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NORGES
ARKTISKE
UNIVERSITET

DEPARTMENT OF PHARMACY

Pharmacology research group

ZEBRAFISH AND SAFETY PHARMACOLOGY

**EFFECTS OF ETHANOL ON EARLY BRAIN
AND EYE DEVELOPMENT.**

Amina Mohammed Ali

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Supervisor: Associate Professor Ingvild Mikkola



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ABSTRACT

Ethanol is well studied with regard to its teratogenic effects in different animal models including humans. Exposure of a fetus to alcohol during pregnancy can lead to fetal alcohol syndrome (FAS), or ethanol mediated toxicity in animal models.

During early embryogenesis, development of the most important organs including the central nervous system (CNS) occurs. Disturbance of any function that contributes to the normal development can lead to defects and dysfunctions of the brain and other important organs.

Zebrafish (*Danio rerio*) is a small fresh water fish increasingly being used as a model system for human disease, drug development and safety pharmacology.

It is also a perfect model for studying the effects of ethanol on molecular level due to the similarities between zebrafish and humans with regards to genetic cascades and signaling pathways during early embryogenesis.

Genes that are members of Pax and Atoh families of transcription factors are expressed in specific parts of the brain. Visualization of gene expression by using *in situ* RNA hybridisation can provide useful information about the brain development of embryos exposed to drugs compared to their normal counterparts. An important part of the project was therefore to isolate RNA and make cDNA, so that the Atoh1a and Atoh1c genes could be cloned and used as probes for *in situ* hybridisation. Probes for *in situ* hybridisation with Pax6.1 and Pax2.1 were already available.

Zebrafish embryos were incubated in different concentrations of ethanol 0.01 %, 0.1 %, 1 % and 2 % for three days. After every 24 hours within the three days the embryos were observed in a dissecting microscope and development changes and mortalities were recorded. Embryos incubated in 2 % ethanol were overall deformed, while embryos incubated in 1% ethanol developed malformations including pericardial edema, yolk sac edema, axial malformations, axial blistering and truncated body axis. Some of the embryos had late development and were inactive. *In situ* hybridisations were done to look for changes in the brain and eye morphology. In addition, changes in Pax6.1 gene expressions were paid extra attention because it is previously reported that ethanol decreases the level of Pax6 expression.

This was confirmed by *in situ* hybridisation of 72 hpf embryos and by Western blot of 24 hpf embryos.

ABBREVIATIONS

ZF- Zebrafish

ZFE- Zebrafish embryo

ICH-International Conference on Harmonization

ADME- Absorption, distribution, metabolism and Excretion

MHB-Midbrain-hindbrain boundary

NCE- New chemical entity

HPF- Hours Post fertilization

MO- Morpholino oligonucleotide

FAS- Fetal alcohol Syndrome

FASD- Fetal alcohol related disorder

ARBD- Alcohol related birth defects

ARND- Alcohol related neurodevelopment disorder

HTS- High throughput-output screening

CNS- Central nervous system

HSPGs- Heparan Sulfate proteoglycan

DPF- days after postfertilization

SHH- Sonic hedgehog

FGF- Fibroblast growth factor

DNA- Deoxyribonucleic acid

RNA- Ribonucleic acid

RT- Room temperature

O/N- Over night

RPM- Round per minute

SAP- Shrimp alkaline phosphatase

RT-PCR- Reverse Transcriptase Polymerase chain reaction

PCR- Polymerase chain reaction

ETOH- ethanol

ISH- *In situ* hybridisation

WB- Western blot

HRS- hours

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1. INTRODUCTION

1.1. Zebrafish and safety pharmacology

Safety Pharmacology studies are defined as those studies that investigate the potential undesirable pharmacodynamics effects of a substance on physiological functions in relation to exposure in the therapeutic range or above (1).

This method is used to find out about unknown or possible adverse effects of drugs before they are out of the market. It is also a way of finding out interaction of a molecular target different from the intended one hence causing toxic effects.

International conference on Harmonization (ICH) approved the S7A guidelines in 2000 for safety pharmacology. This requires the pharmaceutical companies to take safety assessments of drugs under (Good laboratory practice) GLP in order to secure on the possible toxic effects of drugs before they are tested on humans (2).

ICH guides provides the battery core that each new chemical entity (NCE) need to be evaluated on, including the Cardiovascular, CNS and the respiratory system (3).

The number of NCE submitted to FDA have been declined about half since 1997. FDA points in a recent report that technological deficits in toxicology as the primary cause of the pipeline problem. New animal models are needed to test the safety novel drug candidates and the FDA report that an estimated 10 % improvement in predicting failures before clinical trials would save US \$100 million per drug in development costs. In addition to outdated technologies toxicology often suffers by being divorced from the drug discovery process. Efforts to discover leads happens simultaneously as the toxicology assessment, some efforts are made to discover the toxicology in early discovery process but much more progress is needed to develop better animal models for toxicological assessment and to discover toxicology earlier in the drug discovery process (4).

Models like mice, rabbits, rats and dogs have been used for drug testing in decades, but handling of the animals is time consuming and expensive.

Mammalian models of absorption, metabolism, distribution, excretion (ADME) pharmacokinetics are expensive, laborious and require large amount of compound. Besides the consideration that it requires legal welfare legislation, there is an increase pressure to limit animal use in situations, which are absolutely necessary, nevertheless that it requires dissection of a whole animal, which makes the zebrafish a promising model for studying toxicology. Most of the drug companies prefer zebrafish as their first model in clinical trials because of its size and less cost. It is also said that the embryos are unlikely to consider pain and sufferings (0-5dpf) (5).

During the past five years new methods for in vivo drug assay have been discovered which make zebrafish the perfect model for studying disease-modeling, lead-target discovery for compounds and toxicology. Once a lead target has been identified in vitro HTS on target binding can be used to identify the novel structures that modify the binding of the target protein. Practically zebrafish is a perfect model because in vitro assays require small quantities of compounds, takes less space and it's a simpler phenotype in comparison to mammalian assay (4).

1.2. The zebrafish and its benefits

Zebrafish (*Danio rerio*) originally predominate from South East Asia and are broadly distributed in parts of Pakistan, India, Nepal, Bangladesh and Mynmar and was introduced as a genetic model organism by George Streisinger in the late 1960s (6, 7).

Zebrafish have emerged in the past twenty years as a useful vertebrate for studying genetics and development of human diseases, and it has several characteristics that make them preferably beneficial instead of other model organisms including:

Small size and less economical costs

The small size of the embryos (1-2 mm long) makes it easier for researching on a 96-well dishes and it requires less costs on reproduction and to maintain them. Their small size makes them more useful for microscopic assays and other types of screening.

One pair of zebrafish can lay 200-300 eggs during spawning and the same pair of fish can be used to yield again after 5-7 days (8).

Rapid generation time

Zebrafish embryos develop quickly from the time the eggs are spawned. Since fertilization is outside the mothers (intra-utero) gastrulation starts at 6hpf and embryogenesis is completed around 96hpf (9), followed immediately by the segmentation stage where the somites are formed. By 24 hpf, somitogenesis is completed and many organ rudiments have been laid down. Embryos are motile and motility has become touch evoked by 28–30 hpf resulting in the first behavior, the startle response. By 5 days post-fertilization (dpf), embryos start feeding suggesting that most organs have reached a functional state by this time (10). At 48 hpf the zebra fish has generated parts of the organs and organogenesis is completed (11).

Optical transparency

Transparency of embryos at early stage of embryogenesis makes them easy to be visualized intact. The optical clarity of the embryo becomes even more useful when combined with fluorescent markers that highlight the locations or activities of specific populations of cells. Optical transparency makes it easier to determine phenotypic changes during mutagenesis screening and finding out toxicity during toxicity assay. Methods like RNA In situ hybridization and immunochemistry can be used to screen for chemical-induced abnormalities in the expression of specific genes (8).

Genetic similarities

Zebrafish share genetic similarities to humans and tractability in forward and reverse genetic screens. Mutations have been screened and over 400 genes have been cloned, Screen for diseases like Polycystic kidney disease, cholesterol processing, tissue generation, heart disease, anemia's, cancer and nervous system (4).

Another thing is high nucleotide sequence similarity in comparison to humans, including morpholino oligonucleotides screen (6, 12).

There is 80% of genes that are analyzed which link zebrafish to human genome (13)

Morpholino oligonucleotides (MOs) are antisense nucleic acid analogs that have ribosides converted to morpho- lines (C₄H₉NO) and a phosphorodiamidate intersubunit linkage instead of phosphodiester linkage. They work by binding to, and blocking translation of specific mRNA. MOs have been shown to successfully knockdown gene expression in zebra fish embryos (8).

By systematically knocking down many genes, it should be possible to identify gene knockdowns that prevent or slow the development of the disease phenotype

Genetic screening is an efficient way of individualizing the roles of different genes in disease process. They possess a beneficial route in identification and validation of novel drug targets (4).

High Through-output screening

The modern concept of High throughput put screening (HTS) involves automatic robotic system that process basic screening procedure, which would involve obtaining zebrafish embryos of the same development stage, loading them into multi-well plates 96, 384 or more, dosing the plates and imaging or otherwise to obtain the data on changed elicited but the drugs. HTS can be used to assess many ADME issues upfront. Phenotype screening have been described as chemical genetic screen, therapeutic screen, transgene assisted screen and pathway reporter screen (14).

There are several levels of images that can apply on HTS toxicity analysis.

White field microscopy which detects growth defects on the embryo development, Including egg coagulation and other deformities. Structural changes implicating specific tissues can be performed using transgenic zebra fish lines harboring fluorescent derivatives of specific proteins.

Transgenic zebrafish lines can be used to identify tissues for specific proteins by using fluorescence assays that monitor the expressions. If the output reflects changes in the fluorescence intensity, giving results that are comparable to real time PCR assays, with the advantage that fluorescence signal can be followed in vivo during the normal development of the individual. This method allows the monitor of primary transcriptional effects, which is useful to determine the mode of action of a given substance or to predict its overall toxicity even at lower concentrations.

The use of cameras combined with appropriate algorithms allows the recording of reaction base phenotypes particularly important when dealing with neuroactive compounds. Similar methods can be used to monitor heartbeat and blood flow (5).

1.3. Zebrafish and toxicology

The zebrafish have been used for researches and it has been very beneficial in finding toxicological effects of different compounds. It has been used to find preclinical effects, safety and efficacy of drugs.

Zebrafish can be used as a model to test different diseases in human such as cardiovascular diseases, renal impairment, cancer, hearing loss, homeostasis and anemia, as well as neurological disorders (15).

It has a very simple nervous system that can be used to study neurotoxicology, and its transparent at early stages makes it even easier to study the neural structures and malformations.

Toxicology in zebrafish requires small quantities of compounds (μg) in comparisons on testing in other organisms like mammals (4)

Similarities between zebrafish and humans:

Different studies have found out that mechanism of how drugs works are being conserved between zebra fish and mammals. Small toxicity responses have been discovered on areas like the endocrine system, reproductive toxicity, behavioral defects, teratogenesis, carcinogenesis, cardio toxicity, ototoxicity, liver toxicity and so on. 50-70 % of the way chemicals affects the cell cycle in mammals is similar to zebra fish cell cycle. This may reflect to high degree of amino acid frequency between zebra fish and human drug targets. Other studies show even great degrees of similarity up to 95 %, this might be cause of the way protein binds to different drug targets (4).

The blood brain barrier in the fish is said to be the same as in humans. Blood brain barrier is regulated by endoepithelial cells in the blood vessels. These are sealed with tight junction which, contains specific transporter molecules and vesicles. It is responsible for impermeability of drugs to the brain. In addition, the mammalian neurotransmitter system like GABA, glutamate, histamine, dopamine, noradrenalin and acetylcholine are also present in zebrafish. The human basal ganglia cells, the purkinje cells and the granule cell layers are

similar in the forebrain of the zebrafish, where genes and specialized markers are expressed as well (7).

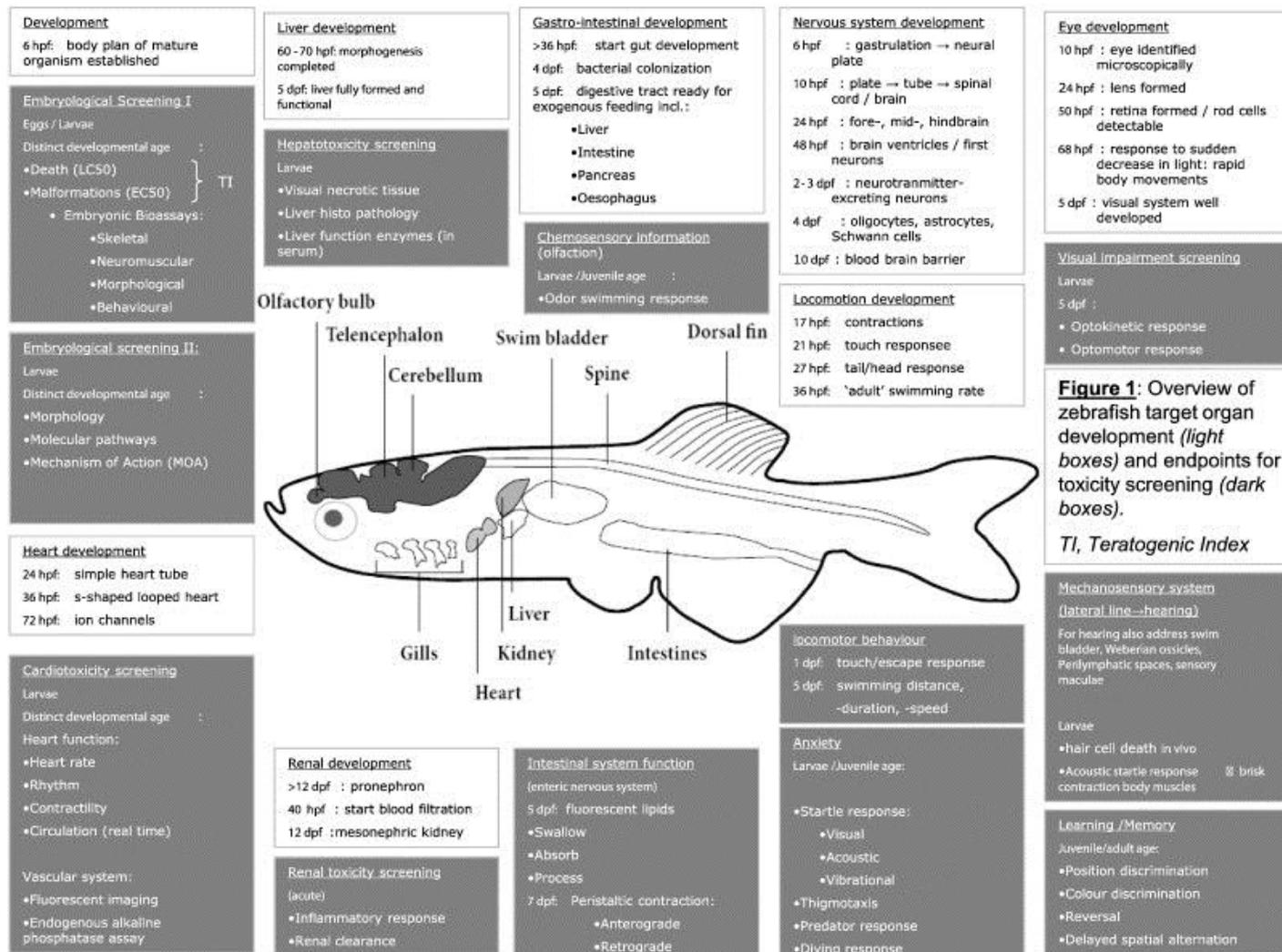


Figure 1: Overview of zebra fish target organ development and endpoints for toxicity screening. This figure is adapted from (7)

Transient genes and transgenics as a toxicological method

Injection of DNA/RNA constructs at 1-2 cell stage can lead to give transient expression of a gene. This allows genes to be easily visualized with fluorescent markers and can restore functional gene expression in mutant embryos. Transgenic lines can be used as toxicological method in two ways. Firstly, once a specific gene has been identified either as a marker for specific tissue or essential part of development pathway, these genes can be assessed for disruption after a chemical exposure. Secondly when a gene has disrupted gene expression or morphology, recovery of normal gene expression can be assessed after application of

therapeutic agents of morpholinos. For example transgenic zebrafish have been used to identify 2,3,7,8-tetrachlorodibenzo-p-dioxin(TCCD) induced neurotoxicity via changes in sonic hedgehog and neurogenic expression in the zebra fish brain (8).

1.4. Eye and CNS physiology

Zebrafish is a good model for investigation of eye development and disease because of its similarity to human eyes. Both zebrafish and humans have evolved eyes for diurnal life including cone-dense retinas, which are also biochemically more similar to human retinas. For example, guanylate cyclase activator 1a is expressed in zebrafish and human retinas (7, 16)

The eye develops from no less than three distinct embryological tissues, *neuroectoderm* which gives rise to the neural retina, pigmented epithelium, optic stalk and ciliary margin; *skin ectoderm*, which is induced to form the lens and subsequently the cornea; and *head mesenchyme* of neural crest cell origin that minimally forms connective tissue of the cornea and sclera (17).

The retina contains three nuclear layers and two plexiform layers. The outer plexiform layer contains cellbodies of photoreceptors. The inner plexiform layer contain cell bodies of horizontal, bipolar and amacrine cells, in addition to the ganglion layers which contain the ganglion cell bodies.

The plexiform layers are found between the nuclear layers and are where the synaptic connections between the retinal neurons take place (18).

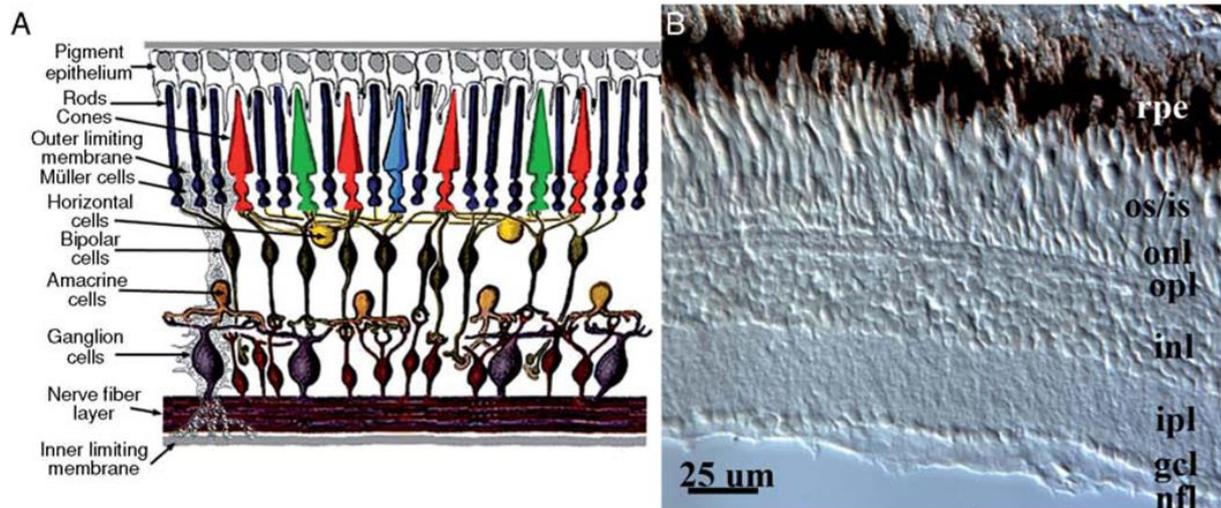


Figure 2: Description of zebrafish retina physiology. On the right (B) Radial cryosection of zebrafish retina, showing retinal pigmented epithelium (rpe), outer and inner segments of photoreceptors (os/is), outer nuclear layer (onl) containing photoreceptor nuclei, outer plexiform layer (opl), inner nuclear layer (inl), inner plexiform layer (ipl), ganglion cell layer (gcl), and nerve fiber layer (nfl). This figure is borrowed from (19)

During 24 hours to 48hpf of embryo development, the zebrafish eye undergoes proliferative changes where the ganglion layers overlaps the inner nuclear cells, then the outer layer of the photoreceptors (20).

The first cells to differentiate in the zebra fish retina are the ganglion cells (~32 hpf) along the vitreal border, followed by the appearance of an inner plexiform layer and amacrine cells. Formation of the photoreceptor layer follows that of inner retinal neurons, at approximately 48 hpf (21).

Transcription factors that are available in the eye are Pax6 which is present in all cells that form the neural retina, lens epithelium and pigment epithelium, whereas Pax2 which is primarily responsible for the cells of the optic stalk.

The absence of these transcription factors can lead to small eye/ectopic eye structures in zebra fish embryos (22).

A variety of intrinsic factors have been demonstrated to influence Pax6 during retinogenesis (23).

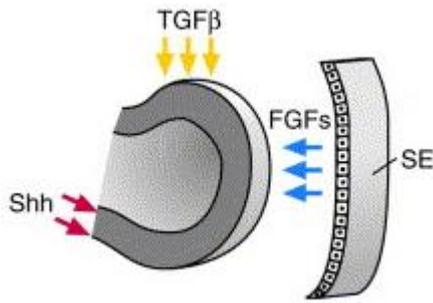


Figure 3: Intrinsic factors that influence retina development. The surface ectoderm (SE) is responsible for the secretion of the FGFs (blue arrows) which promote the neuroretina (NR) differentiation while while a transforming growth factor β ($TGF\beta$) family member secreted from the mesenchyme (yellow arrows) is a candidate for promoting retina pigmented epithelium RPE cell fate. Finally sonic hedgehog (Shh) emanating from the ventral forebrain (red arrows) promotes formation of the optic stalk from the ventral portion of the optic vesicle OV. The initial patterning of the optic vesicle to distal Neuroretina (NR) and proximal retina pigmented epithelium domains is mediated by the head surface ectoderm (SE) and surrounding mesenchyme. This figure is adapted from (23)

Central nervous system

The central nervous system starts to develop after 6hpf during the gastrulation stage. The neural plate is formed which is converted to neural tube during 9-10 hpf. After the gastrulation stage during 24 hpf the CNS is subdivided into several parts including the forebrain (telencephalon and diencephalon), the midbrain, the hindbrain and the spinal cord. At 48 hpf the embryo starts to develop touch stimuli. And during 72 hpf the embryo is fully developed and can feed by itself (7).

1.5. Alcohol

Alcohol is a widely consumed substance worldwide. Prenatal alcohol exposure can lead to Fetal Alcohol syndrome (FAS) in humans and alcohol-mediated development toxicity in zebra fish embryos causing fetal development disorder like mental retardation, growth delay, face abnormalities including small eyes and apoptosis within the developing nervous system. Children with FAS show a variety of ophthalmic defects ranging from microphthalmia, coloboma of iris, optic nerve hypoplasia, and visual impairment, to minor anomalies such as strabismus. Retinal function is known to be affected by prenatal ethanol exposure (21).

Nationwide, FAS occurs has an estimated prevalence 8 for every 1,000 child births (24).

During embryogenesis intake of alcohol can result into birth of children with a smaller brain size and thinner cerebral cortex. Ethanol interferes with ontogenic phases of brain development affecting crucial processes like neurogenesis, neuronal migration, apoptosis and gliogenesis (25).

Lower concentrations or shorter durations of alcohol exposure can lead to alcohol related birth defects (ARBD) or alcohol related neurodevelopment disorder (ARND) (26)

National Organization on Fetal Alcohol Spectrum (NOFAS) in 2004 agreed to use the term Fetal Alcohol Disorder as a supportive expression, not as a diagnostic category but to incorporate various others diagnostic categories such as FAS, ARBD and ARND (27).

Zebrafish share many similar cellular and physiological characteristics with mammals thereby they provide an excellent vertebrate model system.

During embryogenesis alcohol is permeable through the chorion which makes it simpler and precise for the alcohol delivery. $1/25^{\text{th}}$ of the external concentration of alcohol can immerse through the eggs (28). Embryos can be placed and removed from alcohol at different times which makes it easier to control the alcohol concentrations and time for exposure (29).

In humans it is difficult to control the participants and determining alcohol dose by taking blood tests, which might be stressful for the mother and the fetus.

The disadvantage of using zebrafish instead of humans is that zebrafish eggs are developed and fertilized externally where as human are maternal (28), which makes it complex to compare the physiology. The lack of a placenta, which might offer some protection for the developing organism in mammals, is one of the most important. In fact, this relies on a completely different way of exposure to the drug (direct versus indirect), and reflects potential differences in drug adsorption, distribution, metabolism and activation capacity in the zebrafish compared to mammals (30), however the external development of the embryos makes it possible for changes in development to be observed in detail without sacrificing the maternal component and it removes the complication of maternal/placental fetal interaction (11). Another thing is lack of knowledge about the development stages of zebrafish and their correlation to stages of human brain development. However the embryonic development of the brain is well known (29).

Alcohol toxicity in zebrafish has not been used as a primary focus for investigation but it has been used as a blunt instrument to perturb a particular development or signaling way of interest. Thereupon Zebrafish embryos have been used to study how alcohol influences different gene expressions (31).

The pathological features of alcohol-mediated toxicity in zebrafish embryos include cognitive defects, delayed cell differentiation, reduced body length, abnormal development of the eye (microphthalmia, cyclopia), neuronal cell death, craniofacial malformation, behavioral impairment (shoaling) and increased mortality (32).



Figure 4: Malformations induced by ethanol toxicity including pericardial edema, yolk sac edema and axial malformation.

1.6. Possible targets for ethanol mediated toxicity

Ethanol affects the GABAergic and GLUTAergic neurons in the CNS by disrupting sonic hedgehog (Shh), fibroblast Growth Factors (fgf 19), fgf3, fgf8 and Atoh1 expressions (33).

When ethanol is consumed at early stages of pregnancy, it can affect the GABA-induced activity on the excitatory activity in immature neurons which indirectly increases intracellular Calcium, a process that could contribute to a normal brain development, circuit formation during neurogenesis, synaptogenesis, differentiation and migration (34).

Sonic hedgehog plays an important role in regulating vertebrates' organogenesis such as growth of digits and the brain. The hedgehog family consists of Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian hedgehog (Ihh). The Sonic hedgehog signal pathway is the one that is most studied (35).

Shh is a signaling system, which is also expressed in the midline of the central nervous system. It regulates the eye development and is crucial for the separation of the eye fields, formation of the optic stalk, dorsal-ventral patterning of the retina, differentiation of both neural retina and pigment epithelium and the normal laminal organization of the retina (36). It is also said to be the key target for prenatal ethanol exposure.

Expression of Sonic hedgehog control the neurogenesis by amacrine cells, it appears to mediate specification of the other retinal neurons and to differentiate the post-mitotic cells between 28 and 32 hpf (17).

