev delay
B-3	0.20%	P	40 mM	30	1 day	2 dpf	6 dpf	24	4.166666667	4.16666667	dev delay
B-3	0.20%	P	40 mM	30	1 day	2 dpf	6 dpf	24	20.83333333	20.8333333	dev delay
B-3	0.20%	P	40 mM	30	1 day	2 dpf	6 dpf	24	0	0	dev delay
B-4	0.20%	P	40 mM	0	1 day	3 dpf	6 dpf	120	93.44	93.44	none
B-4	0.20%	P	40 mM	30	1 day	3 dpf	6 dpf	120	56.67	56.67	none
B-4	0.20%	P	40 mM	0	2 day	3 dpf	6 dpf	24	91.66666667	91.6666667	none
B-4	0.20%	P	40 mM	10	2 day	3 dpf	6 dpf	24	83.33333333	83.3333333	none
B-4	0.20%	P	40 mM	10	2 day	3 dpf	6 dpf	24	95.83333333	95.8333333	none
B-4	0.20%	P	40 mM	20	2 day	3 dpf	6 dpf	24	45.83333333	45.8333333	none
B-4	0.20%	P	40 mM	20	2 day	3 dpf	6 dpf	24	70.83333333	70.8333333	none
B-4	0.20%	P	40 mM	0	1 day	4 dpf	6 dpf	24	100	100	none
B-4	0.20%	P	40 mM	10	1 day	4 dpf	6 dpf	24	100	100	none
B-4	0.20%	P	40 mM	20	1 day	4 dpf	6 dpf	24	95.83333333	95.8333333	none
B-4	0.20%	P	40 mM	30	1 day	4 dpf	6 dpf	24	66.66666667	66.6666667	none
B-4	0.20%	P	40 mM	60	1 day	4 dpf	6 dpf	24	70.83333333	70.8333333	none
B-4	0.20%	P	40 mM	100	1 day	4 dpf	6 dpf	24	33.33333333	33.3333333	none
B-5	0.20%	P	40 mM	0	1 day	3 dpf	6 dpf	142	88.7	88.7	None
B-5	0.20%	P	40 mM	30	1 day	3 dpf	6 dpf	288	59.3	59.3	None
B-6	0.20%	P	40 mM	0	2 day	3 dpf	6 dpf	75	97.3	97.3	None
b-6	0.20%	p	40 mM	10	2 day	3 dpf	6 dpf	73	69.8	69.8	none

Primer list

Primer	Forward primer seq	reversel primer seq	PCR band	Sequcing result	qPCR meltcurve	qPCR band	primer efficency
SOD1	CGTCGGAGACCTGGGTAATG	TTCCTCATTGCCACCCTTCC	NB	-	-	-	-
SOD2 (2)	TAGGTGCTGTGACCTCCAGA	TCAATGCAGGCTGAAGGGAG	SB	G	G	G	98%
SOD3A	CCAAGTCCTCTTCAGGCAGG	AGAAACCGCCGGATAAGTCC	SB	RP			
SOD3B	GACAGTCTGGCCCAAAGGAA	GTTGCAGGGGATTCCAGTGA	SB	RP			
GAPDH	TGAGGTTAAGGCAGAAGGCG	AAGTAGCACCTGCATCACCC	SB	G	G	G	107%
HPRT1(1)	TATGCGGCTGACTTAGAGCG	TTGAGGCGGATGAAGTCCAC	NB	-	-	-	-
