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Touchdown PCR combined with semi degenerate primers for rapid amplification of HOXD9 loci in humans

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Abstract

Higher eukaryotes possesses a large genome with a high level of gene sequence identity from other genomic DNA and is therefore difficult in assessment and time-consuming. Here we report on an efficient approach for rapid isolation and amplification of HOXD9 loci of human genome via touchdown PCR using semi degenerate primers. For the semi degenerate primers,they were designed based on conserved gene coding regions of consensus sequences. The effects of the universal primer-template matches on the efficiency of standard PCR amplification were investigated after assembly of sequences from different primers matches amplifying the same HOXD9 loci. Touchdown PCR increased both specificity and yield by high and low annealing temperatures in two consecutive amplifications on various gel concentrations. This approach was fast, easy and cost-effective for amplification of consensus sequences in very large gene sequences.

Keywords: Touchdown PCR, degenerate primers, HOXD9 loci, DNA, Genome, Consensus sequences.

INTRODUCTION

Most animal homeotic genes encode transcription factor proteins that contain a region called the homeodomain and are called Hox genes [1].HOXD9 proteins belong to a superfamily that regulates the development and control of many cellular processes [2], including proliferation [3] apoptosis [4], cell shape [5] and cell migration [6]. HOXD9 can also function as an oncogene in several cancer cells [7]. Besides their role in organizing structures along the main body axis, HoxA and HoxD cluster genes are required for proper development of both limbs and external genitalia [8]. PCR is probably the single most important methodological invention in molecular biology to date that tracks the polymorphism and evolutionary process [9]. Since its conception in the mid-1980s, it has rapidly become a routine procedure in every molecular biology laboratory for identifying and manipulating genetic material, from cloning, sequencing, mutagenesis, to diagnostic research and genetic analysis [10]. The fast and easy availability of these genes isessential for the study of functional genomics, gene expression, protein structure–function relationships, protein–protein interactions, protein engineering, andmolecular evolution [11].Primers with degeneratenucleotide positions every third base may be synthesized in order to allow for amplification of targets where only the amino acid sequence is known [12]. In this case, earlyPCR cycles are performed with low, less stringent annealing temperatures, followedby later cycles with high, more stringent annealing temperatures [13].One potential drawback to touchdown PCR is complexity of the programming on thermal cyclers and optimization of primer concentration [14]. Because of the numerous annealingtemperatures used, a large segment of the programming capacity of conventional thermal cyclers can be encumbered. Also, attempts to adjust the annealing temperaturerange can involve considerable reprogramming. Most housekeeping genes, tumor-suppressor genes, and approximately 40% of tissue-specific genes contain G+C sequences in their promoter region that were very difficult to amplify[15]. Some newer thermalcyclers avoid these problems by permitting the programming of automatic incremental temperature changes in progressive cycles [16].

MATERIAL AND METHODS

Isolation of DNA

Genomic DNA from whole blood is extracted with a combination of Proteinase K and SDS followed by protein degradation with organic reagent such as phenol and chloroform[17].Additional purification steps such as precipitation with a saturated solution of sodium chloride, rinsed in 70% ethanol and air-dried briefly and resuspended with 0.2-0.5 ml of TE buffer. DNA concentrations were determined by absorbance readings at 260 nm. All genomic DNA stocks were stored at 4^0C until further use.

Analysis of template integrity

Single-stranded integrity of a template DNA preparation is qualitatively assessed using alkaline agarose gel electrophoresis in 50mMNaCl, 1mM EDTAwith 0.3–0.5% agarose gels to visualize from 2 to > 30-kb single-stranded DNA.Gel is kept for presoak in 1X alkaline running buffer for 30 min to ensure pH equilibration. Gel is run at 0.5–1.8 V/cm (e.g., 3.5–5 h) and neutralized by gently shaking in 0.1 M Tris-HCl, pH 8.0, 1mM EDTA for 30 min, and then stained with 0.5 µg/mL ethidium bromide in TAE buffer.

T_m Predictions

The temperature at which half the molecules are single-stranded and half are double-stranded is called the $T_m$of the complex[18]. Because of the greater number of intermolecular hydrogen bonds, higher G+C content DNA has a higher $T_m$than lower G+C content DNA. Often, G+C

content alone is used to predict the $T_m$of the DNA duplex, however, DNA duplexes values. A simple, generic formula with the same G+C content may have different Tm = 4(G+C) + 2(A+T) °C. Software packages for calculating the Tmvalues are available to perform more accurate T predictions using sequence information (nearest neighbor analysis) and to assure optimal primer design, e.g., Oligo [19], Primer-BLAST [20],or "Tp" algorithm [21].

Primer design

Touchdown semi degenerate primer sequences as shown in Table 1,were selected using Primer-BLAST (NCBI-NIH, USA), which screens GenBank sequences from the target genome (in this case, the human genome) for the "uniqueness" of primer sequences[22]. Program parameters were set to screen for primers of 17-23 nucleotides, with a minimum G + C content of 60% and a minimum melting temperature of 60^0C. Many of these selected primers did work at an annealing temperature of 68^0C as described below.

PCR Programming

For touchdown PCR programing [23], 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.

