

Chapter 2

Scanning for DNA Variants by Denaturant Capillary Electrophoresis

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Abstract Analysis and detection of DNA variation is important in any field of biology. Hence, numerous methods have been developed to analyze DNA. A simple yet effective way of analyzing DNA is by denaturant capillary electrophoresis (DCE). The method is in theory applicable to 95% of the human genome. The method involves three steps; fragment design, PCR amplification and allele separation. The allele separation can in principle be performed with any DNA capillary sequencing instrument.

Keywords DGGE (denaturant gradient gel electrophoresis), CDCE (constant denaturant capillary electrophoresis), CTCE (cycling temperature capillary electrophoresis), PCR (polymerase chain reaction), dsDNA - double strand DNA, ssDNA - single strand DNA

1 Introduction

Analysis of DNA variation, either somatic or inherited mutation, is important in all fields of biology. As all life forms, from virus to humans, are products of their genetic code, any minor alteration in this code may induce severe or life-threatening changes in the organism. Alternatively, adaptation and survival advantage in a given environment may ensue. Thus, analysis of DNA variation has become a major field of research in biology. In 1953, Watson and Crick postulated the structure of DNA [1], which may be regarded as the beginning of the genomic era. This was followed by the description of PCR in 1971 by Kleppe et al. [2], demonstration of Sanger et al. sequencing reactions with termination nucleotides in 1977 [3], and the separation of base substitutions by denaturant gradient gel electrophoresis (DGGE) in 1983 [4, 5]. The familiar PCR amplification, using thermostable polymerases, was first demonstrated in 1985 [6–8]. This discovery accelerated analysis of DNA and development of methods able to analyse DNA. One type of method widely used is the melting gel technique, which has been reviewed extensively by Bjørheim and Ekstrøm

[9, 10]. The method is based on a theoretical calculation of DNA melting by statistical mechanics calculation [11] and separation of variant DNA during electrophoresis, where the migration of less stable fragments (i.e., heteroduplexes) are retarded as compared to the wild-type sequence. The method, in theory, is applicable to about 95% of the human genome, where the melting temperature is below the stability of the artificial high melting domain (*see Note 1*), known as the GC-clamp [12–14] (*see Note 2*). The method, which initially was developed in “slab” gel form, has been transferred successfully to six different commercial DNA capillary sequencing instruments. DCE requires three steps, design of the appropriate fragment (software simulation), PCR amplification, and allele separation by electrophoresis.

Under the conditions for which DNA fragments are appropriately designed for DCE, PCR amplifiable, and given appropriate denaturing conditions (temperature and chemical), heteroduplexes are separated from the wild-type fragments. Therefore, DCE is an excellent method for scanning DNA for unknown variants.

2 Materials

2.1 *Statistical Mechanics Calculation of DNA Melting Probabilities*

Online Internet services, <http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html> (*see Note 3*).

2.2 *PCR*

1. 10 × buffer IV (ABgene, Epsom, UK) (*see Note 4*).
2. 25 mM MgCl₂.
3. 4 × 2.5 mM dNTPs.
4. Taq polymerase (ABgene) (*see Note 5*).
5. Primers.
6. DNA template.

2.3 *Allele separation*

Commercial capillary DNA sequencing instrument (*see Note 6*).

3 Methods

Scanning DNA for variants by DCE requires three steps: fragment design, PCR amplification of the target sequence, and allele separation by capillary electrophoresis. Evaluation of the DNA sequence of interest by use of statistical mechanics calculates the melting properties of the DNA and provides information as to whether the fragments can be analyzed by DCE and where the high melting temperature tail, the GC-clamp (*see Note 2*), should be attached. Furthermore, the simulation of allele melting temperatures yields information on their expected separation by DCE. The purpose of PCR is to produce enough copies of the target sequence, incorporating the label and attaching the high temperature melting domain. DCE for allele separation alleles was first performed with in-lab assembled instruments, where optical parts like mirror, lenses, laser, and detectors were mounted onto an optical bench [15–19]. Thus, all handling, gel replacement, sample loading, and recording of electrophoresis were done manually. However, the manual handling and technical skill of the users resulted in detection of mutants fractions down to 10^{-6} [20, 21]. This detection limit is superior to most methods. With the introduction of automated capillary DNA sequencing instruments, more samples could be processed without intervention from the operator [22–35], while upholding a limit of detection of 10^{-3} [22, 25, 26].

