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METHODS AND SOFTWARE FOR PREDICTING PCR FAILURE RATE IN LARGE GENOMES

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LIST OF ORIGINAL PUBLICATIONS

The current dissertation is based on the following publications referred to in the text by their Roman numerals:

I. Kaplinski L, **Andreson R**, Puurand T, Remm M (2005). MultiPLX: automatic grouping and evaluation of PCR primers. *Bioinformatics* 21(8): 1701–2.

II. **Andreson R**, Reppo E, Kaplinski L, Remm M (2006). GENOMEMASKER package for designing unique genomic PCR primers. *BMC Bioinformatics* 7:172.

III. **Andreson R**, Puurand T, Remm M (2006). SNPmasker: automatic masking of SNPs and repeats across eukaryotic genomes. *Nucleic Acids Research* 34:W651–5.

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My contribution to the articles referred in the current thesis is as follows:

Ref. I created and performed tests with auxiliary program *gt4multiplx* and also participated in creation of the web client for the main program;
Ref. II conducted this study, carried out different tests on various methods, validated the package and was responsible for drafting the manuscript;
Ref. III conducted this study, created and performed tests with the application and was responsible for drafting the manuscript;
Ref. IV conducted this study, created primers for the experiments, analyzed the data and was responsible for drafting the manuscript.

LIST OF ABBREVIATIONS

CEPH	Centre d'Etude du Polymorphisme Humain
CPU	central processing unit (in computers)
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide-triphosphate
e-PCR	electronic (*in silico*) PCR
IUPAC	International Union for Pure and Applied Chemistry
n-mer	short substring with the length of n
LINE	long interspersed nuclear element
LTR	long terminal repeat
PCR	polymerase chain reaction
RAM	random access memory (in computers)
SINE	short interspersed nuclear element
SNP	single nucleotide polymorphism
T_a	primer annealing temperature
T_m	primer melting temperature

INTRODUCTION

Modern genomic technologies allow studying thousands of genomic regions from each DNA sample. Many of these technologies rely on methodology called polymerase chain reaction (PCR) that allows amplification of specific DNA sequences (gene detection for example). The genome-wide genotyping of single nucleotide polymorphisms (SNP), microarray experiments for gene expression, re-sequencing methods – all these depend directly on the efficiency of the PCR reaction. The high-throughput assays require designing simultaneously thousands of PCR primers for the experiments. Therefore, careful estimation of the PCR primer properties is crucial for the success of primer design. Many studies in the past have been focused on optimizing the reagents of the PCR reaction such as concentration of reaction buffer components and PCR protocols. On the other side there is a primer design process. The basic oligonucleotides properties and their optimal combinations are well studied by many scientific groups in order to maximize the amplification efficiency. However, the in-depth examination of the repeats and the uniqueness of PCR primers in large genomes are still under discussion.

The first part of the present thesis gives a brief overview of the PCR method, both biochemical and sequence-based factors influencing the PCR reaction and studies to measure the effects of these factors. The second major topic of the literature review concentrates on the eukaryotic repeats, their classification and methods to detect them. Third part gives an overview of the current electronic PCR (e-PCR) methods that are available today.

The research part of this dissertation entails the following topics: (i) creation of the fast and efficient repeat-masking methodology designed for PCR applications, (ii) creation of the fast and brute-force method to predict PCR products for already designed PCR primers and (iii) discovery of the important factors that affect the PCR failure rate and create statistical models to predict the failure rate of PCR reaction.

1. REVIEW OF LITERATURE

1.1. Polymerase chain reaction

1.1.1. The essence of PCR

The Polymerase Chain Reaction (PCR) technique, conceived by Kary B. Mullis, allowed scientists to make millions of copies of a slight amount of DNA (Saiki *et al.*, 1985, Mullis *et al.*, 1986). This technique, in vitro DNA amplification procedure, has been optimized, improved and perfected in the following years (Saiki *et al.*, 1986, Scharf *et al.*, 1986, Mullis and Faloona, 1987, Saiki *et al.*, 1988, Lawyer *et al.*, 1989, Olson *et al.*, 1989, Erlich *et al.*, 1991). Furthermore, the PCR has revolutionized many aspects of the research ever since and *Science* has nominated in 1989 the DNA polymerase to be the "Molecule of the Year" based on the accomplishments of PCR method (Guyer and Koshland, 1989), for which Kary Mullis was awarded the 1993 year's Nobel prize in Chemistry.

The PCR reaction itself is based on the cyclic synthesis of both DNA chains. A standard PCR amplification involves three following steps: heat denaturation of double-stranded DNA, annealing of the two primers (short oligonucleotides) to their complementary sequences and extension of the annealed primers with thermostable DNA polymerase. An ideal ordinary PCR result is one specific PCR product that is generated in high yield, with minimal cycles containing the fewest number of polymerase-induced errors. The amount of amplified PCR product is doubled in each successive cycle causing the exponential accumulation of given specific fragment (Saiki *et al.*, 1988).

Nowadays there are more advanced PCR technologies, such as Real-Time PCR (Higuchi *et al.*, 1993, Heid *et al.*, 1996), that are commonly utilized in current research projects. In classical PCR the same amount of product is produced independently of the initial amount of DNA template molecules. In real-time PCR however, the number of amplification cycles required to obtain a particular amount of DNA molecules is registered by monitoring the fluorescence of dyes or probes introduced into the reaction (Kubista *et al.*, 2006). This data can be analyzed by computer to calculate the amount of product formed during each reaction cycle. Nevertheless, classical PCR technique is still widely used on many fields due to its efficiency, robustness and fidelity (Vollenhofer *et al.*, 1999, Kurg *et al.*, 2000, Nugent and Saville, 2004, Budowle *et al.*, 2005, Yancy *et al.*, 2005).

1.1.2. The estimation of PCR success

Although the PCR methodology is evolved and protocols are optimized by decades now, the behavior of the reaction is not completely predictable for each new primer-template combination. The non-successful results of a classical PCR include non-targeted products, smear bands or no bands at all. The alternative products are mostly caused by non-unique PCR primers that amplify additional regions from template DNA. The reasons for other non-successful results may be either sequence-based or experimental errors. A closer look to these factors is given in the next chapter.

In order to achieve a high PCR success rates, primers need to be selected carefully. In the beginning of the PCR "era", researchers were amplifying sequences from less complex organisms such as microbes and viruses. Today, with the advancement of genome sequencing project, the genomic DNAs of several higher organisms (like mammals, plants) are available and therefore the specificity of PCR primers requires much closer attention. Even though the cost of single PCR reaction is comparatively low, it is becoming an issue in high-throughput methods for genomic applications.

The prediction of the success of PCR has been studied previously by many groups. Rubin and Levy published a study, where they investigated the relative effects of various parameters on the amplification of non-targeted PCR products (Rubin and Levy, 1996). The most significant factor affecting the PCR specificity is the mismatch tolerance during primer annealing to the template, followed by primer length, template size and product size limits. Beasley with her colleagues have analyzed a thousands of primer pairs and examined the primer characteristics that can cause a false priming or failure to amplify template DNA (Beasley *et al.*, 1999). They have found that the primer length, primer GC content and GC content of the 3' half of the primers were strongly associated with the success rate of PCR. Yuryev along with his workgroup developed statistical scores to evaluate various parameters for predicting the success of primer extension reaction that includes many factors related to PCR primers and products (Yuryev *et al.*, 2002). The statistical prediction (single-plex) model included following PCR-related factors: primer GC content, the number of ambiguous bases and repeats in PCR product, the product structure around PCR primer annealing sites and the nucleotide combinations in last 3 bases at the 3' end of the PCR primers in addition to two product bases next to primer annealing sites. The PCR success can be predicted by the regionalized GC content within the template DNA (Benita *et al.*, 2003). Benita with the co-authors has published a detailed analysis of the template DNA using a sliding window of 21 nucleotides to calculate GC nucleotides in each window. Region was considered significant when it contains >61% GC nucleotides for at least ten consecutive windows. These threshold values gave more precise discrimination between 'good' and 'failed' experiments than any other parameters they have used. A critical examination of oligonucleotides properties has been

published lately (Chavali *et al.*, 2005). The authors propose that the efficiency and accuracy of the PCR are determined by correct calculation of the primer melting temperature (T_m) and secondary structures. They compare several freely available programs and provide suggestions to use different tools depending on the template GC content. The factors affecting cross-species primers and their success in PCR has been studied by Housley and her colleagues (Housley *et al.*, 2006). They have identified three factors with significant impact on the efficiency of PCR: the number of index-species mismatches, GC content of the template and the degree of relatedness between two organisms.

