Republic of Turkey

Hacettepe University

Faculty of Medicine

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CLONING and DETERMINATION of TISSUE SPECIFIC EXPRESSION PATTERN of *Astacus leptodactylus* Na^{+} / Ca^{2+} EXCHANGER GENE

MASTER THESIS

Bora ERGİN

ANKARA

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Prof. Dr. Nuhan PURALI

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APPROVAL

Department:	**Biophysics**
Program:	**Biophysics**
Dissertation Title:	**CLONING and DETERMINATION of TISSUE SPECIFIC EXPRESSION PATTERN of *Astacus leptodactylus* Na^+/Ca^{2+} EXCHANGER GENE**
Name of the Student:	**Bora ERGIN**
Date of Dissertation:	**30.12.2014**

This study has been accepted and approved as a Master Thesis in the program of Biophysics by the examining committee, whose members are listed below.

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APPROVAL

This dissertation has been approved by the committee above in conformity to the regulations and bylaws of Hacettepe University Graduate Programs and has been accepted by the Board of Directors of the Institute of Health Sciences.

Ersin FADILLIOGLU M.D. Prof.
Director

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ABSTRACT

ERGIN, B., Cloning and determination of tissue specific expression pattern of *Astacus leptodactylus* Na^+ / Ca^{2+} exchanger gene, Hacettepe University Faculty of Medicine, Department of Biophysics, Master Thesis, Ankara, 2014. Cytosolic Ca^{2+} plays an essential role in various cellular functions. Changes in cytosolic Ca^{2+} concentration is important for different cellular activities like muscle contraction and neurotransmitter release. A set of channels and exchangers are responsible for maintenance of resting calcium concentration and the generation of the calcium transient. Na^+/Ca^{2+} exchanger can move Ca^{2+} in either direction depending on net electrochemical driving force acting on the exchanger. Structure and function of Na^+/Ca^{2+} exchanger is conserved within the animal kingdom. In the crayfish there are some recent functional studies indicating the presence of Na^+/Ca^{2+} exchanger. However, there is no report focusing onto genetic and molecular properties of the crayfish exchanger yet. To reveal properties of the crayfish Na^+/Ca^{2+} exchanger gene, various molecular techniques have been employed by considering the homology observed among closely related species. As a result, the complete coding sequence of the putative crayfish Na^+/Ca^{2+} exchanger gene has been revealed. Corresponding amino acid sequence has been found to be 58 – 65 % similar to known Na^+/Ca^{2+} exchangers, sharing characteristics of the exchanger protein family. Tissue specific expression pattern of crayfish Na^+/Ca^{2+} exchanger gene indicates that exchanger is expressed in excitable or biologically active tissues like ganglia, muscle and antennal gland while its expression is almost absent in gill which passively filters the neighboring fluid. Future efforts will be dedicated to define structure-function relationship of the discovered gene.

Keywords: Crayfish, Na^+/Ca^{2+} exchanger, nervous system, cloning, quantitative PCR

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ÖZET

ERGİN, B., *Astacus leptodactylus* Na^+ / Ca^{2+} karşı değiştirici geninin klonlanması ve doku spesifik ifade deseninin belirlenmesi, Hacettepe Üniversitesi Tıp Fakültesi, Biyofizik Anabilim Dalı, Yüksek lisans tezi, Ankara, 2014. Sitozolik Ca^{2+} birçok hücresel fonksiyonda hayati rol oynar. Sitozolik Ca^{2+} derişimindeki değişiklikler, kas kasılması ve nörotransmitter salınımı gibi hücresel aktiviteler için önemlidir. İstirahat kalsiyum derişiminin idamesinden ve kalsiyum geçişlerinin gerçekleştirilmesinden bir takım kanallar ve karşı değiştiriciler sorumludur. Na^+/Ca^{2+} karşı değiştirici, üzerine etkiyen net elektrokimyasal sürücü güç uyarınca Ca^{2+}'u iki yönlü taşıyabilir. Na^+/Ca^{2+} karşı değiştiricinin yapısı ve fonksiyonu hayvanlar âlemi içerisinde korunmuştur. Kerevitte Na^+/Ca^{2+} karşı değiştirici varlığını işaret eden bazı fonksiyonel çalışmalar yakın zamanda gerçekleştirilmiştir. Buna rağmen, kerevit karşı değiştiricisinin genetik ve moleküler özelliklerine ilişkin bir rapor henüz mevcut değildir. Kerevit Na^+/Ca^{2+} karşı değiştiricisi geninin özelliklerini açığa çıkarmak amacıyla, yakın türler arasında gözlenen benzerlikler göz önüne alınarak moleküler teknikler kullanılmıştır. Bu çalışmanın sonucunda, kerevit Na^+/Ca^{2+} karşı değiştiricisinin kodlayan bölgesinin sekansının tamamı ortaya çıkarılmıştır. İlgili amino asit sekansı bilinen Na^+/Ca^{2+} karşı değiştiricileri ile % 58 – 65 oranın benzerlik göstermekte ve karşı değiştirici protein ailesinin özelliklerini taşımaktadır. Kerevit Na^+/Ca^{2+} karşı değiştirici geninin doku spesifik ifade deseni, karşı değiştiricinin sinir düğümü, kas ve antennal bez gibi uyarılabilen ya da biyolojik olarak aktif olan dokularda ifade edilirken etrafındaki sıvıyı pasif olarak filtreleyen solungaç gibi dokularda ifade edilmediğini göstermektedir. Devam edecek çalışmalarda bulunan genin yapı-fonksiyon ilişkisinin belirlenmesi amaçlanacaktır.

Anahtar Sözcükler: Kerevit, Na^+/Ca^{2+} karşı değiştirici, sinir sistemi, klonlama, kantitatif PCR

Destekleyen Kuruluşlar: TÜBİTAK (#113 S 555), TÜBİTAK BIDEB 2210 ve Hacettepe Üniversitesi Bilimsel Araştırma Fonu (#014D08101006 ve #013D03101003).

CONTENT

ABBREVIATIONS

$[Ca^{2+}]_{in}$: Cytosolic Ca^{2+} concentration

AMV: Avian myeloblastosis virus

ATP: Adenosine tri-phosphate

Bp: base pair

BLAST: Basil local alignment search tool

cDNA: Complementary deoxyribonucleic acid

ddNTP: 2',3' dideoxy nucleoside triphosphate

dNTP: deoxy nucleoside triphosphate

HIV: Human immunodeficiency virus

MMLV: Moloney murine leukemia virus

mRNA: Messenger ribonucleic acid

NCX: Na^{+} / Ca^{2+} exchanger

ORF: Open reading frame

qPCR: Quantitative polymerase chain reaction

RACE: Rapid amplification of cDNA ends

SNP: Single nucleotide polymorphism

Taq: *Thermus aquaticus*

TMS: Transmembrane segment

FIGURES

TABLES

1. INTRODUCTION

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{in}$) plays an essential role in various cellular functions. Changes in cytosolic Ca^{2+} concentration is essential for different cellular activities like muscle contraction or neurotransmitter release. In a typical myocyte or neuron resting level of $[Ca^{2+}]_{in}$ is very low at about 10^{-7} M. Once the cell is excited $[Ca^{2+}]_{in}$ may rapidly rise 100 fold and decays relatively slowly as the excitation terminates (1,2) . Rapid rise and following decay in $[Ca^{2+}]_{in}$ is defined as the calcium transient (3) . A set of channels and exchangers are responsible for maintenance of resting calcium concentration and the generation the calcium transient (Figure 1). Calcium influx, through the voltage gated or receptor operated channels and release of calcium from intracellular stores like endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) are the major mechanisms responsible for the generation of rapidly rising part of the calcium transient (2) . Sequestration of Ca^{2+} into the intracellular stores and Ca^{2+} extrusion across the cell membrane is responsible for the termination of the calcium transient (1-3) . In some type of cells as in skeletal muscle, contribution of intracellular calcium stores is superior to the other pathways (4) . However, in a neuron opposite is observed. Membrane bound ATP-driven Ca^{2+} pump and Na^{+}/Ca^{2+} exchanger achieves calcium extrusion across the cell membrane. Although the pump binds Ca^{2+} with high affinity; its turnover rate is low (100 s^{-1}). The other mechanism, the Na^{+}/Ca^{2+} exchanger can move Ca^{2+} in either direction depending on net electrochemical driving force acting on the exchanger. Thus, the mode of Na^{+}/Ca^{2+} exchanger may change during the activity of cell (*i.e.* neuronal firing). Na^{+}/Ca^{2+} exchanger has 10 fold lower affinity as compared to that of ATP-driven Ca^{2+} pump. However, it has 10 – 50 fold higher turnover rate (5) .

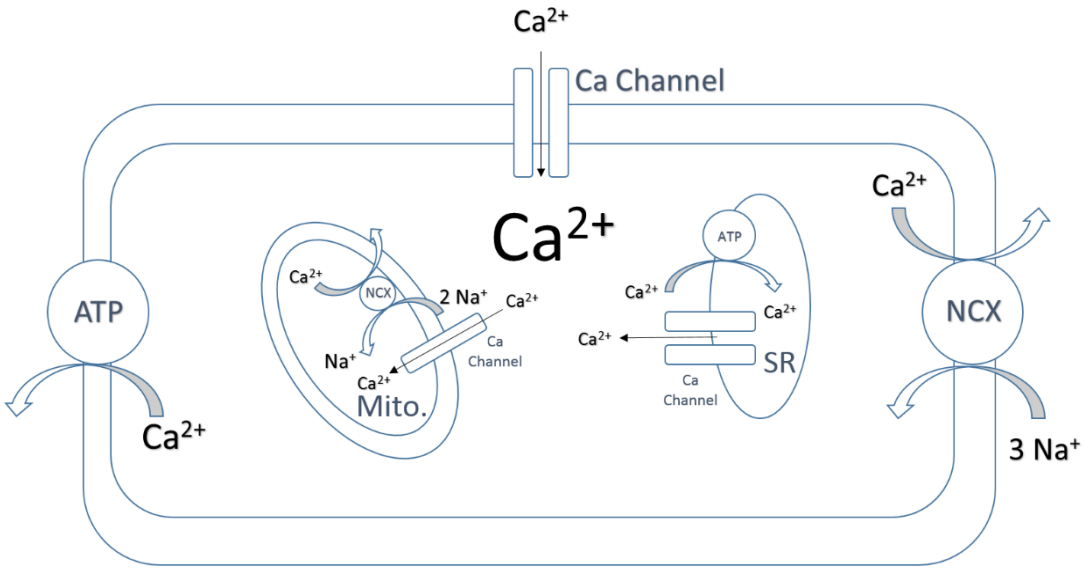


Figure 1.1. **Pathways taking part in cellular calcium homeostasis**

When localizations of ATP-driven Ca^{2+} pump and Na^{+}/Ca^{2+} exchanger are compared, it is clearly observed that unlike a uniform distribution of ATP-driven Ca^{2+} pump, Na^{+}/Ca^{2+} exchanger appears to be confined solely to the specific regions of plasma membrane. For example in a neuron, exchanger is expressed in presynaptic nerve terminals at higher concentration as compared to that observed in other parts of the cell (6) . At nerve terminals, ATP-driven Ca^{2+} pumps are localized in the vicinity of the vesicle docking sites whereas exchangers are distant (7) . Distinct distribution of the two Ca^{2+} transport systems suggests that a different role in Ca^{2+} trafficking should have been assigned to each component.

Currently four distinct Na^{+}/Ca^{2+} exchanger genes have been reported: NCX1, NCX2, NCX3 and NCX4 (8) . NCX1, NCX2 and NCX3 genes are found in mammals whereas NCX4 has recently been explored in amphibians. Mammalian NCX1 expression is widely observed in almost all kinds of tissues or organs (*i.e.* heart, skeletal muscle, smooth muscle). However, NCX2 and NCX3 have only been identified in brain and skeletal muscle. In invertebrates, genes homologous to mammalian NCX1 and NCX2 genes have been reported (5) . However, presence of NCX3 and NCX4 is yet to be investigated.

Na^+/Ca^{2+} exchangers present in invertebrates (Figure 1.3) are homologous to vertebrate NCX1. In crayfish there are some functional studies indicating presence of Na^+/Ca^{2+} exchanger in nervous and muscle tissue (Figure 4.1). However, there is no report available related to its genetic and molecular properties yet. Thus the aims of the present work were;

1. to explore the Na^+/ Ca^{2+} exchanger gene(s) expressed in the crayfish,
2. to identify ORF of the exchanger gene(s),
3. to obtain the amino acid sequence of the putative exchanger protein(s),
4. to determine tissue specific expression pattern of the explored gene(s),
5. to analyze the molecular characteristics of the putative gene(s) and corresponding protein(s).

To achieve those goals described above RNA extraction, cDNA synthesis, PCR, sequence analysis and qPCR methods has been employed by using a set of degenerate and specific primers designed by taking into account the apparent homology observed among closely neighboring species.

The Na^+/Ca^{2+} exchanger consists of 5 N-terminal and 6 C-terminal transmembrane segments separated by a large intracellular loop (Figure 1.2) (9) . N-terminal is located at the extracellular side of the cell while the loop and C-terminal are intracellularly located. Several consensus phosphorylation sites are determined within the loop indicating that exchanger function can be modulated by phosphorylation. Further, cytosolic ions such as Ca^{2+}, Na^+ and H^+ may bind to this part. Intracellular loop is also site for alternative splicing that can produce different isoforms. It is known that there are at least 32 distinct tissue specific NCX1 isoforms (10) .

