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An *in silico* approach to design T-arms primers for VDR locus to detect osteoporosis

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Abstract

Osteoporosis is a skeletal disorder characterized by an increased risk of fractures. Studies have shown that BMD and bone turnover are under strong genetic control. The polymorphisms in the Vitamin D receptor (VDR) have been implicated in many studies as a cause of osteoporosis. We aimed at developing a molecular diagnostic kit to detect osteoporosis. We started with designing multiplex primers and finally designed T-ARMS PCR Kit for the VDR locus. We used the currently available softwares for primer designing like PerlPrimer, Primer3, Primer1, BatchPrimer3, WASP, Primer-BLAST and AutoDimer. Our T-ARMS PCR Kit for the VDR locus included the penultimate primer position mismatch to increase the specificity and a universal sequence and tag to assist successful amplification of all primers within the desired annealing temperature range. Our osteoporosis detection kit is an in-silico approach and has to be validated through *in vitro* PCR amplicifactions.

Keywords: PCR, VDR, Osteoporosis, T-ARMS, Universal sequence.

1. Introduction

Osteoporosis, a multifactorial skeletal disease is an increasing health problem worldwide and is characterized by a reduced bone mineral density (BMD) and increased fracture risk particularly in postmenopausal women. Genetic revelations have started elucidating the complex associations of vitamin D signalling and bone health and cellular proliferation and differentiation [1,2]. The biological actions of vitamin D are considered to be exerted through the nuclear vitamin D receptor (VDR) a member of the nuclear hormone receptor superfamily which acts as a ligand-inducible transcription factor which mediates the effects of 1, 25(OH) 2D3 by regulating the transcription of a number of different cellular genes [3]. The VDR gene is present on chromosome 12p and has a complex intron/exon structure. This gene contains nine exons and spans approximately 75 kb of genomic DNA. Exons 1a–1f encodes the 5' untranslated regions (UTR) of the VDR messenger RNA (mRNA) and seems to be alternatively spliced. Exons 2 and 3 encode the translation start site, which is the DNA binding domain and consists of two zinc finger motifs (one in each domain). Exons 4–9 encode the overlapping ligand binding region and strong heterodimerization domains. Exon 9 also contains the entire 3' UTR region [4, 5].

Vitamin D Receptor gene (VDR) has six Single Nucleotide Polymorphisms (SNPs) namely CDX-2 (G-A), FokI (C-T), BsmI (G-A), Tru9I (G-A), ApaI (C-A),TaqI (T-C).These SNPs appear in literature as CDX-2 (rs2238136), FokI (rs2228570), BsmI (rs1544410), Tru9I (rs757343), ApaI (rs7975232), and TaqI (rs731236) [6]. The sequence surrounding each of these SNPs can be obtained from the dbSNP[7]. CDX-2 is located in the promoter region and can

affect gene regulation. The start codon polymorphism of the FokI (rs2228570) site in exon 2 alters the start codon leading to a 424 amino acid protein instead of 427 amino acids (figure 1). The VDR gene TaqI polymorphism (rs731236) is an RFLP at codon 352 in exon 9 of the VDR gene and is a synonymous mutation. The ApaI (rs 7975232) and the BsmI (rs 1544410) polymorphisms are RFLPs in intron 8 at the 3' end of the VDR gene [8].

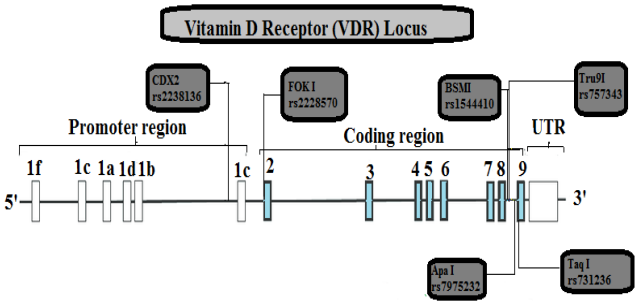

Figure 1: The Vitamin D Receptor (VDR) locus

Osteoporosis is a serious disease all over the world and it affects more than 75 million people in Europe, Japan and the USA. [9] In India the number of people affected with osteoporotic fracture above the age of 50 is approximately 163 million. This number is expected to increase to 230 million in 2015 and such more in the years to come [10]. If osteoporosis is detected earlier then the probability of fracture can be minimised by starting a prophylactic therapy.

