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Enrichment of meiotic recombination hotspot sequences by avidin capture technology²

Daniel Camara Teixeira¹, Sridhar A. Malkaram^{1,3}, and Janos Zempleni¹

¹Department of Nutrition and Health Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583-0806

Abstract

About 40% of the hotspots for meiotic recombination contain the degenerate consensus sequence 5'-CCNCCNTNNCCNC-3'. Here we present a novel protocol for enriching hotspot sequences from digested genomic DNA by using biotinylated oligonucleotides and streptavidin-coated magnetic beads. The captured hotspots can be released by simple digestion with restriction enzymes for subsequent characterization by second generation sequencing or PCR. The capture protocol specifically enriches hotspot sequences, judged by using fluorophore-conjugated synthetic oligonucleotides and synthetic double-stranded oligonucleotides in combination with PCR. The capture protocol enriches single stranded DNA, denatured double-stranded DNA, and large fragments (>3,000 bp) of digested plasmid DNA with good efficacy. No false positive and false negatives were detected when enriching digested DNA from human cell cultures and primary human cells. The protocol can probably be adapted to enriching sequences other than the hotspot sequence by altering the sequence in the capture oligonucleotide. We intend to apply this protocol in studies assessing effects of micronutrient status on meiotic recombination events in human sperm.

Keywords

DNA; isolation; meiosis; recombination

1. Introduction

In mammalian meiosis, maternal and paternal homologous chromosomes assort randomly to form haploid gametes, i.e., sperm and oocytes (Cheung et al., 2010). Parts of homologous chromosomes are exchanged in recombination events, thereby creating genetic variation and diversity. A minimum number of recombinations is needed for meiosis to proceed, and an abnormally decreased frequency of these events is a leading cause of infertility,

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Correspondence to: Janos Zempleni.

¹To whom correspondence should be addressed. Phone: (402) 472 3270 / Fax: (402) 472 1587 / jzempleni2@unl.edu. Postal address: Department of Nutrition and Health Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583-0806.

³Present address: Department of Biology, West Virginia University, Institute, WV 25112-1000. Author disclosures: D. Camara Teixeira, S.A. Malkaram and J. Zempleni, no conflicts of interest.

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miscarriages, and developmental disabilities (Lynn et al., 2002; Martini et al., 2006; Cheung et al., 2010). Aneuploidy can result if recombination occurs too close to a telomere or centromere, leading to abnormal chromosome separation. Therefore, successful reproduction and the health of the offspring depend on the appropriate number of recombinations to occur in the appropriate genomic regions.

Both DNA sequence and epigenetic marks are implicated in the regulation of recombination hotspots. In support of this theory, a degenerate sequence of 13 basepairs (CCNCCNTNNCCNC) can be found in ~40% of human recombination hotspots (Myers et al., 2008); a series of recent high-profile papers revealed a role for the histone H3 lysine-4 (K4) methyltransferase Prdm9 in promoting crossover during meiosis through creating H3K4me3 marks (Baudat et al., 2010; Cheung et al., 2010; Myers et al., 2010; Parvanov et al., 2010). These observations, along with work in our laboratory, link cell nutrition with the regulation of meiosis. Specifically, we have demonstrated that the repression of long terminal repeats (LTRs) depends on a sufficient supply with the micronutrient biotin and a normal activity of the histone biotin ligase holocarboxylase synthetase (HLCS) (Chew et al., 2008; Pestinger et al., 2011; Rios-Avila et al., 2012). Apparently, these effects are mediated by physical interactions between HLCS and the eukaryotic histone methyl transferase EHMT1, which catalyzes the methylation of K9 in histone H3 to create abundant H3K9me gene repression marks (Li et al., submitted). The interactions between HLCS and EHMT1 are strengthened by HLCS-dependent biotinylation of K161 in EHMT1. Both biotin depletion and HLCS knockdown decrease biotinylation marks and H3K9me marks to a similar extent, and cause severe phenotypes such as short life span (Landenberger et al., 2004), low fertility (Landenberger et al., 2004; Camporeale et al., 2006), and low resistance to heat stress in *Drosophila melanogaster* (Camporeale et al., 2006), and de-repression of LTRs in humans, mammalian cell lines, and in *Drosophila* (Chew et al., 2008; Pestinger et al., 2011; Rios-Avila et al., 2012). Importantly, de-repression of LTRs provides a mechanistic rationale for linking biotin deficiency with aberrant meiosis, because the majority of mammalian LTRs contain the 13-bp consensus motif located in hotspots for meiotic recombination (Myers et al., 2008). This theory is also consistent with our observation that biotin deficiency lowers fertility in *Drosophila* (Landenberger et al., 2004).

