Molecular mechanisms of cardiac arrhythmia

Kim Anh Nguyen
Institute of Molecular and Cell Biology, A*STAR

IMCB Supervisors:
Vladimir Korzh & William Go

ECM Supervisor:
Julien Leclaire

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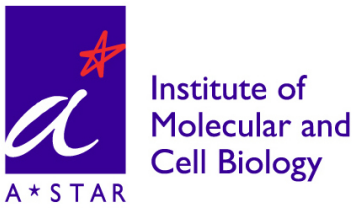

Acknowledgements

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Abstract

Deoxyribonucleic acid (DNA) is the molecule that contains the genetic information of all known living organisms. Its double helix structure and its composition in nucleotides remains the same in all living cells. The information carried by DNA is held in the sequence of genes and encodes the genetic instructions used in the development and functioning of the organism. Each gene codes for a protein, which itself has a specific function.

As the basic mechanisms of cell differentiation are similar among different species, the study of zebrafish genes is very helpful in understanding human hereditary diseases. The zebrafish, although very different from a human being at a first glance, shares around 80% of its genes with us.

This report deals with FGF13 (also known as FHF2a), which belongs to a huge protein family of fibroblast growth factor (FGF), known for its broad functions in mitosis, cell survival and regulation of a variety of biological processes, including embryonic development. In human its function is not well-understood but it is expressed in the slow cardiac conduction system (CCS). In zebrafish, the suspected homolog is fgf13a (cardiac-specific type) and was found to be expressed in the sino-atrial (SA) node. SA-node contains the purported cardiac pacemaker for initiation of cardiac conduction signals. Our goal is to understand what function this protein performs by being expressed at that location.

We will try to show that the protein fgf13a binds to some proteins in the atrium lysates. Once the binding partner has been identified, the main goal is to prove that the binding initiates the electric signal responsible for the atrium contraction. The main steps of our project are:

1. DNA cloning: We've cloned a full-length cDNA for fgf13a-cardiac isoform.
2. Protein expression: We've expressed and purified fgf13.
3. Heart extraction: We've dissected zebrafishes to collect enough atrium lysates (which contains the SA node) to do a pulldown.
4. Pulldown: Atrium lysates and FGF13 are put together. We've then sent the samples to the mass spectrophometer laboratory for analysis to identify the binding partner of FGF13 and to study this binding.

Unfortunately, the results are still being processed at the moment and we can not reach to a straightforward conclusion.

Key words : Molecular Biology, Cardiac arrythmia, FGF13, DNA cloning, Protein expression.

Résumé

L'acide deoxyribonucléique (ADN) est la molécule qui contient toute l'information génétique de tout être vivant. Sa structure en double hélice et sa composition en nucléotides restent les mêmes dans toutes cellules vivantes. L'information portée par l'ADN réside dans la séquence des gènes (une région d'ADN, support de l'hérédité qui influence une caractéristique particulière de l'organisme) et porte l'information génétique, notamment les informations nécessaires au développement et au fonctionnement de l'organisme. Chaque gène code pour une protéine, qui elle-même a une fonction spécifique. La corrélation entre gène, protéine et fonction est le domaine de la biologie moléculaire, combinée avec la biochimie et la génétique.

Comme les mécanismes de base de la différenciation cellulaire sont les mêmes chez les différentes espèces d'êtres vivants, l'étude des gènes de zebrafish permet de mieux comprendre les maladies héréditaires chez l'homme. Le zebrafish, bien que très différent de l'être humain à première vue, partage près de 80% de ses gènes avec nous.

Ce rapport traite de FGF13 (également connu sous le nom de FHF2a), qui fait partie de la grande famille de protéines appelée facteur de croissance des fibroblastes, et qui est reconnue pour ses fonctions principales dans le processus de la mitose, la survie cellulaire, et la régulation de divers processus biologiques dont le développement embryonnaire. Sa fonction chez l'homme n'est pas encore totalement comprise mais le gène s'exprime dans certains tissus en relation avec le système de conduction cardiaque. Chez le zebrafish, le supposé hommologue est fgf13a. Il se trouve que celui-ci s'exprime dans le noeud sino-auriculaire, qui agit comme pacemaker à l'origine des signaux de conduction cardiaque. Notre but est de comprendre quelle fonction ce gène occupe en étant exprimé à cet endroit particulier.

Nous allons nous proposer de montrer que la protéine fgf13a se lie à un partenaire dans le lysat de l'oreillette. Une fois que nous aurons identifié ce dernier, l'objectif principal sera de démontrer que cette liaison est responsable du déclenchement du signal électrique à l'origine de la contraction de l'oreillette. La démarche sera la suivante :

1. Réplication de l'ADN : nous clonons un cDNA complet afin d'obtenir un isoforme cardiaque fgf13.

2. Expression de protéine : nous exprimons et purifions fgf13.

3. Extraction du coeur : nous disséquons des poisson-zèbres afin d'obtenir suffisamment de lysats d'oreillette (contenant le noeud SA) et d'effectuer un pulldown.

4. Pulldown : le lysat d'oreillette et fgf13 sont mis en contact. On envoit ensuite ces échantillons au laboratoire de spectrophotométrie de masse afin d'analyser et d'identifier le partenaire de liaison de fgf13 - puis nous l'étudions.

Malheureusement, les résultats sont encore en cours de traitements à l'heure actuelle, et nous ne pouvons aboutir présentement à une conclusion définitive.

Key words : Biologie moléculaire, Arrythmie cardiaque, FGF13, clonage d'ADN, Expression de protéines.

Contents

Nomenclature

E. coli *Escherichia coli*

A Adenine

BA Bulbus Arteriosus

C Cytosine

CCS Cardiac Conduction System

cDNA complementary DNA

DNA DeoxyriboNucleic Acid

dNTP DeoxyNucleoside TriPhosphates

dpf day post fertilization

FGF Fibroblast Growth Factor

G Guanine

LB *Lysogeny Broth*

PCR Polymerase Chain Reaction

T Thymine

Tm Melting Temperature

Part I

Presentation of A*STAR, IMCB and the mission

Chapter 1

Personal introduction

The following sections are quite similar to the official and formal description of A*STAR so if you need a quick personal sum up, here it goes:
A*STAR can be considered as the Singaporean equivalent of the French CNRS. It is a huge entreprise gathering around 3.000 scientists from graduate students to senior experts coming from 63 different countries. A*STAR covers every research fields in technology: biology, chemistry, physics, informatics... The research subjects are so various that they include robotics as well as material mechanics or optics. The entreprise is divided into research institutes and councils which are themselves subdivided into units and laboratories. I belong to the Institute of Molecular and Cell Biology and more precisely to the Zebrafish Translational Unit, led by Dr Vladimir Korzh.
My supervisors and co-workers are almost all Singaporean people but all of them have roots in another culture. Regardless of whether they come from China, Malaysia or Europe, they are very open-minded, probably because they are, for most of them if not all, bilingual by birth and used to mix different cultures. A*STAR is a very international company which employs a lot of foreigners, especially PhD students. They have specific programs for those who want to do a thesis in their home country and Singapore at the same time with many advantages (financial for example). A*STAR tries to encourage international students to come because they are usually cheaper. The cosmopolitan aspect of my laboratory and of Singapore in general is one of the strong thing I will remember of my internship.
Singapore is becoming a scientific eldorado : for 10.000 employees, there are 100 researchers. The Singaporean government chose to invest massively in the research field and the difference can be quickly felt between the French laboratories always struggling for some government or industrial grants and the almighty rich Singaporean laboratories. Contrary to CNRS, they don't really have budget issues.
Money is one thing, but the government also promotes and supports both foreign students and researchers to set up in Singapore. There are almost 20% of non-permanent residents in Singapore. It is very interesting to discover day after day the different cultures from all over the world and to try from times to times to add a french touch to the lab. If you have an adventurous spirit and if you are attracted to the Asian culture, you definitely should give it a try. A*STAR is a good company to work at and it will be sure a life-changing experiment.

Chapter 2

A*STAR

2.1 Overview

A*STAR stands for Agency for Science, Technology and Research and was called after the best mark you can get in Singaporean graduation system. A*STAR is the lead agency for fostering world-class scientific research and talent for a vibrant knowledge-based and innovation-driven Singapore. A*STAR oversees 14 biomedical sciences and physical sciences and engineering research institutes, and six consortia & centers, located in Biopolis and Fusionopolis as well as their immediate vicinity.

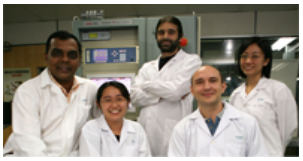
A*STAR supports Singapore's key economic clusters by providing intellectual, human and industrial capital to its partners in industry. It also supports extramural research in the universities, hospitals, research centers, and with other local and international partners.
Most of A*STAR is located in the three towers in One-North, which is so called because it lies 1 degree north of the Equator. The biology departments, like IMCB, are in Biopolis.

Figure 2.1: A*star building in One-North

2.2 Strategic thrusts

A*STAR strives to help Singapore develop into a world-class scientific research hub by building up three types of capital: human, intellectual and industrial.

Human capital



Developing human capital by promoting manpower training and development in the areas of science, engineering and technology (SET); and by undertaking the promotion of SET to increase public awareness and understanding of the importance of science and technology in Singapore.

Intellectual capital

Enhancing and strengthening Singapore's knowledge-creation and innovative capability by directing and undertaking research and development in SET through the research institutes.

Industrial capital

Promoting the commercial application of scientific knowledge and technology in Singapore through industry engagement and collaboration, R&D investment promotion, and active commercialization of intellectual property.

2.3 A*STAR entities

- BMRC supports, oversees and coordinates public sector biomedical research and development activities in Singapore.
- SERC supports, oversees and coordinates public sector research and development in the physical sciences & engineering.
- A*JC promotes and supports inter-disciplinary research programmes that can span the fields of biomedical sciences and physical sciences & engineering.
- A*GA advances human capital development through scholarships, fellowships and collaborations with universities.
- The Corporate Group comprises eight operational departments that supports the whole of A*STAR in achieving its mission.

2.4 BMRC Research Institutes & Consortia

The Biomedical Research Council (BMRC) oversees seven research institutes (RIs) and other research units that serve to support key industry clusters in Biomedical Sciences such as pharmaceuticals, medical technology, biotechnology and healthcare services.

The BMRC research institutes focus on building up core biomedical capabilities in the areas of bioprocessing; chemical synthesis; genomics and proteomics; molecular and cell biology; bioengineering and nanotechnology; and computational biology.

In addition, A*STAR also places great emphasis on translating new knowledge and technologies created at the "benches" into new clinical applications for diagnosis and treatment that can one day be delivered at the "bedsides" of our hospitals and disease centres.

For this reason, the Institute of Medical Biology (IMB) and Singapore Institute for Clinical Sciences (SICS) were established to focus on translational and clinical research. BMRC has also established various consortia to coordinate and drive translational research at the national level in strategic thematic areas.

These consortia aim to consolidate existing research activities and scientific expertise, optimise the use of critical research resources, fill in gaps in research capabilities, as well as build sufficient synergy and critical mass to make Singapore's efforts in these areas internationally competitive.

List of the Biomedical Research Institutes & Consortia

- Bioinformatics Institute (BII)
- Bioprocessing Technology Institute (BTI)
- Clinical Imaging Research Centre (CIRC)
- Experimental Therapeutics Centre (ETC)
- Genome Institute of Singapore (GIS)
- Institute of Bioengineering and Nanotechnology (IBN)
- Institute of Medical Biology (IMB)
- **Institute of Molecular and Cell Biology (IMCB)**
- A*STAR - Duke-NUS Graduate Medical School Neuroscience Research Partnership (NRP)
- Singapore Bioimaging Consortium (SBIC)
- Singapore Institute for Clinical Sciences (SICS)
- Singapore Immunology Network (SIgN)
- Singapore Stem Cell Consortium (SSCC)

Chapter 3

Institute of Molecular and Cell Biology (IMCB)

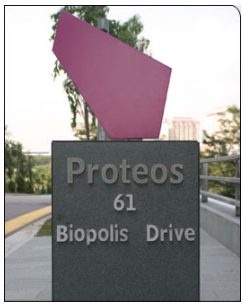
3.1 IMCB

The Institute of Molecular and Cell Biology was established in 1987 at the National University of Singapore (NUS) before becoming an autonomous research institute (RI) of A*STAR and moving to Biopolis in 2004. Today, it has 34 research groups with more than 400 staff members and is led by Executive Director, Professor Hong Wanjin.

IMCB has established itself as a world-class research institute in biomedical sciences with a focus on six major fields: Cancer Biology, Cell Biology, Developmental Biology, Infectious Diseases, Structural Biology and Translational Research.

