# Reference gene expression profiling in peripheral blood mononuclear cells of Rheumatoid Arthritis patients

Dissertation submitted in partial fulfilment for the degree of

Master of Science in Biotechnology

Submitted By

**Anisha Saha**

School of Biotechnology (Campus 11)

KIIT Deemed to be University

Bhubaneswar, Odisha, India

Under the Supervision of

**Dr. Bhawna Gupta**

Assistant Professor

School of Biotechnology

KIIT Deemed to be University

Bhubaneswar, Odisha

School of Biotechnology
Kalinga Institute of Industrial Technology (KIIT)
Deemed to be University
(Established U/S 3 of UGC Act, 1956)
Bhubaneswar, Odisha, India

# CERTIFICATE

This is to certify the dissertation entitled "**Reference gene expression profiling in peripheral blood mononuclear cells of rheumatoid arthritis patients**" submitted by **Anisha Saha** in partial fulfilment of the requirement for the degree of Master of Science in Biotechnology, KIIT School of Biotechnology, Kalinga Institute of Industrial Technology (KIIT), Deemed to be University, Bhubaneswar bearing Roll No. **1661038** & Registration No. **16529950264** is a bonafide research work carried out by her under my guidance and supervision from **15.12.2017** to **12.05.2018.**

Dr. Bhawna Gupta
Assistant Professor
School of Biotechnology
KIIT

'A' Category as per notification of Ministry of HRD, Govt. of India | 'Tier I' Accreditation (Washington Accord) by NBA for Engineering Streams | Accredited by NAAC in 'A Grade' | Accredited by IET, U.K. (B.Tech. Programme)
At/PO: KIIT Campus- 11, Bhubaneswar-751 024(Odisha), India
Tel:91-674-2725466,2725349 Fax:91-674-2725732 E-mail: institute@kiitbiotech.ac.in Website: www.kiitbiotech.ac.in

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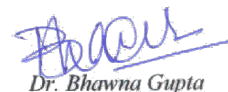
This is also to certify that the above said work has not previously submitted for the award of any degree, diploma, fellowship in any Indian or foreign University.

*Date: 12.05.2018*

*Place: Bhubaneswar*



*Dr. Bhawna Gupta*

## DECLARATION

I hereby declare that the dissertation entitled **"Reference gene expression profiling in peripheral blood mononuclear cells of rheumatoid arthritis patients"** submitted by me, for the degree of Master of Science to KIIT Deemed to be University is a record of bona fide work carried by me under the supervision and guidance of **Dr. Bhawna Gupta, Assistant Professor, School of Biotechnology, KIIT Deemed to be University, Bhubaneswar, Odisha, India.**

Date: 14/05/2018
Place: Bhubaneswar

Anisha Saha
Anisha Saha

## ABSTRACT

Rheumatoid Arthritis (RA) is one of the most prevalent autoimmune, systemic, chronic, inflammatory diseases. The aetiology of the disease is unknown and predictive biomarkers to assess disease predisposition and progression are scarce; that's why several better treatment strategies fail to generate any response.

The foundation of all molecular processes is Central dogma. Efficient translation of RNA is important to produce required proteins in adequate amounts. Any discrepancy in translation processes due to mutation in genomic transcript and non-availability of transcription factor can lead to aberrant proteins. Hence, looking into expression levels of key genes will help in designing new therapeutics. Quantitative real- time PCR is one of the widely used techniques to assess RNA expression levels, where all candidate genes expression levels are checked and compared with a housekeeping or reference gene.

Housekeeping genes are typically constitutive genes that are required for the maintenance of basic cellular function, and are expressed in all cells of an organism under normal and patho-physiological conditions at constant rate. But in disease condition like rheumatoid arthritis, we found widely used housekeeping genes like β-actin, β-globin and GAPDH are not expressed at relatively constant rate. Hence, for the first time we tried to identify a potent housekeeping gene which can be used as a reference gene for transcript analysis as well as protein quantification for other target genes or proteins.

For this study, PBMCs were collected from the blood of rheumatoid arthritis patients followed by isolation of RNA by TRIzol method and quantification using Nano-drop. cDNA was prepared and used for qPCR experiments. Seven housekeeping genes SDHA, TBP, RPL13A, HPRT1, PBGD, RPS9 and β-actin have been used in our study. Careful examination of the melting curve, dissociation curve, melting peaks and $C_t$ values have been compared between patients and healthy controls. This resulted in identification of two potent genes PBGD and SDHA as housekeeping genes for any RNA expression studies involving rheumatoid arthritis.

## ACKNOWLEDGEMENTS

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Date: 14/05/2018

Place: Bhubaneswar

Anisha Saha

Signature

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## LIST OF ABBREVIATIONS/ACRONYMS

1. mRNA – Messenger RNA
2. PBMC- Peripheral Blood Mononuclear Cells
3. qPCR- Quantitative PCR
4. DNA - Deoxyribonucleic acid
5. RNA- Ribonucleic acid
6. RF- Rheumatoid Factor
7. ESR- Erythrocyte Sedimentation Rate
8. C-RP- C-Reactive Protein
9. ACCP-Anti Cyclic Citrullinated Peptide
10. RA-Rheumatoid arthritis
11. cDNA- Complementary DNA
12. RT- Reverse Transcriptase
13. PBS- Phosphate Buffer Saline
14. MCT- Micro centrifuge tube
15. RBC- Red blood cells
16. UTR - Untranslated region
17. µg – Microgram
18. µl – Microliter
19. ml – Millilitre
20. mg – Milligram
21. °C - Degree Celsius
22. nm – Nanometer
23. KCl- Potassium chloride
24. NaCl- Sodium Chloride
25. KCl- Potassium Chloride
26. $Na_2HPO_4$- Sodium hydrogen phosphate
27. $KH_2PO_4$- Potassium dihydrogen phosphate
28. dNTPs- deoxyribonucleotide triphosphate

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# CHAPTER 1

## INTRODUCTION

### 1.1 BACKGROUND AND CONTEXT:

An autoimmune disease is caused due to abnormal immune response of our immune system against self-antigens. These diseases result from failure of the host's cellular and humoral immune systems to differentiate self antigens from non-self, resulting in attack on self-cells and organs by auto antibodies. An autoimmune disease is a condition in which immune system erroneously attacks body. The immune system normally guards against germs like bacteria and virus. Open these sense unfamiliar attackers, it sends out an army of fighter cells to attack immune cells. The immune system can tell the difference between foreign cells and own cells. In an autoimmune disease the immune system mistakes component of our body-like joints or skin as foreign. Autoimmune cells release proteins called as autoantibodies that attack healthy cells.[1]

Rheumatoid Arthritis (RA) is one of the most prevalent autoimmune, systemic, chronic, inflammatory diseases. It primarily involves joints. RA mostly affects women as compared to men and prevalence increases with age most commonly presenting between 50 & 70 years. RA is an autoimmune disorder which involves migration of immune cells to the synovium of joints. Synovium is the part which surrounds the joint and is filled with synovial fluid for easy movement of the joints. When immune cells drift into the joints they produce cytokines which causes joint damage and inflammation.[2]

## 1.2 SCOPE AND OBJECTIVES:

As the housekeeping genes are constitutively active gene in our body which are typically required for the maintenance of basic cellular function, and are expressed in all cells of an organism under normal and disease conditions. But, recent studies on housekeeping genes were deficient in RA patients and healthy people. So, our main aim of study was to find out the housekeeping gene whose expression profile is constant in all individuals.

## 1.3 ACHIEVEMENT:

This study is performed to evaluate and identify a stable housekeeping gene in PBMCs of RA patients and healthy controls. We analyzed the mRNA expression patterns of housekeeping genes in the RA; an autoimmune disease in the Indian case-control population. We created collaboration with clinicians, streamlined blood collection protocols performed qPCR. From the study we were able to identify stable housekeeping gene which can be used in future for every gene expression analysis studies involving RA and HC PBMCs using qPCR.

## 1.4 OVERVIEW OF DISSERTATION:

Blood sample was collected from RA patients; healthy age and sex matched individuals. PBMCs were isolated using ficoll based density gradient centrifugation. RNA was isolated by TRIzol method and quantified using Nano drop. Subsequently, cDNA was synthesized by taking 500ng of total RNA. For transcript analysis quantitative real time PCR was done. After getting $C_t$ value data was analyzed using Graph Pad prism 5. Seven housekeeping genes including SDHA, TBP, RPL13A, HPRT1, PBGD, RPS9 and β-actin were taken in our experiment. Melting curve, dissociation curve and melting peaks for every gene were analyzed by using Roche software. $C_t$ value of each gene of patients was compared with the $C_t$ value for healthy donors in Prism software. Similar expression of PBGD and SDHA genes for both patients and controls was observed. Depending on the experiment it was concluded that PBGD and SDHA can be used as best housekeeping gene for RA.

# CHAPTER 2

## REVIEW OF LITERATURE

### 2.1 AUTOIMMUNE DISEASE

The immune system malfunctions and a breakdown in self tolerance occurs. It could be caused by a sudden inability to distinguish between self and non-self by a causing an immune attack on host tissues. This condition is called autoimmunity.

The most common areas in the body which are targeted by autoimmune diseases are the thyroid gland, stomach, adrenal glands and pancreas, systemic autoimmune diseases.

Different autoimmune diseases often co-exist within family members which point out, that common genes underlie multiple autoimmune diseases, and several diseases may share similar pathogenic pathways[3].

This definition includes a variety of diseases which can be described by the irregular functioning of the immune system that causes an individual’s immune system to generate antibodies which attack their own body tissues., The development of autoimmune disease occurs as a result of an overactive immune response to body material and tissues present in the body. The body attacks its own cells.

An individual’s immune system protects one from disease and infection. If a person has an autoimmune disease, their immune system inaccurately attacks healthy cells in their body. There are currently more than eighty various kinds of autoimmune diseases, and many of them have alike symptoms.

SMOKING AND AUTOIMMUNE DISEASES:

Tobacco smoking is one of the most powerful environmental factors that could prompt autoimmune diseases. Smoking has been associated with Systemic lupus erythematosus. The prevalence of smoking with rheumatoid arthritis is even more established. Smoking was found to be related to the incidence of seropositive rheumatoid arthritis[4].

