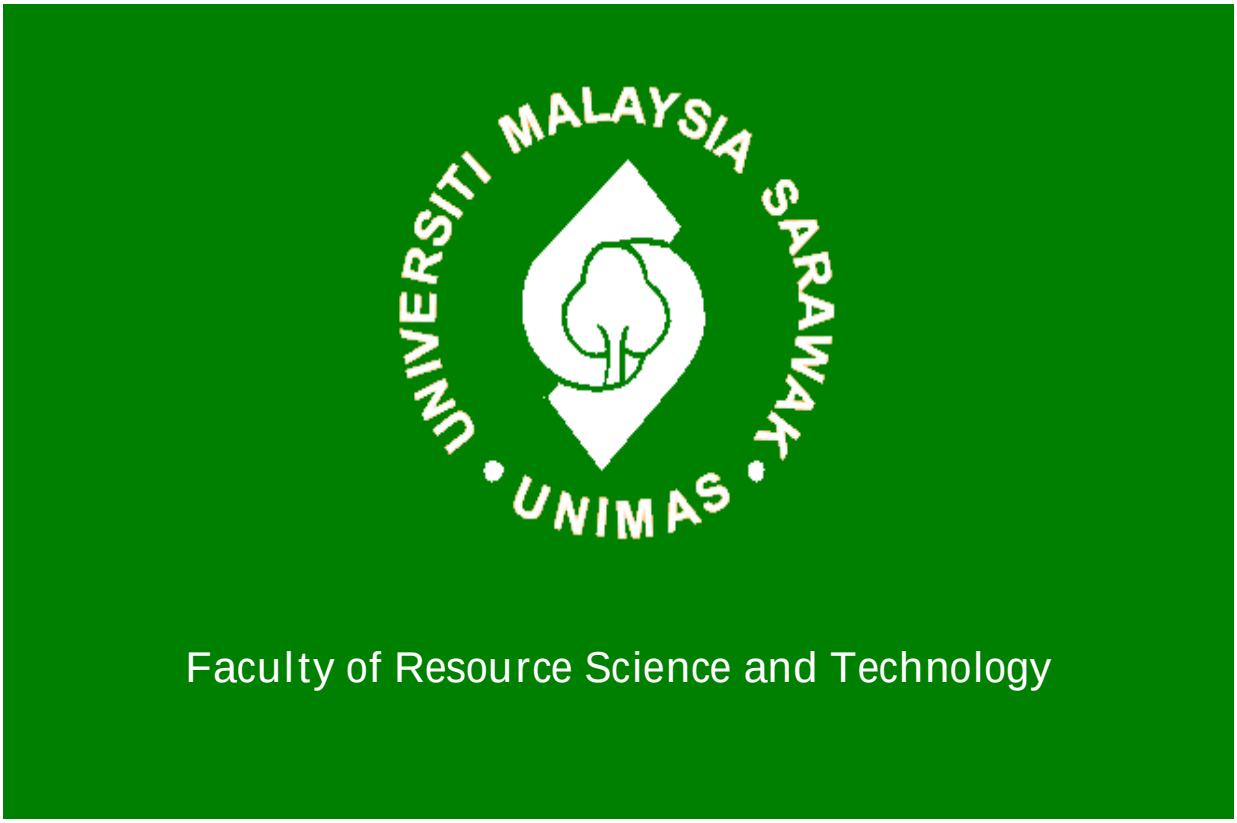

Molecular Cloning and Expression Study of Proline-rich Ena/VASP Ligand 2b (PREL-2b) Gene in Zebrafish Development

SU SIN LING (22438)

Bachelor of Science with Honours
(Resource Biotechnology)
2011

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SU SIN LING
(22438)

This project is submitted in partial fulfilment of the requirement for the degree of
Bachelor of Science with Honours
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2011

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UNIVERSITI MALAYSIA SARAWAK

DECLARATION OF FINAL YEAR PROJECT REPORT/THESIS

TITLE: Molecular Cloning and Expression Study of Proline-rich Ena/VASP Ligand 2b (PREL-2b) gene in Zebrafish Development

ACADEMIC SESSION : 2010/2011

I **SU SIN LING**

confirm that:

√	This thesis represents my own work.
√	The contribution of any supervisors and others to the research and to the thesis was consistent with normal supervisory practice.
√	External contributions to the research are acknowledged.