Here, we sought to develop a protocol for enriching DNA sequences that contain the hotspot 13-mer for subsequent identification of recombination events. This protocol is based on capturing 13-mer sequences by using avidin chromatography (Fig. 1). We envision applying this protocol in future studies aimed at detecting subtle changes in meiotic recombination events in gametes deficient of micronutrients such as methyl donors and biotin.

2. Materials and methods

2.1. Principle of the capture assay

Recombination hotspot sequences were enriched by using a combination of biotinylated synthetic oligonucleotides, streptavidin-coated magnetic beads, and a magnetic particle sorter (Fig. 2). Briefly, an oligonucleotide was biotinylated at its 5' end during chemical synthesis (denoted "oligo 1"); all oligonucleotides in this paper were synthesized by IDT DNA, Inc. The base pairs in oligo 1 were complementary to the sequence in the 3'-terminal half of second synthetic oligonucleotide ("oligo 2"), which also had a sequence complementary to the degenerate hotspot motif extending from its 5'-end for capturing sequences containing 5'-CCNCCNTNNCCNC-3'. Equimolar concentrations of oligos 1 and 2 were prepared in 10 mM Tris-HCl (pH 7.5), containing 1 mM EDTA and 50 mM NaCl ("annealing buffer"). Oligos were denatured by heating at 95°C for 5 min in a thermocycler, followed by annealing at 22°C for 1 h. The annealed oligos have an *EcoRI* restriction site (Fig. 2). Importantly, *EcoRI* does not cut the 13-mer recombination motif. The oligo 1/2

dimer may be stored at 4°C, and all subsequent steps were carried out at 4°C. The dimer is immobilized using streptavidin-coated magnetic beads (Dynabeads m-280, Invitrogen). Digested DNA containing the 13-mer consensus sequence was captured by hybridization with the 5'-end extension in oligo 2, and the captured sequences were released by digestion with *EcoRI*.

2.1 Capture of a fluorescein-labeled oligonucleotides

A fluorophore-conjugated oligonucleotide was used to optimize the protocol and to demonstrate feasibility. A degenerate oligonucleotide containing the 13-mer consensus sequence (5'-CCNCCNTNNCCNC-3') was labeled at its 3'-prime with Fluorescein 6-FAM™ during oligonucleotide synthesis (5'-GCG TAC CNC CNT NNC CNC ATA CGC-3'-FAM, denoted "oligo-3-FAM"). Nine microliters of a 1 micromolar solution of oligo-3-FAM were mixed with 22 µL of a mixture of oligos 1 and 2 (10 µM each) to ensure a molar excess of the capture sequence. The oligonucleotides in the mixture were denatured, annealed, and chilled as described above. Thirty 30 µL of Dynabeads in their original suspension were pre-washed three times with annealing buffer and re-suspended in 30 µL of annealing buffer (4°C). The suspension was mixed with the solution containing the three oligonucleotides. Note that the biotin-binding capacity of Dynabeads was titrated in preliminary studies to ensure that the binding capacity exceeded the mass of biotin in oligo 1 by using a TEX 615-labeled oligo 2 as tracer, in the absence of oligo-3-FAM (data not shown). The suspension was incubated at 4°C for 2 h with gentle rotation. The beads were collected at the bottom of the tube by using the magnetic particle sorter (DiaMag02, Diagenode, Denville, NJ) and the supernatant was collected using a micropipette. Beads were washed three times with 180 µL of annealing buffer (4°C) after removing the tube from the sorter. Each time, the tube was re-inserted into the sorter for collection of the supernatant. Next, beads were suspended in 20 µL of 1x FastDigest® buffer (37 °C) and digested with 2 µL of *EcoRI* (Fermentas) to release oligo-3-FAM through cleaving the *EcoRI* site in oligo1/2; the supernatant was collected. Beads were washed three more times with 180 µL of annealing buffer (4°C) and supernatants were collected. The fluorescence of supernatants and beads before and after treatment were quantified in a 96-well plate using a FLUORstar OPTIMA fluorescence plate reader (emission = 485 nm, excitation = 520 nm). In control experiments, the non-specific binding of oligo-3-FAM to Dynabeads was monitored in the absence of oligos 1 and 2. All samples were protected against exposure to light.