1.1.3. The factors influencing PCR

There are many factors that affect the success of the PCR and can be generally divided into two subgroups: experimental or biochemical and sequence-based factors.

1.1.3.1. Experimental and biochemical factors

The optimal selection of PCR reaction components is crucial for running a successful experiment. The correct annealing of two sequences (PCR primer and DNA template) to each other does, however, depend on the physical and chemical solution conditions under which the reaction takes place. The recommended PCR buffer reagents and their concentrations have been published previously (Innis and Gelfand, 1990). Although the modern formulations may differ considerably, they are generally comparable. Magnesium ion concentration influences many things in the reaction: primer annealing, T_m of template (strongly influences ΔS), product and primer-template associations (high magnesium will enhance the stability of mismatched primers), the enzyme activity and fidelity (important cofactor for Taq DNA polymerase). A titration should be performed with varying [Mg^{++}] with all new template-primer combinations as the results can differ markedly even under the same conditions of concentrations and cycling times/temperatures. Primer and deoxynucleotide triphosphates (dNTPs) concentrations should also not be too high; 0.2μM should be more than sufficient for homologous primers and <50μM for each dNTP (Innis and Gelfand, 1990, Beasley *et al.*, 1999).

The PCR cycle includes 3 steps: denaturation of double-stranded DNA, annealing of the primer sequences to single-stranded DNA template and synthesis of a new complementary strand on the template. A typical DNA template denaturing temperature is set between 93º and 96ºC for every cycle of an amplicon. In the denaturation step the Taq DNA polymerase is inactivated and eventually will lose its activity. After 10 cycles the amplified product acts as a template and therefore it is unnecessary to use same temperature during later cycles. For short amplicons it is proposed that the denaturation temperature

should be lowered to 87º–90ºC after five to ten initial cycles (Yap and McGee, 1991). The increase in denaturation temperature and decrease in time may also work (96^{o}C for 15 sec) (Innis and Gelfand, 1990).

The annealing temperature (T_a) of the PCR primer is related to the T_m and one should aim the T_a about 5^{o}C below the lowest T_m of the pair of primers. Thus, the correct T_m prediction is needed in order to get the precise T_a for the given primer sequence (SantaLucia, 1998, von Ahsen *et al.*, 2001). Too low T_a increases a chance that one or both primers will anneal to sequences other than the true target**,** as internal single-base mismatches or partial annealing of the primers may be tolerated. Too high T_a, on the other hand, can cause the deficiency of the synthesized product, as the likelihood of primer annealing is reduced.

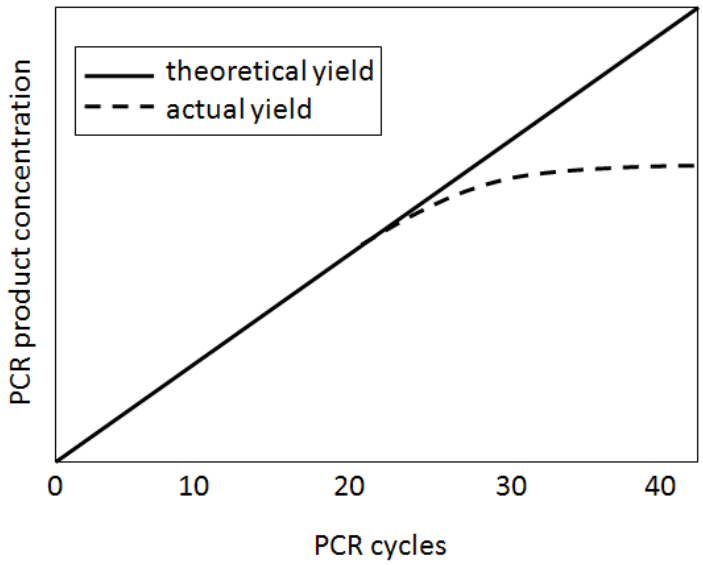

Figure 1. "Plateau effect" in PCR amplification. The attenuation in the exponential rate of PCR product accumulation happens in the late stages of a PCR due to degradation of reactants or reactant depletion.

The total number of cycles depends on the concentration of target molecules: from 40–45 cycles to amplify 50 target molecules to 25–30 to amplify 3×105 molecules to the same concentration. On both cases the exponential growth of the product will diminish (Figure 1) at some stage caused by degradation of reactants (dNTPs, enzyme) with short products, reactant depletion (primers, dNTPs) with long products or competition for reactants by non-specific products (Innis and Gelfand, 1990).

1.1.3.2. Sequence-based factors

The sequence specific factors affecting the PCR success rate can be divided additionally into following groups: PCR primer and product properties (e.g. length, GC content), PCR primer secondary binding sites, primer homology on the target DNA and the number of alternative PCR products. In case of primer design the calculation of the properties in first two groups is an order of magnitude faster than providing the uniqueness of primers and products with whole genomes (data not shown). Furthermore, it requires more computing power, space and sophisticated algorithmic approaches to accomplish latter tasks. Therefore, the special chapters are devoted for masking repeats and counting PCR products afterwards.

PCR primer properties

The first PCR primer property, PCR primer length, is dependent on the base composition and the melting temperature set by the researcher. A prime consideration is that the primers should be complex enough so that the likelihood of primer annealing to sequences other than the chosen target is very low and as short as possible to lower the cost of the primer synthesis. The primer sequence containing sixteen nucleotides will statistically be present only once in every 4^{16} bases (>4 billion) and should be theoretically unique in human genome. Furthermore, it is shown that primers with lengths between 21–26 nucleotides give higher success rates than shorter (18–20 nt) (Beasley *et al.*, 1999).

The extreme GC content (>80 or <20%) of the full primer sequence and in the 3' half of the primer are known to increase the probability of self-complementarities and secondary binding sites. Beasley *et al.* and Haas *et al.* recommend to use primers with GC content close to 50% (Haas *et al.*, 1998, Beasley *et al.*, 1999).

The successful elongation of a primer depends also on the stability at its 3' end (Onodera and Melcher, 2004, Miura *et al.*, 2005). It is shown that primers with a G or C in the last base at 3' end are more likely to succeed in PCR (Li *et al.*, 1997, Onodera and Melcher, 2004). More specifically, the last base should not be a Thymine (T) because of its ability to form non-Watson-Crick base pairs (mismatch tolerance) and increase the probability of secondary binding sites (Kwok *et al.*, 1990). As DNA polymerases are known to form a duplex between not identical primer and template sequences, there are programs available that evaluate the duplex energy of 3' half of the primer candidates (Haas *et al.*, 1998, Rozen and Skaletsky, 2000, Chen *et al.*, 2003, Miura *et al.*, 2005).

The short simple repeats in primer sequence can cause a higher probability of having stable secondary binding sites in genomic DNA (Haas *et al.*, 1998). Figure 2 shows some examples of primers containing simple repeats; a more detailed overview of the repeats and masking methods is given in the next chapter.

```
>primer1
CTGCTTaaaaaaaaaGTaaaaaaaa

>primer2
AATCAAGActctctctctctctGAA

>primer3
ctgctgGTTCAGGCAActgctgctgC
```

Figure 2. Examples of short repeats in primer sequences. Lower-case letters mark the simple repeats.

The avoidance of the formation of primer-dimer artefacts (Figure 3A and 3B) and stable self-complementary hairpin loops (Figure 3C) that compete with the correct primer-template target binding are important to increase the specificity of PCR primers (Chavali *et al.*, 2005).

