INTERNATIONAL JOURNAL OF PHARMACEUTICAL, CHEMICAL AND BIOLOGICAL SCIENCES

Available online at www.ijpcbs.com

Review Article

DIFFERENT TYPES OF PCR TECHNIQUES AND ITS APPLICATIONS

S. Rajalakshmi

Pharmaceutical Analysis Department, Santhiram college of Pharmacy,
Nandyal, Kurnool, Andhra Pradesh, India.

ABSTRACT

Polymerase chain reaction is a biological technology to produce ample number of DNA copies of a particular sequence. Three primary steps involved are de-naturation, annealing and extension. PCR techniques has a lot of applications in plant biology, diagnosis of influenza- human brucellosis- Salmonellae, cloning purpose, in the field of Dentistry, microbiology, forensic science etc., There are many types of PCR techniques such as RT-PCR, touchdown PCR, real time PCR, nested PCR, multiplex PCR, semi quantitative PCR, assembly PCR, asymmetric PCR, LATE- PCR, dial out-PCR etc., This paper is an attempt to give a brief idea about the various types of PCR techniques

Keywords: PCR-Technique, Applications of PCR, Review of PCR.

INTRODUCTION

Polymerase Chain Reaction also called as 'Molecular Phototyping 'means a technique used to amplify small and targeted segmentsof DNA to produce millons of copies of a specific gene fragment. This technique was developed in 1983 by Kary Mullis, he was awarded Nobel prize in 1993 for his work in PCR along with Michael Smith[2,3].

The common requirements for a PCR process are thermo cyclers, DNA template, two primers, Taq polymerase, nucleotides, buffers etc[2]., PCR process is commonly carried out in 10-200 l in small reaction tubes in which uses Peltier effects. Each cycle of PCR have 3 main important steps such as de-naturation, alignment of specific primers (or) annealing and final extension(5). Before going deep into PCR the basic concepts of PCR to be understood. This PCR process will result in 1—2—4---8---16---32 and so on doubling copies.

For that process 4 nucleotide bases such as Adenine, Thymine, Cytosine and Guanine are necessary[3]. This PCR technique results in the amplification of good quality of DNA fragments even though the DNA source is available for PCR process. Many infectious agents and strains of specific genes can be identified by PCR technique. A single PCR process can be completed in 30-35 cycles which consumes 2 hours time to amplify to produce usable amount of DNA fragments. Many types of PCR process with slight modifications can be used to produce better results such as multiplex-PCR, RT-PCR, Nested PCR,inverse PCR, colony PCR, asymmetric PCR helicase PCR, ligation-mediated PCR etc[5].

ESSENTIAL COMPONENTS OF PCR

The following are the essential components of PCR

- Themal cyclers (thermocyclers)
- Target DNA (DNA template)
- Two primers(forward and reverse primers)
- Taq polymerase (themus aquaticus)
- Buffers
- Deoxy nucleotide triphosphates (d NTP's)
- Monovalent \bivalent cation
- Nucleotides (A\T\G\C)
- Water

Thermocyclers

PCR reaction is carried out in 0.2-0.5 ml volume thermo cyclers. It heats and cools the reaction tubes to achieve the temperature required. Many thermo cyclers have heating lids to prevent condensation at the top of the reaction tube. Past thermo cyclers lack a heating lid instead of which a small ball of wax was placed inside the tubes priorly.

Target DNA and nucleotides

It consists of the DNA to be amplified. The segment represents a small part of a large and

complex mixture of a specific DNA of a genome[3]. The shape of DNA is a double helical structure which consists of nucleotides that wind around each other in a helical shape[6]. PCR requires a template molecule (i.e.) DNA\RNA. The 4 nucleotide components are like 4 bricks or building blocks used to construct genome molecules. The nucleotide bases are adenine, thymine, cytosine and guanine which also needs a small amount primer.