Funded primarily by the Biomedical Research Council (BMRC) of A*STAR, IMCB's research activities focus on four major fields: Animal Models of Development and Disease, Cancer Genetics and Therapeutics, Cell Biology in Health and Disease, and Structural Biology and Drug Discovery.

3.2 Zebrafish Translational Unit (ZTU)



The Zebrafish Translational Unit's aim is to develop novel in vivo test systems and approaches that address efficacy of novel drug delivery platforms, drugs screening and testing, and validation of their biocompatibility. The ZTU personnel has experience in developing transgenics and mutants of specific genes as well as analysis activity of signaling pathways during development of multiple systems of organs, in particular, the central nervous system and cardiovascular system. Exposure of a panel of transgenic embryos to a specific chemical may help to address its potential toxicity and/or effect on development of most, if not all, tissues.

Researchers of the ZTU have generated and/or maintain a number of transgenic lines with expression of different fluorescent proteins as cytosolic, membrane and nuclear markers in different cell lineages and tissues, including, but not limited to the heart (endocardium, myocardium, pericardium, conduction system, valves, etc), blood vessels, CNS (brain, ventricular system, spinal cord), PNS (sensory ganglia, lateral line, mechanoreceptors, support and mantle cells), swimbladder, liver, pancreas, epithelium, limbs, intestine, kidney, immune system, etc. These were used to establish several in vivo models including but not limited to some forms of cancer, oxidative stress, hemorrhagic stroke, and so on. In addition to reporter strains, multiple mutant lines with developmental defects in various cell lineages and/or organs, including cilia, immune cells, etc, were developed too. This opens a possibility for embryos with reduced gene doses to be used in drug screens as sensitized backgrounds or in screens for compounds that attenuate the severity of the respective phenotypes.

3.3 Dr. Robinson's research

Dr. Robinson's laboratory seeks to gain detailed knowledge of the mechanisms behind pathogenicity and disease through elucidating structures of key components involved in the progression of these disorders. The laboratory is interested in all areas of aberrant function and misregulation of proteins in conditions arising from genetic mutations or external challenges. One central, but not limiting, theme of the laboratory is the harnessing of force-generating polymerization machines in (mis)driving critical biological processes.

Part II

The project

Chapter 1

Context

1.1 Zebrafish model

Why the Zebrafish ?

At first glance, Zebrafish seems to be a strange comparator to humans but like us they are vertebrate and we share a common ancestor. Although one can easily think of other animals like big mammals and especially monkeys which are known as genetically very similar to the human being, Zebrafish is largely used in molecular biology for its strong likeness with us. As described in [2], zebrafish models have many advantages: first, zebrafish is reamarkably biologically similar to people (it has been demonstrated that 70% of protein-coding human genes are related to genes found in the zebrafish and that 84% of genes known to be associated with human disease have a zebrafish counterpart). As it shares the majority of its genes with humans, it is an important model for understanding how genes work in health and disease.

Also, embryos are transparent allowing the visualization of all developmental stages with great clarity. Most importantly, development (especially embryonic development) is rapid: all common vertebrate specific body features can be seen within two days of development and an adult fish takes only a couple of months to grow.

Another positive aspect is the drug administration directly to fish water or by microinjection, which is much easier than other vertebrate research models.

Zebrafish's genome has been completely sequenced and expression of many genes have been described during development, which allows many possibilities for the study of gene function and diseases. Today only two other large genomes are studied to this extent: the human genome and the mouse genome.

Zebrafish really cheaper to grow comparing to other animals such as cats and dogs. Moreover they reproduce very easily and quickly. They are of course quiet, clean and well-behaved. No need to train them and when one is done with his experimentations, one can feed the catfishes with the remaining zebrafish. With little fishes which are doomed, we can grow something bigger. In this world of compromises and sustainability, this method doesn't waste anything, while conserving the chain of food.

Limitations
However, one can't study everything on zebrafish and apply it to human because there are still some differencies. Since fishes' heart only has two chambers instead of four, it has to be a rearrangement of blood vessels.

Zebrafish's heart

The heart of Zebrafishes is really simple. It is basically the half of a human heart but the principle remains the same. It is made up of two chambers: the atrium and the ventricle. The atrium pumps the blood into the ventricle, which in turn pumps the blood into the conus, an elastic compartment which does not pump, but has the ability to stretch and squeeze. From the conus the blood travels straight to the gills where it is enriched with oxygen. The oxygenated blood then flows through the body and returns to the atrium. Zebrafishes have the amazing particularity of being capable of regenerating its cells and especially its heart.

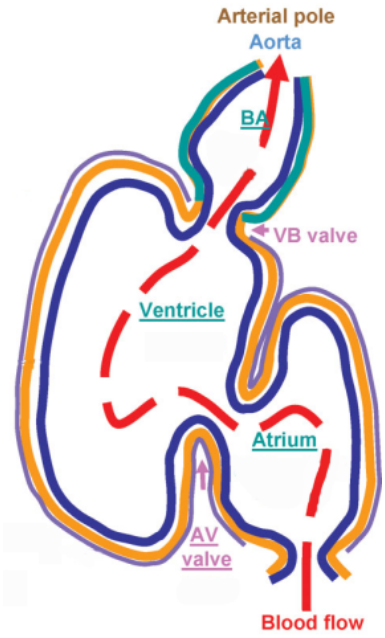


Figure 1.1: Heart of Zebrafish

The role of the heart is universal: pump the blood and make it circulate through the body. A heart can keep working outside the body for a couple of hours if put in appropriate environment, which shows that it doesn't need an external command to beat. This automaticity is due to natural pacemaker and conduction system and is the thing we try to understand.

1.2 Heart's functioning

General functioning
The heart contracts and relaxes in rhythm according to the same cycle of events: it is called the heartbeat. The cardiac cycle consists in two phases: the diastole phase and the systole phase.

1. In the diastole phase, the whole heart is relaxed. The ventricle is expanding and filling with blood. The AV valve is open, the sinoatrial (SA) node, which starts cardiac conduction, contracts causing atrial contraction, the atria empty blood into the ventricles. The semilunar valve is closed preventing back flow into the atria.

2. In the systole phase, the AV valve is closed and the semilunar valve is open, the ventricle contracts and pump blood into the arteries.

The sounds heard when listening to a heart beating is due to the contraction of the ventricles and the closing valves.
The automaticity comes from the SA node: the electric signal that makes the atrium contract is initiated in this part of the heart.

Conduction system
In order to coordinate all the different parts of the heart, the individual myocardial fibers must contract and relax in a rhythmic way. This the role of the conduction system: the conduction system of the heart consists of cardiac muscle cells and conducting fibers that are specialized for initiating impulses and conducting them rapidly through the heart. The electrical impulse is initiated in the leading pacemaker site in the sino-atrial node, propagated rapidly through the differentiated myocardial cells of the atria, after which the atria contract synchronously to pump the blood through the AV canal into the ventricle. Due to slow conduction in the AV canal, the electrical impulse is delayed to allow complete filling of the ventricle. During subsequent activation and contraction of the ventricle, the AV canal myocardium remains contracted as a result of slow relaxation, thus functioning as a sphincter valve. After ventricular contraction, the outflow tract contracts and relaxes slowly, preventing back flow of blood into the ventricle.

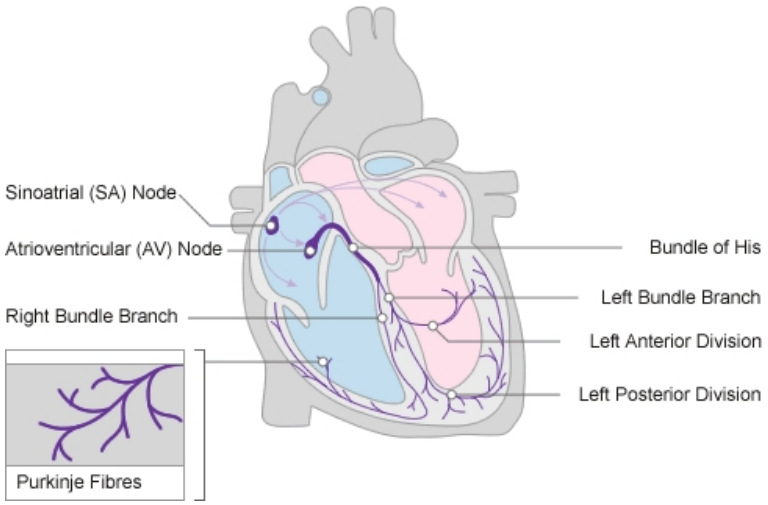


Figure 1.2: Cardiac conduction system

FGF13 and sodium channels
Cardiac muscle cells (and all excitable cells) depend on channels such as Na^+ and K^+ to regulate their bioelectric membrane potential to initiate, regulate and maintenance of cardiac cycle. The picture is a very complicated interplay between various charged ions (Na^+, K^+, Ca^+, Cl^-), each has their own set of ion-specific channels express at specific cell types for performing specific function. We are focusing on $Na_v1.5$, which is the most common sodium channel in the myocardium and encoded by the gene Scn5a. Specific FGF13 isoforms bind to $Na_v1.5$ and modulate its function and membrane expression. As a result, it changes the cardiac conduction properties. As shown in [4], FGF13 knockdown is associated with abnormal cardiac conduction properties because of reduces Na^+ channel current density and delayed recovery from inactivation.
Not all the heart is concerned by $Na_v1.5$:

1. **Atrium**: contains the pacemaker, expresses Fgf13
2. **Ventricle**: no pacemaker, expressing Fgf13, interacting with Scn5a aka Nav1.5 and junctophilin-2
3. **Bulbus Arteriosus**: no pacemaker, not expressing Fgf13, used as negative control for our experiments

1.3 Mission

The objective is to study the functioning of FGF13 protein by finding its binding partners.
Our method is articulated along four big parts: DNA cloning of the gene FGF13, protein expression, heart extraction and protein pulldown. It will be explained in further details in the resuls part of this report.

Part III

Results

Chapter 1

DNA cloning

The aim of the following experiments are to obtain a specific gene from embryos' DNA. The target gene is fgf13a. It will be then expressed in protein.
First, RNA is isolated from embryos and the specific gene is transcripted in cDNA. Then it is amplified to get millions of copies of fgf13a DNA molecules. After an A-tailing, the gene is inserted in a vector and transformed in *E. coli* competent cells. Finally, the target gene is released by digestion.

1.1 Isolation of RNA from Zebrafish's embryos

The first step in cloning a gene is to isolate the DNA from the organism that contains the desired gene. The method is described in annexe A.4 (page 45).
5 dpf-embryos were used since the embryonic heart is more or less matured at that stage and SA-node is functional.
RNA is a very strong molecule, that's why it resists to pipeting up and down through the needle, whose size is really important: too big would not be efficient enough and too small would smash everything.
At the end of the manipulation, we have enough RNA to proceed to the following experiments.

1.2 cDNA

RNA is very sensitive to ribonucleases which destroy it, that's why manipulations of RNA can be tough. To avoid this problem, we need to make a copy of the sequence into DNA which is more stable, so that it can be amplified and purified. Complementary DNA is a doublestranded DNA version synthetized from mRNA template.

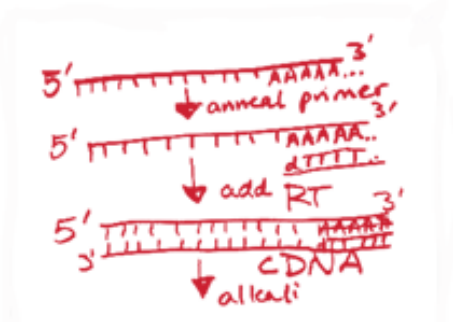


Figure 1.1: Functioning of the reverse transcriptase

Total RNA was extracted from target embryos and reverse transcribed using reverse transcriptase with oligo(dT) primer which targets matured mRNA with poly(A)tail. Oligo(dT) is a short sequence of deoxythymine nucleotides. mRNA has a long sequence of A nucleotides at the end, which can therefore be used as a primer site for reverse transcription. Oligo(dT) binds to the poly(A)tail making possible the extension by reverse transcriptase to create a complementary DNA strand.

Then the mRNA is removed by a RNAase enzyme, leaving the single stranded cDNA alone, which is converted into a double stranded DNA by a DNA polymerase.