SIGNATURE

Date: 24 May 2011

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LIST OF ABBREVIATIONS

BPS	Between PH and SH2
DPF	Day post fertilization
EVH1	Ena/VASP Homology 1
EVL	Ena/VASP like
EVL	Enveloping layer
HPF	Hour post fertilization
LAIX	LB/Ampicillin/IPTG/X-gal
LPD	Lamellipodin
MRL	Mig10/RIAM/Lpd
PH	Pleckstrin Homology
PREL-2	Proline rich Ena/VASP Ligand 2
RA	Ras-association
RAPH1	Ras-associated and pleckstrin homology domains containing protein 1
RIAM	Rap1-GTP interacting adapter molecule
RT-PCR	Reverse transcriptase Polymerase Chain Reaction
SH	Sarcoma Homology 3 domain
VASP	Vasodilator-stimulated phosphoprotein

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Molecular Cloning and Expression Study of Proline-rich Ena/VASP Ligand 2b (PREL-2b) Gene in Zebrafish Development

Su Sin Ling

Resource Biotechnology
Faculty of Resource Science and Technology
University Malaysia Sarawak

ABSTRACT

Proline rich Ena/VASP Ligand 2 (PREL-2) is known as Lamellipodin (Lpd) and Ras-associated and pleckstrin homology domains-containing protein 1 (RAPH1). Proline rich Ena/VASP Ligand 2 (PREL-2) is constituted from Ras-Association (RA), Pleckstrin Homology (PH) domain and a long proline stretch at C-terminal. PREL-2 was found to have significant functions on cell signaling and direct migration, promoting axon guidance, affecting focal adhesion and regulating lamellipodial dynamics. PREL-2 gene in zebrafish is duplicated into PREL-2a and PREL-2b due to genome duplication. To establish the expression pattern of PREL-2b gene in developing zebrafish, total RNA was isolated from 10 embryonic stages of developing zebrafish. Reverse Transcriptase-PCR revealed that PREL-2b gene was expressed along the embryonic development, started from 3hpf and remained until 5dpf. Amplified cDNA sequences were confirmed by nucleotide sequencing, given an output result of 652bp. The gene was later cloned. The findings provide an insight that PREL-2b gene plays an important role during zebrafish embryonic development.

Key words: PREL-2, PREL-2b, zebrafish, RT-PCR, gene expression

ABSTRAK

Proline rich Ena/VASP Ligand 2 (PREL-2) dikenali sebagai Lamellipodin (Lpd) dan Ras-associated dan pleckstrin homology domains-containing protein 1 (RAPH1). PREL-2b gen terdiri daripada Ras-Association (RA), Pleckstrin Homology (PH) domain dan satu regangan proline yang panjang di C-terminal. PREL-2 mempunyai fungsi yang penting dalam mengisyarat sel dan migrasi langsung, mempromosi bimbingan axon, mempengaruhi adhesi focal dan mengawal dinamik lamellipodial. PREL-2 pada zebrafish diduplikasikan kepada PREL-2a dan PREL-2b disebabkan genom duplikasi. Untuk menyelidik pola ekspresi bagi PREL-2b gen dalam zebrafish, RNA telah diekstrak daripada 10 tahap pertumbuhan zebrafish yang berlainan. Data RT-PCR menunjukkan bahawa PREL-2b gen mempunyai ekspresi pada 10 tahap yang dipilih. Urutan cDNA telah ditentu dan disahkan oleh sequencing nukleotida dengan 652bp. Gen tersebut diklonkan. Keputusan yang diperolehi mencadangkan bahawa PREL-2b memainkan peranan yang penting dalam pertumbuhan embrio zebrafish

Kata kunci: PREL-2, PREL-2b, zebrafish, RT-PCR, gen ekspresi

1.0 INTRODUCTION

The zebrafish is referred to as *Danio rerio, Brachydanio rerio* and zebra danio (Robin *et al*., 1991). It has emerged as an important and powerful vertebrate model in numerous experimental approaches and biological studies.