A. primer-dimer

```
dG: -5.4 kcal/mol
    5'-AGCTTGGGAGCTAGCCAAAC-3'
            ||||
3'-CAATAGTAGCCCTACCTTG-5'
```

B. primer-dimer 3' ends

```
dG: -5.8 kcal/mol
5'-ATGGCTTCTAGAGGACCAATG-3'
                  |||||
               3'-GTTACCTTTAACGCAGGTATC-5'
```

C. hairpin

```
dG: -1.64 kcal/mol
                T
               / \
        5' AGCT   G
           ||||   |
3' CAAACCGATCGA   G
               \ /
                G
```

Figure 3. Examples of the secondary structures of PCR primers. The free energies of these secondary structures are calculated using following conditions: DNA at 37°C $[Na^{+}]$ = 0.05 M, $[Mg^{++}]$ = 0.0015 M. The cross dimer (A and B) structures and energies were calculated with MultiPLX (Ref. I) and the hairpin (C) calculated with MFOLD web server (Zuker, 2003).

The important characteristic for the calculation of correct annealing temperature is the melting temperature of the primer as noted above. The choice of a non-optimal temperature can lead to the amplification of false regions. The proper calculation of T_m using latest Nearest-Neighbor thermodynamic formulas (Owczarzy *et al.*, 1997, SantaLucia, 1998, SantaLucia and Hicks, 2004, Panjkovich and Melo, 2005) requires exact concentrations of the molecules from the reaction protocol (von Ahsen *et al.*, 2001, Chavali *et al.*, 2005), thus making the T_m experimentally dependent sequence-based factor.

PCR product properties
The previous studies have shown that the amplicon length is not a critical factor affecting the result of PCR (Beasley *et al.*, 1999, Benita *et al.*, 2003). However, the GC content of the PCR product is more informative (Benita *et al.*, 2003). It has been found that DNA templates with very high or low GC content can be difficult to amplify (Varadaraj and Skinner, 1994). The stable secondary structures of the target DNA to which the primers bind are also important to look after as they obstruct the DNA denaturation and the progress of the polymerase (Fedorova *et al.*, 1992, Dong *et al.*, 2001). Finally, the repetitive elements located on the PCR products are increasing the primer mispriming (Haas *et al.*, 1998).

1.2. Key concepts for masking repeats

1.2.1. Repeats in eukaryotes

Most of the eukaryotic organisms comprise a large fraction of repetitive motifs in their genomic DNA. Current estimates are that 46% of the human and 38% of mouse genomes are occupied by various repeats (Lander *et al.*, 2001, Waterston *et al.*, 2002). These repetitive motifs can be divided roughly into three categories: simple (duplications of simple sets of DNA bases (typically 1–13bp) or minisatellites (14–500bp)), tandem (duplications of more complex 100–200 base sequences) and interspersed repeats (SINEs, LINEs, LTRs and DNA transposons) (Richards and Sutherland, 1994, Prak and Kazazian, 2000, Nagashima *et al.*, 2004).

Although repetitive motifs were once called as a residual “junk DNA”, that opinion is about to change today. It is even argued that repeats probably play an important role of developing the species through genome modifications (Kazazian, 2004). Therefore the role of repeats are noted often as “symbiotic” rather than “parasitic” and the research on this field is an emerging area in evolutionary biology (Zhi *et al.*, 2006).

In addition, accurate identification and classification of repeats is important for developing sequence assembly and genome comparison methods (Edgar and

Myers, 2005), understanding diseases caused by repeats (Deininger and Batzer, 1999) and homology searches and oligo design to avoid the explosion of unnecessary or non-unique results (Kreil *et al.*, 2006).

1.2.2. The masking of repeats

At first we must define "masking" to go further. For example let's say that repeat is one string containing 16 nucleotides and it is presented more than 100 times in genomic DNA in several places. How to mark those places on genomic DNA to make sure that we could recognize them later on? The easiest way to mark them is to replace all nucleotides in length of the repeated string by some other symbol (e.g. "N") than ATGC. When the genomic DNA is scanned through and all repeated strings are replaced, we can say that our sequence is masked.

The main obstacle for masking sequences is the volume of eukaryotic DNA. We cannot simply scan large genomic DNA by brute-force and count or find *de novo* repetitive motifs as it is too time consuming. The other important criterion is the sensitivity of the method. Ideally, all motifs that are defined by given rules as repeats should be found. The sensitivity is a problem of methods, whose algorithms are based on some heuristics. To accept these challenges the specific algorithms are needed.

1.2.3. The methods for finding and masking repeats

There are two separate approaches to locate repeats in biological sequences: using predefined or experimentally verified libraries or trying to find repeats directly from nucleic acid sequence without prior knowledge.

The most widely used program is definitely RepeatMasker (Smit, AFA, Hubley, R and Green, P. http://www.repeatmasker.org/) which uses precompiled representative repeat libraries to run homology search with query sequence. There is also a helper application to speed up RepeatMasker called MaskerAid (Bedell *et al.*, 2000). Instead of using CrossMatch application to find homology between sequence and RepBase repeat library (Jurka, 2000), MaskerAid utilizes WU-BLAST (Gish, W. (1996–2004), http://blast.wustl.edu) for a given task. WU-BLAST is an enhanced version of the original NCBI BLAST (Altschul *et al.*, 1990, Altschul *et al.*, 1997). Replacement of CrossMatch with MaskerAid/WU-BLAST increases the speed of masking more than 30-fold without losing the sensitivity (Bedell *et al.*, 2000). CENSOR (Kohany *et al.*, 2006) is a new tool for identification of both interspersed and tandem repeats using similarity searches with NCBI BLAST or WU-BLAST against RepBase.

DUST (ftp://ncbi.nlm.nih.gov/pub/tatusov/dust/) is a program for filtering low complexity regions from nucleic acid sequences. It catches all repeats of unit length 1 or greater that are repeated at least 4 times. For detecting and masking tandem repeats a program called TandemRepeatFinder is developed by Gary Benson (Benson, 1999). It searches tandem repeat patterns using short substrings (n-mer matches), requires no predefined size and number of the patterns (instead it is using a probabilistic model to calculate them) and determines a single consensus pattern for the smallest repetitive motif in the tandem repeat. The program will find all repeats with period size between 1 and 2000.

All the methods described above are specialized for masking repeats. There exist many alternative applications for finding *de novo* repetitive motifs that do not require predefined repeat libraries. In some decades ago Hugo Martinez and Devereux with his colleagues developed the earliest repeat finding algorithms for molecular biologists (Martinez, 1983, Devereux *et al.*, 1984), but the main problem with those tools was the strict limit on the maximal length of the input sequence they were capable to analyze (Kurtz and Schleiermacher, 1999).

In the present day it is recommended that the programs are able to handle complete genomic DNA when detecting repeats. RepeatMatch (Delcher *et al.*, 1999) performs a maximal unique match decomposition of the two closely related genomes using suffix trees combined with the longest increasing sub-sequence and Smith-Waterman algorithm (Smith and Waterman, 1981). REPuter (Kurtz *et al.*, 2001) can handle effectively large genomes by finding exact repeats in linear space and time using a revised implementation of suffix trees. In the second step the exact matches are used as a seeds and extending them allowing mismatches, insertions and deletions, program guarantees that all repeats will be found according to the user input parameters. RepeatFinder (Volfovsky *et al.*, 2001) is a program designed to find, output detailed classification and statistics of all repeats for partial or complete genomes. The gathering of initial set of exact repeat hits is performed using efficient suffix tree data structure. The second stage is a merging procedure that joins overlapping repeats or repeats with limited distance together. Third step is the classification of newly formed combined repeats and the last (optional) step allows the user to WU-BLAST all similar but non-exact repeats against all others. After the final step repeat classes will be updated and program can build repeat map of the whole genome sequence. The authors of Recon (Bao and Eddy, 2002) propose that the repeat families collected by their application can be used as the basis of creating higher quality libraries such as RepeatMasker library. The algorithm is forming the multiple alignments of repeats with WU-BLAST. FORRepeats (Lefebvre *et al.*, 2003) is using a heuristical approach to minimize the search time and space requirements when using large genomes. Lefebvre and his colleagues are using specific heuristical data structure called *factor oracle* (an automaton) that allows them to perform faster pair-wise alignments of exact repeats. The second step is the extension of exact hits that is