Autoimmunity is defined as the development of immune system reactivity in the form of auto-antibodies and T-cell responses to self structures. A variety of diseases which can be described by the irregular functioning of the immune system that causes an individual’s immune system to generate antibodies which attack their own body tissues[4].

FAMILY HISTORY OF AUTOIMMUNE DISORDERS:

Numerous studies have shown that the tendency to develop autoimmune disorders can be genetic. If a family has members who have an autoimmune disorder, others within the family have increased chances of getting the same disorder or one that is closely related[5].

Some examples of autoimmune diseases:

Rheumatoid Arthritis, Systemic Lupus Erthematosus, Reactive Arthritis, Sjogren's Syndrome, Diabetes Mellitus Type1, Multiple Sclerosis.

**RHEUMATOID ARTHRITIS**:

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by abnormal immune responses to self-antigens. It is a chronic, systemic inflammatory illness that may affect many tissues and organs, but primarily attacks synovial joints, resulting in pain, stiffness and swelling of the joints. The disease produces a surplus of synovial fluid. The pathology of the disease process often leads to the severe damage of articular cartilage and ankylosis of the joints.

SIGNS AND SYMPTOMS

Symptoms include pain, swelling, stiffness; joints become inflamed for unknown reasons. There is no permanent treatment for rheumatoid arthritis. RA is characterized by symmetrical polyarthritis, which is caused by chronic inflammation in the synovial membranes leading to cartilage and joint destruction[6]. Early diagnosis and aggressive treatments are key if the damage caused by this disease is to be controlled.

RA usually lasts many years or an entire lifetime. For some people, RA can last for only a few months to a few years with treatment, although this is rare.

Though the pathogenesis of RA is not yet fully elucidated, research has shown that it can be induced by environmental factors on a genetically susceptible background. This condition leads to abnormality in antigen recognition and presentation, lymphocyte activation as well as differentiation ultimately resulting in enhanced production of pro-inflammatory cytokines and auto-antibodies which eventually cause damage to joints.

RA is less common than other kinds of arthritis such as osteoarthritis. More than 1 million people in the United States have RA. Women are more likely to have RA than men. About 7 out of every 10 people with RA are women. Although RA can happen at any age, it usually develops between ages 50 and 70.

RA is an autoimmune disease of unknown aetiology that primarily targets synovial tissues, cartilage and bone, and is the most common form of immune-mediated arthritis. Although the armamentarium of therapies to treat RA is extensive, optimal symptom control and management of the disease have been elusive. High disease activity, joint damage and disability remain serious issues for patients and physicians and result partially from variable patient responses to therapy. The uniqueness of each patient requires an equally unique treatment regimen that is specifically tailored to that patient. Tailored therapy must follow the general principle of optimizing response by reducing symptoms and stopping further damage[7].

RA is defined using the American Rheumatoid Association (ARA) 1987 criteria. Patients with RA develop a symmetrical polyarthritis, which characteristically affects the small joints of the hands and feet (sparing the distal interphalangeal joints), along with involvement of other joints as the disease progresses.

Although useful for classification, the ARA criteria should not be used for initial diagnosis, because some ARA diagnostic features are found more in advanced disease.

Rheumatic diseases are considered public health problems affecting millions of people worldwide resulting in high and rising health-care costs. For example, in 2003, the United States of America spent a total of 128 billion US$ on rheumatoid arthritis (RA), which is just one type of rheumatic disease. Of this sum, 3.6 billion US$ were spent on drug therapies and about US$ 14,000 on cardiovascular disease associated with an increased prevalence of coronary artery disease present in RA. In the period from 2007 to 2009, US statistical data indicate an increase of approximately a million of new RA cases per year. RA affects approximately 0.5-1% of the world population, being more common in women than in men, and in ages between 40 and 60 years. RA is the most common systemic autoimmune disease in the world, and it causes functional disability and

premature death. Approximately 70% of patients have irreversible joint destruction and 80% of active young adults in the labor market are affected by stiffness and devastating pain. This situation generates a big loss of daily activities and vocational productivity resulting in significant reduction in quality of life. Regarding the etiology of the disease, cigarette smoking is described as a possible risk factor for the onset or worsening of RA. Smoking is associated with a worse prognosis of the disease. In contrast, little has been said about better eating habits or the benefits of physical activity in this population. The implementation of programs of physical training and nutrition education could provide greater independence and quality of life to RA patients. It is necessary to explore these parameters within a public health system. The clinical approach to diagnosis RA is based on the standard procedure established by the ACR (American College of Rheumatology) in 1987. However, this has been widely criticized due to the lack of sensitivity for early detection of the disease. The ACR and European League Against Rheumatism (EULAR) has developed a new set of criteria to classify RA based on new laboratory parameters such as anti-citrullinated peptide antibodies (anti-ACPA), C-Reactive protein (CRP), and Rheumatoid factor (RF). The new set of criteria aims to identify early RA patients in order to institute early drug therapy, thereby reducing the functional disability and articular lesions. High concentrations of CRP, RF, and ACPA in serum are associated with unfavorable outcomes, including persistent disease, joint destruction, and functional.

**SYSTEMIC LUPUS ERTHEMATOSUS (SLE)**

SLE is a chronic autoimmune disease that can affect almost any organ system. Patients with lupus have unusual antibodies in their blood that are targeted against their own body tissues. Lupus can cause disease of the skin, heart, lungs, kidneys, joints, and nervous system. When only the skin is involved, the condition is called discoid lupus. When internal organs are involved, the condition is called systemic lupus erythematosus (SLE). Up to 10% of persons with discoid lupus (lupus limited to the skin) eventually develop the systemic form of lupus (SLE)[8].

SLE (lupus) is a disease that can damage many parts of the body, like joints, skin, kidneys, heart, lungs, blood vessels, and brain. It is a chronic, inflammatory, connective

tissue disease that can affect the joints and many organs, including the skin, heart, lungs, kidneys, and nervous system. It can cause many different symptoms not everyone with SLE has all of the symptoms. It is also called as lupus and SLE. Lupus occurs when the immune system, which normally helps protect the body from infection and disease, attacks different parts of the body[9]. It can have phases of worsening symptoms that alternate with periods of mild symptoms. Most people with SLE are able to live a normal life with treatment.

SIGNS AND SYMPTOMS

Some of the symptoms of SLE are:-Severe fatigue, hair loss, joint pain and swelling, headaches, anaemia, blood clotting problems, fingers turning white or blue and tingling when cold, which is known as Raynaud's phenomenon, a rash on the cheeks and nose, which is called as "Butterfly rash" other symptoms depend on the part of the body the disease is attacking[10].

**REACTIVE ARTHRITIS**

Reactive arthritis referred to as Reiter's syndrome, is a form of arthritis that affects the joints, eyes, urethra (the tube that carries urine from the bladder to the outside of the body), and skin. The disease is recognized by various symptoms in different organs of the body that may or may not appear at the same time. Reactive arthritis is a type of arthritis that an infection in the body can trigger. Most commonly, a sexually transmitted infection or bacterial infection in the intestines triggers development of reactive arthritis. It primarily affects sexually active males between the ages of 20 and 40. Those with HIV (human immunodeficiency virus) are at a particularly high risk. The cause of reactive arthritis is still unknown, but research suggests the disease is caused, in part by a genetic predisposition. Approximately 75% of those with the condition have a positive blood test for the genetic marker HLA-B2[11].

The first symptoms of reactive arthritis are painful urination and a discharge from the penis if there is inflammation of the urethra. Diarrhea may occur if the intestines are affected. This is then followed by arthritis 4 to 28 days later that usually affects the fingers,

toes, ankles, hips, and knee joints. One or a few of these joints may be affected at one time[11].

**MULTIPLE SCLEROSIS**

Multiple sclerosis (MS) is a potentially disabling disease of the brain and spinal cord (central nervous system). In MS, the immune system attacks the myelin sheath that covers nerve fibers and causes communication problems between your brain and the rest of your body. The disease can cause the nerves themselves to deteriorate or become permanently damaged.

SIGNS AND SYMPTOMS

It is vary widely depend on the amount of nerve damage and which nerves are affected. Some people with severe MS may lose the ability to walk independently or at all, while others may experience long periods of remission without any new symptoms[12].There are no cure for multiple sclerosis. Multiple sclerosis signs and symptoms may differ greatly from person to person and over the course of the disease depending on the location of affected nerve fibers. They may include:

Numbness or weakness in one or more limbs that typically occurs on one side of your body at a time, or the legs and trunk, Partial or complete loss of vision, usually in one eye at a time, often with pain during eye movement, prolonged double vision, tingling or pain in parts of your body, electric-shock sensations that occur with certain neck movements, especially bending the neck forward (Lhermitte sign),Tremor, lack of coordination or unsteady gait, Slurred speech, Fatigue, Dizziness, Problems with bowel and bladder function[13].

**SJOGREN'S SYNDROME**

Sjogren's syndrome is a chronic autoimmune disorder of the exocrine glands with associated lymphocytic infiltrates of the affected glands. Dryness of the mouth and eyes results from involvement of the salivary and lacrimal glands. The accessibility of these glands to biopsy enables study of the molecular biology of a tissue-specific autoimmune process. A new international consensus for diagnosis requires objective signs and symptoms of dryness including a characteristic appearance of a biopsy sample from a

minor salivary gland or autoantibody such as anti-SS-A. The most difficult challenge in diagnosis and therapy is patients with symptoms of fibromyalgia (arthralgia, myalgia, fatigue) and oral and ocular dryness in the presence of circulating antinuclear antibodies[14].

**TYPE II DIABETES**

Diabetes mellitus (DM) is probably one of the oldest diseases known to man. It was first reported in Egyptian manuscript about 3000 years ago[15]. In 1936, the distinction between type 1 and type 2 Diabetes was clearly made. Type 2 Diabetes was first described as a component of metabolic syndrome in 1988.Type 2 DM (formerly known as non-insulin dependent DM) is the most common form of DM characterized by hyperglycemia, insulin resistance, and relative insulin deficiency. Type 2 Diabetes results from interaction between genetic, environmental and behavioral risk factors[16].

Type 2 diabetes mellitus (T2DM) is a metabolic disorder and typically results from excess of caloric intake over energy expenditure. It is characterized by a progressive insulin secretory defect due to insulin resistance, which increases the body's demand for insulin in order to retain glucose homeostasis. If pancreatic β-cells fail to secrete enough insulin to compensate for increasing insulin demand, the blood glucose level will be elevated gradually[17]. Chronic hyperglycemia is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels resulting in increasing levels of morbidity and mortality[18].