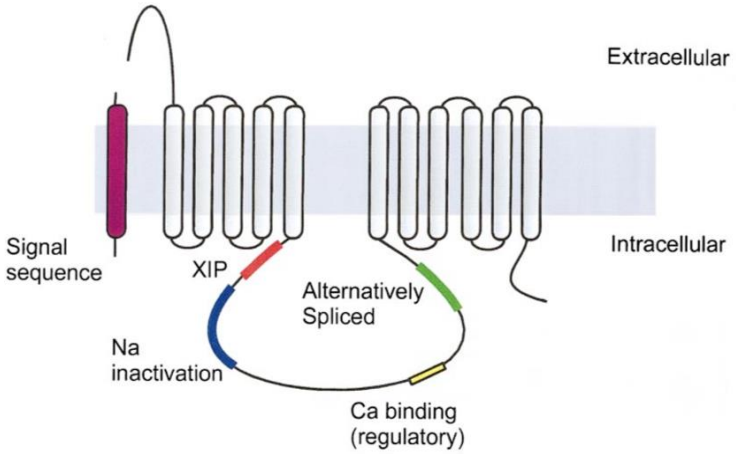


Figure 1.2. Schematic structure of Na^{+}/Ca^{2+} exchanger.

With refer to a phylogenetic analysis, it is observed that NCX is present and its structure is highly conserved within the animal kingdom (Figure 1.3). In mammalian and non-mammalian species size of the known NCX protein is in the range of 861 and 973 amino acids including the isoforms and splice variants (8) . Comparison of NCX sequences from different species indicates that the transmembrane regions of the sequences display more apparent homology as compared to that observed for the intracellular loop. Those similarities are important clues for our experimental purposes to reveal new members of NCX gene family.

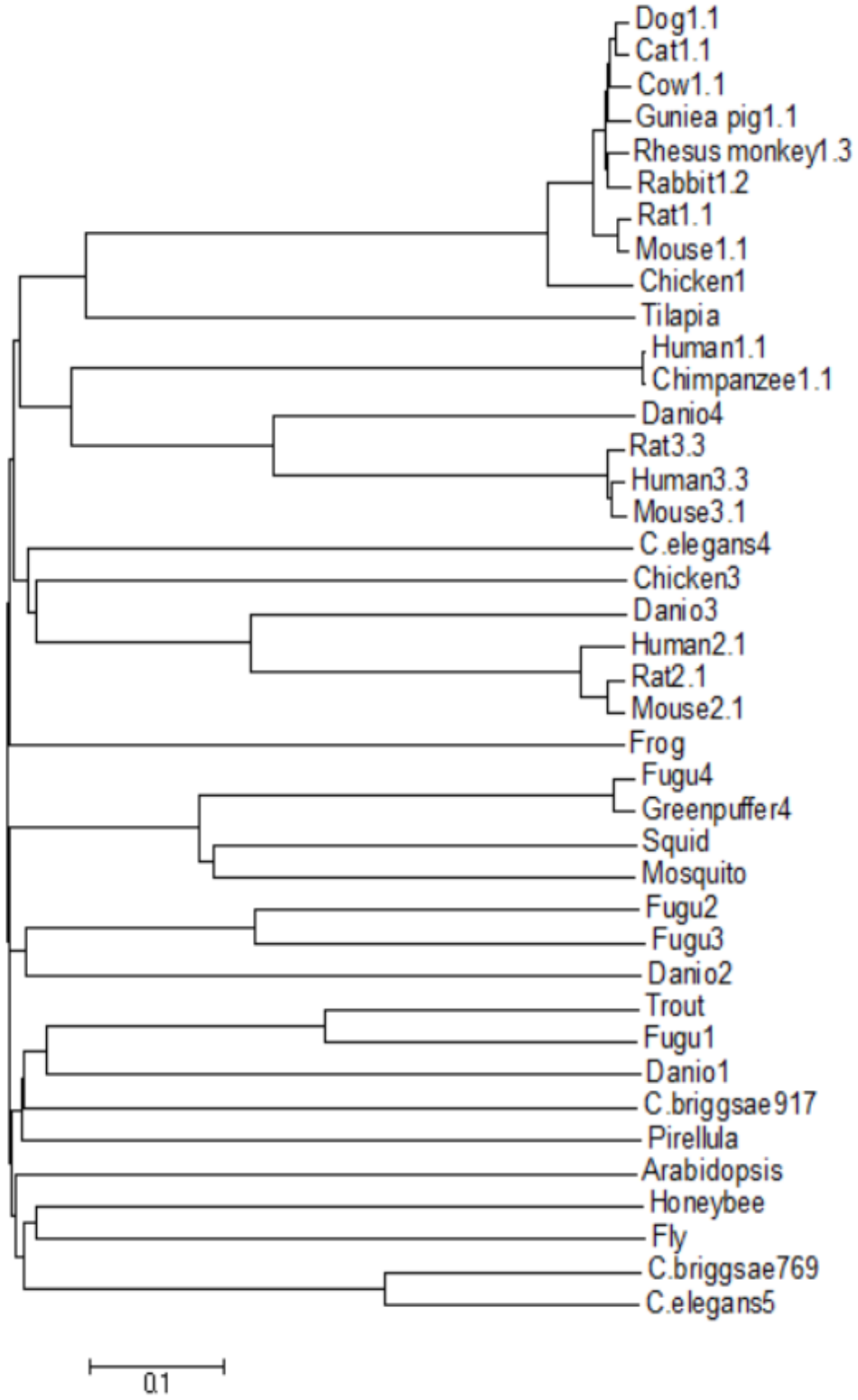

Dog1.1
Cat1.1
Cow1.1
Guniea pig1.1
Rhesus monkey1.3
Rabbit1.2
Rat1.1
Mouse1.1
Chicken1
Tilapia
Human1.1
Chimpanzee1.1
Danio4
Rat3.3
Human3.3
Mouse3.1
C.elegans4
Chicken3
Danio3
Human2.1
Rat2.1
Mouse2.1
Frog
Fugu4
Greenpuffer4
Squid
Mosquito
Fugu2
Fugu3
Danio2
Trout
Fugu1
Danio1
C.briggsae917
Pirellula
Arabidopsis
Honeybee
Fly
C.briggsae769
C.elegans5
0.1

Figure 1.3. **Phylogenetic analysis of NCX gene.** The evolutionary history was inferred using the Neighbor-Joining method (11) . The optimal tree with the sum of branch length = 10.79752216 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (12) and are in the units of the number of amino acid differences per site. The analysis involved 40 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 432 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (13) .

2. MATERIALS AND METHODS

2.1. Animals

The experiments were carried out on the crayfish, *Astacus leptodactylus* tissue samples. Experimental animals were collected in the lakes of Central Turkey and kept in an aerated aquarium at 18-20 ^{o}C and fed on an alternating carrot and fish diet once a week. In the use of experimental animals the guidelines by Hacettepe University have been followed and ethics committee approval has been obtained.

2.2. Decapitation of the Animals and Excision of Tissues

Inter-molt animals were taken from the aquarium and kept in ice for 10 minutes. Animal was quickly decapitated and different tissues of the animal are collected using sterile lancets and scissors. Collected tissues were kept in control solution (14) .

2.3. Total RNA Isolation

Total RNA of tissues were isolated by using the *RNeasy Mini Kit* (Qiagen). Excised tissues were adjusted to weigh 15-30 mg. Fresh homogenization buffer *RLT* - B-mercaptoethanol mixture were prepared in 100:1 ratio, just before the tissue homogenization. Tissue samples were homogenized by using a glass homogenizer or single use plastic pestles for 1.5 ml microcentrifuge tubes (Starlab). Homogenate has been transferred to spin column. All the isolation steps have been carried out according to manufacturer's instructions (for details see 6.1.).

2.4. Quantification of Isolated Total RNA

A 1.5 µl sample of the isolated total RNA have been quantified with *NanoDrop*. ng/µl, 260/280 and 260/230 nm values were recorded (for details see 6.2.).

2.5. cDNA Synthesis

Once total RNA has been isolated and quantified it has immediately been reverse transcribed into cDNA to prevent any degradation.

For different experimental purposes different cDNA synthesis kits were available. In the present study; for quantitative purposes *Quantitect Reverse Transcription Kit* (Qiagen); for determination of long gene fragments *LongRange 2Step Reverse Transcription Kit* (Qiagen) and to obtain complete coding sequence of gene of interest *SMARTer RACE 5' / 3' Kit* (Clontech) have been used (for details see 6.3.).

2.5.1. cDNA Synthesis by using *Quanitect Reverse Transcription Kit*

After quantification of total RNA the procedure given below has been followed. All the steps were carried out on ice.

Table 2.1. Step I of cDNA synthesis by using *Quantitect Reverse Transcription Kit*.

	Volume
Total RNA	Variable (According to NanoDrop read; up to 1 µg)
dH_2O	Variable
Genomic DNA Wipeout Buffer 7x	2 µl
Total	**14 µl**

Mixture from Step I has been mixed and incubated at 42 ^{o}C for 2 minutes.

Table 2.2. Step II of cDNA synthesis by using *Quantitect Reverse Transcription Kit*.

	Volume
Mixture from Step I	14 µl
Quantiscript RT Buffer 5x	4 µl
RT Primer Mix	1 µl
Quantiscript Reverse Transcriptase	1 µl
Total	**20 µl**

Reaction mix from Step II has been mixed and incubated at 42 ^{o}C for 15 minutes and at 95 ^{o}C for 3 minutes. cDNA's were stored at – 20 ^{o}C.

2.5.2. cDNA Synthesis by using *LongRange 2Step Reverse Transcription Kit.*

For determination of long fragments of gene of *interest LongRange 2Step Reverse Transcription Kit* has been used which could reverse transcribe up to 12.5 kilobase pair mRNA, which is longer than most of the known genes. After quantification of total RNA; following procedure has been used. All the steps were carried out on ice.

Table 2.3. Reaction mix for cDNA synthesis by using *LongRange 2Step Reverse Transcription Kit.*

	Volume
LongRange RT Buffer, 5x	4 µl
dNTP Mix, 10 mM each	2 µl
Oligo-dT, 20 µm	1 µl
LongRange RNase Inhibitor, 4 Units / µl	0.2 µl
LongRange Reverse Transcriptase	1 µl
dH_2O	Variable
Total RNA	Variable (According to NanoDrop read; up to 4 µg)
Total	**20 µl**

Reaction solution was mixed and incubated at 42 ^{o}C for 90 minutes and at 85 ^{o}C for 5 minutes. cDNA's were stored at – 20 ^{o}C (Table 2.3).

2.5.3. cDNA Synthesis for *RACE Reaction by using SMARTer RACE 5' / 3' Kit.*

Complete coding sequence of a gene could be determined with Rapid Amplification of cDNA Ends (RACE) protocol if a part of the gene were known (15) . To be able to use RACE protocol at least two gene specific primers (one sense and one anti-sense) were needed. Preparation of 5' and 3' RACE cDNA sample was the first and probably the most important step of all since it directly affected efficiency of the downstream procedures.

To obtain 5' and 3' RACE cDNA, following procedure has been used. All the steps were carried out on ice.

Table 2.4. Preparation of Buffer Mix by using *SMARTer RACE 5' / 3' Kit.*

	Volume
5X First Strand Buffer	4 µl
DTT (100 mM)	0.5 µl
dNTPs (20 mM)	1 µl
Total	**5.5 µl**

In separate tubes template and primer mixtures were prepared.

Table 2.5. Preparation of template and primer mix.

5' RACE cDNA		**3' RACE cDNA**	
	Volume		Volume
RNA	1 - 10 µl	**RNA**	1 – 11 µl
5' –CDS Primer A	1 µl	**3' –CDS Primer A**	1 µl
Sterile dH_2O	0 - 9 µl	**Sterile dH_2O**	0 – 10 µl
Total	**11 µl**	**Total**	**12 µl**

Content of the template and primer mix was incubated at 72 ^{c}C for 3 minutes and then at 42 ^{c}C for 2 minutes. Tubes were spun down to collect contents at the bottom. 1 µl *SMARTer II A Oligonucletide* reagent was included only when 5' RACE reaction was conducted.

Table 2.6. Preparation of enzyme master mix.

	Volume
Buffer Mix from Step 1	5.5 µl
RNase inhibitor (40 U / µl)	0.5 µl
SMARTScribe Reverse Transcriptase (100 U)	2 µl
Total	**8 µl**

Enzyme, template and primer mixtures were combined and 20 µl cDNA synthesis reaction was constructed. Tubes were incubated at 42 ^{c}C for 90 minutes and then at 70 ^{c}C for 10 minutes. 10 µl of Trycine-EDTA buffer has been added to cDNA sample and stored at – 20 ^{o}C.

2.6. Polymerase Chain Reaction (PCR).

Polymerase chain reaction is a method that is used to synthesize a new copy of the template DNA. It is a rapid, specific, sensitive and flexible reaction that can readily be modified to the purpose of interest. Synthesis of the new copy is provided by the activity of an enzyme, *DNA polymerase,* and by addition of free dNTPs in accordance with the template (for details see 6.4).

For a polymerase chain reaction to be effective, the template concentration, Mg^{+2} concentration, annealing temperature and extension time should be optimized. However, the crucial part is the design of gene specific primers.

2.6.1. Primer Design

Primers are short synthetic oligonucleotide sequences that can be used for PCR, sequencing or even as a probe for hybridization studies. Primer design is the first and most essential step of a PCR experiment. There are several criteria that a primer should fulfill for effective functioning (16) :

1) Primer length should be between 17 – 28 nucleotides.
2) Percentage of GC content of a primer should be between 50 – 60 %.
3) 2 out of 3 nucleotides at the 3’ end of a primer should be either G or C to ensure specific priming.
4) Tm of the primers should be in the range of 55 – 80 ^{o}C.
5) 3’ ends of primers should not base pair with each other (Primer Dimers).
6) Primers should not be self-complementary (*i.e.* Hairpin).
7) Runs of 3 or more nucleotides should be avoided.
8) Primers must not show homology to regions other than the target

9) Primer should end with a G or C; but presence of more than 3 G or C's at last 5 bases of 3'end should be avoided.