3.1 Target Selection

1. Find DNA sequence of interest in a public database (*see Note 7*).
2. Select primers for target sequence. The length of the target sequence should be in the range of 80–210 bp (*see Note 8*). Primers can be selected using the following website: <http://frodo.wi.mit.edu/primer3/input.htm>.
3. Cut and paste the sequence to be amplified into a DNA melting simulation bioinformatics tool (*see Note 3*). Observe the theoretical melting profile of the target sequence; simulate GC-clamp (*see Note 2*) and known variants (*see Note 9*). Figure 2.1 demonstrates the 50% melting probability of the Kras gene exon 1, simulated by four computer programs. Please note the difference in melting temperatures; this is a result of algorithm approximations and differences between salt concentration and dissociation constant settings in each program.

3.2 PCR

1. Optimize the PCR reaction, by use of concentration combinations of Mg^{2+} , primers, and dNTPs (*see Note 10*). This should be performed in combination with different annealing temperatures.

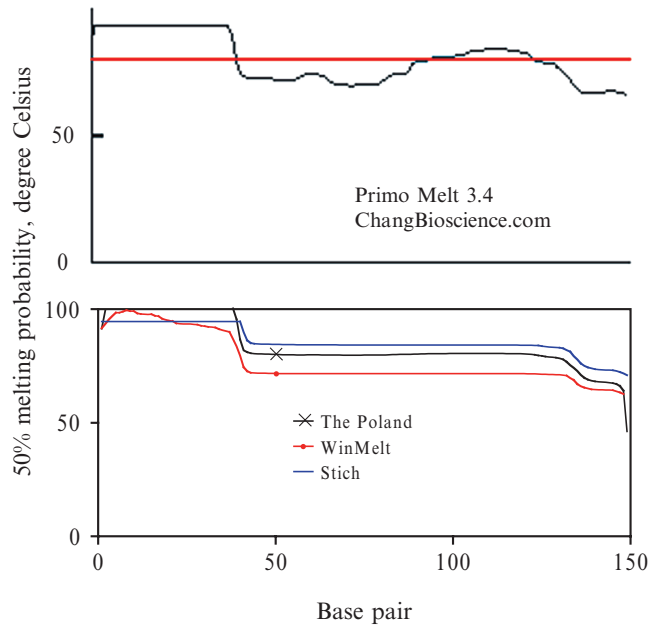


Fig. 2.1 Statistical mechanics calculation of DNA melting, Kras exon 1, obtained with four programs

2. Amplify samples by PCR, using the conditions identified by optimization. Table 2.1 provides an example of the composition of a “standard” PCR reaction mix (*see Note 11*). Perform PCR as follows: 95 °C, 5 min; 35 cycles of 95 °C, 30 s, annealing temperature, 30 s, 72 °C, 60 s (the annealing temperature varies between target sequences and is dependent on the primer design).
3. Create heteroduplexes by denaturing the PCR product at 95 °C for 2 min, followed by reannealing at 65 °C for 30 min (*see Note 12*) (this step can be programmed at the end of the amplification thermocycling program).

Table 2.1 20- μ L PCR mix, volume, and concentrations of reagents

	Concentration	Volume (μ L)	Desired concentration
H ₂ O		12.2	
10 \times buffer	10 \times	2	1 \times
MgCl ₂	25 mM	2	2.5 mM
Primer 1	5 μ M	1.2	0.3 μ M
Primer 2 (6-fam-gc)	5 μ M	0.6	0.15 μ M
DNTP (2.5 mM of each)	10 mM	0.8	400 μ M
DNA	50 ng/ μ L	1	2.5 ng/ μ L
Taq DNA polymerase	5 U/ μ L	0.2	0.05 U/ μ L

3.3 Denaturant Capillary Electrophoresis

1. Set the running temperature of the capillary electrophoresis instrument according to the theoretical melting temperature of the low melting domain of the target sequence (*see Note 13*). For instruments with inappropriate temperature control, temperature cycling (cycling temperature capillary electrophoresis) can be used [29, 36].
2. Prepare the instrument for electrophoresis by replacing the polymer using a syringe pump or by applying high-pressure nitrogen (instrument dependent).
3. Inject the PCR product by applying a high voltage for a short period of time, such as 10kV for 20s (*see Notes 14 and 15*).
4. Perform electrophoresis and record the fluorescent signal.
5. Inspect the electropherogram as raw data in the sequencing analysis software supplied with the instrument. Figures 2.2 and 2.3 are two examples of allele separation and repeatability.
6. Using the internal standard (*see Note 15*), call the genotypes. Figure 2.4 demonstrates genotyping by DCE.