Proline rich Ena/VASP Ligand 2 (PREL-2) gene is one of the proteins that play a significant role in cell migration and cell signaling. It is called Lamellipodin (Lpd) in mammalians. PREL-2/Lpd was identified by Krause *et al.* (2004) as an Ena/VASP binding protein due to the regulation of lamellipodial protrusion by Ena/VASP proteins. Besides, Proline-rich Ena/VASP Ligand 2 (PREL-2) is also known as Ras-associated and pleckstrin homology domains-containing protein 1 (RAPH1) in human.

The structure of PREL-2 gene is constituted by several domains. The N-terminus of PREL-2 is highly charged and is followed by a putative coiled-coil motif, Ras-Association (RA) and Pleckstrin Homology (PH) domain. Besides, it also possesses a long proline stretch at C-terminal (Krause *et al.*, 2004). The C-terminus is proline-rich, harboring eight potential SH3 binding sites, three potential Profilin binding sites and four clusters containing a total of six putative EVH1 binding sites (Krause *et al.*, 2004).

According to Krause *et al.* (2004), PREL-2 gene can bind to Ena/VASP protein directly both *in vitro* and *in vivo.* The studies of Krause *et al.* at 2004 found out that all the six potential EVH1 binding sites at C-terminal in PREL-2 showed medium to strong binding to Ena/VASP like (EVL) protein. In addition, PREL-2 and Ena/VASP proteins can co-localize at the tips of filopodia and lamellipodia. Notably, over-expression of PREL-2

gene can lead to the increasing of lamellipodial protrusion velocity. Knockdown of PREL-2 may impair the lamellipodial formation and reduce the velocity of residual lamellipodial protrusion.

PREL-2 gene was found to have significant functions on cell signaling and direct migration, promoting axon guidance (Chang *et al.*, 2006) and regulating lamellipodial dynamics (Krause *et al.*, 2004). PREL-2 also functions in the mechanism whereby certain pathogens "hijack" the host actin polymerization machinery to infect or move within host cells (Krause *et al*., 2004, cited in Holt and Daly, 2005). Furthermore, Krause *et al.* (2004) also proposed that PREL-2 can negatively regulating adhesion.

Proline-rich Ena/VASP Ligand 2 gene family contains two distinct genes, including Proline-rich Ena/VASP Ligand 2a (PREL-2a) and Proline-rich Ena/VASP Ligand 2b (PREL-2b), which were resulted from genome duplication event. PREL-2a and PREL-2b gene were found to exhibit distinctive complementary during somite development in zebrafish (Lee, 2008).

Northern blot analysis showed high expression of PREL-2 in mouse's organs such as brain, heart, ovary as well as developing embryos, while for its isoform, it is expressed in embryo, ovary and liver (Krause *et al*., 2004). Since the expression of PREL-2 gene on organs has been studied, more efforts are needed to study the expression of PREL-2 gene at embryonic stages. The expression studies of PREL-2b gene on zebrafish embryos had been carried out until 34 hour post fertilization (hpf). However, no follow-up expression study has been conducted on later developmental stages after 34hpf in developing

zebrafish. Therefore, a study has to be conducted in order to identify and establish the expression pattern of PREL-2b gene at different stages of zebrafish development.

In this research study, we focused on the molecular cloning and the expression studies of Proline-rich Ena/VASP Ligand 2b (PREL-2b) gene in developing zebrafish. The main objective for this research study was to clone the Proline-rich Ena/VASP Ligand 2b gene and then observe, identify and establish its expression in developing zebrafish through the application of RT-PCR.

2.0 LITERATURE REVIEWS

2.1 Proline-rich Ena/VASP Ligand 2 (PREL-2) Gene

2.1.1 Proline-rich Ena/VASP Ligand 2 (PREL-2) Gene

Proline-rich Ena/VASP Ligand 2 (PREL-2) belongs to PREL gene family. PREL-2 is also known as Ras-associated and pleckstrin homology domains-containing protein 1 (RAPH1). In human, it is known as Lamellipodin (Lpd). It is a part of MRL protein family (Mig10/RIAM/Lpd).