To date there are 22 Fibroblast growth factor ligands in vertebrates which activate transmembrane receptor kinase at the cell surface. This signaling activates the small GTPase Ras and several cascades mostly the MAP kinase cascade (through Ras, the serine/threonine kinase RAF and the MAP/ERK kinase MEK). This activation can lead to specific phosphorylation and activation of the key transcription factors (37).

Fibroblast growth factor is needed for the correct forebrain patterning (in the anterior neural border dorsal telencephalon and the diencephalon). Fgf3 and Fgf8 changes quickly in the developing forebrain, they are implicated in telencephalic and diencephalic ventral patterning, and their loss of function leads to defects in neuronal differentiation. Fgf8 is responsible for the telencephalon, midbrain and cerebellum while Fgf3 is expressed in the forebrain and the hindbrain. Fgf3 and Fgf8 are implicated in the regional patterning of the brain where Fgf8

regulates its own transcription and the transcription of Fgf3 in the forebrain. Fgf19 is also critical for the development of the ventral region of the telencephalon and diencephalon and is implicated in the specification of γ -aminobutyric acid (GABA)ergic interneurons and oligodendrocytes in the telencephalon and diencephalon (38).

The expression of Shh seems to be dependent on the signaling by Fgf3 and Fgf8 on the hypothalamus and the forebrain, it is said they have an intimate relationship and a positive feedback loop (37).

Heparan Sulfate proteoglycan (HSPGs) are cell surface and extracellular matrix protein that mediate a diverse range of crucial functions during vertebrate and invertebrate development. These functions include regulation of cell growth and differentiation of axon outgrowth in the developing CNS. HSPGs also modulate Fgf-mediated axon growth in the retinotectal system. Agrin is a HSPG that was initially discovered and characterized functionally based on its essential role in neuromuscular synaptogenesis. Agrin is expressed by spinal cord, motor neurons and skeletal muscle, as well as other neural and non-neural tissues that include lung, kidney, brain and eye. Agrin is essential for retina development and it's characterized by its neurogeneal synaptogenesis.

Agrin is needed for the correct Shh signaling for eye development. Since prenatal ethanol exposure can lead to agrin knockdowns in eye development this can lead to problems with optic nerve formation probably due to the fact that agrin modulates Fgf mediated axon outgrowth on retinal ganglion axons. In addition fgf2 mediated formation of the *Xenopus* retinotectal pathway is also HSPG dependent. Agrin knockdowns lead to impaired Midbrain Hindbrain Boundary formation (39).

Agrin and Fgf are important for the optic nerve growth and eye development. Several studies show that Pax6 and ath5 is perturbed after agrin knockdown in zebrafish embryos (40).

Several studies have shown that both purkinje cells and external granule cell is reduced by ethanol exposure, depending on the timing of exposure (33).

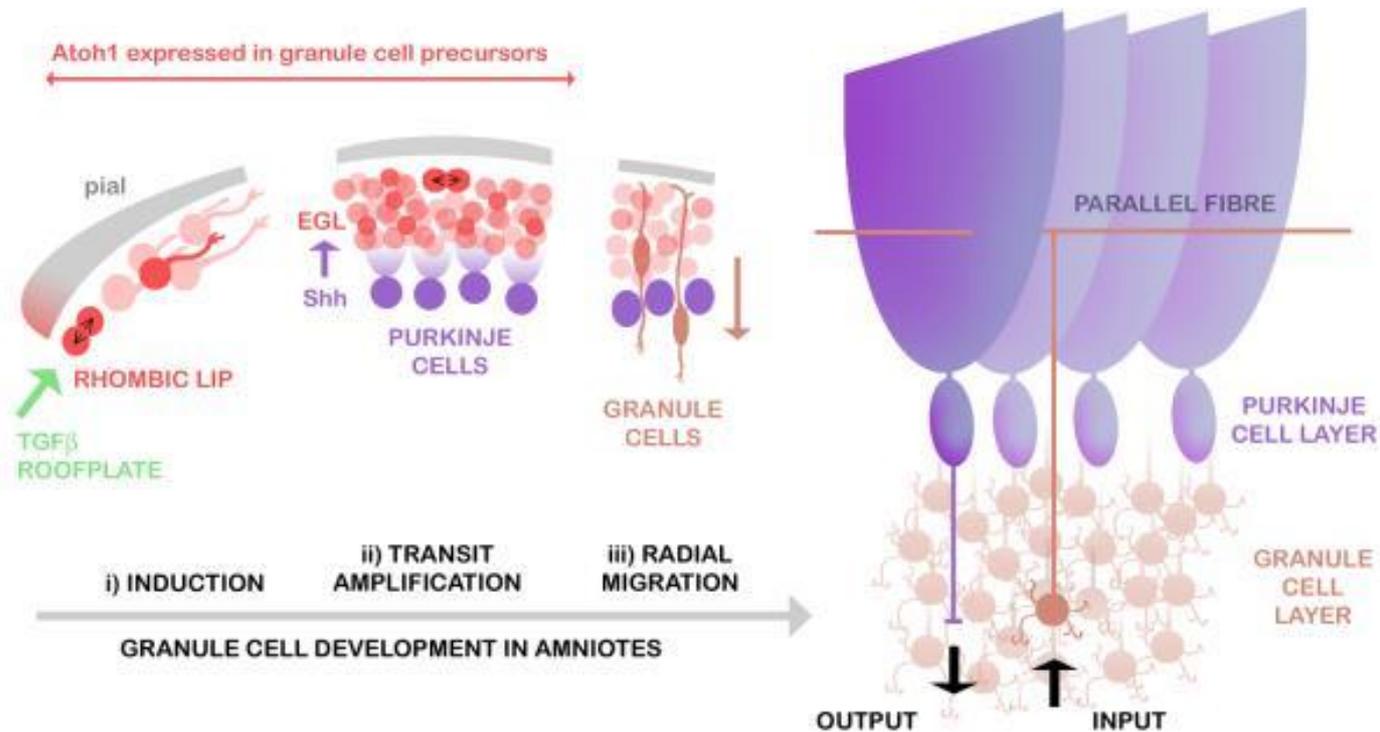


Figure 5: Description of granule cell development in amniotes:Granule cell precursors (red) are initially induced at the rhombic lip by TGFβ signals (green) from the adjacent roofplate (i). Precursors migrate tangentially over the sub-pial cerebellar surface and divide again within the transient EGL (ii). Proliferation is regulated by Shh secreted from underlying Purkinje cells (purple). After their last cell division, postmitotic granule cells (brown) radially migrate into a layer below Purkinje cells (iii). In the mature circuit, glutamatergic granule cells receive inputs from precerebellar neurons and project T-shaped axons (parallel fibres) into an almost cell body-free (molecular) layer, where they synapse on the dendrites of GABAergic Purkinje cells. Purkinje cell outputs directly and indirectly regulate the activity of the vestibular system, thalamus and sub-cortical motor centres (41).

These are possible targets for the ethanol effects on neuron development of zebrafish embryo. Raising the possibility that ethanol-mediated reduces in forebrain Fgf gene expressions may be caused by ethanol disrupting Shh function.

1.7. Pax6 and Pax2 roles in ethanol mediated toxicity

Pax genes come from a family of 9 evolutionary conserved transcription factors (Pax1-Pax9) and is divided into four subgroups based on similarities in structural domains (paired domain, homeo-domain and octapeptides) whereas paired domain and homeo-domain recognizes a specific DNA sequence (42).

Pax6 and *Pax2* are transcription factors that are extremely important in the development of the zebra fish embryo eye and the brain.

Pax6 was initially cloned from human, mice, zebrafish and quail. The *Drosophila* eyeless gene was shown to be a *Pax6* homolog and *Pax6* homologs have now been described in other invertebrates such as flatworm, ribbonworm, *C.elegans*, squid, sea urchin and ascidian.

Pax6 is expressed from the earliest stages of eye morphogenesis in the optic vesicle, giving rise to the retina and pigment retina, as well as in the overlying ectoderm that later forms the lens and the cornea. However, *Pax6* is also expressed in the nasal epithelium, in specific regions of the brain and the spinal cord, and not exclusively in eye primordia (43).

Pax6 is said to be the key regulator of eye development. Over-expression of *Pax6* in mice results to a severe eye phenotype called *small eye*, whereas reduction of *Pax6* activity in humans results to *aniridia* (43).

Ethanol can reduce proliferation and neuronal differentiation radial glial cells through decrease of *Pax6* transcription factor. *Pax6* is a target of several signaling pathways, and phosphorylation sites for p38, ERK and homeo-domain interacting protein kinase 2 are identified (44, 45).

Due to a duplication of the teleost genome million years ago, there are two copies of *Pax6* and *Pax2* genes in zebrafish. *Pax6* genes in zebrafish are divided into *Pax6.1* and *Pax6.2* whereby *Pax6.2* is said to show stronger transactivating capability than *Pax6.1* and both genes are said to induce ectopic eye structure, where by *Pax2* is divided into *Pax2.1* and *Pax2.2* (46).

Pax2.1, which is expressed in the junction between the forebrain and hindbrain, also called midbrain (isthmus) decides the fate of development of the other parts of the brain during gastrula stage. It is also expressed in the CNS, in the developing eye, ear and the kidney (47).

Pax2.1 is localized at the midbrain-hindbrain border and is usually recognized by a stripe (furrow separating the midbrain and the hindbrain). Zebrafish *Pax2* probe can detect this at

very early stage of embryo development, at later stages of embryo development the tectal ventricle enlarges, the tissue between the furrow separating the midbrain and the hindbrain gets considerably thinner (48).

Midbrain-hindbrain boundary (MHB) is a signaling center, acting to pattern and establish neural identities within the brain. MHB originally identified in chick consists of cells that influence the fate of neighboring cells to adopt either a mesencephalic (midbrain) or metaencephalic (hindbrain) fate through expression of transcription factors and soluble signaling molecules. A cascade of signaling (Fgf8) and transcription (Pax2/5/8, engr1/2) factors within Otx2/Gbx1/2 boundary induces formation of the MHB and subsequent interplay between these factors is critical for maintenance of the MHB. Disturbance of any of these factors lead to severe functional disruption in the formation of the isthmus organizer (49).

It's only seen through the first 48hrs of development and then fuses with the forebrain. Loss of the midbrain identity can cause expansion of the forebrain territory and hindbrain territory (50).

Fgf8 is the main organizer of the Pax2.1 expression in the MHB (51).

2. AIM OF THIS THESIS

The aim with the thesis was to see if zebrafish embryos can be used to study the effect of ethanol on early brain development. This was done by:

1. Generation of tools for the study of specific parts of the brain, zAtoh1a1, zAtoh1a2 and zAtoh1c genes which are expressed in the cerebellum will be isolated from zebrafish cDNA and cloned to be used for *in situ* hybridisation .
2. Incubation of zebrafish embryos with different concentrations of ethanol to look for changes in overall morphology and survival. Studies on the molecular level, including *in situ* hybridizations and Western blot of protein extracts are also to be included.

3. MATERIALS

E3 medium had a pH between 6.8-6.9 and contains:

- 5mM NaCl
- 0.17mM KCl
- 0.33 mM CaCl₂
- 0.33 mM MgSO₄
- 0.00001% w/v methylene blue

S.O.C Medium

- 2 % Tryptone
- 0.5 % Yeast Extract
- 10 mM NaCl
- 2.5 mM KCl
- 10 mM MgCl₂
- 10 mM MgSO₄
- 20 mM glucose

Table 1: Materials used

Glycerol	Catalog no: 49782-1L Sigma Aldrich
Tween 20	Catalog no: P1379-500ml Sigma Aldrich
Magic marker	Catalog no: LC5602 Novex
Sea blue Plus 2 prestained	Catalog no: LC5925 Novex
Protinase K 20mg/ml	Catalog no : 25530-049 Invitrogen
NCB/BCIT	Catalog no: 11697471001 Roche
Anti-Digoxigein Fab fragments	Catalog no : 11093274910 Roche
Ethanol absolute	Catalog no : 322221 Sigma Aldrich
Methanol	Catalog no : 32213-K Sigma Aldrich
Sea kem [®] Agarose	Catalog no : 50004 Loriza
Blocking Reagent	Catalog no : 1096176 Roche
Paraformaldehyde	Catalog no : 200-001-8 Merck Schuchardt
Formamide	Catalog no: F-9037 Sigma Aldrich
Heparin 5000U/ml	Catalog no : L6510 Biochrom
tRNA 500µg/ml	Catalog no : 10109223001 Roche
Hydrogen peroxide	Catalog no : 203626 Apotekforeningen NAF
Anti-pax6 rabbit polyclonal	Catalog no : AB2237 Millipore
Donkey anti-rabbit IR-Dye	Catalog no : 92668023 Li-COR,Odyssey
Trizol [®] Reagent	Catalog no : 15596018 Ambion
DIG RNA labeling kit	Catalog no : 11175025910 Roche
LDS-Sample buffer(4x)	Catalog no : NP0008 NUPAGE
Reducing agent	Catalog no : NP0009 NUPAGE
Isopropanol/2-propanol	Catalog no : 59300-1L Sigma Aldrich
Chloroform	Catalog no : 1-2445-1 Merck
PCR-Cloning kit	Catalog no : L:44-0302 Invitrogen
Qiagen Plasmid Mini-kit	Catalog no: 12125
Anti-actin	Catalog no : A2066 Sigma
Qiagen Spin Mini-prep kit	Catalog no: 27106
Qiaquick PCR-purification	Catalog no: 28106

4. INSTRUMENTS

Biofuge Fresco Cold centrifuge

NanoDrop ND-1000 Spectrophotometer

Peltier Thermal Cycler (PTC-200) DNA engine from MJ Research

MJ Research PTC 200 Peltier Thermal Cycler block

MJ Research PTC 200 Peltier Thermal Cycler tubes, Temperature gradient

Stuart Block heater, SBHI30DC

Biofuge Fresco Pico centrifuge

Grant Broekel Blockheater

Unitorn shaker

MERK eurolab Hedolf UNIMAC 2010 rocking platform

Bioruptur sonicating machine

Femars heating cabinet

3130 XL and 3530 XL Genetic analyser(ABI)

High Performance Ultraviolet Transilluminator

Li-COR *Odyssey* Machine

Grant, Waterbath

5. METHODS

5.1. Gel Electrophoresis

Agarose gel 0.7 %

This is a method used to separate the DNA, RNA and proteins fragments by length and size estimation. The agarose (0.7 %) is made by weighing 1.05 g Agarose and adding 150 ml 1x TAE on a conical flask. The mixture is warmed on a microwave for about 2 minutes. The solution must be mixed homogeneously and thoroughly in between until when it starts to bubble. The temperature is reduced to 60 °C, by cooling the conical flask on running water, thereafter placed in a Fermarks heating cabinet.

A sample comb and casting tray is chosen, placed on a holder. 50 µl 1 mg/ml ethidium bromide is placed on a gel casting tray and agarose is poured to cover the combs about 1cm from the tray. After the gel is solidified about 20 minutes, the comb is removed and the casting tray with the gel is placed on an electrophoresis chamber and is covered with the buffer TAE.

The DNA samples are mixed with 6XT and applied to the wells of agarose gel 1kb plus DNA molecular weight standard is used as reference ([Figure 6](#)). The lid is placed on, the positive and negative electrode on the right spots and the current is allowed to flow. Current flow is set up at 90V and flow time is 30-40 minutes. Bubbles appearance on the electrodes confirms the flow of current.

Pictures were taken by using transilluminator which was connected to the camera and computer.

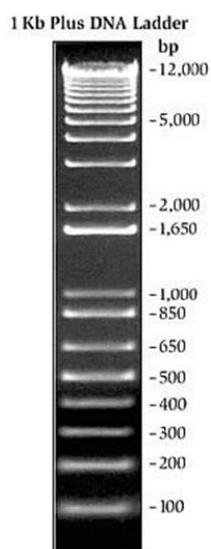


Figure 6: Molecular weight standard for gel electrophoresis obtained from Invitrogen (52)

6XT

0.25 % bromphenole blue
60 mM Na₂EDTA (pH 8.0)
0.6 % SDS
40 % (w/v) sucrose in water

TAE (50X Stock solution)

242 g Tris base in water,
57.1 ml glacial acetic acid,
100 ml of 500mM EDTA (pH 8.0) solution
Adjust to 1 liter

1 kb plus ladder
10 mM Tris-HCl (pH 7.5)
1 mM EDTA
50 mM NaCl

5.2. Isolation of RNA from zebra fish embryos

120 embryos were placed on two eppendorf tube on ice, 60 embryos on each tube. All media was removed. 300 µl Trizol (Ambion) was added to two new microcentrifuge tubes and approximately 200 µl was used in a 1ml syringe with 25G cannula. The embryos were homogenized by mixing them back and forth with the syringe (3X). The remaining 100 µl of Trizol was then used to rinse the syringe and added to the homogenized embryo's. At this point the embryos could have been stored at -80 °C.

An additional amount of 600 µl Trizol was added to the embryos and left in room temperature for 5 minutes. 200 µl of Chloroform (Merck) was added and the samples were placed on ice for 20 minutes. The tubes were inverted several times every 2 minutes.

The samples were then centrifuged in a cold centrifuge at 9000 rpm for 30 minutes

The water phase on the top was transferred to another new tube (approximately 500 µl).

500 µl of Isopropanol was added to the samples and then incubated for 10-15 minutes in 4°C

The samples were centrifuged at 12000 rpm at 4°C for 30 minutes

The supernatant over the RNA pellet was removed and 1 ml ice cold. 80 % ethanol was added for washing the pellet. Centrifugation was done at 12000 rpm for 5 minutes

The supernatant was removed and the tubes were left to dry in the hood until all ethanol had evaporated

The RNA-pellet was re-suspended on 60 µl RNA-ase free water.

The concentration of the isolated RNA was measured by using NanoDrop ND-1000 Spectrophotometer, Saveen Werner

5.2.1. Synthesis of cDNA

RNA was treated with Heat & Run gDNA removal kit (Articzymes), to eliminate genomic DNA.

2 µl HL-dsDNAase and 2 µl 10x Reaction buffer per 10 µl RNA was mixed with the RNA.

Incubated at 37 °C for 10 minutes and thereafter 55 °C for 5 minutes. RNA was placed on ice.

To every tube 6 µg RNA, 2 µl 0,25 µg/µl Random Hexamer Primer (pd(N)₆), 2 µl 10mM δNTP, and RNAase free water was added to a total volume of 26 µl. The mixture was placed on a blockheater (Stuart) at 65°C for 5 minutes and then cooled on ice for minimum 1 minute.

The samples were then centrifuged for about 5 seconds on Eppendorf minispin to collect the samples at the bottom. Then the following was added to each tube 8 µl 5 x First-Strand Buffer (Invitrogen), 2 µl 0,1M DTT (Invitrogen), 2 µl RNAase OUT Recombinant Ribonuclease Inhibitor (Invitrogen), and 2 µl Superscript III RT

Samples were placed on a PCR-machine with a following program

1. 25 °C for 5 minutes
2. 50 °C for 55 minutes
3. 70 °C for 15 minutes
4. 4 °C until the program ends manually.

5.2.2. Optimization

PCR BLOCK GRADIENT.

A mastermix was made containing (Total amount 60 µl)

3 µl DNA

24 µl H₂O

1.5 µl primer zAtoh1a.RT5

1.5 µl primer zAtoh1a. RT3.1

30 µl 2X DyNAzyme

The same procedure was done using primers.

- zAtoh1a.RT5+ zAtoh1a.RT3.2
- zAtoh1c.RT5+ zAtoh1c.RT3

PCR reaction had a following procedure

1. Denaturation at 94 °C for 2 minute
2. Denaturation at 94 °C for 30 seconds
3. Annealing at 56 °C (lowest) and 60 °C (highest) for 1 minute
4. Elongation at 72°C for 1 minute
5. Returning to 2nd Stage for 34 cycles
6. Elongation at 72 °C for 10 minutes
7. 4 °C forever.

20 µl of PCR products were mixed with 4 µl 6XT (Loading buffer)

PCR products were set up for gel electrophoresis for 30 minutes at 90V

5.2.3. PCR-reaction with (Phusion)

Templates were used to generate PCR-products with blunt ends

- zAtoh1a1
- zAtoh1a2
- zAtoh1ac

Reverse primer T7 and Forward primer T3 were used in consideration with the research of DIG-marking probe.

Reaction was set up in the following procedure

Table 2: PCR-reaction set up

Primer	Template
Atoh1a. FWT3+Atoh1a.RT7	zAtoh1a1
Atoh1a. FWT3+Atoh1a2.RT7	zAtoh1a2
Atoh1c. FWT3+Atoh1c.RT7	zAtoh1ac

- 10 µl 5X Phusion Buffer (Biolabs)
- 1 µl δNTP mix (10mm)
- 2.5 µl Forward primer (10µm)
- 2.5 µl Reverse primer (10µm)
- 2,5 µl zAtoh1a1
- 2,5 µl zAtoh1a2
- 2,5 µl zAtoh1ac
- 31 µl H₂O
- 0,5 µl Phusion (Biolabs)

Total 50 µl

PCR-block was set on 98 °C with a following program

1. Denaturation at 98 °C for 40 seconds
2. Denaturation at 98 °C for 10 seconds
3. Annealing at 58 °C for 20 seconds
4. Elongation at 72 °C for 30 seconds
5. Returning to 2nd Stage for 29 cycles
6. Elongation at 72 °C for 5 minutes
7. 4 °C forever.

PCR block was first warmed up to 98 °C, before the samples were loaded.

PCR products were set up for gel electrophoresis for 30 minutes

10 µl of PCR products were mixed with 2 µl 6XT (Loading buffer)

Primer sequences

Table 3: Overview of primer sequences used.

Name	Sequence	PCR-(bp)
zAtoh1a.RT5	5'-GAACTCGACGTCCAGCATTTC-3'	553 bp
zAtoh1a.RT3.1	5'-CCGTTTCTAACACGTTGGCA-3'	
zAtoh1a.RT5	5'-GAACTCGACGTCCAGCATTTC-3'	909 bp
zAtoh1a.RT3.2	5'-GCAACCCATTACAAAGCCCA-3'	
zAtog1c.RT5	5'-ATGCCCCATCCGGACACCCCTTTTGG-3'	615 bp
zAtog1C.RT3	5'-CTATTTTACACCATTGTTTCCTTTCCA-3'	
zShh.RT5	5'-GCAAACCTCCGATGCGTTAT-3'	768 bp
zShh.RT3.1	5'-TCGCAGCAACCGAATTTTCT-3'	
zAtoh1a.FWT3	5'-CATTAAACCCTCACTAAAGGGAAGAAGACTCGACGTCCAGCATTTC-3'	553 bp
zAtoh1a.RT7	5'-TAATACGACTCACTATAGGGCCGTTTCTAACACGTTGGCA-3'	
zAtoh1a.FWT3	5'-CATTAAACCCTCACTAAAGGGAAGAAGACTCGACGTCCAGCATTTC-3'	909 bp
zAtoh1a2.RT7	5'-TAATACGACTCACTATAGGGGCAACCCATTACAAAGCCCA-3'	
zAtoh1c.FWT3	5'- CATTAAACCCTCACTAAAGGGAATGCCCCATCCGGACACCCCTTTT GG-3'	615bp
zAtoh1c.RT7	5'- TAATACGACTCACTATAGGGCTATTTTACACCATTGTTTCCTTTCCA - 3'	

5.3. CLONING

DNA cloning allows a DNA fragment with a particular nucleotide sequence to be separated from a complex mixture of fragments with many different sequences.

Plasmids are circular, double-stranded DNA (dsDNA) molecules that are separate from a cell's chromosomal DNA. Plasmids are actually 1.2-3 kb in length and they contain a replication origin, a gene that is resistance to a particular drug and exogenous DNA (promoter lac Z and multiple cloning site).

Transformation of plasmid in bacteria involves uptake and expression of foreign DNA (53).

Two different plasmids were used which are PCR[®] blunt vector with linearized blunt ends (Kanamycin resistant) and pbluescript SK+/- (Ampicillin resistant).