Protocol used was from Invitrogen Superscript III kit, the method is described in annexe A.5 (page 47)

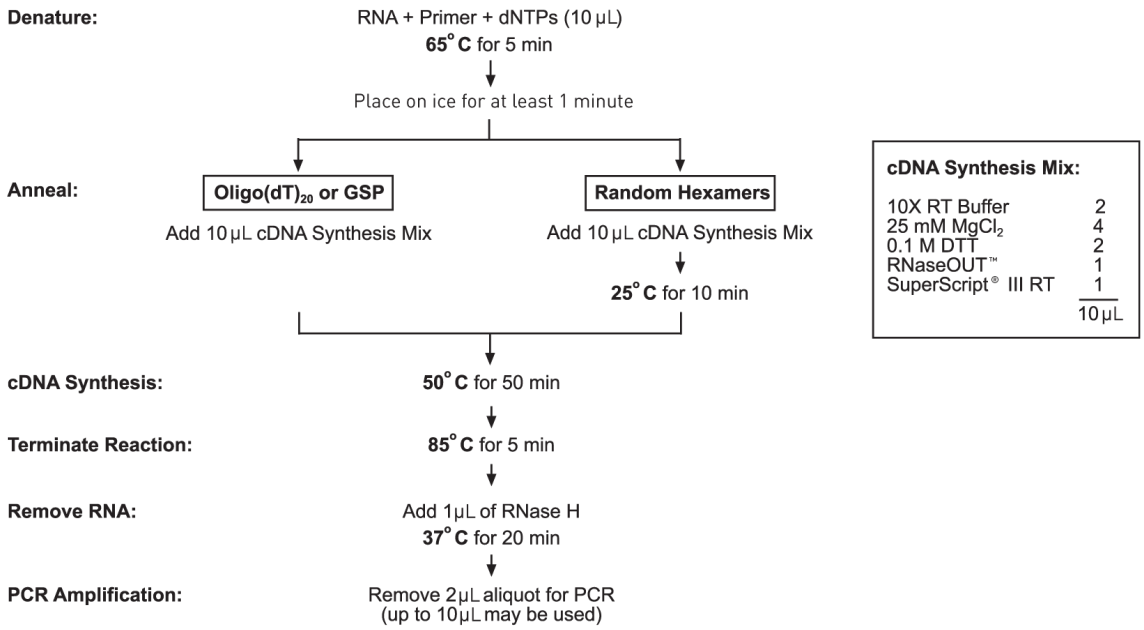


Figure 1.2: Summary of procedure

With this cDNA, we can perform rescue experiments by using it as template for synthesis of sense fgf13a mRNA and inject together with knockdown morpholino of fgf13a and see if by replacing normal copies fgf13a mRNA, it may rescue the animal from knockdown phenotypes that was described above. That way we can know for sure that fgf13a is indeed required for cardiac functions. This is a form of control for

morpholino-dependent knockdown. Morpholino sometimes have non-specific effects. Also, the cDNA can be used for in situ hybridization staining of fgf13a mRNA in the embryo to understand its expression pattern.

1.3 KOD-PCR amplification and purification

1.3.1 PCR amplification

From a small amount of cDNA, we would like to duplicate the DNA sequence *in vitro* in order to obtain a large number of copies. PCR amplification has a multiplication factor of around one million in a couple of hours, which makes it very effective.
The reaction takes place in a PCR tube that is placed in a programmable PCR machine. You can choose the succession of time and temperature. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA.
The basic list of the components for a PCR amplification includes :

- ✓ cDNA template
- ✓ Forward and reverse primers which are specific to the DNA target. We try with two different forward primers to check which one is the most suitable. The primers are UTR-F and F1 for the forward primer and R1 for the reverse primer.
- ✓ KOD DNA Polymerase, which enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides.
- ✓ dNTPs which are the building-blocks from which the DNA polymerase synthesizes a new DNA strand.
- ✓ Buffer solution which provides a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- ✓ Water to top up to a sufficient volume (here it is 50 µL).

The reaction is divided in 4 steps (the temperatures and times used to described the reaction are the ones listed in annexe A.6.1 (page 49)):

- **Initialization**: the reaction is heating to a temperature of 95 °C for 2 min. This step is required by the DNA Polymerase which needs a heat activation (it is a hot-start polymerase). It also allows to separate the two strands of DNA, to break the secondary structures, to homogenize the reaction medium by thermic agitation and to denaturate other enzymes that could be in the solution, like Inverse Transcriptase.
- **Denaturation**: It consists of heating the reaction to 95 °C for 20 s. At this temperature, weak bonds, especially the hydrogen bonds between complementary bases that ensure the cohesion of the doublestrand of DNA, are disrupted, yielding single-stranded DNA molecules.

- **Annealing**: The reaction temperature is lowered to 55-57 °C for 10 s. The temperature is a compromise between several parameters and it is calculating accordingly to the length and sequence of the target gene. Typically, it is about 3-5 °C below the Tm of the primers used. As the PCR machine can heat different tubes at different temperature at the same time, we test 55, 56 and 57 °C. As a result, the optimal temperature is something between 56 and 57 °C. At the end of the PCR amplification, we combine these two tubes for the purification. This step allows to form DNA-DNA hydrogen bonds between the cDNA template and the primers, which are much shorter and in higher concentration in the reaction sodium.

- **Elongation**: Once the primers are fixed to the gene, the temperature rises to 70 °C for 30 s. The temperature depends on the DNA Polymerase (for example, Taq Polymerase has its optimum activity at 75 °C). At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template. The primers serve as departure point for the DNA Polymerase. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified (order of magnitude: polymerisation of one thousand bases per minute). The polymerisation doesn't stop when you want, as a result the copies are longer that the original gene.

- **Cycles**: These last three steps are repeated 24 times. The number of cycles can be increased if you need more copies. The amount of DNA is doubled at each cycle, leading to exponential (geometric) amplification of the specific DNA fragment.

- **Final hold**: Cool down at 4 °C for an indefinite time for short-term storage.

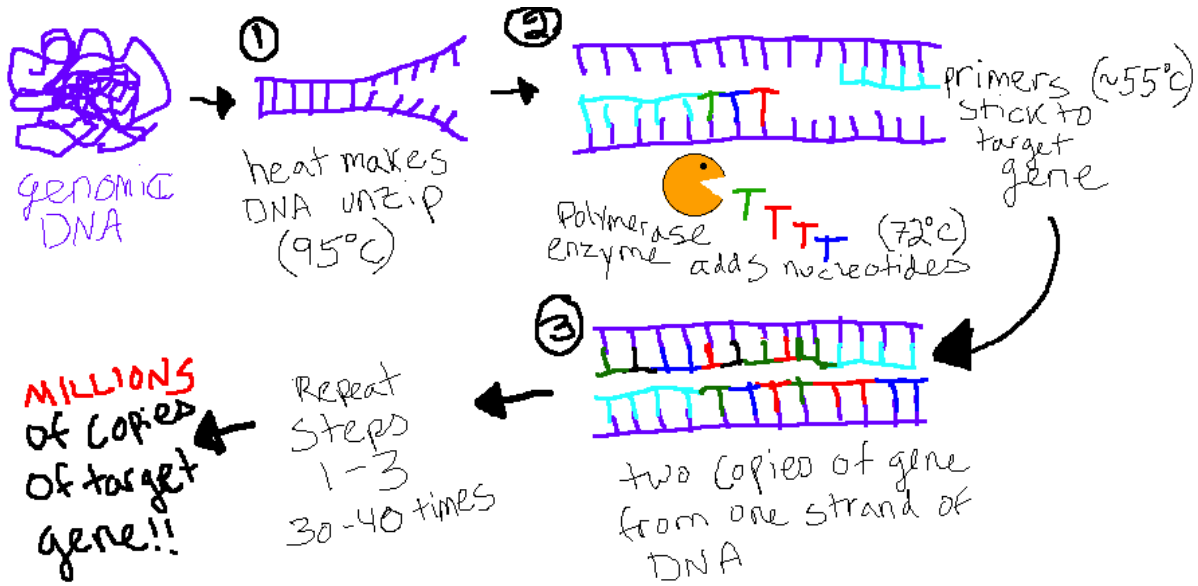


Figure 1.3: Steps of PCR amplification

1.3.2 PCR purification

To purify the product after the PCR amplification, we use the QIAquick PCR Purification Kit and the method described in annexe A.6.2 (page 49). After a PCR reaction, we need to eliminate the remaining dNTP, primers and other components using ethanol while the wash buffer providing in the Qiagen Kit contains high salt which helps DNA bind to the column. The elution buffer contains low salt, which somehow elutes the DNA from the column.

Figure 1.4: Steps of PCR purification

1.4 A-tailing reaction

A-tailing is required in this case since we used KOD DNA polymerase for the amplification which produces blunt-ended PCR product. Unlike Taq Polymerase, it doesn't automatically add an "A" to the end of PCR product. And since we want to clone this product into a vector which is meant for TA-cloning (i.e. pGEM-T easy vector), an additional step for adding "A" to the blunt-end product is necessary for ligation to work. Thus the PCR fragment is tailed by incubating it with dATP and Taq Polymerase as described in annexe A.7 (page 50). This reaction adds an A-tail (AAAAAAAAAAA...) to the 3' end of the gene.

1.5 Ligation reaction with pGEM-T easy vector

A cloning vector is an agent (a DNA molecule) that can carry a DNA fragment from a foreign organism into a host cell. It then replicates itself inside the bacterial cells and produces many copies of itself and the foreign DNA. Therefore, the vector contains one or several cloning sites that make really convenient to insert or remove a DNA fragment in or out of the vector, for example with a restriction enzyme, which cuts the vector and allows the insertion of the foreign DNA by ligation. The most versatile vectors contain a cloning site that can be cut by many different restriction enzymes.
There several types of cloning vector. The most commonly used is the genetically engineered plasmids. A plasmid is an extrachromosomal doublestranded circular DNA molecule that autonomously replicates inside the bacterial cell (the cloning limit is from 100 to 10,000 base pairs or 0.1-10 kilobases).

The one we use is the pGEM-T easy vector. It is a linearized vector, where 3'-end T has been chemically added to the blunt end digested vector. This additional 'T' greatly improves the efficiency of the ligation of PCR products by preventing the recircularization and so the self-ligation of the vector.
The protocol is described in annexe A.8 (page 50). After an overnight incubation at 4 °C, we proceed to the transformation.

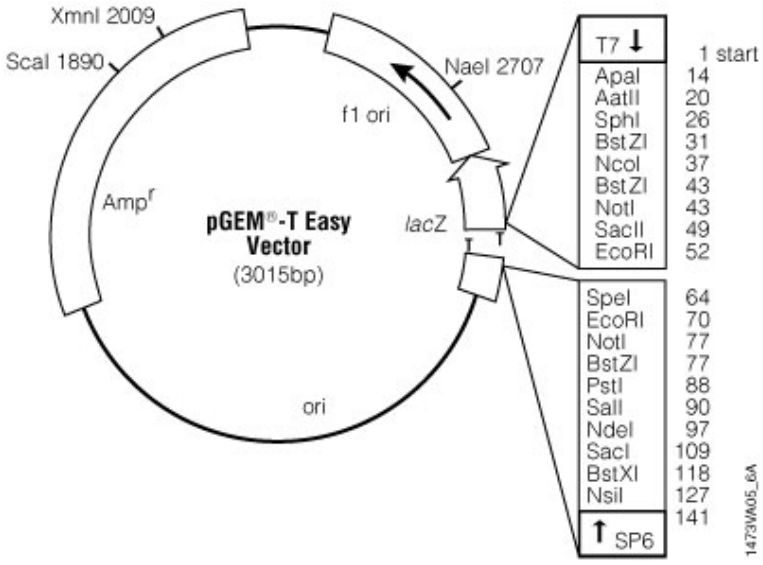


Figure 1.5: Map of pGEM-T Easy vector

1.6 Transformation to *E. coli* competent cells

This step consists in putting the newly constructed plasmid into bacterial cells. The method used is named the heat shock method and is described in annexe A.9 (page 51). Transformation is the process that occurs when a cell ingests foreign DNA from its surroundings. This phenomena can occur in nature in certain types of bacteria. Here, it is artificially reproduced via the creation of pores in bacterial cell membranes. Bacterial cells that are able to take up DNA from the environment are called competent cells. We use One Shot Mach1 T1 Phage-Resistant Chemically Competent *E. coli*. [3]

Principle of the heat shock method
Heat shock transformation uses an environment that provides a lot of divalent cations, such as $CaCl_2$ or $MgCl_2$. These cations prepare the cell walls to become permeable to plasmid DNA by counteracting the electrostatic repulsion between the plasmid DNA and the bacterial cellular membrane.
Then, a sudden increase of temperature (42 °C for 45 s) creates pores in the membrane of the bacteria and allows plasmid DNA to enter the bacterial cell.