Hyperglycemia is a major symptom in T2DM. Other typical symptoms of T2DM include polyuria, polydipsia, fatigue, and weight loss and urine glucose. Diabetes is usually diagnosed based on plasma glucose criteria. The most widely accepted T2DM diagnostic tests are the Fasting Plasma Glucose (FPG) and the Oral Glucose Tolerance Test (OGTT). Both FPG (diagnostic of diabetes at plasma glucose level ≥ 126 mg/dL or 7.0 mmol/L) and 2-hour OGTT (diagnostic of diabetes at plasma glucose level ≥ 200 mg/dL or 11.1 mmol/L) are commonly used diagnostic tests[19].

**PERIPHERAL BLOOD MONONUCLEAR CELLS**

In blood there are three main types of mononuclear cells which help body to fight diseases including infectious diseases, cancers, and leukemia's. They are monocytes, lymphocytes, and macrophages.

Lymphocytes include around 20% of the peripheral white blood cells and get from the basic lymphoid progenitor cell in the bone marrow. They have a huge, relatively round nucleus encompassed by an extremely particular measure of cytoplasm. The unmistakable quality of this normal nucleus has prompted them being alluded to as mononuclear leukocytes, or in more extensive terms, peripheral blood mononuclear cells (PBMCs)[20].

The most imperative normal for these cells is their capacity to explicitly perceive foreign particles and wreck it.

Three major lymphocytes are:

1. T-Cell (Thymus derived)

Cell surface contains immunizer like receptors that see sections of antigens on the surface of contaminated or malignant cells.

2. B-Cell (Bone Marrow derived)

Create antibodies that basically snare outside antigens circling in the circulation system.

3. Natural Killer (NK Cells)

Important player in the innate arms of the immune system and they provide an early defense against pathogens and tumor transformed cells[21].

Other PBMCs in humans:

1. Monocytes, which form into macrophages, inundate and process the pathogen and present parts on their surfaces as a flag. The flag is then gotten by different cells in the versatile invulnerable framework.

2. Dendritic Cells are antigen giving cells a one of a kind capacity to initiate primary immune reactions. They catch and exchange data from the outside world to the cells of the adaptive immune system.

There are promising immune activities in PBMCs that reach well beyond fighting infection. Research is being conducted to understand their role in cancer and other diseases. By identifying surface and cellular markers, it will be easier for researchers to identify the immune populations that would most benefit from this type of cell therapy[22].

**HOUSEKEEPING GENE**

The origin of the term "housekeeping gene" remains obscure. Literature from 1976 used the term to describe specifically tRNA and rRNA. For experimental purposes, the expression of one or multiple housekeeping genes is used as a reference point for the analysis of expression levels of other genes. The key criterion for the use of a housekeeping gene in this manner is that the chosen housekeeping gene is uniformly expressed with low variance under both control and experimental conditions. Validation of housekeeping genes should be performed before their use in gene expression experiments such as RT-PCR[23].

Housekeeping genes are typically constitutive genes that are required for the maintenance of basic cellular function, and are expressed in all cells of an organism under normal and patho-physiological conditions. Some housekeeping genes are expressed at relatively constant rates in most non-pathological situations, the expression of other housekeeping genes may vary depending on experimental conditions. Housekeeping genes are involved in basic cell maintenance and therefore, are expected to maintain constant expression levels in all cells and conditions. Identification of these genes facilitates exposure of the underlying cellular infrastructure and increases understanding of various structural genomic features. In addition, housekeeping genes are instrumental for calibration in many biotechnological applications and genomic studies. Advances in our ability to measure RNA expression have resulted in a gradual increase in the number of identified housekeeping genes. Here, we describe housekeeping gene detection in the era of massive parallel sequencing and RNA-seq. We emphasize the importance of expression at a constant level and provide a list of 3804 human genes that are expressed uniformly across a panel of tissues. Several exceptionally uniform genes are singled out for future

experimental use, such as RT-PCR control genes. Finally, we discuss both ways in which current technology can meet some of past obstacles encountered, and several as yet unmet challenges[24].

Control genes, which are often referred to as housekeeping genes, are frequently used to normalize mRNA levels between different samples. The expression level of these genes may vary among tissues or cells and may change under certain circumstances. So, the selection of housekeeping genes is critical for gene expression studies[25].

**Q-PCR**

Real-time polymerase chain reaction (Real-Time PCR) is also known as quantitative polymerase chain reaction (qPCR) which is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR, i.e., in real-time, and not at its end, as in conventional PCR. Real-time PCR can be used quantitatively (quantitative real-time PCR), and semi-quantitatively i.e., above or below a certain amount of DNA molecules (semi quantitative real-time PCR).

Two common methods for the detection of PCR products in real-time PCR are:

(1) Non-specific fluorescent dyes that intercalate with any double-stranded DNA, and

(2) sequence-specific DNA probes consisting of oligo-nucleotides that are labelled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence[26].

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines propose that the abbreviation qPCR is used for quantitative real-time PCR and that RT-qPCR is used for reverse transcription–qPCR. The acronym "RT-PCR" commonly denotes reverse transcription polymerase chain reaction and not real-time PCR, but not all authors adhere to this convention.

Real-time PCR is carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of at least one specified wavelength and detect the fluorescence emitted by the excited fluorophore. The thermal cycler is also able to rapidly

heat and chill samples, thereby taking advantage of the physicochemical properties of the nucleic acids and DNA polymerase.[27]

The PCR process generally consists of a series of temperature changes that are repeated 25–50 times. These cycles normally consist of three stages: the first, at around 95°C, allows the separation of the nucleic acids double chain; the second, at a temperature of around 50-60°C, allows the binding of the primers with the DNA template; the third, at between 68-72°C, facilitates the polymerization carried out by the DNA polymerase. Due to the small size of the fragments the last step is usually omitted in this type of PCR as the enzyme is able to increase their number during the change between the alignment stage and the denaturing stage. In addition, in four step PCR the fluorescence is measured during short temperature phase lasting only a few seconds in each cycle, with a temperature of, for example, 80°C, in order to reduce the signal caused by the presence of primer dimers when a non-specific dye is used. The temperatures and the timings used for each cycle depend on a wide variety of parameters, such as: the enzyme used to synthesize the DNA, the concentration of divalent ions and deoxyribonucleotides (dNTPs) in the reaction and the bonding temperature of the primers[28].

A primer is a short strand of RNA or DNA (generally about 18-22 bases) that serves as a starting point for DNA synthesis. It is required for DNA replication because the enzymes that catalyze this process, DNA polymerases, can only add new nucleotides to an existing strand of DNA. The polymerase starts replication at the 3′-end of the primer, and copies the opposite strand.

Basic rules to design primers:

i. Primer length: The optimal length of PCR primer is 18-22bp. This length is long enough for adequate specificity and short enough for primers to bind easily to the template at the annealing temperature.

ii. Primer melting temperature: Melting Temperature ($T_m$) is the temperature at which one half of the DNA duplex will dissociate to become single stranded and indicates the

duplex stability. Primers with melting temperatures in the range of 52-58$^{o}$C generally produce the best results. Primers with melting temperatures above 65$^{o}$C have a tendency for secondary annealing. The GC content of the sequence gives a fair indication of the primer $T_m$.

iii. Primer annealing temperature: The primer melting temperature is the estimate of the DNA-DNA hybrid stability and critical in determining the annealing temperature. Too high Ta will produce insufficient primer-template hybridization resulting in low PCR product yield. Too low Ta may possibly lead to non-specific products caused by a high number of base pair mismatches. Mismatch tolerance is found to have the strongest influence on PCR specificity.

iv. GC content: The GC content (the number of G's and C's in the primer as a percentage of the total bases) of primer should be 40-60%.

v. GC clamp: The presence of G or C bases within the last five bases from the 3' end of primers (GC clamp) helps promote specific binding at the 3' end due to the stronger bonding of G and C bases. More than 3 G's or C's should be avoided in the last 5 bases at the 3' end of the primer.

vi. Primer secondary structures: Presence of the primer secondary structures produced by intermolecular or intramolecular interactions can lead to poor or no yield of the product. They adversely affect primer template annealing and thus the amplification. They greatly reduce the availability of primers to the reaction. Hairpins: It is formed by intramolecular interaction within the primer and should be avoided.

vii. Repeats: A repeat is a di-nucleotide occurring many times consecutively and should be avoided because they can misprime. For example: ATATATAT. A maximum number of di-nucleotide repeats acceptable in an oligois 4 di-nucleotides.

viii. Runs: Primers with long runs of a single base should generally be avoided as they can misprime. For example, AGCGGGGGATGGGG has runs of base 'G' of value 5 and 4. A maximum number of runs accepted is 4bp.

ix. 3’End stability: It is the maximum ΔG value of the five bases from the 3' end. An unstable 3' end (less negative ΔG) will result in less false priming.

x. Avoid template secondary structure: A single stranded Nucleic acid sequences is highly unstable and fold into conformations (secondary structures). The stability of these template secondary structures depends largely on their free energy and melting temperatures($T_m$). Consideration of template secondary structures is important in designing primers, especially in qPCR. If primers are designed on secondary structures which are stable even above the annealing temperatures, the primers are unable to bind to the template and the yield of PCR product is significantly affected. Hence, it is important to design primers in the regions of the templates that do not form stable secondary structures during the PCR reaction. Our products determine the secondary structures of the template and design primers avoiding them.

xi. Avoid cross homology: Improve the specificity of the primers it is necessary to avoid regions of homology. Primers designed for a sequence must not amplify other genes in the mixture. Primers are designed and then BLAST to test the specificity. Our products offer a better alternative. You can avoid regions of cross homology while designing primers. You can BLAST the templates against the appropriate non-redundant database and the software will interpret the results. It will identify regions significant cross homologies in each template and avoid them during primer search[29].

# CHAPTER-3

## AIM AND OBJECTIVES:-

1. Blood sample collection
2. To isolate PBMCs from blood sample
3. To analyze the expression of various housekeeping genes in PBMCs of RA patients for identification of an appropriate reference genes for qPCR.

# CHAPTER-4

## MATERIAL AND METHODS:-

OBJECTIVE 1: Blood sample collection

Blood sample was collected from RA patients and healthy control.