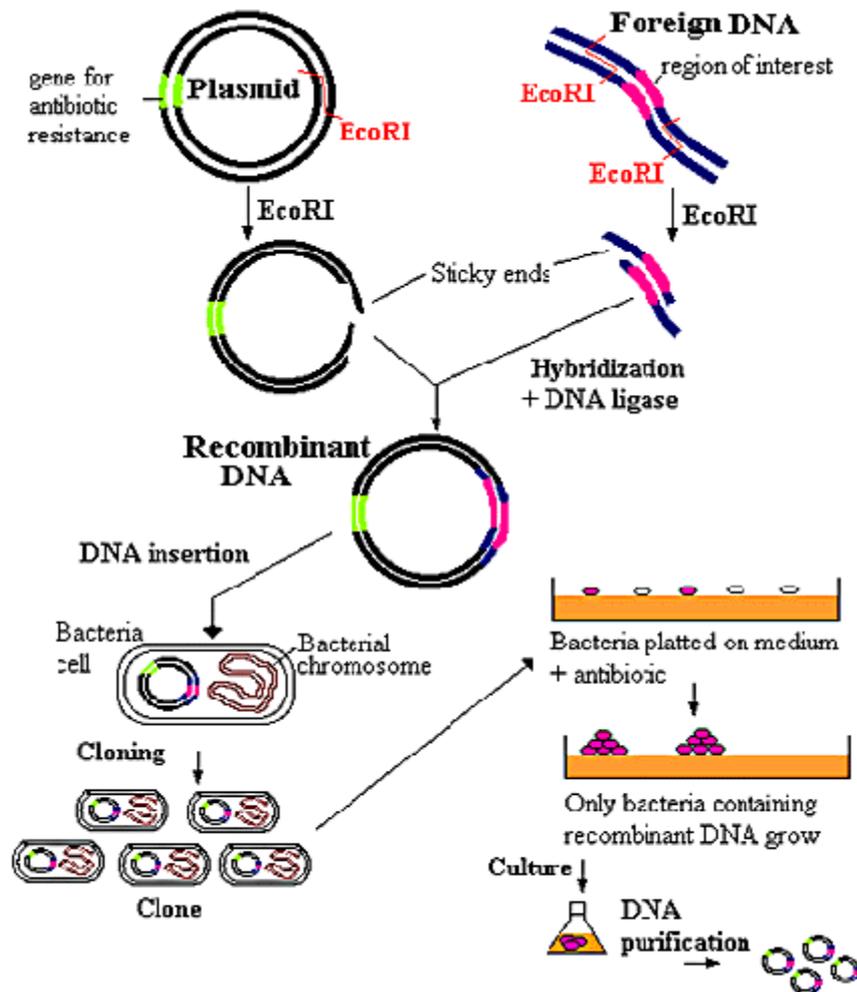
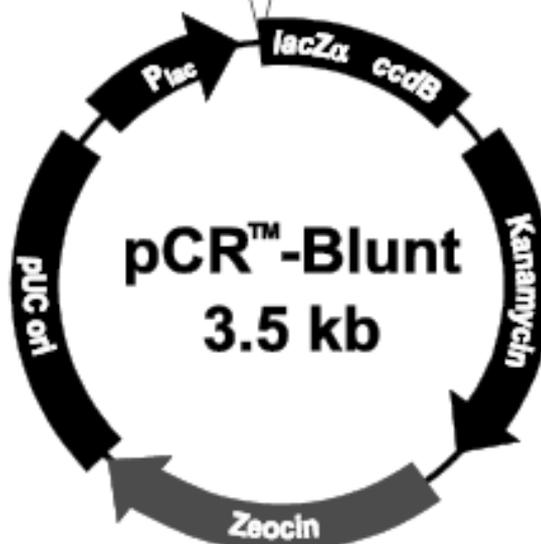
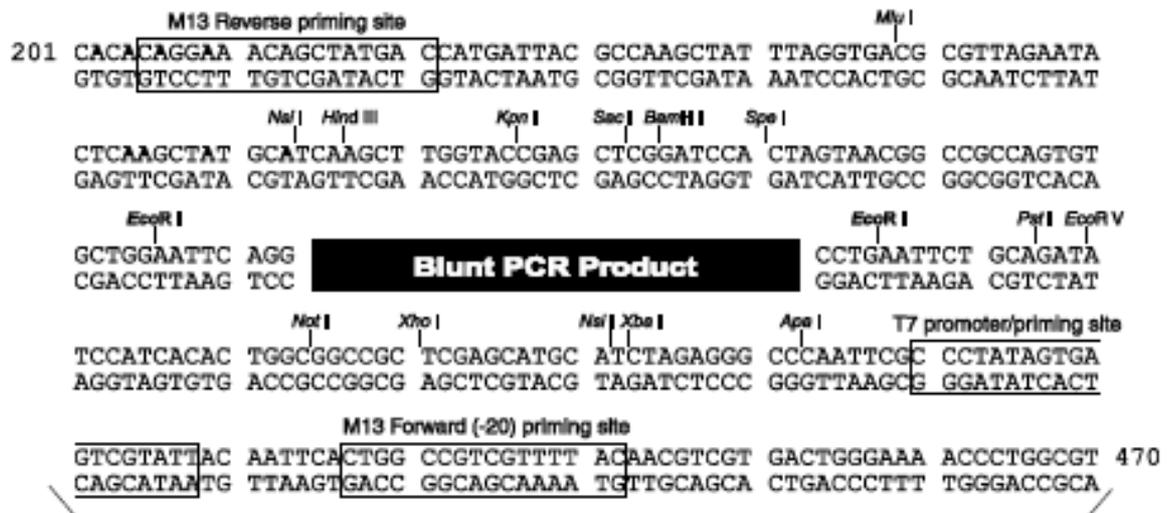


Figure 7: Illustration of cloning zebra fish DNA in plasmid (54)

PCR[®]-Zero-blunt vector

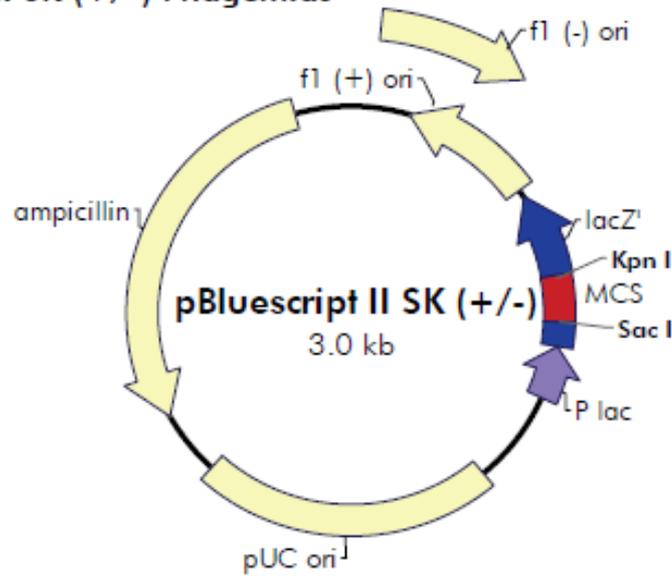


Comments for pCR[™]-Blunt 3512 nucleotides

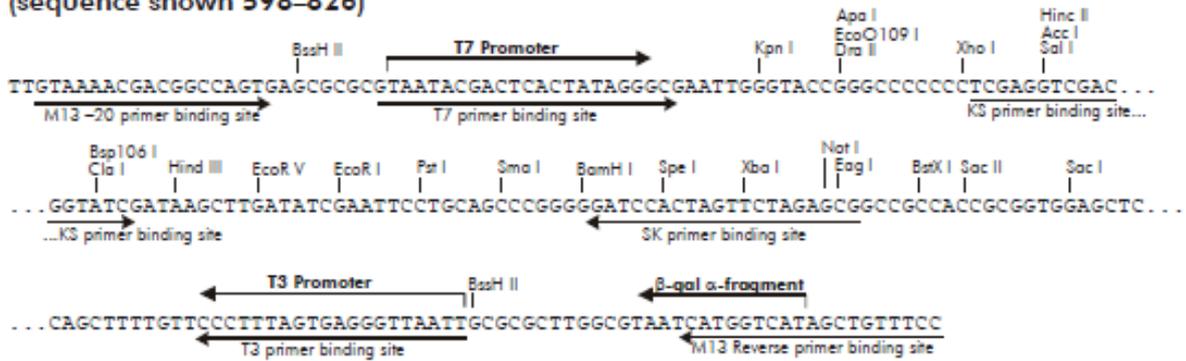
Lac promoter/operator region: bases 95-216
 M13 Reverse priming site: bases 205-221
LacZ-alpha ORF: bases 217-570
 T7 promoter priming site: bases 400-419
 M13 Forward (-20) priming site: bases 427-442
 Fusion joint: bases 571-579
ccdB lethal gene ORF: bases 580-882
 Kanamycin resistance ORF: bases 1231-2025
 Zeocin resistance ORF: bases 2231-2605
 pUC origin: bases 2673-3386

Figure 8: PCR[®]-Zero Blunt vector chart sequence and map (55)

pBluescript II SK (+/-) Phagemids



pBluescript II SK (+/-) Multiple Cloning Site Region (sequence shown 598–826)



Feature	Nucleotide Position
f1 (+) origin of ss-DNA replication [pBluescript SK (+) only]	135–441
f1 (-) origin of ss-DNA replication [pBluescript SK (-) only]	21–327
β -galactosidase α -fragment coding sequence (<i>lacZ'</i>)	460–816
multiple cloning site	653–760
T7 promoter transcription initiation site	643
T3 promoter transcription initiation site	774
<i>lac</i> promoter	817–938
pUC origin of replication	1158–1825
ampicillin resistance (<i>bla</i>) ORF	1976–2833

Figure 9: pBluescript SK+/- vector chart sequence and map (56)

5.3.1. RT-PCR

cDNA was used as a template with the following primer sequence:

- zAtoh1a.RT5+ zAtoh1a.RT3.1
- zAtoh1a.RT5+ zAtog1a.RT3.2
- zAtoh1c.RT5+ zAtoh1c.RT3

Each PCR-reaction had a total volume of 40 μ l with 2 μ l of cDNA, 16 μ l of H₂O, 1 μ l zAtoh1a.RT5 +1 μ l zAtoh1a.RT3.1 and 20 μ l of 2 x DyNAzyme Mastermix. The same procedure was repeated with other primers.

PCR reaction had a following procedure

1. Denaturation at 94 °C for 2 minutes
2. Denaturation at 94 °C for 30 seconds
3. Annealing at 56 °C for 1 minute
4. Elongation at 72 °C for 1 minute
5. Returning to 2nd Stage for 34 cycles
6. Elongation at 72 °C for 10 minutes
7. 4°C forever.

10 μ l of PCR products were mixed with 2 μ l 6XT (Loading buffer)

PCR products were set up for gel electrophoresis for 30 minutes at 90V

5.3.2. Blunt cloning of PCR-product

Zero Blunt PCR-Cloning Kit from Invitrogen was used

Blunt vector 1 μ l

PCR product 2 μ l of each

5X Ligase Buffer 2 μ l

H₂O 4 μ l

T4 DNA Ligase 1 μ l

Total 10 μ l

The mixture was left in room temperature for 30 minutes. 2 μ l of the total 10 μ l mixture was mixed with 50 μ l of DH5 Competent Bacteria cells in a 15ml Falcon tube. Mixture was left on ice for 1 hour. Heat shocked for 30 seconds at 42 °C. Placed vial on ice for 90 seconds. 250 μ l of S.O.C medium was added and the mixture was placed for 1 hour at 37 °C at

225 rpm (Unitorn shaker). 100 µl bacteria was spread on LB plate with 50 µg/ml Kanamycin by using sterile bacteria techniques. Plates were left to dry and turned upside down, then placed at 37 °C overnight (O/N).

Plates were stored at 4°C. Colonies were picked randomly from each plate with a sterile toothpick and placed in a 15 ml Falcon tube containing LB medium with 3 ml Kanamycin (50 µg/ml) and incubated at 37 °C, 225 rpm O/N

5.3.3. Cloning with pBluescript

pBluescript ks+/- was digested with EcoRV (Biolabs)

7 µl of DNA, 2 µl 10xTA, 1 µl EcoRV, and 10 µl H₂O was placed on a Block heater 37 °C for 90 minutes.

1 µl SAP (Biotec Pharmacon) was added and the mixture was incubated again at 37 °C for 30 minutes.

20 µl DNA and 4 µl 6XT were set up for electrophoresis 90V for 30 minutes.

Spin Mini-prep kit was used. The procedures were the same as in Mini-Prep (5.3.8).

5.3.3.1. Qiagen (gel purification)

DNA fragment from agarose gel was excised with a clean sharp scalpel with help of UV-light. The gel slice is weighed in a colorless tube. Added 3 volumes of buffer QG to one volume of gel (100 mg~100 µl) QG. Incubated the mixture on a blocking heater at 50 °C for 10 minutes. Within 2-3 minutes the mixture was flipped to help dissolve the gel. Assured that the mixture is yellow (similar to buffer QG without dissolved agarose). The whole mixture was transferred to a spin column and spin for 1 minute at 13000 rpm.

500 µl was added, spin for 1 minute at 13000 rpm, 750 µl PE was added centrifuged for 1 minute at 13000 rpm, centrifuged for 1 minute at 13000 rpm. 50 µl Eluting Buffer was added and left to absorb for 1 minute, centrifuged for 1 minute at 13000 rpm.

5.3.3.2.Ligation

13 µl H₂O

2 µl pBluescript (EcoRV digested, gel purified)

2 µl PCR product (zAtoh1a, zAtoh1b, zAtoh1c)

2 µl 5xLigation buffer

1 µl T4 DNA-ligase

Ligation was set up for O/N (16°C)

5.3.4. Transformation of bacteria

Bacteria were transformed with pBluescript vector and pZero-blunt (Invitrogen). 50 µl of DH5 competent bacteria was placed in a 15 ml falcon tube and 2 µl of ligation mixes containing either pBluescript or pZero-blunt vectors were added to the bacteria. Placed on ice for

30 minutes. Heat shocked for 45 seconds at 42 °C, placed on ice for 2 minutes. 250 µl S.O.C was added at RT and the mixture was incubated for 1 hour at 37 °C at 225 rpm (Unitorn shaker). 100 µl was spread on LB plates containing 100 µg/ml Ampicillin for the bacteria containing pBluescript plasmid, and on plates containing 50 µg/ml Kanamycin for the pZero-blunt transformed bacteria. Plates were left to dry and turned upside down, and then placed at 37 °C O/N.

Miniprep light procedure was done.

5.3.5. Miniprep “light”

1.5 ml bacteria cultures were transferred to 1.5 ml eppendorftubes. Centrifuged for 20 seconds and supernatant removed. Pellet was re-suspended in 100 µl Buffer P1 with RNA-ase and vortexed. 100 µl Buffer P2 was added and tubes were mixed by turning up and down 4-6 times. Tubes were left on RT for 4 minutes. 140 µl Buffer N3 was added and tubes were mixed by inverting 4-6 times. Tubes were centrifuged for 10 minutes, 13000 rpm in room temperature (RT). Supernatant was transferred to new tubes. 400 µl isopropanol was added; tubes were mixed by inverting 4-6 times. Tubes were left on RT for 2-3 minutes. Tubes were centrifuged for 5 minutes at 13000 rpm at RT. Supernatant was discarded and the DNA-pellet was washed by adding 500 µl 70 % EtOH. Tubes were centrifuged for 5 minutes, 13000 rpm at RT. Supernatant was removed. Tubes were dried on the bench for 10-15 minutes. DNA-pellet was dissolved in 50 µl TE. DNA was set up for gel electrophoresis (0.7 % Agarose gel):

- 2 µl DNA for each DNA-prep tube
- 3 µl H₂O
- 1 µl 6XT

5.3.6. Digestion of DNA with restriction enzyme (pZero-Blunt vector)

Mastermix was made containing:

- 28 µl 10X TA
- 7 µl *EcoR I*(Biolabs)
- 35 µl H₂O

Total 70 µl →5µl placed in 12 new eppendorftubes

5.3.7. Digestion of DNA with restriction enzyme (pBluescript vector)

A mastermix with p-bluescript vector was made containing

- 28 µl 10X TA
- 7 µl *EcoR I*(Biolabs)
- 35 µl H₂O
- 14 µl Hind III(Biolabs)

Total 84 µl →6µl placed in 12 new eppendorf tubes with 15 µl DNA

15 µl DNA was placed in each tube and tubes incubated at 37 °C for 1 hour.

For each DNA-prep tube 4 µl 6XT was added and set up for gel electrophoresis for 40 minutes at 90V.

5.3.8. Mini PREP

Approximately 100 µl from tubes containing bacteria with insert was placed in 15 ml Falcon tube and 3 ml LB-medium Kanamycin 50 µg/µl was added. Tubes were incubated in a shaker 225 rpm O/N at 37°C. 1,4ml of the overnight cultures were centrifuged at 4000 rpm for 2 minutes in eppendorftubes. 250 µl Buffer P1 with RNAase cold from (Qiagen Kit) was added and vortexed. 250 µl Buffer P2 was added and tubes were mixed by inverting 4-6 times left for maximum 5 minutes. 350 µl Buffer N3 was added and tubes were mixed inverting 4-6 times. Tubes were centrifuged for 10 minutes at 13000 rpm

Supernatant was transferred to a spin column. Spin Column was centrifuged for 1 minute at 13000 rpm

Supernatant was removed and 500 µl PB was added and centrifuged for 1 minute at

13000 rpm

Supernatant was removed and 750 µl PE Buffer was added and centrifuged for 1 minute at 13000 rpm. Supernatant was discarded and the spin column centrifuged for 1 minute at 13000 rpm

Colon was placed in 1.5 ml eppendorf tube. 50 µl Elution buffer was added, left to rest for 1 minute. Colon was centrifuged at 13000 rpm for 1 minute

DNA was set up for gel electrophoresis (0.7 % Agarose gel):

- 2 µl DNA for each DNA-prep tube
- 3 µl H₂O
- 1 µl 6XT

5.3.9. Sequencing for (pZero-blunt)

Two tests were done on each group marked 1a, 4a, 5b, 7b, 11c and 12c.

The same procedure as for miniprep (5.3.8) was done to cleanse the minipreps for sequencing. Measured the DNA by using by using NanoDrop ND-1000 Spectrophotometer, Saveen Werner

A sequence reaction was set up for 1a, 5b and 12c using PCR-products, Big dye, M13R primer, Sequencing buffer and H₂O

(A=zAtoh1a1, B=zAtoh1a2, C=zAtoh1c)

Table 4: Measurements of DNA

	1a	4a	5b	7b	11c	12c
260/280	1,84	1,86	1,86	1,86	1,86	1,85
260/230	1,93	1,94	2,08	1,92	1,44	1,92
DNA	200,5 ng/µl	142,5 ng/µl	173,3 ng/µl	162,2 ng/µl	150,5 ng/µl	187,3 ng/µl

Table 5: Sequence reaction

	1a	5b	12c
DNA	3 µl	4 µl	4 µl
Sequencing buffer	4 µl	4 µl	4 µl
M13 R primer	1 µl	1 µl	1 µl
Big dye	2 µl	2 µl	2 µl
H₂O	10 µl	9 µl	9 µl
Total	20 µl	20 µl	20 µl

A following sequencing program was set up

1. Denaturation at 94 °C for 1 minute
2. Denaturation at 94 °C for 1 minute
3. Annealing at 63 °C for 2 minutes
4. Elongation at 72 °C for 2 minutes
5. Returning to 2nd Stage for 35 cycles
6. Elongation at 72 °C for 10 minutes
7. 4 °C forever.

The sequence reaction was then sent for sequencing at the medicine department.

A tool method called BLAST (Basic Local Alignment Search Tool, <http://www.ncbi.nlm.gov/BLAST>) was used to search if the zebra fish genomic DNA was conclusive as expected.

5.3.10. A new miniprep “light “ was done using zAtoh1c in pZero-blunt and PCR2.1 TOPO, zAtoh1a1/2 in pZero-Blunt and PCR 2.1 TOPO, and zAtoh1a1/2 in pBluescript

5.4. ZEBRAFISH HUSBANDRY

5.4.1. Mating

Zebrafish are stored in a fish laboratory in containers marked with date of birth and when lastly spawned. The tank system used is called *Zeb Tec* which changes the water continuously and maintains the right temperature on the osmotic water, pH and conductivity. The maintenance temperature is about 28 ± 0 °C, pH of 7-8 (6).

The adult zebrafish are fed with Artemia (adult brine shrimp) two times per day and Gemma-Micro 300 (Skretting) three times per day.

The female zebra fish has a round bottom while the male zebra fish is more slender, streamlined and darker in color. The male fish has reddish-yellow hue around the blue stripes while the female blue stripes alternate with the silver stripes (57).

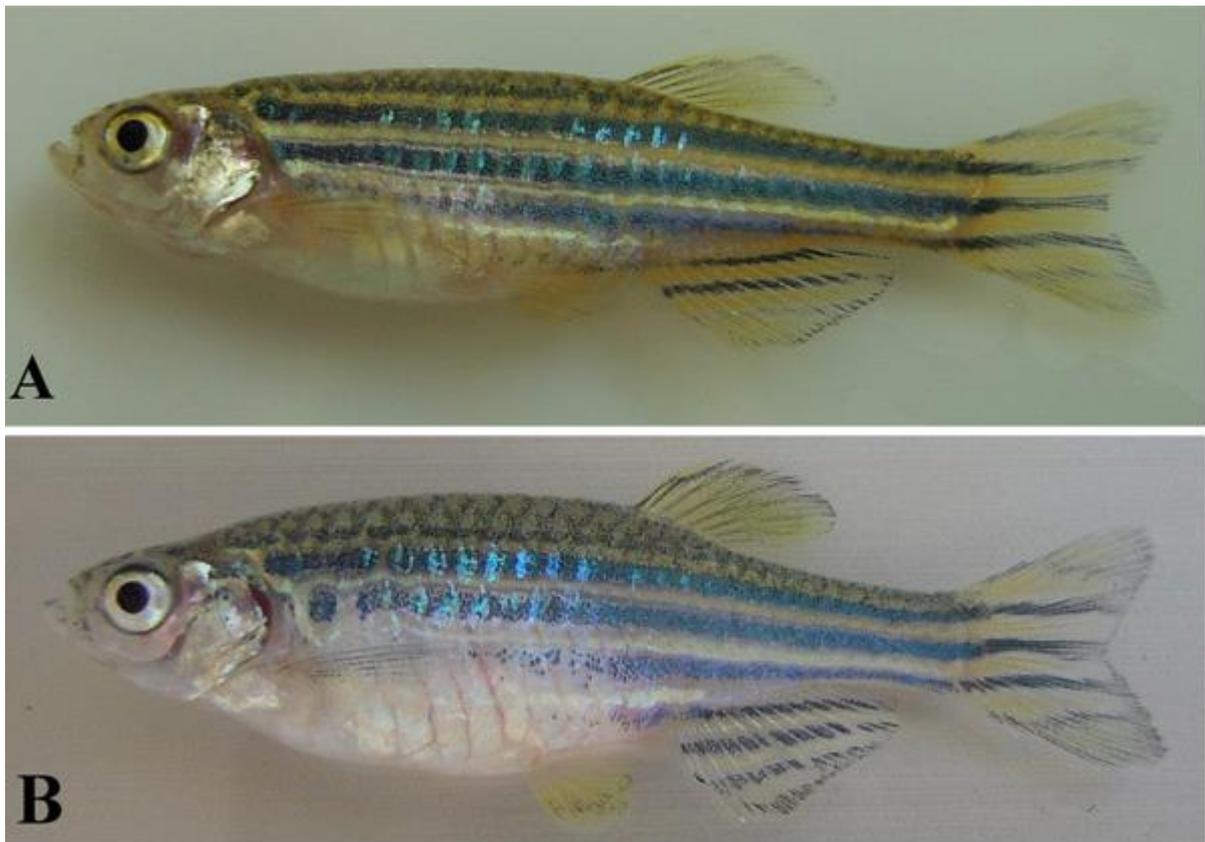


Figure 10: A: Male ZF, B: Female ZF. Female zebrafish has a round bottom while the male is more slender. The male zebrafish has a yellowish-hue around the blue stripes while the female alternate with silver stripes along the blue stripes (58)

Reproduction usually occurred during the photoperiod at the first few hours of daylight from 08:45 am where by the separated females and males are combined.

The female zebra fish were separated from the male fish during the afternoon 15 p.m (the day before spawning). The main reason for separating them is to prepare them for fertilization.

Oviposition is divided into three parts the initiatory, receptive and spawning. The release of gluconorides in the water induces ovulation for the females. During the initiatory phase the males swim towards the female and touch their tails with their noses/head, circling around the females or in front of them, at this phase the females swim beside the males. During spawning the females release hormones that tempt male mating behavior (6). The females swim beside the males in such a manner that their genital pores are aligned to each other, where the male performs tail oscillations around the females triggering oviposition and simultaneously releasing sperms. Typically 5-20 eggs are released at a time (57).

Several methods were used during mating including marble technique, where by marbles were placed at the bottom of special breeding tanks. When fish spawn over the marbles, the eggs drop into the spaces in between and preventing eggs cannibalism (6).

There could be a possibility that the fish could be squeezed in between the marbles thereby the marbles were replaced by artificial green seaweeds and a dark layered cover was placed under the tank.

A special breeding tank (8L) is filled with osmotic water and a small plastic mating cage with a mesh bottom is placed inside the breeding tank. Fish pairs approximately 2 females and 2 males are placed in the mating box during the afternoon. The females are separated from the males with a wall in between the cage. The wall is then removed during the first hours of daylight (photoperiod).

These methods may be effective in some extent but cannot be used in production of large quantity of eggs.

A method used in large breeding is a 70 liters tank is pawn with a net (separator) in between where by separating the female from the male with equal quantity during the evening. And the fish pairs are combined during the first few hours of daylight and left to spawn for almost 2 hours. When the fish spawn the fertilized eggs fall through the floor of the container and beneath them there is another net protecting the eggs from cannibalism by adults. The fish pairs are usually removed and placed back on their original containers before the collection of the eggs.

The large tank has a tap where when opened and the water runs through and the eggs falls throughout the net filter placed under the tap .A rod is usually used to stir up the water so as the eggs that fastens on the bottom of the container are mixed up with the water that flows through the tap.



Figure 11: Ispawn (*Zebtec*) breeding tank

After spawning the eggs are usually collected and cleaned using E3 medium and thereafter kept in E3 medium for further use.

5.4.2. Quality of embryos

The quality of the embryos was checked by observing them on the microscope. Discoloration of embryos to whitish precipitation was an indication that the embryos were dead.

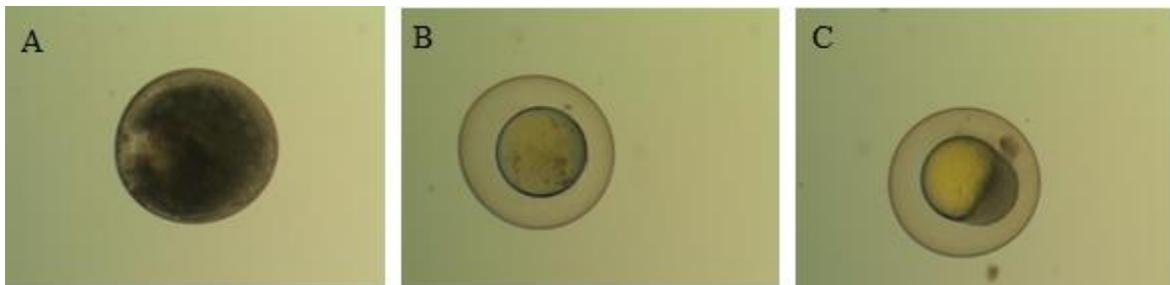


Figure 12: Description of quality of embryos, A-Dead zebrafish embryo, B: Unfertilized embryo and C: Fertilized embryo.

The embryos that were unfertilized were recognized by lack of development.

Incubation of embryos in different concentration of alcohol

99.8% of Ethanol absolute was diluted to 1 % and 2 % with Embryo medium to 50 ml

1 % Ethanol

$$C_1V_1 = C_2V_2$$

$$99.8 \% * V_1 = 1 \% * 50000 \mu\text{l}$$

$V_1 = 505 \mu\text{l}$ (Ethanol absolute) and then diluted to 50 ml with E3-Medium

2 % Ethanol

$$C_1V_1 = C_2V_2$$

$$99.8 \% * V_1 = 2 \% * 50000 \mu\text{l}$$

$V_1 = 1010 \mu\text{l}$ (Ethanol absolute) and then diluted to 50 ml with E3-Medium

Dilution from 1 % Ethanol

0.1 % Ethanol

$$C_1V_1 = C_2V_2$$

$$1 \% * V_1 = 0.1 \% * 50000 \mu\text{l}$$

$V_1 = 5000 \mu\text{l}$ (Ethanol absolute) and then diluted to 50 ml with E3-Medium

Dilution from 0.1 % Ethanol

0.01% Ethanol

$$C_1V_1 = C_2V_2$$

$$0.1 \% * V_1 = 0.01 \% * 50000 \mu\text{l}$$

$V_1 = 5000 \mu\text{l}$ (Ethanol absolute) and then diluted to 50 ml with E3-Medium

Embryos were incubated in a 6 well petridish with approximately 20 embryos in each plate that contained different concentrations of ethanol. The number of embryos incubated varied from experiment to experiment. The well plates used were marked with the time of incubation and type of medium used. The embryos were incubated at 28.4 °C in Fermaks heating cabinet.

