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Sex Determination Using Polymerase Chain Reaction (PCR): A Research Report

Kaushal Richa

Department of Life Sciences, University of Bedfordshire, Park Square Campus, Luton LU1 3JU Bedfordshire, UK

***Abstract*: To govern the sex of given unknown samples by firstly amplifying the SRY gene, which was required for the testis differentiation, using polymerase chain reaction (PCR) technique and then attaching specific primers. Gel electrophoresis was undertaken for separating DNA fragments and visualisation using UV light to get images of bands for comparison of length and results by DNA analysis through spectrophotometry. In this experiment agarose gel was used because of its simple nature, fastness and nontoxicity. Conclusive results were obtained and the analysis was successful.**

***Keywords:* genotyping, sex determining gene, DNA replication, SRY gene, PCR primers, Spectrophotometry.**

I. INTRODUCTION

Conversion of chromosomal sex at the time of fertilisation into gonadal and somatic sex is responsible for sexual differentiation. In several cases like where the sex of father and child is to be matched, where application of medical and genetic organization is of huge prominence, here genotyping is essentially useful, also for the analysis of genetic disorders, criminal accusations. Thus, sex determination act as an invariant tool of utmost value for genetics, criminal sciences, for putting advanced technology in noble usage [1]

Using the present information of molecular basics and genetics, we can determine sex in mammals SRY gene, is the mammalian sex determining gene, which was isolated in 1990. Testes are responsible for production of all the necessary hormones for male phenotype completion. So sex determination in mammals can be done detection of testis differentiation which further requires the presence of protein, which is encoded by SRY gene .Human SRY gene is 223 amino acid proteins .The central one third of this gene encodes a conserved motif of 79 amino acids which are related with DNA, includes Mc protein and nuclear non histone high mobility group (HMG) protein. As a result this SRY conserved motif is referred as HMG box [6]

In maternal plasma cell free foetal DNA is present allowing advanced approaches for gender identification as it is clear from prior research that its concentration is higher in maternal plasma in early pregnancy stages. However, to identify the sex is not that easy, it involves many technical advancements. Now days we can evaluate using PCR protocol, most popular method in cell and molecular biology for genotyping [10] It was first designed by Mullius and colleagues at Cetus Corporation [5].Here the key trick is presence and absence of SRY gene in Y chromosome. In this method amplification of SRY gene is done in PCR product. Here enzyme used is Taq DNA polymerase for DNA replication as it is heat stable. Buffer used should contain Mg+ as cofactor. It would generate many copies of DNA sequences. For analysis, X and Y PCR products would be present only in male DNA. While female has no SRY gene amplification. PCR makes it possible to isolate DNA fragments from genomic DNA by selectively amplifying a specific part of DNA [7].This technique needs to contain an internal control for the verification of absence of SRY gene which may result from PCR's amplification failure.

Specially designed two sets of PCR primers are provided for GAPDH and SRY gene amplification. Primer is of forward nature having 20-30 bases and reverse nature matched for GC content. These PCR primers get submerged and mixed in PCR products. Spectrophotometry is done for DNA determination and the information obtained through it can be useful in measuring the concentration and purity of DNA for each of three samples by observing their absorption spectra at 260

and 280nm, so after DNA quantification we checked the obtained values using this parameter of purity and sex detection occurs by comparison of the given samples using DNA ladder [6]

II. MATERIALS REQUIRED

A. ***Chemicals used-*** Buffer ATL, Proteinase K, Water, Ethanol, Buffer AW1, Buffer AE, Primer mix for SRY and GAPDH dNTPs, taq DNA polymerase, DNA samples, Ethidium bromide, 1.5%agarose, TAE electrophoretic buffer, DNA ladder.

B. ***Apparatus-*** Pipette, Tubes, cuvettes, eppendrof tube, incubator, qlamp mini spin column centrifugator, ice bath, thermocycler, mini gel electrophoresis system, gel documentation system to record images.