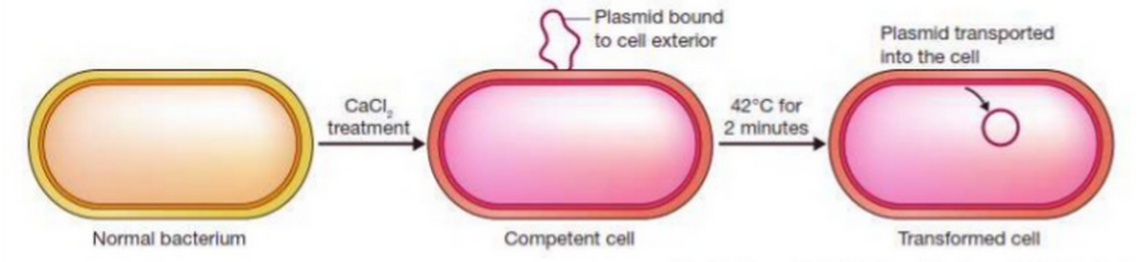


Figure 1.6: Heat shock method

Bacterial growth

Under favorable conditions, growing bacterial population doubles at regular intervals: this is called exponential growth and is not representative of the normal pattern of growth of bacteria in nature. The plate is incubated at 37 °C as suggested in [1]. The growth of cells in culture can be modeled with 4 different phases as shown in the curve below:

- **Lag phase**: Immediately after innoculation of the cells into fresh medium, the population remains temporarily unchanged. Actually, cells are growing in volume and in mass but no cell division is observed. During lag phase, bacteria adapt themselves to the new environment. The length of the lag phase directly depends on the previous growth condition of the organism: indeed if the bacteria which were growing in a rich medium, are inoculated into nutritionally poor medium, it will take them more time to adjust to the new environment because they will have to synthesise the necessary proteins, co-enzymes and vitamins needed for their growth. Conversely, an organism, which was growing in a poor medium and put in a rich medium, will adapt very quickly. It can sometimes start the cell division without any delay, and therefore will have a shorter lag phase.

- **Exponential or Log phase**: During this phase, the bacteria are rapidly growing and dividing regularly by binary fission at a constant rate: it is a balanced growth. The growth medium is exploited at its maximal rate. The number of bacteria increases exponentially (it has a geometric progression): a single cell divides into two, which replicate into four, eight, sixteen, thirty two and so on.

- **Stationary phase**: When the cells in cultures occupy all the available substrate and have no room left for expansion or when they exceed the capacity of the medium to support further growth, cell proliferation is greatly reduced or ceases entirely. Stationary phase results from a situation in which growth rate and death rate are equal. Finally bacteria stopa their division completely.

- **Death phase**: If incubation continues after the population reaches stationary phase, a death phase follows, in which the viable cell population declines because of depletion of nutrients and the subsequent accumulation of metabolic waste products and other toxic materials in the media. Cells die and the number of dead cells exceeds the number of live cells.

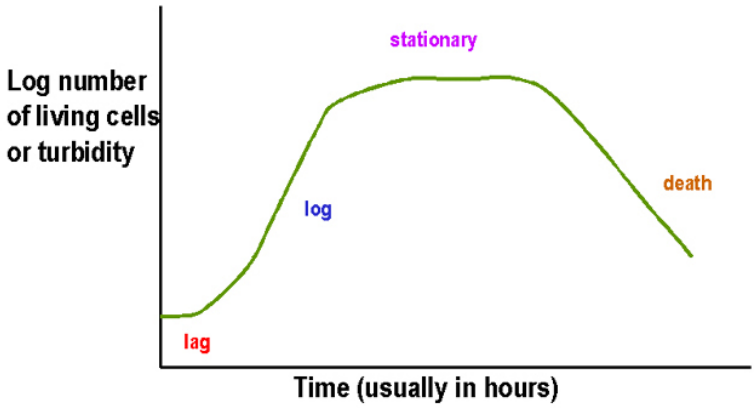


Figure 1.7: Growth curve

We want to pick up colonies right after the beginning of the stationnary phase, or if not possible during the stationnary phase. We are using One Shot Mach1 T1 Phage-Resistant Chemically Competent *E. coli* which are the fastest growing chemically competent cells currently available. Colonies are clearly visible within only 8 h of plating the transformation mix.

Blue/white screening

Since the transformation efficiency of plasmids in *E. coli* is very low, most *E. coli* cells that proliferate in the medium would not contain the plasmids. Therefore, one must find a way for transformed *E. coli* to be distinguished from the ones that don't have the insert.

The blue/white screening is the fastest and most convenient method to be sure to pick up colonies which has the insert: cells transformed with vectors that contain the recombinant DNA will produce white colonies, whereas those which only contains the plasmid will be blue. The colonies are grown in presence of X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside), a colorless analog of lactose, which is used to test for the presence of an enzyme, the β-galactosidase when it is in its active state. The color diffrenciation relies on the principle of α-complementation of the β-galactosidase.

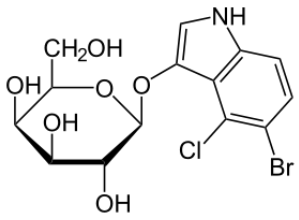


Figure 1.8: X-Gal

β-galactosidase is a protein encoded by the *lacZ* gene. In its active state, this protein is homotetramer. The bacteria *E. coli* carries a mutant β-galactosidase with its N-terminal residues 11—41 deleted (termed the ω-peptide). This mutant protein is named *lacZ*ΔM15 and is unable to form a tetramer, it is therefore inactive. However, in the presence of an N-terminal fragment of the protein, the α-peptide, the mutant protein may return fully to its active tetrameric state. This phenomena is called the α-complementation.

The bacteria *E. coli* carries the *lacZ* deletion mutant, while pGEM-T easy vector (alone) has the sequence that encodes the α-peptide, so when they are expressed together, they form a functional β-galactosidase enzyme. But when the ligation is successful, the α-complementation process is disrupted. Indeed, within the *lacZ* gene, there is a multiple

cloning site which is cut when the foreign DNA is inserting in the vector. As a result,, in cells containing the vector with an insert, no functional β-galactosidase may be formed. The presence of active β-galactosidase is then detected by X-gal within the agar plate. X-gal is cleaved by β-galactosidase to form 5-bromo-4-chloro-indoxyl, which then spontaneously dimerizes and oxidizes to form a bright blue insoluble pigment 5,5'-dibromo-4,4'-dichloro-indigo. Finally, the cells containing a functional β-galactosidase (so it means they contain a vector with an uninterrupted *lacZ*α, therefore no insert) are blue, whereas the ones with the insert (the ones we are looking for), are white because X-gal is not hydroloyzed.

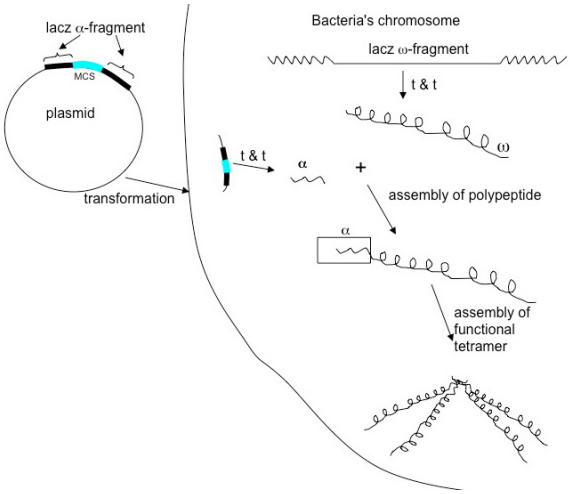


Figure 1.9: Transformation fo a mutant *E. coli* strain with a plasmid that doesn't have the insert

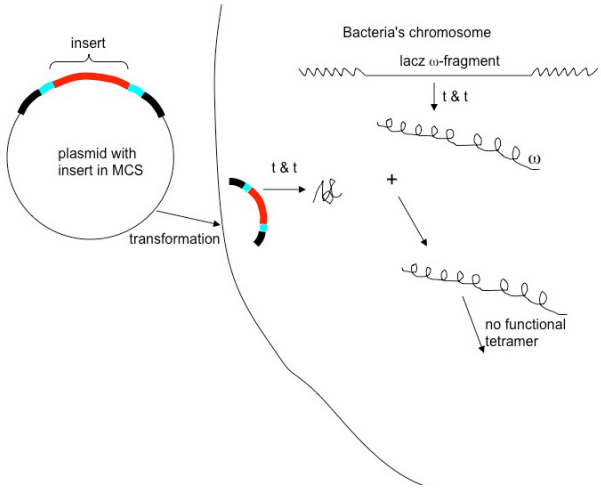


Figure 1.10: Transformation fo a mutant *E. coli* strain with a plasmid that carries the insert

1.7 MiniPrep of plasmid DNA

After the transformation, we would like to analyse the plasmid contained in white colonies, which are supposed to contain the correct insert. For that, the plasmid DNA has to isolated from the bacteria and then purified. Since, we at first just need a small amount of DNA for analysis, we proceed with a MiniPrep (to purify DNA after a transformation, several kits exist, they are named by size of bacterial culture and corresponding plasmid yield: in increasing order, these are the MiniPrep, MidiPrep, MaxiPrep, MegaPrep, and GigaPrep). After confirmation of the success of our experimentations, we will scale up to MidiPrep.

Overnight incubation
A well-separated colony (to make sure it won't be mixed with other bacteria around) is taken from the transformation plates and used to inoculate 1 mL of LB-Amp broth. For the MiniPrep procedure, bacteria should be in stationary phase so the tube is incubated with shaking overnight at 37 °C to obtain a saturated culture. If everything goes right, he solution becomes cloudy after a night of incubation.

Lysis of the bacteria
The kit used is AxyPrep Plasmid Miniprep Kit. All details can be found here: `http://catalog2.corning.com/Lifesciences/media/pdf/protocol_AP_MN_P.pdf`
The method is based on the lysis of the bacteria under alkaline conditions (pH=12-12.5) so that the bacteria can release the DNA.

Purification

Neutralization : An acetic acid solution brings back the pH to neutrale. DNA strands, which was denatured during lysis, can renature. The white pellet after centrifugation contains only the plasmid DNA, small fragments of chromosomal DNA, and RNA.

Wash : Two washes with alcohol are sometimes necessary. The first one uses isopropanol. This alcohol rapidly precipitates nucleic acids. However, if one wait too much before centrifugation, proteins will also precipitate. Thus it is important to time this step for a quick precipitaion before centrifugation. The second wash uses ethanol. An ethanol wash helps remove salts and any remaining buffer.

Eluent : Tri-HCl buffers the DNA solution.

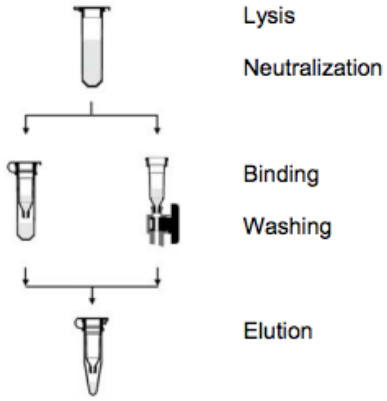


Figure 1.11: MiniPrep principle

1.8 Digestion on Miniprep plasmids

To release the target gene from the plasmid, we cut it with the restriction enzyme Not I as described in annexe A.11 (page 52). As seen on the map of pGEM-T easy vector, Not I attacks each side of the insert so no other restriction enzyme is needed here. Once the insert is free, we run a gel to check its size.

After the cutting process, we prepare the sample for sequencing, as described in annexe A.12 (page 53), to determine the exact sequence of the DNA.

1.9 Scale up to MidiPrep

Now that we are sure that we have to right insert, we can scale up to MidiPrep. MidiPrep is the same principle as MiniPrep but in a larger scale (annexe A.13 (page 53)). The yield of the MidiPrep is: $1,283.7$ mg/mL.

1.10 Digestion with Spe I

The clone needs to be first linearised by Spe I restriction enzyme. This restriction enzyme can recognize the specific nucleotide sequence A/CTAGT and cut at the location indicated by /. The DNA fragment can then be analysed or used for other reactions. After the reaction, an agarose gel is run to check the cutting. The uncut DNA will come out before the DNA fragment that has been cut by Spe I because it is heavier. As you can see on the following picture of the gel, some uncut DNA remains in the solution but with no consequences.