1.1 Techniques to detect polymorphisms in the VDR locus:

1.1.1 PCR - RFLP

The VDR SNP polymorphisms can be detected by PCR-RFLP, a polymerase chain reaction amplification followed by a restriction fragment length polymorphism system with the restriction enzyme. Flugge *et al.*, 2007 [6] and Douroudis *et al.*, 2003 [11] have used a PCR RFLP method to detect the polymorphisms in the VDR region. This is a cumbersome, time-consuming, expensive procedure, and some methodological alternatives have already been described. Since the number of individuals to be tested to prove association is typically high, simple and less labour-intensive methods are desirable [12].

1.1.2 T-ARMS PCR

T-ARMS, an improvement to Multiple Allele Specific (MAS) PCR[15] a tri primer system and Multiplex ARMS PCR[16] a tetra primer amplification refractory mutation system has proved to be rapid, simple and economical method to genotype SNPs. Genotyping requires only a single PCR followed by electrophoresis separation as with multiplex ARMS. It is based on the principle of combining two outer primers and two allele specific inner primers. The novelty here is a mismatch base to increase the specificity of the reaction introduced at the 3′ end of SNP primer at position-2 (second to the terminal) from 3' terminus of the same allele-specific primer. Mismatch created through T-G or C-A transversions could increase the allele specificity [17]. This extra mismatch destabilizes the base paring between the primers and their corresponding non-targets templates and have been found to increase the specificity of the reaction by eliminating false-positives results [18]. Zhang *et al.*, in 2013 developed a T-ARMS PCR, using eight primers, to simultaneously detect two cervical and four breast cancer mutations [16].

The known mutations in VDR locus can be identified by various technologies of which PCR-RFLP is common. Usually starting with the polymerase chain reaction (PCR), additional assay steps are performed based on the type of mutation. But PCR-RFLP is a cumbersome and techniques like SNaPshot [19-21] is an expensive procedure for large scale genotyping so we aimed at developing a Multiplex PCR and finally arrived at T-ARMS PCR kit to detect VDR gene polymorphisms for osteoporosis from the available online primer designing softwares.

2. Materials and Methods

2.1 Design of Multiplex PCR

The criteria for a good primer include many parameters that are essential to a successful PCR amplification. Some of the considerations for an optimal primer set for the gene of interest with high specificity and yield are listed below (Table 1).

Table 1: Primer parameters for a successful PCR

S.N	Primer Parameters	Description of Primer Parameters
1	**Primer Length**	18-30 nucleotides.
2	**Melting Temperature (Tm)**	55°C- 65°C, Tm variation between a primer pair is 3°-5°C.
3	**GC content**	40 and 60%. Balanced distribution of GC-rich and AT-rich domains
4	**3'-End Sequence**	Critical for the control of mis-priming. 3' of a primer ending in C or G promotes binding
5	**Secondary Structures**	Should not contain complementary sequences within themselves or form hairpins. Intra and inter primer homology to be avoided. Runs of 4 or more of one base, or dinucleotide repeats to be avoided.
6	**Specificity**	Should be specific to the target sequence and is dependent on primer length.

2.2 Primer Designing Software used in this study

2.2.1 PERLPRIMER

PerlPrimer developed by Marshall in 2004[22] is an open source GUI application written in Perl that designs primers for generic, bisulphite PCR, QPCR and sequencing primers. It allows the user to specify the concentration of Mg2+, dNTPs and primers, or use standard PCR conditions [23].

2.2.2 Primer-BLAST

Primer-BLAST developed by Ye *et al*., 2012 [24] combines BLAST with a global alignment algorithm to ensure a full primer-target alignment in designing target-specific primers. It is sensitive enough to detect targets that have a significant number of mismatches to primers, and also checks the specificity of pre-existing primers. [25]

2.2.3 Primer3:

Primer3 developed by Untergasser *et al*., 2012 [26] can analyze primers for PCR and real time PCR experiments. It constitutes some important features like primer detection, cloning, sequencing and Primer listing. The sequences downloaded from dbSNP are used to design PCR primers to amplify fragments harbouring each SNP [27].