III. METHODS USED

A. Pipetting practise:

In this session we tried to get familiar with the pipette for optimum amount of accuracy and precision which is required for the success of the experiment. We used 1-20 µl for pipetting 20µl of water on a piece of Para film for about five times. We also took care of the fact that we need to change and use a new tip each time we pipette out the water. We measured the diameter of drops every time and recorder it for analysing our accuracy. Diameter came to be point 0.4 cm of 4 times out of five consistently which convinced us that we would have less chances of error in latter session of the practical.

B. DNA purification from tissue:

180µl of buffer ATL was added into 1.5ml eppendrof tube after homogenising the tissue. Added 20 µl of proteinase K which was then vortexed to mix thoroughly and after that incubated at 56° C for about approximately 24 hours so that the tissue is lysed fully. In the incubation period the sample was vortexed at regular intervals for complete dispersion of sample. Then, the setting of water bath was done at 70° C for ten minute of duration. 100% ethanol of 200µl concentration was added to the sample and mixing was done by 15 seconds of vortexing. The sample obtained, referred as lysate, was transferred carefully in to new QlAmp mini spin column. After closing the cap centrifugation was done for 1 minute at 13000rpm. Above written step was repeated to pass the solution completely through the column. A new collection tube was taken for transferring the column and tube have filtrate was discarded. 500µl buffer AW1 was added to this tube and centrifuged at 8000rpm for 1 minute. This column was then introduced to a new 2ml tube and just like former step the tube containing filtrate was discarded. Centrifugation of tube was done at 13000 rpm after the addition of 500µl buffer AW2 for three minute. The column was placed back in the same tube after discarding the filtrate. Centrifuged this tube at 13000rpm for 1 minute and then transferred the column into new 1.5ml tube. To this column 200µl of buffer AE was added a gestated at room temperature for 1 minute. It was then centrifuged at 8000rpm for 1 minute without discarding the filtrate afterwards. Now the column was castoff and 1.5ml tube was left on ice for the PCR fixed up.

C. PCR:

In this process, a reaction master mix is prepared properly in a tube, containing the entire important reagent needed for PCR reaction to run smoothly which are as follows:-

PCR buffer, primers mix, d NTP mix,Mg^{2+}, taq polymerase, sterile PCR water, care should be taken that primer mix must contain forward and reverse primers for each SRY and GAPDH. A total concentration of 23µl mixture is then aliquot into tube for unknown and control samples. Added DNA samples to each reaction tube to make final volume of 20µl and at last they are kept in thermo cycler after labelling as “M”, “F”,” 1”,”2”,”3” and “-“ and group number.

Table 1: Then samples were stored at 20° C before later usage

Tube	Master mix volume(µl)	DNA sample	Sample volume(µl)	Final tube volume (µl)
M	23	SRY + Control	2	25

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F	23	SRY-Control	2	25
1	23	Unknown 1	2	25
2	23	Unknown 2	2	25
3	23	Unknown 3(isolated from tissue)	2	25
-	23	Sterile water	2	25

D. Agarose gel electrophoresis:

Second session of practical comprises of a technique done on PCR products obtained from the previous session. Gel electrophoresis is a method used for separating DNA fragments from PCR products. Some kinds of safety measures and precautions should be kept in mind before performing this technique. Firstly, dye used here for the visualisation purpose is highly toxic and mutagenic in nature, present in agarose gel. So gloves are strictly required to be worn while using the gel, handling samples and during viewing images. Lid should always be kept above electrophoresis tank before switching on and it should be removed after switching off the power. Secondly, while dealing with geldoc system provided here gives exposure to UV radiations which can cause serious damage to eyes, skin, so gloves are mandatory. In order to start, 8 sample comb is positioned in mini gel electrophoresis system, gel sol(1.5% agarose),is decanted to depth of 5mm and allowed to settle for 15 minutes. During settling process, 5µl of sample loading buffer is supplemented to each of 25µl of DNA in each 0.2ml tube got from PCR reaction. After setting of gel, comb is removed and TAE (electrophoresis buffer) is added by 0.5cm.In each well, 20µl of all samples tube M, F, 1, 2, 3 etc. is added with 4µl DNA ladder addition to a different well. Now, power is switched on to run the gel for nearly 40 minutes till the dye runs down gel solution at approximately 600bp.After running time is completed fully, gel is viewed under UV light using Geldoc system.