5.4.3. Dechorionisation

Two different methods were used for dechorionisation the fish embryos. One of them is manually by using two needles 25G and trying to remove the chorions from the embryos by making a tear on the chorion with one syringe and removing the chorions with the other syringe (59).

The second method used was by treating the embryos with a dilute solution of pronase (2 mg/ml in E3-medium). Pronase makes the chorions brittle and easier to remove. The reaction was stopped by removing the pronase and washing the embryos as soon as the first embryo starts to come out of the chorions. The pronase treated embryos were washed up with E3-medium at least 3-4 times to remove the entire enzyme (3).

5.5. DIG-labeling of probes for *in situ* hybridisation.

5.5.1. Restriction enzyme digest for linearization of plasmid

1. 2.5 µg plasmid was linearized with a correct restrictions enzyme

Table 6: Set up for enzyme digestion for linearization of plasmid

Pax 6.1	Pax 2.1
327.6 ng/µl plasmid	412 ng/ µl plasmid
5 µl 10x TA	5 µl 10x TA
2 µl enzyme	2 µl enzyme
35 µl H ₂ O	37 µl H ₂ O
50 µl	50 µl

2. Plasmid was digested at 37 °C for 3-5 hours
3. The products were set up for Gel electrophoresis: 5 µl plasmid DNA + 1 µl 6XT
4. The DNA was correct so the further procedures were proceeded

5.5.2. Cleaning and precipitation of DNA

45 µl of cut plasmid DNA was with 155 µl H₂O and 200 µl Phenol: Chloroform. The tubes were vortexed and centrifuged at 13000 rpm for 1 minute. The upper phase was transferred to two new eppendorf tubes. 200 µl of phenol:chloroform was added and vortexed, then centrifuged at 13000 rpm for 1 minute. The top phase was removed once again to new tubes.

20 µl 3mM NaOAc and 450 µl of 100% Ethanol absolute was added and vortex, then left at -20 °C for 1 hour. The tubes were centrifuged in a Cold centrifuge 13000rpm 4 °C for 30 minutes. Supernatant was removed and DNA pellet washed in 500 µl ice cold 80 % Ethanol. The mixtures were centrifuged again for 15 minutes at 13000 rpm at 4 °C. DNA pellet was left to air dry on the bench for 10 minutes.

5.5.3. DIG-labeling of Pax6.1 and Pax2.1 Probe

Pellet was re-suspended in 13 µl nuclease free H₂O and the following was added:

2 µl 10x Transcription buffer (Roche)

2 µl DIG labeling mix (Roche)

1 µl RNA-se inhibitor

2 µl T7 polymerase

The mixture was then incubated for 2 hours at 37 °C. 2 µl 0,5M EDTA, 1 µl glycogen (20 mg/ml), 2 µl 4M LiCl, 66 µl 100 % EtOH was added, the mixture was mixed thoroughly and then stored over night at -75 °C.

The tubes were centrifuged for 30 minutes at 13000 rpm at 4 °C. Then washed in 300 µl 70 % ice cold EtOH, centrifuged for 15 minutes at 13000 rpm at 4 °C. Left to air dry in the RNA-hood. Then diluted in 20 µl DEPC H₂O.

1 µl probe was mixed with 4 µl DEPC H₂O then warmed at 80 °C for 10 minutes. 1 µl 6XT was added and the samples were run on agarose gel. Gel tub was washed with RNA ZAP.

1 x TAE buffer was used, 80V for 30 minutes

5.5.4. DIG LABELING of zAtoh1a1, zAtoh1a2, zAtoh1c

PCR products from zAtoh1a1, zAtoh1a2 and zAtoh1ac generated from probes containing SP6 (reverse) and T7 (forward) sites were used for DIG labeling with T7 polymerase. After purification of PCR products using PCR purification kit from Qiagen. The DIG labeling protocol was followed as described above.

5.6. SPOT ASSAY TESTING

The Control sample from DIG-labeling kit was diluted to 20 ng/ μ l

DIG-labeled Samples (Pax2.1 and Pax6.1) were diluted (50x) by adding 2 μ l DIG-labeled RNA to 98 μ l nuclease free H₂O

The following dilution series were made

Table 7: Dilution series

<i>CONTROL</i>	A	B	C	D	E	F
<i>Pax2.1</i>	A ₁	B ₁	C ₁	D ₁	E ₁	F ₁
<i>Pax6.1</i>	A ₂	B ₂	C ₂	D ₂	E ₂	F ₂

A: 2 μ l of 20 ng/ μ l in 38 μ l DEPC H₂O1 ng/ μ l

B: 5 μ l of dilution A in 45 μ l DEPC H₂O.....100 pg/ μ l

C: 5 μ l of dilution B in 45 μ l DEPC H₂O.....10 pg/ μ l

D: 5 μ l of dilution C in 45 μ l DEPC H₂O.....1 pg/ μ l

E: 5 μ l of dilution D in 45 μ l DEPC H₂O.....0.1 pg/ μ l

F: 5 μ l of dilution E in 45 μ l DEPC H₂O.....0.01 pg/ μ l

1 μ l of the diluted probe and Control test (A-F) were spotted in a membrane with the control reference on the bottom row. Thereafter crosslinked by being exposed to UV-LIGHT for 10 minutes.

The membrane was then washed in washing buffer in a 10 cm petridish. Thereafter incubated with blocking solution for 30 minutes at room temperature, sat at a rocking plate form at 33 rpm. Membrane was incubated in anti-DIG-alkaline phosphatase for 30 minutes in room temperature, rocked at 33 rpm. Ratio of 1:2000 was used since a blocking solution at 10 ml made.5 μ l of DIG-alkaline phosphatase was added and diluted in a 1 x MAB buffer.

Membrane was washed with washing buffer (2 x 15 minutes) and rocked at 33rpm.

Washing buffer was removed and staining detection buffer was added for 2 minutes. 5 ml of staining buffer was mixed with ½ a tablet NBT/ NBCI (Roche) which was packed in aluminum foil to protect from the light. The staining solution was then added and the membrane was packed with foil and left for 15-30 minutes.

Incubation was stopped by washing the membrane with tap water.

Washing buffer:

Blocking solution (10 %)

1 g Blocking reagent (Roche)

10 ml MAB-buffer

5.7. *In situ* RNA hybridization (SARS PROTOCOL)

24 hpf, 48 hpf and 72 hpf embryos (approximately 80 embryos per tube) were used

1. Embryos of the correct developmental stage were fixed in 1 ml 4 % paraformaldehyde-PBS over night at 4 °C
2. Dehydrated in 1 ml methanol for 10 minutes at RT, again in methanol at -20 °C. The embryos could be stored at -20 °C for several months.
3. Rehydrated in the following procedure
 - 75 % methanol/ 25 % PBS for 5 minutes
 - 50 % methanol/ 50 % PBS for 5 minutes
 - 25 % methanol/ 75 % PBS for 5 minutes
 - PBST for 5 minutes
4. Optional bleaching was done on embryos >48hpf. Approximately 1 ml of bleaching solution was placed in tubes that contained the embryos. The tubes were then exposed to light so as the bleaching reaction can take place. The embryos were observed in between to make sure that they don't over bleach. The reaction time wasn't supposed to exceed 30 minutes.
5. Digestion: 1 ml 10 µg/ml Proteinase K for embryos < 48hpf was used and incubated for 10 minutes at RT, 25 µg/ml for embryos >48hpf
6. Reaction was stopped by rinse twice in 2 mg/ml Glycine in PBST
7. Embryos were prefixed with 4% paraformaldehyde for 20 minutes at RT
8. Rinsed 5 x 5 minutes in PBST
9. Prehybridization:
 - 500 µl hybridization buffer was inserted carefully when embryos sank to the bottom.
 - Replaced with fresh hybridization buffer and incubated at 65 °C for 10 minutes.
 - Replaced hybridization buffer and incubated for 3 hours at 65 °C.

10. 10 µg Pax2.1 probe and 20 µg Pax6.1 probe per 6.5 ml Hybridization solution was warmed up for 5 minutes at 70 °C before use to denature. Embryos were incubated with the DIG-probes O/N at 65 °C.

11. Washing: All the solutions used were warmed up in net wells (65 °C)

- 75 % formamide, 2 x SSC for 10 minutes
- 50 % formamide, 2 x SSC for 10 minutes
- 25 % formamide, 2x SSC for 10 minutes
- 2X SSC for 10 minutes
- 0,2X SSC for 2x30 minutes

12. Blocking: Hybridized embryos were transferred to wells and incubated in

- 1 ml MAB for 5 minutes
- 1 ml MAB+BMB for 1 hour
- 500 µl MAB+BMB+HI-FCS for 3 hours

3 µl antibody (Anti-Digoxigenin-AP Fab Fragments Roche) was added in a tube marked “AB” with control embryos. Thereafter 600 µl MAB+BMB+LS was added to make a dilution of 1:2000 and rocked to preabsorb while embryos are blocking.

720 µl MAB+BMB+LS was added in each tube then 80 µl of “AB” and incubated while rocking O/N at 4 °C

13. Washing: Embryos were washed with MAB(5X 20minutes)

- Then 3 x 5 minutes with alkaline phosphatase buffer solution
- Transferred to 24 well dish

14. Stained: ½ a tablet NBT/BCIP was dissolved in 5 ml MAB-solution and 800 µl was transferred to each well. The 24 well dishes was covered with aluminum foil and checked regularly for staining. Typically the staining is stopped after 2 hours but for some experiments the staining was prolonged O/N at 4 °C.

15. Staining was stopped by rinsing twice in PBST and then fixing with 4 % PFA O/N at 4 °C

16. To take pictures embryos were rinsed in 25 % Glycerol for 10 minutes, 50 % glycerol for 10 minutes and the 100 % Glycerol (Sigma Aldrich) for 10 minutes

17. A microscope slide was used, three layer of tape were placed on the ends of each slide cut 1 cm x 2 cm. Several drops of glycerol were placed in the middle and the embryo was placed on the middle. Cover slip was placed on top and pictures were taken by

using Nikon camera. Cover slip was moved back and forth to make any adjustments while positioning the embryo for the right posture (lateral or dorsal)

Buffers used

PFA

4 % paraformaldehyde

0.8 g PFA (Merk Schuchardt)

16 ml H₂O

2 ml 10 x PBS

1-2 drops (50-100 µl) 2M NaOH

Adjusted the volume to 20 ml.

Place on a waterbath at 65 °C for 20-30 minutes, shaken in between.

20X SSC

175.3 g of NaCl

88.2 g of Sodium Citrate

800 ml H₂O.

Adjust the pH to 7.0 with a few drops HCl.

Adjust the volume to 1 liter with ultrapure water.

Sterilize by autoclaving.

Optional bleaching (10ml)

2.5 % 20 x SSC

5 % Formamide (Sigma Aldrich)

92.5 %, 10 % H₂O₂ (NAF)

MAB

100 mM Maleic acid

150 mM NaCl

pH adjusted 7.5

0.1 % Tween 20

MAB+BMB (2 % Boehringer Blocking Reagent)

Block (dilute 5x from a 10 % stock solution)

in 1 x MAB

MAB+BMB+20 % HI-FCS (Heat indicated Fetal Calf serum)

2 % Blocking solution (10 %)

20 % HI-FCS

in 1 x MAB

Hybridization buffer

50 % Formamide

5X SSC

50 µg/ml Heparin (Biochrom)

500 µg/ml tRNA (Roche)

0.1 % Tween-20

92 µl of 1M Citric acid (pH 6,0)

H₂O to 10 ml

Alkaline phosphatase buffer

100 mM TrisHCL pH 9.5

50 mM MgCl₂

100 mM NaCl

0.2 % Tween-20 (Sigma aldrich)

0.2 % Triton-X100

Staining buffer

10 ml Alkaline phosphatase buffer

1 (NBT/BCIP) tablet (Roche)

5.8. Western blot analysis

Western blot is a method used in research to separate and identify proteins. In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with antibodies specific to the protein of interest (60).

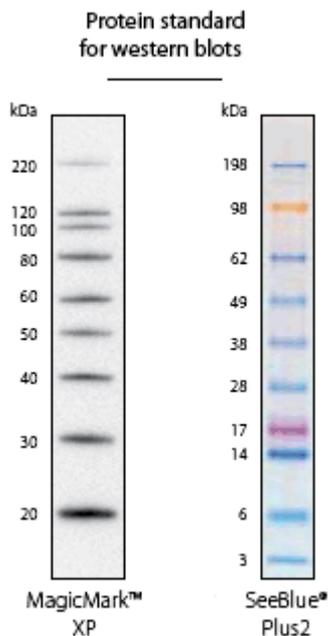


Figure 13: Protein molecular standards for Western blot analysis obtained from Invitrogen on the left Magicmark™ and on the right prestained seeblue® plus (61).

2 x 25 embryos that were incubated in E3-medium, 1 % EtOH and 2 % EtOH at 24 hpf, 48 hpf and 72 hpf .The embryos were dechorionised and placed on ice.

A mastermix was made containing

105 µl NuPAGE® LDS Sample Buffer (4X)

42 µl NuPAGE® Reducing Agent (10X)

273 µl Ionized H₂O

420 µl

50 µl of mastermix was pipetted on the embryos and mixed thoroughly 10x by using a pipette and then warmed up at 100 °C for 5 minutes.

Samples were first sonicated for 2.5 minutes and centrifuged for 2 minutes.

NuPAGE® Bis-Tris Gels 4-12 % with NuPAGE® MES SDS with 12 wells was used. The gel was removed from the pouch and the comb was smoothly removed. Peeled the tape bottom of the cassette. Rinsed the gel-well with 1x SDS running buffer (Nupage). Put together the two gels in the Mini-Cell such that the notched “well” side of the cassette faces inwards toward the buffer core. Placed the gels on the bottom of the mini-cell and lock into place with the gel tension wedge. The inner chamber was filled up with running buffer to check for tightness of the seal. SDS running buffer (Nupage) was filled on the outer chamber and the samples were loaded into the wells. 5 µl of the zebra fish protein samples were loaded. 1 µl Magicmarker (Nupage) and 4 µl See Blue Breeze (Nupage) were used as molecular weighing protein standards (Figure 13). Electrophoresis was performed at 200V for 45 minutes.

A nitrocellulose membrane was cut 7 cm x 7.5 cm and two pieces of filter paper About 700 ml Transfer Buffer was used to soak the pads until they were saturated. Two soaked blotting pads were placed into the cathode (–) core of the blot module.

The gel cassette was opened carefully by using a gel knife.

A piece of pre-soaked filter paper was placed on top of the gel,

Filter paper saturated with the transfer buffer and all trapped air bubbles were removed by gently rolling over the surface using a roller

The plate was turn over so the gel and filter paper are facing downwards over a gloved hand or clean flat surface.

A Gel Knife to push the foot out of the slot in the plate, and separate the gel from the plate.

After placing the gel on a flat surface, the foot of the gel was cut off with the Gel knife. The pre-soaked transfer membrane was positioned on the gel, ensuring all air bubbles have been removed.

Another pre-soaked filter paper was placed on top of the membrane. Trapped air was removed. Placed blotting pads such that the gel is closest to the surface of the cathode core.

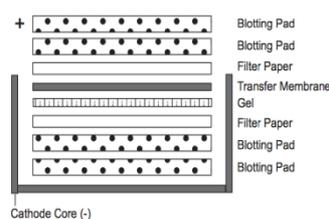


Figure 14: Illustration of sandwich set up for blotting procedure(62)

Added two more pre-soaked blotting pads and placed the anode (+) core on top of the pads. The gel/membrane assembly was held securely between the two halves of the blot module ensuring complete contact of all components. Positioned the gel/membrane assembly and blotting pads in the cathode core of the XCell II™ Blot Module to fit horizontally across the bottom of the unit. The blot module was held together firmly and slide into the guide rails on the lower Buffer Chamber. The Gel Tension Wedge was placed and locked (62).

The gel was blotted for 2 hours on 20V.

The membrane was transferred to a 50 ml tube with 5 ml of Blocking buffer (Li-COR) was left to block overnight at 4 °C.

Replaced with 2 ml of blocking buffer and 2.5 µl of Tween 20®, 2.5 µl Anti-pax6 rabbit (1:1000) (Millipore).

Incubated while rolling for 45minutes.Placed 2.5 µl of anti-actin (Sigma) and incubated again for 45minutes.

Membrane was washed 4x5mins with 1xTBST.

10 ml of 1x TBST was placed and 1 µl of anti-rabbit 680(Li-COR,Odyssey) was added. The tube was wrapped with aluminum foil and incubated for another 1 hr. Thereafter washed 4 x 5 minutes in 1 x TBST.

Membrane was scanned using ODESSEY LI-COR machine.

5.8.1. Western blot assessment using DDT AND SDS

In one of the researches 2 x SDS and 1M DTT (Dithiothreitol) were used for making the samples for Western blot analysis. 160 µl 2x SDS and 40 µl DDT was mixed and 30 µl was pipetted on 15 embryos. The same procedure was done as on the previous analysis. 20 µl of the protein sample was used on each well. After blotting, and blocking the membrane was double stained with primary antibody 5 µl anti-pax6 (sheep) and 2.5 µl anti-actin (rabbit) for 45 minutes. Washed 4 x 5 min with 1 x TBST. Thereafter incubated in 1µl anti-sheep 800(1:10000) and 1µl anti-rabbit 680 (1:20000)

Buffers used

Blocking buffer

PBS (phosphate-buffered saline) contained 0.1% sodiumazide.

1 x Running buffer

Diluted form 20 x SDS Running buffer, which contained

50 mM MES

50 mM Tris Base

0,1 % SDS

1 mM EDTA

pH 7.3

Transfer buffer

29 g trisebase

144 g glycine

1 liter methanol

H₂O til 6 liters

1 x TBST

10 x TBS

0.1 % Tween 20

TBS

20 mM Tris, pH 7.5

500 mM NaCl

2X SDS(Sodium dodecyl sulfate)

5 ml 1M Tris-HCl, pH 6.8

10 ml 10 % SDS,

20 ml 50 % Glycerol

0.1 g bromphenol blue.

Distilled water to 50

1M DDT (Dithiothreitol)

1.54 g Dithiothreitol

33.3 μ l 3M NaOAc, pH 5.2

Distilled water to 10 ml

6. RESULTS

The main goal was to clone cDNA to zAtoh1a1, zAtoh1a2 and zAtoh1c to make probes that could be used as markers in cerebellum, in order to study the effects of alcohol during brain development. The first part of the results describes Cloning experiments while the second part contains incubation of embryos in different concentrations of ethanol, *In situ* hybridisation of probes and Western blot analysis.

6.1. Part 1

Embryos at 24 hpf were used to extract RNA from zebrafish embryos and cDNA was synthesized. PCR was then used for amplification of the appropriate target genes from cDNA with specific primers from the zAtoh1a1, zAtoh1a2 and zAtoh1c genes. Shh was used as positive control. RT-PCR (5.3.1) showed no product of the Atoh primers (Figure 15A).

Optimization (5.2.2) was done with PCR block gradient, since no conclusive results were obtained from RT-PCR. PCR block gradient showed amplification only on Lane 2 at 615 bp at 58° C. Rest of the bands showed no amplification (Figure 15B).

A new PCR-reaction with Phusion (5.2.3) was done and PCR products of expected size respectively; zAtoh1a1 at ~553 bp, zAtoh1a2 at ~909 bp and zAtoh1c at ~ 615 bp were observed and thereafter were used for cloning (Figure 15C).

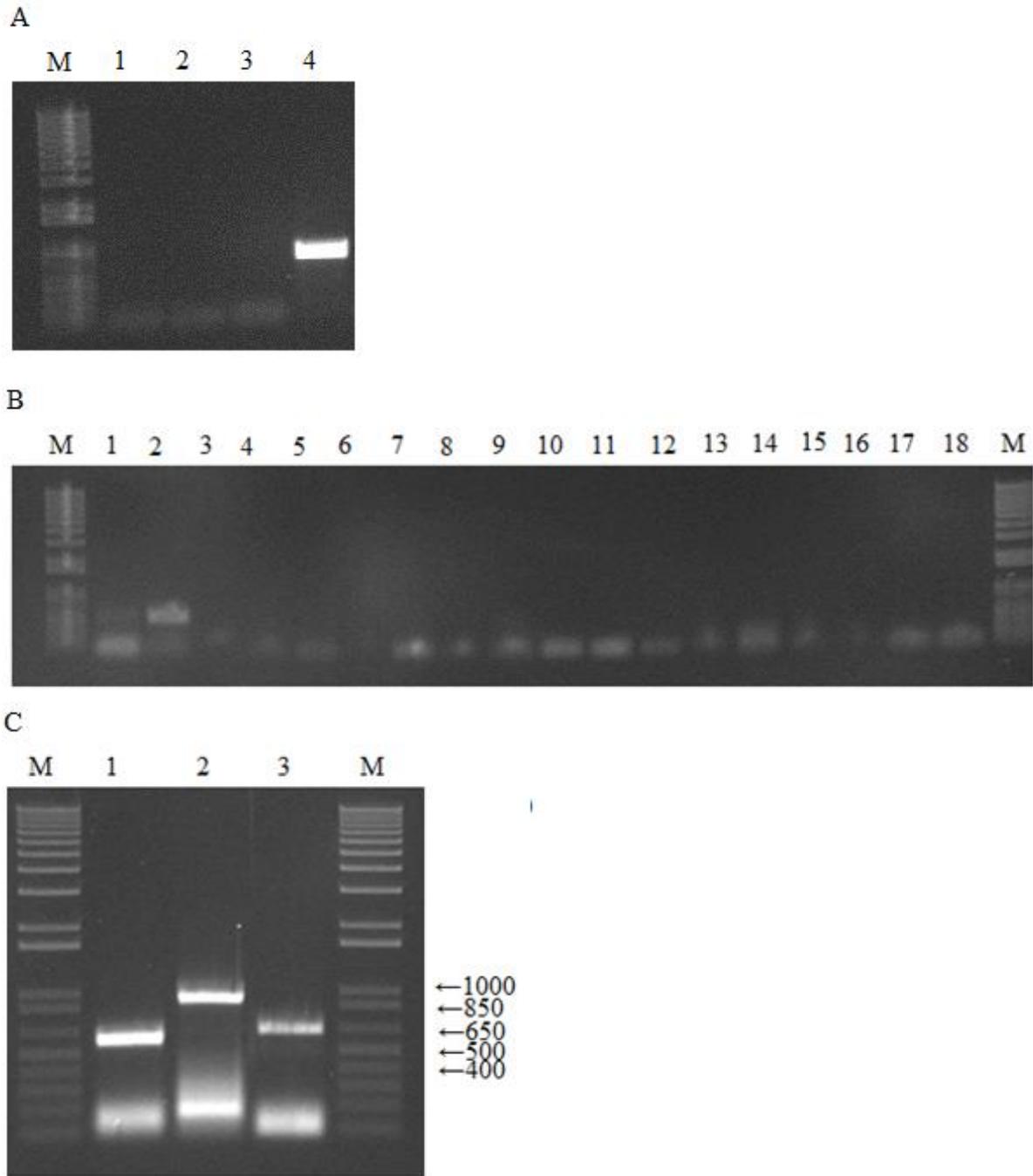


Figure 15: Optimization of the PCR conditions was required for the successful amplification of the zAtoh1a1, zAtoh1a2 and zAtoh1c genomes from cDNA. Lane M, 1kb plus ladder, Figure A, Lane 1 zAtoh1a1, Lane 2 zAtoh1a2, Lane 3 zAtoh1c, Lane 4 zfShh.1 at 768bp (Positive control) Optimization with PCR block gradient at 58 °C (Figure B), from left lane 1-3 zAtoh1a1, Lane 4-6 zAtoh1a2, Lane 7-9 zAtoh1c. Amplification only on lane 2 at ~615 bp. Figure D zAtoh1a1 at ~553 bp, zAtoh1a2 at ~909 bp and zAtoh1c at ~615 bp

6.1.1. Cloning of PCR® Zero-blunt vector and pbluescript KS +/-

After PCR amplification of zAtoh1a1, zAtoh1a2 and zAtoh1c cDNA, the PCR products were purified and several attempts were done to clone them into the pZero-blunt vector. Due to the difficulty in cloning of PCR-blunt vector pBluescript was used. Linearization of pBluescript with *EcoRV* (5.3.3) gave a bright single band (Figure 16) which was cut out from the gel and purified by Qiagen gel purification kit (5.3.3.1).

PCR products from amplification were used for ligation of pBluescript digested with *EcoRV* and pZero-blunt. Ligation mixtures from pBluescript and pZero-blunt were added to DH5 competent bacteria cells. Bacteria cell culture was spread on LB plates containing 100 µg/ml Ampicillin and 50 µg/ml Kanamycin. A miniprep light was done (5.3.5) to obtain DNA. DNA products were digested with restriction enzymes; *EcoRI* and *Hind III*. DNA was set up for gel electrophoresis with 0.7 % agarose gel for 40 minutes at 90V. Results of pZero-blunt from Lane 1-12 show weak bands with insert in plasmid but they are not of the right size (Figure 17).

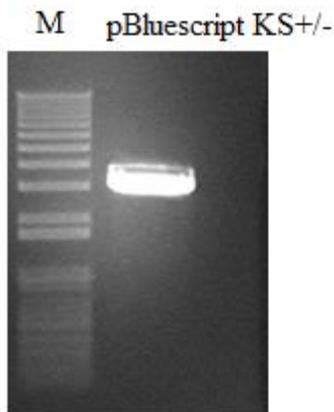


Figure 16: Linearized p-bluescript with *EcoRV*

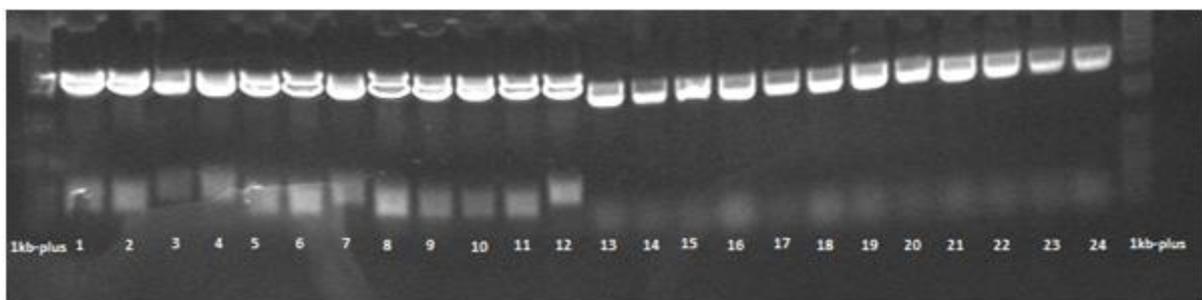


Figure 17: Cloning of pZero-blunt showed insert which was digested with *EcoRI*. From left lane 1-4 zAtoh1a1 with pZero-blunt, Lane 5-8 zAtoh1a2 with pZero-blunt, Lane 9-12 zAtoh1c with pZero-blunt. Lanes with pBluescript digested with *EcoRI* and *Hind III* shows no insert at all. Lane 13-16 zAtoh1a1 with pBluescript, Lane 17-20 zAtoh1a2 with pBluescript 2 and Lane 1-24 zAtoh1c with pBluescript.