2.2.4 BatchPrimer3:

BatchPrimer3 developed by You *et al*., 2008 [28] uses Primer3 core program as a major primer design engine and can be used to design several types of primers including hybridization oligos, generic primers, SSR primers, SNP genotyping primers (like SNaPshot primers, SSP primers, and T ARMS primers), as well as DNA sequencing primers combined with a new score-based primer selection module. It can also be used to select position-restricted primers. [29]

2.2.5 WASP:

WASP (a Web-based Allele Specific Primer designing tool) designs multiplex allele-specific (AS) primers for detecting SNPs and was developed by Wangkumhang *et al*., 2007 [30]. WASP enables users to design MAS primers for SNPs in the local SNP database including dbSNP, Hap map, and JSNP. The Penultimate Primer Position Mismatching is done to improve reaction specificity additional mismatches at the penultimate base of the AS primers. Since different mismatches have different destabilizing effects, it is essential to consider terminal and penultimate mismatch together. This guarantees that the PCR hybridization will stop when it encounters another mismatch at the penultimate location.[31]

2.2.6 PRIMER1:

Primer1 software was developed by Collins and Ke, 2012 [32] provides for T-ARMS PCR. The Primer1 program is implemented and the algorithm used for calculating complementary is similar to Rozen and Skaletsky, 2000 [33]. The program follows the Little 1997 [34] rules for selecting the additional mismatch base. First, it identifies all of the possible inner forward and inner reverse primers. An 'optimal' inner primer pair is identified form this set which has the closest match to the input Tm and also the minimum Tm difference between the two primers. It then proceeds to select the outer reverse and outer forward primers matching the mean Tm of the two inner primers [35].

2.2.7 AUTODIMER:

AutoDimer was developed by Vallone and Butler [36] can screen sets of preselected PCR primer pairs for potential cross-reactivity. While Primer3, PerlPrimer and Primer-BLAST can screen singleplex primers for secondary structures and compatibility, Primer1, Wasp, and BatchPrimer3 can screen upto three to four primers simultaneously. But AutoDimer can check form singleplex primers upto 1000 different primer sequences simultaneously [37].

We used the whole genome sequence (>gi|568815586:c47908762-47845054 Homo sapiens chromosome 12, GRCh38.p2) downloaded from NCBI and the Reference Sequence (rs11568820, rs2228570, rs1544410, rs7975232, rs731236) for Vitamin D Receptor (VDR) from NCBI-dpSNP to design a T-ARMS multiplex PCR to detect osteoporosis.

3. Result and Discussion

We selected four SNPs ApaI, TaqI, BsmI and FokI for this study because of the association of these SNPs to osteoporosis in published literature [38, 39]. We wanted to use the robustness of T-ARMS particularly its specificity at the 3'terminus to independently identify both the mutant and wild type allele and its increased sensitivity by including the penultimate mismatch.

3.1 Primer3/WASP/Primer1 Set of Primers for VDR locus:

First we used Primer3 software for finding primers to design a multiplex primer set for the VDR locus to detect osteoporosis [26, 27]. Primer3 picked up five sets of primers for each of the four VDR polymorphisms. For each locus Primer3 gave a set of primers for wild type and mutant which could detect either wild or mutant homozygotes. These different sets of primers were tried through AutoDimer software for primer compatibility in a multiplex reaction [36, 37]. AutoDimer gave only the compatibility of the primers based on secondary structure and GC content. Base pair sizes of the products from different locus were not checked for compatibility. On manually checking for amplicon size compatibility we found that both wild and mutant types could not be detected at the same time because base pair sizes were nearly same only with a few nucleotide differences between the wild

type and mutant type. So Primer3 could detect only one homozygote either the wild or the mutant type and did not suit our purpose for developing a multiplex for VDR locus.