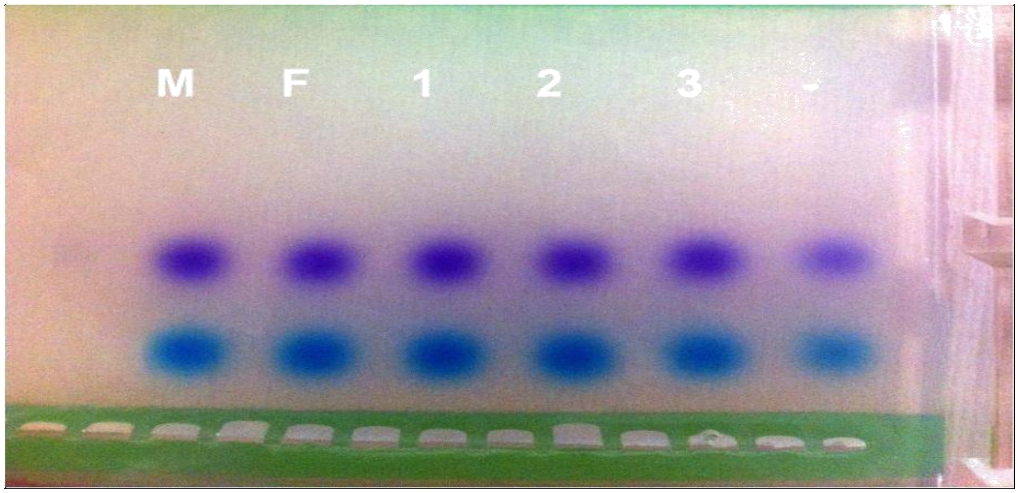

Figure 1: Image above shows electrophoresis gel system with comb within the gel buffer solution

E. DNA determination:

For determining DNA conc.in the respective given samples we used spectrophotometry. This technique can be used by specific DNA absorbance of UV radiation to characterize particular features of DNA.1ml of water is poured into a cuvette and is then moved to spectrophotometer. The equipment is set at 'zero' before doing latter steps. After doing the settings, 200µl of DNA sol.is added to cuvette one by one having 1ml of water. Mixed thoroughly and 'run' button is pressed to get readings at 260 and 280nm for the characteristic distinction and estimation of protein contamination respectively. Their ratio is also recorded for calculations purposes. Data obtained is used for determination of DNA content given in 3 unknown samples 4a,4b,4c.The dilution factor is taken as 20µl of DNA +980µL of water for each 3 samples.

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IV. RESULTS

Table 2- Data obtained from spectrophotometry and calculations

	DNA 4a	DNA4b	DNA 4c
Abs260	0.019	0.148	0,114
Abs280	0.011	0.084	0.071
Ratio abs260/abs280	1.727	1.762	1.606
DNA concentration in cuvette (µg DNA ml)	0.95	7.4	5.7
The dilution factor for sample	50.0	50.0	50.0
DNA concentration in original sample(µg DNA ml)	47.5	370.0	285.0
Amount of DNA in the 20µl sample(µg)	0.95	7.4	5.7

The above given table containing data obtained from spectrophotometry and calculations, help in concluding that DNA 4b has the highest sample concentration when compared with other samples. Also, DNA 4b sample has the highest degree of purity amongst all other given DNA samples. The standard absorbance ratio of a pure DNA is 1.8, using this information if we compare all 3 given DNA samples; we notice that DNA 4b sample has absorbance most closely to this fact i.e. 1.762 as given in table shown above. Thus, DNA 4b is very much suitable and most preferred for use in PCR technique.