Two primers

They are forward and reverse primers which are usually 16-30 nucleotides[10] in length. Primers limit the DNA sequence to be replicated and results in the amplification of a particular DNA sequence[7]. Primers are short, artificial DNA strands not more than 50 nucleotides, which determines the beginning and the end of the region to be amplified, the polymerase synthesizes the complementary sequence from each primer. If template contains A nucleotide, enzyme adds on T nucleotide to the primer and if template contains G nucleotide, enzyme adds on C nucleotide to the primer[8]. Two components that are considered for a primer are length of the primer and actual sequence of the primer[4]. The primers depend upon

- Primer length
- Melting point
- Specificity
- Complementary primer sequences

The length of the primer should be as short as possible. The annealing temperature should below at least 5 C than the melting point temperature

Taq polymerase

Many micro-organisms can live in inhospitable conditions or in the presence of salt\acidic concentrations. The banteria synthesises at the rate of 35-100 nucleotides\sec. the DNA polymerase from Thermus Aquaticus is stable at 95'C which is a thermophile. Both the cloned gene and the native Taq obtained from Thermus aquaticus are available commercially to serve as a standard reagent for PCR reaction(4). They also grow in the geysers over 110' C and can withstand heating to 94' C and presence of extreme salt/acidic concentrations(9). They have contributed greatly for the stimulation, specificity, automation of PCR process. The bacteria synthesizes at the rate of 35-100 nucleotides/sec[10].

Buffers

It consists of Magnesium chloride which supplies Mg divalent cations required as a co-factor. It is needed in 1-5 mM concentration and Mg joins to nucleotides to be recognized by the polymerase enzyme. It also acts as a co-factor for the enzyme. The most commonly used buffer is 10mM Tris at pH 8.3, 50 mM kcl, 1.5-2.5 mM Mgcl . the other PCR buffers used are DMSO, PEG 6000, Glycerol formamide etc.

Fig. 1: Origin of Thermus aquaticus

Fig. 2: Structure of Taq polymerase

D NTP'S

These deoxy nucleotide triphosphates consists of nucleotides floating in liquid and it supplies the nucleotides to Taq polymerase enzyme to synthsise a new strand of DNA. These NTP'S consist of \Y phosphates which serve as a source of energy to PCR reaction. The concentration of d NTP's should be 20-200 m

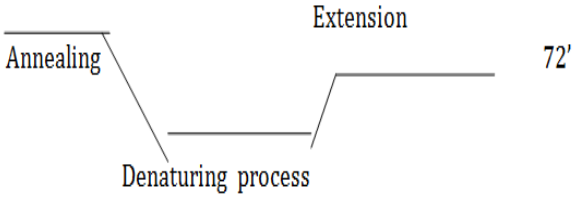
PROCESS INVOLVED IN PCR

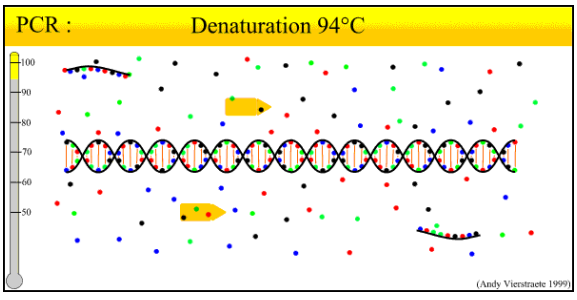
There are 3 main processes naely

- Denaturing \ melting
- Annealing
- Elongation\ extension step

Denaturing \ melting

The first step involved is heating to a temperature of 94-96 'C for 10-20 min which causes 2 complementary strands to separate. The whole time consumed is about 5 min. the thermally stable Taq polymerase enzyme is used here.





Annealing (hybridization)

The annealing temperature should be chosed in such a way that, it should 2' C lower than melting\ denaturing temperature. This process takes place in about 1-2 min, the temperature is lowered hence the primers attach to single DNA strands. The temperature is lowered to 50-65 C. each strand of DNA molecule becomes annealed with an oligo- nucleotide primer complementary to either and of target sequence.