6.1.2. Sequence for PCR® Zero-blunt vector

Since cloning of pZero-blunt indicated that there was an insert of PCR products in vector but not at the right size plasmid DNA was extracted from pZero-blunt bacteria cultures with miniprep (5.3.8) and DNA concentration was measured (Table 4). The mini-preps with the appropriate amount of DNA approximately 500 ng/μL, ratio of purity (260/280) nm of ~1.8-2.0 and secondary measure of nucleic acid purity (260/230) nm of 2.0-2.2 were set up for sequence reaction (Table 5).

The sequencing results showed that it was not the nucleotide sequences of Atoh genes that had been cloned. Due to a suspected contamination direct sequence of PCR products was set up to find out whether the contamination came from transformed bacteria cell or from the PCR products. The results showed that PCR-products contained the right zebrafish zAtoh1a1, zAtoh1a2, zAtoh1c genes (Appendix 1). DH5 bacteria culture was streaked directly on a LB-agar plate containing Kanamycin (50 μg/ml) or Ampicillin (100 μg/ml) but there was growth on both plates, which confirmed that the bacterium was contaminated.

6.1.3. Cloning of PCR® Zero-blunt vector, PCR2.1 TOPO and pBluescript KS+/-

A new experiment was done using other types of vectors as mentioned above (5.3.10)

No results when obtained from this experiment either, the segments that are seen are larger than the expected segments after enzyme digestion. The large segments are determined to be the size of vectors (Figure 18); respectively pZero-blunt vector around ~3.5 kb as seen on Lane 1-15, however Lane 12 has a segment over 4.4 kb which is the size of vector probably with the insert but not digested. Lane 16-23 shows the size of PCR2.1 TOPO ~3.9 kb used for cloning without insert while Lane 24 shows pBluescript with insert around ~3900 kb but not digested.

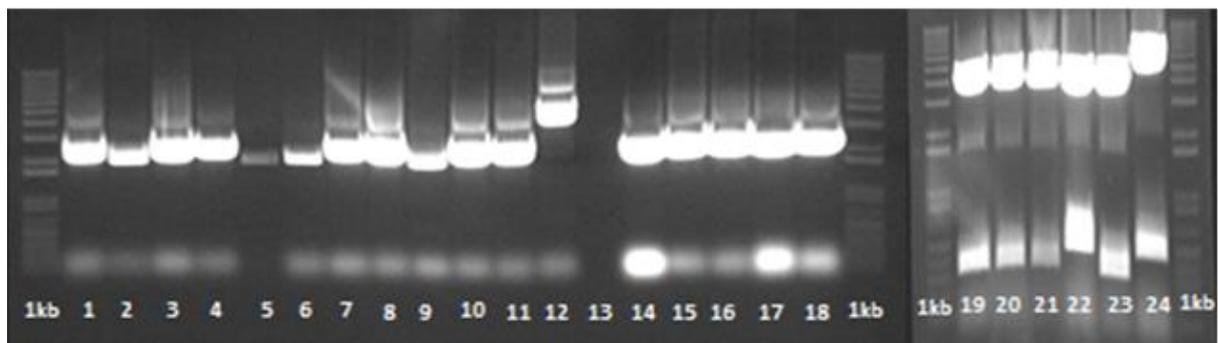


Figure 18: After digestion of Ecor I and Hind III, cloning of PCR® Zero-blunt vector, PCR2,1 TOPO and pBluescript KS +/- in zAtoh1a1, zAtoh1a2, zAtoh1c results show no cloned insert in vector. First lane -1kb plus ladder, Lane 1-5 zAtoh1c cloned with pZero-blunt 6-10 zAtoh1a2 with pZero-blunt 11-15 zAtoh1a1 with pZero-blunt, Lane 16-20 zAtoh1a1 with PCR2,1 TOPO, Lane 21-23 zAtoh1a2 with PCR2.1 TOPO and Lane 24 zAtoh1a2 with pBluescript.

6.1.4. DIG-probe direct from PCR-products

Since there were problems with the cloning of PCR products containing zAtoh1a1, zAtoh1a2 and zAtoh1c genes, it was impossible to make plasmids to be used for ISH probe generation. However a method using PCR-products as templates for DIG-labeling has been described (5.5.4) is possible. To do this, PCR primers with SP6 and T7 binding sites are required. The PCR probes successfully used to amplify the Atoh genes were therefore re-ordered with a SP6 sequence in the 5' end of the forward primer, and a T7 sequence in the 5' end of the reverse primer. After the PCR products were generated and purified, the T7 DNA polymerase was used to make DIG-labeled antisense transcripts (= ISH probes).

Labeling of DIG-probe from PCR-products was successful. A strong band of zAtoh1a2 around 900 bp, and two weaker bands for zAtoh1a2 and zAtoh1c.

The labeled probes were used for RNA *in situ* hybridization as described in the next section of the thesis.

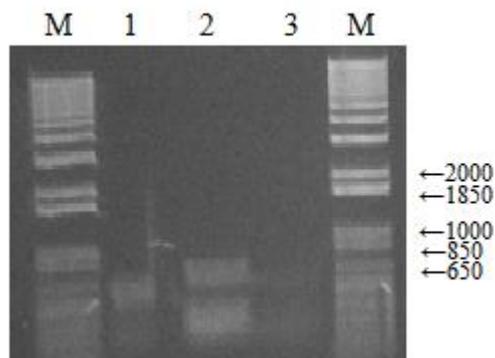


Figure 19: DIG labeled probe Lane M 1kb-plus ladder, Lane 1 zAtoh1a, Lane 2 zAtoh1a2 and Lane 3 zAtoh1c. Weak bands of lane 1 and 3, A slightly strong band at ~900bp.

6.2. Part II

Zebrafish embryos obtained from mating were incubated in different concentrations of ethanol 0.01 %, 0.1 %, 1 %, 2 % and E3-medium for 24 hours, 48 hours and 72 hours. The embryos were observed and dead embryos were counted and removed daily. Whitish precipitate in the chorion determined that the embryos were dead (5.4.2). Remaining embryos were dechorionised. Changes on morphology were registered (10.2) and photographed. A Nikon Digital Sight DS-U3 camera was used to photograph the embryos.

Some embryos were fixed with 4 % PFA and stored at -20°C for *in situ* hybridization, while the rest were used to make protein extracts for Western blot analysis and stored at -80 °C.

6.2.1. Zebrafish and mortality

The percent of death decreases in relation with time of incubation because dead embryos were removed daily.

The number of death varied a lot. There are several factors that influenced mortality such as

- Quality of the embryos that were used
- Suffocation of embryos due to lack of oxygen and this could be caused by a large number of embryos being incubated in a small petridish.

Referring to the reasons mentioned above as the cause of mortality for the embryos incubated in E3-medium.

Over all the embryos that were incubated in 2 % ethanol had the highest rate of death from 24 hpf of incubation up to 72 hpf of incubation (Figure 20). Mortality increased gradually over time with longer incubation period. This could be to the fact that incubation of embryos in ethanol makes it impossible for embryos to develop thereby causing death.

Embryos that were incubated in 1 % ethanol showed a large number of deaths as well, during 24 hrs to 48 hrs of incubation. During this period of time the development of the most important organs likes the CNS and the brain occurred (7).

Embryos that were incubated in 0.01 % had a large number of deaths during 48 hpf, approximately 24 % in comparison with embryos incubated in 24 hpf. While embryos incubated in 0.1 % ethanol had higher number of death during 24 hpf approximately 22 % (Table 8).

Table 8: Total number of death

	E3-Medium	0.01 % Ethanol	0.1 % Ethanol	1 % Ethanol	2 % Ethanol
24 hpf (n=1380)	24 %	22 %	22 %	22 %	28 %
48 hpf(n=1380)	18 %	25 %	12 %	17 %	29 %
72 hpf(n=420)	24 %	-	-	24 %	25 %

N: Total number of embryos incubated per group

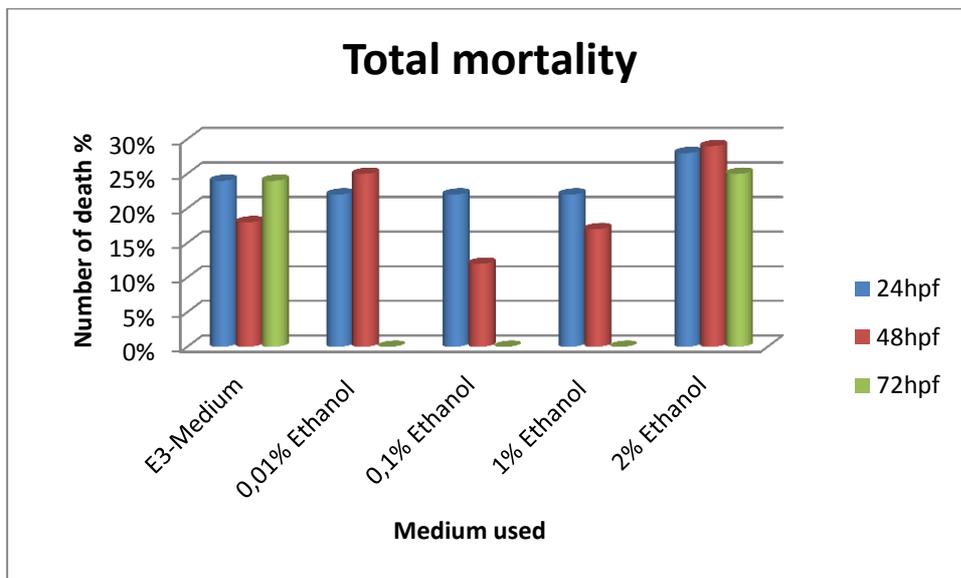


Figure 20: Number of mortality with time (hours), x-axis shows the mediums used for incubation while the y-axis show the total number of death in percent. Embryos incubated in 24 hpf had a raise in lethality according to higher concentration of ethanol. 48 hpf had fluctuation in mortality, but the highest number of death is in 2 % ethanol.

6.2.2. Morphological changes in 24 hpf embryos.

Embryos that were incubated in 1 % ethanol do not show much morphological changes, this could be because it is very early in the development stage to conclude if ethanol affects embryogenesis since during that period the embryos are still developing or the changes are comparatively small and not visible. Embryos that are incubated in 2 % ethanol were seriously deformed (Figure 22C) some embryos from 2 % ethanol had yolk sac edema and pericardial edema (Table 9).

Embryos incubated in 1 % ethanol had axial malformation (truncated tail and body axis) (Figure 22B). They were inactive in comparison with embryos in E3 medium. Some embryos were pale, which could be caused by a delay in development compare to other embryos.

Table 9: Overview of deformations and morphological changes in 24hpf embryos

N=140	Mortality after 24 hrs	D	SD	PE	YSE	AM	Embryo remaining
E3	19	-	-	-	-	-	121
1 % EtOH	17	13	4	-	-	13	123
2 % EtOH	39	17	24	5	3	3	101

AM: Axial Malformation, YSE: Yolk Sac Edema, PE: Pericardial Edema, D: deformed, SD: seriously deformed

N: Total number of embryos

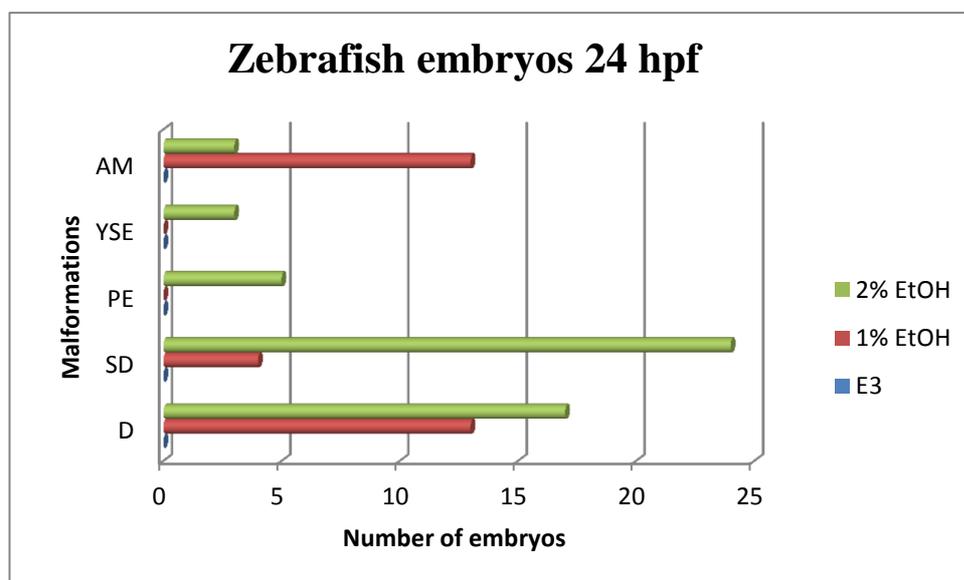


Figure 21: Graphical view of different kind of malformations at 24 hpf in control, 1 % and 2 % ethanol. Embryos incubated in 2 % ethanol were seriously deformed (SD) and deformed (D) as the graph shows. While embryos incubated in 1 % ethanol had a higher rate of axial malformations (AM) and other deformations like yolk sac edema (YSE), pericardial edema (PE).

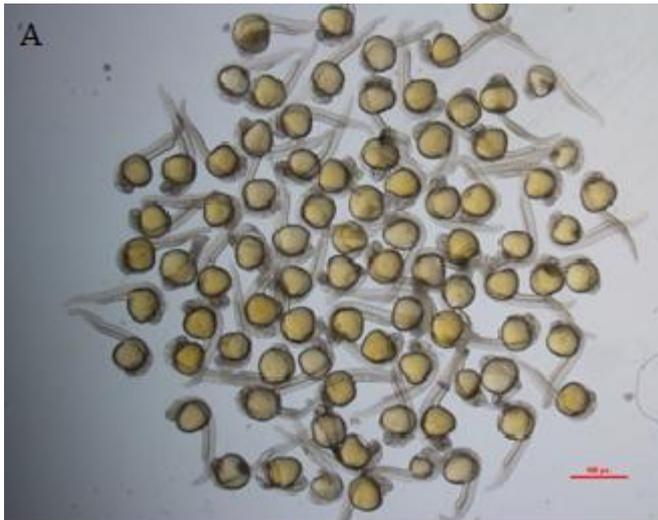


Figure 22: Overview of embryos incubated for 24 hours in A: E3-medium, B: 1 % Ethanol and C: 2 % Ethanol. Embryos incubated in 1 % ethanol (Figure B) shows axial malformation including truncated tail and body axis while embryos in 2 % ethanol (Figure C) show severe deformation.

6.2.3. Morphological changes in 48 hpf embryos

Embryos that were incubated in 1 % ethanol showed different malformations including pericardial edema, yolk sac edema, and axial malformation (Figure 24B, Table 10). 2 % ethanol embryos were not active they appeared to have reduced locomotive senses; they had no actual movements and shoaled when touched on the tail. Embryos had axial blistering around the tail and their body sizes were reduced, shortened length and had bowed body axis (Figure 24 C). They also developed pericardial edema, yolk sac edema and truncation around the head area. Most of the embryos in E3-medium and 1 % ethanol showed a sign of delay in development, which could be caused by lack of oxygen (a large number of embryos incubated in a small dish) or effects of ethanol (Figure 24A, B).

Table 10: Overview of deformations and morphological changes in 48 hpf embryos

N=140	Mortality after 24 hrs	Mortality after 48 hrs	D	SD	PE	YSE	AM	Embryo remaining
E3	18	-	-	-	-	-	-	122
1 % EtOH	31	2	5	4	21	20	6	109
2 % EtOH	41	21	13	44	8	9	103	78

AM: Axial Malformation, YSE: Yolk Sac Edema, PE: Pericardial Edema D: deformed, SD: seriously deformed

N: Total number of embryo

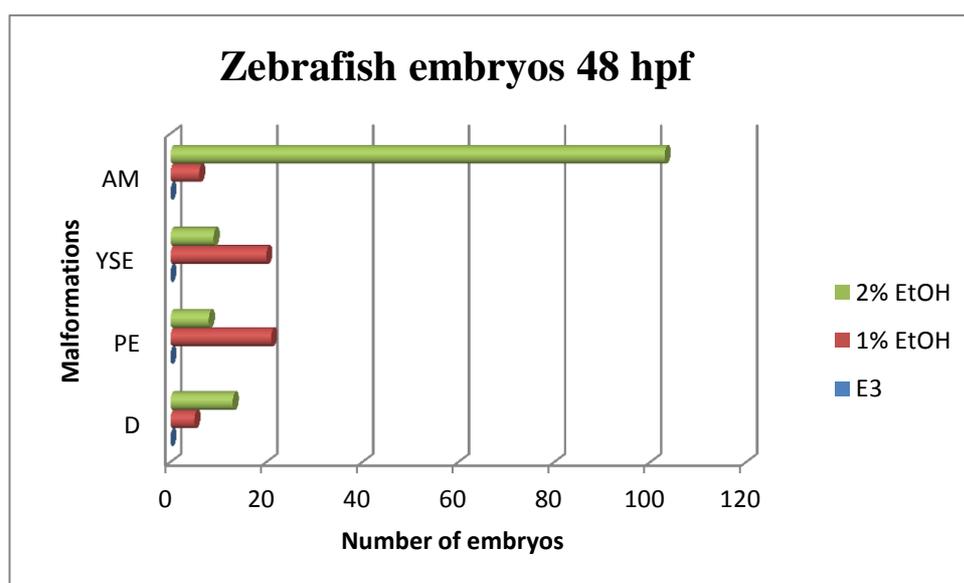


Figure 23: Graphical view of different kind of malformations at 48 hpf in control, 1 % and 2 % ethanol. Deformations (D) arose frequently in 2 % ethanol embryos. Axial malformation (AM) is dominating in 2 % ethanol embryos. While yolk sac edema (YSE) and pericardial edema (PE) occurs currently in embryos incubated in 1 % ethanol.



Figure 24: Overview of embryos incubated for 48 hours in A: E3-medium, B: 1 % Ethanol and C: 2 % Ethanol. Embryos had delay in development (Figure A and B). By a random look embryos in 1 % ethanol developed pericardial edema (PE) and yolk sac edema (YSE). Some few embryos survived through incubation in 2 % ethanol (Figure C) they shows different deformations, including truncation/swelling on head area, PE, YSE, shortened body length and axial blistering around the tail.

6.2.4. Morphological changes in 72 hpf embryos

Embryos that were incubated in 1 % ethanol showed the same kind of deformities as embryos from 48 hpf (Table 11) Embryos that were incubated in 2 % ethanol had deformations and the few that weren't deformed had other kind of malformation including yolk sac edema (YSE), axial malformation (AM), axial blistering (AB) and pericardial edema (PE)(Figure 26C).

Table 11: Overview of deformations and morphological changes in 72 hpf embryos

N=140	N=Mortality after 24 hrs	N=Mortality after 48 hrs	N=Mortality after 72 hrs	D	S D	P E	Y S E	A M	Embryo remaining
E3	31	1	2	-	-	-	-	-	106
1 % EtOH	26	2	6	3	-	39	30	5	106
2 % EtOH	35	12	12	4 5	11	49	49	49	81

AM: Axial Malformation, YSE: Yolk Sac Edema, PE: Pericardial Edema, D: deformed, SD: seriously deformed
N: Total number of embryos

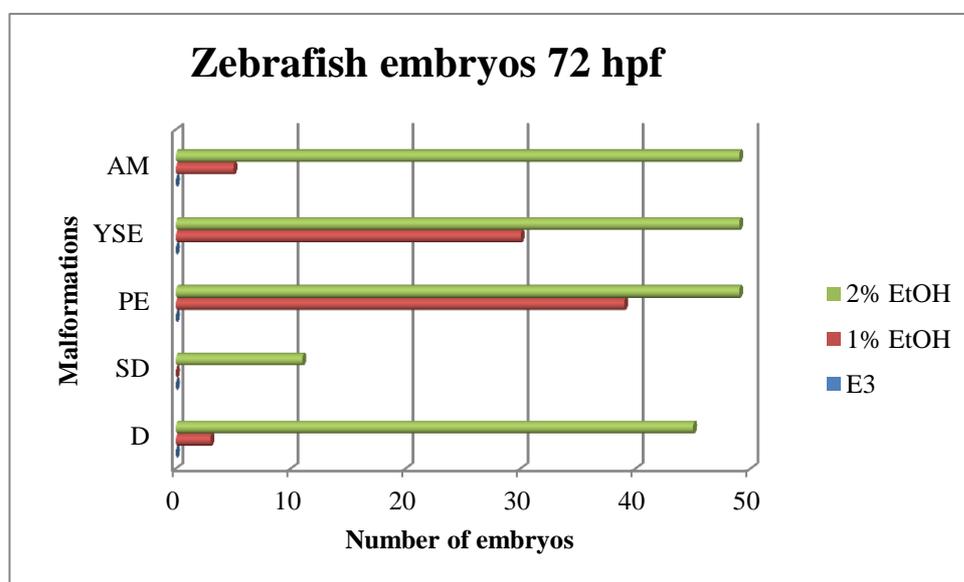


Figure 25: Graphical view of different kind of malformation at 72 hpf in control, 1 % and 2 % ethanol. Embryos incubated in 2 % ethanol indicated persistent occurrence in variety of malformations including axial malformation (AM), yolk sac edema (YSE), pericardial edema (PE). A higher number in the embryos seriously deformed (SD) and deformed (D). While 1 % ethanol embryos has diversity in deformity, with severity in pericardial edema (PE) and yolk sac edema (YSE).

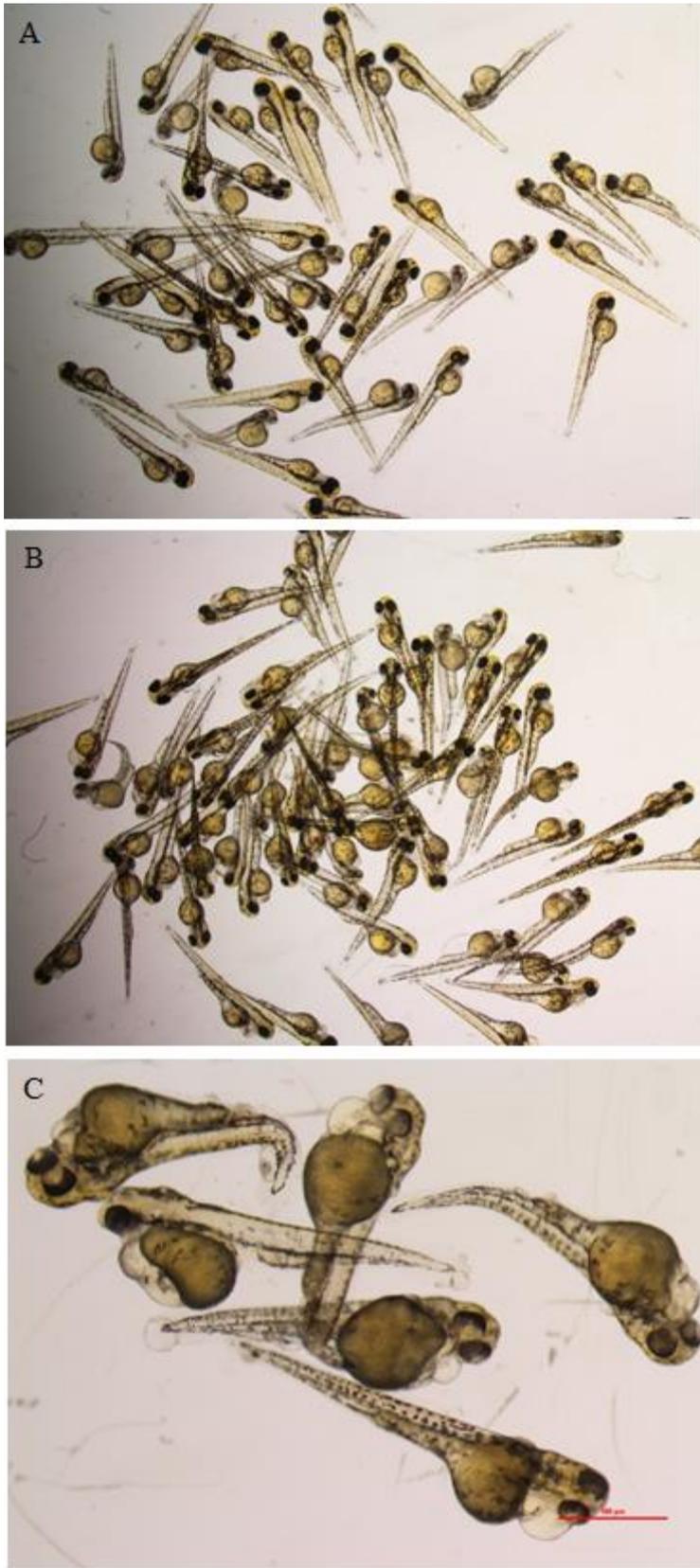


Figure 26: Overview of embryos incubated for 72 hours in A: E3-medium, B: 1 % Ethanol and C: 2 % Ethanol. Embryos incubated in 1 % ethanol developed pericardial edema (PE) and yolk sac edema (YSE) and truncated body axis. Some few embryos survived through incubation in 2 % ethanol (Figure C) they shows different deformations, including truncation/swelling on head area, PE, YSE, shortened body length and axial blistering (AB) around the tail.

Embryos were incubated too in 0.01 % and 0.1 % ethanol. There were not any changes observed from the embryos apart from late development and shoaling. (Figure 27F).



Figure 27: Closer view of embryos that were incubated in different concentrations of ethanol. A and C: 48 hpf 1% ethanol, B: 72 hpf 1 % ethanol, D: 48 hpf 2 % ethanol, E: 48 hpf 0.01 % ethanol and F: 48 hpf 0.1 % ethanol. Most of the embryos incubated in 0.1 % (Figure F) ethanol appeared to be pale, had less pigmentation.

Use of DIG-Labeled probes to visualize structures in the eyes and brain in normal embryos and embryos exposed to ethanol.

DIG-labeled PCR-products zAtoh1a1, zAtoh1a2 and zAtoh1c (6.1.4) were supposed to be used for *in situ* hybridization to visualize structures of the eyes and brains. Cloning was not successful and DIG labeled of PCR products (Figure 19) did not give results when used for *in situ* hybridization experiments (results not shown).

Therefore Pax6.1 and Pax2.1 DIG labeled probes were used as replacement.