Gene specific primers could easily have been designed according to above mentioned criteria whenever the target sequence was known. However, if the target region were unknown it has been roughly estimated by analyzing the conserved regions of the genes in related species. In such a situation, reverse translation of conserved amino acid sequence would reveal a set of nucleotide sequences that would cover all possible matches. During the design of degenerate primers, all the possible nucleotide differences at each position were covered. As a result a mixture of primers has been obtained and at least one of those primers would be complementary to the estimated target region. The reaction conditions were optimized since its efficiency would be inferior to that obtained by using a pair of gene specific primers.

2.6.2. PCR Procedures.

In the present study, have been used three different PCR kits to serve different purposes:

HotStart Taq DNA Polymerase (Qiagen) has been used for amplification of target regions up to 2000 base pairs in length. *HotStart Taq Polymerase* has been conjugated to a specific antibody and was not active at laboratory conditions. For activation of the enzyme the reaction mix was kept at 95 ^{o}C for 15 minutes. The inactive enzyme would not let mis - priming unless activated. Thus, the enzyme was superior to other kits.

The following procedure has been used to prepare a reaction mix. All the steps were carried out on ice.

Table 2.7. Reaction mix for *HotStart Taq DNA Polymerase*.

	Volume
dH_2O	38.3 µl
10x Reaction Buffer (Contains 15 mM $MgCl_2$)	5 µl
dNTPs (10 mM each)	0.5 µl
Forward Primer (10 mM)	2 µl
Reverse Primer (10 mM)	2 µl
Template (cDNA or gDNA)	2 µl
Taq Polymerase (5 Units / µl)	0.2 µl
Total	**50 µl**

Table 2.8. Thermal Cycling protocol for *HotStart Taq DNA Polymerase.*

Temperature	**Duration**	
95 °C	15 minutes	
95 °C	30 seconds	35 Cycles
55 – 65 °C	30 seconds	
72 °C	2 minutes	

Platinium Taq DNA Polymerase (Invitrogen) has been used for amplification of large gene fragments. The enzyme enabled the amplification of the templates up to 12 kilobase pairs in length. Reaction buffer did not contain $MgCl_2$. Thus, concentration of Mg^{+2} required an optimization step.

Table 2.9. Reaction mix for *Platinium Taq DNA Polymerase*.

	Volume
dH_2O	35.3 µl
10x Reaction Buffer (without $MgCl_2$)	5 µl
$MgCl_2$ (50 mM)	3 µl
dNTPs (10 mM each)	0.5 µl
Forward Primer (10 mM)	2 µl
Reverse Primer (10 mM)	2 µl
Template (cDNA or gDNA)	2 µl
Taq Polymerase (5 Units / µl)	0.2 µl
Total	**50 µl**

Table 2.10. Thermal cycling protocol for *Platinium Taq DNA Polymerase*.

Temperature	**Duration**	
95 °C	15 minutes	
95 ° C	30 seconds	35 Cycles
55 – 65 °C	30 seconds	
72 °C	4 minutes	

RACE PCR experiments have been run on cDNA samples, constructed from ganglion tissue, as instructed in the user manual to obtain full length of the gene to both ends. Anti-sense primer of *SMARTer II A oligo* has been paired with a specific primer to obtain gene specific amplifications in either direction. *SeqAmp DNA Polymerase* (Clontech) has been used to perform those long polymerase chain reactions.

Table 2.11. Reaction mix for *SeqAmp DNA Polymerase*.

	Volume
dH_2O	15.5 µl
2X SeqAmp Buffer	25 µl
5' or 3' GSP (10 mM)	1 µl
10x UPM	5 µl
5' or 3' RACE cDNA	2.5 µl
SeqAmp DNA Polymerase	1 µl
Total	**50 µl**

Table 2.12. Optimized "touch-down" cycling protocol for *SeqAmp DNA Polymerase.*

Temperature	**Duration**	
94 °C	2 minutes	
94 °C	30 seconds	5 Cycles
72 °C	4 minutes	
94 °C	30 seconds	5 Cycles
70 °C	30 seconds	
72 °C	4 minutes	
94 °C	30 seconds	25 Cycles
68 °C	30 seconds	
72 °C	4 minutes	

2.6.3 Quantitative PCR.

Quantitave PCR (qPCR) is a variation of conventional PCR method, developed by Higuchi et al. (17) . qPCR has potential applications in forensic sciences, analysis of chromosome aberrations, single nucleotide polymorphism (SNP) analysis, pathogen detection and gene expression studies (18) . It has been shown to be a

powerful method for quantification of transcription levels in real time due to its high sensitivity and reproducibility (19) .

In the present study, *Quantitect SYBR Green PCR Kit* (Qiagen) has been used. Reaction mixture contained both *SYBR Green I* dye and *ROX* passive reference dye for normalization to prevent possible tube variations. Kit also contained *Hot Start Taq* DNA polymerase system to provide specific amplifications (for details see 6.5.).

The following procedure has been used to prepare reaction mix. All the steps were carried out on ice.

Table 2.13. Reaction mix for *Quantitect SYBR Green PCR Kit*.

	Volume
dH_2O	3.9 µl
2x QuantiTect SYBR Green PCR Master Mix	5 µl
Forward Primer (10 mM)	0.3 µl
Reverse Primer (10 mM)	0.3 µl
Template cDNA	0.5 µl
Total	**10 µl**

Table 2.14. Thermal cycling protocol for *Quantitect SYBR Green PCR Kit*.

Temperature	**Duration**		
95 °C	15 minutes		
95 °C	15 seconds	45 Cycles	
60 °C	30 minutes		
72 °C	30 seconds		Data Acquisition for SYBR Green I and ROX
Melt Analysis (Ramp from 72 °C to 95 °C, rising by 1 °C each step, wait for 5 seconds at each step)			

2.7. Agarose Gel Electrophoresis.

After each polymerase chain reaction success, specificity and efficiency of the products needed to be examined. Agarose gel electrophoresis is a fast, easy and reliable technique for separating the components of PCR products. To visualize DNA fragments within the gel, a fluorescent tag called Ethidium Bromide has been added into the gel solution. Ethidium bromide binds specifically to double stranded DNA and absorbs UV light at 285 nm at its bound form. Ethidium bromide is a carcinogen, specific measures were taken while working with Ethidium bromide (20) .

For quality control of PCR products, 1% agarose gels with Ethidium bromide were produced as follows: first, 100 ml of 0.5x TBE was added to 1 gram of agarose (20) . Mixture was heated in microwave oven until homogenous and clear solution was obtained. Solution was let to cool down for 5 minutes in room temperature. Before pouring it into electrophoresis tray; 5 µl of Ethidium bromide solution (10 mg / ml, SNP Biyoteknoloji) was added. After the combs were placed the mixture has been let to cool down on the tray for 30 minutes.

The samples were loaded to wells provided by combs. To increase the density, samples were mixed with 2X *Gel Loading Solution* (Dr. Zeydanlı) in 1:1 ratio. Molecular weight marker (*GelPilot Ladder*, Qiagen) was loaded to one or two wells in each gel (for details see 6.6.).

2.8. Purification of PCR Product.

After a polymerase chain reaction has been completed and examined in agarose gel, PCR product needed be purified for downstream processes. PCR purification was particularly important if PCR product were going to be used for sequence analysis. Un-incorporated primers, dNTPs and Mg^{+2} may interfere to the following sequencing reaction. Hence, they needed to be removed from reaction mix.

For purification of PCR product *QIAQuick PCR Purication Kit* (Qiagen) has been used. All the steps were carried out according to manufacturer's instructions.

2.9. Sequencing.

Sequencing of obtained PCR products has been performed by using *Sanger Sequencing* (Chain Termination) method. Basically Sanger Method is a second PCR on purified PCR product sample, with the use of only a single sense or anti-sense primer (for details see 6.7.).

BigDye Terminator Cycle Sequencing Kit (Life Technologies) has been used for sequencing reactions. The following procedure was followed to prepare reaction mix. All the steps were carried out on ice.

Table 2.15. Reaction mix for *BigDye Terminator Cycle Sequencing Kit.*

	Volume
dH_2O	5.4 µl
BigDye Reaction Mix	3 µl
Forward or Reverse Primer (10 mM)	0.6 µl
Purified PCR Product	1 µl
Total	**10 µl**

Table 2.16. Thermal cycling protocol for *BigDye Terminator Cycle Sequencing Kit.*

Temperature	**Duration**	
95 °C	20 seconds	50 Cycles
55 °C	25 seconds	
60 °C	4 minutes	

3. RESULTS.

3.1. Quantification of Isolated Total RNA

Table 3.1. Quantification of total RNA isolated from a male animal

	ng / µl	260 / 280	260 / 230
Heart	26.3	2.07	2.49
Muscle	24.2	1.54	2.27
Ganglion	14.2	2.1	1.92
Intestine	59.4	1.51	2.04
Gill	152.3	1.99	2.09
Kidney	37.1	2	2.07
Stomach	120.6	1.3	2.11
Sperm Duct	101.6	2.36	2.12
Liver	274	2.14	2.09

All the samples have been adjusted to 14.2 ng/µl. 10 µl total RNA from each sample has been reverse transcribed.

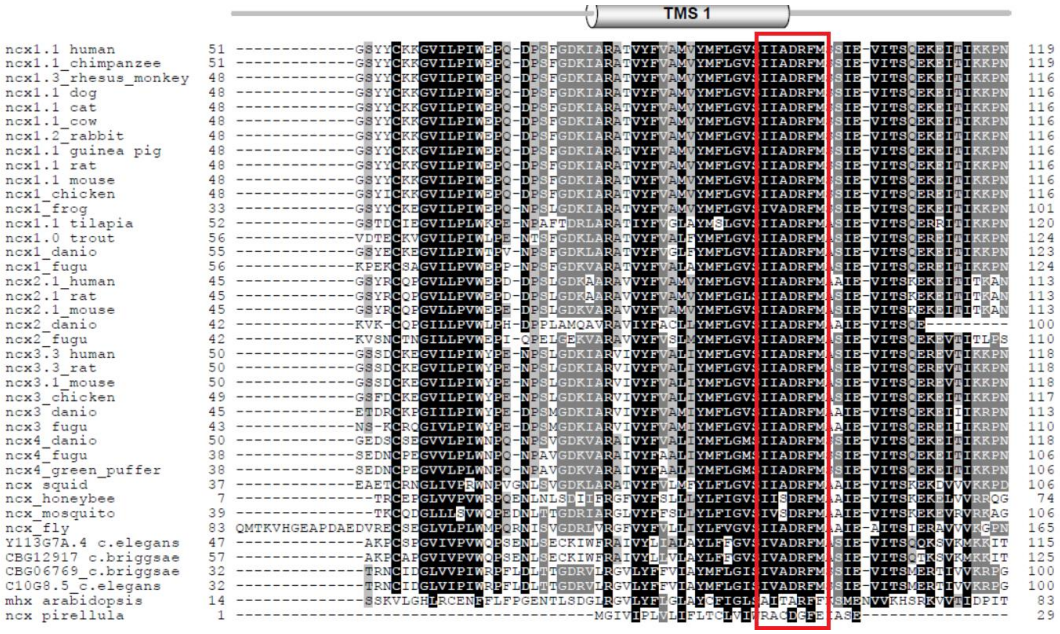
Table 3.2. Quantification of total RNA isolated from a female animal

	ng / µl	260 / 280	260 / 230
Heart	101.3	2.29	1.81
Muscle	49.4	2.02	3.2
Ganglion	94.8	2.03	2.2
Intestine	162.6	2.05	2.45
Gill	87.8	1.96	2.6
Kidney	168.8	2.13	2.03
Stomach	889.4	2.13	2.19
Ovary	350.9	2.68	2.54
Liver	1054.2	2.13	2.2

All the samples have been adjusted to 49.4 ng/µl. 10 µl total RNA from each sample has been reverse transcribed.

3.2. Determination of Conserved Amino Acid Sequences for Degenerate Primer Design.

Known NCX sequences of various organisms were retrieved from databases and aligned with the help of ClustalW2 algorithm. Once the alignment was completed several preserved domains has been determined. Those regions were used to design degenerate primer sets. It has been noted that transmembrane domains were highly conserved through different organisms (8) .