REQUIREMENTS:-

- Icebox
- Syringes (DispoVan)
- Tourniquet
- Spirit
- Cotton swab
- Heparin coated Vials

THEORY-

Collection of blood samples from patients is a tough job as it involves ethical issues, so blood was collected by a trained medical practitioner after a written consent duly signed from the donor. We prepared a pro forma that mentions about various clinical factors to be considered including, disease duration, number of actively inflamed joints, SGPT,ESR (Erythrocyte Sedimentation Rate) and medications etc. To prevent blood coagulation we used heparin coated vials.

**Heparin** is an anticoagulant; it binds to anti thrombin which then inactivates thrombin and other coagulation factors and inhibits coagulation of blood.

**Single use syringe** is used to prevent infection and other transferrable diseases. Icebox is used to maintain a stable temperature for sample storage.

PROTOCOL

- After receiving duly signed consent form along with a pro-forma (as designed by the clinicians and used in our lab) filled up by the doctors, the nurse tied tourniquet around the patients arm.
- The patient was pricked with the help of a single use syringe, three milliliters to five milliliters of blood was collected and kept in a Heparin (lithium) coated vacutainer.

- The vial was inverted 2-3 times slowly to mix the blood with heparin.
- The vial was kept inside an icebox.
- The syringe was discarded according to the hospital protocol.
- Blood samples from healthy controls were collected from the blood bank.
- The samples were immediately brought to the lab for processing.

| **Clinical and para clinical variables** | **RA patients (n=3) Mean $\pm$ SEM** |
|---|---|
| Gender (F/M) | 2/1 |
| Age (years) | 34.00 $\pm$ 5.03 |
| Disease duration (years) | 4.66 $\pm$ 0.88 |
| DAS | 7.0 $\pm$ 0.57 |
| Swollen joint count | 4.3 $\pm$ 3.2 |
| Tender joint count | 6.2 $\pm$ 3.2 |
| ESR (mm/1st h) | 20.33 $\pm$ 2.60 |
| SGPT (mg/L) | 23.00 $\pm$ 1.73 |
| RF positive (%) | 100 |
| Anti-CCP (%) | 100 |

Table 4.1: Characteristics of 3 patients with rheumatoid arthritis included in the study; DAS: Disease activity score, ESR: Erythrocyte sedimentation rate, RF: Rheumatoid factor, CCP: Cyclic citrullinated peptide.

## OBJECTIVE 2: Isolation of PBMCs from blood sample

REQUIREMENTS:-

- Blood sample
- Ficoll
- PBS
- RBC lysis buffer
- Freezing media (for storage of cells)

- Pipettes (1ml, 100ul)
- Falcon tubes (15ml)
- Autoclave MCTs (1.5ml)
- MCT stands
- Discards (liquid & solids)
- Tissue paper
- 70% Ethanol
- Gloves
- Parafilm

THEORY:

Peripheral blood is the primary source of lymphoid cells for investigation of the human immune system. Its use is facilitated by Ficoll-Hypaque density gradient centrifugation-a simple and rapid method of purifying peripheral blood mononuclear cells (PBMC) that takes advantage of the density differences between mononuclear cells and other elements found in the blood sample. Thus, cells are distributed in the solution in layers based on the differences in their density/size. Additional purification methods can be employed as the mononuclear cell sample can be purified from monocytes by adherence or by exposure to L-leucine methyl ester; these methods are described for both procedures. Cord blood and peripheral blood from infants contain immature cells, including nucleated red cells, which can result in significant contamination of the mononuclear cell layer, and removal of these cells requires additional steps that are described. The isolation procedures presented here can also be applied to cell populations derived from tissues.

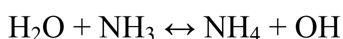
FICOLL is a neutral highly branched high mass hydrophilic polysaccharide that separates blood in different layers by gradient centrifugation. It separates blood into a top layer of plasma.

PHOSPHATE-BUFFERED SALINE (PBS) is water based salt solution that contains sodium hydrogen phosphate ($NaHPO_4$), potassium hydrogen phosphate ($KHPO_4$), sodium chloride (NaCl) and potassium chloride (KCl). It is isotonic solution which is nontoxic to

cells. The ion concentration and the osmolarity of this buffer solution are same as that of human body. It disengages the attachment and clump of cells.

RBC LYSIS BUFFER is used for the lysis of the erythrocytes. The basic mechanism of hemolysis by isotonic method is as follows: $NH_4$ (Ammonium) is in equilibrium with $NH_3$ (Ammonia). The $NH_2$ diffuses across the cell membrane. Inside the cell, the ammonium/ ammonia equilibrium is re-established.

This can be explained by the equation:



$H_2O + NH_3 \leftrightarrow NH_4 + OH$

$NH_3$ diffuses freely through the cell membrane and increases the concentration of intracellular $OH^-$. $OH^-$ reacts with intracellular $CO_2$ to form $HCO_3^-$. In red blood cells the intracellular $HCO_3^-$ is exchanged with extracellular $Cl^-$ through the $Cl^-/HCO_3^-$ transmembrane anion exchanger of RBCs i.e. the band 3 anion channel which is specifically present in the RBCs and not in other lymphocytes. The result is an influx of $NH_4Cl$ inside RBCs, which causes cellular swelling and eventually rupture of the cell membrane of RBCs.

Biosafety cabinet used here is a Class II laminar air flow hood designed for work involving BSL-2. It protects the working environment from dust and other airborne contaminants by maintaining a constant, unidirectional flow of HEPA-filtered air over the work area. It also has UV light system to maintain sterile condition.

PROTOCOL

<u>PREPARATION OF PBS:</u>

For 10X PBS for 500ml with pH 7.4

| **Chemicals** | **Amount(in gm)** |
|---|---|
| Sodium chloride (NaCl) | 40 |
| Potassium chloride(KCl) | 1 |
| Disodium phosphate | 7.2 |
| Potassium dihydrogen phosphate($KH_2PO_4$) | 1.2 |

TABLE: 4.2: Requirements for 10X PBS

All four chemicals were dissolved in 400 ml of purified water by stirring in a beaker.

- The pH was adjusted to 7.4 with 1N HCl.
- The solution was transferred to graduated cylinder and volume was adjusted to 500ml with autoclaved water.
- It was then sterilized by autoclaving.
- 1X concentration of PBS was prepared from the 10X PBS stock and filtered with 0.22um nylon filter for use in 50 ml aliquots.

PREPARATION OF RBC LYSIS BUFFER:

For preparing 10X RBC Lysis Buffer for 100ml

| Chemicals | Amount (in gm) |
|---|---|
| Ammonium chloride($NH_4Cl$) | 8.02 |
| Sodium bicarbonate ($NaHCO_3$) | 0.84 |
| Disodium EDTA | 0.37 |

TABLE: 4.3: Requirements for RBC lysis buffer

- The required amount of the three chemicals were weighed and taken in a beaker.
- To it autoclaved distilled water was added and then stirred.
- It was then transferred to a measuring cylinder and the volume was then made to 100 ml.
- 1X concentration of RBC Lysis buffer was prepared from 10X stock and filtered with 0.22μ nylon filter for use in 50 ml aliquots.

ISOLATION OF PBMCS:

- First the hood was cleaned properly with 70% ethanol and then it was UV treated for 20 minutes.
- The blood sample was poured into 15ml tube and diluted with PBS in 1:1.
- It was slowly layered over equal amount of Ficoll without intermixing blood and Ficoll.
- It was then subjected to density gradient centrifugation at 1200g for 20min at 25°C.

- Four distinct layers were visible: upper straw coloured plasma, followed by white buffy coat comprising of PBMCs, a clear Ficoll layer and RBC pellet at the bottom.
- The plasma was collected in 1.5ml MCTs and marked with patient details.
- The white buffy coat or PBMC layer was transferred to fresh 15ml tube and diluted with RBC lysis buffer and kept it for 10min and centrifuged at 500g for 5 minute at 25°C.
- The supernatant was discarded after centrifugation without disturbing the cell pellet.
- The pellet was washed and resuspended in 1ml 1X PBS and collected in MCTs.
- It was again centrifuged at 500g for 5minutes at 25°C.
- The supernatant was discarded and divided the cells into two MCTs and marked them one as PBMC and another one as TRIzol.
- Those were washed again and the supernatant was discarded then added freezing media and TRIzol.
- The cells were stored in -80°c until the use.

<u>COUNTING OF ISOLATED PBMCS CELLS WITH THE HELP OF AUTOMATED CELL COUNTER</u>

REQUIREMENTS:

- Countess automated cell counter (Invitrogen)
- Countess cell counting chamber slide (Invitrogen)
- Trypan Blue
- 1X PBS

THEORY:

Trypan blue is a used to selectively color cells for microscope. It is a diazo dye. Live cells with intact cell membranes are not colored and the dead cells are stained blue. Since live cells have their membrane intact and are selectively permeable, in a viable cell trypan blue is not absorbed; however, it easily passes through the membrane in a dead

cell. Hence, dead cells appear as a distinct blue color entity under a microscope. This staining method is also known as dye exclusion method of staining.

PROTOCOL:

- First the cells were centrifuged at 500g for 5 minutes at RT.
- The supernatant was discarded and then pellet was dissolved in 500µl PBS.
- It was again centrifuged at 500g for 5 minutes at RT.
- After centrifugation the supernatant was discarded and the pellet was reconstituted in 150µl of PBS.
- 2µl of isolated PBMCs sample was taken.
- To it 4µl of 1X PBS and 4µl of trypan blue was added.
- Then it was loaded on to the cell counting chamber.
- It was then inserted into the opening of the Countess automated cell counter and the cells were counted.

OBJECTIVE 3:To analyze the expression of various housekeeping genes in PBMCs of RA patients for identification of an appropriate reference genes for qPCR

RNA isolation from PBMCs

REQUIREMENTS:

1. TRIzol
2. Chloroform
3. Isopropanol
4. 75% Ethanol
5. Filter pipette tips
6. RNAseZap

THEORY:

TRIzol is a monophasic chemical reagent that is used for separation of DNA, RNA and proteins. It is composed of phenol and guanidium isothiocyanate. This helps in maintaining the integrity of RNA while the homogenization occurs. The function of guanidium isothiocyanate is to lyse the cells and the viral particles. In addition to this it also prevents the activity of the RNase and DNase enzymes by denaturing them and ensuring that the RNA extract remains unharmed. Phenol denatures the protein and separates DNA to the organic phase. Addition of chloroform causes phase separation due to which the mix separates into organic and aqueous phases. The RNA remains in the aqueous phase and the DNA and proteins migrate to the organic phase. Isopropanol addition precipitates the nucleic acid that has been recovered from the aqueous phase. 75% ethanol precipitates only RNA and dissolves all the contaminating proteins and only RNA is recovered at the end as the pellet after centrifugation.