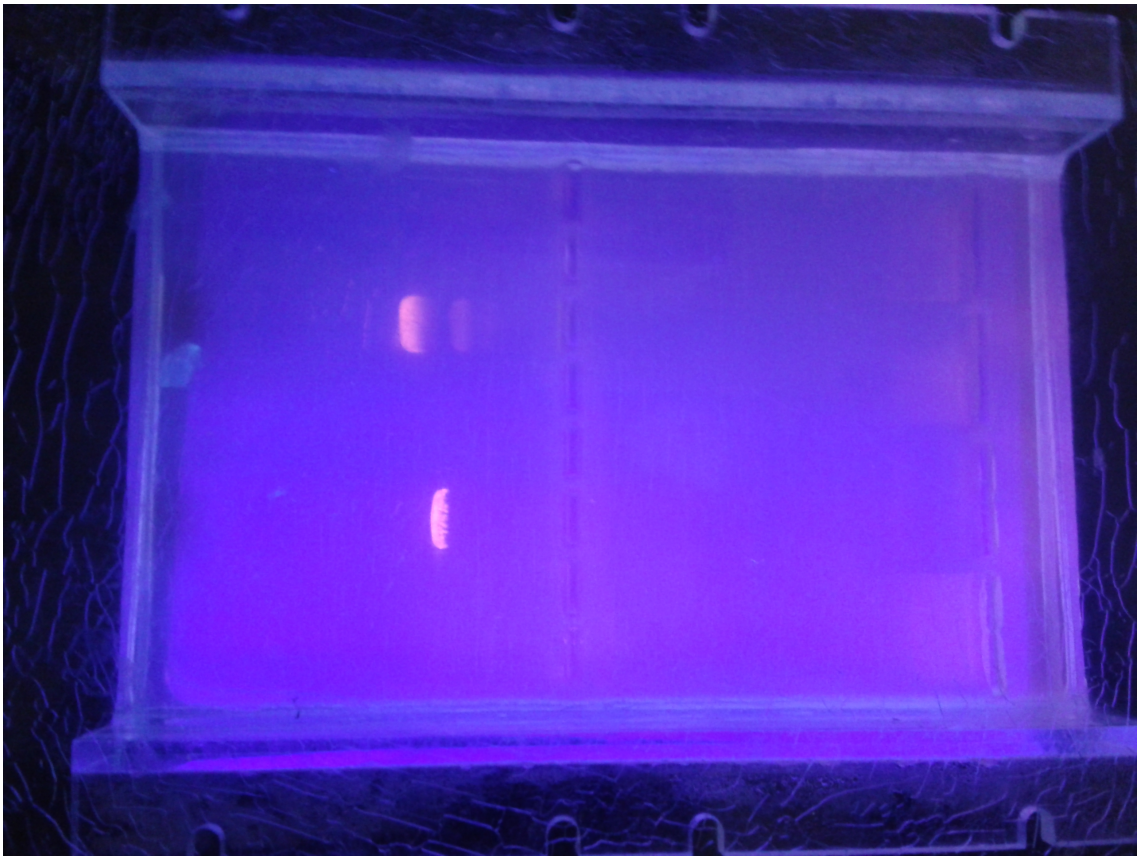


Figure 1.12: Gel to check Spe I cut

1.11 *In vitro* transcription reaction with DIG label

Transcription is the process to produce RNA from template DNA using gene promoter activation and something called "DNA-dependent RNA polymerase and *in vitro* transcription is just a way to replicate such reaction in test tubes as oppose inside nucleus of cell where they naturally take place. This can be easily achieved using commercially available kit (such as T7/SP6 Megascript by Ambion). The product of such reactions produces gene specific, labelled RNA which we usually use as probe to target specific mRNA by in situ hybridisation to produce gene expression pattern in fixed animal tissues, such as zebrafish embryos. Detectable epitopes like DIG can be integrated into the RNA via T7 transcription.

Chapter 2

Protein expression and purification

After the DNA has been transcripted into mRNA, the mRNA is then translated into polypeptide chains, which are ultimately folded into proteins. The isolation of a recombinant protein of interest from bacteria starts by the introduction of the gene into the host cell.
Various host cells can be used, but in this project, BL21 (DE3) competent *E. coli* cells have been used as the expression host, which easily produces a large amount of protein after IPTG induction.
Protocol is described in annexe A.16 (page 55).

BL21(DE3) *E. coli* are chemically competent *E. coli* cells suitable for transformation and routine protein expression. This strain expresses the T7 RNA polymerase (RNA polymerase enables transcription of the gene from DNA to RNA, RNA can then be translated to protein) upon addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) which induces a high-level protein expression from T7 promoter driven expression vectors. In other words, the *E.coli* cells have this RNA polymerase but it is in an inhibited state. You need the addition of the IPTG to de-repress this inhibition.

2.1 Protein expression

The gene of interest that has been cloned into a T7 expression vector is first transformed into the compentent cells.
The process of protein expression can start. It involves two main steps:

- **Cell growth**: The cells are grown in highly enriched medium, such as Luria Broth (LB), until there is enough material. During the initial phase of intensive biomass production, protein expression is limited to its minimum, due the toxicity of the protein to the cells.
- **IPTG induction**: BL21(DE3) *E. coli* strains harbor a genomic copy of the gene for T7 RNA polymerase under the control of the lac repressor. Under repressive conditions, ie when the phage T7 RNA polymerase gene inhibits the T7 RNA polymerase expression, T7 RNA polymerase is not produced, and transcription of the target gene is negligible. But when inducer is added, T7 RNA polymerase

is expressed and becomes dedicated to transcription of the gene of interest. Most of the cellular protein synthesis machinery will be devoted to producing the target protein.

It is advisable to induce protein expression at mid-to-late log phase of the growth curve to ensure maximal yield while avoiding the problems associated with cells going into stationary phase such as induction of proteases.

2.2 Lysis of cells

Lysozymes are added to bacterial cultures to enable lysis of the tough bacterial cell wall so that the expressed protein can be released from the bacterial cells. Then, the sonication can begin. Cells are lysed by liquid shear and cavitation. DNA is also sheared during sonication, so it is not necessary to add DNase to the cell suspension. The main problem is controlling the temperature. During the sonication, it is important to keep the sample as close to 4 °C as possible. A bucket of ice will keep the protein lysate very cold, allowing thorough sonication, with minimal protein degradation due to heat.

2.3 Purification by affinity chromatography (AC)

We use the HisTrap affinity columns (which contain Nickel) to purify our proteins because our proteins are His-tagged. Affinity chromatography principle relies on a reversible interaction between a protein and a specific ligand attached to a chromatographic matrix. The target protein(s) is specifically and reversibly bound by a complementary binding substance (in our case, the ligand is Nickel). The sample is applied under conditions that favor specific binding to the ligand. Unbound material is washed away, and the bound target protein is recovered by changing conditions to those favouring desorption. Elution is performed specifically, using a competitive ligand, or non specifically, by changing the pH, ionic strength or polarity. Here, we change to an elution buffer. Proteins are concentrated during binding and collected in a purified, concentrated form. The key stages in a separation are shown in Figure 5.

Chapter 3

Protein extraction

Our goal is to understand fgf13a's role by finding its the binding partners in the atrium and more specifically in the SA-node, which is known for being the natural pacemaker of the heart, that's why we proceed to a heart extraction and then protein extraction to obtain the lysate. Lysate is a general descriptive term for the protein extract we obtained from animal tissues. Biochemical procedure usually required to break (or lyse) the cells to release its protein and other biomolecule content in the cells for further analysis. In this case, they can be further clarified using other techniques to isolate specific proteins, depending on what the target. We are only interested in the atrium but we proceed with all three parts of the heart. The other tissues (ventricle and BA) are used as negative controls.

3.1 Heart extraction

The first step is to extract the heart from the fish and then separate the different parts of the heart: atrium, ventricle and bulbous arteriosus (BA).

3.1.1 Dissection of zebrafish

The method is inspired by the protocol in annexe A.1.1 (page 38).
The tricaine solution used to make the fish "sleep" before the dissection has to be concentrated enough to make the fish stop moving and breathing in a couple of minutes time. The fish has to be alive enough for its heart to keep beating, so that it is easy to identify, but sleepy enough to avoid any last kicks or egg laying while you are dissecting it. The nervous system of fishes is more developped than ours, that's why they still can move, even after their heart has been extracted.
Heart is a quite solidaire muscle but the atrium may sometimes be cut off accidentally during the extraction because it is not as solidly attached to the ventricle as the bulbous arteriosus. The pourcentage of lost atrium is estimated around 10%.
After a brief demonstration by my supervisor, my first fish was a catastrophy that lasted a good half an hour. But after a while of practicing, I was able to dissect a fish in less than 2 minutes without the microscope (average calculated with preparation and cleaning time).

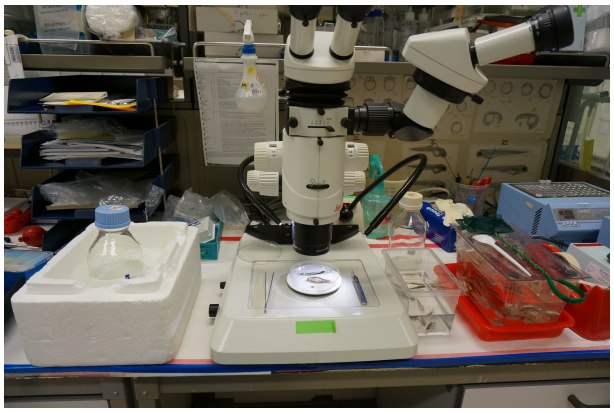


Figure 3.1: Work station

Figure 3.2: Heart extraction

3.1.2 Cut

This is the tricky part of the manipulation. The atrium is the smallest and softest part of the heart. It doesn't really have a determined shape, which makes the cutting tough when it is covered with blood. As we are interested in the atrium, we choose to cut slightly in the ventricle if the junction is too messy to be broken precisely.

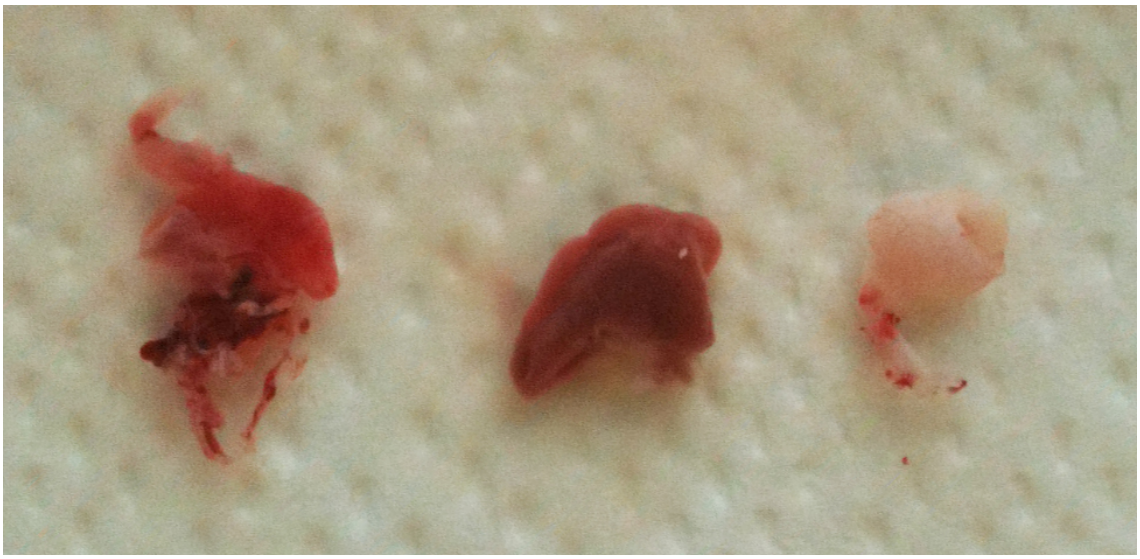


Figure 3.3: The 3 pieces of a carp's heart. From left to right: atrium; ventricle; BA

3.2 Total protein extraction

3.2.1 Extraction

To extract the protein from the different parts of the heart, we use the total protein extraction kit Millipore Cat# 2140 with the protocol described in annexe A.2 (page 41).

The first choice we have to make is the selection of the buffer, according to the stability of the target protein with respect to pH and the bufferring compound and the following step to avoid time and protein loss caused by an additional buffer exchange step.
The Total Protein Extraction Kit is composed of two buffers: TM and 50X PI (which has to be diluted to 1X PI).

Buffer	**Component**
TM Buffer	HEPES (pH7.9), MgCl2, KCl, EDTA, Sucrose, Glycerol, Sodium deoxycholate, NP-40, Sodium OrthoVanadate
50X PI	A cocktail of protease inhibitors

The quantities of 1X PI used are shown in the table 5.1.

Table 3.1: Preparation of Diluted Albumin (BSA) Standards - In our case

	Weight of tissues (mg)	Amount of 1X PI Buffer (µL)
Atrium	22.4	56
Ventricle	69.6	174
BA	36.6	91.5

3.2.2 Weight

To determine the weight of protein we have extracted, we use the Thermo Scientific Pierce BCA Protein Assay Kit as described in annexe A.3 (page 42). This assay is based on the very sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) using a unique reagent containing bicinchoninic acid (BCA). This cation comes from the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium and the purple color is due to the chelation of two molecules of BCA with one cuprous ion, and more precisely it has been shown that the macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are responsible for color formation with BCA. The complex formed by Cu^{+} and BCA is water-soluble and exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000 µg/mL).
The BCA method is not a true end-point method; indeed the final color continues to develop. However, following incubation, the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed together. Better work fast though.
However, as the amount of protein is really limited, all the volumes are divided by 10: the volume of each standard is reduced to 30 µL instead of 300 µL (exact used volumes are in the table 4.1); for the working reagent, we take 2353 µL of reagent A and 47 µL of reagent B; for the mix, we take 10 µL of sample + 200 µL of working reagent. Since the purple color is really strong (much darker than the standards), the three samples are diluted until the color matches the one of the standards.