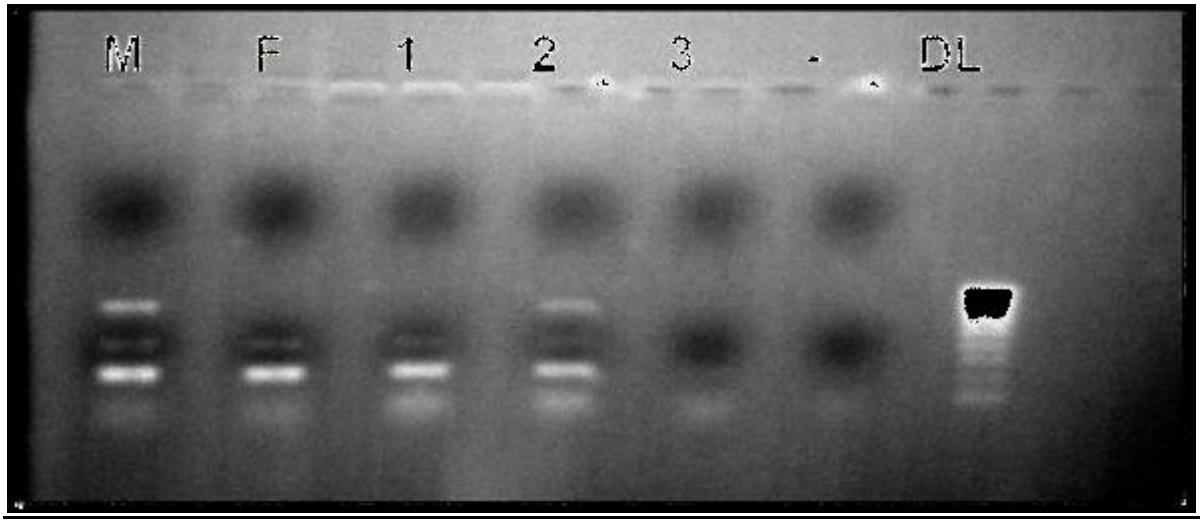

Figure 2

M – Positive SRY control
F- Negative SRY control
1 –Unknown sample 1
2 – Unknown sample 2
3 – Unknown sample 3
DL – DNA ladder

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V. DISCUSSION

A primer is a short synthetic nucleotide used in molecular genomics. Designing of primers is very important for the efficiency of PCR reaction in genotyping. The usage of specific primers for PCR amplification was first reported many years ago. Basically primers are designed for the identification of conserved regions [3] There are a number of factors that determine the estimation of a given sample with amplification. They comprises of GC-content of gene and amplified regions, degree of conserved protein which are encoded, CpG dinucleotide in primer region, length of primers. It is very well known that higher the number of incongruities amid primer and a aim, the lesser probability is of target to PCR-amplify. Primers having length of 24-30 bps are the best suited as they do not dime rise nor they attach themselves irrelevantly at different sites as in the case of too lower and higher number of bps respectively [3]They are non-reactive, inert to themselves and to other primers as well. There is also corollary link between higher gene density and GC-richness in mammalian genomes [5]

Figure 3 N Priming of base pairs

3’ end of primers should be a G or C, or CG or GC: this checks "breathing" of ends and upsurges effectiveness of priming; melting temperature (Tm) between 55-80^{o}C are desired; 3’-ends of primers must not be complementary as or else primer dimers will be synthesised favourably to any other product. Turns of three or more Cs or Gs’ at the 3'-ends of primers may endorse mispriming at G or C-rich sequences (due to steadiness of annealing), and must be eluded [5]. Diffusion of bands in gel images can occur due to dimerization of primers [5] Extra bands which are not required are sometimes obtained because of nonspecific priming or due to presence of primers in unequal molar amounts [2]Primers used in this practical were good as my experiment was a successful one.

SRY gene is expressed in sertoli cells, it plays a useful role in development of gonads [4], induces proliferation of cells in gonads and developing vasculature patterning of XY gonads It has been investigated by scientists that human SRY gene introduces and is responsible for testis determination by following ways:-.

