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RESEARCH ARTICLE

TOUCHDOWN PCR FOR INCREASED SENSITIVITY AND SPECIFICITY IN AMPLIFICATION OF MOUSE COGNATE HOMEOBOX

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Abstract

Mammalian system have a large genome with a high level of gene sequence identity from other genomic DNA, making assessment difficult and time-consuming.Our findings describe a simple method for rapidly isolating and amplifying HoxA loci in the mouse genome using degenerate primers. For the semi degenerate primers,they were designed based on cognate gene coding regions of consensus sequences. After assembling sequences from different primer matches amplifying the same HoxA loci, the effects of the universal primer-template match on the efficiency of standard PCR amplification were investigated. Touchdown PCR increased specificity and yield in two consecutive amplifications on different gel concentrations by using high and low annealing temperatures. This method was quick, simple, and inexpensive for amplification of consensus sequences in very large gene sequences.

Introduction:-

Hox genes in mammals play a crucial role in development of both limbs and external genitalia depends on the HoxA and HoxD cluster genes in addition to their function in organizing structures along the main body axis [1-3]. The single most significant methodological advancement in molecular biology to date is undoubtedly PCR [4].Since its inception in the middle of the 1980s, it has quickly established itself as a standard approach in every molecular biology lab for locating and modifying genetic material, from cloning to sequencing to mutagenesis to diagnostic research and genetic analysis [5-7]. For the study of functional genomics, gene expression, protein structure-function correlations, protein-protein interactions, protein engineering, and molecular evolution, these genes must be quickly and easily accessible[8].It is possible to create primers with degenerate nucleotide positions every third base in order to amplify targets for which just the amino acid sequence is known [9]. Here, lower, less rigorous annealing temperatures are used during early PCR cycles, followed by higher, more stringent annealing temperatures during later cycles [10]. The intricacy of the thermal cycler programming is one potential disadvantage of touchdown PCR [11]. A significant portion of the programming capacity of typical thermal cyclers may be occupied because to the various annealing temperatures employed [12].Additionally, attempts to change the annealing temperature range may necessitate extensive reprogramming [13]. By allowing the programming of automatic incremental temperature adjustments in progressive cycles, some more recent thermal cyclers circumvent these issues [14]. The promoter region of the majority of housekeeping genes, tumor-suppressor genes, and over 40% of tissue-specific genes have G+C sequences that were extremely challenging to amplify [15].

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Methods:-

Isolation of DNA

Proteinase K and SDS are used to extract the genomic DNA from whole blood, and then organic chemicals like phenol and chloroform are used to break down the proteins. Additional purification procedures include precipitation with a saturated sodium chloride solution, rinsing in 70% ethanol, quickly drying in the air, and resuspension with 0.2–0.5 ml of TE buffer. Readings of absorbance at 260 nm were used to calculate DNA concentrations. Prior to usage, all genomic DNA stores were kept at 40°C [16].

DNA purity check and analysis

Using alkaline agarose gel electrophoresis in 50 mM NaCl, 1 mM EDTA, and 0.3-0.5% agarose gels to visualise from 2 to > 30-kb single-stranded DNA, the single-stranded integrity of a template DNA preparation is qualitatively evaluated. To achieve pH equilibration, the gel is presoaked in 1X alkaline running buffer for 30 min. Gel is run at 0.5-1.8 V/cm (for example, 3.5–5 h) then neutralised with gentle shaking in 0.1 M Tris-HCl, pH 8.0, 1 mM EDTA for 30 min. The gel is then stained with 0.5 g/mL ethidium bromide in TAE buffer [17].

T_m Predictions

The Tm of the complex is the temperature at which half of the molecules are single-stranded and half are double-stranded. Higher G+C content DNA has a higher Tm than lower G+C content DNA because there are more intermolecular hydrogen bonds. The G+C content alone is frequently used to forecast the Tm of the DNA duplex, but DNA duplex values are also important [18]. Tm = 4(G+C) + 2(A+T) °C may vary for a straightforward, general formula with the same G+C content. To ensure the best primer design and to do more accurate T predictions utilising sequence information (nearest neighbour analysis), software programmes like as Oligo, Primer-BLAST, or Melt are available (Mt. Sinai School of Medicine, New York, NY).

Primer design

Touchdown degenerate primer sequences HoxFP: ATGGGATCCCTCGCCAA and HoxRP:TTAGAACTGGATGTTGGAGTTGT were designed using Primer-BLAST (NCBI-NIH, USA) to check the "uniqueness" of primer sequences against GenBank sequences from the target genome (in this case, the mouse genome). The program's parameters were set to look for primers between 17 and 23 nucleotides, with at least 60% G + C and a melting point of 60^0C. Many of these chosen primers did function as detailed below at an annealing temperature of 68^0C [19].