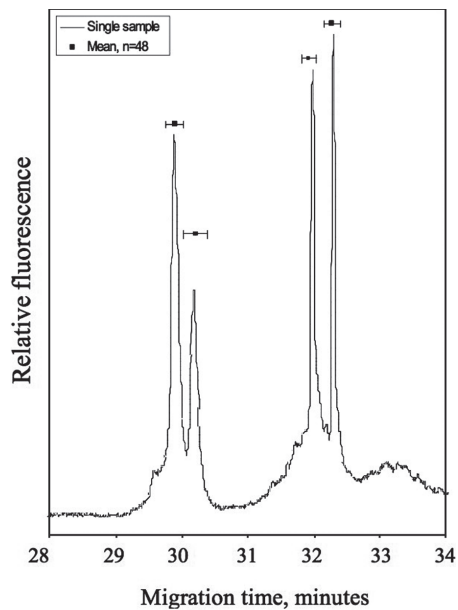


Fig. 2.2 Allele separation and repeatability of a one base pair deletion in the PPRA gamma gene (OMIM #601487). DCE was performed with temperature cycling between 50 °C and 47 °C, repeated 20 times [29, 36]. The average migration time of 48 runs (black square) is shown with 1 standard deviation for each duplex. Migration times were normalized to the sum of the four-migration times of each peak maximum [36]

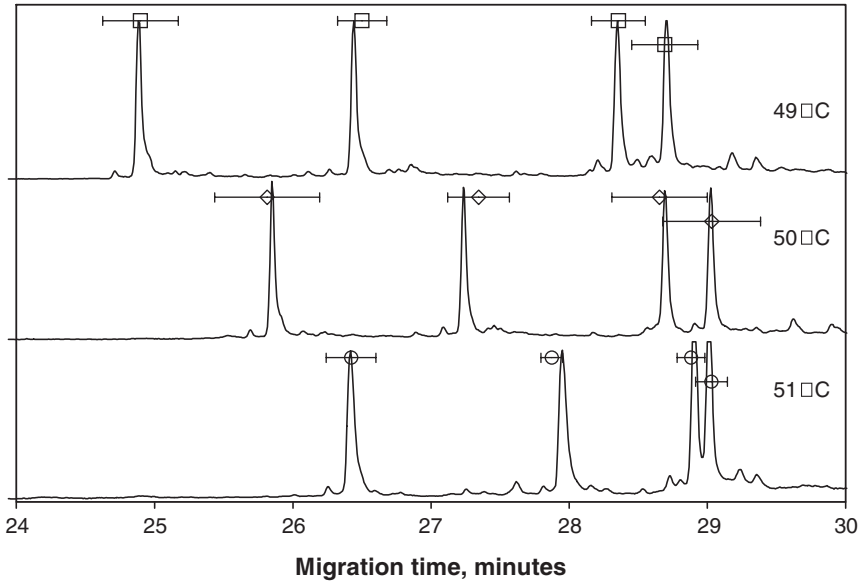


Fig. 2.3 Separation of homoduplexes and heteroduplexes (NCBI ref SNP ID: rs10887), using the CEQ 8000 instrument (Beckman Coulter) at different temperature settings. The CEQ8000 instrument hardware was used without modification, and standard CEQ capillary arrays (33 cm), polymer, and separation buffer were used. The sample injection was standardized to 2.5 kV for 15 s while the separation was performed under the standard voltage setting of 4,000 V. Replicates ($n = 32$) of the samples were analyzed at three temperature settings. The average migration time of 32 runs is shown with 1 standard deviation for each peak (homoduplex and heteroduplex). Please note the baseline separation between the homoduplexes at all temperatures

4 Notes

- Figure 2.5 depicts the distribution of 50% melting probabilities in 58440bp from chromosome 1. Here, 95% of the melting temperature is below 77 °C, which is below the stability of the GC-clamp. Please note that the resolution on the x-axis is 0.1 °C.
- Standard GC-clamp with 6-Fam label:
5'-6-FAM-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGC
CCG-3'