similar to BLAST. On the other hand, Pevzner with his colleagues have shown that neither pair-wise (RepeatMatch, REPuter) nor multiple alignment (RepeatFinder, Recon) methods alone are so successful of classifying repeats as their RepeatGluer (Pevzner *et al.*, 2004). Instead, they are using A-Bruijn graphs to eliminate the "mosaic" nature of the sub-repeats (smaller repeats that are overlapping or part of the bigger repeats). The program creates the matrix of input sequence, constructs the A-Brujin graph and removes bulges, whirls and zigzag patterns from the graph. PILER (Edgar and Myers, 2005) is a program package that is using different search methods for several repeat classes. For finding local and multiple alignments PALS (Edgar and Myers, 2005) and MUSCLE (Edgar, 2004) are used respectively. The output of the PILER is an annotation of the input sequences giving locations of intact, isolated copies of repeated elements and a library containing one consensus sequence for each family. RepeatScout (Price *et al.*, 2005) builds a set of repeat families by using high frequency of short substrings with fixed length as seeds. The next step involves the greedy extension of each seed to a longer consensus sequence. Those sequences are aligned against the genome to locate all repeats.

RAP (Campagna *et al.*, 2005) and WindowMasker (Morgulis *et al.*, 2006) are applications that are based purely on a word-counting algorithms. This is an alternative way to find repeats and rely on the statement that a sequence containing frequent words is very likely a repeat. The former program allows using discontinuous words whereas latter program uses exact words only. The algorithm of both programs is divided into two separate parts: at first it count alls n-mers and then the sequence masking (WindowMasker) or visualization of repeats (RAP) will occur. The input sequence will be scanned two times in both cases. These methods are optimized for short word sizes (16 or less), but WindowMasker counts required word size dynamically unlike RAP, where the user defines it manually. A novel method for finding fragmented repeats is called Greedier (Li *et al.*, 2008). This method is using separate iterations to locate transposons: 1) identifies the local similarities between predefined repeat library and target sequence and 2) computes a fitness value for each match separately to tag repeat motifs. Experiments show that Greedier is twice as effective as WindowMasker or RepeatMasker for finding true positive transposon bases and avoiding false positives.

To conclude the overview of different repeat finding and masking methods, the question how to represent all repeats in genomic sequence is still open. As the Bao and Eddy wrote in their paper (Bao and Eddy, 2002), "The problem of automated repeat sequence classification is inherently messy and ill-defined and does not appear to be amenable to a clean algorithmic attack." Current methods approach differently to the problem, but yet there is no ideal solution or common understanding how to classify and draw borders between repeat candidates. Additionally, there are many programs available for finding and/or masking repeats, but only few of them (DUST, RepeatMasker, WindowMasker)

are practically usable in a large-scale whole genome primer or probe design studies. Therefore the need for fast and specialized tools still exists.

1.3. The electronic PCR

Ten years ago, Gregory Schuler introduced to the scientific community a new term called electronic PCR (e-PCR) (Schuler, 1997). The closer definition for this term is the following: e-PCR is the process of recovering sequence-tagged sites (STSs) in DNA sequences by searching for subsequences that closely match the PCR primers and are in the correct order, orientation and spacing to be consistent with the PCR product size. We are widening the definition of e-PCR by saying that e-PCR is the process of counting all binding sites of PCR primers and possible PCR products they may produce in a given sequence within a certain distance.

1.3.1. The relevant e-PCR methods

Many current probe and PCR primer design applications use various mechanisms to exclude non-unique oligo candidates from the regions of interest. Some of them are executing BLAST application for e-PCR: PrimerMaster (Proutski and Holmes, 1996), PRIMO (Li *et al.*, 1997), PRIMER3 (Rozen and Skaletsky, 2000), MEDUSA (Podowski and Sonnhammer, 2001), PrimeArray (Raddatz *et al.*, 2001), GST-PRIME (Varotto *et al.*, 2001), PIRA-PCR (Ke *et al.*, 2001), OligoArray (Rouillard *et al.*, 2002), PRIMEGENS (Xu *et al.*, 2002), GenomePrimer (van Hijum *et al.*, 2003), GenomePRIDE (Haas *et al.*, 2003), PUNS (Boutros and Okey, 2004), ROSO (Reymond *et al.*, 2004), GenoFrag (Ben Zakour *et al.*, 2004), MPrime (Rouchka *et al.*, 2005), SNPbox (Weckx *et al.*, 2005), Primaclade (Gadberry *et al.*, 2005), DualPrime (Andersson *et al.*, 2005) and FastPCR (http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm). However, the low speeds of BLAST or inability to process large genome sizes are the bottlenecks for these applications.

The high-speed methods applicable to large-scale projects are becoming more important with the increasing number of available full genome sequences. To overcome that problem alternative sequence search and alignment methods are required. MEGABLAST (Zhang *et al.*, 2000) is the upgrade of BLAST that is specifically designed to search highly similar matches. It is using a greedy algorithm when extending the alignment diagonals and achieves 10 times faster execution times than BLAST. MPBLAST (Korf and Gish, 2000) is a small subsidiary method that fastens the BLAST search by concatenating short query sequences into relatively few long sequences. The maximum speed improvement is about 10-fold using MPBLAST. SSAHA (Ning *et al.*, 2001) and

BLAT (Kent, 2002) are both indexing the sequence database in a similar way. Both programs build up and index of n-mers and their positions in the database. Unlike SSAHA that is using always a single perfect match as a seed, BLAT implements "unsplicing" logic – a very quick algorithm for finding short multiple nearby perfect matches. Multiple nearby matches offer much greater specificity for a given level on sensitivity than the perfect matches as shown by Jim Kent (Kent, 2002). Despite of the fact that these programs are relatively fast, they are not optimized for finding short oligonucleotides and there is a need of specific parsers to interpret the output (to count the primer binding sites and predict possible products).

The e-PCR (Schuler, 1997) program is the first application specifically designed for the prediction of all possible PCR products from given genomic sequences. It is using a word-based (7 nucleotides from the primer 3' end) strategy to speed up the search process. Program also allows using mismatches, but only in 5' end of the primer sequence. This limitation is based on the assumption that the mismatches cannot be tolerated in the 3'end of the primers (Sommer and Tautz, 1989). A web-based tool VPCR (Virtual PCR) (Lexa *et al.*, 2001) processes PCR primers, obtains BLAST search results and prints out potential PCR products. PRIMEX (Lexa and Valle, 2003) is an upgrade of previous program that is using word-based lookups from pre-indexed array of n-mers instead of BLAST searches. Sven Rahmann introduces alternative method that is using a suffix tree and the longest common substring approach for selecting the candidate oligonucleotides (Rahmann, 2003). Kevin Murphy with his co-workers have modified the original e-PCR algorithm to perform more accurate and faster string searches with their new method called me-PCR (Murphy *et al.*, 2004). The upgrade includes: the increase of maximum hash word size, hash word can be any substring of a given primer (in e-PCR it was strictly at 5' end) and multithreading for computers with several CPUs. Osprey (Gordon and Sensen, 2004) is a software package for the selection of unique and optimal oligonucleotides for microarrays and DNA sequencing. The package includes a novel computational method for the identification of alternative binding sites using position-specific scoring matrices that can be used to encode the thermodynamic profile of a sequence. This methodology is advantageous over pair-wise alignment approaches because the match and mismatch scores depend on the Nearest-Neighbor (SantaLucia, 1998) thermodynamics and therefore the secondary binding site calculation is context sensitive. This allows a more detailed evaluation of primer candidates in the oligo design process. SPCR (Cao *et al.*, 2005) can assess the similarity between primer and template using the vectors of hydrogen bond numbers after sequence conversion. This similarity (or dissimilarity) between primer and template can be used as a probability estimation of annealing site selection and annealed structure stability. Additionally, SPCR algorithm tolerates any type and number of mismatches in primer-template interaction. BISEARCH (Aranyi *et al.*, 2006) is a nice and efficient web application to design PCR primers and run e-PCR. It is

using hashing of 16-mer oligonucleotides and their permutations to identify all alternative primer locations on native genomic or bisulfate treated genomic DNA. In-Silico PCR (isPCR) (http://www.soe.ucsc.edu/~kent/src/) is a great tool created by Jim Kent for predicting PCR products using UCSC Genome Browser (http://genome.ucsc.edu/) genomic data.