2.3 Enrichment of 13-mer sequences from a mixture of double stranded (ds)DNA fragments

The capture protocol was combined with semiquantitative PCR to assess whether the capture protocol can enrich the 13-mer sequence from a mixture of dsDNA. Briefly, a synthetic 100-mer containing the 13-mer hotspot sequence (5'-AAT TCA ATT TGA AAC TTG TGG TAG ATA TTT TAC TAA CCA ACT CTG CCN CCN TNN CCN CTC ACC AAA TTG TTC TTT TAA CCG CAT TCT TTC CTT GCT TTC G-3' forward strand; hotspot sequence underlined) and its complement was used as a target for enrichment, and a 50-mer not containing the hotspot sequence (5'-TAT TAT TAT TAT TAT TAT TAG ATA TTT TAC TAG GCG GCG GCG GCG GCG GCG-3') and its complement was used as control. After annealing the 100-mer oligonucleotides and the 50-mer oligonucleotides in separate reactions, equimolar concentrations of the two double-stranded sequences were subjected to separation by Dynabeads as described above. Fractions were collected and amplified by PCR (13 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 90 s), using an equimolar mixture of the following primers in a 96-well plate PCR format: 5'-AAT TCA ATT TGA AAC TTG TGG TAG-3' (forward) and 5'-CGA AAG CAA GGA AAG AAT GCG GTT-3' (reverse) for amplifying the 100-mer, and 5'-TAT TAT TAT TAT TAT

TAT-3' (forward) and 5'-CGC CGC CGC CGC CGC CGC-3' (reverse) for amplifying the 50-mer. The primers were designed to amplify the entire length of the 100-mer and the 50-mer. PCR products were run on a 2% agarose gel and visualized using ethidium bromide (Wiedmann et al., 2003).

In a control experiment, the hotspot sequence was moved from the 100-mer to the 50-mer to formally exclude amplicon size as a confounder. The 50-mer double-strand that contained the 13-mer hotspot sequence had the sequence 5'-TTT TAC TAA CCA ACT CTG CCN CCN TNN CCN CTC ACC AAA TTG TTC TTT TA-3' (forward strand; hotspot sequence underlined), and the 100-mer that did not contain the hotspot sequence had the sequence 5'-ATT TTT TAA CCA ATA GGC CGA AAT CGG CAA AAT CCC TTA TAA ATC AAA AGA ATA GAC CGA GAT AGG GTT GAG TGT TGT TCC AGT TTG GAA CAA GAG TCC A-3' (forward strand). The following PCR primers were used in this control experiment: 5'-TTT TAC TAA CCA ACT CTG C-3' (forward) and 5'-TAA AAG AAC AAT TTG GTG AG-3' (reverse) for amplification of the 50-mer, and 5'-ATT TTT TAA CCA ATA GGC CG-3' (forward) and 5'-TGG ACT CTT GTT CCA AAC-3' (reverse) for amplification of the 100-mer.

2.4 Capture of long dsDNA containing the 13-mer hotspot sequence

The enrichment of human DNA containing the 13-mer hotspot sequence will begin with the digestion of samples with *EcoRI*, which does not cut into the 13-mer sequence. We conducted an *in silico* digestion to predict the number of fragments containing the 13-mer hotspot and the size distribution of the fragments. The digestion was conducted using the University of California, Santa Cruz build of the human reference genome (Genome Browser, 2009). The results of this analysis suggest that complete digestion of the human genome (22 autosomes plus 2 heterosomes) with *EcoRI* creates 289,757 fragments containing the hotspot sequence (Table 1). Seventy-five percent of these fragments are larger than 3 kb if the DNA is digested solely with *EcoRI*. Should one aim at creating smaller fragments, conducting a double-digestion with *EcoRI* and *AluI* is recommended, which will result in more than 99% of the fragments being < 3 kb (online supplementary Table 2). *AluI* does not cut into the hotspot sequence and does not alter the overall concept of the assay including the release of fragments from the beads by using *EcoRI* (Fig. 2). In this proof-of-principle study, digestions were limited to using *EcoRI* and fragments sized 50 to 3,000 bp.