Anti-sense probes were DIG-labeled by linearization of the plasmid and application of T7-DNA polymerase for transcription of antisense strand. Pax6.1 and Pax2.1 containing plasmids were digested with *EcoRI* (Figure 28A) for 3-5 hours at 37 °C (5.5.1), thereafter DIG labeled to generate the Pax6.1 and Pax2.1 anti-sense probes (Figure 28B).

The bands obtained shows low intensity, which reflects that the amount of probes was low (Figure 28B). This indicates that larger amount of probe is needed per experiment during *in situ* hybridization (ISH). Spot test assay was used to estimate the concentration of probes to be used (Figure 29).

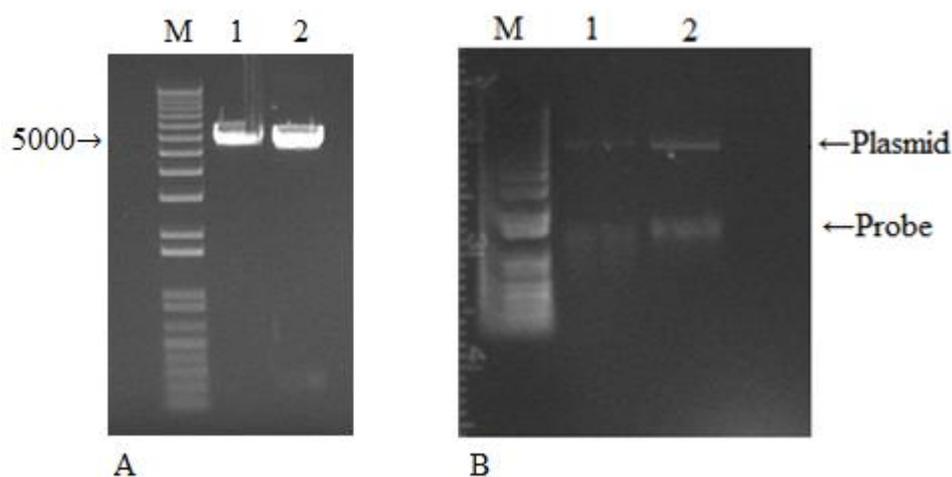


Figure 28: Lane M 1kb plus ladder, Figure A shows linearization of plasmid after *EcoRI* digestion Lane 1 Pax6.1 and Lane 2 Pax2.1, Figure B DIG-labeled probe with plasmid, Lane 1 Pax6.1 and Lane 2 Pax2.1

6.3. Spot test assay

Spot test assay was used to estimate the concentration of probes to be used for *in situ* hybridisation experiments (Figure 29)

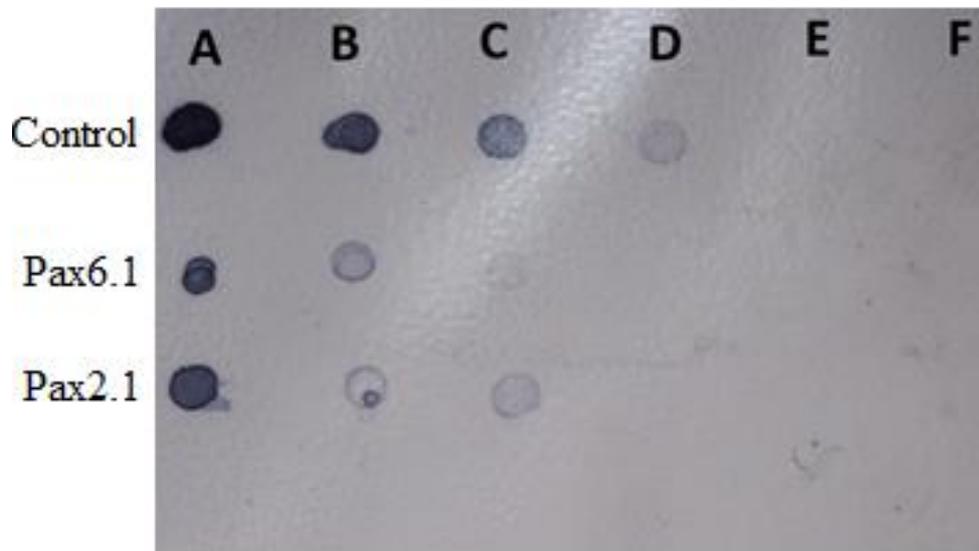


Figure 29: Spot test OF DIG-labeled probes, Upper row: Control Probe, Middle row: Pax6.1, Lower row: Pax2.1. Based on comparison with the control Dilutions the amount of probe was estimated to be 100 ng/ μ l (Pax2.1 and Pax6.1). From the membrane antibody/alkaline phosphatase was spotted at 100 pg/ μ l $100 \text{ pg/ } \mu\text{l} * 1000 = 100 \text{ ng/ } \mu\text{l}$. As thumb of rules use 0.5 -1 μ g probe for *in situ* hybridisation, thereby 500 ng is needed which is approximately 5 μ l per ml hybridization buffer.

6.4. Overview of Pax6.1 and Pax2.1 Probe after RNA-*in situ* hybridisation

Anti-DIG antibody conjugated to the enzyme alkaline phosphatase was used to bind the DIG-labeled probes hybridized to the RNA transcribed from the target genes.

Pax6.1 and Pax2.1 (Figure 30) ISH probes were used as positive controls. *In situ* RNA hybridisation was used to visualize the gene expression pattern in the embryos incubated with ethanol. Pax2.1 is localized at the midbrain-hindbrain border and is usually recognized by a stripe (furrow separating the midbrain and the hindbrain) and around the optic stalk at early stages of embryonic development (48) (Figure 30A). While Pax6 is expressed in a uniform manner in whole eye at 24 hours of development excluding the anterior part of the optic stalk (63), including the retina, lens and cornea (Figure 30B). In addition it is expressed in the hindbrain. Pax6.1 and Pax2.1 labeled probes were pooled together to be able to stain the eye and the stripe at the midbrain-hindbrain border (MHB) and the hindbrain simultaneously.

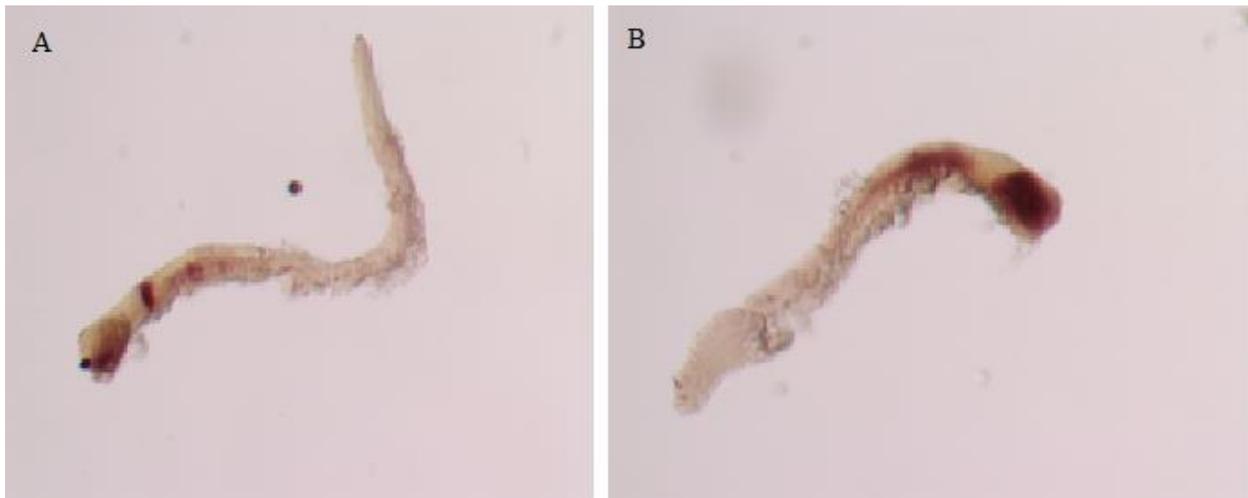


Figure 30: Pax6.1 and Pax2.1 were used as controls to visualize regions of the brain and eyes in 24 hpf embryos. Figure A, 24 hpf with Pax2.1 staining, shows stripe around the midbrain and hindbrain and weak staining around the eye (Optic stalk), Figure B with 24 hpf, Pax6.1 show intense staining around the eye and the neural tube (hindbrain).

6.4.1. Zebrafish embryos (24 hpf) with Pax6.1 and Pax2.1 Probe

Embryo incubated in 1 % and 2 % ethanol doesn't show much difference in comparison to the embryo from E3-medium (Figure 31A). Since it was difficult to observe the morphology changes staining of the embryos was an option used in order to observe the changes and measure the size of the brain structure. No clear visible reduction of eye size or reduction in staining. For embryos incubated in 2 % ethanol, the staining clearly indicated abnormal patterning of the brain with no defined MHB (Figure 31C).

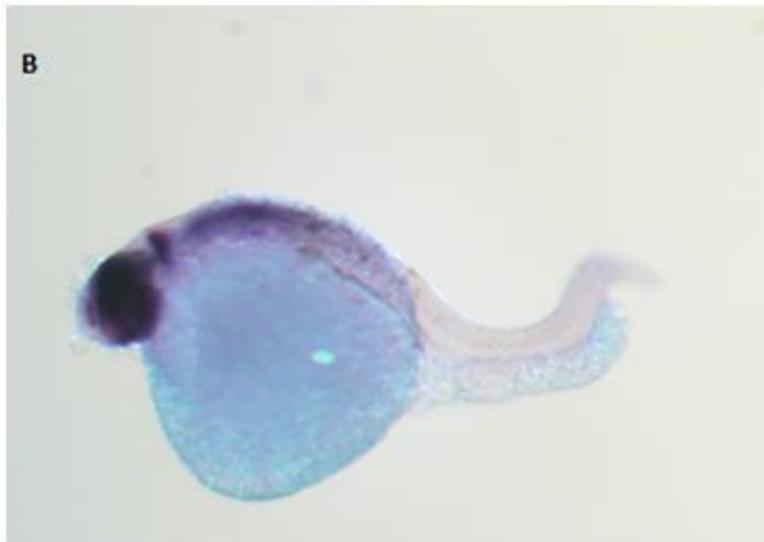
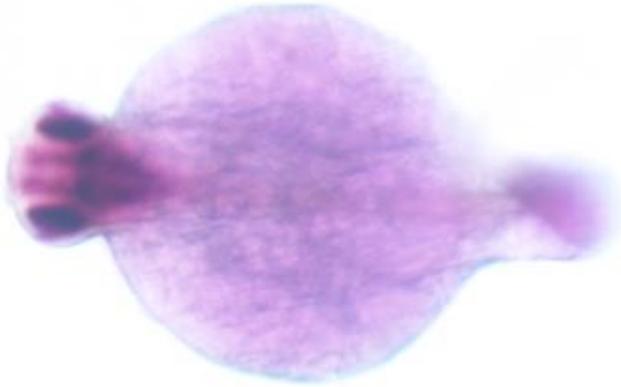


Figure 31: Lateral view of 24 hpf embryos with whole mounted probes against Pax6.1 and Pax2.1 were used to visualize regions of the brain and eyes in 24 hpf embryos incubated with 1 % and 2 % ethanol; A: E3-Medium, B: 1 % Ethanol, C: 2 % Ethanol.

6.4.2. Zebrafish embryos (48 hpf) with Pax6.1 and Pax2.1 Probe

Embryo incubated in 2 % ethanol (Figure 32C) had the weakest staining and this shows us that there is less Pax6.1 gene expressed in the eyes when embryos are exposed to this concentration of ethanol. Pax6 is said to be the master gene for eye development (43) and reduction of it can cause suppression in retina neurogenesis and reduced lens size. Pax2.1 is visible in a closer look at 2 % ethanol but weaker too. Control embryo (Figure 32A) shows strong intensity of staining around the eye, when compared to embryo incubated in 1 % ethanol, (Figure 32 B) which indicates weaker staining.

A



B



C

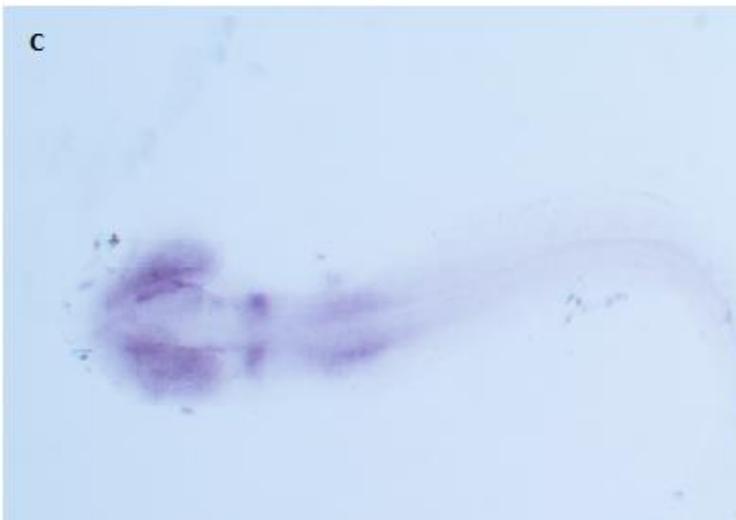


Figure 32: Dorsal views of 48 hpf whole-mounted embryos with probes against Pax6.1 and Pax2.1 A: E3-Medium, B: 1 % Ethanol, C: 2 % Ethanol, Stronger staining in Figure A and B in comparison with Figure C, which indicates reduction of Pax6 expression around the eye. Pax2.1 clearly visible in Figure C.

6.4.3. Zebrafish embryos (72 hpf) with Pax6.1 and Pax2.1 Probe

Embryo incubated in E3-medium ([Figure 33A](#)) shows no changes in the retina and the lens in comparison to the embryo incubated in 1 % ethanol ([Figure 33B](#)) and 2 % ethanol which showed weaker Pax6.1 expressions with higher concentration of ethanol that could influence development of the retina and the lens. It wasn't able to see the Pax2.1 in this stage of development 72 hpf because the embryos brain is fully developed and the tectal ventricle enlarges, thus the tissue between the furrow separating the midbrain and the hindbrain gets considerably thinner (48).

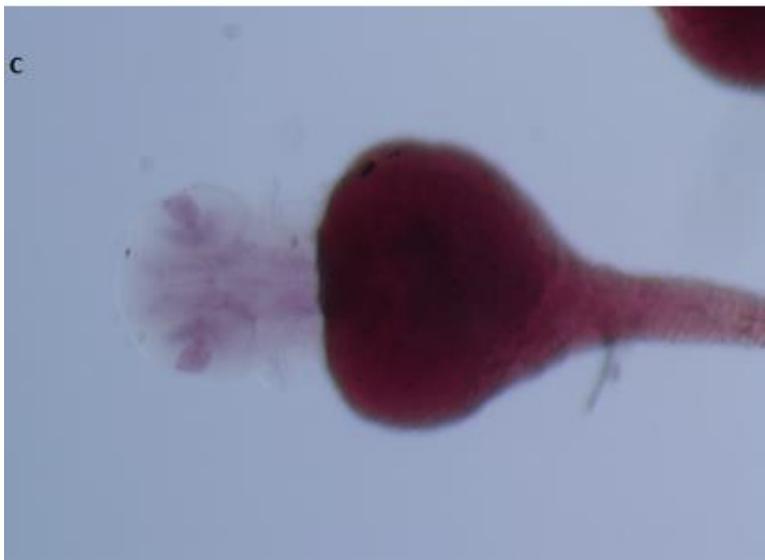


Figure 33: Dorsal views of whole-mounted embryos 72 hpf labeled with RNA probes against Pax6.1 and Pax2.1 (A-C) A: Control, B: 1 % Ethanol, C: 2 % Ethanol. Alcohol induced reduction of the retina and lens size.

6.5. Pax6.1 and Pax2.1 probe measurements

Since there were no obvious differences in the in situ staining pattern in embryos incubated in the lower concentrations of ethanol compared to the control, measurements were done to see if ethanol influences the eye size. The junction between the midbrain and hindbrain will become the cerebellum. We were interested to see if there were disturbances in the development of this area of the brain at this early stage. To do this the staining of Pax2.1 in the stripe marking the midbrain-hindbrain border (MHB), and the staining of Pax6.1 expression in the hindbrain was used. The gap between these staining was measured to see if there were changes in the presence of ethanol. Measurements on the eye were done also to see if ethanol influences the eye development.

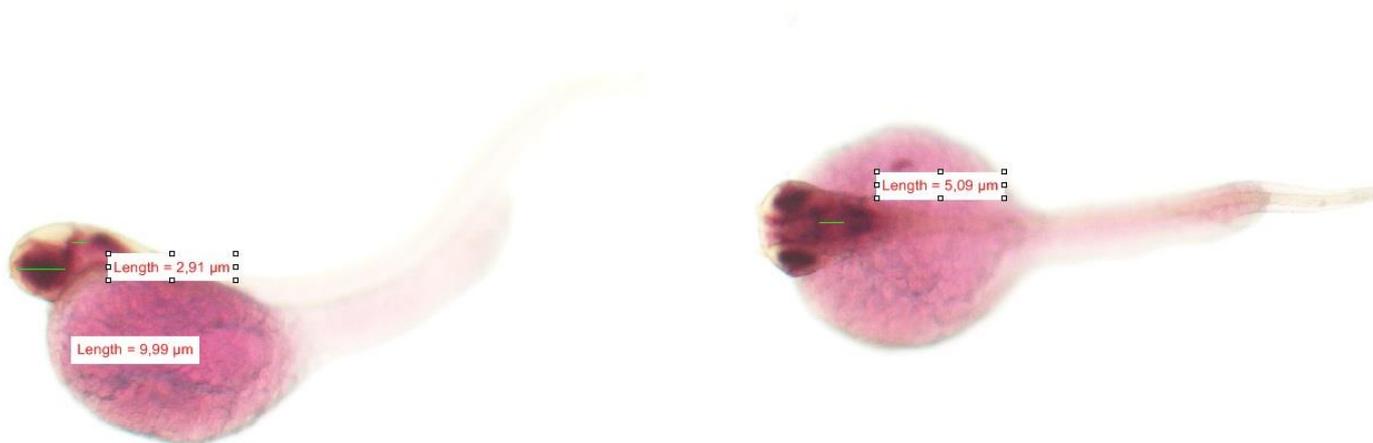


Figure 34: An overview of how the lateral and dorsal measurements were done. Measurements were done on the computer using Nikon Digital Sight DS-U3 camera and a program called NSCEI.

Evaluation of data

Data were evaluated as the mean \pm standard error of mean (S.E.M) and analyzed for statistical significance by using p-value was calculated by using Excel (Student t-test, two sided, assuming unequal variance).

According to my calculation of t-test, there was a lot of variation on the results. I conclude that there is a significance difference whether the measurements of the junction between the brain was done lateral or dorsal. However the lateral and dorsal measurements of 24hpf shows inconsistency in the p-value where E3-medium and 1 % ethanol incubated embryos had a $p > 0.05$ and 0.1 % ethanol had $p < 0.05$. Measurements for the 48 hpf embryos show a significant difference $p < 0.05$, which could be caused by imprecise positioning of embryos

while taking measurements; this could be a factor influencing variety in the measurements. I nevertheless decided to use both the lateral and dorsal measurements for further discussion.

6.5.1. Measurements of the eye and the distance between midbrain/hindbrain in 24hpf embryos

A t-test was done to compare if there was significance difference in the embryos incubated in E3-medium with those in 0.1 % ethanol and 1 % ethanol.

Lateral and dorsal measurements of the previously described brain area in embryos incubated in 24 hpf 1 % showed a p-value > 0.05 while the measurements of embryos incubated in 24 hpf 0.1 % ethanol shows significance difference with a p<0.05. This shows us that ethanol influences the size of MHB in early embryogenesis, by reducing the gap between the midbrain and the hindbrain ([Figure 35](#))

The eye size in 24 hpf 1% ethanol and 0.1% ethanol, shows a p-value > 0.05, which paraphrases that ethanol, does not reduce the eye size in 24 hpf hence no significance difference. To conclude, the only significant difference observed with these measurements were the lateral/dorsal measurement distance of the midbrain /hindbrain junction for embryos at 24 hpf incubated with 0.1 % ethanol compared to embryos incubated with E3.

Table 12: Average measurements of the eye and the distance between the midbrain/hindbrain for 24 hpf

	Mean eye size (μm) \pm STD	Mean lateral Midbrain/hindbrai n distance (μm) \pm STD	Mean Dorsal Midbrain/Hindbrain distance (μm) \pm STD
E3medium	13.3 \pm 2.0(n=4)	5.1 \pm 0.4	5.2 \pm 0.5
0.1% EtOH	12.8 \pm 1.0(n=4)	4.1 \pm 0.4	5.9 \pm 0.3
1% EtOH	12.5 \pm 2.3(n=5)	4.5 \pm 1.2	4.5 \pm 0.4

STD: Estimated standard derivation

n:reflects the number of embryos that were measured for visualization of the Pax6.1 around the eye and Pax2.1 around the junction of the midbrain/hindbrain.

p-value given < 0.05 as significant, p< .001 given as highly significant

Details on the measurements is given in the Appendix([10.3.1](#))

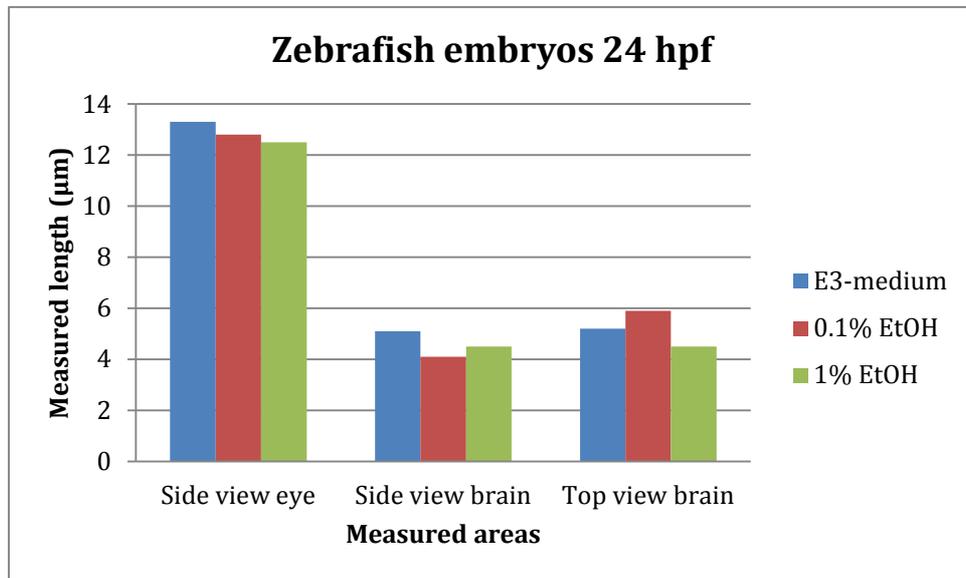


Figure 35: Measurement of the distance between midbrain/hindbrain and eye size in 24 hpf embryos incubated in E3-medium, 0.1 % ethanol and 1 % ethanol. Eye size measurements of 1 % ethanol and 0.1 % ethanol has $p > 0.05$. Midbrain hindbrain lateral/dorsal distance in 0.1 % ethanol has $p < 0.05$ and 1 % ethanol has $p > 0.05$

6.5.2. Measurements of the eye and the distance between midbrain/hindbrain in 48 hpf embryos

Measurement of the lateral and dorsal (MHB) in embryos incubated in 48 hpf 0.1 % and 1% shows no significance difference with a p-value > 0.05. Although the overview of the graph ([Figure 36](#)) shows that there is a reduction on the size of MHB especially in the embryos incubated in 1 % and a slight reduction in the eye size.

In general graphical interpretation measurements of the eye size shows no significance difference. The measurements of the eye size on embryos incubated in 0.1 % ethanol and 1 % ethanol for 48 hpf has a p>0.05.

Table 13: Average measurements of the eye and distance between the midbrain/hindbrain for 48 hpf

	Mean eye size (μm) \pm STD	Mean lateral Midbrain hindbrain distance (μm) \pm STD	Mean Dorsal Midbrain hindbrain distance (μm) \pm STD
E3medium	9.3 \pm 1.6 (n=10)	3.8 \pm 0.5	4.4 \pm 0.7
0,1 % EtOH	9.3 \pm 1.6 (n=10)	3.7 \pm 0.4	4.6 \pm 1.1
1 % EtOH	8.93 \pm 1.6(n=13)	3.5 \pm 0.7	4.5 \pm 1.0

STD: Estimated standard deviation

n:reflects the number of embryos that were measured for visualization of the Pax6.1 around the eye and Pax2.1 around the junction of the midbrain/hindbrain.

p-value given < 0.05 significant, p<0,01 given as highly significant

Details on the measurements is given in the Appendix([10.3.1](#))

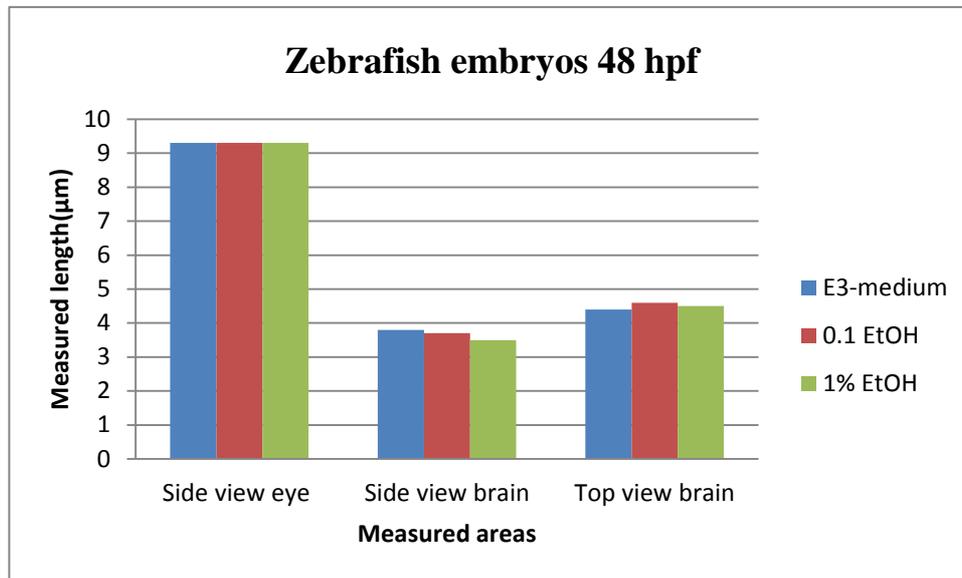


Figure 36: Measurement of the distance between the midbrain/hindbrain and eye size in 48 hpf embryos incubated in E3-medium, 0.1 % ethanol and 1 % ethanol. Measurements on the eye size show no significance difference $p > 0.05$, while measurements of the lateral /dorsal midbrain hindbrain junction $p > 0.05$.