So we used WASP is for designing MAS PCR primers which gave three primers: wild type, mutant type and common primer which can detect could again detect only homozygotes [30,31]. We also applied the penultimate primer position mismatching as suggested by Little [34]. WASP selected four sets of primers when reference sequence was given as input and five sets for the FASTA sequence of the whole genome for each of the four polymorphic loci. These different sets of primers were run through AutoDimer software for primer compatibility in a multiplex reaction. Again after checking for amplicon size compatibility we could not arrive at a multiplex primer set for VDR because heterozygotes could not be identified. This was because the one primer either forward or reverse was specific to the wild/mutant type while the other primer was common to both. To detect the heterozygotes these primer sets were not sufficient and did not suit our purpose for developing a multiplex for VDR locus.

Since we encountered the above mentioned difficulties in designing a multiplex primer set for VDR locus in the next step we tried using Primer1 to develop a T-ARMS primer set [32,33]. Primer1 gave us ten sets of primers with four primers in each set: outer forward, outer reverse and inner forward, inner reverse for each set. But a full T-ARMS set could not be developed because Primer1 was able to pick up primer sets only for FokI and ApaI of the VDR locus. We also noted that Tm was in the range of 65-72°C. So we checked the GC content using Thermo scientific Multiple Primer Analyzer [40] and found that some primers had GC content above 60%.

In the next step we combined Primer3, WASP and Primer1 outputs and tried to compare the primers from all three sets and to find good primer sets. We arrived at the following primer set after checking for amplicon size manually and parameters like length, Tm and GC content though Thermo scientific Multiple Primer Analyzer software. The penultimate primer position mismatching was done to improve reaction specificity additional mismatches at the penultimate base of the AS primers was introduced and again checked through AutoDimer. Table 2 gives the Primer3/WASP/Primer1 set of T-ARMS primers for VDR locus. But we found that though we had corrected for most of the parameters required for a good multiplex primer set the annealing temperatures were incompatible for successful amplification and there were overlapping amplicon sizes of the products which were still a problem for successful detection of all twelve genotypes of the four VDR loci of our interest to detect osteoporosis.

Table 2: Primer 3/ WASP/ Primer1 set of T-ARMS primers for the VDR locus to detect osteoporosis.

S.N	Locus Name	Primer Name	Primer Type	T-ARMS FROM MAS PRIMERS	Base Pair	Tm	GC Content
1	**FOK I**	P2	IF	CTTGCTGTTCTTACAGGTAC	1476	55	45
			OR	TGCAGCCTTCACAGGTCATA		60	50
			OF	ATCTGCACCACTTCTTTCCC	152	60	50
			IR	TGCTGGCCGCCATTGCCTACT		72	63
2	**BSM I**	P3	IF	TGGGGCCACAGACAGGCCTTCA	184	78	73
			OR	ACTTCCTCTTCGGCCTTTTC		60	50
			OF	GGAAATACCTACTTTGCTGGT	235	55	43
			IR	CAGAGCCTGAGTATTGGGAAGGG		62	48
3	**APA I**	P5	IF	AAGGCACAGGAGCTCTCAGCTGGACC	203	72	62
			OR	GATCATCTTGGCATAGAGCAGGTGGCTG		72	54
			OF	GGTCTGGATCCTAAATGCACGGAGAAGTCA	244	72	50
			IR	GGGGTGGTGGGATTGAGCAGTGAAGT		72	58
4	**TAQ I**	P6	IF	TGCAGGACGCCGCGCTGATA	166	73	65
			OR	CTGCTTGGAGTGCTCCTCAT		61	55
			OF	GGATCCTAAATGCACGGAGA	236	60	50
			IR	CGGTCCTGGATGGCCGCC		65	67
IF-INNER FORWARD,OR-OUTER REVERSE,IR-INNER REVERSE,OF-OUTER FORWARD							

3.2 Primer-BLAST/PerlPrimer Set:

PerlPrimer and BatchPrimer3 were also tried to design T-ARMS primers [22,23,28,29]. The PerlPrimer software had the same limitations of Primer3 in developing multiplex primer set for VDR locus. BatchPrimer3 could not pick primers from the given rs (reference SNP) of the VDR locus which are the input format so this software is not suitable for T-ARMS PCR primer design of the VDR locus. This could be probably because the length of input sequence was insufficient to pick up a primer pair and parts of Genomic DNA FASTA sequence around the locus of interest which had a longer length than the rs sequence was not accepted by the software.

