Method

High throughput DNA sequence variant detection by conformation sensitive capillary electrophoresis and automated peak comparison

Helen Davies [a], Ed Dicks [a], Philip Stephens [a], Charles Cox [a], Jon Teague [a], Chris Greenman [a], Graham Bignell [a], Sarah O'Meara [a], Sarah Edkins [a], Adrian Parker [a], Claire Stevens [a], Andrew Menzies [a], Matt Blow [a], Bill Bottomley [a], Mark Dronsfield [b], P. Andrew Futreal [a], Michael R. Stratton [a], Richard Wooster [a,*]

[a] *The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK*
[b] *Applied Biosystems, Lingley House, 120 Birchwood Boulevard, Warrington WA3 7QH, UK*

Abstract

We report the development of a heteroduplex-based mutation detection method using multicapillary automated sequencers, known as conformation-sensitive capillary electrophoresis (CSCE). Our optimized CSCE protocol detected 93 of 95 known base substitution sequence variants. Since the optimization of the method, we have analyzed 215 Mb of DNA and identified 3397 unique variants. An analysis of this data set indicates that the sensitivity of CSCE is above 95% in the central 56% of the average PCR product. To fully exploit the mutation detection capacity of this method, we have developed software, canplot, which automatically compares normal and test results to prioritize samples that are most likely to contain variants. Using multiple fluorescent dyes, CSCE has the capacity to screen over 2.2 Mb on one ABI3730 each day. Therefore this technique is suitable for projects where a rapid and sensitive DNA mutation detection system is required.
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Introduction

The reference human genome sequence and genome sequences of other species provides a resource that has had a significant impact on the discovery of disease-causing genes. One of the rate-limiting steps in the gene discovery process is identification of DNA sequence variants or mutations. Another is the collection of suitably characterized sets of samples. Furthermore, following their discovery, many disease genes are screened in large numbers of susceptible individuals to determine mutation spectra and prevalence. Screening for known sequence variants can be performed in many ways; however, DNA sequencing is the final point in the discovery of novel variants. Even with recent developments in sequencing technologies, this can be slow and expensive and the data are

complex [1]. An alternative approach is to rapidly screen the candidate region and then sequence only those areas with evidence of putative mutations. There is, therefore, a requirement for mutation-screening techniques that are rapid, sensitive, and cost effective.

There are many examples of rapid DNA screening techniques, such as denaturing gradient gel electrophoresis [2], chemical cleavage of mismatches [3], single-stranded confirmation polymorphism [4], the protein truncation test [5], denaturing high-performance liquid chromatography (DHPLC) [6], and conformational-sensitive gel electrophoresis (CSGE) [7]. CSGE is one of the most widely used of these methods and sensitivity comparisons with other techniques favored CSGE [8–10]. CSGE has been developed further with the use of fluorescent labels and slab gel automated sequencers [11,12] and has been transferred to single-capillary sequencers [13], where it was named conformation-sensitive capillary electrophoresis (CSCE). CSCE has been transferred to multiple-

capillary sequencers [14–16]; however, these studies analyzed no more than four genes with at most 120 sequence variants. Here we report the optimization of CSCE on multicapillary DNA sequencers together with software to view and prioritize the traces. In a screen of 215 Mb of DNA for novel sequence variants, we identified 3397 unique variants, making this the first demonstration of a truly high-throughput application of CSCE.

Results

Protocol development and evaluation of mutation detection sensitivity

A set of 45 PCR products (STSs) containing a total of 95 single-nucleotide polymorphisms (SNPs) were used to establish the most sensitive conditions for CSCE on both ABI PRISM 3100 and ABI3730 genetic analyzers. The PCR products ranged in size from 190 to 450 bp and SNPs were located 45 to 174 bp from the ends of the PCR products. The sequence of the 45 STSs was determined for 24 DNA samples (Coriell Cell Repositories DNA Polymorphism Discovery Resource Collection, M24PDR) by DNA sequencing to identify all sequence variants. These samples were used in the subsequent optimizations.