PREL-2 structure is constituted by several domains. The N-terminus of PREL-2 is highly charged and is followed by a putative coiled-coil motif, Ras-association (RA) and PH domain (Figure 2.1). The PH domain is a protein module of 100-120 residues that bind to the phosphoinositides. Indeed, the PH domain in PREL-2 gene can bind to phosphatidylinositol (3, 4) bisphosphate (PtdIns (3, 4) P2). The specific binding of PREL-2 gene and PtdIns (3, 4) P2 can be a mechanism by which Ena/VASP proteins localize at leading edge (Krause *et al.*, 2004).

PREL-2 gene shares both the PH and RA domain with the Grb7 family protein and RIAM as well as *C.elegans* Mig-10 (Krause *et al*., 2004; Lafuente *et al*, 2004). Besides, it also contains a long proline stretch at C-terminal. The C-terminus is proline-rich, harboring eight potential SH3 binding sites, three potential Profilin binding sites and four clusters containing a total of six putative Ena/VASP Homology 1 (EVH1) binding sites. (Krause *et al*., 2004).

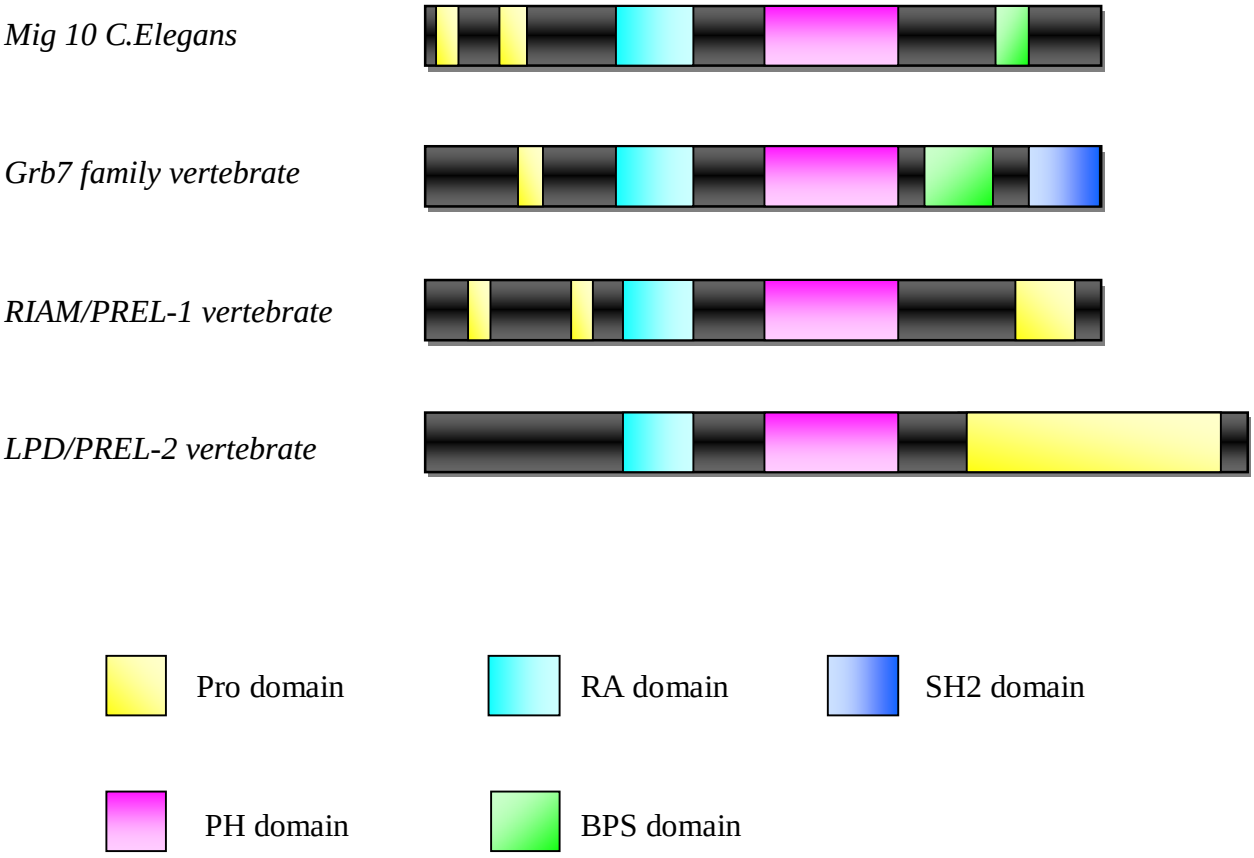

Figure 2.1: Domain structure of vertebrate Grb and PREL family proteins. RIAM/PREL-1, Lamellipodin/PREL-2 and Mig-10 share a conserved domain structure and are related to the Grb7 family proteins. Pro, proline-rich domains; RA, Ras-association domain; PH, pleckstrin-homology domain; SH2, Src-homology-2 domain; BPS, between PH and SH2.

(Adopted and modified from Lee, 2008; Legg and Machesky, 2004)

PREL-2/Lamellipodin is first identified by Krause *et al.* at 2004 due to its ability to co-localize with the Ena/VASP proteins in lamellipodia and filopodia. It is being identified as an Ena/VASP binding protein because it regulated lamellipodial protrusion (Krause *et al*., 2004). PREL-2 has multiple FPPPP motifs, a Ras/Rap Tapes association domain, and a profiling binding proline-rich domain (Chang *et al*., 2006). It contains six motifs that conform to the consensus binding site for the EVH1 domain of Ena/VASP proteins (Krause *et al.*, 2004, Lafuente *et al.*, 2004, Jenzora *et al*., 2005, cited all in Holt and Daly, 2005).