Table 3.2: Preparation of Diluted Albumin (BSA) Standards - In our case

Vial	Volume of Diluent (µL)	Volume and source of BSA (µL)	Final BSA concentration (µg/mL)
A	0	30.0	2000
B	7.5	22.5	1500
C	15.0	15.0	1000
D	18.75	11.25	750
E	22.5	7.5	500
F	26.25	3.75	250
G	28.125	1.875	125
H	29.625	0.375	25
I	30.0	0	0 = blank

3.3 Results and conclusion

Results As no one knows how much lysate there is in the atrium of an adult zebrafish's heart, we are completely blind about the number of fishes required for the following step, which is the pulldown, that is why we begin with the 50 biggest animals we have. They were all born in december 2011 and are really fat which makes the extraction a bit tricky because of all the fat envelopping the heart.
Here is the table showing the amount of lysates we extracted from each part of the 50 hearts.

Table 3.3: Results of weight of extracted protein

	Dilution	Absorbance	Concentration (mg/mL)	Volume (µL)	**Weight** (mg)
BA	3X	0.126	8.2	59	**0.4838**
Ventricle	6X	0.176	8.5	167	**1.419**
Atrium	6X	0.114	7.7	42.4	**0.3265**

Problem For the next (the pulldown), we need at least 5 mg of lysate contained in the atrium and each experiment requires a control.
After the total protein extraction process, only 300 µg of lysate are collected, which means about 1,500 zebrafishes have to be sacrificed in total. We face here one of the major limitations of the zebrafish as an experiment animal: its small size (compared to the mouse for example).
The dissection of 1,500 zebrafishes takes a lot of time. Moreover, as we only can use adult fishes that people don't need anymore, we are depending on the fishes available. At a pace of about 50 dissected fishes every morning to gather enough material for one single experiment, we need to find a shortcut. But in the meantime, we still keep dissecting as many zebrafishes as we can.

Solution To counter this problem of small scale, some other animals have been considered, like other similar species of fishes, chicken or pigs. Finally, our choice fell on

carps. To make sure we have enough material for a pulldown experiment, we combine atrium and ventricles.
After the protein extraction process, we have 7 mL with a concentration of 11.7 mg/mL, which makes 8.19 mg of lysate.

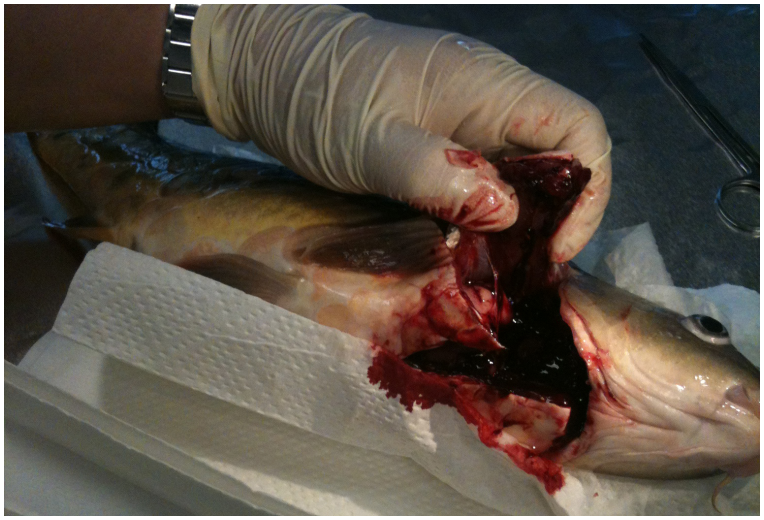


Figure 3.4: Heart extraction of a carp

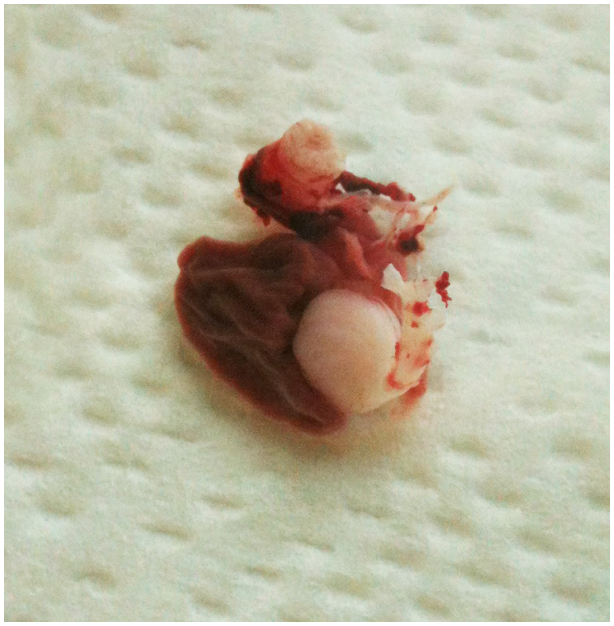


Figure 3.5: Heart extracted

The four carps we dissected are hybrids of Asian ordinary carps and European ordinary carps. They come from Temasek (a laboratory next to NUS). They measure between 30 and 40 cm and weight around 2 kg. Killing them with tricaine powder was really sad because they can't be eaten afterwards.
Carp's genome is not sequenced but, as a fish, we hope that its proteins' behaviour will be the same as the zebrafish.

Chapter 4

Protein pull-down

The pull-down assay is an in vitro method used to determine a physical interaction between two or more proteins. Pull-down assays are useful for both confirming the existence of a protein-protein interaction predicted by other research techniques and as an initial screening assay for identifying previously unknown protein-protein interactions. The principle is similar to immunoprecipitation except that the antibody function is replaced by an affinity system of Ni2+ beads, which can bind recombinant proteins that carry a His tag with good efficiency.
In this project, the pull-down assay is a small-scale affinity purification technique that enables the immobilization of histidine-tagged zebrafish FGF13 and the purification of binding partners within the carp heart lysate, which can then be analyzed and identified by mass spectrometry. Protein extraction from the heart of the carps resulted in approximately 80mg of total protein. For mass spectrometry (MS) analysis, at least 5mg of protein was required per experiment. Due to the good yield obtained, pulldowns of triplicates could be carried out so as to minimize errors or deviations. The pulldowns were carried out with protein baits of :

full-length zFGF13a (FL zFGF13)	x3
shorter deletion construct of zFGF13a (short zFGF13)	x3
with no bait at all (beads only) as a control	x3

Protocol is described in annexe A.17 (page 56).
The pull-down involves a four-day process :

- 1st day: The bait (either FL or short zFGF13) which is tagged with poly-His fused to its N-terminus is immobilized to the Ni_2^+ beads. After a round of washing of the beads, the carp heart lysate is added to the beads and the prey proteins are allowed to bind to the bait overnight with rotation at 4 °C.

- 2nd day: Unbound proteins are washed away and the beads are then incubated with benzonase overnight to remove background signals. Benzonase is used for viscosity reduction and removal of nucleic acids from protein preparations.

- 3rd day: After washing of the beads, the beads are incubated with Precision protease overnight to allow cleavage of the tagged-bait with the bound protein interactors from the beads.

- 4th day: A short spin allows separation of the beads from the supernatant. SDS loading dye is added to the supernatant and then sent to another lab facility that analyzes the data via a mass spectrophotometer. The process of analysis takes about 3-4 weeks by the facility.

The beads will be left in the microcentrifuge tube and can be disposed. But as a practice, we also add SDS loading dye to the beads and then run a gel to check that most of the bait has cleaved from the beads.

Unfortunately, we only managed to purify recombinant proteins and isolate the proteins from the carp at the very last minute, that's why we don't have the final results yet. We will get the results from the mass spectrophometer laboratory in at least three weeks, so we have nothing to conclude yet, but an expectation is that we will find some Fgf13 interactors that were identified previously (eg. Scn5a, etc). If this expectation will be confirmed, it means that the experiment was successful and we may analyze interactors other than Scn5a to study their role during formation and function of the slow CCS..

Part IV

Appendices

Appendix A

Protocols

A.1 Dissection of zebrafish heart

A.1.1 Adult heart

This protocol describes a quick and easy method of removing intact heart (bulbus arteriosus, atrium and ventricle) from an adult zebrafish.

Subject collection

1. Remove fish from habitat tank using net and place in 0.2% MS222 to anesthetize.
2. Place anesthetized fish in ice water for 15 min to euthanize.
3. Transfer fish to a 90 mm Petri dish for dissection.

Measuring fish

1. Measure single fish in petri dish, half-filled with fresh PBS, laying on its right side.
2. Measure length of fish from snout to base of tail (not including tail fins) with a digital calliper. Record this as standard length (SL).
3. Before dissection, label 1.5 mL microfuge tube with solution suitable for subsequent application. Fresh PBS works fine.
4. If you have fish with transgenic background, e.g. Tg(cmlc2:gfp), perform dissection under UV scope. Trim away non-fluorescent tissues.

The following protocol works best for adult zebrafish longer than 12 mm SL.

Dissection

1. Orientate fish left side face up in petri dish with fresh PBS.
2. Using a pair of sharp-ended forcep, flip open the gill cover and remove this gill cover for better view of body cavity (Fig 1; Step 1).
3. Using the pair of forceps, hold onto the cleithrum and snap break the cleithrum bone by applying some pressure on the bone itself (Fig 1; Step 2).
4. With another pair of sharp-ended forceps, clamp onto the left pectoral fin and pull open the fish belly to open up the body cavity (Fig 1; Step 3), exposing the silvery pericardium tissue (Fig 1. Red triangle).
5. Once remove this silvery tissue, the heart will be exposed. Flush away any blood from the body cavity with ice-cold PBS if necessary.
6. Cut the artery which connects the bulbous arteriosus to the gills, located superior to the heart. Once this artery is cut, put the forceps tip underneath the atrium, and scoop out the heart from the cavity using the forceps. Any extra tissues that tag along with the dissected heart can be trimmed away later.
7. Non cardiac tissues can be removed by holding the heart on one side with forceps, and trim away using needles and sharp-ended forceps. The epicardial lining (membrane with black pigmentation) can be removed from the outside of the heart if desired by gently scrapping the tissue with a needle.
8. Contain dissected heart in microfuge tube with fresh ice-cold PBS.
9. Remove as much PBS before snap-freezing the tissues with liquid nitrogen for storage.
10. Alternatively, proceed with standard TRIzol RNA extraction protocol without freezing.

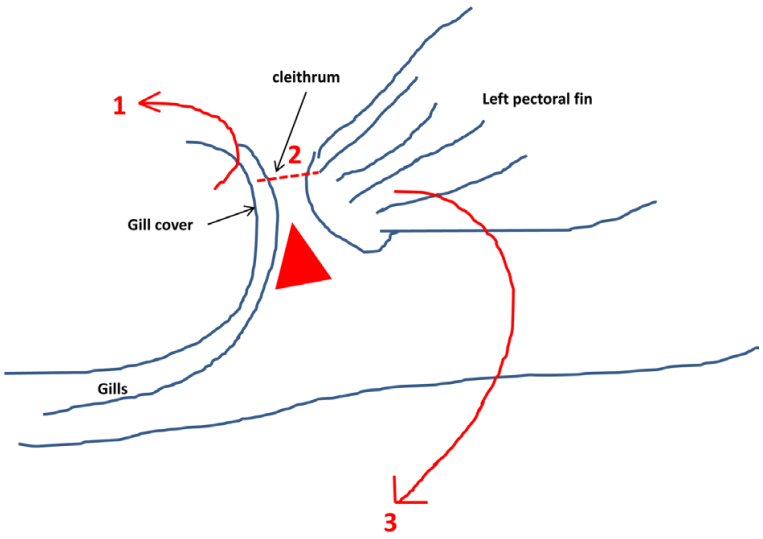


Figure A.1: Heart extraction from adult Zebrafish

A.1.2 Young embryos and larvae

Taken from "Comparative genomics identifies genes mediating cardiotoxicity in embryonic zebrafish heart, Chen et al., Physiol. Genomics (2007)33:148-158" & "Purification of hearts from zebrafish embryos, Burns and MacRae, BioTechniques (2006)40:274-282"

Materials

- Zebrafish with GFP-labelled hearts, e.g. Tg(cmlc2::GFP) line
- 0.02% Tricaine
- Embryo Disruption Medium (EDM): Lebovitz's L15 medium supplemented with 10% FBS 1.5 mLl microfuge tubes
- 18, 19 & 23-gauge syringe needles
- 100 µm sterile cell strainer (BD Falcon cat# 352360)
- 40 µm sterile cell strainer (BD Falcon cat# 352340)
- 30 mmPetri dishes
- Ice
- Dry ice
- Liquid Nitrogen
- Trizol Reagent (Ambion # 15596)

Method

1. Before heart extraction, anesthetise $\sim$ 200 embryos were with 0.02% Tricaine.
2. Transfer embryos in a microcentrifuge tube, and add 1 mL of EDM.
3. Position tip of needle to 100 µL mark of 1.5 mL microfuge tube.
4. Separate hearts of zebrafish embryos from the bodies by sheer force generated by drawing and repelling the embryos through a syringe needle. Repeat for 30 times at 1 s per syringe motion. (33 hpf: 23G; 48 – 96 hpf: 19G needle).
5. Apply fragmented embryos to 100 µm strainer and collect flow through in 30 mm Petri dish.
6. Add additional EDM to wash syringe, needle and nylon mesh.
7. Subsequently, apply the flow-through to a 40 µm nylon mesh (Step 7 to 9 is optional if there isn't.