The CSCE separation medium was initially optimized for the ABI3100. A number of variables were assessed in the optimization. Compared to a 50-cm capillary, a 36-cm capillary had no significant effect on the separation or resolution of heteroduplexes but gives shorter run times. Increasing the run temperature from 18 to 30°C reduced the run time but also decreased the resolution of heteroduplex peaks, lowering variant detection efficiency. Increasing the concentration of CAP polymer from 4 to 7% increased the run time but had no beneficial effect on heteroduplex resolution or detection. The inclusion of 2-pyrrolidinone (1–5%), ethanediol (10%), or glycerol (5%) had no positive effects on heteroduplex separation or sensitivity. A taurine-based buffer gave a slight improvement compared to TAPS-based buffer. The inclusion of urea from 1 to 5.5 M, however, had a dramatic effect on the separation of heteroduplex peaks. Urea had a beneficial effect on heteroduplex peak detection in 27% of STSs tested. The optimum urea concentration was 4 M. Higher concentrations of urea up to 5.5 M had a detrimental effect on fragment resolution, particulary on fragments larger than 300 bp.

On the ABI3730, an even spatial calibration across the array is required to achieve even signal intensities from all 96 capillaries. The ABI3100 protocol produced a very poor spatial calibration profile on the ABI3730. The refractive index of POP7, a separation medium recommended for the ABI3730, was 1.3995, whereas 4 M urea, 5% CAP in 1× TTE (the optimal ABI3100 mutation detection medium) had a lower refractive index of 1.3812. Including 5% sucrose increased the refractive index of the separation medium to 1.3911 and increased the length of the run time but had no effect on detection sensitivity. The concentration of sucrose was titered between 5 and 10% to determine the concentration that would produce an even spatial

calibration profile on the ABI3730 while keeping the run time to a minimum. The following conditions produced the best mutation detection sensitivity for ABI3100 and ABI3730: 36-cm capillaries, 18°C run temperature, 5% CAP polymer, 4 M urea, 1× TTE, and 7.5% sucrose (ABI3730 only).

Another factor in the optimization of the protocol was the design of the STSs. The sensitivity of detection in CSGE is thought to be lower toward the ends of the STS; hence, the 3′ ends of the PCR primers were placed at least 50 bp away from the sequence to be screened. One STS (stCP250012) was unable to detect a T to G substitution 48 bp from the end of the amplimer (the optimal position for the PCR primer dictated a 48-bp extension). The PCR primers were redesigned so the variant was 77 and 101 bp from the end of the amplimer (STSs stCP250059 and stCP250060, respectively). In both of the redesigned assays, the variant was clearly detected. However, another variant, a G to A, which was undetectable when 50 bp from the end of the amplimer (stCP250032), remained undetectable despite redesigning of the PCR primers to position the variant 95 bp from the end of the amplimer (stCP250062). STS information is available from http://www.sanger.ac.uk/genetics/CGP/.

Fragment size is also an important factor in STS design. Five of the 45 STSs used for the optimization produced fragments greater than 400 bp. All 10 SNPs present in these five fragments were detected by CSCE. However, a very subtle shift in a 412-bp fragment (stCP250043) was more obvious when the primers were redesigned to reduce the fragment size to 320 bp (stCP250061). For both the ABI3100 and ABI3730, 93 of the 95 sequence variants were detected in the optimization set, giving a sensitivity of 98% (Table 1). The two sequence variants that were not detected were a G to A in a 398-bp amplimer (stCP250062) and G to A in a 350-bp STS (stCP250031). The GC content of these fragments was 46.5 and 42.3%, respectively.

Sequence variant detection in 215 Mb of DNA sequence

Following the optimization, 215 Mb of tumor DNA was screened for novel sequence variants. Approximately 80% of the data was generated on the ABI3100, with the remainder coming from the ABI3730. These data have been combined for further analysis as both platforms gave identical sensitivities. A total of 12,687 different STSs were analyzed in one or more

Table 1
Base substitutions in the CSCE optimization set

Substitution	Total	Detected	Missed
C/T G/A	70	68	2
A/C T/G	14	14	0
C/G G/C	4	4	0
T/A A/T	7	7	0
Total	95	93	2

Note. Distribution of single nucleotide variants according to the four nucleotide substitution classes for the 95 SNPs used in the CSCE optimization set. The number of SNPs detected and missed by CSCE for each of the substitution types is shown.

samples from a set of 272 DNAs. Sequencing 6170 putative variants suggested by CSCE identified 4369 true variants, with 1801 sequences being wildtype (giving a false-positive rate of 29.2%). A total of 3397 unique variants were identified in 1711 STSs, giving an average of one variant per 1300 bp of DNA screened.