1) Activation of gene expression through consensus binding sites (A/T) AACAAT
2) Helps in bending of DNA
3) Represses a suppressor of testis enhancing factor
4) Involvement in pre-m RNA splicing

The resulting bands obtained from gel electrophoresis of PCR products readily reveals that for SRY gene amplicon is nearly above 700 bp while ideal is 741 bp and bands for GAPDH gene is almost above 200 bp as in for ideal is 218 bp when compared with bases in DNA ladder in ideal data in genotypic PCR of SRY gene [9].For the analysis, we first consider well having solution of “-“ tube, as it has no DNA sample so no bands are attained when visualised .The bands of sample marked “F” which is of female as its bp length is above 200 bp which is for GAPDH gene, present in it but no SRY gene amplified band is present here. In “M” marked sample there are 2 band amplification one for GAPDH and other for SRY gene above 700 bp and 200 bp respectively giving information that it is male. We can observe in the image of unknown sample “2” that there are 2 bands, one at sequence 700 bp and another near 200 bp representing that its male genotype. The sample marked”3” reveals its female genotype when compared to female control as it has a band near 200 bp sequence length only.

Technology advancements due to different research projects have increased the outcomes of success in positive manner in these techniques and also added to the proficiency, accuracy and precision in this field .The kindliness of PCR, marks it more reachable for the DNA sequence amplification from embryo's cells observed in amniotic fluid in today's world but still there is a chance of contamination in the fluid of maternal cell. In Duchenne muscular dystrophy, the cells of foetus perceived in pre natal diagnosis which is screened for abortion, therefore maternal cells contamination in this, fallouts in misdiagnosis. Thus, this type of examination is done with utmost precision, efficiency, caution and vigility [5]

Now days, PCR equipment is constantly revising its levels of working and is reducing its portions of errors. DNA microarrays, genomic libraries, real time PCR, and several others have paved the ways of new augmentation and elucidated the benefits from these methods to positive bounds since discovery. The new advancements in biomedical science since last century has helped in widespread understanding of knowledge regarding network interaction of several gene module which carry out co-ordination with cellular function integration at molecular level of contrivance of phenotypic expression of genotype. The functionality of genome's main part is statically not known. For the complete understanding of metabolic regulation we require more information of DNA recognition protein, transcription factor gene expression. The profile of gene expression has been commonly used for addressing corollary between ecologically influenced and patters of cellular expression. Detection technologies which are PCR based utilised primers of specific species and prove their value as tools of research with specific consideration to aetiology, ecology. In common lab projects, experience with nested PCR gives higher sensitivity and improvise robustness. Whereas methods which are PCR based are commercially developed for detection and quantification of mRNA in many organism. Majority of them have attractive substitute due to high copy number in cell. Nested PCR has higher specificity which makes it favourites for scientists having keen interested gene expression dynamics of plant/microbe interaction [8]. Earlier PCR discovery revolutionised the scientific era and now the possibility to amplify low volume nucleic acid and analysed on the single chip has fascinated keen interest. 'Lab on a chip' has been steadily advancing over last several years. This microanalysis has been introduced for the elimination of cross contamination and proper control of temperature and flow to the solution. In several experiments, to amplify some nucleic acids for the analysis is next to impossible. For their amplification the conventional PCR is not enough .The improvisation of PCR can be done by increasing the rate of heat transfer and reducing the thermal mass. Due to upcoming micro-electro-mechanical systems (MEMS) knowledge, the miniaturized PCR chips have been able to develop [2] The miniaturized PCR devices have various benefits like low reagent ingestion, fast heating/cooling rates, short assay time, with huge potential for integration of multiple processing module to lower the size and consumption of power.

VI. CONCLUSION

The main aim in genomics is to grasp the knowledge that how regulation of genes occur in various diseases, tissues, developmental stages and species. Recent discovery of high-throughput investigation technique, using massively parallel signature sequencing (MPSS), has come in to picture. Mapping DNase hyper sensitive (HS) sites present with in nuclear chromatin is various useful and powerful techniques. This method of testing relates chemistry of PCR along with detection fluorescent probes (taqMan probes, FRET hybridisation probes) of amplified product in the vessel in which reaction occurs [6].So, as both PCR and detection of amplified product comes to completion in less time, quicker than the traditional PCR recognition methods. Real time PCR assays give specificity and indifference with reduced risk of losing the nucleic acids which are amplified [4]. The perfect blend of superb accuracy, specificity, not as much of risk of contamination, sensitivity, and good speed has built an enormous appeal for PCR technology for immune assay testing method based on it, for infectious diseases diagnosis. Thus, PCR technique used in this experiment for the amplification proves to be very righteous.

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