8. Add additional EDM to rinse the Petri dish that collected the 100 µm flow-through and apply to the 40 µm mesh.

9. Invert the 40 µm mesh and wash off the retained material with EDM into a new 30 mm Petri dish.

10. Follow up by size fraction of the disrupted embryos with manual retrieval of the individual green fluorescent protein (GFP)-expressing hearts with a p20 pipette tip under epifluorescence. Keep preparation on ice.

11. Spin down the hearts (13000 rpm, 3 s) and remove supernatant.

12. Add 500 µL of Trizol (do this in fume hood) to the tube.

13. Snap freeze the heart suspension with liquid nitrogen and store at -80 °C for both RNA (Section 2) and Protein extraction (Section 3).

14. If you are working with multiple samples, you can temporary store the heart suspensions in dry ice and transfer to -80 °C altogether.

Note: Total RNA (1 µg/500 hearts at 72 hpf) can be extracted with Qiagen RNeasy mini kit. Every young larva heart is composed of $\sim$ 300 cells. Heart-RNA extraction is optimal at 72 hpf. Successful hybridization of Affymetrix zebrafish arrays requires RNA from $\sim$ 400 hearts.

A.2 Total protein extraction

Kit component

- 13 mL TM Buffer (protein buffer)
- 260 µL 50X PI Buffer (cocktail of protease inhibitors) (stored at -20 °C)

This kit contains enough reagents for the isolation of protein from 5 g of tissue or cultured cells. It is easy to use: no scraping, no freeze-thaw cycles and no sonication.

Method

Preparation

1. Aliquot 50X PI on ice and store at -20 °C.

2. Prepare 1 mL 1X PI Buffer/aliquot 20 µL 50X PI + 980 µL TM Buffer.

Always make fresh working solutions before isolation of proteins.

Protocol

1. Dilute 50X PI solution to 1X PI in TM Buffer keeping the solution on ice.
2. Weigh certain tissues and chope them into small pieces.
3. Keep the tissues on dry ice.
4. Add 1X Pi to the tissue at 2.5 mL/g of tissue or per 25 millions cells and put in ice for 5 min.
5. Homogenize the tissue or cells for 20 s and then put on dry ice for 15 s.
6. Homogenize the tissue or cells for the second 20 s. (A third 20 s homogenization may be required if the tissue or cells are not well homogenized.)
7. Rotate the homogenized tissue or cells at 4 °C for20 min.
8. Centrifuge at 11,000 rpm at 4 °C for 20 min.
9. Collect the supernatant.
10. Determine the concentration of the total protein. Bradford Assay may be used.

A.3 Determine the concentration of protein with Pierce BCA Protein Assay Kit

The BCA method is not a true end-point method, ie the final color continues to develop (better work fast).

A.3.1 Preparation of standards and working reagent

Preparation of Diluted Albumin (BSA) Standards
Use Table 1.1 as a guide to prepare a set of protein standards. Dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably using the same diluent as the sample(s). Each 1 mL ampule of 2 mg/mL Albumin Standard is sufficient to prepare a set of diluted standards for either working range suggested in Table 1.1.

Table A.1: Preparation of Diluted Albumin (BSA) Standards - Dilution scheme for standard test tube protocol and microplate procedure (working range = 20 - 2000 µg/mL

Vial	Volume of Diluent (µL)	Volume and source of BSA (µL)	Final BSA concentration (µg/mL)
A	0	300 out of stock	2000
B	125	375 out of stock	1500
C	325	325 out of stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = blank

Table A.2: Preparation of Diluted Albumin (BSA) Standards - Dilution sscheme for enhanced test tube protocol (working range = 5 - 250 µg/mL

Vial	Volume of Diluent (µL)	Volume and source of BSA (µL)	Final BSA concentration (µg/mL)
A	700	100 out of stock	250
B	400	400 of vial A dilution	125
C	450	300 of vial B dilution	50
D	400	400 of vial C dilution	25
E	400	100 of vial D dilution	5
F	400	0	0 = blank

Preparation of the BCA Working Reagent (WR)

1. Use following formula to determine the total volume of WR required :
   $(\#standards + \#unknowns) \times (\#replicates) \times (volume of WR per sample) = total volume WR requir$
   Example: for the standard test-tube procedure with 3 unknowns and 2 replicates of each sample:
   $(9 standards + 3 unknowns) \times (2 replicates) \times (2mL) = 48mL WR required$
   Note: 2 mL of the WR is required for each sample in the test-tube procedure, while only 200 µL of WR reagent is required for each sample in the microplate procedure.

2. Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). For the above example, combine 50 mL of Reagent A with 1 mL of Reagent B.
   Note: When Reagent B is first added to Reagent A, turbidity is observed that quickly disappears upon mixing to yield a clear, green WR. Prepare sufficient volume of WR base on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).

A.3.2 Test-tube procedure (sample to WR ratio = 1:20)

1. Pipette 0.1 mL of each standard and unknown sample replicate into an appropriately labeled test tube.

2. Add 2 mL of the WR to each tube and mix well.

3. Cover and incubate tubes at selected temperature and time:

   - Standard protocol: 37 °C for 30 min (working range = 20 - 2000 µg/mL)
   - RT protocol: RT for 2 h (working range = 20 - 2000 µg/mL)
   - Enhanced protocol: 60 °C for 30 min (working range = 5 - 250 µg/mL

   Notes:

   - Increasing the incubation time or temperature increases the net 562 nm absorbance for each test and decreases both the minimum detection level of the reagent and the working range of the protocol.
   - Use a water bath to heat tubes for either Standard (37 °C incubation) or Enhanced (60 °C incubation) protocol. Using a forced-air incubator can introduce significant error in color development because of uneven heat transfer.

4. Cool all tubes to RT.

5. With the spectrophotometer set to 562 nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples within 10 min.
   Note: Because the BCA assay does not reach a true end point, color development will continue even after cooling to RT. However because the rate of color development is low at RT, no significant error will be introduced if the 562 nm absorbance measurements of all tubes are made within 10 min of each other.

6. Substract the average 562 nm absorbance measurement of the Blank standard replicates from the 562 nm absorbance of all other individual standard and unknown sample replicates.

7. Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

A.3.3 Microplate procedure (sample to WR ratio = 1:8)

1. Pipettel 25 µL of each standard or unknown sample replicate into a microplate well (working range = 20 - 2000 µg/mL).
   Note: If sample size is limited, 10 µL of each unknown sample and standard can be used (sample to WR ratio = 1:20). However, the working range of the assay in this case will be limited to 125 - 2000 µg/mL.

2. Add 200 µL of the WR to each well and mix plate thoroughly on a plate shaker for 30 s.

3. Cover plate and incubate at 37 °C for 30 min.

4. Cool plate to RT. Measure the absorbance at or near 562 nm on a plate reader.
Notes:

   - Wavelengths from 540 - 590 nm have been used successfully with this method.
   - Because plate readers use a shorter light path length than cuvette spectrophotometers, the Microplate Procedure requires a greater sample to WR ratio to obtain the same sensitivity as the standard Test Tube Procedure. If higher 562 nm measurements are desired, increase the incubation time to 2 h.
   - Increasing the incubation time or ratio of sample volume to WR increases the net 562 nm measurements for each well and lowers both the minimum detection level of the reagent and the working range of the assay. As long as all standards and unknowns are treated identically, such modifications may be useful.

5. Substract the average 562 nm absorbance measurement of the Blank standard replicates from the 562 nm measurements of all other individual standard and unknown sample replicates.

6. Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.
Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a pure linear fit. If plotting results by hand, a point-to-point curve is preferable to linear fit to the standard points.

A.4 RNA extraction from Zebrafish's embryos by trizol

A.4.1 Phase separation

1. Add about 30 dechonrionated larvae (5dpf) into 1.5 mL tube. Remove egg water and rinse 2 times with PBS. Remove all PBS and add 500 µL Trizol.

2. Homogenize tissue samples by passing through a 23G needle for 5-7 times.

3. Incubate homogenized samples for 5 min at room temperature (to allow dissociation of nucleoprotein complex)

4. Add 100 µL of chloroform to samples. Cap the tube securely.

5. Shake tubes vigorously by hands for 30 s.

6. Incubate for 3 min at room temperature.

7. Centrifuge sample at 10000 rpm for 15 min at 4 °C.
   Note:mixture separates into a lower red phenol-chloroform phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase is approximately 50% of the total volume.

8. Remove the aqueous phase of the sample (∼350 µL) by angling the tube at 45° and pipetting the solution out.
   Note: avoid drawing out any of the interphase or organic layer into the pipette when removing aqueous phase.

9. Place aqueous phase into a new tube (RNase-freetube).

10. Remove the interphase and save the organic phenol-chloroform phase for protein extraction.

11. If necessary, store the organic phase in -80 °C.

A.4.2 RNA isolation

1. Add 0.8 volume of 100% isopropanol to the aqueous phase.

2. Incubate at room temperature for 10 min.

3. Centrifuge at 10000 rpm for 10 min at 4 °C.
   Note: RNA is often invisible prior to centrifuge and forms a gel-like pellet on the side and bottom of tube.

4. Remove supernatant, leaving only the pellet.

5. Wash the pellet with 750 µL of 75%ethanol (RNase-free).
   Note: RNA can be stored in this state at least 1 year at -80 °C.

6. Vortex sample briefly, then centrifuge the tube at 13000 rpm for 10 min at 4 °C. Discard supernatant.

7. Air dry the RNA pellet for 5 min. Do not allow RNA to dry completely, because pellet will lose its solubility.

8. . Resuspend RNA pellet in 30 µL RNase-free water by passing the solution up and down several times through a pipette tip.

9. Incubate in water bath or heat block set at 55-60 °C for 10 min.

A.5 Create gene specific cDNA from extracted total RNA

Official manual can be found here: `http://tools.invitrogen.com/content/sfs/manuals/superscriptIIIfirststrand_pps.pdf`.
The following procedure is designed to convert 1 pg–5 µg of total RNA or 1 pg–500 ng of poly(A) + RNA into first-strand cDNA.

1. Mix and briefly centrifuge each component before use.

2. Combine the following in a 0.2- or 0.5- mL tube:

Component	Amount
up to 5 µg total RNA	n µL
Primer $*$50 μM oligo(dT)20, *or* 2 μM gene-specific primer (GSP), *or* 50 ng/µL random hexamers	1 µL
10 mM dNTP mix	1 µL
DEPC-treated water	to 10 µL

3. Incubate the tube at 65 °C for 5 min, then place on ice for at least 1 min.

4. Prepare the following cDNA Synthesis Mix, adding each component in the indicated order.

Component	1 Reaction	10 Reactions
10X RT buffer	2 µL	20 µL
25 mM MgCl2	4 µL	40 µL
0.1 M DTT	2 µL	20 µL
RNase OUT (40 U/ µL)	1 µL	10 µL
SuperScript III RT (200 U/ µL)	1 µL	10 µL

5. Add 10 µL of cDNA Synthesis Mix to each RNA/primer mixture, mix gently, and collect by brief centrifugation. Incubate as follows.

   Oligo(dT) 20 or GSP primed: 50 min at 50 °C
   Random hexamer primed: 10 min at 25 °C, followed by 50 min at 50 °C

6. Terminate the reactions at 85 °C for 5 min. Chill on ice.

7. Collect the reactions by brief centrifugation. Add 1 µL of RNase H to each tube and incubate the tubes for 20 min at 37 °C.

8. cDNA synthesis reaction can be stored at -30 °C to -10 °C or used for PCR immediately.