2. PRESENT INVESTIGATIONS AND DISCUSSION

2.1. Aims of the present study

The main goal of the present study was to investigate the factors affecting the PCR success and create the effective methodology for finding and masking repeats in large genomic DNA sequences.

The specific aims for the current thesis were following:

1. to create and test a fast and efficient repeat-masking methodology suitable for applications using PCR. This methodology should be usable in large- and small-scale projects, wherein researchers are amplifying regions from genomic DNA. (Ref. II, III)
2. to create a fast and brute-force method to count the binding sites of PCR primers and predict PCR products for already designed oligonucleotides. Given methodology would allow us to examine and understand the links between word-based search methodology and PCR success rate. (Ref. I, II)
3. to find main factors related to the primer sequence that allow to predict the failure rate of PCR and compare statistical models of different complexity for their ability to predict PCR failure rate in genomic DNA sequences. (Ref. IV)

2.2. GENOMEMASKER package (Ref. II)

We have got involved with the primer design problems a several years ago by participating a large-scale genotyping project covering the human chromosome 22 (Dawson *et al.*, 2002). This study included the design of specific PCR primer pairs to amplify regions around 1278 single-nucleotide polymorphisms (SNPs). We wanted to analyze given primers from that project to study the effect of the secondary binding sites to PCR reaction success amongst other primer properties. The long running times of the whole genome database searches with current applications (BLAST) or the inability to use large input size (VPCR) gave us a reason to develop our own method (GenomeTester) for counting primer binding sites and predicting products.

The alternative approach to design unique PCR primers is to pre-mask the repeats on the template DNA. There were published no such exhaustive and fast repeat-masking tools specialized for PCR primer design. The goal was not to write another detailed annotation program of repeats for genomic DNA, but to create an application capable of finding out all short oligonucleotides in given length that are present too many times in genomic sequence. By finding and marking those short sequences primer design programs can use that for

excluding non-unique primer candidates from the template sequence. Although widely used applications for masking DNA databases and genome sequences were still RepeatMasker/MaskerAid, TandemRepeatFinder and DUST, the speed or low level of sensitivity were the main drawbacks of these programs. The application called GenomeMasker is dedicated to mask repeated primer binding sites efficiently in large genomes.

2.2.1. GenomeMasker application

2.2.1.1. Algorithm

The efficiency of both parts of the GENOMEMASKER package – Genome-Tester (GT) and GenomeMasker (GM) – is based on the usage of specific hash-like data structure for genomic sequences. The hash structure in GM application contains a list of all repeated sequence motifs with given length. All words (motifs) are encoded to binary form (into 32-bit integers) and sorted to speed up the search process and reduce the size of the hash structures. The word size can be defined between 8 to 16 nucleotides in current implementation (by default it is 16).

The workflow of the GM is described graphically on Figure 1A in Ref. II. The first part of the application creates list of repeated motifs and second part masks the over-represented words in input file. The motif becomes over-represented when it appears more times in given genome as special user-defined cutoff (e.g. 1, 2, 3, etc.). The search itself is based on the binary search algorithm explained briefly in Ref. II (pg. 4). The second advantage of our method is the on-demand memory-mapping technique that will help to achieve fast search times for both small and large input data (Ref. II in pg. 4).

The third part of the GM application is a modified PRIMER3 program published lately (Koressaar and Remm, 2007). The improvements include: new formulas for calculating melting temperature and a salt correction, calculation of the effects of divalent cations and the ability to recognize and use the lower-case masked sequence for primer design. The program rejects primer candidates containing lower-case letters in 3' end. The lower-case masking preserves the DNA sequence and allows primers to be designed that partly overlap the masked region.

2.2.1.2. Sensitivity and specificity

We have tested the sensitivity of the GM and compared it with widely used program RepeatMasker (RM) at similar sensitivity level. For that we have selected 1000 random regions from human genome (1000 nt each). All these sequences were masked with both programs separately. Although the sensitivity of GM and RM was very similar (37% and 41% respectively), the sequence masking with RM is less detailed (Figure 2AB in Ref. II). The reason for this is

the incompleteness of the RepBase libraries in case of short repeats. In some cases, the DNA sequences are extensively masked by RM and the primer design in these complicated regions is impossible (Figure 2A in Ref. II). The exhaustive masking with GM will find and mask all short repeated motifs where undesired primer hybridization can occur.

To compare the specificity of different masking programs we have tested several repeat-masking programs (Table 1 in Ref. II). The primers design was attempted with PRIMER3 with combination of each masking program for all those random sequences created previously. The results clearly show that neither DUST, TandemRepeatFinder nor PRIMER3 built-in repeat library are sufficient to exclude non-unique primer candidates. RM is a good method in avoiding most of the repeats, but it is too stringent on many sequences (31% of 1000 sequences are excluded). Only 7% of the sequences masked by GM are unsuitable for primer design, thus, making the GM more suitable for PCR applications.

We have studied the effect of the primers overlapping the repeat sites. So far we have believed that masking one nucleotide from 3' end of the primer candidate is enough to guarantee the unique PCR primer. So the question is, whether GM should mask the whole repeat motif or the one nucleotide from 3' end is sufficient? Additionally, does the 5' end of the primer affect the outcome of PCR when overlapping repeat motif? To ask these questions we have selected a region from human genome that contains one repetitive sub region (words occur more than 10 times in human genome) and designed several primers overlapping the flanks of this sub region (Figure 4). We have used the GM to locate that repeat region with following parameters: word size is 16 nucleotides, masking type is ‚forward' (sense strand only) and masked only one base from 3' end of each repeat motif. There are 19 different sense primers and one antisense primer in each PCR reaction: eleven primers for testing the 3' end and eight primers for 5'end theory. The PCR protocol for these experiments is described in the Ref. IV. As shown in Figure 4, masking only one nucleotide from motifs 3' end is not enough for successful PCR reaction. It is best to mask the whole word instead of fraction of that word. This can be easily achieved with GM by defining special parameter (-nbases). The other part of this experiment gave also very interesting results. It seems that we should mask not only the motif area, but some additional nucleotides after the 3' end as well. This may show that primer whose 5' end overlaps with repeat can possibly still bind to secondary sites and therefore create alternative products by itself. Currently, the masking from that direction is not implemented in GM, but will be done in future releases.