VDAC3	ACGGCAAAACACGCAAACAT	TGGTCTCAAGGTTTCCAGCG	SB	RP			
PINK1 (3)	GGGAAAGGTTGTAACGCAGC	GAAGCAGAAAGTCGAAGCGG	SB	G	G	G	91%
PRKN	GCGAGTGTGTCTGAGCTGAA	CACACTGGAACACCAGCCACT	NB	-	-	-	-
TH	TACATACGGCACGCTTCCTC	CAGTCCTGCTCCATACGCTT	SB	G	G	G	93%
B2M	ACTGCTGAAGAACGGACAGG	GCTGCCCGGTTTGGATTTAC	MB	-	-	-	-
SKP1	TGGAGAGATGTTCGAGGTGGA	CGGCGTTTACATTGGGCAG	SB	G	Ng	-	-
SRSF7A	GAATCGCCGTTTCCTCCTTTC	CGAGAGGAACTCCGGGATGA	SB	RP			
PARK7	CGAGAGGAACTCCGGGATGA	GTTCTGGGCTCCAAGCAAAC	NB	-	-	-	-
SLC6A3 (2)	GCCTGGTTTTACGGAGTGGA	GGAGGATTGAAGGTGGCGAA	SB	G	G	MB	-
TIGARB	GTAGGGATTACACACCGCCC	GATAAACGCCCCGTGACTGA	NB	-	-	-	-
SLC18A2 (1)	ATTGCATTTGTGCCAGCCAG	TCAGCAATGGCGTACACACT	SB	NG	-	-	-
SLC18A2 (2)	ATTGGGATTGCACTGGGAGG	GGAACCTGCTGCAATGAGGA	NB	-	-	-	-
SLC18A2 (3)	ATTCTTTGCGTGCCTCTTGC	ATGATGGTCATGAGCCAGGG	SB	G	NG	-	-
NR4A2B (1)	CAAAAGTGCCTGGTCGTTGG	ACAGGGATATCCTGCGGACT	NB	-	-	-	-
NR4A2B (2)	GGTGCTCAACGGCATTTGTT	TGTCCCATACTGAGCCTGGA	MB	-	-	-	-
NR4A2B (3)	AAATCCCCGGCTTTACCGAG	GGGCTCCTTTAGTCCGTGTC	MB	-	-	-	-
NR4A2B (4)	CATTCCCTAAACGTCGGGCA	TCTGCACCGTGCGCTTAAA	NB	-	-	-	-
NR4A2B (5)	CCCATTCCCTAAACGTCGGG	CTGCACCGTGCGCTTAAAGA	SB	DNC			
NR4A2B (6)	ACGAGTCCGGAGTATCACAGT	CTGGCAGGTTGGATCTGTACG	NB	-	-	-	-
SLC18A2 (4)	GACGGTGGTCGTGCCTATT	TGGTCATCTGCTTTGGGACA	NB	-	-	-	-
SLC18A2 (5)	GCATGGGTTTTGCTTTAGGTCC	GCCATCTTTTCCTCGTTGGC	NB	-	-	-	-
SLC18A2 (6)	ATCCTCATTGCAGCAGGTTCC	CGGCAAAGATGTTGGTCCCT	NB	-	-	-	-
SLC18A2 (7)	TCATCAAGCTACACGCTGCT	TGCCAAATGGAGGACCAACTAA	NB	-	-	-	-
SLC18A2 (8)	ACATCCTCATTGCAGCAGGTT	TAAGTGAGCACAGCCATCTCC	NB	-	-	-	-
SLC18A2 (9)	ATGATGGAGACCATGTGCCC	TTTGCAAGAGGCACGCAAAG	SB	NG	-	-	

SLC18A2 (10)	GCATGCTGCTGGTTGGAATC	ACCTGCTGATGGACCTAAAGC	SB	NG	-	-	-
SLC18A2 (11)	TGACGGTGGTCGTGCCTATT	GAGGCAAACAACAGACCCAC	SB	G	G	G	92%
SLC18A2 (12)	CTCACAAAATGGGGAGATGGC	AAAACCCATGCAAAAGGCGA	SB	G	G	G	95%
SLC6A3 (3)	GCCACCTTCAATCCTCCCAA	CCAGTGATGGAGCGTGAACT	SB	ng	-	-	-