RESULTS

Isolation of genomic DNA

Blood samples were collected, and genomic DNA was extracted by the silica column (PureLink™ Genomic DNA Purification Kit, Thermo Scientific). 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 in Figure 1. Nucleic acid molecular weight marker, 1 Kb DNA Marker (Thermo Scientific, SM0243). The highest DNA yield was from fresh blood treated with deionized water; its purity was close to 1.8.

HOXD9 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 9 to 11 genes arranged in tandem. This gene is one of several homeobox HOXD genes located at 2q31-2q37 chromosome regions as shown in Figure 2. Deletions that removed the entire HOXD 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, Jul 2008].

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 [24].Ten different primer pair were retrieved from the database using Primer-Blast which allow primers to amplify mRNA splice variants.

Primer	Sequence 5’ - 3’
HOXD9 (F)	ATGGGATCCCTCGCCAATTG
HOXD9 (R)	TTAGAACTGGATGTTGGAGT

Table 1. Overview of Forward and Backward oligonucleotides

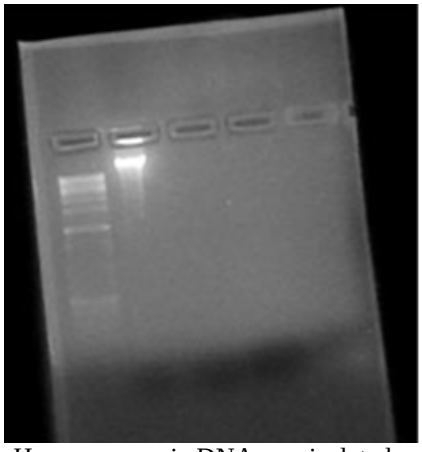

Figure 1. Human genomic DNA was isolated and stained with ethidium bromide

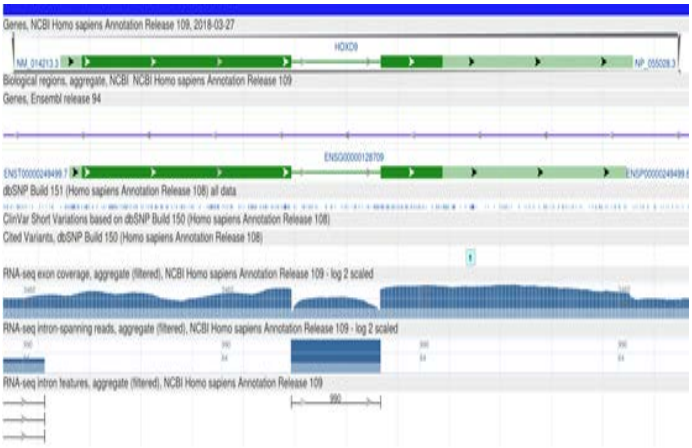

Figure 2. Homeobox genes located on human chromosome retrieved from NCBI

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.

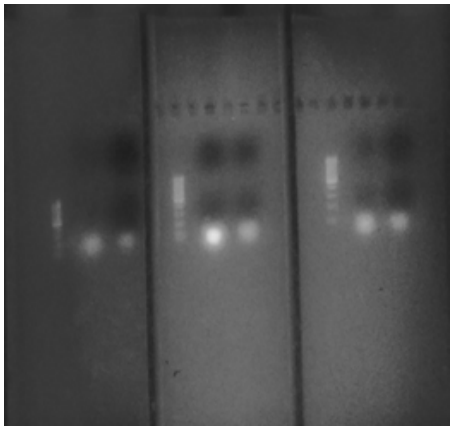

Figure 3. PCR amplicon were loaded into wells (Well 1-SDPCR*and Well 2-TDPCR*) and analyzed by agarose gel electrophoresis with 1%, 1.5% and 2% gels along with 1kb ladder

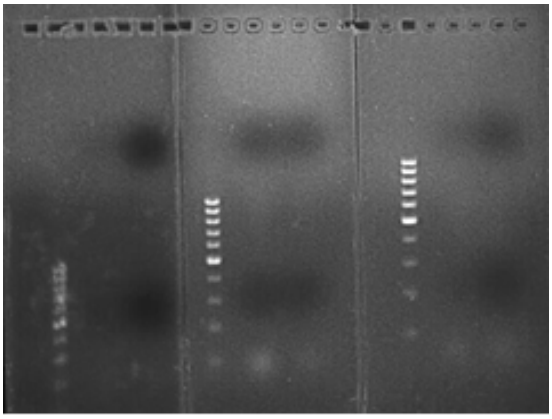

Figure 4. PCR amplicon were loaded into wells (Well 1-SDPCR*and Well 2-TDPCR*) and analyzed by agarose gel electrophoresis with 1%, 1.5% and 2% gels along with 100bp ladder
*SDPCR – Standard PCR
*TDPCR – Touchdown PCR

Product Analysis

PCR products are determined using precast agarose gels of 0.5%, 1%, 1.5% and 2% (Himedia, Mumbai) run in either TAE or TBE. 10ul of amplified sample is loaded into wells along with 1kb ladder as shown in Figure 3 or 100bp as shown in Figure 4 as a convenient marker for size estimates of the products. The resolved DNA bands are detected by staining the gels with 0.5 µg/mL of ethidium bromide, followed by destaining with water. Depending on the level of resolution needed, gels are run at 1.5 V/cm for up to 6 h or at 5 V/cm for 1–2 h and finally photographed under UV illumination.

CONCLUSION

We used this method in a case where we had designed primers to amplify only the HOXD9 loci in the human 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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