If this sequence is used as it is on the “forward” or “reverse” primer, a standard 20-mer primer with a different label can be used to make the fragment-specific internal standard (*see Note 15*). For large-scale studies featuring many fragments, primer cost reduction can be achieved by in-PCR attachment of a GC-clamp and labeling. This is illustrated in Fig. 2.6. The purpose of the GC-clamp is to prevent strand dissociation during electrophoresis. When the low melting

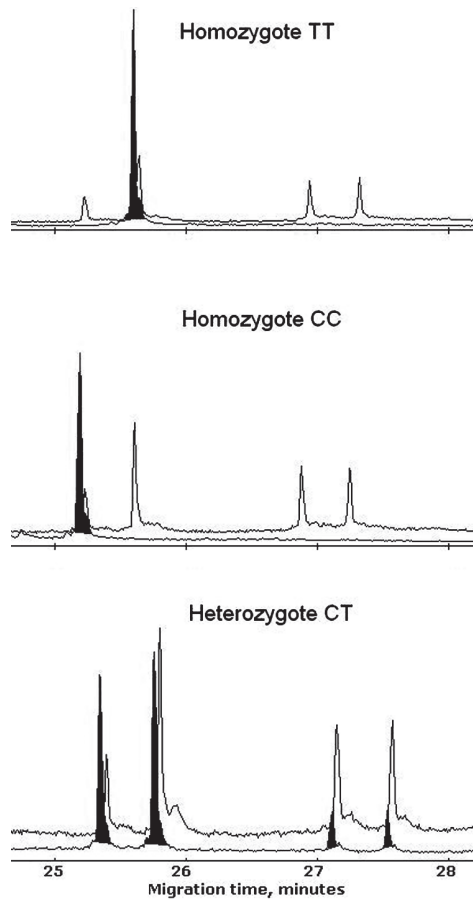


Fig. 2.4 Genotyping according to an internal standard. Three samples are genotyped (solid peaks) and scored against an internal standard (open peaks) for the SNP with NCBI reference number rs375384. The four peaks in the internal standard are the two homoduplexes and the two heteroduplexes (mismatch of Watson and Crick strands) formed during PCR and heteroduplex formation, respectively. However, when using DCE for genotyping, the user should be aware that any new or additional events in the target sequence may alter the peaks positions in the electropherogram

domain is subjected to the appropriate denaturing condition (chemical and temperature), this part of the fragment will be in a state of equilibrium between dsDNA and ssDNA. Because of the branching, the DNA fragment will be retarded during electrophoresis. Therefore, fragments with minor differences (substitution, deletion, or insertion) generally have a different average velocity through the polymer from the consensus sequence, which results in the observed separation of the alleles.

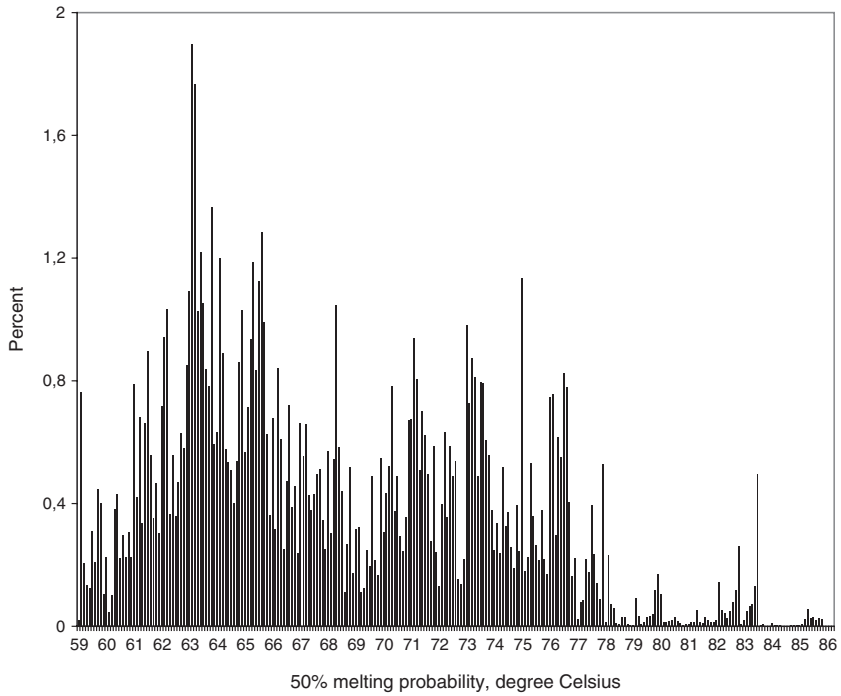


Fig. 2.5 Melting probabilities of 54440 bp from chromosome 1

In PCR labeling and GC extension, Kras exon 1 amplification and allele separation by CTCE

