

Bioinformatic Tools Identify Chromosome-Specific DNA Probes and Facilitate Risk Assessment by Detecting Aneusomies in Extra-embryonic Tissues

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Abstract: Despite their non-diseased nature, healthy human tissues may show a surprisingly large fraction of aneusomic or aneuploid cells. We have shown previously that hybridization of three to six non-isotopically labeled, chromosome-specific DNA probes reveals different proportions of aneuploid cells in individual compartments of the human placenta and the uterine wall. Using fluorescence in situ hybridization, we found that human invasive cytotrophoblasts isolated from anchoring villi or the uterine wall had gained individual chromosomes. Chromosome losses in placental or uterine tissues, on the other hand, were detected infrequently. A more thorough numerical analysis of all possible aneusomies occurring in these tissues and the investigation of their spatial as well as temporal distribution would further our understanding of the underlying biology, but it is hampered by the high cost of and limited access to DNA probes. Furthermore, multiplexing assays are difficult to set up with commercially available probes due to limited choices of probe labels. Many laboratories therefore attempt to develop their own DNA probe sets, often duplicating cloning and screening efforts underway elsewhere. In this review, we discuss the conventional approaches to the preparation of chromosome-specific DNA probes followed by a description of our approach using state-of-the-art bioinformatics and molecular biology tools for probe identification and manufacture. Novel probes that target gonosomes as well as two autosomes are presented as examples of rapid and inexpensive preparation of highly specific DNA probes for applications in placenta research and perinatal diagnostics.

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INTRODUCTION

The human placenta is a vital organ anchoring the fetus to the mother via the uterus and providing an interface for the transport of nutrients, gases and waste. The overwhelming number of chromosomal studies of the placenta has been performed on cells biopsied from floating villi, which were cultured for several days to obtain metaphase spreads for conventional chromosome banding analysis. We decided to perform investigations on uncultured interphase cells using fluorescence in situ hybridization (FISH), since cell viability or proliferation are minor concerns when using FISH [1-5]. Probes for our initial studies of aneuploidy in extra-embryonic tissues were obtained from a commercial source (Abbott, Des Moines, IL) [6, 7]. Probe sets were comprised of three to four chromosome enumerator probes (CEPs) targeting chromosome types, X, Y, 16 or 18, or locus-specific probes (LSPs) for chromosome 13 or 21 [7]. Studying the chromosomal make-up of cells in different compartments of

anchoring villi and the uterine wall also referred to as 'basal plate', we found that the karyotypes of these extra-embryonic cells were mostly unrelated to the karyotype of the fetus [5, 7, 8]. The most common abnormality we have observed was a gestational age-related gain of chromosomes affecting invading cytotrophoblasts (iCTB's) [7]. For a more comprehensive analysis and to be able to increase the number of chromosome types that can be scored simultaneously in a single FISH experiment, we had to develop our own custom sets of chromosome-specific DNA probes.

While the DNA probe development efforts described in the present communication were prompted by the need to develop a novel probe set for more comprehensive cytogenetic analyses of normal placental tissue compartments from uncomplicated pregnancies [6], DNA probes selected in a similar fashion are likely to find widespread application in investigations of unusual conditions such as spontaneous abortions [9, 10] or confined placental mosaicism (CPM) [11-14], the cytogenetic analysis of human preimplantation embryos [15-21], perinatal analysis [22-24], tumor research and diagnosis [1-5, 25-27] as well as radiobiological or environmental studies [28-40]. Thus, the description of our probe selection approach combining bioinformatics tools for data mining of genomic databases with deeply redundant recom-

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binant DNA clone libraries, which follows the brief review of the more conventional techniques for DNA probe selection, may provide useful information for a diverse group of researchers in the life sciences and enable the average research lab to prepare chromosome-specific custom DNA probes at a very affordable cost.