TRIzol solubilisation and extraction is a relatively recently developed general method for deproteinizing RNA. This method is particularly advantageous in situations where cells or tissues are enriched for endogenous RNases or when separation of cytoplasmic RNA from nuclear RNA is impractical. TRIzol (or TRI Reagent) is a monophasic solution of phenol and guanidinium isothiocyanate that simultaneously solubilizes biological material and denatures protein. After solubilization, the addition of chloroform causes phase separation (much like extraction with phenol: chloroform: isoamyl alcohol), where protein is extracted to the organic phase, DNA resolves at the interface, and RNA remains in the aqueous phase. Therefore, RNA, DNA, and protein can be purified from a single sample. TRIzol extraction is also an effective method for isolating small RNAs, such as microRNAs or endogenous, small interfering RNAs. However, TRIzol is expensive and RNA pellets can be difficult to resuspend. Thus, the use of TRIzol is not recommended when regular phenol extraction is practical.

TRIzol extraction is a straight forward way to prepare RNA from cells or tissues. Although TRIzol removes large DNA molecules efficiently, it does not remove plasmid DNA or DNA fragments efficiently, which can be problematic for subsequent

polymerase chain reaction (PCR) applications. However, when used appropriately, the RNA yield is quantitative. An alternative method for Purification of RNA by SDS Solubilization and Phenol Extraction is available, as are procedures for Preparation of Cytoplasmic and Nuclear RNA from Tissue Culture Cells, and Ethanol Precipitation of RNA and the Use of Carriers. Further information is also available on Determining the Yield and Quality of Purified RNA.

A phenol-chloroform extraction is a liquid-liquid extraction. A liquid-liquid extraction is a method that separates mixtures of molecules based on the differential solubilities of the individual molecules in two different immiscible liquids. Liquid-liquid extractions are widely used to isolate RNA, DNA, or proteins.

The extraction of nucleic acids involves adding an equal volume of phenol-chloroform to an aqueous solution of lysed cells or homogenized tissue, mixing the two phases, and allowing the phases to separate by centrifugation. Centrifugation of the mixture yields two phases: the lower organic phase and the upper aqueous phase.

Chloroform mixed with phenol is more efficient at denaturing proteins than either reagent is alone. The phenol-chloroform combination reduces the partitioning of mRNA into the organic phase and reduces the formation of insoluble RNA protein complexes at the interphase. Moreover, phenol retains about 10-15% of the aqueous phase, which results in a similar loss of RNA; chloroform prevents this retention of water and thus improves RNA yield. Typical mixtures of phenol to chloroform are 1:1 and 5:1 (v/v). At acidic pH, a 5:1 ratio results in the absence of DNA from the upper aqueous phase; whereas a 1:1 ratio, while providing maximal recovery of all RNAs, will maintain some DNA present in the upper aqueous phase. Isoamyl alcohol is sometimes added to prevent foaming (typically in a ratio of 24 parts chloroform to 1 part isoamyl alcohol). Guanidinium salts are used to reduce the effect of nucleases.

Protocol:

- Isolated cell pellet was dissolved in 500 µl of TRIzol, it was then mixed by pipetting gently and was incubated for 5 minutes at RT.

- 200 µl of chloroform was added to it and was shaken gently so as to mix the contents of each tube.
- It was again incubated for 5 minutes at RT.
- After incubating the tubes were subjected to centrifugation at 12000g for 20 minutes at 4°C.
- Centrifugation yielded three distinct layers in the tubes. The top aqueous layer contained the RNA the interphase contained the DNA and the bottom organic phase contained the proteins.
- The top aqueous layer was carefully pipetted out by tilting the tube at 45° and was transferred to fresh tubes.
- To it equal volume of 100% Isopropanol was added and was mixed gently and incubated overnight in the -20°C.
- The next day the tubes were centrifuged at 12000g for 20 minutes at 4°C.
- The supernatant was removed and the pellet was dissolved in 500 µl of 75% ethanol.
- It was slowly pipette to mix the pellet and then it was centrifuged at 12000g for 20 minutes at 4°C.
- After centrifugation the supernatant was discarded and the cell pellet recovered was RNA.
- The tubes were then inverted inside the hood and left to dry for about 2 hours.
- After drying a gel like pellet was observed in the bottom of the tube and this gel like pellet was the RNA.
- The pellet was then reconstituted in 15 µl of Nuclease Free water.

<u>Quantification of RNA extracts Requirements:</u>

1. Nanodrop
2. KIM wipes
3. 70% ethanol
4. NF water
5. RNA

THEORY:

A nanodrop is an instrument that is used to quantify and qualify RNA, DNA and protein. A nanodrop works on the Lambert Beer Law by absorbing light in the UV-visible spectral region. It functions by combining fiber optic technology and natural surface tension properties to capture and retain minute amounts of sample. The system employs shorter path lengths, which result in a broad range of nucleic acid concentration measurements. Reducing the volume of sample required for spectroscopic analysis. It divides the absorbance at 260 nm and 280 nm to give purity of the extract. 0.4 to15000 ng/µl is the concentration range that it can detect. It is able to give results with as little as 2 µl of extract. The assessment of the purity of a nucleic acid sample is often performed by a procedure commonly referred to as the A260/A280 ratio. The A260/A280 ratio is only an indication of purity. Pure DNA and RNA preparations have expected A260/A280 ratios of $\geq$1.8 and $\geq$2.0 respectively and are based on the extinction coefficients of nucleic acids at 260 nm and 280 nm. The absorbance value of nucleic acid is 260nm where as protein shows highest absorbance at 280nm so, the ratio A260/A280 can precisely indicates the purity of nucleic acid. When the ratio of 260/280 is below 1.8 there is protein contamination in the sample. And when the ratio is above 2.0 there is RNA in the sample ethanol contamination.

PROTOCOL:

- First the nanodrop was cleaned properly with tissue paper so as to clean any dust present on it.
- Then it was switched on and RNA was selected from the interface after that the lid of the nanodrop was removed.
- 2µl of NF water was added to the loading spot and the lid was closed, this was to clean the loading spot.
- The lid was then opened and the loading spot was wiped with KIM wipes.
- Then 2µl of NF water was added and then blank was selected then measure option was selected from the interface this was done to set the blank.

- The samples were then loaded onto the loading spot and it was measured for each of the samples.
- The machine gave the concentration of RNA (ng/µl) and its purity (260/280).

Preparation of cDNA by reverse transcription PCR Requirements:

1. PCR Tubes
2. Thermal Cycler (ProFlex PCR system, applied biosystems by life technologies)
3. 10X RT buffer
4. dNTP
5. Random primer
6. Reverse Transcriptase enzyme
7. NF water

THEORY:

RNA molecules are single-stranded nucleic acids composed of nucleotides. RNA plays a major role in protein synthesis as it is involved in the transcription, decoding, and translation of the genetic code to produce proteins. RNA stands for ribonucleic acid and like DNA, RNA nucleotides contain three components:

- A Nitrogenous Base
- A Five-Carbon Sugar
- A Phosphate Group

RNA nitrogenous bases include adenine (A), guanine (G), cytosine (C) and uracil (U). The five-carbon (pentose) sugar in RNA is ribose. RNA molecules are polymers of nucleotides joined to one another by covalent bonds between the phosphate of one nucleotide and the sugar of another, these linkages are called phosphodiester linkages. Although single-stranded, RNA is not always linear. It has the ability to fold into complex three-dimensional shapes and form hairpin loops. When this occurs, the nitrogenous bases bind to one another. Adenine pairs with uracil (A-U) and guanine pairs with cytosine (G-C). Hairpin loops are commonly observed in RNA molecules such as messenger RNA (mRNA) and transfer RNA (tRNA). RNA molecules are produced in

the nucleus of our cells and can also be found in the cytoplasm. The three primary types of RNA molecules are messenger RNA, transfer RNA and ribosomal RNA.[30]

Messenger RNA (mRNA) is a subtype of RNA. An mRNA molecule carries a portion of the DNA code to other parts of the cell for processing. mRNA is created during transcription. During the transcription process, a single strand of DNA is decoded by RNA polymerase, and mRNA is synthesized. Physically, mRNA is a strand of nucleotides known as ribonucleic acid, and is single-stranded[31]. Mature mRNA is used as a template for preparing cDNA. This conversion is brought about by reverse transcriptase. Reverse transcriptase is a RNA-dependent DNA polymerase. It acts on a single strand of mRNA and uses this mRNA as a template; reverse transcriptase produces its complementary DNA based on the pairing of RNA base pairs. This enzyme executes reactions in the same way as DNA polymerase. It also requires a primer with a free 3′-hydroxyl group.

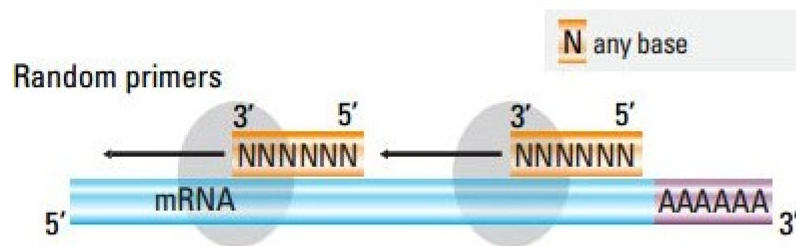



FIGURE4.1.1: Above figure shows the annealing of random primers to the RNA for DNA synthesis[32].