Extension

Here the DNA polymerase fills the missing strands, the temperature involved will be dependent upon the stability of Taq polymerase (Thermus aquaticus polymerase). The temperature involve is 72 C for 5-15min and Taq polymerase starts filling the missing nucleotides in the 3' 5' direction away from each of the primer. DNA polymerase polymerises 1000's of bases\ min. The amplification is done on logarthimic scale and amplified product of PCR process called as amplicon. One cycle takes 1-3 min and the whole process takes place about 45 min and would generate millions of copies

ANALYSIS OF PCR PRODUCT

There are many ways to analyse PCR products. They are

a. Staining of amplified DNA product with a chemical dye like ethidium bromide[14]
b. Labelling of PCR primers and nucleotides with fluorophores before PCR amplification[14]. SYBR Green Imay also be used instead ethidium bromide(1). It has the advantage so that it can be used with various pairs of different primers, it also costs less expensive than a probe.
c. **Agarose Gel Electrophoresis**
Agarose gel consists of 0.9% agarose in 40mM tris-base pH-8.3, 20mM acetic acid, 1mM purified from 0.9% agarose gels using QIA (quick gel extraction kit). After gel electrophoresis technique, gel is soaked in a buffer containing a de

that specifically stains DNA[12]. After the complation of all of the 3 process, it is visualized b UV transilluminatikon[8]. If the bands is present, it indicates the target sequence of original DNA sample and absence of any bands indicate the absence of original DNA sample.

DIFFERENT TYPES OF PCR TECHNIQUES

1. Nested –seminested PCR
2. Multiplex PCR
3. RT-PCR
4. Touchdown PCR
5. Inverse PCR
6. Allele specific PCR
7. Asymmetric PCR
8. Arbitrary PCR
9. Core sample PCR
10. Degenerate PCR
11. Assembly PCR
12. Dial-out PCR
13. Digital PCR
14. Traditional PCR
15. Hot start PCR
16. In-silico PCR
17. Inter sequence PCR
18. Ligation-mediated PCR
19. Methylation- specific PCR
20. Miniprimer PCR
21. Nano particle PCR
22. Overlap-extension PCR
23. Quantitative PCR
24. Solid phase PCR
25. Suicide PCR
26. Thermal asymmetric interplaced PCR
27. Semiquantative PCR
28. Conventional PCR
29. Colony PCR
30. After exponentional PCR
31. Standard PCR
32. Qualitative PCR

Quantitative PCR techniques

It is also referred to real time PCR. It gives an idea about how much DNA amount present in the sample.

Qualitative PCR techniques

When PCR techniques is used for detecting a specific DNA segment, it is called as quantitative PCR method. PCR techniques are used in the identification of genes of bacteria and virus (9). Only qualitative PCR technique can detect whether the individual has been reinfected with a different but related pathogen. It is fast and simple, inexpensive technique.

Conventional PCR

This defined as a normal PCR process. Here the primers bind specifically to each other with 2 DNA strands. Primers also limit the sequence to be replicated and a particular DNA sequence is amplified with billions of copies.

All that needed for the PCR process are PCR tubes made up of aluminium blocks, DNA polymerase, buffer, and target DNA, primers. The whole process takes place within 35-40 minutes repeatedly and viewed by gel electrophoresis technique. PCR's process can be done using Prime STAR HS kits (!6). Normally 50 micro liters of PCR reaction mixtiure consists of 10 micro litres of genomic DNA content 2% agarose gel is used with ethidium bromide dye for analysing the samples.

Multiplex PCR

Multiplex Pcr technique detects different pathogens in a single sample, used to identify exonic/intronic sequence[5] in specific genes. The designing of primers are different because they are meant to adhere to specific DNA sequence. Here in multiplex PCR the base pair lengths should be different to form distinct bands because varying sizes of different DNA genes are targeted in a single reaction to avoid higher expenses, time consumption and recognizes many pathogens at once[11].

This technique is used to detect viral/bacterial and other infectious agents[17]. The presence of one or more primer pair increases the risk of primer-dimer amplification and discrimination of DNA laer fragments[10]. In addition to buffers multiplex Pcr contains Taq polymerase additive, which decreases the competition among amplicons.