Based on this observation, we considered it important to demonstrate that large fragments do not escape detection when analyzed by using avidin capture technology. Briefly, *pBluescript II sk* (+) vector (GenBank accession no. **X52328.1**) was digested with *EcoRI* to release a 2961-bp fragment. A 13-mer in the fragment (5'-AAG ACG ATA GTTA-3', bp 1511-1523 in the vector) was selected and a degenerate oligonucleotide (5'-TAN CTN TNN TCN T-3', denoted "oligo-Blue") was synthesized with a sequence complementary to the Bluescript 13-mer. Note that oligo-Blue has the same degree of degeneration as oligo 2 in the regular avidin capture protocol, and that the degenerate nucleotides in oligo-Blue are in the same positions as they are in oligo 2. An avidin capture assay was conducted as described above, but oligo-Blue was substituted for oligo 2. Semiquantitative PCR (24 cycles of 94°C for 30 s, 52°C for 30s, and 72°C for 3.5 min) was conducted to amplify the entire 3-kB fragment in all sample fractions, using primers 5'-TAT CAA GCT TAT CGA TAC CGT CGA-3' (forward) and 5'-AGC CCG GGG GAT CCA CTA GTT CTA-3' (reverse).

2.5 Enrichment of 13-mer hotspot sequences from human cells

HEK293 human embryonic kidney cells, MCF-7 human mammary carcinoma cells, and IMR90 primary human fibroblasts were obtained from the American Type Culture

Collection (Manassas, VA) and cultured following the vendor's recommendations. Genomic DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA), and 2 μ g of genomic DNA extracted from each cell lineage was digested with high concentration (HC) *EcoRI* (Promega, Madison, WI) according to manufacturer's instructions. Two hundred-fifty nanograms of digested DNA, dissolved in 2.5 μ L of the digested aliquot, were mixed with 50 μ L of a solution of oligos 1 and 2 (10 μ M each); samples were denatured (95°C, 5 min) and annealed (22°C, 1 h). Sample enrichment was conducted as described above, using 60 μ L of pre-washed streptavidin beads. We conducted an *in silico* analysis to identify six human DNA sequences of 500 bp that are flanked by *EcoRI* sites; three of these fragments contained the 13-mer hotspot sequence and the three other sequences did not contain the sequence (online supplementary Table 1). Nested PCR reactions were conducted using primers F1 and R1 for the first reaction (25 cycles) and primers F2 and R2 with μ L of product from the first reaction as a template for the second reaction (27 cycles). Amplicons were visualized using agarose gel electrophoresis as described above.

3. Results and discussion

3.1 Fluorescein labeled oligonucleotide capture

Oligonucleotides containing the hotspot sequence are retained by the streptavidin beads and released by treatment with *EcoRI*. Oligo-3-FAM was mixed with streptavidin-immobilized oligos 1 and 2, and beads were subjected to sequential washes. The fluorescence signal in the supernatant after loading the capture oligonucleotides was ~1,000 units (sample 1, Fig. 3) and decreased to ~200 units in subsequent washes prior to treatment with *EcoRI* (samples 2 – 4). The fluorescence signal increased by ~20 fold when beads were treated with *EcoRI* (sample 5), and returned to baseline levels during subsequent washing steps (samples 6 – 8). Sample 9 depicts the fluorescence of streptavidin beads after the final wash. We speculate that the protocol can be modified to capture any sequence of interest by adjusting the capture sequence in oligo 2. We also propose that the temperature stability of the complex can be increased by increasing the length of oligos 1 and 2. For our particular case, we would not have benefitted from using longer capture oligos, because the hotspot motif with its length of 13 bp was the limiting factor with regard to denaturing the complex by heat.