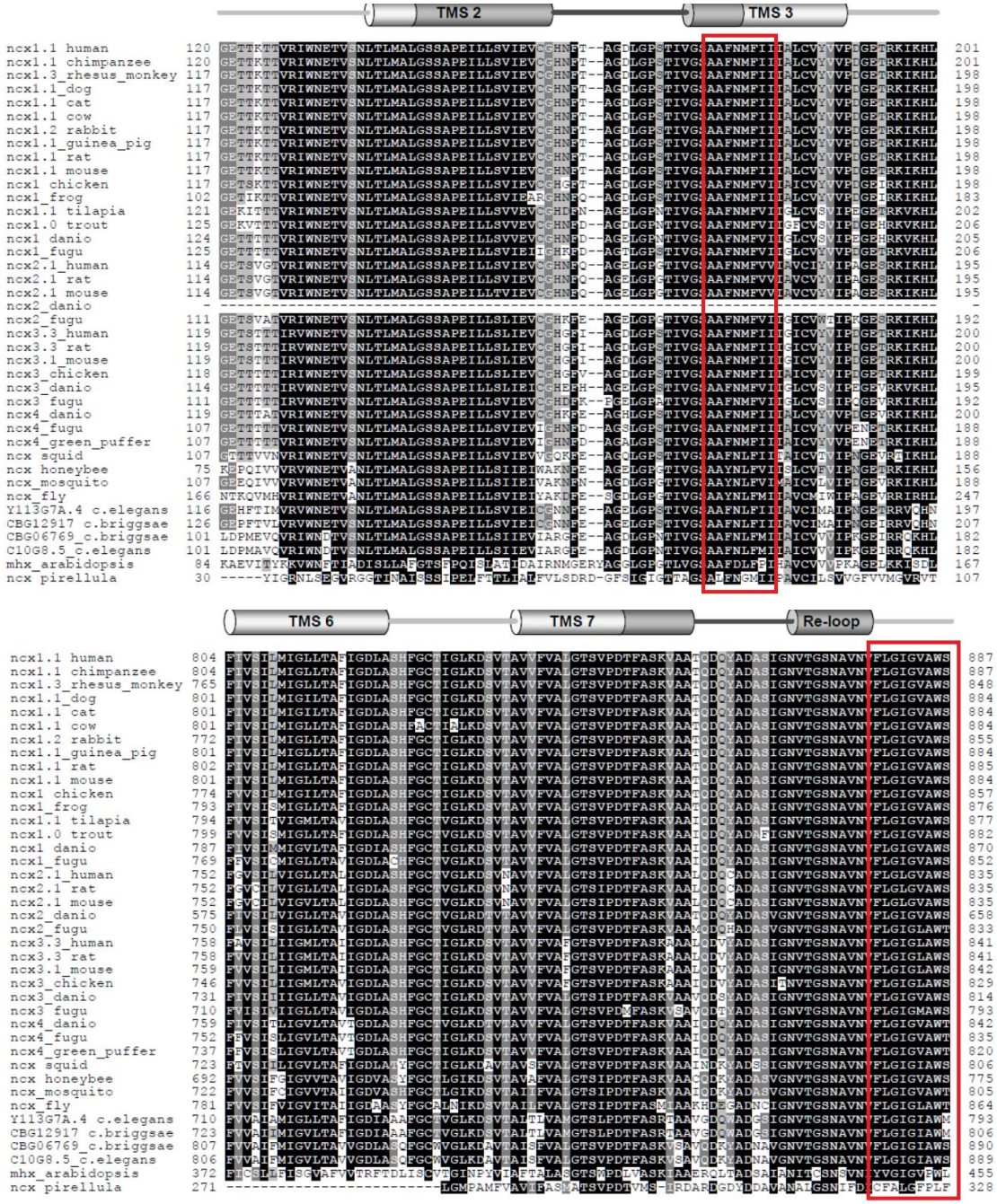


Figure 3.1. Identification of conserved domains among NCX family. Several conserved domains determined by a ClustalW2 analysis. Color intensity represents the degree of similarity at each residue. Red frames represent the candidate regions for primer design.

After these regions were determined; degenerate primers with the least possible variations were designed (Table 3.3).

Table 3.3. Degenerate Primers Used in the Study.

Primer Name	Amino acid sequence	Corresponding primer sequence (5' to 3')
DPNC_1F	IIADRFM	ATH ATH GCN GAY MGN TTY ATG
DPNC_1R	FNLFIII	ATD ATN ATR AAN ADR ATR AA
DPNC_2F	LGIGIAW	TNG GNA THG CAN THG CNT GG
DPNC_2R	FSVTMF	AAC ATN GTN CAN GWR AA
DPNC_3F	WKVLFA	TGG AAR GTN YTN TTY GC
DPNC_3R	GIGIAW	CCA NGC DAT NGC DAT NTC
DPNC_4F	WAKKMND	TGG GCN AAR AAR TGG AAY GA
DPNC_4R	WIAGMKG	CCY TTC ATN CCN GCD ATC CA

Further, a part of the nucleotide sequence of NCX gene was known for a very closely related organism, *Procambarus clarkii*. In order to increase the probability of the success a set of specific primers for *P. clarkii* NCX gene has also been designed (Table 3.4).

Table 3.4. Specific Primers Used in the Study.

Primer Name	Sequence (5' to 3')
Clarki_1F	AGCGAAAGTATTGGTATGATGGAGTTG
Clarki_1R	GCAGGCCAATCATACTCATAAAAATCG
Clarki_2F	AGATCATCGAGGAGGACAGCTACGAG
Clarki_2R	GCTTCTCCTCACCTCCTTTATCTTCG
Clarki_3F	CTCTCTCTAAGAAGGAGGAGGAGGAACG
Clarki_3R	GCTTCTCCTCACCTCCTTTATCTTCGTC

3.3. Polymerase Chain Reaction

The relevancy of each one of the above mentioned primers has been systematically tested in PCR on crayfish cDNA samples. Either a long or a short extension time has been used depending on the estimated size of the fragment. One degenerate primer pair successfully amplified a fragment of crayfish cDNA with a long PCR method and in another experiment one specific primer pair gave a positive result under routine PCR condition. Thus, under two different PCR conditions two different bands obtained from crayfish ganglion cDNA (Figure 3.2).

Table 3.5. First successful primer pairs for initial amplifications.

Primer Pair	Size of the amplicon
DPNC_1F – DPNC_2R	≈ 2000 bp
Clarki_2F – Clarki_3R	≈ 350 bp

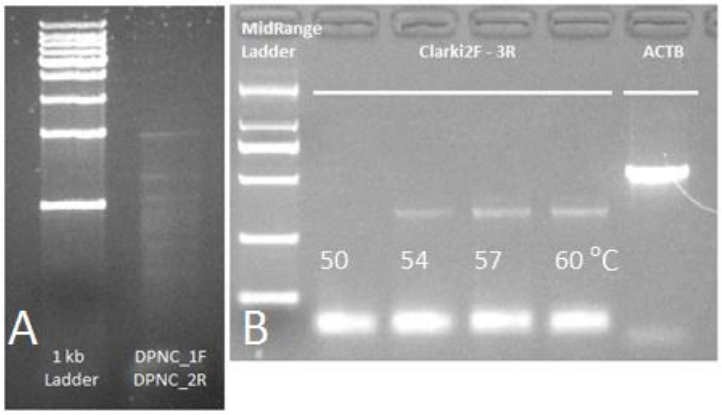
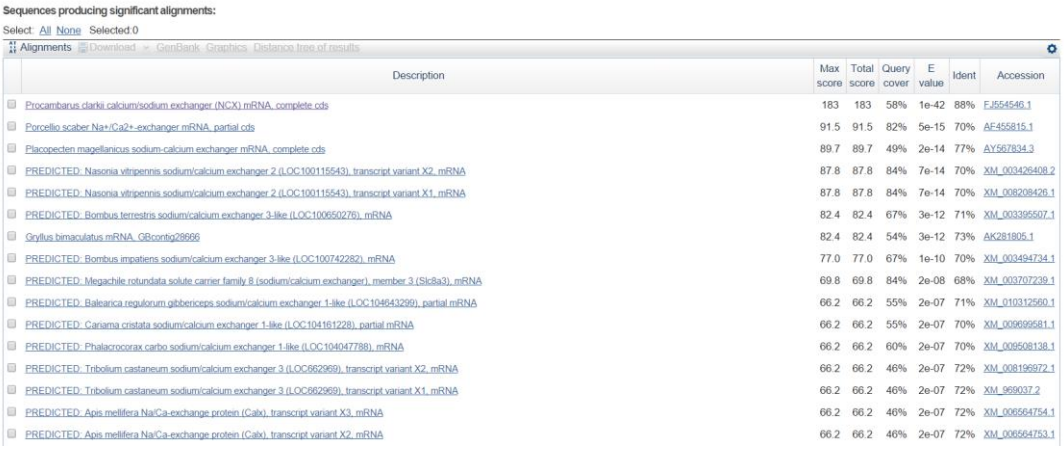


Figure 3.2. Gel Photographs of two different PCR products on crayfish ganglion cDNA. A) Products of PCR on LongRange cDNA sample by using primer pair DPNC_1F – DPNC_2R. B) Products of the Optimization PCR set by using the primer pair Clarki_2F – Clarki_3R.

Amplicon obtained by using the degenerate primers was weak and had non-specific amplifications which were inappropriate for a sequencing reaction. PCR product from primer pair Clarki_2F – Clarki_3R has been sequenced to verify the correctness of the amplification.

When the obtained sequence was loaded to Nucleotide BLAST (blastn) for comparison, it provided high resemblance with NCX sequences from other organisms (Figure 3.3).



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Procambarus clarkii calcium/sodium exchanger (NCX) mRNA, complete cds	183	183	58%	1e-42	88%	FJ554546.1
Porcellio scaber Na+/Ca2+-exchanger mRNA, partial cds	91.5	91.5	82%	5e-15	70%	AF455815.1
Placopecten magellanicus sodium-calcium exchanger mRNA, complete cds	89.7	89.7	49%	2e-14	77%	AY567834.3
PREDICTED: Nasonia vitripennis sodium/calcium exchanger 2 (LOC100115543), transcript variant X2, mRNA	87.8	87.8	84%	7e-14	70%	XM_003426408.2
PREDICTED: Nasonia vitripennis sodium/calcium exchanger 2 (LOC100115543), transcript variant X1, mRNA	87.8	87.8	84%	7e-14	70%	XM_008208426.1
PREDICTED: Bombus terrestris sodium/calcium exchanger 3-like (LOC100650276), mRNA	82.4	82.4	67%	3e-12	71%	XM_003395507.1
Gryllus bimaculatus mRNA, GBcontig28666	82.4	82.4	54%	3e-12	73%	AK281805.1
PREDICTED: Bombus impatiens sodium/calcium exchanger 3-like (LOC100742282), mRNA	77.0	77.0	67%	1e-10	70%	XM_003494734.1
PREDICTED: Megachile rotundata solute carrier family 8 (sodium/calcium exchanger), member 3 (Slc8a3), mRNA	69.8	69.8	84%	2e-08	68%	XM_003707239.1
PREDICTED: Balearica regulorum gibbericeps sodium/calcium exchanger 1-like (LOC104643299), partial mRNA	66.2	66.2	55%	2e-07	71%	XM_010312560.1
PREDICTED: Cariama cristata sodium/calcium exchanger 1-like (LOC104161228), partial mRNA	66.2	66.2	55%	2e-07	70%	XM_009699581.1
PREDICTED: Phalacrocorax carbo sodium/calcium exchanger 1-like (LOC104047788), mRNA	66.2	66.2	60%	2e-07	70%	XM_009508138.1
PREDICTED: Tribolium castaneum sodium/calcium exchanger 3 (LOC662969), transcript variant X2, mRNA	66.2	66.2	46%	2e-07	72%	XM_008196972.1
PREDICTED: Tribolium castaneum sodium/calcium exchanger 3 (LOC662969), transcript variant X1, mRNA	66.2	66.2	46%	2e-07	72%	XM_969037.2
PREDICTED: Apis mellifera Na/Ca-exchange protein (Calx), transcript variant X3, mRNA	66.2	66.2	46%	2e-07	72%	XM_006564754.1
PREDICTED: Apis mellifera Na/Ca-exchange protein (Calx), transcript variant X2, mRNA	66.2	66.2	46%	2e-07	72%	XM_006564753.1

Figure 3.3. Blastn result for Clarki_2F – Clarki_3R amplicon sequence.

Sequence from Clarki_2F is loaded to Blastn. Similar sequences are listed. Loaded sequence produced significant alignments with other sodium / calcium exchangers, highest alignment was with sodium / calcium exchanger of *Procambarus clarkii*.

Once a fragment of the crayfish specific sequence has been obtained specific primers were designed (Table 3.6).

Table 3.6. Specific NCX primers designed for the Crayfish.

Primer Name	Sequence (5’ to 3’)	Amplicon Size
qNCX_1F	ATGGTTGACAAGCTGGTGAAGAAGG	90 bp
qNCX_1R	GACGGTGATAGCCTCAGTAAACTGC	
qNCX_2F	CTCGCACCCAGATCAGAATCAAGG	157 bp
qNCX_2R	TCGTCATCGTCTCCTTCAGCTTGG	

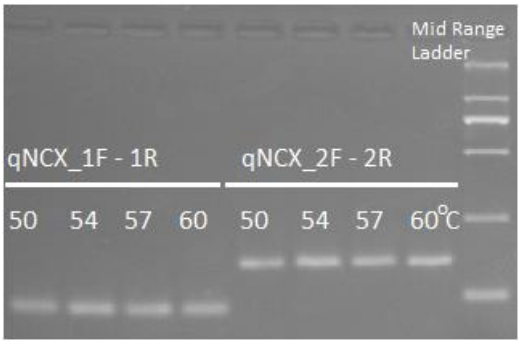


Figure 3.4. **Photograph of PCR products.** PCRs of two different specific primer pairs were optimized.

By using the specific primers it became possible to run RACE reactions to reveal the rest of the sequence.

3.4. 5’ RACE Reaction.

For 5’ RACE Reaction; qNCX_2R and *Universal Primer Mix* from *SMARTer RACE Kit* has been used on RACE cDNA obtained from crayfish ganglion tissue. First reaction gave a distinct band with the size of ≈2200 bp (Figure 3.5).

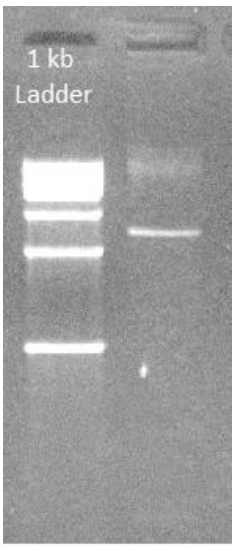


Figure 3.5. **Result of 5’ RACE Reaction.** Polymerase chain reaction on 5’ RACE cDNA with qNCX_2R and Universal Primer Mix produced a distinct band with the size ≈ 2200 bp.

5’ RACE amplicon is sequenced with Sanger’s Method starting with qNCX_2R primer. With this method up to 500 bases could be sequenced in each reaction. To cover the entire length of the amplicon, new sequencing primers needed to be synthesized after each reaction. 9 different sequence primers were used to reveal whole sequence (Table 3.7). At the final step of the sequencing reaction start codon of the gene has been revealed.

Table 3.7. Primers used for 5’ RACE sequencing.