Protocol:

- First calculations for amount of template to be taken for concentration of 500ng of template were done.
- If high concentration of RNA was present then it was diluted so that it would be easy to pipette and no pipetting error was there.
- Now the mastermix was prepared for 8 tubes.

| Reagent | Amount |
|---|---|
| RT buffer | 2µl |
| dNTP | 0.8µl |
| Random primer | 2µl |
| Reverse Transcriptase enzyme | 1µl |
| RNA (template) | 500ng |

TABLE 4.4: Mastermix for cDNA synthesis

- Calculations were done for 8 tubes; all the components were added to a single tube labelled as mastermix. It was made sure that the reverse transcriptase enzyme was added at the end.
- 8 PCR tubes were taken and were labelled accordingly. The calculated amount of templates was added to the labelled PCR tubes. The volume was made upto 14.2µl by NF water.
- 5.8µl of mastermix was added to each tube making the total volume 20µl (total volume of PCR reaction).
- Then the tubes were taken to the PCR system and the following program was selected

| Steps | Temperature(°C) | Duration(hh:mm:ss) |
|---|---|---|
| 1 | 25 | 0:10:00 |
| 2 | 37 | 2:00:00 |
| 3 | 85 | 0:05:00 |
| 4 | 4 | Hold |

TABLE 4.5: Program for cDNA synthesis

- Then the program was run in the PCR system.
- After the program came to an end the samples were taken out of the PCR system and then were stored in the -20°C refrigerator.

**Transcript expression analysis by real time PCR**

REQUIREMENTS:

- SYBR Green Master Mix I
- Template (cDNA)
- Primer
- 384well plate
- Sealing foil
- Roller
- Roche LightCycler 480 instrument

**THEORY**:

In conventional PCR the amplified DNA product is detected in an endpoint analysis. Real time PCR is a technique where the accumulation of PCR products is measured as the reaction proceeds, in real time, with product quantification after each cycle. The real-time polymerase chain reaction (PCR) uses fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR reaction. qPCR monitors the Real Time amplification of a targeted cDNA molecule during the PCR thus provided quantitative assessment of the transcript copy numbers. Two common methods for the detection of PCR products in real-time PCR are: (1) nonspecific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence. Here we use the first method i.e. the fluorescent dye method. SYBR Green is dsDNA-binding dye and the detection is monitored by measuring the increase in fluorescence throughout the cycle. SYBR Green has an excitation and emission maxima of 494 nm and 521 nm respectively. It is thought to bind in the minor groove of dsDNA and upon binding increases in fluorescence over a hundred fold. It is compatible with PCR up to a point, at very high concentrations it starts to inhibit the PCR reaction. In the LightCycler Instrument, SYBR is monitored in channel F1. The biggest advantage of SYBR is that it

binds to any dsDNA; there is no designing and optimizing of probes required. . The biggest disadvantage of SYBR is that it binds to any dsDNA; the specific product, nonspecific products and primer dimers are detected equally well. There are a number of ways to handle this problem. Careful optimization of the PCR reaction can usually reduce primer dimers to a level that is only important for very low copy detection. Once the melting point of the product has been determined the LightCycler Instrument's flexible programming allows the user to acquire fluorescence above the melting temperature of the primer dimers, but below the melting temperature of the product. The qPCR process generally consists of a series of temperature changes that are repeated 25-40 times. These cycles normally consist of three stages: the first, at around 95°C, allows the separation of the nucleic acids double chain; the second, at a temperature of around 50-60°C, allows the binding of the primers with the DNA template; the third, at between 65-72°C, facilitates the polymerization carried out by the DNA polymerase. Once polymerization of a new DNA strand is initiated from the primers, SYBR green molecules intercalates, the fluorescence is detected and measured in real-time and its geometric increase corresponding to exponential increase of the product is used to determine the quantification cycle ($C_t$) in each reaction.

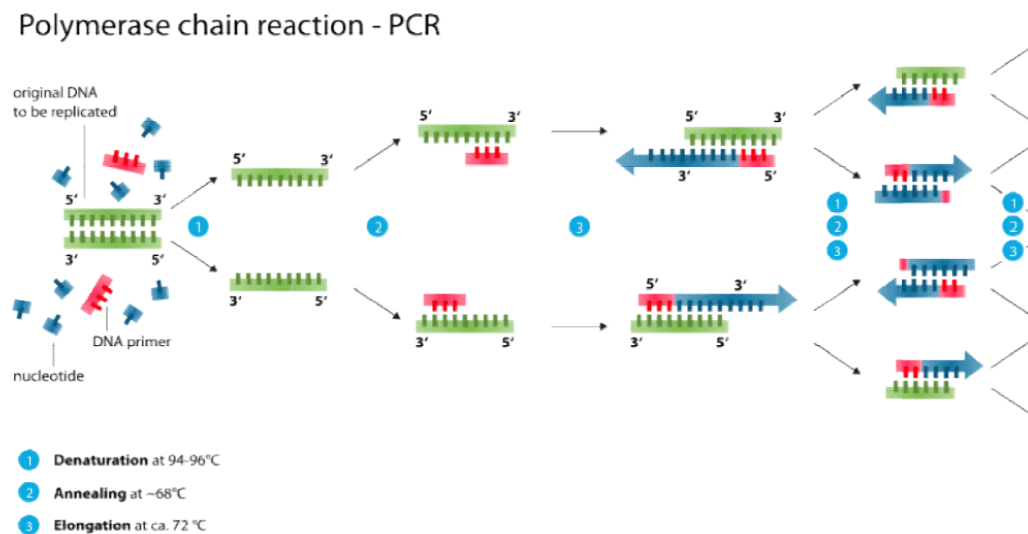



FIGURE4.1.2: Above figure shows different steps of qPCR.

**Melting curve**: It is graphical representation of melting temperature verses fluorescence in arbitrary unit. The melting curve analysis assess whether their intercalating dye in PCR/qPCR assays have produced single, specific products. As every DNA fragment has different melting point so this curve helps in detecting the sensitivity and specificity of the PCR reaction. A melting peak shows whether same sample from different wells denature at same temperature or not where as dissociation curve shows complete dissociation of the DNA double strands.

**Amplification Curve**: During qPCR the initial PCR cycles produce low fluorescent signals that cannot be detected by the CCD camera. The linear portion of each curve is in the exponential phase of PCR, where the amount of product, and therefore the signal, doubles after each cycle. The top portion of the curves shows minimal signal increase, as PCR slows due to the depletion of reaction components, such as primers and dNTPs. The curves should be smooth during the exponential phase of the PCR. Any spikes in the curves may be the result of unstable light sources from the instrument, or sample preparation problems, such as the presence of bubbles in the reaction wells.[33]

**$C_t$ value:** It is a graphical representation of cycle number verses fluorescence in arbitrary unit. It shows at which cycle the cDNA begins to bind with the primer. More the $C_t$ value less is the expression of mRNA as low expressed RNA does not bind to the primer rapidly.

**qPCR controls**: In order to negate and normalize any changes in qPCR results due to technical errors different controls are included in the qPCR reaction. The qPCR reaction is carried out using a No Template Control (NTC) that includes all the reagents except the cDNA. It is taken as a negative control and a house keeping gene like β-globin is takes as positive control as is a gene which will present in every condition .This ensures any errors due to contamination in the reagents. Though equal quantity of RNA was used to prepare cDNA yet any pipetting error is analyzed and considered using a House Keeping Gene control. In the present experiments we used two housekeeping genes β-actin and β-globin. These genes express cytoskeletal elements and do not drastically

change their expression during experimental conditions. Moreover all the qPCR reactions are carried out in triplicates to further avoid any technical error.

**Protocol:**

- The desired primers were chosen for the real time PCR analysis.
- Calculations for the required amount of primers were done based on the following

| Reagents | Amount |
|---|---|
| Nuclease free water | 4.8µl |
| Primer | 0.67µl |
| SYBR Green Master Mix I | 2.5µl |

TABLE: 4.6. Requirements for qPCR

- A 384 well qPCR plate was prepared with 8ul of master mix/well.
- Then 2ul of cDNA template was added to each well.
- Same numbers of wells were prepared for β-actin and three wells with only master mix as NTC.
- The plate was covered with a film and then placed in the 384 well slot of qPCR machine (Roche Light Cycler 480) and the program was started.

**<u>Gene Specific Primer Design</u>**

For SDHA, TBP, IPO8, RPL13A, HPRT1, PBGD, RPS9, β-actin the primers were designed using Roche Life Science Software and NCBI data base.

**PROTOCOL**

- Gene for SDHA Homo sapiens was searched.
- Click on the name SDHA was done which show another page.
- Click on NCBI Reference Sequence show a page where we should click on mRNA icon to get the mRNA sequence.
- Then, we found the FASTA file.
- Now another site i.e. Universal probe Library-Assay Design-Roche Life Science was opened.

- Homo sapience was selected as the organism.
- The FASTA file was uploaded in the space provided and then it was allowed to design.
- It gave both the forward and reverse primer sequences which were then given to the company (GCC Biotech) for primer synthesis.
- This procedure was done for all the primers.
- The company gave both the primers separately as live attenuated form at room temperature with all the data which are given bellow. s
- The primers were stored at -20°c refrigerator after dilution for future use.

| GENES | NAME | FUNCTION |
|---|---|---|
| SDHA | succinate dehydrogenase complex flavoprotein subunit A | This gene encodes a major catalytic subunit of succinate-ubiquinone oxidoreductase, a complex of the mitochondrial respiratory chain. The complex is composed of four nuclear-encoded subunits and is localized in the mitochondrial inner membrane. Mutations in this gene have been associated with a form of mitochondrial respiratory chain deficiency known as Leigh Syndrome. |
| TBP | TATA-box binding protein | Initiation of transcription by RNA polymerase II requires the activities of more than 70 polypeptides. The protein that coordinates these activities is transcription factor IID (TFIID), which binds to the core promoter to position the polymerase properly, serves as the scaffold for assembly of the remainder of the transcription complex, and acts as a channel for regulatory signals. TFIID is composed of the TATA-binding protein (TBP) and a group of evolutionarily conserved proteins known as TBP-associated factors or TAFs |
| RPL13A | ribosomal | Ribosomes, the organelles that catalyze protein synthesis, consist of |