Some examples of multiplex PCR techniques which are employed in medicinal field are

Brucella diagnostics based on perosamine synthetase gene.

Application of PCR technique using two sets of primers B4/B5-JPF/JPR for the diagnosis of active human brucellosis in Egypt.

- Also used in the identification of major species of genes Brucella targetting bcsp 31, omp 2b, omp2a, omp 31.
- A 19-primer multiplex PCr specifically identified B.neotomae, B.ceti,B.microti.
- Several multiplex PCR's have been described for the simultaneous detection of M.tuberculosis and Brucella species.

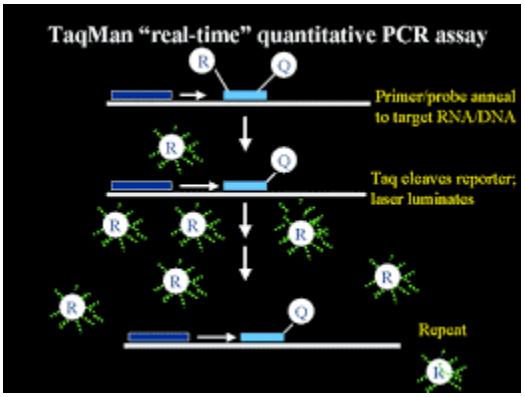
Nested-semi nested PCR

Two sets of primers are used here for a single locus point. The first set is an amplified sequence and the second set is complementary to the first sequence which will be shorter than

the first amplified product. Nested PCR is used because it intends to reduce the contaminations in products due to the amplification of unexpected primer binding sites.
It has drawbacks like risk of contamination and needs great care while being performed. These contaminations can be controlled by adding ultra-pure oil of two mixtures,[5] and by using primers designed to anneal at different temperatures.
Nested/seminested PCR were used in identifying Brucella in human blood samples. This method is more specific but has disdvantages like 'primer and dimerisation'[17] cross reaction. No single specific species yet cross- reacted with PCR products.

Standard PCR
This is simple effiecient and sensitive technique. This method is carried out with one pair of primers which amplifies the targeted genomic sequence of Brucella species. It also helps in the early diagnosis Brucella. Standard PCR technique is used to determine the number of leucocytes DNA/ heamo compounds.

Quantitative/semiquantitative-real time PCR
Fluorescent dyes like SYER Green master mix are used for the identification of samples and probes are used to measure the amount of amplified product in real- time[2]. cDNA is obtained by RT-PCR for a RNA sample. ApoA1/Bactin are used as markers followed by gel electrophoresis process with ethidium bromide dye staining procedure. Here the main disadvantage is the generation of non-specific hybridisation. All the reactions were performed in quadruplicates using the DNA stocks.



Real-time PCR
It is also called as quantitative PCR(q PCR)[5], highly reproducaable,rapid, sensitive and specific technique for automating data. Brucella species has been developed for targeting 16S-23S genea by means of this technique. It has lower risk of contamination. It has 2 techniques for detection

1. SYBER Green dye(or) fluorochromes (or) inter calculating agents
2. Taq man probes (or) fluroprobes.

Other forms of probes include

a. Minor groove DNA binder probes
b. Hybridisation probes
c. Sunrise probes
d. Scorpion probes
e. Molecular beacons
f. FRET (fluorescent resonance energy transfers)[5].

These fluoroprobes are more complex and expensive than intercalculating agents. The increase in the DNA in each cycle reflects a propotional increase in emitted fluorescence and also propotional to the hybridisation of probes.

All probes except molecular beacons uses identical performance characteristics[12]. The real time PCR has many advantages like

1. Quantitative quality control of input target DNA.
2. Fast/ efficient real-time PCR on 96/384-well plates leading to short turn around time.
3. Elimination of post amplication steps.
4. Standardised PCR protocols with uniform amplification specifications which leads to high reproducability.
5. Availability of stable and quality-controlled PCR reagents contributing high reliability.