Primer Name	Sequence (5’ to 3’)
5RACESeq_1	AGAGCACGTCCTTCTCGTAGCTGTC
5RACESeq_2	CGTAAACAAGTTCGCCTTCAGCTGC
5RACESeq_3	CAGGATCGAAGAATATACGGATGGTG
5RACESeq_4	CACTTCAAAGATACCCACGTTCTCC
5RACESeq_5	ATAAGTTAAATGCTGCCGAACCGAC
5RACESeq_6	CAGCCAAACATATGCAAACACAGAG
5RACESeq_7	CACACCATTCCCACGAAATAAACG
5RACESeq_8	TTCTTCACTGTAACTTCCTTCTCCTG
5RACESeq_9	TGATAACTCTCTGCAGTCCGGTCTC

3.5. 3’ RACE Reaction.

For 3’ RACE Reaction; Clarki_2F and *Universal Primer Mix* from *SMARTer RACE Kit* has been used on RACE cDNA obtained from crayfish ganglion. A nested PCR with qNCX_2F and *Short Primer* from *SMARTer RACE Kit* produced a shorter second band (Figure 3.6). The sequence analysis was performed on this shorter band sample after it was retrieved from the gel.

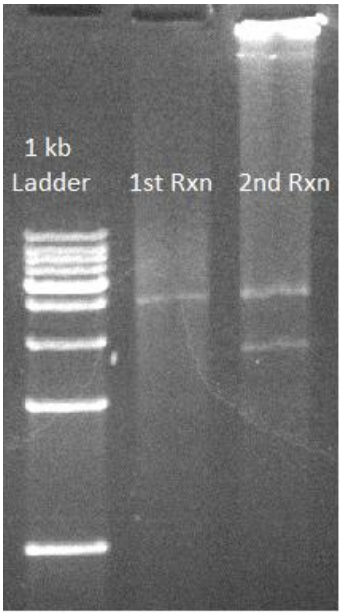


Figure 3.6. **Result of 3' RACE Reaction.** On 1st reaction, PCR on 3' RACE cDNA with Clarki2F and Universal Primer Mix. On 2nd reaction, polymerase chain reaction on first band with qNCX_2F and "Short Primer Mix" produced two different bands.

3' RACE amplicon has been sequenced with Sanger's Method starting with gene specific primer. With this method up to 500 bases can be read at each reaction. To cover all of the amplicon, new sequencing primers were synthesized after each reaction. 5 different sequence primers were used to reveal whole sequence (Table 3.8). At the final step of the sequence stop codon of the gene has been revealed.

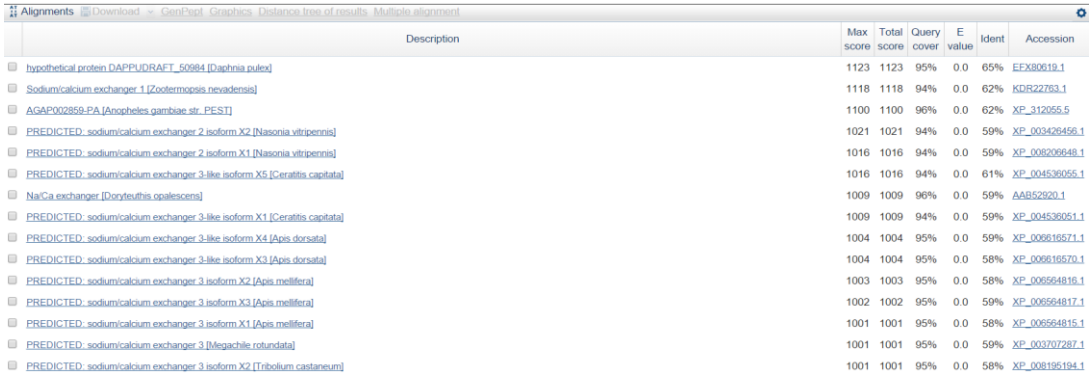
Table 3.8. Primers used for 3' RACE sequencing.

Primer Name	Sequence (5' to 3')
PRI3F_1	CTGCACTATCAGCCTTAAAGACTCCG
PRI3F_2	TCCGTGAAGACTATCTCCATTGC
PRI3F_3	TTAAAGTTCTACCCGGCAACCTGGC
PRI3F_4	CACTTCATCACCGTCTTCTGGAAGG
PRI3R_1	AATACCCAGGAAGACGTTGACGGAG

When all sequence fragments were aligned an ORF of 2665 bp that corresponds to a sequence of 884 amino acids was obtained (Figure 3.7).

MAEHKASYSHWGMHLLGFSVLVIILCSPVSAAEGNETEDTFAKCIDGLIIPVWSPQDNLTIGDRVARAIVYFV
GMVWLFIGVSIIADRFMGSIEMITSQEKEVTVKKPNGETQIIVVQVWNETVANLTLMALGSSAPEILLSVVEI
FARNFEAGDLGPGTIVGSAAFNLFVIIGICVYVIPDGEARYIKHLRVFFVTAFFSVFAYVWLYLILAEISYGIVEF
WEAFLTFLFFPLTVGLAYIADRRLLFYKYMTKEYRLGKRGVIIETEGADVEMGKKDEGGPMNDENVDESVRE
FEEHRKEYMAVIREMRQKHPNIGMDNLESMAREEIINRGPKSRAFYRIQATRKLTGGGNIMKKAREEIKPEK
EIAELNKKDEDTIRIFFDPGHYTVMENVGIFEVTVVREGGDLNTTVLVDYKTEDGTANADGDYVGAEGTLVF
LPGETQKNFKLEVIDDDVFEE,DEHFYVRLSNMRLGTQDGTAVTNAVNGDAGQPKEPIKMELAAPYVATIM
ILDDDHGGIFNVSEKDVEIAETIGTYELKIVRWSGARGRVTVPYKTEDGTAKSGKDYEAAEGELVYENNETEK
TISIQIIEEDSYEKDVLFYLDIGEPVASGGFEFASKDSALTEEEKIALLGKPKLGNATRTQIRIKENKEYRNMVDK
LVKKANASMLVGSSSWKEQFTEAITVQAEGDDEEEEDGGEEKLPSCMDYVMHFITVFWKVLFAFVPPTDR
QGGWSCFVISIAAIGFLTALIGDVAIHFGCTISLKDSVTAISIVALGTSVPDTFASKVAAQQDPYADASVGNVT
GSNAVNVFLGIGIAWTMAAIYHNVKGNEFKVLPGNLAFSVTLFCVEALVAIALMMVRRHPSIGGELGGPRK
SKIATSCFLIFS*

Figure 3.7. **Putative Crayfish NCX amino acid sequence.** Translation of ORF produced a peptide sequence in 884 amino acid length.



Alignments Download GenPept Graphics Distance tree of results Multiple alignment

Description	Max score	Total score	Query cover	E value	Ident	Accession
hypothetical protein DAPPUDRAFT_50984 [Daphnia pulex]	1123	1123	95%	0.0	65%	EFX80619.1
Sodium/calcium exchanger 1 [Zootermopsis nevadensis]	1118	1118	94%	0.0	62%	KDR22763.1
AGAP002859-PA [Anopheles gambiae str. PEST]	1100	1100	96%	0.0	62%	XP_312055.5
PREDICTED: sodium/calcium exchanger 2 isoform X2 [Nasonia vitripennis]	1021	1021	94%	0.0	59%	XP_003426456.1
PREDICTED: sodium/calcium exchanger 2 isoform X1 [Nasonia vitripennis]	1016	1016	94%	0.0	59%	XP_008206648.1
PREDICTED: sodium/calcium exchanger 3-like isoform X5 [Ceratitis capitata]	1016	1016	94%	0.0	61%	XP_004536055.1
Na/Ca exchanger [Doryteuthis opalescens]	1009	1009	96%	0.0	59%	AAB52920.1
PREDICTED: sodium/calcium exchanger 3-like isoform X1 [Ceratitis capitata]	1009	1009	94%	0.0	59%	XP_004536051.1
PREDICTED: sodium/calcium exchanger 3-like isoform X4 [Apis dorsata]	1004	1004	95%	0.0	59%	XP_006616571.1
PREDICTED: sodium/calcium exchanger 3-like isoform X3 [Apis dorsata]	1004	1004	95%	0.0	58%	XP_006616570.1
PREDICTED: sodium/calcium exchanger 3 isoform X2 [Apis mellifera]	1003	1003	95%	0.0	58%	XP_006564816.1
PREDICTED: sodium/calcium exchanger 3 isoform X3 [Apis mellifera]	1002	1002	95%	0.0	59%	XP_006564817.1
PREDICTED: sodium/calcium exchanger 3 isoform X1 [Apis mellifera]	1001	1001	95%	0.0	58%	XP_006564815.1
PREDICTED: sodium/calcium exchanger 3 [Megachile rotundata]	1001	1001	95%	0.0	59%	XP_003707287.1
PREDICTED: sodium/calcium exchanger 3 isoform X2 [Tribolium castaneum]	1001	1001	95%	0.0	58%	XP_008195194.1

Figure 3.8. **BLAST result of the putative amino acid sequence.** Whole amino acid sequence is loaded to Blastn.

ATGGCTGAACACAAAGCTAGCTATTCCCACTGGGGAATGCATTTGCTAGGTTTTAGTGTTTTAGTG
ATAATTCTCTGCAGTCCGGTCTCTGCTGCTGAAGGTAACGAAACTGAAGACACCTTCGCAAAATGT
ATCGATGGTCTTATCATCCCAGTATGGTCGCCTCAGGACAACCTGACTATTGGCGACCGCGTGGCT
CGTGCCATCGTTTATTTCGTGGGAATGGTGTGGCTTTTCATTGGTGTGTCAATCATCGCCGATCGT
TTCATGGGTTCCATTGAAATGATCACTTCACAGGAGAAGGAAGTTACAGTGAAGAAACCAAACGG
TGAGACGCAGATCATCGTGGTACAAGTATGGAACGAGACAGTAGCCAACCTCACTCTAATGGCGC
TGGGGTCCTCTGCTCCTGAGATCCTACTGTCCGTTGTTGAGATATTTGCCAGAAACTTCGAAGCTG
GAGACCTCGGTCCAGGAACCATTGTCGGTTCGGCAGCATTTAACTTATTTGTCATCATTGGTATTT
GTGTTTATGTAATTCCTGATGGTGAGGCGCGATACATCAAACACCTCCGAGTGTTCTTCGTTACAG
CCTTCTTCTCTGTGTTTGCATATGTTTGGCTGTATTTAATCCTGGCTGAAATCTCATACGGTATTGT
GGAGTTCTGGGAAGCTTTCCTGACTTTCCTCTTCTTCCCTCTTACCGTTGGTCTTGCTTACATTGCTG
ATCGCCGTCTTCTTTTCTACAAATATATGACAAAGGAATACCGCTTGGGCAAACGTGGTGTTATCA
TTGAAACTGAAGGTGCTGATGTTGAGATGGGGAAGAAAGATGAAGGAGGTCCTATGAACGATG
AAAATGTGGATGAGTCTGTTAGGGAATTTGAAGAGCACCGTAAGGAATACATGGCTGTCATTCGT
GAGATGCGGCAGAAGCATCCTAACATAGGAATGGATAACCTGGAGTCTATGGCCCGTGAGGAAA
TTATTAACCGAGGCCCCAAGTCTCGGGCCTTCTACCGTATTCAGGCTACCAGGAAGTTGACTGGTG
GTGGTAATATCATGAAGAAAGCAAGAGAGGAAATTAAACCAGAAAAAGAAATTGCAGAGTTAAA
CAAGAAAGACGAAGACACCATCCGTATATTCTTCGATCCTGGCCACTACACAGTTATGGAGAACG
TGGGTATCTTTGAAGTGACAGTTGTCCGTGAAGGAGGAGACCTCAATACCACTGTTCTCGTTGACT
ACAAAACTGAAGACGGTACAGCAAATGCTGATGGGGATTACGTTGGTGCCGAAGGAACCCTGGT
TTTTCTGCCTGGTGAAACCCAGAAAAACTTTAAGCTTGAAGTTATTGACGACGATGTTTTCGAGGA
AGATGAGCATTTTTATGTCCGACTCTCCAACATGCGACTGGGCACTCAAGACGGGACTGCAGTAA
CCAACGCCGTCAACGGAGATGCTGGCCAACCCAAAGAGCCCATCAAGATGGAACTGGCAGCTCC
ATACGTCGCCACTATTATGATCCTCGACGATGATCACGGTGGTATCTTTAACGTCAGTGAAAAGGA
CGTAGAAATTGCGGAGACCATTGGTACATACGAGCTGAAGATTGTGCGTTGGTCTGGCGCACGT
GGCCGCGTCACTGTGCCCTACAAGACGGAGGACGGCACTGCCAAATCCGGCAAGGACTACGAAG
CAGCTGAAGGCGAACTTGTTTACGAAAACAACGAAACTGAGAAAACCATATCGATCCAGATCATC
GAGGAGGACAGCTACGAGAAGGACGTGCTCTTCTACTTGGACATCGGCGAGCCTGTGGCTAGTG
GAGGCTTCGAGTTCGCCAGCAAGGACTCGGCACTGACGGAAGAAGAAAAGATCGCACTCCTAGG
CAAGCCTAAGCTTGGCAACGCCACTCGCACCCAGATCAGAATCAAGGAAAACAAAGAGTACCGG
AACATGGTTGACAAGCTGGTGAAGAAGGCCAACGCTTCCATGCTCGTCGGCTCCTCCTCTTGGAA
GGAGCAGTTTACTGAGGCTATCACCGTCCAAGCTGAAGGAGACGATGAGGAAGAAGAAGATGG
AGGTGAGGAGAAGCTGCCCTCCTGCATGGACTACGTGATGCACTTCATCACCGTCTTCTGGAAGG
TTCTCTTCGCCTTTGTTCCACCCACTGACCGGCAAGGTGGATGGTCGTGTTTTGTAATCTCTATAGC
AGCGATTGGATTTTTGACTGCATTAATCGGCGATGTGGCCATTCATTTCGGCTGCACTATCAGCCT
TAAAGACTCCGTGACGGCTATATCCATTGTGGCCCTCGGGACTAGCGTGCCAGACACCTTCGCCTC
CAAGGTGGCAGCCCAGCAGGACCCCTACGCTGACGCCTCCGTCGGTAACGTCACGGGCTCCAAC
GCTGTCAACGTCTTCCTGGGTATTGGTATTGCGTGGACGATGGCTGCCATCTATCACAACGTTAAG
GGAAATGAATTTAAAGTTCTACCCGGCAACCTGGCTTTCTCCGTGACGCTGTTCTGTGTGGAGGC
GCTGGTAGCCATCGCTCTCATGATGGTTAGACGACACCCGAGTATTGGCGGCGAACTGGGCGGTC
CCAGGAAGTCGAAGATTGCAACGTCATGCTTCCTGATCTTTAGTTAG

Figure 3.9. Nucleotide Sequence of Putative NCX Gene.