PCR Programming

For touchdown PCR programing, thermal cycler was set to denature for 1 min at 94°C, anneal for 2 min, and primer extend for 3 min at 74°C. Follow the cycling program with a 7-min primer extension step and a 4°C soak step, annealing stage was set for 2 cycles/°C beginning at 55°C and decreasing at 1°C increments to 41°C (i.e., 30 total cycles in 15 steps) to be followed by 10 additional cycles at 40°C [20].

Product Analysis

Agarose gels of 0.5%, 1%, 1.5%, and 2% (Genei, Bangalore) run in either TAE or TBE, PCR products are identified. A marker for size estimations of the products, such as a 100 bp or 1 kb ladder, is put into wells with 10ul of the amplified sample. The resolved DNA bands are detected by staining the gels with 0.5 µg/mL of ethidium bromide, followed by destaining with water and are operated at 1.5 V/cm for up to 6 hours or at 5 V/cm for 1-2 hours, depending on the amount of resolution required, and are then photographed under UV light [21].

Results:-

Isolation of genomic DNA

Blood samples were collected, and genomic DNA was extracted by the silica column (Genei, Bangalore). This method involves rapid and efficient purification of genomic DNA from a variety of samples such as mammalian cells and tissue, mouse tails, E. coli, and yeast. The whole genomic DNA obtained was analyzed by agarose gel electrophoresis, and the results are shown. Nucleic acid molecular weight marker, 1 Kb DNA Marker (Genei, Bangalore). The highest DNA yield was from fresh blood treated with deionized water; its purity was close to 1.8.

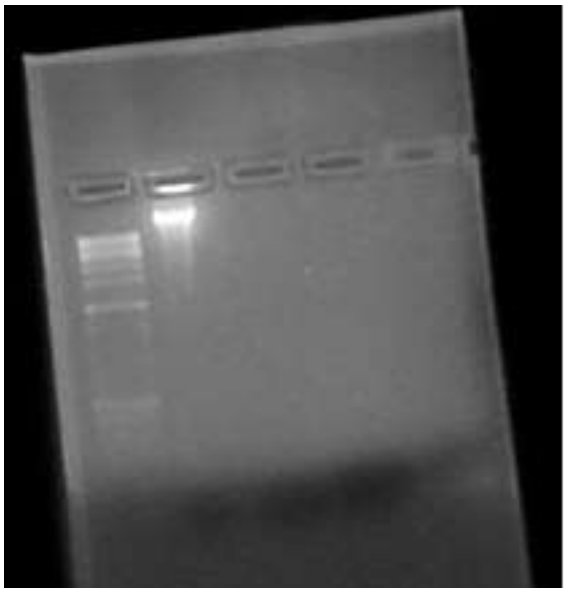

Fig:- Mouse genomic DNA was isolated and stained with ethidium bromide.

HOXA homeobox

The homeobox genes encode a highly conserved family of transcription factors that play an important role in morphogenesis in all multicellular organisms. Mammals possess four similar homeobox gene clusters, HoxA, HoxB, HoxC and HoxD, located on different chromosomes, consisting of 7 to 13 genes arranged in tandem. This gene is one of several homeobox HoxA genes located at 2q31-2q37 chromosome regions. Deletions that removed the entire HoxA gene cluster or 5' end of this cluster have been associated with severe limb and genital abnormalities. The exact role of this gene has not been determined. [provided by RefSeq, Sep, 2011].

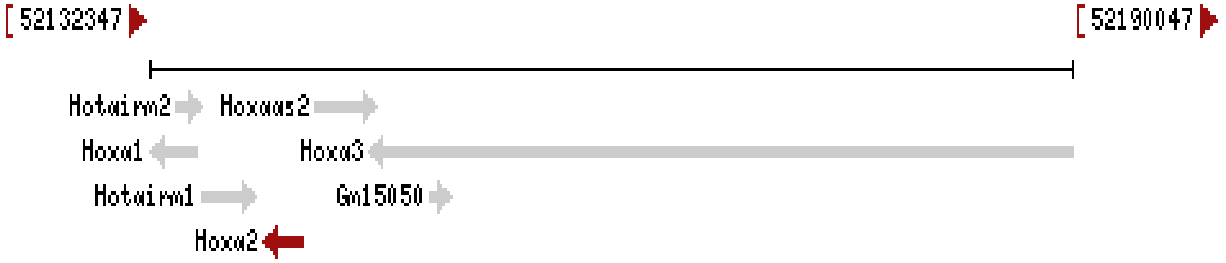

Fig 1:- Homeobox genes located on mouse chromosome retrieved from NCBI.