In this data set, only those samples with putative mutations were sequenced. It is possible to compare the general characteristics of the STSs and the variants that were detected but not directly compare CSCE and DNA sequencing. The GC content of the DNA sequence in the STSs that did not yield any variants (range 20–76%, average 47.40%, SD = 10.90) was similar to the GC content of STSs in which variants were found (range 24–71%, average 47.75%, SD = 10.60) and the complete STS set (range 20–76%, average 47.44%, SD = 10.83). A subset of 1131 single-basepair substitutions (237 detected on the ABI3730 and 894 detected on the ABI3100) from 794 STSs were used for a more in-depth analysis of mutation detection features of CSCE. In this set, 129 variants were shown to be somatic mutations by sequencing matched normal DNA, whereas 1002 were germ-line variants. Of the germ-line variants, 612 were in the portion of the STSs that covered the coding sequence. Table 2 is a comparison of the distribution of the germ-line coding sequence variants detected by CSCE with the distribution of previously published genome-wide coding sequence variants.

The heteroduplex method exhibited a greater empirical sensitivity toward the central portion of the STSs. To estimate this sensitivity, a probability of mutation detection was calculated as a function of the percentage distance of a mutation from the nearest end of the STS in question. This was modeled as a cumulative beta distribution (see Materials and methods). A buffer at each end of the STS can then be designed to ensure the greatest sensitivity across the region of interest. As seen in Fig. 1, designing buffers of 22% at each end resulted in an estimated detection rate in excess of 95% between the buffers.

CSCE trace analysis

The CSCE method has the ability to produce approximately 10,000 traces per ABI3730 per day. To prioritize traces for further analysis, a trace comparison program, canplot, was developed to identify samples with putative sequence variants
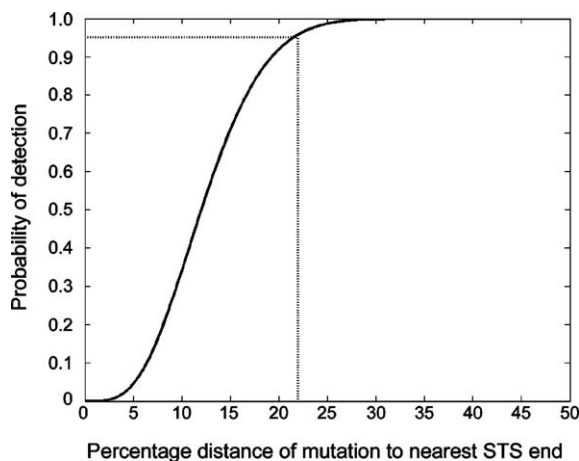

Fig. 1. Estimated probability of mutation detection. Buffers of 22% at each end of the STS provide an estimated mutation detection rate of at least 95% within the buffers.

by comparing traces from a reference and test sample. Furthermore, canplot interacted with our laboratory information management system to enable the seamless tracking of data. A visual examination of the trace data from the initial optimization experiments suggested that samples with the same sequence had very similar trace profiles, whereas those with a sequence variant had either additional peaks or one or more shoulders on the primary peak. Canplot used two tests, the presence of additional peaks above the background and the presence of shoulders in the primary peak, to prioritize traces for visual inspection. Ultimately all traces were examined to minimize the loss of data. Canplot also aided in the inspection of the data by clipping the trace to present only the region with the heteroduplex peaks and rescaling the control and test peaks to simplify visual comparison (Fig. 2). In an analysis of 808,618 tumor traces, canplot suggested that 159,062 (20%) were likely to contain sequence variants. After human review, 31,985 of these traces were considered sufficiently different to warrant sequencing. Human review identified a further 7028 traces for sequencing in the 649,556 traces that canplot called normal. Therefore, this set of 808,618 CSCE experiments led to 39,013 sequencing experiments, representing a 95% reduction in the sequencing load. Canplot successfully prioritized 80% of the trace data containing 82% of the samples that were sent for sequencing.