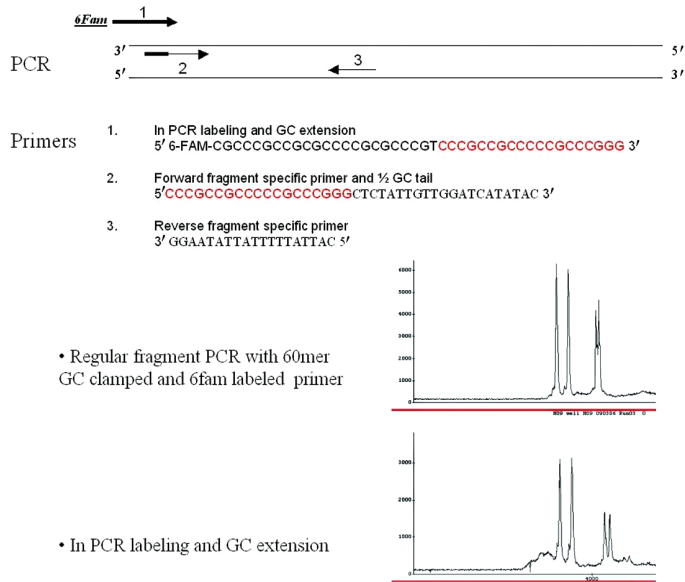


Fig. 2.6 In-PCR labeling and GC extension

3. Calculation of the melting properties of the DNA of interest can be performed by various software packages or online Internet programs. Information about commercial software or online bioinformatics tools can be found via the following Internet links:
<http://bioweb.pasteur.fr/seqanal/interfaces/dan.html>.
<http://www.changbioscience.com/primo/primomel.html>.
http://stitchprofiles.uio.no/ctemp_prof.htm.
<http://bioinformatics.org/meltsim/wiki/Main/HomePage>.
<http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html>.
<http://www.medprobe.com/uk/sequencher-demo.html>.
We have been successfully using WinMelt (Medprobe, Oslo, Norway) to simulate changes in thermodynamics and used the theoretical melting temperature as a guide for the temperature setting during electrophoresis. We found a linear relationship between stability changes and separation achieved by DCE [36].
4. The buffer and MgCl_2 are prepared in the laboratory to keep the PCR cost as low as possible. As a $10 \times$ PCR buffer, we regularly use sterile-filtered 750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM $(\text{NH}_4)_2\text{SO}_4$, with 0.1% (v/v) Tween 20 added to the buffer after sterile filtration. This buffer is the same as ABgene's buffer IV (ABgene, Epsom, UK). MgCl_2 is added into the reaction mixture from a separate vial, because this facilitates PCR optimization with respect to Mg^{2+} concentration.
5. The selection of enzyme should be based on the detection limit needed for the study. Using Taq polymerase gives an error rate in the range of 1×10^{-4} to 1×10^{-5} misincorporations per bp per cycle, while PFU (Stratagene, La Jolla, CA) has been shown to produce tenfold fewer errors [37]. Therefore, studying low mutant fractions requires the use of PFU polymerase. However, we successfully combined the two enzymes (ratio 10:1, Taq:PFU), preserving the processivity of TAQ and the fidelity of PFU.
6. We tested six capillary DNA sequencing instruments, from different manufacturers, and found that alleles can be sufficiently separated by DCE with ABI 310 and ABI 3100 (Applied Biosystems, Foster City, CA), CEQ 8000 Genetic Analysis (Beckman Coulter, Fullerton, CA), MegaBACE 1000 and MegaBACE 4000 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), and SCE 2410 (Spectrumedix, State College, PA) [22–27, 29, 30, 32–35, 38, 39]. Standard capillaries and sieving matrix for each instrument were used, making this method readily available to any user with access to such instruments.
7. An example of a bioinformatics tool that can be used to access a nucleic acid sequence is found at <http://snpper.chip.org/>. This “database” presents gene search, dbSNP data, sequence search options, and annotated sequences (with intron, exon, coding, and SNP) in a very straightforward format. For illustration purposes, annotated “epidermal growth factor receptor isoforma” can be found at the following link: <http://snpper.chip.org/bio/show-sequence/?TYPE=U&GENE=17905>.
8. Primers can be selected using the following website: <http://frodo.wi.mit.edu/primer3/input.htm>