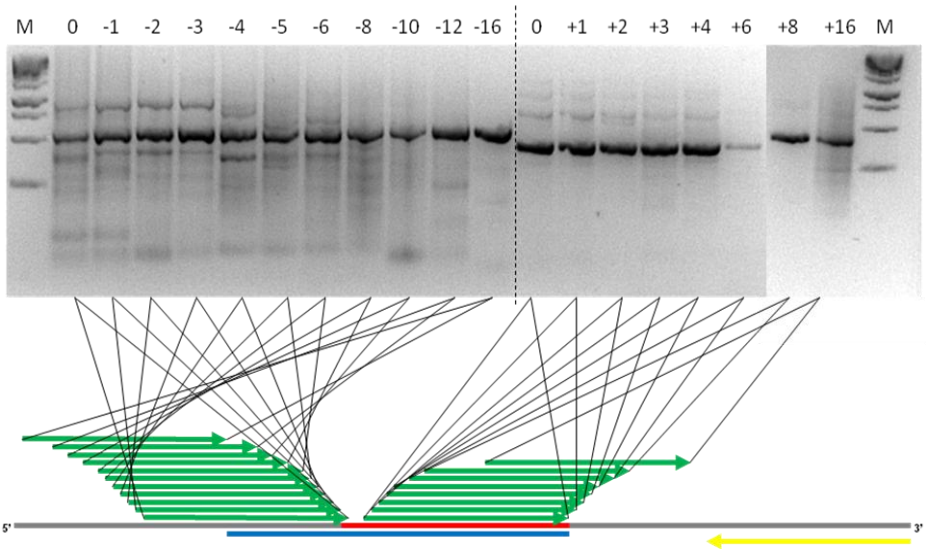

Figure 4. The effect of the repeat position in PCR primer. There are 19 different sense primers (green arrows) and on antisense primer (yellow arrow) involved with these experiments. Red line shows the repeat region masked with GenomeMasker (*wordsize=16, maskingtype=forward, nbases=1*) and blue line shows the actual repeat region. Numbers above the bands define the primer 3' end positions according to the red line. The antisense primer was the same in each reaction with different sense primer. The band with –16 above is the control experiment in which both primers are not overlapping with the actual repeat region (blue line).

2.2.1.3. Performance

One of the important aspects of evaluating program efficiency is its speed. Both methods, GM and RM, gave good results of filtering out non-unique binding sites. Therefore we decided to compare the computational performance of these two programs with different input sizes, sensitivity parameters (RM) and word lengths (GM). Even with the „rush job" (-qq) setting enabled, the RM is still at least ten times slower (Figure 3 in Ref. II). Although there is a speed-up called MaskerAid available, it makes RM even slower with the least sensitive mode than native version (Bedell *et al.*, 2000). The newer versions of RM utilize the WU-BLAST algorithm natively, without the need for MaskerAid.

2.2.2. GenomeTester application

2.2.2.1. Algorithm

The GT application counts and locates all potential binding sites of the PCR primer pair in the genome and predicts the location of all PCR products these primers can generate. The GT is based also on pre-indexed genome sequences like GM, but the main difference is in the structure of the index. Whereas GM stores only the words that are over-represented in the genome, the GT stores all locations of each word (with length defined by the user) and store them similarly to the sorted binary files. The index files (one for each letter: A, C, G, T) stores 8 bytes of data for each word occurring in genomic DNA. The first 4 bytes contain the word sequence and last four the location of the word in given chromosome/genome.

The workflow of the GT is described graphically on Figure 1B in Ref. II. Before searching primer binding sites and products with GT, one must create the binary indexes of the genomic sequences. The second part of the GT application creates a list of primer binding site coordinates and detects possible products with given length. The search itself is using the same binary search algorithm as GM to find those binding sites quickly.

2.2.2.2. Performance

The speed of the e-PCR methods working with eukaryotic genomes is the most important factor followed by memory requirement. We have created 5 different randomly selected primer datasets from human genome and tested the efficiency of several methods suitable for e-PCR (Figure 4 in Ref. II). The well-known homology search programs like BLAST and MEGABLAST are more than 100-fold slower than the newer methods. SSAHA, me-PCR and isPCR are more effective with large datasets, but GT is effective with both large and small datasets. The me-PCR is designed to predict PCR products only and in our tests, some of the products were lost with non-unique primer pairs (we were using default margin ‚M' value). The increase of this parameter will slow down the program. The memory requirements for these calculations on human chromosomes were ranging from 1 GB (SSAHA) to 300 MB (all other methods except GT) and our method was between of them allocating 500 MB of computer RAM.

2.3. Implementation for MultiPLX (Ref. I)

Large-scale studies pose the complex requirements on primer design and also on selecting primers into groups to mix them into one PCR reaction (multi-plexing). Primers in the mix must be specific to their targets and work under the

same reaction conditions. The simple string comparisons are unlikely to give accurate predictions of real interactions, therefore more advanced methods are required using Nearest-Neighbor thermodynamic alignment computation (Kaderali and Schliep, 2002). MultiPLX is designed to perform an automatic grouping of PCR primers using thermodynamic approach and can handle large datasets very efficiently. Program estimates the primer-primer and primer-product interactions, difference in T_m and product length and predicts the risk of primers generating secondary products from the template DNA. The speed of the MultiPLX algorithm is reasonable even with the larger primer sets, although the computation of primer-product interactions with very large data sets may take some time (Table 1 in Ref. I).

The calculation of the primer and product compatibility scores is implemented internally to the MultiPLX program. However, program allows the import of a custom user-specified score to help selecting optimal multiplex groups. One possibility to calculate the custom scores is to test the uniqueness of primers from different pairs that can generate alternative PCR products when multiplexed together. Therefore, we have created a special application called GT4MULTIPLX (http://bioinfo.ut.ee/gt4multiplx/), which is based on the GT algorithm described in previous chapter. It is using the similar input file as the MultiPLX (tabulated text file with ids and primer sequences) and generates all possible primer combinations of them. When GT detects one or more possible PCR products, the IDs and number of product(s) will be stored. Output of this program helps to eliminate wrong PCR product within all multiplex groups as the number of products can be thought as a specific score. User can also define a cutoff to this custom score in the MultiPLX grouping module with the parameter “maximum allowed score”.

2.4. Implementation for SNPmasker (Ref. III)

The discovery, validation and allele determination of single nucleotide polymorphisms (SNPs) can be conducted with different technologies available today (Syvanen, 2005). These methods require mostly high-quality PCR primers or probes to analyze SNPs and attention has to be paid to the repeats and variations when dealing with the genomic DNA. It is shown that the closely located SNPs are causing the lower performance on large-scale genotyping assays in the HapMap Project (Koboldt *et al.*, 2006). To overcome those problems with repeats and SNPs one should mask the template sequence before starting to design primers on it. There are several web services, which provide masking SNPs and repeats simultaneously (Table 1 in Ref. III). However, none of them allow the retrieval of masked sequence by both chromosomal coordinates and homology search. We have developed a web service called SNPmasker designed to mask SNPs from recent dbSNP database (Sherry *et al.*, 2001),

repeats with two alternative programs (GM and RM) and to offer population-specific substitution of SNP alleles using HapMap frequency tables. SNPmasker supports currently information about two organisms: human and mouse.

The implementation of SNPmasker involves three following steps: the localization of input sequences, masking of SNPs and masking of repeats. The most time consuming process is the homology search with MEGABLAST, if the exact location of the input is not defined by the user. After the sequence has been localized or retrieved from database, all SNPs (except deletions and insertions) will be masked in that region. In addition to several masking types (IUPAC,"N", custom symbol) SNPmasker provides unique option to modify the sequence by replacing SNP positions with the most frequent nucleotide (major allele) in given population (CEPH, Japanese, Chinese and African). It might be useful in studies, which are working with the individuals from specific population only as the 25% of the SNP positions (~900000 nucleotides in total) present the minor allele in the current human genomic sequence (data not shown). The masking of repeats is optional, but recommendable. There are various masking options for GM and also the possibility to use the RM (Figure 1 in Ref. III).

The masking style depends on the requirements of given study. For example, to amplify a region around SNP on could use strand-specific lower-case GM repeat-masking and replace all SNPs with "N" letter (Figure 2B in Ref. III). This kind of masking allows finding more primer candidates in highly repetitive regions. Some might want to use the RM masked sequence (e.g. for hybridization probe design) (Figure 2C in Ref. III). The usefulness of a population specific masking is already described above (Figure 2D in Ref. III).

2.5. Predicting the PCR failure rate (Ref. IV)

The statistical modeling in the field of primer design is a good possibility to estimate the weights of various molecular and sequence-specific mechanisms affecting the PCR assays. The values for these mechanisms, factors from now on, can be calculated with several software implementations available today. Given study was focused on refining the previous repeat-masking algorithm of GenomeMasker application by finding the most significant sequence-based factors causing the PCR failure.