Discussion

The reference human genome sequence and its annotation provide many more candidate genes and other sequence features than ever before for the study of genetic diseases. Screening candidate sequences for novel variants and mutations requires methods that are fast, sensitive, specific, and economical. There are a number of emerging sequencing technologies that may satisfy all of these criteria at some point in the future [17]. In the meantime, Sanger dideoxysequencing is the final step in determining novel variants. An alternative strategy is to adopt a rapid screening approach followed by direct sequencing of

Table 2
Comparison of single nucleotide variants in the coding regions of the human genome and germ-line coding variants detected by CSCE

Substitution	Variants in the coding genome (%)	Variants found by CSCE (%)
C/T G/A	42,448 (70.1)	442 (72.2)
A/C T/G	9188 (15.2)	104 (17.0)
C/G G/C	6292 (10.4)	47 (7.7)
T/A A/T	2660 (4.4)	19 (3.1)

Note. The distribution of single nucleotide variants in the four nucleotide substitution classes for the coding region of the human genome as a whole (from Ensembl, http://www.ensembl.org) and those germ-line coding variants detected using CSCE.
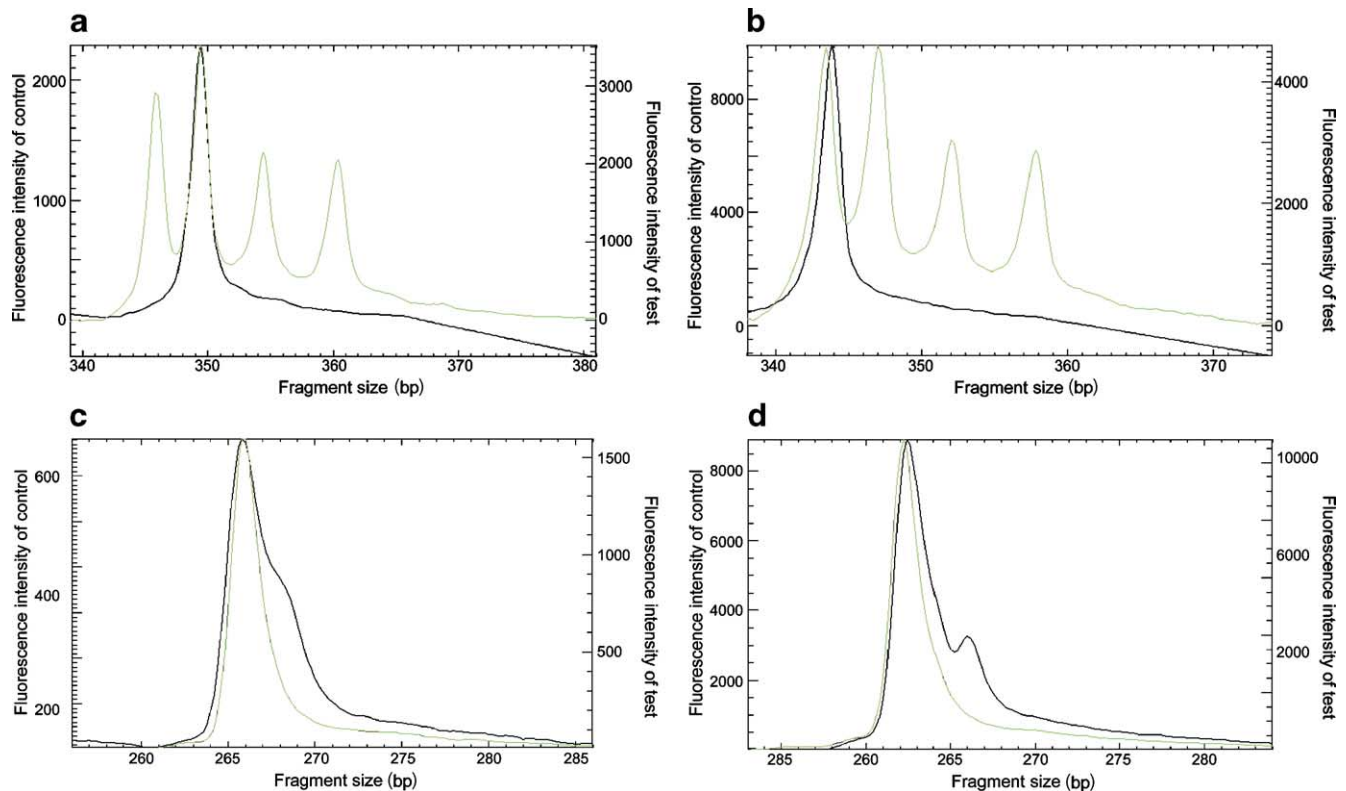
Fig. 2. CSCE traces as presented by canplot software. The control trace is shown in black and the test trace in green. The scale on the left-hand side indicates the intensity of the control trace and the test is on the right-hand side. (a) Example of an A to G substitution 126 bp from the end of a 331-bp fragment analyzed on an ABI3100. This fragment resolves into four peaks representing both homoduplexes and both species of heteroduplex. (b) The same 331-bp fragment analyzed on an ABI3730. (c) A 265-bp STS with a C to T substitution 86 bp from the end. (d) The same 265-bp fragment as in (c) analyzed on an ABI3730.

putative variants. The heteroduplex process, in various forms, is one of the most widely used rapid screening methods.