3.2 Synthetic dsDNA capture followed by PCR amplification

The ultimate goal of this assay is to enrich 13-mer sequences from digested DNA in which only some fragments will contain the 13-mer recombination hotspot sequence. Digested DNA is a more challenging sample matrix than the oligo-3-FAM based on the following unique properties of DNA compared with an oligonucleotide. First, DNA is double-stranded (whereas oligo-3-FAM is single-stranded), i.e., self-annealing competes with the binding to the capture sequence in oligo 2. Second, nucleotides will extend from the 5'- and the 3'-end in DNA digests; these nucleotides will not bind to the capture sequence in oligo 2 and may cause sterical hindrance with regard to binding. Third, the 13-mer hotspot can be found in only about half the fragments in digested DNA whereas 100% of oligo-3-FAM is complementary to oligo 2, i.e., there is competition in the context of non-specific binding of DNA fragments.

The capture protocol enriches dsDNA containing the hotspot sequence from a mixture of dsDNA. In a first experiment, a 100-mer of dsDNA fragment containing the hotspot sequence was mixed with a 50-mer not containing the sequence and samples were processed using the capture protocol (Fig. 4A). The number of washing steps prior to treatment with *EcoRI* was increased to nine washes in order to minimize the background signal produced by sensitive PCR amplification of samples. A signal was detectable for both 100-mer and 50-mer in the first wash (Fig. 4B, fraction 1); the signal decreased to background levels for

the 100-mer after about 3 washes while no signal was detectable for the 50-mer starting with wash fraction 2. Treatment with *EcoRI* after the ninth wash produced a strong signal for the 100-mer in fraction 10, but produced no signal for the 50-mer. These observations can be interpreted as follows. First, the capture oligos lose some of the bound material despite taking great care during sample preparation, judged by the small amounts of the 100-mer detectable in fractions 2 and 3. Alternatively, the capture oligos might have been saturated with hotspot sequences in the 100-mer. Second, dsDNA not containing hotspot sequences does not bind to the capture oligos, judged by the absence of a 50-mer signal in fractions 2 – 10. Third, detectable amounts of hotspot sequences are released by treatment with *EcoRI*, judged by the 100-mer signal in fraction 10. Collectively, the capture protocol enriches hotspot sequences from a mixture of dsDNA. These observations are not artifacts caused by the distinct sizes of oligos. When the hotspot sequences was moved from a 100-mer to a 50-mer, the protocol enriched the 50-mer (Fig. 4A and 4B).

The capture protocol efficiently enriches large fragments of DNA, judged by the enrichment of a 2,961-bp fragment released by digestion of *pBluescript II sk (+)* vector (Fig. 5A). When the digested vector was subjected to enrichment by using the oligo-Blue capture oligonucleotide, the fragment bound to the beads and was released by treatment with *EcoRI* (Fig. 5B). Specifically, the fragment was detectable in the bead supernatant after loading (fraction 1) and was not detectable in subsequent washing steps (fractions 2 – 9); treatment of beads with *EcoRI* caused a release of detectable amounts of the fragment (fraction 10). Enrichment of large fragments by the capture protocol is an important feature of this protocol, because ~99% of human DNA containing the hotspot sequence 5'-CCN CCN TNN CCN C-3' and double digested with *AluI* and *EcoRI* will be 100 – 3,000 bp in size.

3.3 Human DNA capture followed by PCR amplification

The capture protocol enriches DNA containing the hotspot sequence from a complex mixture of DNA fragments generated by digestion of human DNA with *EcoRI*. DNA from human MCF-7 cells was digested to release approximately hotspot-containing 300,000 fragments per set of autosomes and the two heterosomes. Using *in silico* protocols, we selected three fragments not containing the hotspot sequence and three fragments containing the hotspot sequence. Fragments were chosen to be about 500 bp long, thereby representing the majority of *EcoRI* digestion products. When digested DNA was analyzed before enrichment by using the capture protocol, all six fragments produced strong signals in analysis by nested PCR (Fig. 6A, input control). After the digested DNA was submitted to enrichment using oligos 1 and 2 in the capture protocol, only fragments containing the hotspot consensus sequence were detectable (Fig. 6B). Note that the amplicon sizes were smaller than the size of the actual fragment, because of the choice of primers for the nested PCR. The numbers above the lanes in the gels correspond to the primers in Online Supplementary Table 1. The selective enrichment of fragments containing the hotspot motif is not cell line specific. Analysis of DNA from HEK293 cells produced the same results as those described for MCF-7 cells (Fig. 6C and 6D).