According to Krause *et al*. (2004), PREL-2 can bind to Ena/VASP protein both *in vitro* and *in vivo.* The studies found out that all the six potential EVH1 binding sites in PREL-2 showed medium to strong binding to Ena/VASP like (EVL). This binding indicated that all the six sites in PREL-2 can bind to the EVH1 domain of Ena/VASP proteins. In addition, PREL-2 and Ena/ VASP can co-localize at the tips of filopodia and lamellipodia. PREL-2 over-expression can lead to the increasing of lamellipodial protrusion velocity. However, knockdown of PREL-2 may impair the lamellipodial formation and cause F-actin depletion, and reduce the velocity of residual lamellipodial protrusion.

PREL-2 was found to have significant functions on cell signaling and direct migration. When Ena/ VASP are over-expressed, PREL-2 contains longer, less branched filaments. Knockdown of PREL-2 with short hairpin RNA showed severe lamellipodia and F-actin depletion, suggesting that PREL-2 might signal to actin regulatory proteins. PREL-2 also functions in the mechanism whereby certain pathogens “hijack” the host actin

polymerization machinery to infect or move within host cells (Krause *et al*., 2004, cited in Holt and Daly, 2005). According to Legg and Machesky (2004), PREL-2 is crucial for Ena/VASP localization at the plasma membrane and for the dynamics of lamellipodial actin assembly. Furthermore, Krause *et al.* (2004) also proposed that PREL-2 can affect focal adhesion. PREL-2 is thought to negatively regulating adhesion, an effect opposite to Rap1-GTP interacting adapter molecule (RIAM).

2.1.2 Expression of PREL-2 gene

According to Northern blot analysis, PREL-2 generally shows strong expression in the organ of mice such as the brain, heart, ovary as well as developing embryos (Krause *et al.*, 2004). While for its isoform, it is expressed in embryo, ovary and liver.

2.1.3 Proline-rich Ena/VASP Ligand 2b (PREL-2b) Gene

There are two members of Proline-rich Ena/VASP Ligand 2 gene, which are denoted as Proline-rich Ena/VASP Ligand 2a (PREL-2a) and Proline-rich Ena/VASP Ligand 2b (PREL-2b). The two genes are arising through genome duplication.

The PH and RA domain of both genes exhibited high sequence identity to the mammalian orthologous member with approximately 87% and 95%, respectively (Lee, 2008). PREL-2a and PREL-2b also exhibited 70% amino acid sequence identity.