The initial optimization of the sensitivity of this protocol was performed using a direct comparison to DNA sequencing. This is in contrast to other reports that compare capillary heteroduplex methods with results obtained from other prescreening techniques such as CSGE [15]. The optimized protocol detected 93 of the 95 SNPs in the original test set. There were no obvious explanations for the 2 SNPs that were not detected in terms of SNP position, STS length, or GC content. Previous experience indicated that insertions and deletions from 1 to 25 bp always resulted in additional peaks in heteroduplex analysis. Therefore, these were not considered during optimization.

It was determined that 48.4% of the 215 Mb of tumor DNA screened was coding sequence. The distribution of single-nucleotide variants discovered in this set matches the known distribution of variants across the whole coding human genome (Table 2). This suggests that there were no major biases in the detection ability of CSCE. Mutation detection sensitivity by heteroduplex methods is thought to decrease in the last 50 bp of the fragment [7] and was the basis of our original design parameters. However, the analysis of the variants detected in the 215 Mb of sequence indicates that the buffer size should be larger, with 95% of variants detected with a 22% buffer, i.e., an 88-bp buffer for a 400-bp amplimer. Previous data suggested that very high or low GC content reduces the sensitivity of heteroduplex methods [7]. CSCE detected sequence variants in

STSs with GC content ranging from 24 to 71%. This suggests that the GC content of the human coding sequence (as determined by cDNA GC content of 30 to 70% with maxima at 42 and 58% [18]) will not hinder CSCE.

CSCE analysis has a specificity of 70.8% where 4369 of 6170 putative variants were confirmed by DNA sequencing. This is a conservative value, as the objective was to find as many variants as possible and hence sequence weak candidates as well as those with a high probability of being mutant. Unlike some methods, for example, DHPLC [6], CSCE does not require either a precalculation of run conditions or an empirical determination using known variants. Hence, a single set of run parameters is used for all fragments. Based on 400-bp fragments and buffers of 22% at each end for maximum mutation detection, up to 2.2 Mb can be screened per day on an ABI3730 or 649 kb per day on an ABI3100. One ABI3730 has the potential to create over 10,000 trace files per day. Canplot was written to identify those samples that are most likely to contain variants. This increased the throughput of CSCE analysis and assisted in the prioritization of experiments.

The discovery of novel DNA variants is continuing to play an important role in our understanding of human disease. CSCE is a sensitive method that allows the swift identification of variant genes in disease, for example, BRAF in cancer [19]. Until it is possible to completely resequence whole genomes, it is likely that CSCE coupled with DNA sequencing will continue to expedite these discoveries.

Materials and methods

PCR

Primers were designed to amplify the coding exons of interest plus 50 bp of intronic sequence at either end of the exon. The maximum PCR fragment size was 400 bp with overlapping STSs for larger exons (minimum of 100-bp overlap). One of the primers in each pair was labeled with FAM, VIC, or NED fluorescent dye (Applied Biosystems). Primer sequences for the initial optimization set of 45 STSs are available from http://www.sanger.ac.uk/genetics/CGP/.

Fifteen-microliter PCRs were performed using the following: 20 ng each primer, 100 μM each dNTP, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 0.3 units Abgene Thermostart *Taq*, 12 ng test DNA, 3 ng control DNA. PCR was carried out in a DT-108 Super Duncan water bath thermocycler (KBiosystems) as follows: 95°C for 15 min, 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 10 min. An additional denaturation step followed by slow cooling was used to maximize the formation of heteroduplexes. This involved heating to 96°C for 10 min followed by a slow cooling of 1.5°C per minute to 60°C; samples were held at 60°C for a further 30 min.