APPLICATIONS OF PCR
PCR can be applied in many fields

- In diagnosis therapy
- In nucleic acid detection assays
- In medical field
- In agricultural sciences
- In mycology-parasitology
- In dentistry
- In virological diagnostics
- In insert analysis
- In molecular systematic evolution
- In cancer therapy
- In therapy resistant assessment
- PCR-as biomarker
- In forensic medicine
- In virology
- In bacteriology
- In phytopathology
- In PCR-fingerprinting
- In the detection of microbilogical gene.

DIAGNOSTIC APPLICATIONS

Quantitative HIV viral load determination is a most important parameter for diseases outcome and used for diagnosis of the diseases[12].

As a bio-marker for diseases processes

Bio-markers are the genes specially induced or down regulated during diseases. They are called as gene sigmatures[12] which identifies a number of neoplastic disease process

Example: Cytokines-chemokines- receptors are selectively induced and used to detect infectious diseases without the presence of pathogens.

In Medicine

Clinical microbiology is a sub-division of Molecular biology in which the micro-organisms are difficult to identify and culture. Many problems like difficulty in culturing and identification are reduced by the usage of PCR technique. Usage of PCR process has led to the selection and assurance of good samples of blood in blood banks.

PCR technique is also used in the characterization of many viruses, including influenza which benefits the patients a rapidity and sensitivity[5]. So Molecular biology allows the identification of mutations, carriers of diseases like diabetes, obesity, neurological disorders, cardiac, metabolic and congenital diseases.

In Forensic Science

Genetic basis of diseases with sudden death can be investigated with molecular methods, forensic molecular pathology involves the applications of molecular biology in medical science to investigate the genetic basis of pathophysiology of diseases[5]. Genetic profile of alleles identified in different regions of DNA marked by genetic marker STR(short Tandem Repeat).

In Plant Research

In plant research it can be used in the following methods, they are

a. Insert analysis b. Phyto-pathology
c. Molecular system and evolution

Insert Analysis

It is routine procedure in molecular biology for screening and analysis of clones in complementary DNA group. It also involves the cultivation of bacteria followed by the isolation of vectors & DNA inserts for further purification.

Phyto-pathology

It helps to detect and monitor infection of plants by virus, bacteria, fungi. Nucleotide sequences are of only limited to very few plants pathogens such as pseudomonas, xanthomonas, mycoplasmas. RAPD is also one of the methods in identifying and differentiating plant pathogens and also to produce set of specific DNA fragments.

Molecular Systematics-evolution

Morphological characters can be based upon the phytogenetic relation and during last decades molecular techniques has become more and more popular for analysis of plant genes[18]. Molecular Systematics can be used in the following ways:

1. Analysis-comparisons of allozymes
2. Isolation-sequencing of proteins
3. Analysis of RAPD fingerprints
4. Analysis of restriction patterns
5. Sequence comparisions of marker genes.

In Agricultural Sciences

Identification of multiple infectious diseases is the most useful application of PCR technique. The following are the milestone achievements in agricultural fields.

a. Identification of a mutated gene EDA with ectodermal dysplasia in Holstein cattle
b. Identification of polymorphism in ABCB1 gene in phenobarbitol responsive-resistant idiopathic epileptica.
c. Deletion of Meq gene (which decreases immunosuppresion in chickens).
d. MTM/ mutated gene with X-linked Myotubular myopathy in dog species.
e. Insertion mutation in ABCD4 with gall bladder mucocele formation in dogs
f. Agricultural techniques can also be used for the identification and charcterisation of specific pathogens of animals.
g. Bursal diseases virus-Avian samples
h. Bovine respiratory syncytial virus
i. Actinobacillus pleuropneumoniae-pgs samples
j. Canine parvo-virus type-2-faecal samples of dogs
k. Feline immunodeficiency virus
l. Feline leukemia virus(FeLV).

In Virology

PCR technique is used to monitor antiviral therapy in HIV-1,HBV,HSV-1,HSV-2. RT-PCR used in the study of infectious diseases. Diagnosis was a big problem due to high cost lab time consuming and of low sensitivity of the results. PCR technology facilitated and improved

the detection, diagnosis of certain number of genes.