A.5.1 One step RT-PCR kit (Qiagen)

Mix

- 5X Rxn buffer: 10 µL.
- dNTP: 2.2 µL
- Forward primer: 3.0 µL
- Reverse primer: 3.0 µL
- RNase inhibitor: 2.0 µL
- Total RNA (Trizolextracted): 2.0 µL
- Enzyme mix: 2.0 µL
- Rnase-freewater: up to 50 µL

Final volume: 50 µL

RT-PCR profile

1. 50 °C, 32 min
2. 95 °C, 15 min
3. 95 °C, 30 s
4. 50 °C, 30 s
5. 72 °C, 1 min
6. 95 °C, 30 s
7. 55 °C, 30 s
8. 72 °C,1 min
9. Go to 6, repeat 34 times
10. 72 °C, 5 min
11. 4 °C, forever

A.5.2 Gel electrophoresis to check the DNA

Mix

- 5 µL DNA template
- 1 µL Loading dye

Run
Run gel at 120 V for 30 min.

A.6 PCR Amplification by high fidelity KOD Hot-start DNA polymerase (Novagen)

A.6.1 PCR Amplification

Mix

- Template cDNA: 2 µL
- Forward primer (10uM): 1.5 µL
- Reverse primer (10uM): 1.5 µL
- DMSO: 2.5 µL
- KOD 2X Mastermix: 25 µL
- PCR grade water: 17.5 µL

Final volume: 50 µL

KOD-PCR profile

1. 95 °C, 2 min
2. 95 °C, 20 s
3. 55 °C, 10 s
4. 70 °C, 30 s
5. Go to Step 2, repeat 24 times
6. 70 °C, 2 min
7. 4 °C, forever

A.6.2 PCR purification

1. Add 1:1 volume of Binding buffer.
2. Mix gently, do not pipet up and down.
3. Transfer up to 800 µL of the solution to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.

4. Add 100 μL of Binding Buffer to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.

5. Add 700 μL of Wash Buffer to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.

6. Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove residual wash buffer.

7. Transfer the GeneJET purification column into a clean 1.5 mL microcentrifuge tube. Add 20 μL of Elution Buffer to the center of the purification column membrane. Centrifuge for 1 min.

A.7 A-tailing for blunt-end PCR product (from KOD-PCR)

1. Take 5 μL of purified PCR fragment and add 1ul Taq DNA polymerase + 10X reaction buffer with MgCl2.

2. Add dATP to final concentration of 0.2mM.

3. Add 5 units of Taq DNA Polymerase.

4. Add deionized water to a final volume of 10 μL.

5. Incubate at 70 °C for 30 min.

6. Use 2 μLl in a ligation reaction with pGEM-Teasy vector(Promega).

A.8 Ligation of the clean DNA with pGEM-T easy vector

Mix

- 6 μL DNA template (from One-Step RT-PCR; 2 μL from A-Tailing rxn)
- 1 μL pGEM-T easy vector
- Distilled water: to final volume of 20 μL
- 10 μL 2X ligation buffer
- 1 μL T4 ligase

Final volume: 41 μL

Incubation
Incubate at 16-22 °C for 2 h / 4 °C for overnight.

A.9 Transformation to Ecoli competent cells

1. Get frozen DH5α competent cells from -80 °C freezer. Cells are aliquoted in 50 µL tubes.
2. Thaw cells on ice for 5 min.
3. Add gently ligation reaction (5 µL) to cells. Gently stir 4-5rounds. Do not mix by pipetting up and down.
4. Incubate cells on ice for 20 min.
5. Prepare 42 °C water bath.
6. Heat shock cells in 42 °C water bath for 45 s only.
7. Immediately transfer to ice for 2 min.
8. Add 250 µL SOC medium prewarmed to 37 °C.
9. Mix by gently flicking the tube.
10. Incubate cells suspension at 37 °C for 1 h with shaking.
11. Spread 100 µL of cells on selection plates [50ug/ml Carbenicillin, IPTG (10 µL) + Xgal (40 µL)].
12. Incubate overnight at 37 °C.

A.10 Mini-prep

A.10.1 Mini-prep preparation

Pick white colonies from plates the next day and grow them in 3 mL LB broth with 50 µg/mL Ampecillin.
Incubate at 37 °C overnight with shaking.

A.10.2 Mini-prep DNA extraction

This step is used to extract plasmid from transformed Ecoli.

1. Collect 1-4 mL of overnight LB culture. Centrifuge at 13.2 rpm for 1 min to pellet the bacteria. Decant or pipette off as much of the supernatant as practical.
2. Resuspend the bacterial pellet in 250 µL of Buffer S1 by vortexing.The pellet must be completely resuspended. S1 must be added RNase before use.

3. Add 250 µL of Buffer S2 and mix by gently inverting the tube 4-6 times. Do not vortex.

4. Add 350 µL of Buffer S3 and mix by gently inverting 6-8 times. Centrifuge at 13.2 rpm for 10 min to clarify the lysate. Do not vortex.

5. Place a Miniprep column into an uncapped 2 mL Microfuge tube. Transfer the clarified supernatant from step 4 into the miniprep column. Transfer the Miniprep column and 2 mL Microfuge tube to microcentrifuge and spin at 13.2 rpm for 1 min.

6. Pipette 700 µL of Buffer W2 into each Miniprep column. Centrifuge at 13.2 rpm for 1 min.

7. Discard filtrate from the 2 mL Microfuge tube. Place the Miniprep column back into the 2 mL Microfuge tube. Centrifuge at 13.2 rpm for 1 min.

8. Transfer the Miniprep column into a clean 1.5 mL Microfuge tube. To elute the purified plasmid DNA, add 60 µL of Eluent to the center of the membrane. Stand for 1 min at room temperature. Centrifuge at 13.2 for 1 min.

A.11 Cut plasmid with Not I

A.11.1 Reaction

Mix

- 5 µL Plasmid
- 1 µL Not I
- 2 µL 10x Buffer 3
- 1 µL BSA
- 11 µL Water

Final volume: 20 µL

Incubation
Incubate reaction at 37 °C for 1 h.

A.11.2 Gel to check the restriction

Mix

- 10 µL DNA Template
- 2 µL Loading dye 6x

Run
Run DNA on 1% agarose gel at 120 V for 30 min.

A.12 Sequence DNA with T7 and SP6 sequencing primers

Mix

- 5 µL DNA Template
- 1 µL Primer
- 4 µL Big dye

Prepare two different tubes with the same DNA template (one with the forward primer and one with reverse primer).
Put the tubes in PCR machine and set up the sequencing program as below.

Sequencing program

1. 95 °C, 4 min
2. 95 °C, 30 s
3. 51 °C, 10 s
4. 60 °C, 4 min
5. Go to step 2 and repeat 34 times
6. 15 °C, forever

A.13 MIDI-prep

A.13.1 Preparation of the MIDI-prep

After checking the sequencing of clones, put the good clones in 50 mL LB broth + 50 µL/mL Amp and grow overnight at 37 °C shaking.

A.13.2 MIDI-prep plasmid extraction

1. Harvest the bacterial cells by centrifugation at 45,000 rpm for 30 min at 4 °C.
2. Resuspend the bacterial pellet in 4ml Buffer P1. (RNase A must be added to Buffer P1)
3. Add 4 mL Buffer P2, mix thoroughly by vigorously inverting the sealed tube 4-6 times, and incubate at room temperature (15-25 °C) for 5 min.

4. Add 4 mL Buffer P3 to the lysate, and mix immediately and thoroughly by vigorously inverting 4-6 times. Do not incubate the lysate on ice.

5. Screw the cap onto the outlet nozzle of the QIAfilter Midi Cartridge. Pour the lysate into the barrel of the QIAfilter cartridge. Incubate at room temperature for 10 min.

6. Equilibrate a QIAGEN-tip 100 by applying 4 mL Buffer QBT, and allow the column to empty by gravity flow.

7. Remove the cap from the QIAfilter cartridge outlet nozzle. Gently insert the plunger into the QIAfilter Midi cartridge and filter the cell lysate into the previously equilibrated QIAGEN-tip.

8. Allow the cleared lysate to enter the resin by gravity flow.

9. Wash the QIAGEN-tip with 10 mL Buffer QC 2 times.

10. Elute DNA with 5 mL Buffer QF. Collect the eluate in a 15 mL tube.

11. Precipitate DNA by adding 3.5 mL room-temperature isopropanol to the eluated DNA. Mix and centrifuge immediately at 4500 rpm for 45 min at 4 °C.

12. Wash DNA pellet with 1 mL of room-temperature 70% ethanol, and transfer to the 1.5 mL microfuge tube and spin at 13 rpm for 10 min at 4 °C.

13. Remove the 70% ethanol and air dry the pellet for 5 min, homogenize the pellet with 100 µL distilled water.

14. Re-sequence the clone for verification.

A.14 Digestion with Spe I

A.14.1 Reaction

Check the DNA concentration. Let be X µL the volume to add to have 5 µg of plasmid.

Mix

- X µL Plasmid
- 4 µL Spe I
- 4 µL 10X Buffer 2
- 2 µL 100X BSA
- $30 - X$ µL Water

Final volume: 40 µL

Incubation
Incubate at 37 °C for 2 h.

A.14.2 Gel to check the cutting

Mix

- 5 µL Template
- 5 µL Water
- 2 µL Loading dye 6x

Run
Run DNA on 1% agarose gel at 120 V for 30 min.

A.15 In vitro transcription reaction

Mix

- 25 µL Template
- 5 µL DIG RNA label
- 2 µL Rnase inhibition
- 4 µL T7 polymerase
- 4 µL Reaction Buffer for T7

Final volume: 40 µL

Incubation
Incubate at 37 °C overnight in the PCR machine.

A.16 Protein expression

A.16.1 Small scale

1. Pick colony (BL21-DE3) and grow up sample in 5 mL LB/Amp overnight at 37 °C
2. Take 1 mL and put in 10 mL LB/Amp. Grow for about 1 h (until OD600= 0.6-0.8)
3. Induce cells with 0.5-1mM IPTG at 25 °C for 3-5 h or overnight
4. Spin down at 4000 rpm, 10 s, 4 °C and pellet cells
5. Resuspend pellet in 1.5 mLl His-binding buffer containing P.I. and lysozyme *check pH= ± 1.5pI of protein

6. Sonicate in 2 mL tube for 30 s - 1 min eg.1 s on, 5 s off, Amp23/24
7. Spin down cells at 13K rpm, 30 s, 4 °C
8. Run gel for pellet and supernatant. For pellet, add 50 µL H_{20} and dye, heat at 95 °C, centrifuge 10 s and load supt
9. Stain gel with Coomassie Blue and take a picture.

A.16.2 Large scale

1. Grow construct in 100 mL LB/Amp overnight at 37 °C
2. Put 20 mL (of 100 mL culture) into 1 L culture (using 2 L flasks) for 3 h or put 5-25 mL culture into 100 mL of fresh LB for 2-3 h and then into 1 L culture
3. Grow till OD600= 0.6-0.8, cool to temperature required for induction and then add 1mM IPTG overnight
4. Spin at 4.2K rpm, 1 h at 4 °C (use big centrifuge tubes)
5. Resuspend pellet in 30 mL His-binding buffer (store at -20 °C or -80 °C)
6. Add protease inhibitor + lysozyme to suspension
7. Sonicate sample (2 min: 5 s on, 10 s off, 45%) or use microfluidics
8. Spin at 19K rpm, 90 min at 4 °C (use ss34 rotor)
9. Filter the supernatant before purification using AKTA system (0.45 µm filter)

A.17 Pulldown

Day 1

1. Centrifuge cells at 2000rpm, for 20 min at 4 °C
2. Wash cells once in 10 mL PBS
3. Resuspend cells in 10 mL lysis buffer
4. Lyse cell membrane with 1B Dounce Homogenizer (2x5 mL each, 15 times)
5. Spin at 10K rpm for 10 min at 4 °C
6. Pool supernatant and measure protein concentration via BCA kit
7. Wash and resuspend beads in base buffer (BB)
8. Using a 15 mL Falcon tube, incubate 400 µg protein (bait) with 10 µL Ni_2^+ beads. Top up volume to 1 mL and incubate on ice for 15 min (flick every 5 min)
9. Centrifuge at 200g (1500rpm) for 3 min at 4 °C to wash beads with 2x1 mL BB

10. Add lysate to beads (5 mg minimum per experiment)
11. Roll overnight at 4 °C

Day 2

1. Wash 3x1 mL BB (centrifuge at 200g for 3 min at 4 °C to wash)
2. Add 10 µL Benzonase (25unit/ µL) to beads with 10 µL BB (to clear background signal)
3. Incubate on ice overnight (keep at 4 °C)

Day 2

1. Wash 2x1 mL BB
2. Add 50 µL loading dye, heat at 100 °C for 5 min
3. Use 5 µL to run the reaction to check and freeze the rest at -80 °C

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