| | | |
|---|---|---|
| | protein L13a | a small 40S subunit and a large 60S subunit. Together these subunits are composed of 4 RNA species and approximately 80 structurally distinct proteins. This gene encodes a member of the L13P family of ribosomal proteins that is a component of the 60S subunit. The encoded protein also plays a role in the repression of inflammatory genes as a component of the IFN-gamma-activated inhibitor of translation (GAIT) complex. As is typical for genes encoding ribosomal proteins, there are multiple processed pseudogenes of this gene dispersed throughout the genome. |
| HPRT1 | hypoxanthine phosphoribosyltransferase 1 | The protein encoded by this gene is a transferase, which catalyzes conversion of hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate via transfer of the 5-phosphoribosyl group from 5-phosphoribosyl 1-pyrophosphate. This enzyme plays a central role in the generation of purine nucleotides through the purine salvage pathway. Mutations in this gene result in Lesch-Nyhan syndrome or gout |
| PBGD | Porphobilinogendeaminase | porphobilinogendeaminase catalyzes the loss of ammonia from the porphobilinogen monomer (deamination) and its subsequent polymerization to a linear tetrapyrrole |
| RPS9 | ribosomal protein S9 | Ribosomes, the organelles that catalyze protein synthesis, consist of a small 40S subunit and a large 60S subunit. Together these subunits are composed of 4 RNA species and approximately 80 structurally distinct proteins. This gene encodes a ribosomal protein that is a component of the 40S subunit. The protein belongs to the S4P family of ribosomal proteins. It is located in the cytoplasm. Variable expression of this gene in colorectal cancers compared to adjacent normal tissues has been observed, although no correlation between the level of expression and the severity of the disease has been found. |

| | | |
|---|---|---|
| β-actin | actin beta | This gene encodes one of six different actin proteins. Actins are highly conserved proteins that are involved in cell motility, structure, integrity, and intercellular signaling. The encoded protein is a major constituent of the contractile apparatus and one of the two nonmuscle cytoskeletal actins that are ubiquitously expressed. Mutations in this gene cause Baraitser-Winter syndrome 1, which is characterized by intellectual disability with a distinctive facial appearance in human patients. Numerous pseudogenes of this gene have been identified throughout the human genome. |

TABLE 4.7:-List of primers and their functions.

| Sl. No. | Primer Name | Primer Sequence 5' to 3' | No. of Bases |
|---|---|---|---|
| 1 | SDHA_F | TGGTTGTCTTTGGTCGGG | 18 |
| 2 | SDHA_R | GCGTTTGGTTTAATTGGAGGG | 21 |
| 3 | TBP_F | GCCCGAAACGCCGAATATAA | 20 |
| 4 | TBP_R | AATCAGTGCCGTGGTTCGTG | 20 |
| 5 | RPL13A_F | CCTGGAGGAGAAGAGGAAAGAGA | 23 |
| 6 | RPL13A_R | TTGAGGACCTCTGTGTATTTGTCAA | 25 |
| 7 | HPRT1_F | GCTTTCCTTGGTCAGGCAGTA | 21 |
| 8 | HPRT1_R | AACACTTCGTGGGGTCCTTT | 20 |
| 9 | PBGD_F | GGCAATGCGGCTGCAA | 16 |
| 10 | PBGD_R | GGGTACCCACGCGAATCAC | 19 |
| 11 | RPS9_F | CTGGATGAGGGCAAGATGAAG | 21 |
| 12 | RPS9_R | GTCTGCAGGCGTCTCTCTAAGAA | 23 |
| 13 | β-actin_F | GCTACGAGCTGCCTGACG | 18 |
| 14 | β-actin_R | GGCTGGAAGAGTGCCTCA | 18 |

TABLE.4.8:-List of primers and their sequences used for quantitative real time PCR.

| Primer Name | MW(g/mol) | GC % | Tm° c | Yeild OD | Yeild µg | Yeild n mol | Vol. for 100p mol/µl | Synthesis Scale (µmol) | Purification |
|---|---|---|---|---|---|---|---|---|---|
| SDHA_F | 5583.6 | 55.6 | 56.0 | 5.5 | 168 | 30.0 | 301 | 0.01 | HPSF |
| SDHA_R | 6563.3 | 47.6 | 57.9 | 6.1 | 172 | 26.2 | 262 | 0.01 | HPSF |
| TBP_F | 6104.0 | 50.0 | 57.3 | 5.7 | 149 | 24.4 | 244 | 0.01 | HPSF |
| TBP_R | 6164.0 | 55.0 | 59.4 | 5.9 | 170 | 27.7 | 276 | 0.01 | HPSF |
| RPL13A_F | 7244.8 | 52.2 | 62.4 | 6.3 | 154 | 21.3 | 213 | 0.01 | HPSF |
| RPL13A_R | 7678.0 | 40.0 | 59.7 | 6.6 | 188 | 24.5 | 245 | 0.01 | HPSF |
| HPRT1_F | 6428.2 | 52.4 | 59.8 | 6.1 | 179 | 27.9 | 279 | 0.01 | HPSF |
| HPRT1_R | 6099.0 | 50.0 | 57.3 | 5.9 | 174 | 28.6 | 285 | 0.01 | HPSF |
| PBGD_F | 4931.2 | 62.5 | 54.3 | 5.2 | 142 | 28.9 | 288 | 0.01 | HPSF |
| PBGD_R | 5782.8 | 63.2 | 61.0 | 5.5 | 154 | 26.7 | 266 | 0.01 | HPSF |
| RPS9_F | 6584.3 | 52.4 | 59.8 | 6.1 | 157 | 23.9 | 238 | 0.01 | HPSF |
| RPS9_R | 7039.6 | 52.2 | 62.4 | 6.3 | 179 | 25.5 | 254 | 0.01 | HPSF |

**Table 4.9:** Oligonucleotide synthesis report as provided by Eurofins

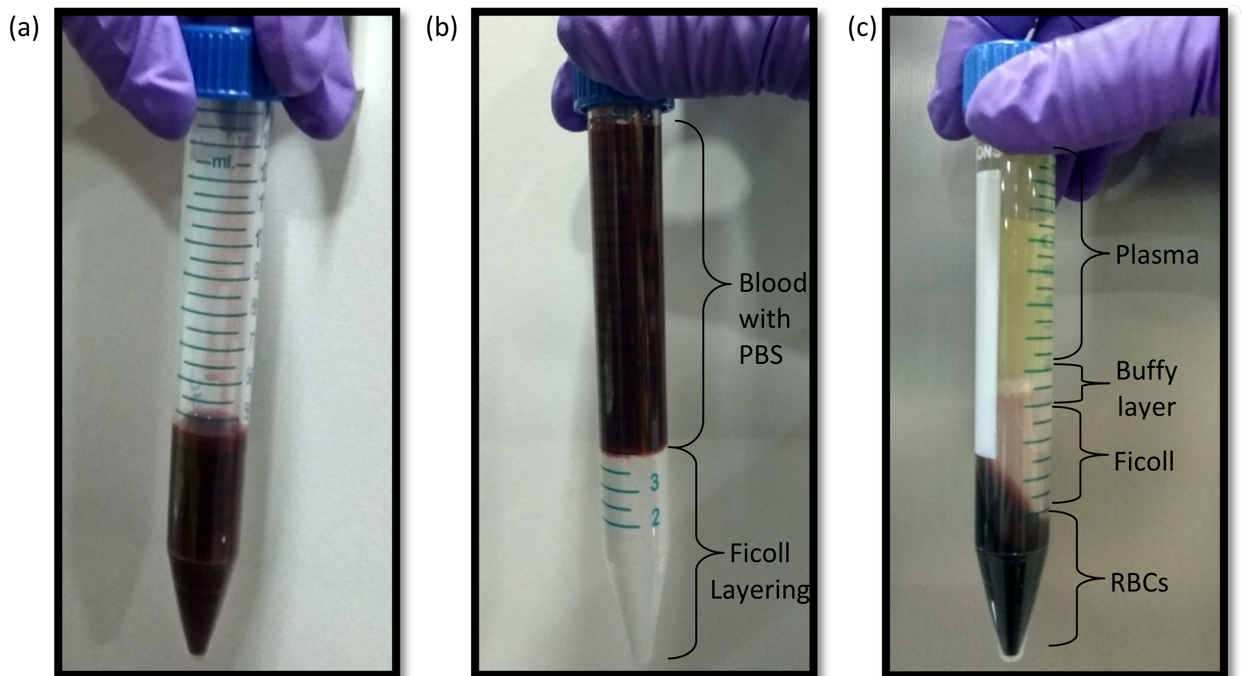
## CHAPTER-5

## RESULTS:

RESULT- 1

ISOLATION AND QUANTIFICATION OF PBMCs FROM PERIPHERAL BLOOD BY FICOLL DENSITY GRADIENT CENTRIFUGATION:

Blood samples were collected from different rheumatic disorder patients (n=3) and healthy individuals (n=3). Within one hour of collection PBMCs were isolated using Ficoll density gradient centrifugation. Figure 1 shows different stages of PBMC isolation. Peripheral blood was taken in 15 ml Falcon tube (Figure a). Before centrifugation blood layered over Ficoll (Figure b). After centrifugation buffy coat above Ficoll, cleared plasma at the top and settled RBC at the bottom of falcon tube was obtained (Figure c). We washed the buffy coat pellet (PBMCs) twice and reconstituted PBMCs with 500ul PBS. We quantified the isolated PBMCs by automated cell counter. Dead and live cells were identified using Trypan blue (Figure d) and total viable cell concentration, total dead cell concentration, percent of viability were calculated simultaneously (Figure e).