In Microbiology

Conventional PCR is the most oftenly used technique . the following species has conventional PCR techniques already developed such as Bacillus anthracis, Variola major, Anthrax species. It has high sensitivity , easy usage and less time consuming.

50-75% of anaerobic bacteria can be identified and classified by conventional PCR. It can also be used for the detection of Lactobacillus, Gadnarella vaginalis, Mycoplasmas horminis, Fuso bacterium species. Many commercial assays are available for different species like M tuberculosis, M avium complex, C trochomatis, N gonorrhea. So molecular detection is a invasion metod which is less time consuming and rapid.

In Dentistry

They can be useful in the following conditions such as

1. Periodontal diseases
2. Dental caries
3. Oral cancer
4. Endodontic infections

Periodontal Diseases

The following viruses can be detected by PCR techniques in periodontal diseases , such as HCMV, EBV, HSV, HPV. QRT-PCR used to examine gene expression invivo in oral cavity. Many scientists has compared conventional PCR and RT-PCR for the detection and quantification of peridonto pathogenic bacteria such as A. actinomycete, M comitans, P. gingivalis, T. dentiola, T. socranskci etc.

Dental caries

Dental health destroying bacterias such as S. cricettus, S.ratti , S. mutans, S. sobrinus, S. downei, S. ferus, S. macacae out of which mutant genes of S. mutans/ S. sobrinus can be easily isolated from human oral cavity. This PCR technique allowed rapidity and efficient results and quick completion of reaction.

Endodontic infections

Important endodontic pathogens include T. denticola, D. pseudomosintes, F. alocis, T. forsythia, T. malthopilum, T. socranskii and P. tannearae . using PCR techniques these strains can be easily detected and identified. PCR techniques has raised the medical microbiology field to another high position by its method.

Oral cancer

By RT-PCR, S. anginosus can be easily detected and diagonised and treated. Prognosis of oral cancers can be done by RT-PCR technique. In carcinoma of head and neck, PCR technique is used to detect EBV virus in nasopharynx cancer and squamous cell carcinoma in lymph nodes.

The PCR technique can also be used to detect breast cancer, follicular lymphoma, stomach cancer, prostate cancer, Ewling's sarcoma.

In Mycology- Parasitology

Conventional PCR is qualitative technique, fluroscent PCR utilizes marked primers/probes to detect fungi in environmental samples. Pneumocystis jiroveci causes severe pneumonia in patients infected with HIV, its detection is done by immune-fluroscence technique[1]. Aspergillus species can also be detected by immune=fluorescent technique. Otherspecies which can be detected are Plasmodium and histopathiological species.

In the field of Infectious Diseases

Hepatitis C virus causes liver inflammation leading to liver cirrohis/ cancer. PCR used for the detection of HCV infection.

In HIV-AIDS

Quantitative PCR is used to detect HIV-AIDS. Three combinations of drugs used against HIV and regular monitoring of these 3 drugs level in the body can be done by PCR technique.

In Cancer-Therapy

Both qualitative and quantitative PCR techniques can be used in detecting diseased strains. p53 gene is helpful in monitoring in cellular divisions, which can be detected by PCR-technique, regardless it out of control leads to cancerous cell production.

PCR technique used to control the cancer diseases by a process called Promoter Methylation(9). Cellular divisions can't be changed but can be switched off. In the DNA region the cell attaches small molecules (-CH3 groups) to the building blocks of DNA. Thus polymerases normally read the genes and produce working copies are no longer allowed to enclose and start to another region. Hence remains silent and no new cell is formed.

CONCLUSION

PCR is a simple technique, widely used process in minutes to produce large amplified no. of copies. PCR would work on larger and larger fragments of plasmid DNA's are produced[13].PCR is moreover a sophisticated technique with high sensitivity, specificity, requires infrastructural

support but it's expensive[10]. PCR technique is also used for inhibitory activities of metal ions[18]. PCR techniques can also be used in the detection of micro-organisms like Salmonella species. The applications of molecular biology PCR techniques has transformed diagnosis-prognosis-treatment of many diseases.PCR used for creations of recombinant clones and can be characterized by colony PCR technique[19]. RT-PCR technique is also one of the accurate, less time consuming technique for the detection of micro-organisms[7].