Selection of DNA Target Sequences and Preparation of Non-Isotopically Labeled DNA Probes for FISH

Briefly, successful cytogenetic analysis by FISH is based on the formation of stable hybrids between the DNA targets inside cell nuclei or metaphase chromosomes and the labeled DNA probes molecules provided by the investigator [41]. The DNA probes can either be marked by a fluorochrome, which can then be detected by eye or a camera attached to a fluorescence microscope, or by a non-fluorescent, non-isotopical hapten, most often biotin, digoxigenin or dinitrophenol, which is detected by a fluorescent moiety such as a fluorochrome-labeled avidin or antibody. Different probe types are available to suit particular applications: whole chromosome painting probes allow the delineation of inter-chromosomal translocations in metaphase spreads [37, 42, 43], while intra-chromosomal rearrangements are detected in metaphase or interphase cells with chromosome band-specific probes [44-47]. In addition, there are DNA probes that target somewhat smaller, gene- or locus-specific regions [34, 48-52].

While the FISH technology found widespread application in research laboratories around the world, its acceptance in clinical settings is still hampered by a limited selection of commercially available, U.S. Food and Drug Administration (FDA)-approved tests and the typically labor-intensive, costly nature of producing DNA probes that perform well in multiplexed assays [53]. While FDA approval may be required for all diagnostic probes that are shipped across state borders in the U.S., the in-house preparation of DNA probes might lead to significant cost savings in research laboratories. Our laboratories have a long-standing track record of production of novel DNA probes and innovative cytogenetic assays, many of which have found their way into contemporary cancer research or preimplantation genetic diagnosis (PGD) analysis [16, 43, 45, 47, 48, 50, 54-60]. To facilitate the distribution of molecular cytogenetic assays and make DNA probes as well as multiplex FISH tests available to the less experienced laboratory, we have undertaken probe production pilot studies which take advantage of the vast resources generated in the course of the Human Genome Project such as physical maps and recombinant DNA libraries.

Our initial studies focused on the preparation of novel DNA probes for chromosome scoring or 'enumeration' in interphase cell nuclei and metaphase spreads, since these seem to remain the most common applications in research and the clinical settings [53, 61]. The vast majority of these CEPs target highly reiterated, tandemly-repeated DNA sequences in order to bind many copies of a rather small probe sequence to a tightly localized area or volume. Different ways of isolating and purifying such DNA probes exist [25, 54, 59, 60, 62-66].

Briefly, up until the 1980's, satellite DNA sequences were enriched, isolated and characterized by a cumbersome, labor-intensive workflow which involved either density gra-

dient centrifugation or timed reassociation of single stranded, thermally denatured DNA followed by enzymatic digestion of single stranded DNA by exonucleases. This was followed by molecular cloning, library screening, clone characterization and DNA sequencing which made this a rather costly enterprise [67-69]. The use of endonucleases to break up large tandemly repeated DNA clusters facilitated the hunt for chromosome-specific heterochromatic, satellite DNA, expedited the cloning-characterization steps and led to major progress in the identification of chromosome-specific high order tandem repeats [62, 70-74].

The breakthrough in the isolation of chromosome-specific DNA polynucleotides and preparation of DNA probes for FISH came with the application of DNA amplification using the polymerase chain reaction (PCR) in the late 1980's: chromosome-specific sequences could be extracted on-line from larger, high order tandem repeats of satellite DNA to define the PCR primer sequences and amplify a specific fragment from genomic DNA [54] (Fig. 1A).

In a variation of this scheme, chromosome-specific sequences could be amplified with consensus PCR primers from template DNA which provided limited sequence variety, such as flow-sorted human or mouse chromosomes [25, 75] (Fig. 1B). In general, DNA probes generated this way still represented a pool of diverse sequences and molecular cloning was required to isolate the highly specific, informative probes [25].

It wasn't until the completion of a first draft of the human genome sequence when new sets of genomic tools became available that would revolutionize the ways individual investigators analyze the human genome in the 1990's and onwards often using no more than their personal computer and an on-line connection to publicly available databases. Large insert, recombinant DNA libraries such as YAC [76, 77], P1 [78, 79] or BAC [66, 80, 81] libraries had been constructed and characterized, clones had been end-sequenced and placed on the larger physical maps by basic sequence alignment procedures [82].

The work of Baumgartner *et al.* (2006) [65] showed that a combination of database searches (to identify BAC clones rich in satellite content) in combination with *in vitro* DNA amplification can expedite the preparation of chromosome-specific DNA probes. However, this approach still requires some *a priori* knowledge of the target sequence to specify the PCR primers [65].