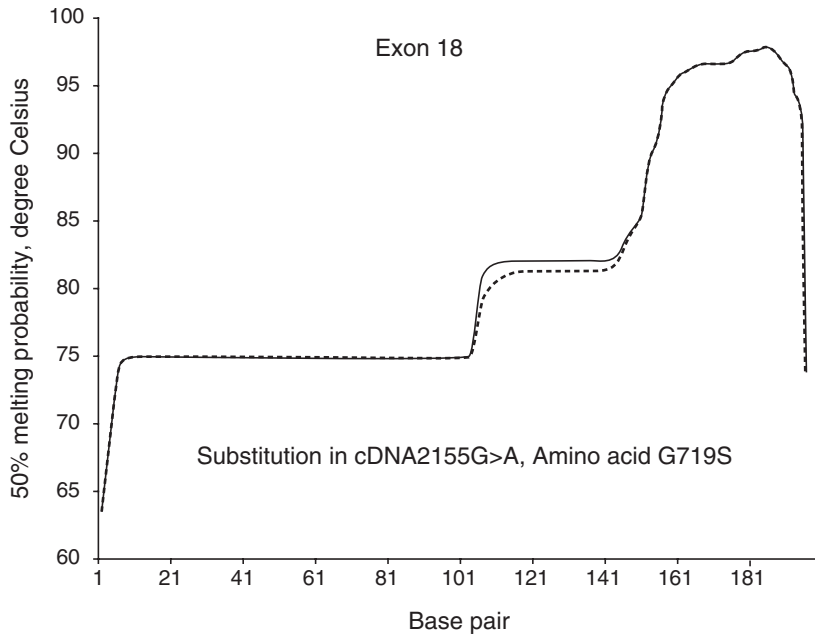
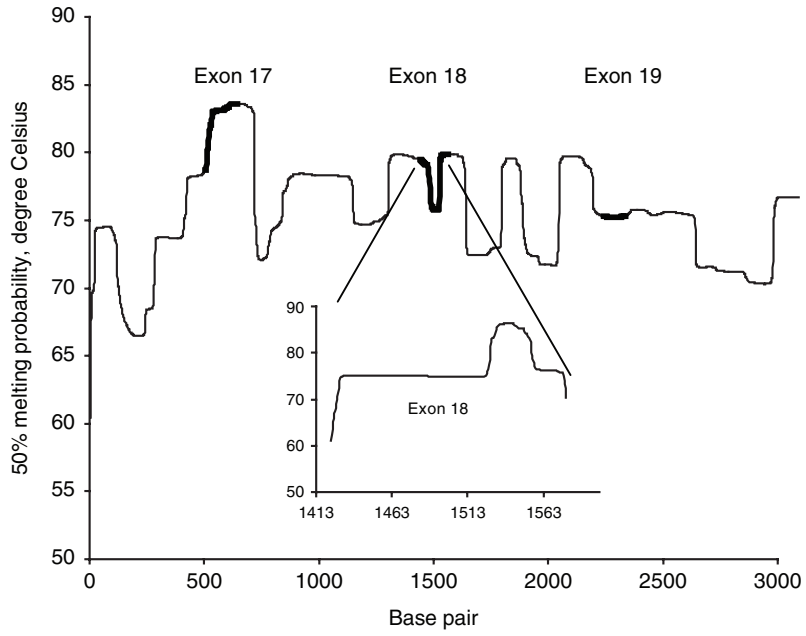


Fig. 2.7 Selection and design of target sequence and stability difference between alleles as calculated by statistical mechanics