2.5.1. Factor and model types

In this study we had the opportunity to analyze 1014 different primer pairs from human chromosome 22 (Dawson *et al.*, 2002) and 300 from random regions around the genome. For each primer pair we have selected and calculated several factors (236 in total) with various tools that may be related to the PCR failure. The important parts of the factors include different modeling of PCR primer binding sites (exact matches, mismatches, thermodynamics). There were also other primer-specific and PCR product related factors present in the statistical analysis (Table 1 in Ref. IV).

The factors are grouped differently into 5 models: GM1, GM1MM, GM2, GM2MM and PCR (Figure 1 in Ref. IV). The first four models (‚GM' can be defined as the abbreviation for GenomeMasker) contain mostly primer binding site counting properties, whereas the last model includes all factors in model building process. The binding site factors in GM1 and GM1MM models are based on the fixed word sizes (exact and with mismatches respectively) and GM2 with GM2MM on the variable word sizes (thermodynamic approach). The complexity and the computing power requirement of the parameter calculation are rising from GM1 to PCR. Although, the variety of factors is higher when building the complex models, the simpler ones are preferred in case of the similar statistical power to make the potential future implementations highly efficient.

2.5.2. Comparison of models and top factors

For each model the four most significant factors were selected and included into final models (Table 2 in Ref. IV). The statistical analysis was performed with the generalized linear models (GLZ). The order of the factors in these models is based on the χ^2 values of over the whole dataset. Interestingly, the most significant factors are the primer binding sites in each model. Other important factors include GC content of primer pairs and number of PCR products along with their length. The difference between exact and mismatched binding site modeling is minor in both, variable (GM2) and fixed (GM1) word sizes. However, comparing the first factors in each model, the thermodynamic approach gave almost two times higher χ^2 values than counting fixed strings. This confirms the arguments about better prediction of primer mispriming sites using thermodynamic modeling (SantaLucia, 2007).

The next obvious question is whether the single best factor is enough to actually eliminate the bad primer candidates or not? To answer that we have generated ten non-overlapping "control" primer sets from the original dataset to analyze the PCR failure prediction efficiency using different number of factors in each model (Figure 2 in Ref. IV). Failure rate of experimentally tested PCR pairs (predictive power of the model) was calculated at increasing sensitivity for

each model. The cutoff values are raised from 0 to given point, where the number of positive (remaining) primers is in predefined model sensitivity level (10%, 20%, 30% etc.). The simpler models, like GM1 and GM1MM, which do not include thermodynamics, were not so successful if only single factor was included into model (Figure 2A in Ref. IV). However, those models gain more power using more than one factor and reduce the difference with complex models (Figure 2B in Ref. IV). The best model GM1 helps to achieve 3-fold decrease in the failure rate of primers in our dataset: from 17% to 6%.

The binding sites with shorter word sizes and primer GC content in simple models (GM1) compensate the absence of mismatches and thermodynamics respectively. The dynamics of failure rate on some of the top factors is shown in (Figure 3 in Ref. IV). The higher number of binding sites raises the failure rate of PCR in all cases (Figure 3A in Ref. IV). High GC content in primer sequences tends to cause the PCR failure with higher probability due to possible false priming with strong energy levels in genomic DNA (Figure. 3B in Ref. IV). The higher number of PCR products (Figure. 3C in Ref. IV) increases also the failure rate, however, adding this factor to the PCR model does not make the model more efficient. Similar effect was seen with product length (fourth factor in PCR model).

2.5.3. Performance of the GM1 model

We have compared the GM1 model efficiency with widely used RepeatMasker and our previous tool GenomeMasker. For that we have selected 1000 random regions around human genome containing 1000 nucleotides each. We have masked these sequences using tools or model named above and executed PRIMER3 to design primer pairs for each region. Masking with GenomeMasker software and GM1 model is done using a special option: mask only last nucleotide from 3' end of the repeat motif. Additionally, 1000 random exonic and intronic sequences were retrieved randomly from all known human genes to compare the overall masking extent in different genomic regions.

Table 3. in Ref. IV shows that GM1 model with strict cutoff level the failure rate is approximately 2.3 times lower comparing to RepeatMasker. However, using given cutoff (10%) the primer design is possible only in 6 or 14% of the random genomic regions. Therefore, increasing the cutoff level to 20%, the primer design possibility is raising to similar level with the RepeatMasker and the failure rate of the PCR is still 1.5 times lower with GM1. Overall genome masking percentage with RepeatMasker was 50%, with GenomeMasker (max 10 binding sites allowed, masking 1 nucleotide) 52% and with GM1 model (with 4 factors, masking only one nucleotide from 3' end of the repeated word) 81% of nucleotides of human genome. Higher masking of exon regions by our GM1 method may reflect the ability of GM1 to take GC-content of primers into

account. Generally GC-rich primers have higher failure rate and therefore GC-rich exon regions are more extensively masked (Figure 3B in Ref. IV).

Although the GM1 model with four factors can reduce the PCR failure rate more than 30 percent, some of the causes of reaction failure remain still undetected. It is said that the optimization of the annealing temperature in thermocycling, salt and primer concentration, the choice of buffer and usage of enhancers can raise the good yield of unique PCR amplicon (Innis and Gelfand, 1990, Beasley *et al.*, 1999) up to 20% (SantaLucia, 2007). Therefore, the wise combination of the masking strategy with improved experimental design principles is a good way to increase the specificity and minimize the necessity of the cost- and time-expensive experimental optimization.

The results in given study demonstrate that GM1, and specifically the binding site modeling using exact matches with fixed word sizes, was similarly efficient as GM2 and PCR model and more than 2 times effective than RepeatMasker for reducing the PCR failure rate. We have compared different binding site modeling possibilities and found that the GM1 model with four factors is efficient enough to use instead of GM2MM or PCR models requiring complicated algorithmic improvements. The significant factors in GM1 model can be implemented in future versions of the GenomeMasker application and the cutoff values for word sizes should be replaced with failure rates to create even more efficient repeat-masking algorithms optimized for PCR assays.

CONCLUSIONS

The summarized results of the study:

1. We have created very fast and efficient repeat-masking and e-PCR applications included in GENOMEMASKER package. GenomeMasker application is able to mask entire human genomic DNA within 6 hours using detailed masking profile. The masking of the repeat motifs is more sensitive and specific compared to other available tools and thus being very useful in primer design assays including three main steps: masking repeats, designing primers with enhanced program modified PRIMER3 and removing primer pairs creating possible alternative PCR products. Additionally, we have created useful web interface called SNPmasker for masking repeats and SNPs with desired locations in mouse and human genomic DNA.
2. The GenomeTester application (the second important part of the GENO-MEMASKER package) locates all binding sites and predicts PCR products with the speed of 1000 primer pairs per minute. The speed of the given application allowed us to create a special procedure for MULTIPLX program called GT4MULTIPLX. This allows user to calculate specific scores for MULTIPLX to achieve more accurate and successful grouping of primer pairs for multiplex PCR. In addition, the fast GenomeTester application made possible in following study to count primer binding sites with different word sizes in reasonable time-scale.
3. The statistical analysis of 236 factors that may affect the outcome of PCR reaction was performed on 1314 primer pairs and their product sequences. The most significant factors in each model we have created in this study were connected to counting primer binding sites. Additionally, the GC content of primer 3' terminus was important factor to increase the power of simpler models. The best model to use in repeat-masking applications should be as effective and easy to compute as possible. We have found that the GM1 model with four factors was similarly effective for predicting the PCR failure rate as more complex models and comparable even with PCR model, which was built including all factors used in study. These results allow us to enhance the future versions of GenomeMasker application and increase the performance of pre-masking repeats even further used in primer design process.