We recently demonstrated that publicly available on-line databases can be analyzed using a suite of simple bioinformatics tools to identify chromosome-specific BAC clones [60]. Specifically, we used our proprietary information of a Y chromosome-specific sequence [83-85] and a DNA sequence alignment program (BLAST) [82] to identify BAC clone RP11-243E13 as a potential DNA probe. Using the Genome Browser program at the UC Santa Cruz (UCSC) Genome Center web site (genome.ucsc.edu), we then identified a BAC clone mapped to the satellite containing centromeric heterochromatin on the human X chromosome (BAC RP11-294C12) [60]. Probes prepared from these two BAC clones showed an impressive better-than-expected performance in FISH experiments by displaying strong, highly specific FISH signals localized exclusively to the target chromosomes (Fig. 2).

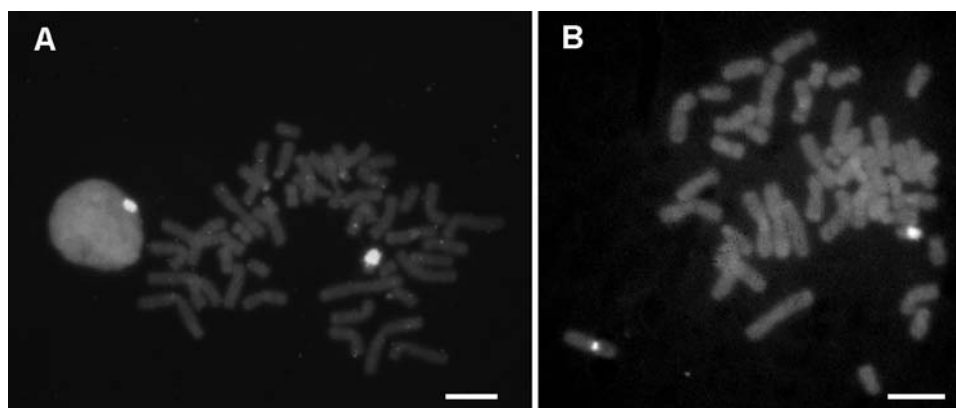


Fig. 1. In situ hybridization of cloned, chromosome-specific PCR products. **A)** Biotinylated DNA prepared from PCR products with chromosome Y -specific oligonucleotide primers bind specifically to the heterochromatic region of the human Y chromosome. **B)** The Y-specific probe shown in **A)** can be combined with a biotinylated probe for the smaller tandemly repeated DNA cluster at the centromeric region of the X-chromosome. Bound probes were detected with avidin-FITC (green fluorescence) on ethidium bromide (red fluorescence) stained chromosomes, here shown as grey scale images. (Bars = 10 μ m).

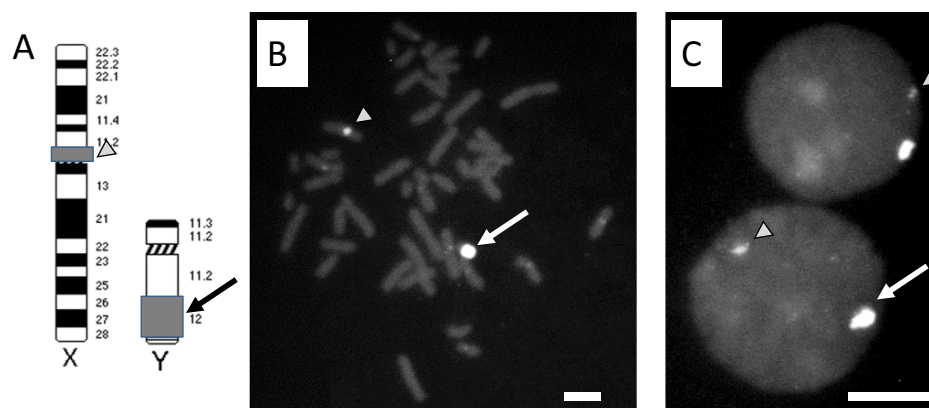


Fig. 2. In situ hybridization analysis of DNA probes prepared from BAC clones. The BAC clones RP11-294C12 and RP11-242E13 hybridized to metaphase spreads prepared from short term cultures of human lymphocytes showed specific hybridization to the target regions on the X (arrowhead) and Y (arrow) chromosome, respectively. **A)** Schematic representation of the FISH target regions on the X and Y chromosome. **B)** Hybridization of both probes to metaphase chromosomes. **C)** Hybridization signals in diploid interphase cell nuclei. (Bars = 10 μ m).