There are similarities and differences between PREL-2a and PREL-2b in terms of expression pattern. No expression was shown for both genes at 4 cell stage and 3hpf. PREL-2a was first expressed in somites at 11hpf where the segmentation begun (Lee,

2008). However, no detection of PREL-2b was observed at this stage. According to Lee (2008), PREL-2a and PREL-2b exhibited different and distinctive complementary spatiotemporal somite expression pattern between 18hpf and 34hpf. While PREL-2a was expressed in the posterior tail, PREL-2b showed strong expression in the anterior tail. When PREL-2a showed weak expression at the presomitic mesoderm of posterior trunk at 34hpf, PREL-2b was observed to have strong expression in the posterior tail region, the domain where PREL-2b was weakly expressed at 24hpf (Lee, 2008).

2.2 Zebrafish

2.2.1 General remarks

Recently, the zebrafish has emerged as a forefront and pre-eminent research model for biologists studying developmental as well as cell biological processes, human diseases and the screening of therapeutic drugs (Penberthy *et al*., 2002; Sumanasa and Lin, 2004, cited in Lawrence, 2007). The zebrafish has several unique characteristics and attributes that make it well positioned for identifying novel regulatory genes in vertebrate development and elucidating their function in the developing animal (Dahm *et al.*, 2005). Specifically, it acts as an important model system for the study of infectious disease and immune function (Sullivan and Kim, 2008), *in vivo* studies of cell death (Pyati, Look and Hammerschmidt, 2007), drug target screening and validation (Sumanas and Lin, 2004), functional aging and very gradual senescence (Kishi et al., 2003).

2.2.2 Background

The zebrafish (*Danio rerio*, formerly also known as *Brachydanio rerio*) is a small tropical freshwater fish; with body length rarely exceeds 40mm (Spence *et al.*, n.d.). *Danio rerio* was first described by Francis Hamilton, a surgeon of British East India Company at the beginning of the 19th Century (Spence *et al.*, n.d.). *D. rerio* was later assigned to its subgenus Brachydanio, therefore so-called *Brachydanio rerio*.

Zebrafish are indigenous to South Asia, mainly distributed at northern India as well as northern Pakistan, Nepal, Myanmar and Bhutan (Dahm *et al.*, 2005; Lawrence, 2007). The zebrafish belongs to the family of the cyprinids (*Cyprinidae*) in the class of ray-finned

fishes (*Actinopterygii*) and within this class to the bony fishes (teleosts or *Teleostei*) to which most extant ray-finned fishes belong (Dahm *et al.*, 2005). According to Wixon (2000), the males are slender and torpedo-shaped, with black longitudinal stripes and a gold colouration on the belly and fins. Females are fat when laden with eggs and have little, if any, gold on their undersides (Wixon, 2000).

The zebrafish is omnivorous, its natural diet consists primarily of zooplankton and insects, although phytoplankton, filamentous algae and vascular plant material, spores and others (Dutta, 1993; McClure *et al.*, 2006; Spence *et al*., 2007a, cited in Spence *et al.*, n.d.).

Courtship behavior in zebrafish consists of a male chasing the female rapidly, often nudging her flanks with his snout and attempting to lead her to a spawning site, swimming around or in front of her in a tight circle, or figure of eight, with his fins raised (Spence *et al.*, n.d.).

2.2.3 Advantages of zebrafish as a model organism

Zebrafish has many favorable attributes to be enumerated individually. Those properties make it ideally and uniquely suited as a model organism.

First of all, zebrafish has several embryological advantages that contributed to its popularity as a research model. Zebrafish exhibit external embryonic development and therefore is accessible to embryos at all developmental stages without any sacrifices. Furthermore, the embryos are sturdy and its size is large enough for experimental manipulations (Dahm *et al.*, 2005). Moreover, the chorion of the zebrafish eggs is

transparent, allowing the continuous observation of the developing embryo under microscopes.

Besides, it is worth to mention that the embryonic development of the zebrafish is synchronous and extremely rapid. Rapid embryonic development facilitates numerous observation and visualization of developmental processes and experimental approaches. The completion of experiments generally can be achieved within a few hours to days. Compared with other vertebrate models for developmental studies, such as the mouse or *Xenopus*, the composition of the zebrafish larvae's organs is relatively simple. They composed of fewer cells, making them easier to study (Dahm *et al.*, 2005).