Capillary electrophoresis

PCR fragments were diluted in 1× PCR buffer in the range of 0 to 1/10 (the dilution was STS specific and was determined by testing two samples). The diluted fragments were pooled (one each of FAM, VIC, and NED) and diluted 1/30 in water to give a final dilution in the range 1/30 to 1/300 of the primary PCR. Prior to loading on the ABI3100 or ABI3730, 2.5 μl of the pooled and diluted PCR product was added to 7.5 μl of water and 0.1 μl GeneScan 500 LIZ size standard (Applied Biosystems Catalogue No. 4322682).

For the ABI3100, the run conditions were as follows: 16 capillaries, 36-cm array, and standard GeneScan36_POP4 module with modifications including a run temperature of 18°C, a run time of 900 s, sample injection of 15 kV for 5 s, dye set G5. The separation medium used contains 5% CAP polymer (Applied Biosystems Catalogue No. 4340379), 4 M urea, 1× TTE (90 mM Tris, pH 9.0, 8.5 mM taurine, 0.5 mM EDTA, National Diagnostics EC-871). For the ABI3730, the run conditions were as follows: 36-cm 96-capillary array and standard GeneScan36_POP7 module with modifications including a run temperature of 18°C, a run time of 720 s, a data delay time of 750 s, sample injection of 15 kV for 10 s, dye set G5. The separation medium was 5% CAP polymer, 4 M urea, 7.5% sucrose, 1× TTE. The running buffer for both methods is 1× TTE and both separation media are stable for at least 2 weeks at 4°C. Data collection software for the ABI3100 and ABI3730 was 3100 Data Collection Software Version 1.0.1 and 3730XL DNA Analyser Data Collection Software Version 1.0, respectively.

Refractive indexes were measured on an Abbe 60 refractometer according to the manufacturer's instructions.

Trace analysis

The trace files from the ABI3100 or ABI3730 were analyzed using proprietary software written in IDL (available from the authors upon request). The sensitivity and specificity parameters for canplot were optimized using the initial set of 45 STSs. Prior to any manipulation, all data channels were smoothed using a 5-point smoothing process. The LIZ-labeled size standard was used to perform a conversion of the x-axis scan index (which is time-like) to fragment size in basepairs using a cubic spline interpolation, followed by a linear re-interpolation onto a regular fragment-size axis scale. Next, all peaks within a data channel were located using a peak model that stipulated that the peak maximum must have positive gradients on the left-hand side and negative gradients on the right-hand side. The peak with maximum intensity was used to define a primary peak, whereas secondary peaks within 400 scan indices of the primary peak were also assigned to the marker-specific signal. The peak-finding process was applied to tumor and normal traces and the two were scaled to one another using the intensities of the primary peaks. If the number of clearly resolved peaks present in the normal and tumor traces were different, the tumor was prioritized for review. If the numbers of peaks were identical, a peak shoulder comparison algorithm was used to assess the symmetry characteristics of the tumor and normal signals. If the extent of any shoulder mismatch was greater than a critical width of 0.5 bp, the trace was prioritized for review. All other traces were given a low priority for review.

DNA sequencing

Samples with putative mutations were re-amplified using unlabeled PCR primers of the same sequence as the original CSCE primers. The PCR products were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's protocol and analyzed on an ABI3100 or ABI3730.

Statistical methods

To quantify the observed decline of the heteroduplex method's ability to detect mutations toward the ends of STSs, a model was fitted as described below.

x denotes the percentage distance of a mutation to the nearest end of the STS containing the mutation. D denotes the event that the mutation is detected. $p(D|x)$ denotes the probability that the heteroduplex method will detect the mutation, given position x. It is assumed that this probability takes the form of a (rescaled) cumulative distribution of a beta random variable,

$$p(D|x) = \int_0^{x/50} \frac{C(a+b)}{C(a)C(b)} z^{a-1}(1-z)^{b-1} dz.$$

This is a two-parameter distribution, the values α and β of which are fitted from the observed mutation positions. These observations derive from the distribution of mutations, given detection, $p(D|x)$. These two distributions are connected via Bayes law, where

$$p(x|D) \propto p(D|x)p(x).$$

Assuming the distribution of mutations $p(x)$ is uniform gives, upon normalization,

$$p(x|D) = \frac{p(D|x)}{\int_0^{50} p(D|x) dx}.$$

The parameters α and β can then be estimated from the data by standard fitting methods. The method of moments was used to fit the curve in Fig. 1, based on 1131 observed mutations.

Acknowledgment

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ygeno.2005.11.008.

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