9. Figure 2.7 represents a point-by-point illustration of the process of fragment design simulation and primer selection, leading to a fragment suitable for DCE analysis. Assume we want to analyze the EGFR gene (OMIM #131550) for mutations that render lung cancer patients susceptible to Iressa treatment. One of the mutational hotspots is sited in exon 18. In Fig. 2.7, the 50% melting probability (in °C) is plotted for the DNA sequence of exon 17 to 19. The bold line in the Fig. 2.7(a) graph indicates the exons. The enlargement of exon 18 contains 17 bp of priming sites in the intron on each side. From this melting profile, decision on the placement of the GC-clamp is made. As a rule of thumb, always place the GC-clamp on the side of the fragment with the “highest” average melting temperature. This prevents “hidden Death Valleys” which can result in pore peak resolution (31). Although the stability of the GC-clamp is high, the length of the low melting domain is limited. Experience has shown that the optimal low melting domain lengths are between 80 and 210 bp. Figure 2.7(b) illustrates a simulation of two different alleles in the target sequence. Note: The high temperature melting domain from the base pair 150 to 180 is the effect of the CG-clamp. By simply changing a base pair (SNP or mutation hot spot), a slightly different melting profile is revealed. This melting difference is sufficient to separate homoduplex alleles.
10. While optimizing the chemical conditions of the PCR is important for most assays involving amplification of DNA, the number of thermocycles also can affect the specificity of the amplification. The number of final copies (with a maximum of 10^{11} copies/ μ l) is a function of the starting copy number (N_0), amplification efficiency (eff) and the number of cycles (C). The following formula can be used to calculate the final copy number after PCR:

$$N = N_0 (\text{eff})^C$$

Because one of the primers is labeled with 6-fam, we generally use twice the amount of the unlabeled primer than the labeled primer. This is to prevent over-amplification of the labeled strand compared to the unlabeled (*see Note 16*). Table 1 is an example of a standard 20- μ L PCR mix.

11. Troubleshooting a PCR reaction is beyond the scope of this chapter. The objective for primer design is to design primers specific for the target sequence, which anneal to the correct sites and facilitate amplification of the desired sequence. Once this is achieved, the labeled PCR products are ready for allele separation by DCE. However, a good laboratory practice is always to optimize each fragment with regard to Mg^{2+} concentration and annealing temperature.
12. Although many minor alterations (base substitution, deletion, and insertion) in DNA results in thermodynamic changes, some changes do not alter the thermodynamics of the target sequence [40]. These changes might be a G:C \rightarrow C:G or a A:T \rightarrow T:A transition, depending on the neighboring base sequence. To be able to detect those changes, a simple heteroduplex formation step (post-PCR) can be performed. Simply by denaturing the dsDNA and annealing the Watson and Crick strands slowly, highly unstable (as compared to matched base pair)

mismatches are formed (Fig. 2.7). A regularly used heteroduplex formation program of 95 °C for 2 min, followed by reannealing at 65 °C for 30 min, is sufficient for heteroduplex formation. Please note that this should be performed only on undiluted PCR products. Figure 2.8 depicts possible strand combination as a result of the denaturation and reannealing.

13. The denaturing temperature has to be adjusted for chemical denaturant in the polymer. Therefore, if the melting domain of the target sequence is theoretically melting at 75 °C and the polymer contains 7 M urea, the denaturing temperature is set to 54 °C [41] ($75\text{ °C} - 21\text{ °C}$, correction for chemical denaturant, 3 °C/M urea). Sequencing polymer regularly contains 6 or 7 M urea. Consequently, the denaturing conditions are corrected accordingly. For sieving matrixes [42] formulated in the laboratory, chemical denaturant can be omitted. However, the temperature limitation or restriction on the various DNA capillary sequencing instruments dictates the use of chemical denaturants. For new fragments, a serial analysis at different temperatures for a heterozygote sample reveals the best separation temperature for the instrument used.
14. Crude or diluted PCR product in water (down to 1:50) is injected electrochemically, following the standard injection procedure recommended by the instrument manufacturer. Figure 2.9 demonstrates the relative small differences in injection efficiency introduced when PCR products are diluted in water. The PCR product was injected by applying 10 kV for 20 s and alleles were separated by CTCE in a MegaBACE 1000. The peaks are two homoduplexes and two heteroduplexes, respectively. Please note the nontemplate addition of adenosine, which is the stutter peak to the right of each duplex. We observed degradation of DNA diluted in water stored in 4 °C. Therefore, the diluted product should be stored