Primer design

Primer pairs are specific to input template as no other targets were found in selected database. Several parameters including the length of the primer, %GC content and the 3' sequence has been optimized for successful PCR. Primer-blast uses stringent parameters that can detect targets having significant number of mismatches to primers. Ten different primer pair were retrieved from the database using Primer-Blast which allow primers to amplify mRNA splice variants.

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	CTACCCAAGCCTAGGCGAAC	Plus	20	1002	1021	60.18	60.00
Reverse primer	CGAAACCCCAAAGCCACTTG	Minus	20	1724	1705	59.97	55.00
Product length	723						

Fig 2:- Primer pair used for amplification of D9 gene designed using Primer design software.

PCR Amplification

The whole genomic DNA obtained was used as the template for PCR analysis.Reaction volume of 10ul is prepared using 2X master mix of 10ul, primers of 1ul each and template DNA of 3ul and final volume to 15ul with sterile water. PCR amplifications is carried out in a programmable thermal cycler (Eppendorf), program the following method: 94°C for 50s (reagent mixing and initial template denaturation); 94°C for 50s (denaturation) and 64-57°C for 50s (increment of 1°C annealing for every 5 cycles); 72°C for 50s (synthesis); 70°C for 10 min (final completion of strand synthesis); 4°C until tubes are removed.

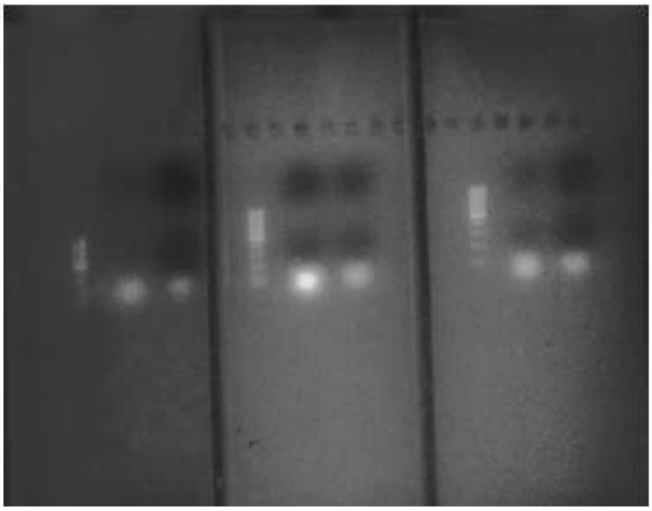

Fig 3:- PCR amplicon were loaded into wells (Well 1-SDPCR*and Well 2-TDPCR*) and analyzed by agarose gel electrophoresis with 1%, 1.5% and 2% gels along with 1kb ladder.

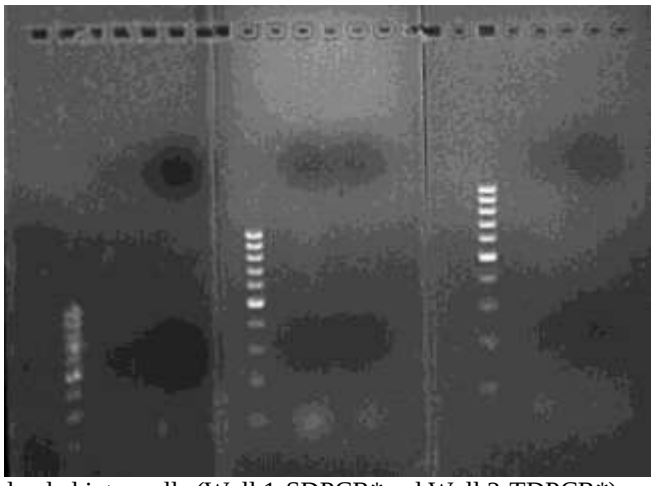

Fig:- PCR amplicon were loaded into wells (Well 1-SDPCR*and Well 2-TDPCR*) and analyzed by agarose gel electrophoresis with 1%, 1.5% and 2% gels along with 100bp ladder.

*SDPCR – Standard PCR

*TDPCR – Touchdown PCR

Conclusion:-

We used this method in a case where we had designed primers to amplify only the HOXA loci in the mouse genome. Touchdown PCR from 100ng of total DNA using primers at an annealing temperature of 55^0C, gave the expected product of 140bp. This was only exacerbated by the traditional processive approach of incrementally raising the annealing temperature every five cycles during the PCR. Using the touchdown strategy the imbalance between correct and spurious annealing was automatically redressed, and allowed amplification of sufficient correct length of amplicon. Even in cases where an appropriate discriminatory temperature has been empirically determined, the touchdown approach could also help avoid secondary problems, such as an inconsistency of well temperatures within or between thermal cycling machines.

Conflict of Interest
Nil.

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