Last but not least, zebrafish are easy to breed and has high fecundity (Lawrence, 2007). This fact, together with the small size and the transparency of zebrafish embryos, zebrafish take advantages of them and therefore being promoted as the most favorable model organism.

2.2.4 Embryonic development of zebrafish

The development of the zebrafish is similar to the embryogenesis in higher-ordered vertebrates, including humans and other mammals (Dahm *et al*. 2005). Kimmel *et al*. (1995) defined seven broad periods of embryogenesis-the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods.

As a vertebrate, embryonic development in the zebrafish is extremely rapid because embryogenesis takes approximately 2 days. At the one-cell stage immediately after

fertilization, the zygote sits on top of a large ball of yolk. Four hours following fertilization (4 hpf), the cells have undergone several rounds of cleavages and division to form a mass of cells located on top of the yolk. Gastrulation begins with epiboly of the cells at 6 hpf. At this stage, the antero-posterior and dorso-ventral axes can be determined. 8 hpf is the onset of thickening in the region of the future head. At 10 hpf, first somite forms elucidated the starting of somitogenesis. Somites continue to develop and can be counted under microscope clearly. At 18 hpf, body plan becomes recognizable, and the first voluntary muscle contractions are observable as movements of the tail. After a day (24 hpf), most vertebrate-specific body features and organs are already recognizable, including a compartmentalized brain, eyes, ears, and all internal organs. However, the majority of the organs are not yet functional. Notably though, the heart starts beating just before the end of the first day of development. Over the next 24 hpf, numerous cell types differentiate, and the organs gradually begin to function. At 48 hpf, zebrafish start to hatch and start swimming very actively. At 5 dpf, larvae swim well and search for food independently.

The speed of zebrafish development is temperature dependent. Zebrafish develop at temperatures ranging from approximately 18 to 32 ◦C (Dahm *et al.*, 2005). In fact, the higher the temperature, the faster the development of the zebrafish.

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Zebrafish

The zebrafish used in this research study were supplied by Dr. Lee Kui Soon and Animal Biotechnology Laboratory (ABL) from Department of Molecular Biology, Faculty of Resource Science and Technology (FRST), University Malaysia Sarawak (UNIMAS).

3.1.2 Embryo medium

1 liter of embryo medium/E3 medium was prepared in 1X and 50X respectively with nutrient formulation as listed below:-

i. 5mM Sodium chloride (NaCl)

ii. 0.17mM Potassium chloride (KCl)

iii. 0.33mM Calcium chloride dihydrate ($CaCl_2.2H_2O$)

iv. 0.33mM Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)

Table 3.1: Chemical composition for 50X embryo medium

Materials	**Molarity (mM)**	**Amounts (g)**
NaCl	250	14.610
KCl	8.5	0.634
$CaCl_2.2H_2O$	16.5	2.426
$MgSO_4.7H_2O$	16.5	4.067

3.1.3 Ampicillin (50 mg/ml)

To prepare Ampicillin stock with concentration of 50 mg/ml, 0.1 g of Ampicillin was added to 2 ml of MiliQ water and was filter-sterilized using 0.2µM syringe filter. The Ampicillin solution was later stored at -20°C.

3.1.4 Isopropyl-b-d-thiogalactopyranoside (IPTG)

To prepare IPTG stock solution with concentration of 24 mg/ml, 0.12 g of IPTG was added to 5 ml of MiliQ water and was filter-sterilized using 0.2µM syringe filter. The IPTG solution was later stored at 4°C until use. It should be stable for many months.

3.1.5 LB/Ampicillin/IPTG/X-gal (LAIX) plates preparation

LB/Ampicillin plates were brought to laminar flow, 100 µl of IPTG was spread onto the plates and let the reagent be absorbed by placing them in an inverted position inside a 37°C incubator for 30 minutes. After this, 20 µl of X-gal was spread onto the plates and let the reagent be absorbed by placing them in an inverted position inside a 37°C incubator for 30 minutes. The plates were stored in 4°C until use.