6.6. Pax6 in Western blot analysis

We wanted to study if Pax6 was influenced by ethanol. A reduction of Pax6 proteins was expected in ethanol-incubated embryos since it has been published that alcohol reduces Pax6 expression and with higher concentration of alcohol Pax6 proteins are almost suppressed (64) Protein extracts were prepared from ethanol incubated embryos and control embryos. Protein samples of 15 embryos were prepared for analysis (5.8) Extracts from 10 embryos were loaded on the gel where the proteins were separated with gel electrophoresis. A nitrocellulose membrane was used to absorb the separated proteins. Thereafter primary and secondary antibodies were used to mark the protein of interest.

Pax6 is identified around 48kDa and actin around 42kDa (Figure 37) .The membrane for Western blot analysis shows a weaker band at 1 % and almost diminished band at 2 % indicating that there is a reduction in the amount of Pax6 protein in the embryos that were incubated in 2 % alcohol. The results from 48 hpf and 72 hpf could not be used because all of the bands on the blot were very blurry. This could be caused by viscosity of the zebrafish protein samples used.

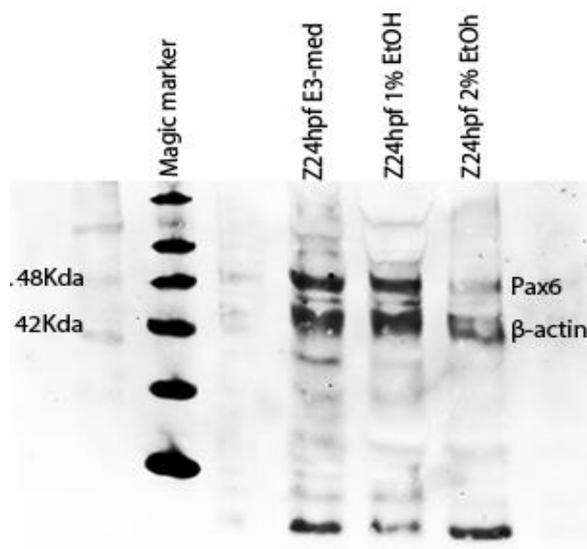


Figure 37: Western blot analysis assessing Pax6 in zebrafish embryos incubated in E3-medium,1 % ethanol and 2 % ethanol for 24 hpf. A weak band with low intensity on 24 hpf 2 % ethanol which shows a reduction in Pax6 protein. Expected molecular weight of Pax6 around 48kDa, and actin at 42kD

Several Western blot assessments were done was redone using NUPAGE LDS buffer and Reducing agent (Figure 38) by changing the amount of protein loaded . An amount of protein equivalent to 2.5 embryos were loaded per well. A clear band of Pax6 is identified but no changes from the samples incubated in ethanol or control samples (E3-medium). A thinner band on 24 hpf E3-medium, 1 % ethanol and 2 % ethanol which could be due to the little amount of protein loaded..

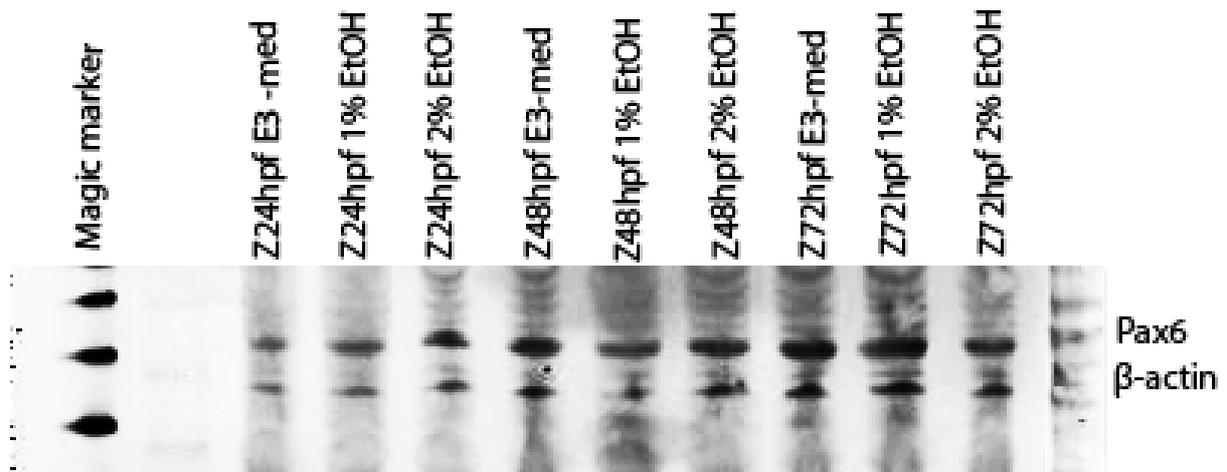


Figure 38: Western blot analysis assessing Pax6 in zebrafish embryos incubated in E3-medium, 1 % ethanol and 2 % ethanol for 24 hpf, 48 hpf and 72 hpf. Weak intensity on the 24 hpf with antibody against Pax6 and actin Pax6 was used as positive control at the last band. Band from 48 hpf and 72 hpf seem to have Pax6 protein but no change on the amount of proteins. Expected molecular weight of Pax6 is around 48kDa and 42kDa

7. DISCUSSION

7.1. Summary of results:

The objective of this thesis was to study how ethanol affects zebrafish embryo development. Tools were generated to study specific parts of the brain, zAtoh1a1, zAtoh1a2 and zAtohlc genes which are expressed in the cerebellum were isolated from zebrafish cDNA and cloned to be used for *in situ* hybridization. Cloning was not successful and DIG labeling of PCR products did not give results. Therefore Pax6.1 and Pax2.1 gene expressions were further used to study ethanol effects on zebrafish embryos.

Zebrafish embryos were incubated with different concentrations of ethanol to look for changes in overall morphology and survival. Studies on the molecular level, including *in situ* hybridizations and Western blot of protein extracts are also to be included.

Incubation of embryos in different concentrations of alcohol in chronological order (24 hours, 48 hours and 72 hours) lead to morphological changes including pericardial edema, yolk sac edema, axial malformations, axial blistering, shortened body length and whole embryo deformations.

Ethanol influenced Pax6.1 and Pax2.1 gene expression depending of the stage of embryo development and concentration used. Clearly Pax6.1 expressions are reduced during 48-72 hpf embryos and this was determined by decreased intensity of staining of *in situ* hybridization probes in the retina and lens. Although the measurements that were done on the eye size confirmed no reduction of the eye size.

Measurements of the eye size for embryos harvested in 1 % ethanol during 24 hours does not appear to be influenced by ethanol. But the measured region just posterior to the midbrain hindbrain boundary (MHB) is reduced in size during the first 24 hours of embryogenesis.

Embryos incubated for 48 hours in ethanol shows no reduction in the junction between the midbrain and the hindbrain, while the eye size is slightly reduced.

Western blots (WB) assessment were done to study if Pax6 was influenced by ethanol. A reduction of Pax6 proteins was expected in ethanol incubated embryos since it has been manifested that alcohol reduces Pax6 expression and with higher concentration of alcohol

Pax6 proteins are suppressed hence induction of ocular abnormalities (64) Our first WB analysis clearly shows (Figure 37) that the amount of Pax6 proteins is decreased in embryos incubated in ethanol however technical difficulties in the experiments led to conflicting results. Due to a number of reasons discussed (7.5) which made it difficult to conclude if Pax6 proteins are affected by ethanol.

General discussion

Children with Fetal Alcohol syndrome (FAS) show a variety of ophthalmic disorders. One common phenotype seen in humans exposed to ethanol in utero is microphthalmia. Microphthalmia is seen in 90 % of children with FAS. Ethanol induced microphthalmia can occur as a consequence of a number of potential mechanisms including general development delay, increased cell death, reduced cell proliferation and reduced cell differentiation with the developing eye (65). During 24 to 48 hours after fertilization the retinal neuroepithelium undergoes rapid proliferation and differentiation to form a laminated structure composed of different retinal cell types (21, 66, 67).

Several studies have revealed on how ethanol affects the development of the brain, however no study has been published yet to show if ethanol influences the development of the stripe with where Pax2.1 is expressed on the junction of the midbrain and the hindbrain.

Various mechanisms are declared as possible targets that can lead to ethanol mediated toxicity (1.6). Predominantly there is an association on mechanisms of signal pathways, which might lead to FAS. Shh seems to be implicated as the midline of these manifestations. It is highlighted that ethanol affects the *GABAergic* and *GLUTAergic* neurons in the CNS by disrupting sonic hedgehog (*Shh*), fibroblast Growth Factors (*fgf 19*), *fgf3*, *fgf8*, and *Atoh1* expressions. Agrin is required for proper Shh signaling in the eye development and for the generation of serotonergic/dopaminergic neurons.

In Fetal Alcohol Syndrome, delay closure of the neural tube is noted. There is a study that shows that alcohol affects the fetal neural stem cells that produce most of the neurons of the adult brain (68) and that it affects serotonergic hydroxytryptamine pathway (HT), by reducing the number and density of 5HT neurons in the developing embryo (69).

Factors emanating the midline and MHB, mediate serotonergic and dopaminergic neuron development. Signal pathways responsible for this are *Shh* emanating from the midline, *Fgf8* emanating from the MHB and *fgf4* from the underlying tissues (70).

Fgf8 depends on the signaling from *Shh* in the brain. This growth factor is defined to be the organizers of *Pax2.1* in the MHB (37) Since ethanol impairs *Shh* signaling, this could affect *Fgf8* and hence *Pax2.1* in MHB, which might cause reduction in the gap between the stripe which separates the midbrain and the hindbrain (4).

Shh signaling seems to be the main target for both *Pax2.1* and *Pax6.1* expressions in the eye and cerebellum. Overexpression of *Shh* leads to depletion of the cells restraining *Pax6* and elevate the number of cells containing *Pax2* in the eyes (22).

It is known that overexpression of *Shh* in zebrafish, ventralises the optic cup, giving an expanded *Pax2.1* domain at the expense of *Pax6* domain and subsequently an expanded optic stalk and reduced retina size (microphthalmia) (21). A mechanism has been found in zebrafish where *Shh* is required to promote the wave front of retinal ganglion cell (RCG) differentiation and induce its own expression. Both *Shh* and *Atonal* (*Ath5*) are first expressed in the differentiating RCGs close to the optic stalk and subsequently this expression spreads (71).

Ethanol suppresses *Pax6.1* and hence induces microphthalmia. Basing on that fact, it gives us as assumption that ethanol induces the expression of *Shh* hence repression of *Pax6* through *Pax2.1* in the eyes (22, 46), However several studies display that craniofacial abnormalities are induced by loss of *Shh*, hence Fetal alcohol syndrome (72, 73).

7.2. Mating and dechorioniation:

The zebrafish laid a lot of eggs. Embryos obtained through mating were incubated in ethanol and E3-medium. After incubation for 24 hours, 48 hours and 72 hours, the embryos were dechorionised and studied.

7.3. Cloning

Cloning of DNA from the zebrafish Atoh genes did not succeed as planned.

Several sources of error could be determined as causes including:- a wrong enzyme used during PCR synthesis, where the DyNAzyme DNA polymerase was used instead of the Phusion. This made it difficult to clone PCR products in the pZero-blunt vector. Since a linearized blunt vector was used which has complimentary blunt ends, using DyNAzyme would make it incompatible since the enzyme generate PCR-productss with an overhang/sticky ends .The Phusion DNA polymerase produce blunt-end PCR products and was supposed to be the preferable enzyme. The DyNAzyme DNA polymerase is used for transformation of vector like PCR TOPO 2.1, which requires a T/A overhang.

Furthermore it turned out that the competent bacteria cells used for transformation of cloned PCR products were contaminated. This caused a lot of “false positives” to be analyzed, and these were shown to be plasmid containing inserts that were not the zAtoh PCR products. Since direct sequencing of the PCR products verified that they were correct, the observed contamination had to come from another step in the cloning procedure. The competent DH5 bacteria cells were tested by streaking them direct on LB-medium agar plates containing antibiotics, but there were growth on both plates which shows that the cells were contaminated with plasmids providing antibiotic resistance.

Another suspicion came up that there might be a contamination on the Cloning kit used; thereby a new cloning kit was used; yet the same results were obtained.

7.4. Whole-mount *in situ* hybridization (ISH)

To study expression of different genes on the ethanol-incubated embryos several experiments were done using the probes that were DIG labeled (zAtoh1a1, zAtoh1a2, zAtoh1c, Pax6.1 and Pax2.1). Staining the embryos with DIG labeled (zAtoh1a1, zAtoh1a2 and zAtoh1c probes) was not successful and this could be due to several reasons.

Maintenance of the right temperature during hybridization and washing after hybridization could be one source of error. During hybridization the temperature of the denatured probes was supposed to be exact as the hybridization buffer that the embryos were incubated in or the probe would not be effective to label/mark the desired areas of the brain/eyes. The temperature of the buffer solutions used during the washing process was supposed to be approximately the same as the temperature of the discarded buffers. Since the whole process

requires precision while pipetting the buffers back and forth temperature management could be an issue.

Another reason could be under-digestion with Proteinase K which was used to permeabilize the embryos, so as to remove proteins that surround nucleic acids (74). Under digestion will not allow the probe to penetrate in the embryos (75), while over digestion will make the embryo disintegrate and alter the morphology (76). Embryos DIG-marked with zAtoh1a1, zAtoh1a2 and zAtoh1c could possibly have been under-digested with Proteinase K, or possibly the low concentration of the probes (6.1.4) hence unable to mark the desired areas. Over-fixation of the embryos is another factor, this process can make the embryos very fragile. Tissue fixation is used to maintain the tissue architecture as well as to ensure the retention of the target RNA or DNA and influence probe penetration to the target genome (74). Over-fixation can make it difficult to mark the probes and diminish the quality of ISH (76).

Although there was an addition of Tween 20® in almost all the buffers, fixed embryos could stick together into clumps, which could avoid penetration of probes, but yet embryos that were not stuck together were available and staining could be detected.

To be able to distinguish between problems due to bad probe quality and problems caused by failure somewhere along the *in situ* hybridization protocol, positive and negative controls were always included. Embryos where no probes were added (but secondary antibody and staining was performed anyway) used as negative control. A probe already used before, where the quality had been approved were used as a positive control. Several experiments were done on treated and untreated embryos to get familiar with the basal staining and inherent variability of the staining (76).

In order to get a strong intensity of staining the embryos were incubated at 4°C overnight with staining buffer, which made the embryos to appear deep blue/purple after the staining.

7.5. Western blot analysis

Furthermore to confirm if Pax6 is influenced by ethanol, Western blot analysis was done on embryos incubated in E3-medium, 1 % and 2 % ethanol for 24 hours, 48 hours and 72 hours. Protein samples of 15 embryos were prepared on the first analysis. Protein samples of 10 embryos was used per well and the results from 24 hpf seemed to be as expected. A reduction, hence suppression of Pax6 proteins was expected in the embryos incubated in high concentration of ethanol (64). But from the second analysis blue fuzzy smear bands appeared on the background of the membranes from most of the experiments and the protein bands on the membrane appeared blurry. This was observed several times.

Different attempts were done to make this protocol successful. A number of causes were suspected including, problems with the LDS loading buffer .A possible explanation could be unstable buffer temperature. The LDS buffer used was stored in the refrigerator while it is supposed to be stored in normal room temperature. LDS buffer contains glycerol, which becomes viscous and sticky when stored at lower temperatures, which might change the concentration of the preferred buffer solution. Several experiments were done by maintaining the correct standards of the protocol but yet the fuzzy smear bands reappeared.

Incorrect use of buffer system instead of NUPAGE® Tris-Acetate SDS Running buffer and NUPAGE® LDS sample buffer on the NUPAGE® Tris-Acetate Gels could result on fuzzy smear bands, but that would not be a source of error in this case, since the right type of buffers were used.

Furthermore another assumption was that the fuzzy smear bands could be caused by overload of protein per well.

Accordingly, adjustment of the tissue samples were done from 20 µl (10 crushed embryos) to 5 µl (2.5 crushed embryos) to assure that there wasn't an overload of proteins in each well yet the results were the same.

Contamination with membranes or DNA complexes in the sample could be a considerable source of error, which could cause streaking of proteins but due to lack of time this (62).

This procedure gives us information of overall reduction of Pax6 expressions but it does not explain in which area the reduction occurs. It can used as an addition method incase several protocols are experimented to help make confirmation of the results.

7.6. Future perspectives

The effects of ethanol during embryogenesis are clearly known but methods on how to reduce and avoid these effects of Fetal alcohol syndrome are still unknown. With further studies on how ethanol affects different transcription factors on the molecular level it will make it possible to find the target genes and provide knowledge on how effects of ethanol can be countered.

Injection of Shh m-RNA simultaneously with alcohol exposure of embryos can result into reduction of alcohol induced toxicity like cyclopia, body length and other body malformations (33, 73). Since Ethanol is known to repress Shh. Shh/fgf3 Morpholino oligonucleotides/transient genes could be injected in embryos before incubating in ethanol and thereafter the embryos can be marked with zAtoh1a/Pax6.1 and zAtoh1c/Pax2.1 so as to study if still ethanol affects these transcription factors or the regions of the brain where these transcription factors are expressed.

8. CONCLUSION

High concentration of ethanol (2 %) lead to gross deformations, while lower concentrations 0.1 % and 1 % lead to ethanol mediated toxicity including microphthalmia and body malformations.

Ethanol (2 %) harvested embryos for 72 hours induced microphthalmia hence reduced retina and lens size. Measurements on the eye size conclude that Pax6.1 expression is not suppressed by alcohol. But Pax2.1 in the cerebellum is reduced during the first 24 hours of development.

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10. APPENDIX

10.1. Appendix 1

9.1.1. Direct sequences from PCR products

LM-12013-12-18

ATSGRCGTRCAKACGAGTACCCACCAGCCTTGGCACTCATGGCCAGCAGTGACCCACGCG
CCTGGCTGGCTCCCGTGCAGGCTGGCACCTGCGCGGCACACGCCGAATACCTGCTGCACT
CGCCCGGCTCGAGCGCGGAAGGCGTGTCTCTGCCTCCAACCTTCAGGAAGAGCAGCAAG
AGTCCTGTCAAAGTACGCGAGCTCTGCCGGCTTAAAGGAGCTGTGGGGGCAGATGAGGG
CAGACAGCGGGCCCCATCCAGCAAATCCACCAACGTCGTGCAGAAACAGAGGCGAATGG
CTGCCAATGCCC GGGAGAGGCGAAGAATGCACGGATTGAACCACGCGTTCGACGAGCTG
CGCAGTGTATCCAGCCTTTGACAACGACAAGAACTCTCCAAGTACGAAACCCTGCAG
ATGGCCCAGATCTACATCAACGCCCTGTCCGACTTACTACAGGGCCCCGGTGTCTAAAGCC
GACCCGCCAAACTGCGACCTGCTGCATGCCAACTKWWAAAAAAAAAMSGG

NCBI-blast confirmed that the nucleotide sequence contained *Atoh1a*

Danio rerio atonal homolog 1a (*atoh1a*), mRNA

Sequence ID: [ref|NM_131091.1|](#)

Length: 906 Number of Matches: 1

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
913 bits(494)	0.0	494/494(100%)	0/494(0%)	Plus/Plus

```
Query 16 AGTACCCACCAGCCTTGGCACTCATGGCCAGCAGTGACCCACGCGCCTGGCTGGCTCCCG 75
          |||
Sbjct 173 AGTACCCACCAGCCTTGGCACTCATGGCCAGCAGTGACCCACGCGCCTGGCTGGCTCCCG 232
Query 76 TGCAGGCTGGCACCTGCGCGGCACACGCCGAATACCTGCTGCACTCGCCCGGCTCGAGCG 135
          |||
Sbjct 233 TGCAGGCTGGCACCTGCGCGGCACACGCCGAATACCTGCTGCACTCGCCCGGCTCGAGCG 292
Query 136 CGGAAGGCGTGTCTCTGCCTCCAACCTTCAGGAAGAGCAGCAAGAGTCTGTCAAAGTAC 195
          |||
Sbjct 293 CGGAAGGCGTGTCTCTGCCTCCAACCTTCAGGAAGAGCAGCAAGAGTCTGTCAAAGTAC 352
Query 196 GCGAGCTCTGCCGGCTTAAAGGAGCTGTGGGGGCAGATGAGGGCAGACAGCGGGCCCCAT 255
          |||
Sbjct 353 GCGAGCTCTGCCGGCTTAAAGGAGCTGTGGGGGCAGATGAGGGCAGACAGCGGGCCCCAT 412
Query 256 CCAGCAAATCCACCAACGTCGTGCAGAAACAGAGGCGAATGGCTGCCAATGCCCGGGAGA 315
          |||
Sbjct 413 CCAGCAAATCCACCAACGTCGTGCAGAAACAGAGGCGAATGGCTGCCAATGCCCGGGAGA 472
Query 316 GGCGAAGAATGCACGGATTGAACCACGCGTTCGACGAGCTGCGCAGTGTATCCAGCCT 375
          |||
Sbjct 473 GGCGAAGAATGCACGGATTGAACCACGCGTTCGACGAGCTGCGCAGTGTATCCAGCCT 532
Query 376 TTGACAACGACAAGAACTCTCCAAGTACGAAACCCTGCAGATGGCCAGATCTACATCA 435
          |||
Sbjct 533 TTGACAACGACAAGAACTCTCCAAGTACGAAACCCTGCAGATGGCCAGATCTACATCA 592
Query 436 ACGCCCTGTCCGACTTACTACAGGGCCCCGGTGTCTAAAGCCGACCCGCCAAACTGCGACC 495
          |||
Sbjct 593 ACGCCCTGTCCGACTTACTACAGGGCCCCGGTGTCTAAAGCCGACCCGCCAAACTGCGACC 652
Query 496 TGCTGCATGCCAAC 509
          |||
Sbjct 653 TGCTGCATGCCAAC 666
```

LM-2-2013-12-18

GRGGKTTAAAGSACACTTTTTATACTTGAACCTATTGGCCGAGGATGCCGCAGYACTGT
GTGTATCCYGCGGWGTTTGCACATGGGCTTTGTAATGGGTTGCACCTGCTGCACTCSCCC
GGYTCGAGCGCGGAAGGCGTGTCTCTGCCTCCAACCTTCARGARGAGCAGCAAGAGTCCT
GTCAAAGTACGCGAGCTCTGCCGGCTTAAAGGAGCTGTGGGGGCAGATGAGGGCAGACA
GCGGGCCCCATCCAGCAAATCCACCAACGTCGTGCAGAAACAGAGGCGAATGGCTGCCA
ATGCCCGGGAGAGGGCGAAGAATGCACGGATTGAACCACGCGTTTCGACGAGCTGCGCAGT
GTCATCCCAGCCTTTGACAACGACAAGAACTCTCCAAGTACGAAACCCTGCAGATGGCC
CAGATCTACATCAACGCCCTGTCCGACTTACTACAGGGCCCCGGTGCTAAAGCCGACCCG
CCAAACTGCGACCTGCTGCATGCCAACGTGTTAGAAACGGACCGATCTCCCAGAGGATCA
CCGGGCGTCTGTTCGGAGAGGCACGGGCGTGGGTTACCCGTACCAGTACGAGGACGGAAC
ATTCAACTCTTTCATGGAGCAAGACCTCCAGTCGCCCTCTGGAACGAGCAAGTCTGGTTC
GGAGGCCAGTAAAGACTCGCCTCGGTTCGAACCGGAGTGATGGAGAGTTCTCGCCTCACT
GCACTTCAGTGACTCAGACGAAACGCACTTGGAGCTGCAGAGTGAAGACGAGCTGTCCG
AACTGAAACTGGCCAAGCGCCGCGCTTTTTAAGAAAACACACCGCCGGACTGTCCCTCAA
TCGTGTCAATCGCATATCTGGGCTGWAAGGGGGGGGGYGC

NCBI-blast confirmed that the nucleotide sequence contained Atoh1a2

Danio rerio atonal homolog 1a (atoh1a), mRNA

Sequence ID: [ref|NM_131091.1](#)

Length: 906 Number of Matches: 1

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
1110 bits(601)	0.0	608/613(99%)	1/613(0%)	Plus/Plus

```
Query 16 AGTACCCACCAGCCTTGGCACTCATGGCCAGCAGTGACCCACGCGCCTGGCTGGCTCCCG 75
      |||
Sbjct 173 AGTACCCACCAGCCTTGGCACTCATGGCCAGCAGTGACCCACGCGCCTGGCTGGCTCCCG 232
Query 76 TGCAGGCTGGCACCTGCGCGGCACACGCCGAATACCTGCTGCACTCGCCCGGCTCGAGCG 135
      |||
Sbjct 233 TGCAGGCTGGCACCTGCGCGGCACACGCCGAATACCTGCTGCACTCGCCCGGCTCGAGCG 292
Query 136 CGGAAGGCGTGTCTCTGCCTCCAACCTCAGGAAGAGCAGCAAGAGTCTGTCAAAGTAC 195
      |||
Sbjct 293 CGGAAGGCGTGTCTCTGCCTCCAACCTCAGGAAGAGCAGCAAGAGTCTGTCAAAGTAC 352
Query 196 GCGAGCTCTGCCGGCTTAAAGGAGCTGTGGGGGCAGATGAGGGCAGACAGCGGGCCCAT 255
      |||
Sbjct 353 GCGAGCTCTGCCGGCTTAAAGGAGCTGTGGGGGCAGATGAGGGCAGACAGCGGGCCCAT 412
Query 256 CCAGCAAATCCACCAACGTCGTGCAGAAACAGAGGCGAATGGCTGCCAATGCCCGGGAGA 315
      |||
Sbjct 413 CCAGCAAATCCACCAACGTCGTGCAGAAACAGAGGCGAATGGCTGCCAATGCCCGGGAGA 472
Query 316 GGCGAAGAATGCACGGATTGAACCACGCGTTTCGACGAGCTGCGCAGTGTTCATCCCAGCCT 375
      |||
Sbjct 473 GGCGAAGAATGCACGGATTGAACCACGCGTTTCGACGAGCTGCGCAGTGTTCATCCCAGCCT 532
Query 376 TTGACAACGACAAGAAACTCTCCAAGTACGAAACCCTGCAGATGGCCCAGATCTACATCA 435
      |||
Sbjct 533 TTGACAACGACAAGAAACTCTCCAAGTACGAAACCCTGCAGATGGCCCAGATCTACATCA 592
Query 436 ACGCCCTGTCCGACTTACTACAGGGCCCCGGTGCTAAAGCCGACCCGCCAAACTGCGACC 495
      |||
Sbjct 593 ACGCCCTGTCCGACTTACTACAGGGCCCCGGTGCTAAAGCCGACCCGCCAAACTGCGACC 652
Query 496 TGCTGCATGCCAAC 509
      |||
Sbjct 653 TGCTGCATGCCAAC 666
```