REFERENCES

1. Maecela Agne, Rafael Lima, Licas Andre, Paulo Roberto, Alessandria de Tavaras and Sergio Crovela. Principles and Applications of Polymerase chain Reaction in Medical Diagnostic fields- A Review. Brazilian Journal of Microbiology. 2009;40;1-11.
2. Tahminur Rahman MD, Muhammed Salahuddin, Razia Sultana, Arumina Moue and Muntahina Setu. Polymerase Chain Reaction-A Short Review. AKMMC. 2013,4(1):30-36.
3. Mohini Joshi and Deshpande JD. Polymerase Chain Reaction-Methods, Principles and Applications: International Journal of Biomedical Research. 2010;1(5):81-97. Tabitha M. Powledge-The Polymerase Chain Resction Breakthroughs in Bioscience-A Series of articles for general andiences.
4. PCR methods have made excellent progress diverse fields of medical sciences, microbiology, mycology, dentistry, parasitology, infectious diseases based upon the above review work made in this article.
5. Waleed A Ibrahim, Abdul Ghany A, Soad A Nasef and Hatem ME. Comparative study on the use of RT-Pcr & standard isolation techniques for the detection of Salmonella in broiler chicks; Internetional Journal of Veterniry Sciences and Medicine. 2014:2.67.
6. Sridhar Rao P. N-Polymerase chain Reaction CPCR, www.micro rao
7. A Download document on polymerase Chain Reaction- explained.
8. Somma M and Querci M. Analysis of food samples for the presence of Genetically Modified organism; JRC-European Commission; Session-6.
9. Downloaded document on Polymerase chain Reaction- explained.
10. Pusterla N, Madiga JE and Leutenegger CM.RT-PCR A Novel Molecular Diagnostic Tool for Equine Infectious diseases.; Journal of Veterinary Medicine. 2006;(20):3-12.
11. Kary Mullis B. The Usual origin of Polymerase Chain Reaction. Scientific American Journal. 1990.
12. Lilit Gariyan and Nidhi Avashia. Research Techniques made simple-PCR;Journal of Invest Dermatol. 2013;133(3):6.
13. Pablo Perez-Pinera, Manuel Menendez Jose and Antonio Vega. Detection of DNA sequences of using PCR based approach; Electronic Journal of Biotechnology. 2006;9(5):22-25.
14. Bria D Mariani, Daniel S Martin, Antonio F Chen, Haruyo Yagi, Shaldon S Lin and Rocky S Taun. PCR-Molecular diagnostic Technology for Monitoring Chronic Osteomyelitis. Journal of Experimental Orthopaedics. 2014;19.
15. Ying Wang, Zhanli Wang, Chunfang Liu, Anma and Hui Yu. PCR-based assays for the diagnosis of human Brucellosis; Annuals of Clinical Microbiology & Antomicrobials. 2014;13:31.
16. Masool Majaz, Parvaiz Hassan, Vijeshwar Verma and Zafar Reshi. Inhibition of Polymerase Chain Reaction by Lithium chloride. International Journal of Life Sciences and Pharma Research. 2012; 2(4).
17. Margaret F Docker, Robert H Delvin, Jon Richard and Jaswinder Khattra. Sensitive & Specific PCR- assay detection of Loma Salmonae (microsporea). Inter search. 1997;29:41-48.
18. Saroj K Dangi, Ajay P Singh and Satyaveer S Dangi. PCR amplification-Cloning of Immunogenic protein NAd-dependent beta hydroxybutryl coA dehydroganase gene of Clostridium Chauvoei; Veterinary World. 2015;7(10):848-51.
19. Thoraia Shinawi. Lab manual of Molecular biology lab.M.Wink, Heidelberg; Bioforum Extra- Prague. 1994;5-17.