Primer-BLAST was used to obtain a set of primers form the FASTA sequences of VDR locus [24,25]. Primer-BLAST gave a list of ten sets for each locus of the VDR

region. The primers picked out by PerlPrimer, also a set a list of ten sets for each locus of the VDR region. We picked up the optimal primer set from the list provided by Primer-BLAST and PerlPrimer by checking primer parameters through Thermo scientific Multiple Primer Analyzer software and amplicon size was checked manually. Table 3 is the set we obtained from the Primer-BLAST/PerlPrimer softwares. Again we ended up with problem of incompatible Tm, 58-64°C. We felt that the annealing temperature was too low and might cause mispriming.

Table 3: Primer-BLAST/PerlPrimer set of T-ARMs primers for the VDR locus to detect osteoporosis.

S.N	Locus Name	Primer Name	Primer Type	T-ARMS FROM SINGLE PLEX PRIMERS	Base Pair	Tm	GC Content
1	**FOK I**	P2	IF	CTTGCTGTTCTTACAGGTAC		58	50
			OR	TCACAGGTCATAGCATTGAA	222	57	40
			OF	GCAACATCTGAAACCAGGCA		59	50
			IR	GCCGCCATTGCCTACA	270	60	66
2	**BSM I**	P3	IF	CACAGACAGGCCTACT		59	62
			OR	AGGAATGTTGAGCCCAGTTCA	143	62	47
			OF	GTTAGGCACCAACAGGGAGAG		62	57
			IR	AGAGCCTGAGTATTGGGAACGC	570	62	50
3	**APA I**	P5	IF	TGGGATTGAGCAGTGAGTGA		63	55
			OR	TTGCCAAACACTTCGAGCAC	453	62	50
			OF	GTGATGAGGTCCAAAGAGGGG		62	57
			IR	AGGAGCTCTCAGCTGTGC	799	63	66
4	**TAQ I**	P6	IF	AGGACGCCGCGCTGATC		65	70
			OR	TTGCCAAACACTTCGAGCAC	378	62	50
			OF	GAGGCTCAAGGAATGGAGATGG		62	54
			IR	CGGTCCTGGATGGCGTCA	1500	64	66
IF-INNER FORWARD, OR-OUTER REVERSE,IR-INNER REVERSE, OF-OUTER FORWARD							

Finally, we tried combining the Primer-BLAST/PerlPrimer set and Primer3/WASP set. These primer sets were again checked through AUTODIMER for multiplexing.

Again the annealing temperatures of certain primers in the set were not within the range for a successful multiplex PCR amplification, Tm 57-65°C. We felt that the annealing temperature was too low and might cause mispriming. So we tried to add the universal primer sequence and tag to the selected T-ARMS primers.

3.3 Universal Tag

The use of universal primer for multiplex T-ARMS PCR and its corresponding tag sequence was to ensure that amplification of a T-ARMS PCR will be successful at lower annealing temperatures and also provide a suitable range of annealing temperature were all the primers can amplify together [16].

After adding the universal primer sequence and checking through AutoDimer we obtained the T-ARMS PCR primer set for VDR locus (Figure 2).

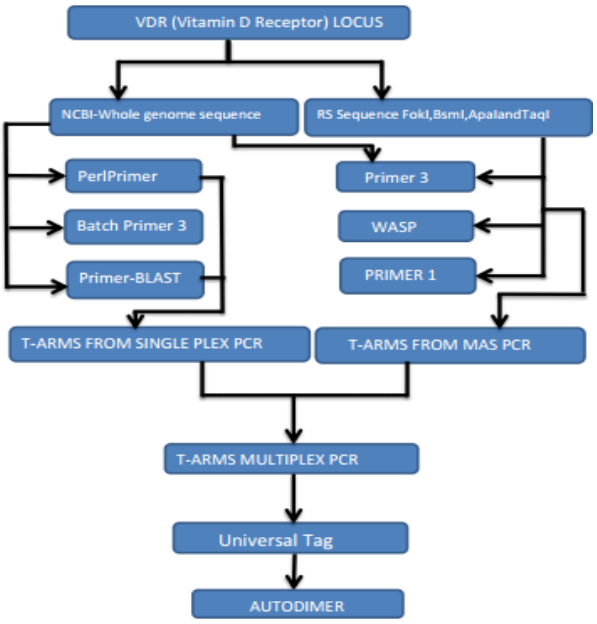

Figure 2: Flowchart of the T-ARMS primer set for the VDR locus to detect osteoporosis.