SLC6A3 (4)	GCCTGGTTTTACGGAGTGGA	CGCCAGTTTGTCACAGAAGC	SB	G	NG	NG	-
SLC6A3 (5)	CGCCTGTATGATGCTCAGGT	CAGTGTCGCAATGGCTTCTG	SB	multi hit blast	-	-	-
SLC6A3 (6)	TGCTACAAGAATGGCGGAGG	GTAGGAGCCCACATACAGCG	SB	NG	-	-	-
SLC6A3 (7)	TATAACCGTGAAGGAGCCGC	CGTGGTCTTGTACGTGTCGT	SB	NG	-	-	-
SLC6A3 (8)	CCCTGGATCCACTGCAACAA	GTCAGGACCACATACGGCAT	SB	NG	-	-	-
SLC6A3 (9)	TACCCAGAAGCCATTGCGAC	TGACGCAGATGAGCGAGATG	SB	NG	-	-	-
HPRT1(2)	TTGCAGTAGCTTGTCGGTGT	CAGACGTTCAGTTCGGTCCA	SB	NG	-	-	-
HPRT1(3)	CCGAGAGTTGAGGACCGTTT	AGACGTTCAGTTCGGTCCAT	SB	G	G	G	98%
HPRT1(4)	CCACGCTAACAGGAAAGAACG	CTGTATCCAACGCTCCTCGG	SB	NG	-	-	-
HPRT1(5)	CCCACAGTGTAACGTGAGGAA	CTCATCGCTGATCACGACAC	SB	RP	DNC	-	-
HPRT1(6)	GGCTCATCATGGACCGAACTG	TCACTGTTGCGATTAAGGGCT	SB	RP	DNC	-	-
HPRT1(7)	CCGCCTCAAGAGTTACCAAAATG	GGTGTCCTCTTCACCAGCAA	SB	RP	DNC	-	-
HPRT1(8)	ACCAAAACACTATGCGGCTG	ACCTGTGGACTGGTCATTTTGG	SB	RP	DNC	-	-
HPRT1(9)	GGACCGTTTTGCAGTAGCTTG	GGCCAGACGTTCAGTTCGGT	SB	RP	DNC	-	-
BACTIN	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC	SB	NG	-	-	-
TUBA	CCTGCTGGGAACTGTATTGT	TCAATGAGTTCCTTGCCAAT	SB	G	NG	-	-
G6PD	GTCCCGAAAGGCTCCACTC	CCTCCGCTTTCCTCTC	NB	-	-	-	-
TPB	CGGTGGATCCTGCGAATTA	TGACAGGTTATGAAGCAAAA CAACA	NB	-	-	-	-
B2M	GCCTTCACCCCAGAGAAAGG	GCGGTTGGGATTTACATGTT G	NB	-	-	-	-
ELFA	CTTCTCAGGCTGACTGTGC	CCGCTAGCATTACCCTCC	SB	G	G	G	99%
18S	TCGCTAGTTGGCATCGTTTATG	CGGAGGTTCGAAGACGATC A	NB	-	-	-	-
cyp19a1b	AAAGAGTTACTAATAAAGATCCACCGGTAT	TCCACAAGCTTTCCCATTTC A	NB	-	-	-	-
cyp1a	GCATTACGATACGTTCGATAAGGAC	GCTCCGAATAGGTCATTGAC GAT	SB	RP			
slc6a3	GCCACCTTCAATCCTCCCAA	CGCCAGTTTGTCACAGAAGC	SB	G	G	G	102%
sod1	TCCACGTCCATGCTTTTGGT	TCAGCGGTCACATTACCCAG	SB	G	G	G	105%
gad1b	TGTGTCCGATGGCTTGAGTC	CACACGGAGGATGGTTCACA	SB	G	G	G	92%

Primers: NB - no bands, SB - single band, MB – multiband, G – good, RP – results pending, NG – not good, DNC – do not continue