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SUMMARY IN ESTONIAN

Meetodid ja tarkvara PCR praimerite töötamise ennustamiseks suurtes genoomsetes DNA järjestustes

DNA oligonukleotiididel põhinevad tehnoloogiad on leidnud biotehnoloogia valdkonnas laialdast kasutust. Üheks levinumaks molekulaarseks meetodiks on DNA polümeraasi ahelreaktsioon (PCR). Tegemist on tsüklilise reaktsiooniga, kus mõlema DNA ahela jaoks sünteesitakse protsessi käigus uus komplementaarne ahel. Lisaks reagentidele, ensüümile ja paljundatavale DNA-le, on protsessi jaoks vajalikud lühikesed oligonukleotiidid ehk PCR praimerid, mis hübridiseeruvad vastavalt komplementaarse DNA ahelaga ja võimaldavad ensüümil pikendada antud puuduvat ahelat. Ideaalseks reaktsiooni tulemuseks on spetsiifiline ja kõrge kontsentratsiooniga paljundatud DNA regioon ehk PCR produkt (Saiki *et al.*, 1988).

Kaasaegsetes suuremahulistes genotüpiseerimise projektides disainitakse ja kasutatakse tuhandeid praimeripaare korraga, et üles amplifitseerida erinevaid regioone iga indiviidi või organismi DNA pealt. Seetõttu on PCR edukust mõjutavate faktorite hindamine praimerite valimise protsessis väga oluline, et vähendada rahalisi kulutusi ja ajakulu. Varasemad uuringud selles vallas on keskendunud rohkem reaktsiooni reagentide optimiseerimisele nagu PCR puhvri komponentide, soola, DNA, oligonukleotiidide jt. kontsentratioonid ning protokolli optimiseerimisele (Innis and Gelfand, 1990, Beasley *et al.*, 1999). Hilisemad uuringud on pööranud tähelepanu ka praimerite järjestuse omadustele nagu GC sisaldus, praimeri pikkus ja sekundaarstruktuurid, mis võivad mõjutada amplifiseerimise efektiivsust (Haas *et al.*, 1998, Rozen and Skaletsky, 2000, Chen *et al.*, 2003, Chavali *et al.*, 2005, Miura *et al.*, 2005). Samuti on uuritud kindlate nukleotiidide või nende kombinatsioonide mõju praimeri erinevates positsioonides (Yuryev *et al.*, 2002) ja PCR produktide järjestusepõhiseid omadusi: GC sisaldus, sekundaarsturktuurid (Varadaraj and Skinner, 1994, Benita *et al.*, 2003). Vähem on uuritud eukarüootsetes organismides leiduvate korduvate motiivide mõju PCR edukusele.

Käesoleva doktoritöö kirjanduse ülevaade keskendub seni teadaolevate PCR reaktsiooni mõjutavate faktorite kirjeldamisele erinevate uurimisgruppide poolt. Eraldi on välja toodud biokeemilised ja järjestusepõhised faktorid. Lisaks on antud lühiülevaade eukarüootide kordusjärjestustest ja nende klassifikatsioonist ning korduste leidmise meetoditest. Viimane peatükk kirjeldab e-PCR metoodikat ja selle rakendusi, mis on tänapäeval kasutuses.

Antud doktoritöö üheks eesmärgiks oli luua kiire ja efektiivne korduste maskeerimise meetoodika, mis on spetsiaalselt optimiseeritud PCR praimerite disainiks. Doktoritöö raames loodi programmide pakett GENOMEMASKER, milles leiduv aplikatsioon GenomeMasker on võimeline maskeerima kõik korduvad motiivid inimese genoomsel DNA-l 6 tunniga. Järjestuste maskeeri-

mine on võrreldes teiste olemasolevate programmidega tunduvalt kiirem, täpsem ja spetsiifilisem. Lisaks on loodud spetsiaalne web'i aplikatsioon SNPmasker, mille abil on kasutajal võimalik maskeerida ära kordused ja ühenukleotiidsed polümorfismid (SNP) nii inimese kui hiire järjestustel. Pakett sisaldab ka modifitseeritud praimerite disaini programmi PRIMER3, mis tunneb ära GenomeMasker poolt maskeeritud DNA järjestuse ja kasutab uuemaid termodünaamika tabeleid ning valemeid.

Teiseks eesmärgiks oli luua meetod, mis võimaldaks kiiresti lugeda kokku PCR praimerite seondumiskohad suurtes genoomides ja ennustada produktide teket. GenomeTester nimeline aplikatsioon GENOMEMASKER paketis võimaldab PCR produkte ennustada 1000 praimeripaari jaoks minutis. Lähtudes aplikatsiooni kiirusest oli võimalik MULTIPLX programmi jaoks kirjutada spetsiaalne web'i tööriist GT4MULTIPLX, mis võimaldab arvutada praimeripaaride jaoks spetsiifilised skoorid, mida on võimalik hilisemal multipleks gruppide moodustamisel arvesse võtta. Lisaks võimaldas efektiivne GenomeTester aplikatsioon järgmises uuringus mõistliku aja jooksul läbi viia erinevate sõnapikkustega praimerite seondumiskohtade ja produktide kokkulugemised.

Käesoleva töö viimases osas uuriti erinevaid faktoreid, mis võiksid vähendada PCR edukust. Uuringus kasutati 1314 praimeripaari katseandmeid (>80000 üksikut katset) ja iga paari kohta arvutati 236 erineva faktori väärtused. Selgus, et kõige enam mõjutab PCR edukust praimeri seondumiskohtade arv genoomis. Lisaks oli oluline primeri 3' otsa GC sisaldus. Oluliste faktorite põhjal koostati 5 erinevat PCR edukust ennustavat statistilist mudelit. Faktorid jaotati mudelitesse arvutusliku keerukuse alusel. Mudelite võrdlemisel selgus, et GM1 (kõige lihtsamini arvutavav mudel), mis sisaldab 4 olulisemat faktorit, ennustab PCR edukust samal tasemel või isegi paremini, kui keerulisemad mudelid (GM2, PCR). Sellele toetudes on võimalik tulevikus tõsta GenomeMasker aplikatsiooni algoritmi efektiivsust veelgi praimerite disaini protsessi parandamiseks.

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PUBLICATIONS

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Scientific work

My research projects are associated with development of specific bioinformatics methods and algorithms for genotyping applications. These include improvements in DNA identity search and repeat masking algorithms. Later on, I have been modeling the prediction of PCR failure rate, identifying the most important sequence-based factors influencing PCR and trying to implement these models into current masking algorithms.

List of publications

1. **Andreson, R**, Möls, T, Remm, M. (2008) Predicting failure rate of PCR in large genomes. *Nucleic Acids Research.* (accepted)
2. **Andreson, R**, Kaplinski, L, Remm, M. (2007) Fast masking of repeated primer binding sites in eukaryotic genomes. *Methods Mol Biol*. 2007;402:201–18. Review.
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4. **Andreson, R**, Reppo, E, Kaplinski, L, Remm, M (2006) GENOMEMASKER package for designing unique genomic PCR primers. *BMC Bioinformatics*, 7, 172.
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2000	Euroopa Bioinformaatika Instituut (EBI), Hinxton, UK, külalisüliõpilane
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Teadustegevus

Teadustöö on seotud bioinformaatika alaselt genotüpiseerimiseks vajalike algoritmide ja meedodite arendamisega. Algselt tegelesin DNA järjestuste sarnasuse ja identsuse otsingu meetodite uurimisega. Hiljem lisandus sellele PCR edukuse hindamine ja spetsiaalsete mudelite loomine eesmärgiga arendada välja efektiivsemad korduste maskeerimise algoritmid.

Publikatsioonid

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2. **Andreson, R**, Kaplinski, L, Remm, M. (2007) Fast masking of repeated primer binding sites in eukaryotic genomes. *Methods Mol Biol*. 2007;402:201–18. Review.
3. **Andreson, R**, Puurand, T, Remm, M. (2006) SNPmasker: automatic masking of SNPs and repeats across eukaryotic genomes. *Nucleic Acids Research*, 34, W651–W656.
4. **Andreson, R**, Reppo, E, Kaplinski, L, Remm, M (2006) GENOMEMASKER package for designing unique genomic PCR primers. *BMC Bioinformatics*, 7, 172.
5. Kaplinski, L, **Andreson, R**, Puurand, T, Remm, M. (2005) MultiPLX: automatic grouping and evaluation of PCR primers. *Bioinformatics*, 21(8), 1701–1702.