LM3-2013-12-18

GTTCGCACATCCGACTGTCGTTTRGACCTCAGAGCAGTGTCCCAAAGCCCCAATGGGCTG
GAGAGAGGAGCASCTGCAGGACAGGGCGAGCTGTGACCCCTGCGCACTCGTCCAGCTCC
GGCTCACTGGCCTTTCATATYCCAGTGAAGAGCAGAGCTCTATCGCCCCGAGCCCGGAGGC
GTCGCAGACTAGCCGCTAACGCCCCGAGAGAGGAGGAGGATGCTGGGCTTGAAYGTGGCY
TTCGACCGCCTGAGGAGTGTATTCTAATGTGGAGAGTGACAGGAAGCTGTCCAAGTCT
GAGACGCTTCAGATGGCACAGATCTACATCAGCACCCCTGAGTGAGCTACTGGAGGACAA
AGAKTGTGACCCARAAA YCCCATAACCCRACTTTGACCATGCAGGATCAGGACATCRCA
AGGGGTTGCCTATGACCGAAGAGACAAAACAGA ACTCAAGAACAATCCCACCTGTAGG
ATAAGATCATATAATGGGAATTTTGGGTGCCTAACTTGSAGTGACCRCAAAAACAGCTGAA
CTCTTAGTGGACTCTCACTGTCTGGAAAGGAACAATGGTGTAAAATAGA

NCBI-blast confirmed that the nucleotide sequence contained Atoh1c

Danio rerio atoh1c mRNA, partial cds

Sequence ID: dbj|AB530457.1

Length: 779 Number of Matches: 1

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
987 bits(534)	0.0	545/556(98%)	0/556(0%)	Plus/Plus

```
Query 28 CTCAGAGCAGTGTCCCAAAGCCCCAATGGGCTGGAGAGAGGAGCASCTGCAGGACAGGGC 87
      |||
Sbjct 82 CTCAGAGCAGTGTCCCAAAGCCCCAATGGGCTGGAGAGAGGAGCAGCTGCAGGACAGGGC 141
Query 88 GAGCTGTGACCCCTGCGCACTCGTCCAGCTCCGGCTCACTGGCCTTTCATATYCCAGTGA 147
      |||
Sbjct 142 GAGCTGTGACCCCTGCGCACTCGTCCAGCTCCGGCTCACTGGCCTTTCATATCCCAGTGA 201
Query 148 AGAGCAGAGCTCTATCGCCCCGAGCCCGAGGGCGTCGCAGACTAGCCGCTAACGCCCCGAGA 207
      |||
Sbjct 202 AGAGCAGAGCTCTATCGCCCCGAGCCCGAGGGCGTCGCAGACTAGCCGCTAACGCCCCGAGA 261
Query 208 GAGGAGGAGGATGCTGGGCTTGAAYGTGGCYTTCGACCGCCTGAGGAGTGTATTCTCTAA 267
      |||
Sbjct 262 GAGGAGGAGGATGCTGGGCTTGAACGTGGCTTTCGACCGCCTGAGGAGTGTATTCTCTAA 321
Query 268 TGTGGAGAGTGACAGGAAGCTGTCCAAGTCTGAGACGCTTCAGATGGCACAGATCTACAT 327
      |||
Sbjct 322 TGTGGAGAGTGACAGGAAGCTGTCCAAGTCTGAGACGCTTCAGATGGCACAGATCTACAT 381
Query 328 CAGCACCCCTGAGTGAGCTACTGGAGGACAAAGAKTGTGACCCARAAA YCCCATAACCCRAC 387
      |||
Sbjct 382 CAGCACCCCTGAGTGAGCTACTGGAGGACAAAGATTGTGACCCAGAAATCCCATAACCCGAC 441
Query 388 TTTGACCATGCAGGATCAGGACATCRCAAGGGGTTGCCTATGACCGAAGAGACCAAAAAC 447
      |||
Sbjct 442 TTTGACCATGCAGGATCAGGACATCGCGAAGGGGTTGCCTATGACCGAAGAGACCAAAAAC 501
Query 448 AGAACTCAAGAACAATCCCACCTGTAGGATAAGATCATATAATGGGAATTTTGGGTGCCT 507
      |||
Sbjct 502 AGAACTCAAGAACAATCCCACCTGTAGGATAAGATCATATAATGGGAATTTTGGGTGCCT 561
Query 508 AACTTGSAGTGACCRCAAAAACAGCTGAACTCTTAGTGGACTCTCACTGTCTGGAAAGGAA 567
      |||
Sbjct 562 AACTTGCAGTGACCACAAAACAGCTGAACTCTTAGTGGACTCTCACTGTCTGGAAAGGAA 621
Query 568 CAATGGTGTAAAATAG 583
      |||
Sbjct 622 CAATGGTGTAAAATAG 637
```

10.2. Appendix 2

10.2.1. Overview of experiments done with incubations with different concentrations of alcohol

Table 14: Experiment 1, 3 and 6 for 24 hpf

	<i>N=Mortality after 24 hrs</i>	<i>D</i>	<i>SD</i>	<i>PE</i>	<i>YSE</i>	<i>AM</i>	<i>Embryo's remaining</i>
E3							
N=40	15 %	-	-	-	-	-	85 %
N=60	63 %	-	-	-	-	2 %	37 %
N=100	13 %	-	-	-	-	-	87 %
1 % EtOH							
N=40	13 %	3 %	7 %	-	-	3 %	87 %
N=60	40 %	8,3 %	2 %	22 %	33 %	18 %	60 %
N=100	12 %	7 %	-	-	-	11 %	88 %
2 % EtOH							
N=40	23 %	8 %	35 %	8 %	8 %	8 %	77 %
N=100	30 %	14 %	10 %	2 %	-	-	70 %

Table 15: Experiments for 48 hpf

	<i>N=Mortality after 24 hrs</i>	<i>N=Mortality after 48 hrs</i>	<i>D</i>	<i>SD</i>	<i>PE</i>	<i>YSE</i>	<i>AM</i>	<i>Failure</i>	<i>Embryo's remaining</i>
E3									
N=40	13 %	-	-	-	-	-	-	-	87 %
N=60	50 %	2 %	-	-	-	-	-	6 %	42 %
N=100	13 %	-	-	-	-	-	-	-	87 %
1 % EtOH									
N=40	18 %	-	-	10 %	28 %	8 %	13 %	-	82 %
N=60	50 %	2 %	7 %	-	10 %	30 %	12 %	8 %	40 %
N=100	14 %	2 %	5 %	-	10 %	17 %	1 %	-	84 %
2 % EtOH									
N=40	23 %	30 %	18 %	10 %	8 %	8 %	8 %	-	47 %
N=100	32 %	9 %	6 %	42 %	5 %	6 %	all	-	59 %

Table 16: Experiments for 72 hpf

	<i>N=Mortality after 24 hrs</i>	<i>N=Mortality after 48 hrs</i>	<i>N=Mortality after 72 hrs</i>	<i>D</i>	<i>SD</i>	<i>PE</i>	<i>YSE</i>	<i>AM</i>	<i>Embryo's remaining</i>
E3									
N=40	20 %	-	-	-	-	-	-	-	80 %
N=100	23 %	1 %	2 %	-	-	-	-	-	74 %
1 %									
EtOH	35 %	-	2 %	3 %	-	33 %	33 %	3 %	63 %
N=40	12 %	2 %	5 %	2 %	-	26 %	17%	4 %	81 %
N=100									
2 %									
EtOH	25 %	15 %	2 %	30 %	8 %	40 %	40 %	40 %	58 %
N=40	25 %	6 %	11 %	33 %	8 %	33 %	33 %	33 %	58 %
N=100									

Table 17: Experiment 2 with 24 hpf

	<i>N=Mortality after 24hrs</i>	<i>Failure</i>	<i>Embryo's remaining</i>
E3 N=60	27 %	30 %	43 %
0.01 % EtOH N=60	22 %	20 %	58 %
0,1 % EtOH N=60	32 %	30 %	38 %
1 % EtOH N=60	25 %	20 %	55 %

Table 18: Experiment for 48 hpf

	<i>N=mortality after 24hrs</i>	<i>N=mortality after 48 hrs</i>	<i>Failure</i>	<i>D</i>	<i>SD</i>	<i>PE</i>	<i>YSE</i>	<i>AM</i>	<i>Embryo's remaining</i>
E3 N=60	40 %	-	5 %	2 %	-	-	-	2 %	55 %
0.01 % EtOH N=60	22 %	3 %	12 %	2 %	-	-	13 %	5 %	63 %
0.1 % EtOH N=60	30 %	-	5 %	-	-	-	23 %	5 %	65 %
1% EtOH N=60	22 %	2 %	5 %	2 %	-	10 %	13 %	8 %	71 %

Experiment 4 og 5

Table 19: Experiment 4 and 5 with 24 hpf

	<i>N=Mortality after 24 hrs</i>	<i>D</i>	<i>SD</i>	<i>PE</i>	<i>YSE</i>	<i>AM</i>	<i>Failure</i>	<i>Embryo's remaining</i>
E3								
N=100	29 %	-	-	-	-	-	-	71 %
N=100	9 %	-	-	-	-	-	3 %	88 %
0,1 % EtOH								
N=100	30 %	1 %	-	-	23 %	1 %	12 %	58 %
N=100	9 %	3 %	-	-	-	4 %	45 %	46 %
1 % EtOH								
N=100	30 %	4 %	1 %	-	36 %	1 %	18 %	52 %
N=100	13 %	4 %	-	-	-	14 %	2 %	85 %

Table 20: Experiments for 48 hpf

	<i>N=Mortality after 48hrs</i>	<i>D</i>	<i>SD</i>	<i>PE</i>	<i>YSE</i>	<i>AM</i>	<i>Embryo's remaining</i>
E3							
N=100	1 %	2 %	-	-	-	-	99 %
N=100	11 %	1 %	-	1 %			89 %
0,1% EtOH							
N=100	-	1 %	-	-	9 %	-	100 %
N=100	12 %		2 %		10 %		88 %
1% EtOH							
N=100	-	4 %	-	7 %	10 %	4 %	100 %
N=100	11 %	1 %	-	7 %	22 %	3 %	89 %

AM: Axial Malformation, YSE: Yolk Sac Edema, PE: Pericardial Edema, D: deformed, SD: seriously deformed

10.3. Appendix 3

10.3.1. Overview of eye measurements and midbrain-hindbrain junction

P-value is for the lateral and dorsal measurements taken between the junction in the midbrain/hindbrain

Table 21: Measurements of the eye for pax6.1 and midbrain-hindbrain junction for pax2.1

	Side view eye (μm)	Side view brain (μm)	Top view brain (μm)
Z24hpf E3-medium	11,10 14,77 12,26 15,01	4,96 4,57 5,55 5,19	5,81 4,62 5,16 5,08
Mean	13,3	5,1	5,2
Standard Deviation	2,0	0,4	0,5
p-value	>0.05 n.s		
0,1% EtOH	12,67 14,03 11,65 12,92	4,58 4,24 3,61 3,92	6,00 5,77 5,54 6,23
Mean	12,8	4,1	5,9
Standard Derivation	1,0	0,4	0,3
p-value	<0,05 significant		
1% EtOH	9,23 13,95 11,14 13,95 14,46	3,23 3,92 5,27 6,16 3,92	4,85 4,15 4,44 5,12 4,15
Mean	12,5	4,5	4,5
Standard derivation	2,3	1,2	0,4
p-value	> 0.05 n.s		

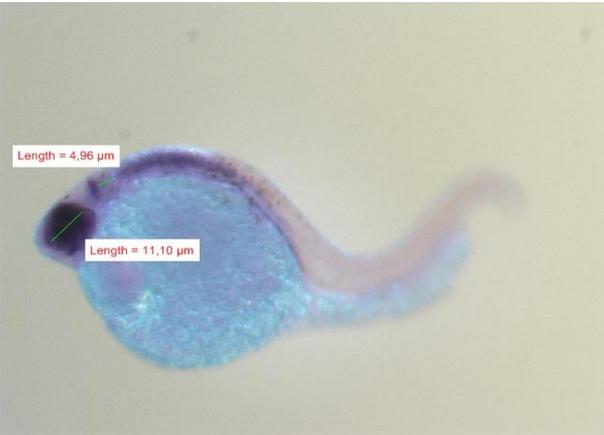
Z48hpf medium	E3-	10,2 8,95 11,77 10,15 9,23 8,77 9,92 7,85 6,92 7,85	3,75 4,16 4,85 3,92 3,69 3,46 3,46 3,69 3,92 3,00	3,53 5,77 4,85 4,03 4,62 4,15 4,97 4,38 4,38 3,46
Mean		9,3	3,8	4,4
Standard deviation		1,6	0,5	0,7
p-value	< 0.05 significant			
0,1% EtOH		10,15 11,08 9,69 10,15 11,54 9,92 7,62 8,77 6,69 7,38	3,69 4,21 4,38 3,43 4,15 3,23 3,00 3,69 3,52 3,70	3,23 6,23 6,46 4,85 3,46 4,15 3,92 3,92 5,54 4,16
Mean		9,3	3,7	4,6
Standard Deviation		1,6	0,4	1,1
p-value	< 0.05 significant			
1% EtOH		9,16 8,08 8,31 11,77 8,31	2,50 3,46 4,38 4,62 3,69	2,29 3,88 4,73 5,08 3,91

	11,08	4,35	4,97
	5,08	3,00	4,38
	8,77	2,54	4,15
	9,46	3,46	3,92
	8,54	3,49	3,61
	9,99	2,91	5,09
	9,23	3,23	6,34
	8,31	3,23	5,54
Mean	8,93	3,5	4,5
Standard derivation	1,6	0,7	1,0
p-value	< 0.05 significant		

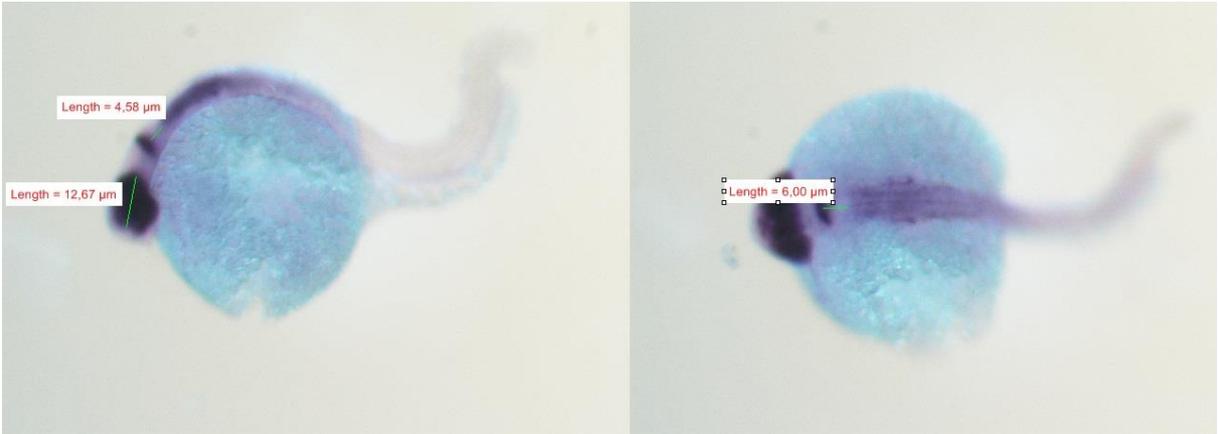
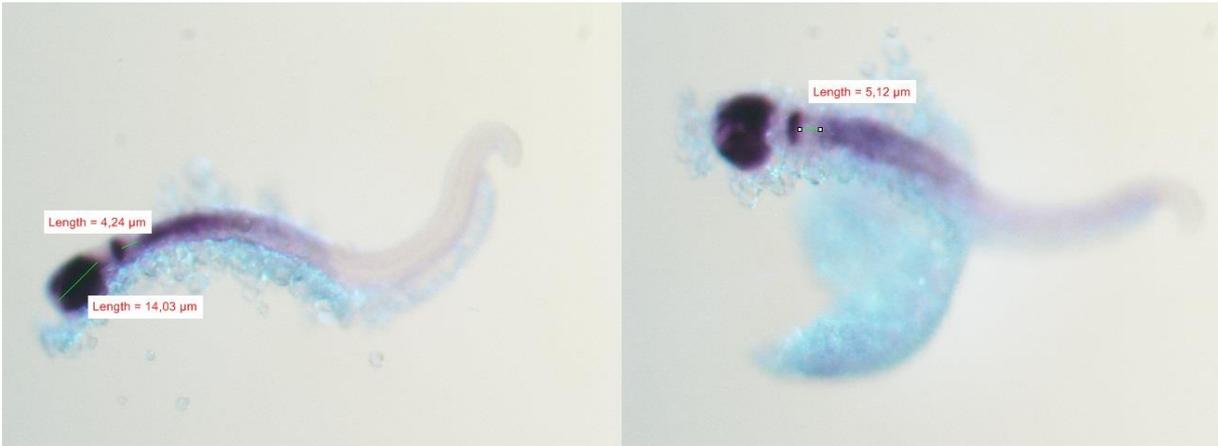
n.s: not significant $p > 0.05$

10.3.2. Pictures for the measurements of the Pax2.1 and pax6.1

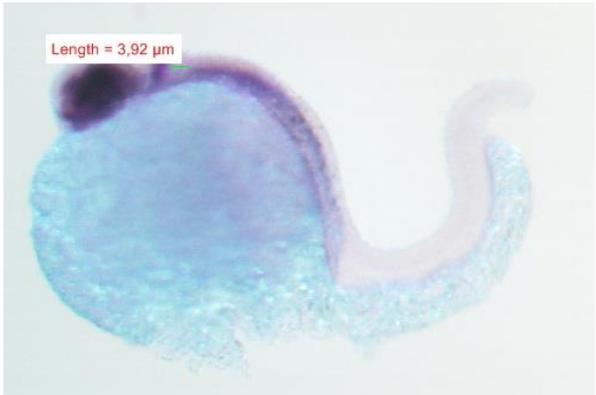
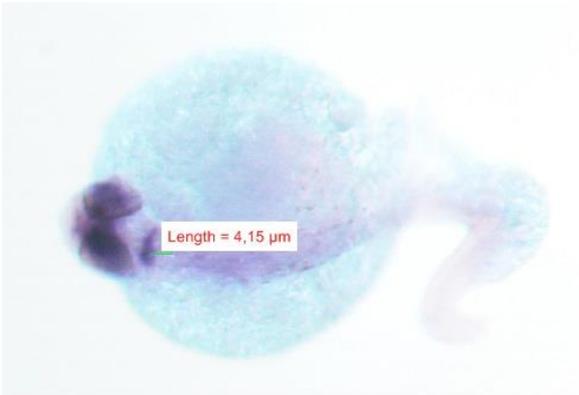
24 hpf zebrafish embryos in E3-medium



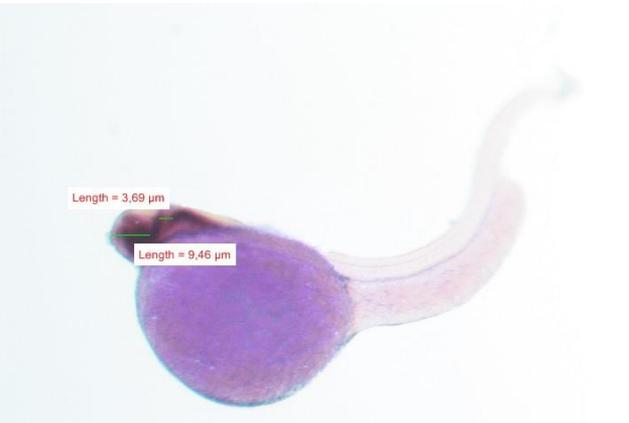
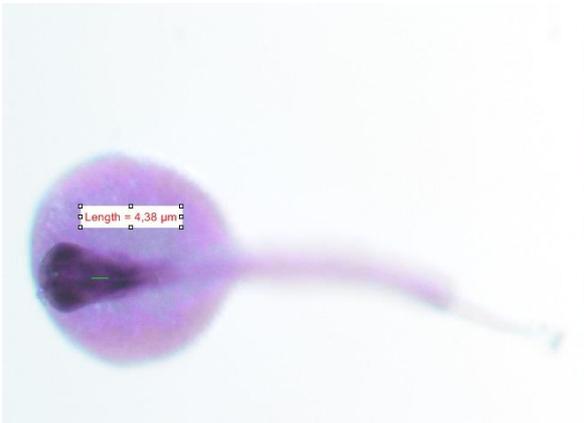
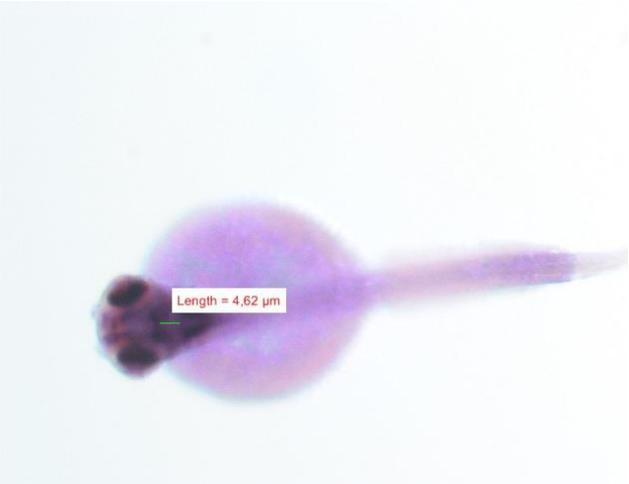
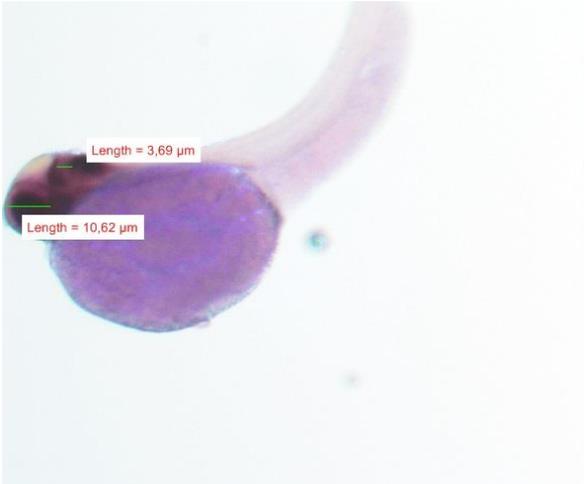
24 hpf zebrafish embryos in 0.1% Ethanol



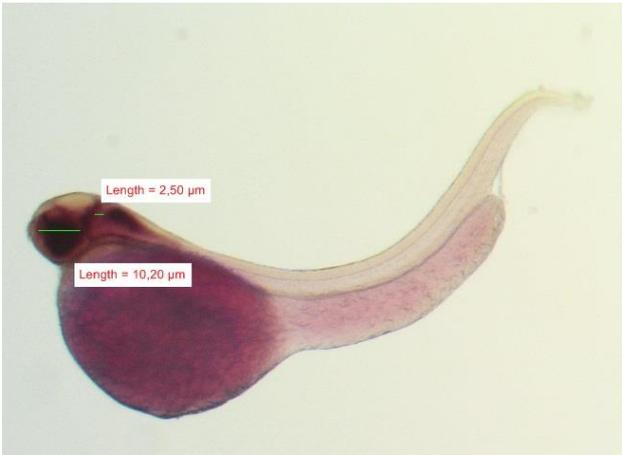
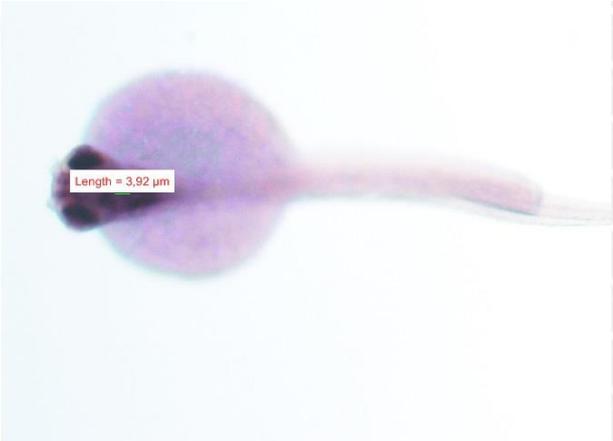
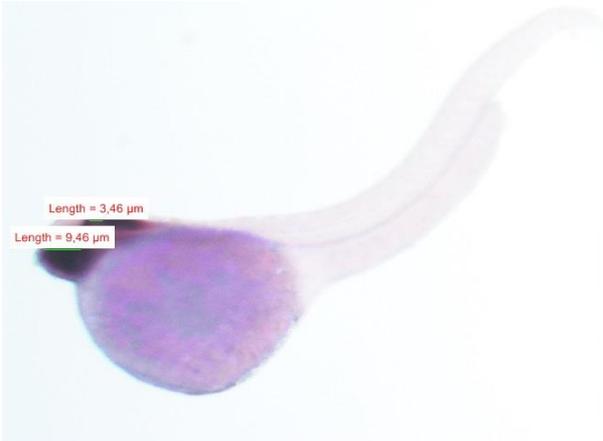
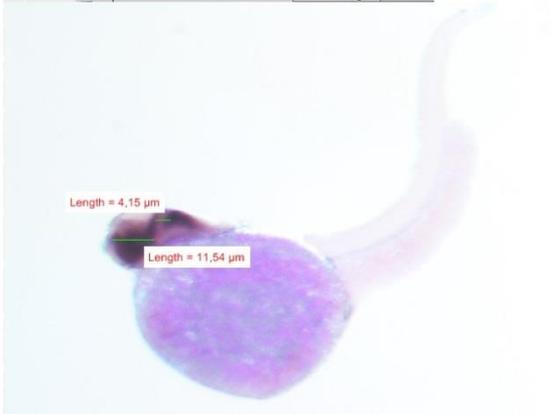
24 hpf zebrafish embryos in 1% Ethanol

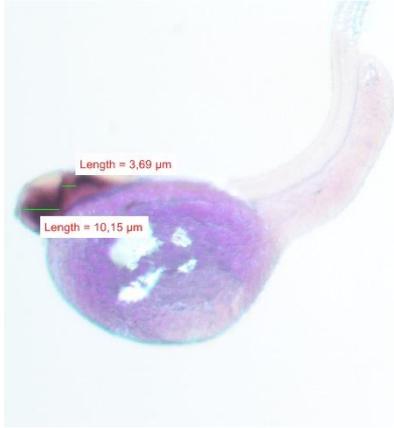


48 hpf zebrafish embryos in E3-medium



48 hpf zebrafish embryos with 0.1%Ethanol





48 hpf zebrafish embryos with 1%Ethanol