FokI and BsmI primers were form Primer-BLAST/PerlPrimer set. The ApaI tetra primers were form Primer1 set. TaqI primers was a combination of reverse outer primer and forward inner primer form Primer-BLAST, reverse inner primer from WASP, forward outer primer form Primer1 set (Table 4).

Table 4: T-ARMS Multiplex Primer sets for the VDR locus to detect osteoporosis.

S. N	Locus Name	Primer Name	Primer Type	T-ARMS MUTIPLEX PRIMERS	Base Pair	Tm	GC Content
1	FOK I	P2	IF	**AGGTGACACTATAGAATA**CTTG CTGT TCTT ACAG GTAC	222	62	39
			OR	**GTACGACTCACTATAGGGA**TCAC AGGT CATA GCAT TGAA		65	44
			OF	**GTACGACTCACTATAGGGA**GCAACATCTGAAACCAGGCA	270	69	49
			IR	**AGGTGACACTATAGAATA**GCCG CCAT TGCC TACA		66	47
2	BSM I	P3	IF	**AGGTGACACTATAGAATA**CACAGACAGGCCTACT	143	64	44
			OR	**GTACGACTCACTATAGGGA**A GGAA TGTT GAGC CCAG TTCA		69	48
			OF	**GTACGACTCACTATAGGGA**GTTAGGCACCAACAGGGAGAG	570	69	53
			IR	**AGGTGACACTATAGAATA**AGAGCCTGAGTATTGGGAACGC		67	45
3	APA I	P5	IF	**AGGTGACACTATAGAATA**AAGGCACAGGAGCTCTCAGCTGGACC	203	71	50
			OR	**GTACGACTCACTATAGGGA**GATCATCTTGGCATAGAGCAGGTGGCTG		71	51
			OF	**GTACGACTCACTATAGGGA**GGTCTGGATCCTAAATGCACGGAGAAGTCA	244	71	49
			IR	**AGGTGACACTATAGAATA**GGGGTGGTGGGATTGAGCAGTGAAGT		70	48
4	TAQ	P6	IF	**AGGTGACACTATAGAATA**AGGACGCCGCGCTGATC	378	69	51
			OR	**GTACGACTCACTATAGGGA**TTGCCAAACACTTCGAGCAC		68	49
			OF	**GTACGACTCACTATAGGGA**GGTCTGGATCCTAAATGCACGGAGAAGTCA	236	71	49
			IR	**AGGTGACACTATAGAATA**CGGTCCTGGATGGCCGCC		71	56
		Tag	F	**AGGTGACACTATAGAATA**		41	33
			R	**GTACGACTCACTATAGGGA**		49	47
IF-INNER FORWARD, OR-OUTER REVERSE, IR-INNER REVERSE, OF-OUTER FORWARD							

Thus by adding the universal sequence and tag we were able to obtain a T-ARMS primer set Tm within a range of 62-71°C, amplicon sizes with a range of 143-570 and GC content with a range of 39-56. This T-ARMS PCR kit for the VDR locus kit is and in-silico approach and has to be validated with osteoporosis cases and normal controls by in-vitro PCR reactions.

4. Conclusion

Compared to PCR-RFLP genotyping T-ARMS PCR is a highly sensitive and specific method to genotype the VDR locus for polymorphisms implicated in osteoporosis. We started with a multiplex PCR design and arrived at T-ARMS PCR. We are sure that the changes we introduced in the original criteria of tetra-primer ARMS-PCR design by adding a universal primer sequence and tag will led to better amplification. Thus, we were able to achieve a specific genotyping method by choosing higher annealing temperatures for the T-ARMS primers. However, we are yet to validate these primers through *in vitro* PCR amplifications.

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