(d)

(e)

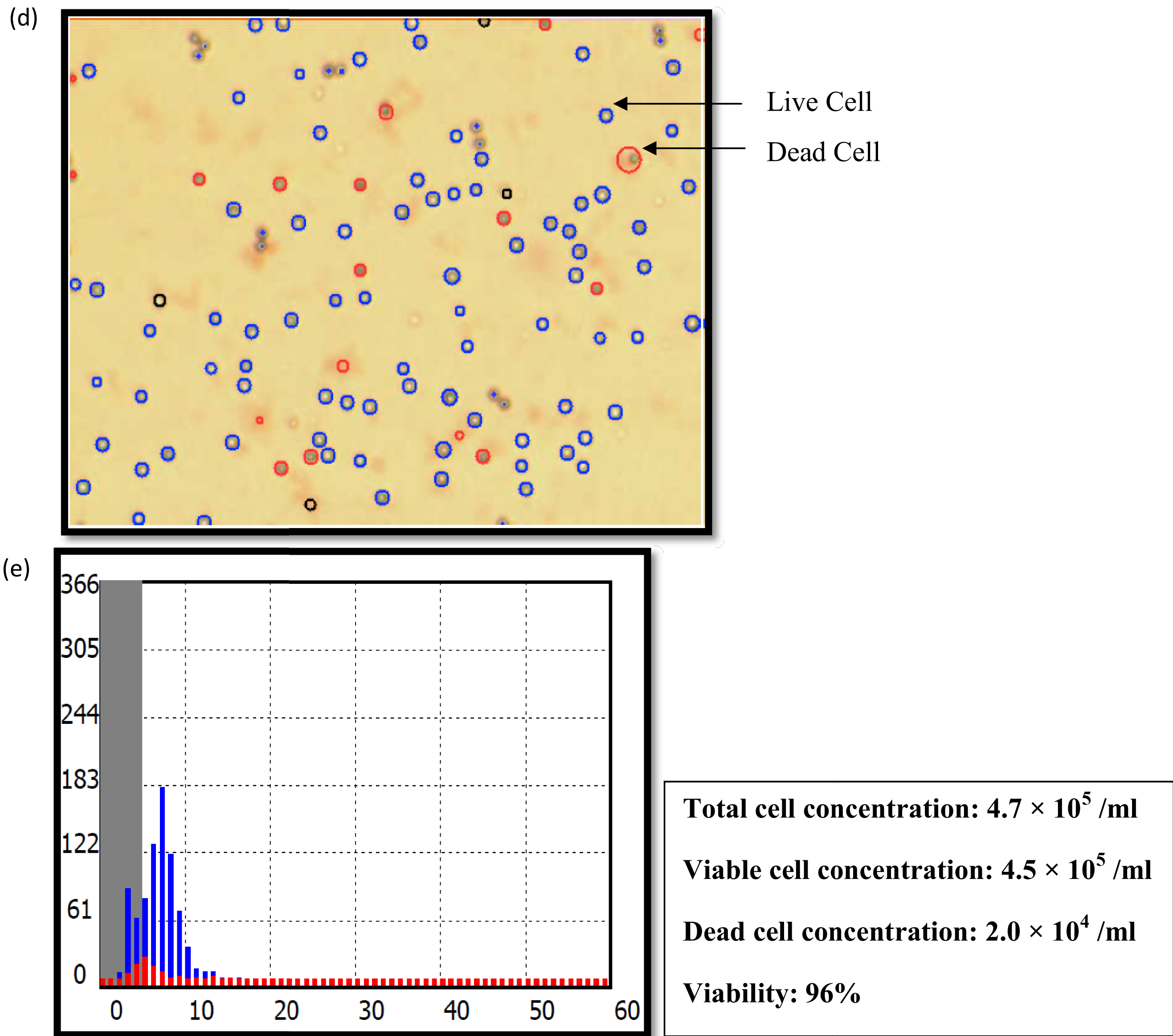



Figure1- (a) Blood sample in 15ml falcon tube. (b) Blood layered over ficoll in 15ml falcon tube. (c) Ficoll density gradient showing different layers of blood. (d) This image captured from automated cell counter shows viable cells encircled by blue line and dead cells by red outline. (e) Graph representing the number of cells against cell size. The maximum numbers of cells have size in between 7-8 μm.

RESULT 2:

PRIMER OPTIMIZATION FOR REAL-TIME PCR:

Total RNA was isolated from all the subjects by TRIzol method. Quantity and purity was checked by nanodrop and 500 ng of RNA used to synthesized cDNA. To optimize all the primer, qPCR was done in triplicate manner in 384 well plate. We observed the same melting peaks and amplification curves for all the triplicates using a particular primer (Figure 2 a-c) for all 7 primers used.

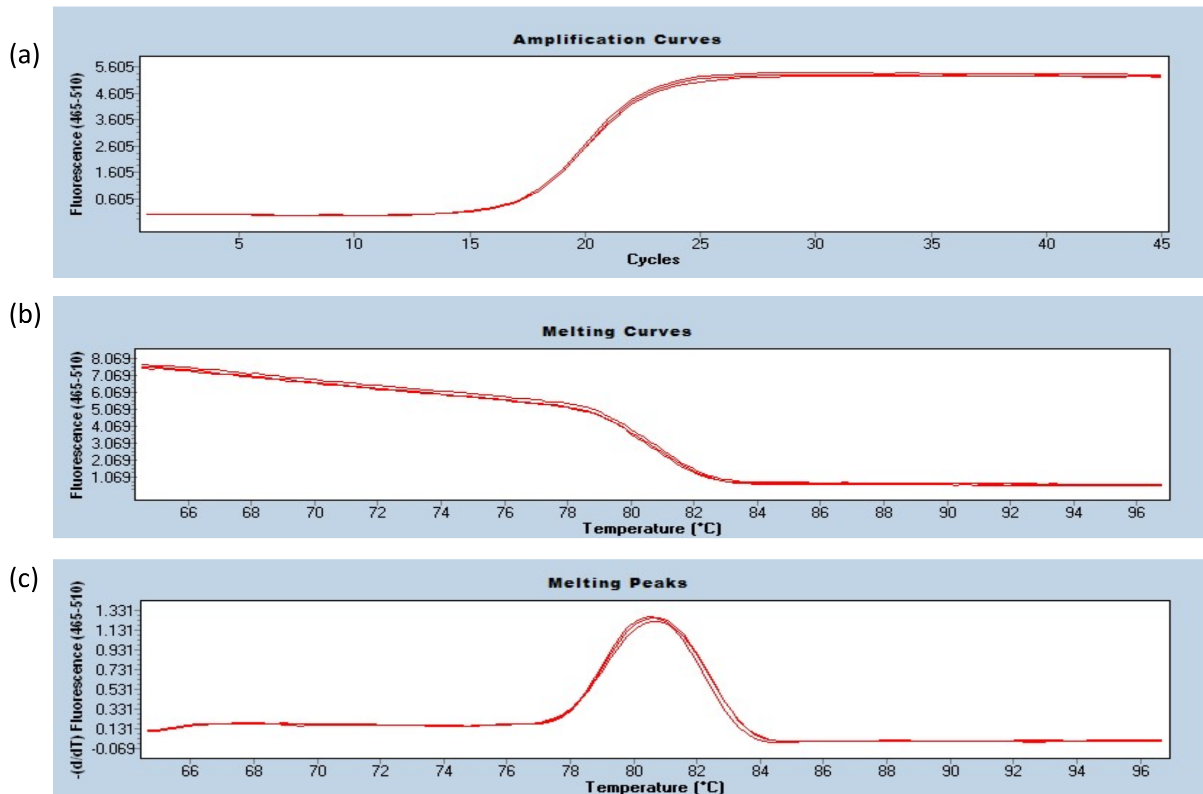



Figure 2-(a) Figure is a representative image of amplification curve of all primers used in our experiment, (b-c) A representative figure of melting curve and melting point of primers used in the experiment.

RESULT-3

For identification of best housekeeping genes for studies involving RA PBMC samples, we have done qPCR with seven different housekeeping gene i.e, SDHA, TBP, RPL13A, HPRT1, PBGD, RPS9 and β-actin of RA samples(n=3) and HC samples(n=3). The results are plotted as $C_t$ values. The genes having minimum difference between the $C_t$ values in RA and HC samples are thus the best housekeeping genes. From our study we observed a difference between $C_t$ values of PBGD and SDHA between RA and HC smaples to be 0.79 and 1.18 respectively. Therefore these two genes can be used as housekeeping genes for further gene expression analysis.

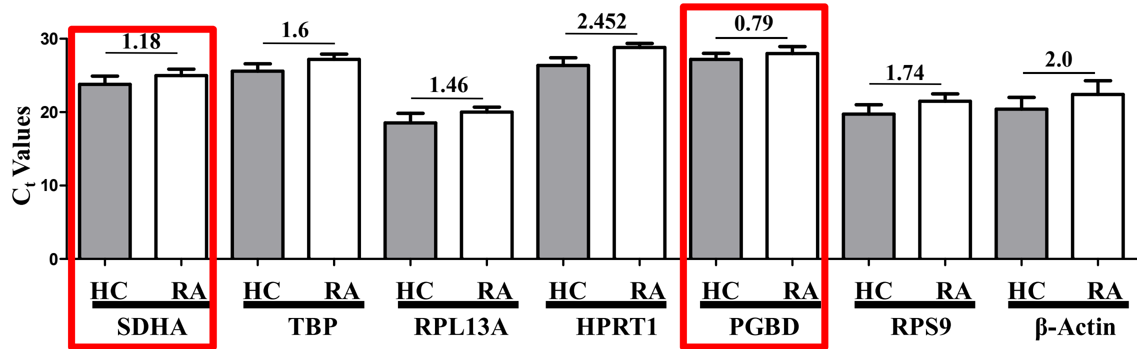



Figure 3-Graph of Housekeeping genes representing the $C_t$ values of RA and HC PBMCs. The grey bars represent HC samples and the white bars represent RA samples. Numerical figures above the graph represents difference between the $C_t$ values of RA and HC smaples for respective genes.

# CHAPTER-6

## DISCUSSION

The present study identifies the role of housekeeping genes in RA disease. This study is totally based on clinical samples. The blood samples were handled properly to avoid any kind of infection and clotting. PBMCs were isolated by density gradient centrifugation. We got good numbers of mononuclear cells with high viability.

Working with RNA is not easy as it is unstable so, it is preferable to synthesize cDNA from total RNA. Isolation of RNA was done under sterile and RNAase free conditions taking proper precautions. Studies have used various housekeeping genes for normalization in vairous experiments which majorly include β-actin, β-globin, but RA being a skeletal and autoimmune disease, the expression of most commonly used housekeeping genes vary significantly. Thus in order to identify an appropriate housekeeping genes, we checked seven housekeeping genesreported by various studies including SDHA, TBP, RPL13A, HPRT1, PBGD, RPS9 and β-actin. We analysed the expression of these genes in RA and HC PBMCs using qPCR.

Analysis of qPCR results indicate PBGD and SDHA as a best housekeeping gene among the seven genes analysed which can be used for normalization purpose in gene expression studies involving RA PBMCs.

## Conclusion:

The qPCR results suggest that PBGD and SDHA genes can be appropriate housekeeping genes for studies involving gene expression analysis in RA PBMCs. However the number of samples involved in the study are less increasing which may help us to find the most appropriate housekeeping gene for RA PBMCs studies.

## Future work:

1) We have planned to collect more number of RA and HC PBMC samples for finding the best housekeeping gene.
2) We have also planned to use the appropriate housekeeping gene in our further studies involving gene expression analysis.

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