We formally excluded the remote possibility that aneuploidy in immortalized cells may produce results different than in normal primary cells. We analyzed digested DNA from primary human fibroblasts (IMR90 cells) using the capture protocol. As described for MCF-7 and HEK293 cells, DNA before enrichment produced a signal regardless whether the hotspot sequence was present in fragments (Fig. 6E) whereas DNA after enrichment produced a signal only for those fragments that contained the hotspot sequence (Fig. 6F).

We conclude that the capture protocol can be successfully applied to enriching degenerate, short sequences such as the 13-bp hotspot consensus sequence for meiotic recombination and possibly other genomic sequences. Enrichment of target sequences prior to sequencing

reduces the sample volume and costs by ~50%, at least in the case of the hotspots for recombination. We intend to apply this protocol in future studies assessing the effects of micronutrient status on meiotic recombination events in human sperm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

An avidin-based protocol for capturing meiotic recombination sequences is proposed.

This capture protocol reduces the cost of subsequent high-throughput sequencing.

The protocol can probably be adapted to capture any DNA sequence of interest.

Figure 1.
Flow chart of the avidin-based protocol for capturing recombination hot spots sequences.

Figure 2.

Principle of the capture protocol. The hybridization of oligos 1 and 2 creates an *EcoRI* restriction site and a 5' end overhang that is complementary to the sequence in the recombination hotspot 5'-CCN CCN TNN CCN C-3'. The annealed capture oligonucleotides are immobilized on streptavidin-magnetic coated beads through the biotin-conjugated 5' end in oligo 1.

Figure 3.

Capture and release of a fluorophore-conjugated oligonucleotide. Oligonucleotide oligo-3-FAM is labeled with fluorescein and contains the hotspot sequence for recombination. The numbers denote the sequential washes of streptavidin beads before and after treatment with *EcoRI*. Fraction 9 depicts the fluorescence in beads after the final wash (N=3; the image represents a representative example).

Figure 4.

Enrichment of a DNA containing the hotspot motif from a mixture of short, double-stranded DNA. (A) Synthetic dsDNA oligonucleotides: 100-mer containing the hotspot sequence for recombination; 50-mer not containing the hotspot sequence. (B) PCR-amplified samples of supernatants from streptavidin beads after loading with oligonucleotides (sample 1), sequential washes of beads (samples 2 – 9), and supernatant after treating beads with *EcoRI* (sample 10). (C) As described for “A” but now the 50-mer contains the hotspot sequence. (D) As described for “B.” Abbreviation: M, marker.

Figure 5.

Capture of long dsDNA with oligo-Blue. (A) *EcoRI* fragment of pBluescript II sk (+), containing a 13-mer with a degree of degeneration similar to the hotspot sequence. (B) PCR-amplified samples of supernatants from streptavidin beads after loading with oligonucleotides (sample 1), sequential washes of beads (samples 2 – 9), and supernatant after treating beads with *EcoRI* (sample 10). Abbreviation: M, marker.

Figure 6.

Enrichment of DNA for fragments containing the hotspot sequence in human cell lines. (A) Select fragments without (lanes 1 – 3) and with (lanes 4 – 6) the hotspot sequence were amplified by nested PCR before enrichment by capture protocol, using *EcoRI*-digested DNA from MCF-7 cells. (B) As described for “A” but after enrichment by using the capture protocol. (C) As described for “A” but using DNA from HEK293 cells. (D) As described for “B” but using DNA from HEK293 cells. (E) As described for “A” but using DNA from IMR90 fibroblasts. (F) As described for “B” but using DNA from IMR90 fibroblasts. Abbreviation: M, marker.

Table 1Fragment frequency of human DNA digested with *EcoRI*^{*}

Size of fragments (bp)	Number of fragments	Percentage (%)
0-100	51	0.02
0-1000	5955	2.06
0-3000	44122	15.23

^{*} Analysis based on 22 autosomes plus X and Y chromosomes. Only fragments containing the 13-mer CCNCCNTNNCCNC hotspot are shown.