Probe Preparation and Fluorescence in Situ Hybridization (FISH) of BAC-derived DNA Probes

The procedures used for hybridization of BAC-derived DNA probes follow pretty much the published procedures for oligonucleotide, plasmid or P1-derived DNA probes [50, 86, 87]. In typical experiments, the BAC DNAs are extracted from overnight cultures following an alkaline lysis protocol [88] or using a BAC DNA miniprep kit (Zymo Research; Irvine, CA). The DNAs are confirmed on a 1% agarose gel and quantitated spectrophotometrically. Probe DNAs are labeled with biotin-14-dCTP or digoxigenin-11-dUTP (Roche; Indianapolis, IN) by random priming using a commercial kit (BioPrime Kit, Invitrogen; Carlsbad, CA). Slides of metaphase spreads of cells are made from short-term cultures of peripheral blood lymphocytes from a karyotypically normal male following published procedures [35].

The slides (metaphase cells, interphase cell nuclei or slides carrying deparaffinized tissue section) are denatured in 70% formamide at 70 °C, dehydrated and overlaid with a hybridization cocktail containing 20-50 ng of denatured probe DNA in buffer containing 10% dextran sulfate and 50-

55 % formamide. Following overnight incubation at 37°C (48 or more hours for deparaffinized tissue sections), slides are washed to remove excess probes and incubated with a fluorochrome-conjugated avidin or corresponding antibodies as required [59, 66, 89]. Finally, the slides are mounted with 4,6-diamino-2-phenylindole (DAPI) (0.1 μ g/ml) in antifade solution coverslipped and imaged on a fluorescence microscope.

BAC-Derived DNA Repeat Probes for Autosomal Targets

We were also interested in whether this concept of knowledge-based probe selection can be extended to probes for human autosomes. In our 2006 paper [65], we had proposed a satellite-rich BAC clone, RP11-469P16, as template for a PCR based probe generation scheme. The UCSC Human Genome Browser at genome.ucsc.edu indicates the presence of a long interspersed repeated DNA sequence (LINE) in the BAC insert, which may lead to undesirable cross-hybridization since LINES are not chromosome-specific, but exist in thousands of copies across the human genome.

According to information provided on the UCSC Genome browser web site, a BAC insert typically consists of 25-350 kb of DNA. During the early phase of a sequencing project, it is common to sequence a single read (approximately 500 bases) at each end of each BAC from a large library. Later on in the project, these BAC end reads are mapped *in silico* to the genome draft sequence. Tracks in the genome browser as shown in Fig. 3 show these mappings in cases where both paired ends could be mapped within. A valid pair of BAC end sequences must be at least 25 kb but no more than 350 kb away from each other. The orientation of the first BAC end sequence must be "+" and the orientation of the second BAC end sequence must be "-". BAC end sequences are placed on the assembled sequence using Jim Kent's *blat* program [90]. Tracks can be used for determining which BAC contains a given gene or DNA repeat clusters using the 'RepeatMasker' program (www.repeatmasker.org). Please note that for the heterochromatic regions, there has been almost no clone validation in place to ensure that the predicted size or location of the BAC probe is correct.

When using a DNA probe prepared from BAC RP11-469P16, FISH results showed cross-hybridization to multiple chromosomes other than chromosome 2 (Fig. 4A, B). However, a DNA probe prepared from BAC clone RP11-100H17 (Fig. 3, arrow), which is expected to bind ~20 kb proximal of RP11-469P16 on the short arm of human chromosome 2, gave strong, highly specific FISH signals on interphase and metaphase cells (Fig. 4C). This can be attributed to the lack of interspersed non-chromosome specific DNA repeats in the insert of BAC RP11-100H17 as well as its composition of DNA tandem repeat units of entirely chromosome 2-specific satellite DNA.