3.6. Tissue Specific Expression of Putative Crayfish NCX Gene.

Previously optimized qNCX_2F – qNCX_2R primer pair has been used on tissue specific cDNA samples on quantitative polymerase chain reactions. Two distinct set of experiments were conducted on tissue samples obtained from one male and one female animal.

Conducting melting analysis on PCR products confirmed the specificity of reactions. While specific PCR products melted at high temperatures, primer dimers and non-specific amplifications melted at lower temperatures. Presence or absence of a PCR product in a sample could be determined by melting analysis.

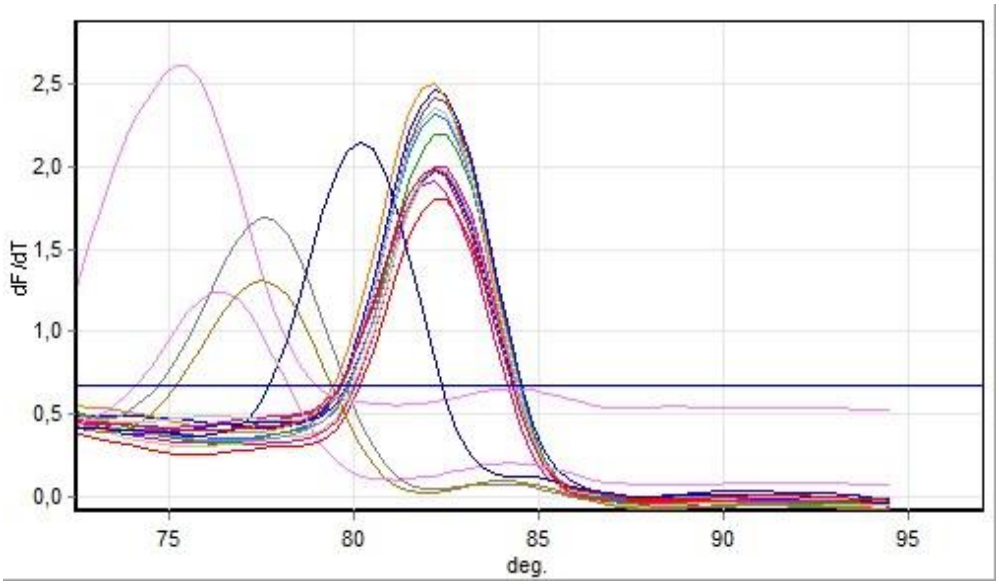


Figure 3.10. Melting curve for the male animal. Melting curve of qNCX_2F – qNCX_2R amplicon from 9 different tissues of male animal. Specific amplicons melt at 82 ^{o}C while there are some primer dimers and non-specific amplifications. Color codes are as in Table 3.9.

Table 3.9. Tm values for different tissues of the male animal.

No.	Colour	Name	Genotype	Peak 1
1		Heart NCX_1		82,3
5		Heart NCX_2		82,2
9		Muscle NCX_1		82,3
13		Muscle NCX_2		82,3
17		Ganglion NCX_1		82,2
21		Ganglion NCX_2		82,0
25		Intestine NCX_1		82,2
29		Intestine NCX_2		82,0
33		Gill NCX_1		77,5
37		Gill NCX_2		75,3
41		Kidney NCX_1		82,3
45		Kidney NCX_2		82,3
49		Stomach NCX_1		82,2
53		Stomach NCX_2		77,5
57		Sperm Duct NCX_1		80,2
61		Sperm Duct NCX_2		82,2
65		Liver NCX_1		82,2
69		Liver NCX_2		76,3

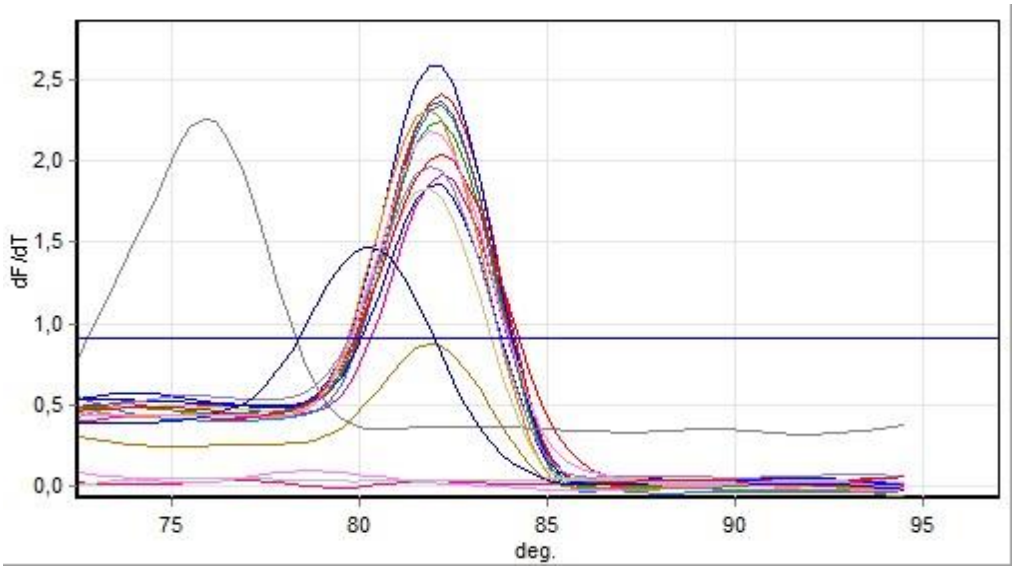


Figure 3.11. Melting curve for the female animal. Melting curve of qNCX_2F – qNCX_2R amplicon from 9 different tissues of female animal. Specific amplicons melt at 82 ^{o}C while there are some primer dimers and non-specific amplifications. Color codes are as in Table 3.10.

Table 3.10. Tm values for different tissue samples of the female animal.

No.	Colour	Name	Genotype	Peak 1
1		Heart NCX_1		82,2
5		Heart NCX_2		82,0
9		Muscle NCX_1		82,2
13		Muscle NCX_2		82,0
17		Ganglion NCX_1		82,0
21		Ganglion NCX_2		81,8
25		Intestine NCX_1		82,0
29		Intestine NCX_2		
33		Gill NCX_1		76,0
37		Gill NCX_2		
41		Kidney NCX_1		82,0
45		Kidney NCX_2		81,8
49		Stomach NCX_1		82,0
53		Stomach NCX_2		
57		Ovary NCX_1		80,2
61		Ovary NCX_2		
65		Liver NCX_1		82,2
69		Liver NCX_2		82,0

4. DISCUSSION

Calcium has a set of essential roles in various cellular functions. As it was already emphasized in the previous sections, $[Ca^{2+}]_{in}$ is strictly regulated. NCX is one of the key mechanisms taking part in that regulation process. However, to date there has been neither functional nor genetic data indicating the presence of NCX in the crayfish. On the contrary, NCX has been shown to be present in various phylogenetically related species (8) . Further, a recent experiment from our laboratory supplied some information indicating that NCX functions in crayfish neurons and participates in calcium homeostasis. As can be followed in Figure 4.1, neural activity induces a calcium transient in sensory neurons. The rapid rise in $[Ca^{2+}]_{in}$ decays relatively slowly when the neural activity terminates. NCX has been calculated to work mostly in exit mode in resting conditions (1) . Thus, NCX is expected to be a major mechanism taking part in the decay of the Ca^{2+} transient. The idea has been supported by the observation that time course of the decay apparently prolonged when the neurons are exposed to 10 mM $NiCl_2$, a well-known NCX blocker (Figure 4.1).

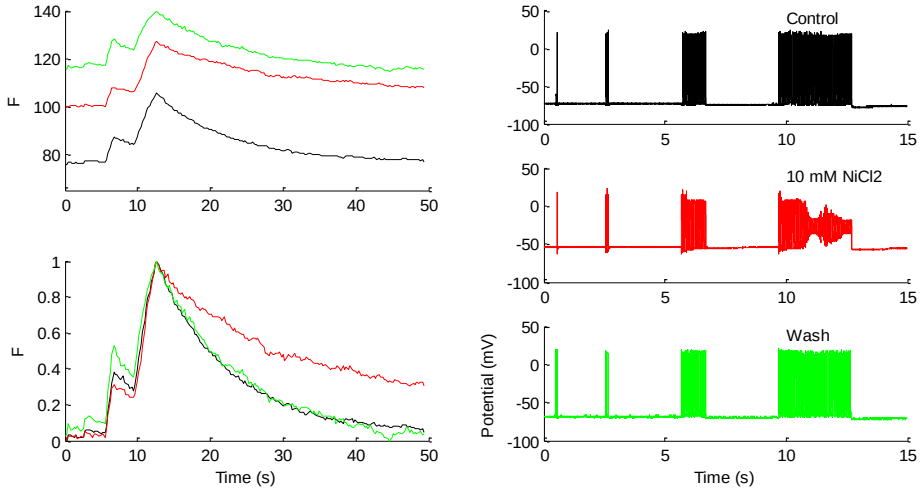


Figure 4.1. **Effects of $NiCl_2$ exposure on the cytosolic calcium signals**. A neuron is stimulated by the same supra-threshold current stimulus in control, 10 mM $NiCl_2$, and control solution. Right column, from top to bottom are shown the evoked potential responses. Top left are superimposed calcium specific fluorescent signals. Bottom left are normalized signals shown above. Scales are as in bottom plots.

Genes coding Na^+ / Ca^{2+} exchanger has been identified in almost all the species spanning from unicellular structures to human (Figure 1.3). Further, a run in BLAST indicated a strong homology within the known NCX gene sequences (Figure 3.3). Thus, it is conceivable to propose that NCX is a common regulatory element in most of the living species (5) . Present results confirm the idea completely that NCX is included within the crayfish genome and has a specific expression pattern (Figure 3.8).

As can be followed from Figure 3.9, a sequence of 2655 base pairs has been cloned by using a set of primers designed in reference to the homology within the phylogenetically related species. Identified gene consists of 2655 nucleotides. An ATG translation initiation codon has been identified at 5' end and a TAG termination codon has been found 2652 bases downstream.

Related amino acid sequence is consisted of 884 residues (Figure 3.7). When amino acid sequence is compared to the other sequences in BLAST it is observed that the sequence is 58 - 65% similar to the other known Na^+ / Ca^{2+} exchangers (Figure 3.8).

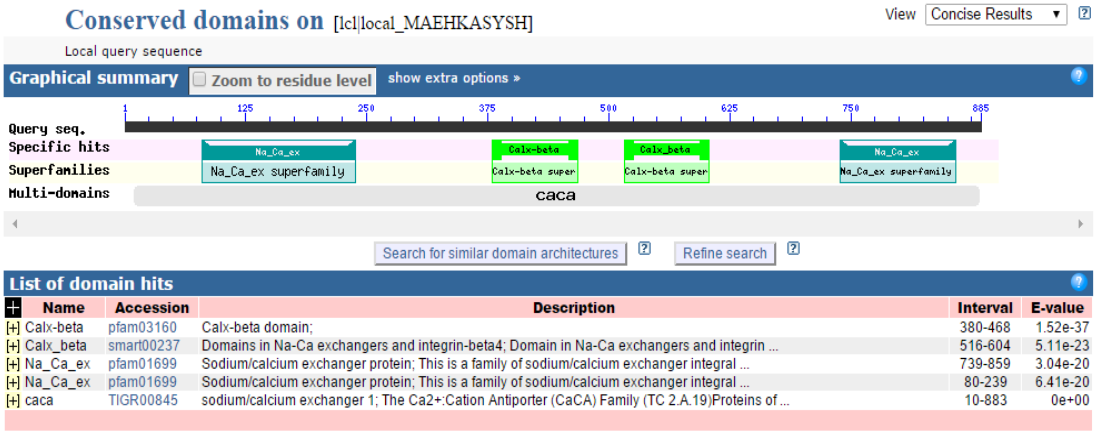
Comparison of the cloned amino acid sequence yields similarities with characteristics of the exchanger family. Some of those similarities are strongly associated with specialized function of the exchanger. For example, two fragments located between residues 80-239 and 739-859 are associated with Na^+ / Ca^{2+} exchanger superfamily integral membrane protein domains. Those are similar to corresponding domains present in cardiac myocytes, epithelial cells, neurons, photoreceptors and smooth muscle cells (Figure 4.2). Present results indicate that initial and terminal segments are located within the membrane and flank a larger intracellular segment. This observation is further supported by the hydrophobicity analysis of the cloned amino acid sequence. Considering data (Figure 4.3), initial and terminal parts corresponding roughly to the segments 1 – 250 and 750 – 885 are compatible with a trans membrane localization of a peptide. However, the fraction flanked by those regions is low in hydrophobicity, and is compatible with the hydrophilic properties of a segment having cytosolic localization. It is conceivable to propose that the ends of the peptide correspond to the trans membrane segments while the latter one may correspond to intracellular Ca^{2+} binding domain of the exchanger. The intracellular domain has crucial role in regulation of the exchanger activity. In myocytes, it binds Ca^{2+} with a K_d of 100 nM and initiates Na^+ / Ca^{2+} exchange (2) . However, extracellular Ca^{2+} concentration of crayfish, 13.5 mM, is several folds larger than that in mammalian species. Thus, resting cytosolic Ca^{2+} concentration is calculated to be slightly larger than that in mammalian cells (*i.e.* 500 – 600 nM). However, with regard to the experiments conducted by using the Ca^{2+} sensitive fluorescent indicator OGBAPTA-1 (Figure 4.1), estimated value of the $[Ca^{2+}]_{in}$ should be less than 500 – 600 nM since K_d of the indicator for Ca^{2+} is about 170 nM. It is apparent that with regard to either one of the approximations resting Ca^{2+} would be larger than those in mammalian cells. When the intracellular domain of the cloned

amino acid sequence is compared with those present in mammalians, poor similarity rates are obtained (52 – 61 %). However, initial and terminal parts have higher rates of similarity through the species (63 – 76% for TMS1; 59 – 73% for TMS2). The observed difference can be interpreted as a functional consequence due to the possible differences in Ca^{2+} binding properties.