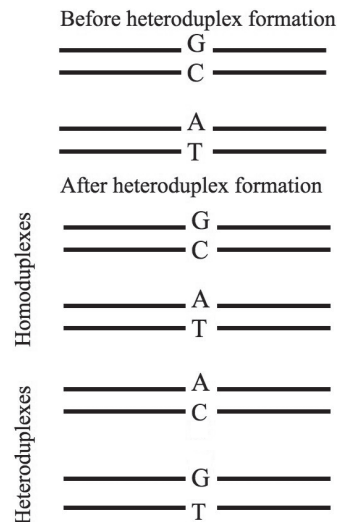


Fig. 2.8 Strand combination as a result of heteroduplex formation

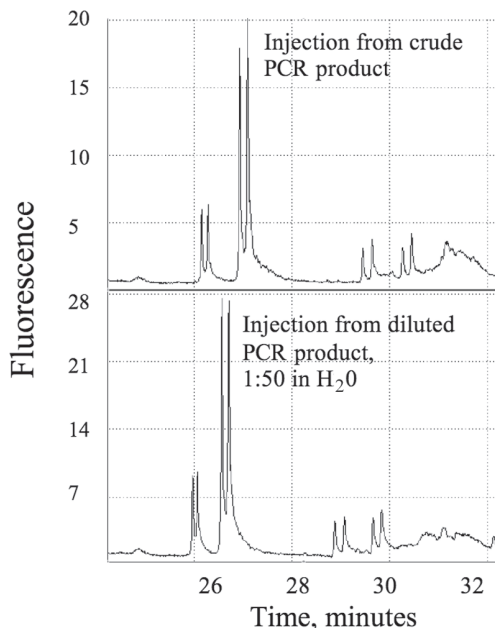


Fig. 2.9 Effect on electrochemical injects by diluting PCR product in H₂O

at -20°C or below when not in use. The dilution of PCR products in “commercial loading solutions” or formamide is not recommended, as this may denature the dsDNA.

15. DCE has been used for the genotyping of DNA variants by separating alleles and comparing the pattern with an internal standard. The internal standard is made from a heterozygote sample by reamplification with a 20-mer primer specific for the GC-clamp (*see Note 2*). This primer has a different fluorophore attached to its 5' end from the primer used to amplify the samples. As the GC-clamp is standardized, the same 20-mer labeling technique can be used for different fragments [27, 28, 30, 33]. Amplification of the internal standard with differential labeling is performed on a 1:1,000, or 1:10,000, diluted heterozygote PCR product in H₂O. Due to the high template copy number, fewer thermocycles are needed (less than 25). Primers:

Forward: 5'-Alternative label-CGCCC GCCGCGCCCCGCGCC3'

Reverse: Unlabeled primer

In Fig. 2.10, the bold lines represent the GC-clamp and the short lines with arrows indicate the forward and reverse primers. The alternative label is instrument dependent but could be, for example, carboxy-X-rhodamine (ROX).

16. We noted that labeled ssDNA molecules have a “tendency” to migrate into the heteroduplex region during DCE. Hence, using unbalanced primer concentrations (see Table 2.1) reduces the risk of amplifying the labeled ssDNA but

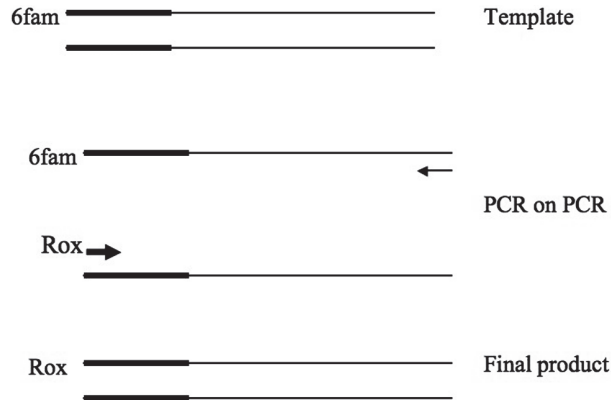


Fig. 2.10 Label “switching” used to construct an internal standard

allows the amplification of unlabeled ssDNA, which has no effect on the analysis.

17. The electrophoresis cost per sample (using standard conditions, as recommended by the instrument manufacturer) is about \$1 (MegaBACE 1000). However, we optimized the DCE method using the MegaBACE 1000 with respect to cost per analysis. By formulating the sieving matrix and running buffer in the laboratory, we got more runs out of the capillary arrays and thereby reduced the polymer cost. Hence, the cost to us of running the electrophoresis with the MegaBACE 1000 is about 5 cents per sample.

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