Since inserts of BAC clones that contain satellite DNA, but no short or long interspersed repeated DNA sequences (SINEs, LINEs) appear to render a high signal-to-noise ratio and strong chromosome specific signals which can easily be scored by eye using a microscope, we prepared a SINE-/LINE-free DNA probe for the short arm of chromosome 4, band p11. The BAC RP11-360M1 carries an insert of an estimated 59846 bp, which is rich in tandemly-repeated satellite DNA repeats, but free of interspersed repeat DNA (Fig. 5).

In situ hybridization of the chromosome 4-specific DNA probe prepared from BAC RP11-360M1 in combination with a differently labeled probe (BAC RP11-294C12) for the centromeric region of the X chromosome to deparaffinized human placental tissue section showed excellent probe performance, i.e., strong and highly specific DNA signals which were easily scored (Fig. 6).

CONCLUDING REMARKS

Molecular cytogenetic analyses using FISH have provided major contributions to our understanding of disease processes including tumorigenesis, cancer progression and metastasis, but also to the existence of aneuploid cell populations or cohorts in seemingly normal tissues [5, 61, 91-96].

For example, with an incidence of one in every 5-6 clinically recognized pregnancies, spontaneous abortions

(SABs) during the first trimester are the most frequent pregnancy complication in women [9]. Causes of SABs have been identified as chromosomal abnormalities, uterine defects, immunological problems, hormonal imbalance and infections [2-6]. While more than half of all first trimester SABs are associated with chromosomal abnormalities, nearly 40% remain unexplained [6]. With no apparent association between placental villous morphology and fetal chromosomal abnormalities, SABs with either euploid or aneuploid conceptuses demonstrated incomplete cytotrophoblast (CTB) differentiation and compromised invasion [7-9]. These observations prompted our studies of the chromosomal make-up of extra-embryonic cells at maternal-embryonic and fetal-maternal interfaces, i.e., the human placenta and the uterine wall. However, as mentioned in the introduction the application of DNA probes described in this review is not limited to investigations of fetal or extra-embryonic tissues.

The novel database mining approach to DNA probe selection described here is a fast and inexpensive solution to the problems of 'probe bottlenecks' in clinical research. Mapping information for BAC clones is publicly available from UCSC or the National Center for Biomedical Information (NCBI)/National Institute of Health, USA, different libraries outside the US, such as the Wellcome Trust Sanger Institute, Hinxton, UK, or the Resources for Molecular Cytogenetics, Dipartimento di Genetica e Microbiologia, Università di Bari, Bari, Italy, as well as several commercial sources are available to purchase these clones. The BAC-derived satellite DNA probes also seem to outperform most of the chromosome enumerator probes that are presently in use in research and clinical laboratories. In summary, the procedures described in the present communication allow a laboratory with typical, non-specialist equipment to prepare chromosome-specific DNA probes in just a few days and thus represent the most efficient, rapid and cost-conscious approach to generation of chromosome-specific DNA probes for cytogenetic studies.

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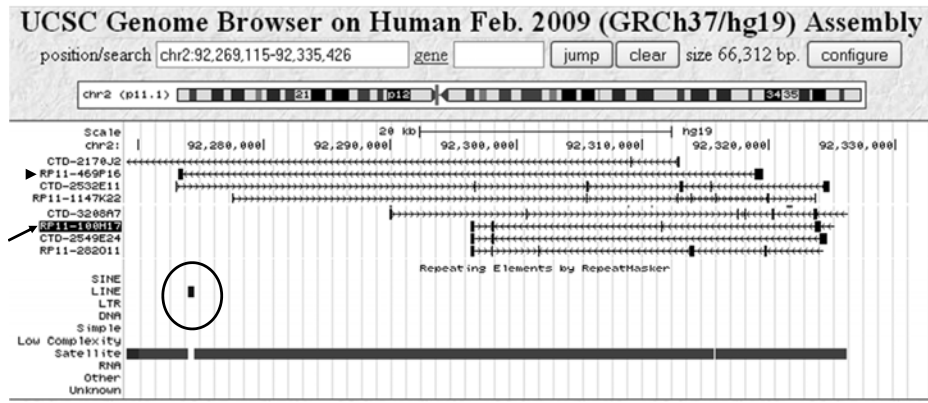


Fig. 3. Screen dump of the UCSC Genome Browser (GoldenPath) display showing the BAC ends mapped to the targeted region of chromosome 2: 92,269,115 bp to 92,335,426 bp. The alignment BAC clone end sequences with the draft sequence of the human genome places BAC RP11-100H17 (arrow) in a region comprised entirely of satellite DNA, while BAC RP11-469P16 (arrowhead) is predicted to contain a cluster of long interspersed repeated DNA sequences (LINEs)(circled).