Present results indicate that the cloned nucleic acid sequence corresponds to a gene which most possibly codes a Na^{+} / Ca^{2+} exchanger. Thus, the exchanger is expected to have a distribution pattern dictated by the functional needs. Considering Figure 3.10 and Figure 3.11, exchanger mRNA is most abundantly expressed in excitable or biologically active tissues like ganglia, muscle and antennal gland (kidney). It is noteworthy to indicate that in gill which passively filters the neighboring fluid, expression of the exchanger is almost absent.

The putative alpha peptide of the crayfish Na^{+} / Ca^{2+} exchanger is closely related with Na^{+} / Ca^{2+} exchangers from other species (Figure 4.4). When its location in phylogenetic tree is considered, crayfish exchanger is genetically closest to exchangers from fugu and puffer fishes which express NCX4 genes. However, crayfish exchanger is relatively distant from mammalian NCX1 and NCX3 genes. The distant relationship between the NCX of aquatic animals and mammalians might be related to the differences in the environment.

In the present work, a crayfish gene coding Na^{+} / Ca^{2+} exchanger protein is firstly cloned at its full length and its molecular properties has been identified together with those properties of the alpha protein. Future efforts will be dedicated to define structure-function relationship of the discovered exchanger.



Name	Accession	Description	Interval	E-value
[+] Calx-beta	pfam03160	Calx-beta domain;	380-468	1.52e-37
[+] Calx_beta	smart00237	Domains in Na-Ca exchangers and integrin-beta4; Domain in Na-Ca exchangers and integrin ...	516-604	5.11e-23
[+] Na_Ca_ex	pfam01699	Sodium/calcium exchanger protein; This is a family of sodium/calcium exchanger integral ...	739-859	3.04e-20
[+] Na_Ca_ex	pfam01699	Sodium/calcium exchanger protein; This is a family of sodium/calcium exchanger integral ...	80-239	6.41e-20
[+] caca	TIGR00845	sodium/calcium exchanger 1; The Ca2+:Cation Antiporter (CaCA) Family (TC 2.A.19)Proteins of ...	10-883	0e+00

Figure 4.2. Domain hits of putative NCX amino acid sequence (21) .

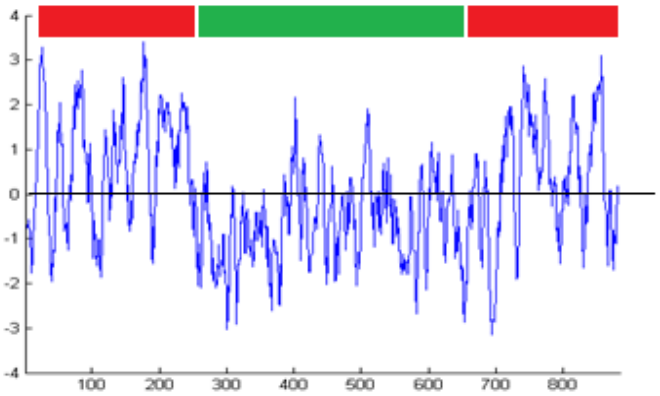


Figure 4.3. Kyte & Doolittle Hydrophobicity plot of Na^{+} / Ca^{2+} exchanger amino acid sequence. Red and green bars indicate transmembrane segments and intracellular loop, respectively.

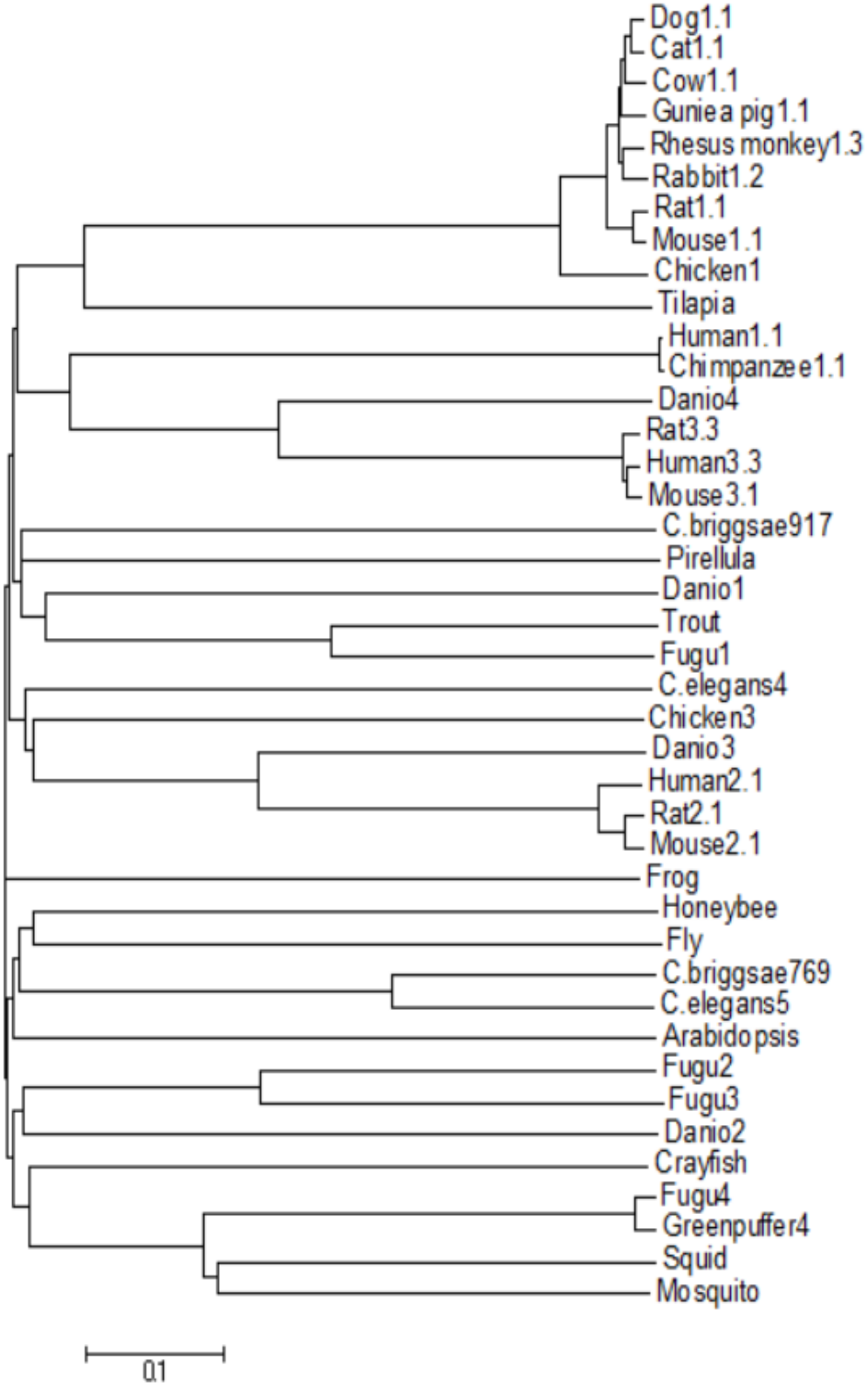

Dog1.1
Cat1.1
Cow1.1
Guniea pig1.1
Rhesus monkey1.3
Rabbit1.2
Rat1.1
Mouse1.1
Chicken1
Tilapia
Human1.1
Chimpanzee1.1
Danio4
Rat3.3
Human3.3
Mouse3.1
C.briggsae917
Pirellula
Danio1
Trout
Fugu1
C.elegans4
Chicken3
Danio3
Human2.1
Rat2.1
Mouse2.1
Frog
Honeybee
Fly
C.briggsae769
C.elegans5
Arabidopsis
Fugu2
Fugu3
Danio2
Crayfish
Fugu4
Greenpuffer4
Squid
Mosquito
0.1

Figure 4.4. **Evolutionary relationships of taxa.** The evolutionary history was inferred using the Neighbor-Joining method (11) . The optimal tree with the sum of branch length = 11.24262033 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (12) and are in the units of the number of amino acid differences per site. The analysis involved 41 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 432 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (13) .

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6. APPENDIX

6.1. RNA Isolation

Total RNA Isolation is performed with *RNeasy Mini Kit* (Qiagen). The method combines the use silica-based membranes and a spin technology. A special high salt buffer system enables the binding of RNA molecules that are longer than 200 nucleotides in size to a silica membrane. The selective isolation enriches the mRNA content of the total RNA since most of the ribosomal RNAs are shorter than 200 nucleotides. Other contaminating molecules are washed away during the procedure. Presence of guanidine-thiocyanate in the isolation buffer inactivates the RNases to prevent sample degradation (22) .

6.2. Quantification of Isolated Total RNA

NanoDrop (Thermo Scientific) is a widely used spectrophotometry device to determine nucleic acid concentration. Nucleic acids absorbs UV light at 260 nm and it is possible to determine the concentration of the sample from its absorbance. Proteins and phenolic compounds are possible RNA contaminants and they absorb UV light strongly at 280 nm. To determine the purity of RNA sample A 260 / A 280 ratio is also calculated. The ratio should be around 2.0 for a pure RNA sample. Another measure of RNA purity is A 260 / A 230 ratio. The ratio should be between 2.0 – 2.2 for a pure RNA sample. Any value lower than 2 indicates presence of contaminants that would absorb UV light strongly at 230 nm (EDTA, carbohydrates, TRIzol etc.) (23) .

6.3. cDNA Synthesis

Isolated and quantified total RNA should be converted into cDNA immediately to prevent any degradation. The enzyme that converts RNA to cDNA is reverse transcriptase and the enzyme is generally isolated from RNA viruses like avian myeloblastosis virus (AMV), Moloney murine leukemia virus (MMLV) or human

immunodeficiency virus (HIV). For production of single stranded cDNA; both activities of reverse transcriptase has been used: RNA – dependent DNA polymerase activity (24) and hybrid dependent exoribonuclease activity (RNase H activity)(25) . RNA – dependent DNA polymerase activity produces cDNA copy of existing RNA molecule where RNase H activity degrades the RNA strand in RNA – cDNA hybrid to prevent the re-use of RNA molecules as templates. Thus, RNase-H activity ensures that each RNA molecule is represented as cDNA, but only for once.

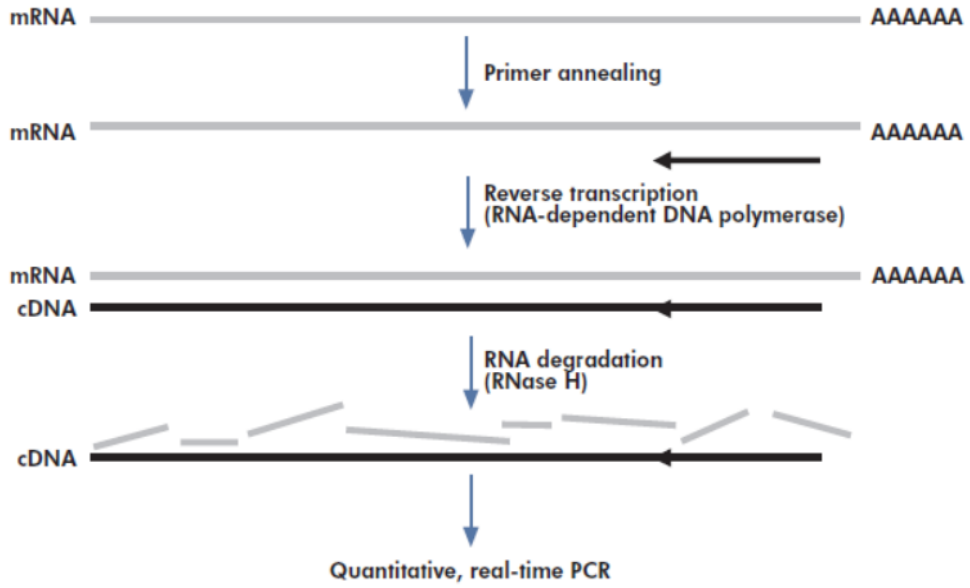

Figure 6.1. First strand cDNA synthesis by Quantitect Reverse Transcription Kit. After primer annealing, cDNA strand is synthesized, as being complementary to mRNA, by a reverse transcriptase enzyme. Then, RNase H activity of the enzyme degrades RNA from RNA: DNA duplex. Resulting cDNA pool is representative of initial RNA pool. (Devised from QuantiTect Reverse Transcription Kit Manual).

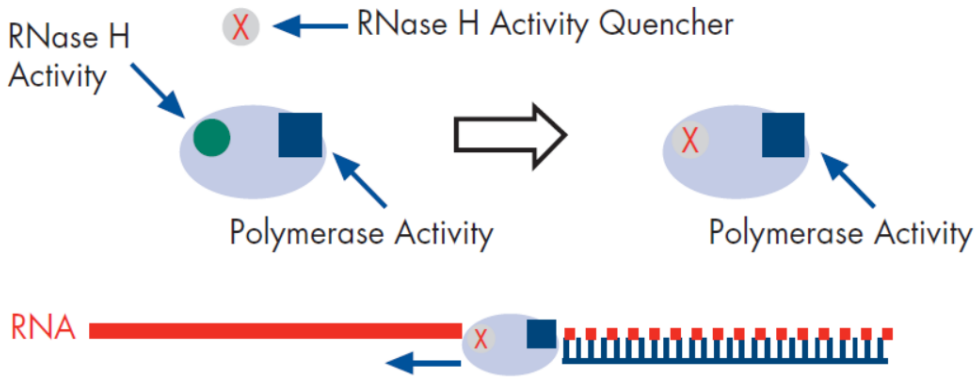

Figure 6.2. **First strand cDNA synthesis by LongRange 2Step Reverse Transcription kit.** RNase H activity of reverse transcriptase is quenched, so RNA template is not degraded. Obtained cDNA pool is not representative for initial RNA pool so this cDNA pool cannot be used for quantitative purposes (Devised from LongRange 2Step Reverse Transcription Kit Manual).

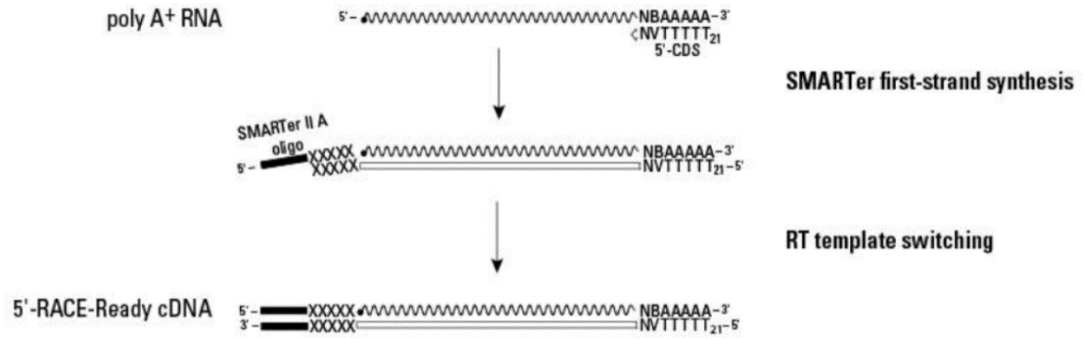

Figure 6.3. **Synthesis of 5' RACE cDNA.** First strand cDNA synthesis initiated from 3' end poly-A RNA with modified oligo-dT primer. SMARTer II A oligo is added to 5' end of complete RNA templates. Template switching of reverse transcriptase produces complete cDNA's with known sequences at 3' end. (Devised from 5' / 3' SMARTer RACE Kit Manual).

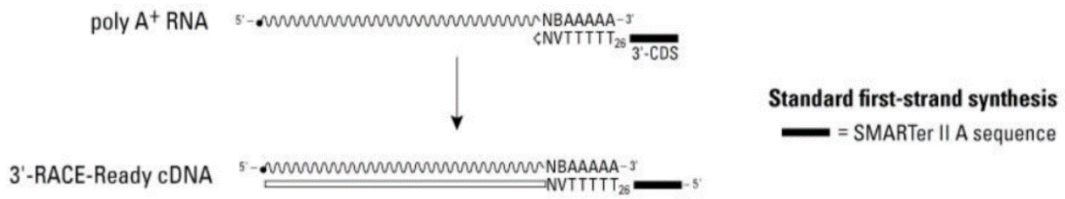

Figure 6.4. **Synthesis of 3' RACE cDNA.** First strand cDNA synthesis initiated from 3' end poly-A RNA with modified oligo-dT primer carrying SMARTer II A sequence at the 5' end. Resulting product have known sequences at 5' end. (Devised from 5' / 3' SMARTer RACE Kit Manual)

6.4. Polymerase Chain Reaction

Polymerase chain reaction is a method that is used to synthesize a new copy of the template DNA (26) . It is a rapid, specific, sensitive and flexible reaction that can be modified according to the purpose. Synthesis of the new copy is provided by the enzyme DNA polymerase by addition of free dNTPs in accordance with the template. Isolation of thermostable DNA polymerases from *Thermus aquaticus* (*Taq* DNA Polymerase) enabled the automatization of whole procedure. With thermostable enzymes and programmable thermal cyclers, PCR is a simple procedure with only several components:

PCR Buffer: Provides an optimal pH and monovalent salt environment for the reaction.

Mg^{+2}: Cofactor for polymerase.

dNTPS: Building bricks of the new copy.

Primer Pair: Provide 3'-OH for DNA polymerase to bind. Specifies the target area to be amplified. Primer pairs should be designed specifically for the target gene.

Template: DNA source to be amplified.

Water: Provides the liquid environment that other components interact and reaction take place. Possible source for contamination so particular attention should be paid.

In programmable thermal cyclers; reaction tubes are subjected to three-step cycling procedure. In first part; reaction mixture is heated up to 94 ^{o}C to denature double stranded DNA. After denaturation; mixture is cooled down to primer annealing temperature; which is generally between 55 ^{o}C and 65 ^{o}C. At this temperature primers bind to their specific target regions. Then the reaction is heated to 72 ^{o}C at which the *Taq* polymerase works optimally. During this step enzyme adds free nucleotides to growing chain of new copy at a rate of 1000 bases / minute.

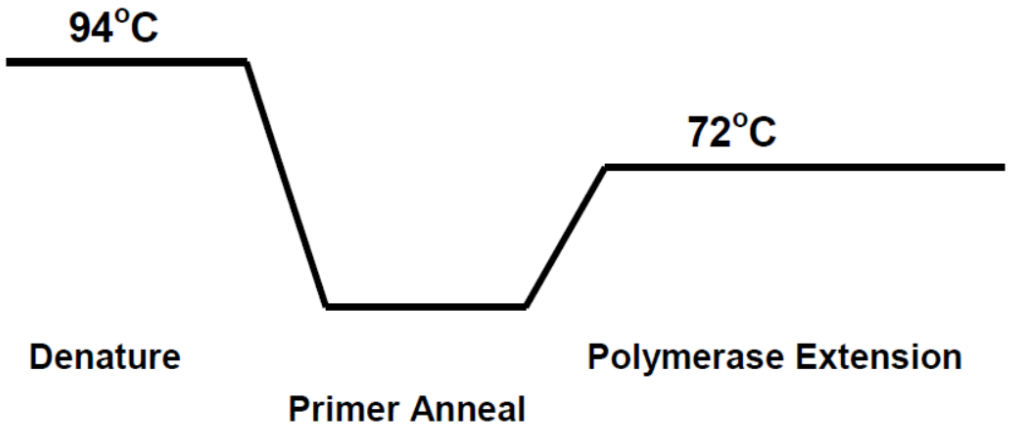

Figure 6.5. Three-step cycling protocol of PCR

6.5. Quantitative Polymerase Chain Reaction

Quantitave PCR (qPCR) is a variation of conventional PCR method developed by Higuchi et al (17) .qPCR has potential applications in forensic sciences, analysis of chromosome aberrations, single nucleotide polymorphism (SNP) analysis, pathogen detection and gene expression studies (18) . It is a powerful method for quantification of transcription levels due to its high sensitivity and reproducibility (19) .

One drawback of PCR is that the amount of product at the end of the reaction is not proportional to the amount of the starting material. For this reason conventional PCR does not give a comparative idea about the target amounts in two

different samples. However in qPCR, specific fluorescence dyes or probes are introduced to the reaction. Monitoring the signals from fluorescence dyes or probes during the course of reaction, it is possible to correlate the amount signal to the amount of starting material (18) .

SYBR Green I is a nucleic acid dye that is commonly used in molecular biology applications. *SYBR Green I* has ability to bind double stranded DNA. Bound dye – DNA complex absorbs light at 497 nm and emits light at 520 nm. These properties make SYBR Green I a suitable dye for real time detection of newly synthesized PCR products. Detection by *SYBR Green I* is easy, cheap and requires less effort as compared to other methods like *Taq Man* probes. But interpretation of results obtained with *SYBR Green I* can be confusing as dye binds nonspecifically to all double stranded DNA molecules, including primer dimers. There are several precautions should be taken to ensure the gene specific signal with *SYBR Green I* (27) :

- Primers should be specific to gene of interest and they should not show any homology to other regions.
- The size of the amplicon should be between 100-200 base pairs.
- Data acquisition from *SYBR Green I* should be performed at extension temperature or at a higher temperature to minimize the interference from primer dimers.
- After the amplification reaction, a melt analysis should be performed to differentiate specific amplicons from primer dimers or other non-spesific amplifications.

6.6. Agarose Gel Electrophoresis

After completion of a polymerase chain reaction, the results should be examined in terms of success, specificity and efficiency. Agarose gel electrophoresis is a fast, easy and reliable technique for visualization of PCR products. Agarose is a polysaccharide polymer extracted from seaweed and it has ability to form a porous matrix that can be used for variety of purposes. Agarose gels can be used for separation of PCR products under the electrophoretic conditions. DNA molecules are negatively charged due to their phosphate groups. There are two phosphate groups for each base pair in a double stranded DNA, so charge to mass ratio is constant for DNA fragments with different sizes. Electrophoresis separates DNA fragments under constant electric force. As the charge to mass ratio is constant; fragments are separated according to their sizes. With the presence of a molecular marker along with sample PCR products, the sizes of the fragments can be calculated. The intensities of the bands give a rough idea about the amount of PCR products. The concentration of agarose can be adjusted according to the size of PCR products to be visualized. The higher the concentration of the gel solution, the higher the resolution is. To visualize DNA fragments within the gel, a fluorescent tag called Ethidium Bromide is used. Ethidium bromide binds to double stranded DNA and absorbs UV light at 285 nm. Ethidium bromide is a carcinogen, specific precautions should be taken while working with Ethidium bromide (20) .

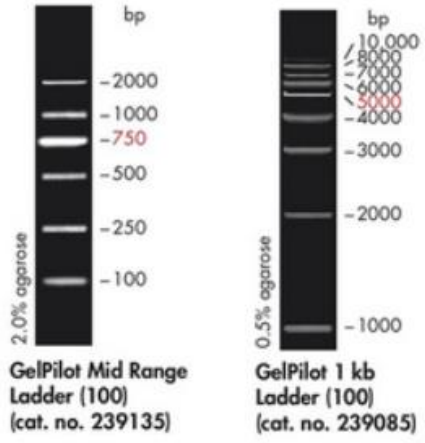

Figure 6.6. Gel photographs of molecular weight markers with different size ranges (28) .

6.7. Sanger Sequencing

Sequencing of obtained PCR products has been performed by using Sanger Sequencing (Chain Termination) method. Basically Sanger Method is a second PCR on a purified PCR product sample, with the use of only a single sense or anti-sense primer. This method has been based on the ability of *Taq* DNA polymerase to incorporate ddNTPs to a growing sequence. As ddNTPs lack –OH group at 3rd carbon, addition of a new nucleotide would be prevented and growth of chain would be terminated. The reaction has been repeated for 50 cycles with both dNTPs and ddNTPs and it is assumed that the reaction would terminate at each position with a ddNTP at least once. ddNTPs has been tagged with four different fluorescent dyes, so all the amplicons become tagged at their 3' ends after chain termination. The reaction mixture would be applied to an analyzer and fragments were separated via capillary electrophoresis. Fluorescently tagged fragments were read by the analyzer and the sequence of the amplicon would be revealed (29) .

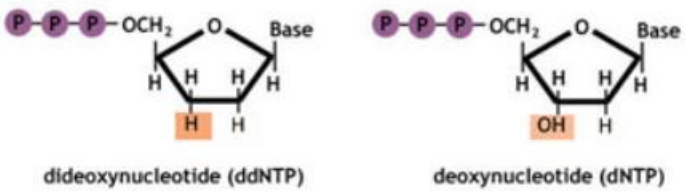

Figure 6.7. **Structural difference between dNTP and ddNTP.** Only difference between ddNTPs and dNTPs is hydroxyl group at 3rd carbon. A ddNTP can be added to a growing DNA chain but after a ddNTP, no more nucleotides can be added and chain growth is terminated (30) .

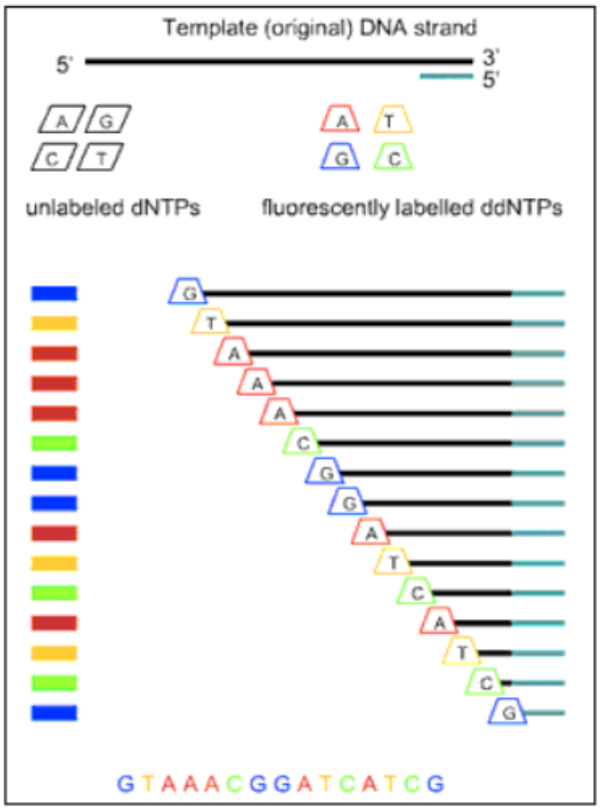

Figure 6.8. **Schematic representation of Chain Termination method.** DNA fragments terminated at each nucleotide are produced in a reaction mixture with dNTPs and ddNTPs. Each fragment is labeled with a different fluorescent tag from their 3' end (31) .