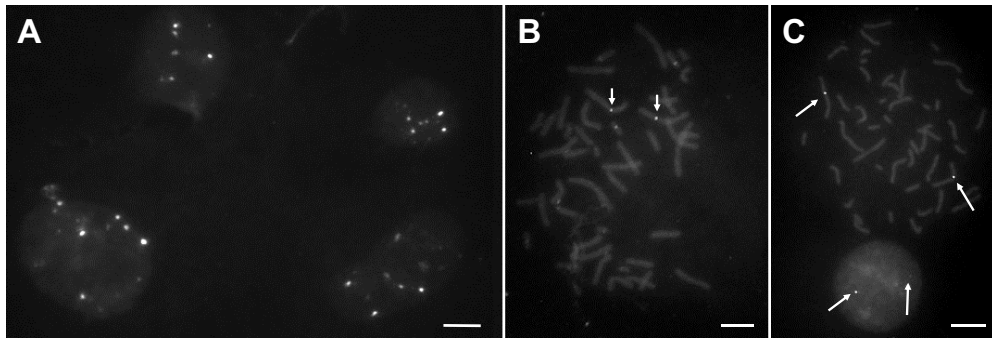


Fig. 4. FISH performance of BAC-derived DNA probes targeting the centromeric heterochromatin of chromosome 2. A-B) A DNA probe prepared from BAC clone RP11-469P16 shows multiple signals in normal interphase cell nuclei (A) or on metaphase spreads (B). Arrows in B) point at the target region on chromosome 2. C) A DNA probe prepared from BAC clone RP11-100H17 binds exclusively to the chromosome 2-specific target region (arrows). (Bars = 10 μ m).

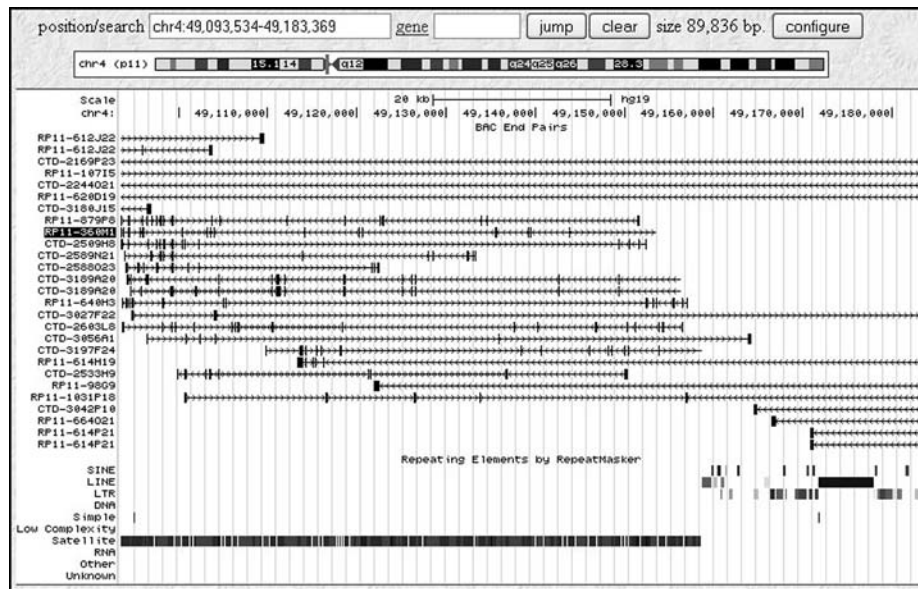


Fig. 5. Screen dump of the UCSC Genome Browser display showing the BAC ends mapped to the region chromosome 4: 49,093,534 bp to 49,183,369 bp. The alignment BAC clone end sequences with the draft sequence of the human genome places BAC RP11-360M1 (highlighted) in a region comprised almost entirely of satellite DNA and completely free of interspersed repeated DNA sequences such as short interspersed repeats (SINEs) or LINEs.

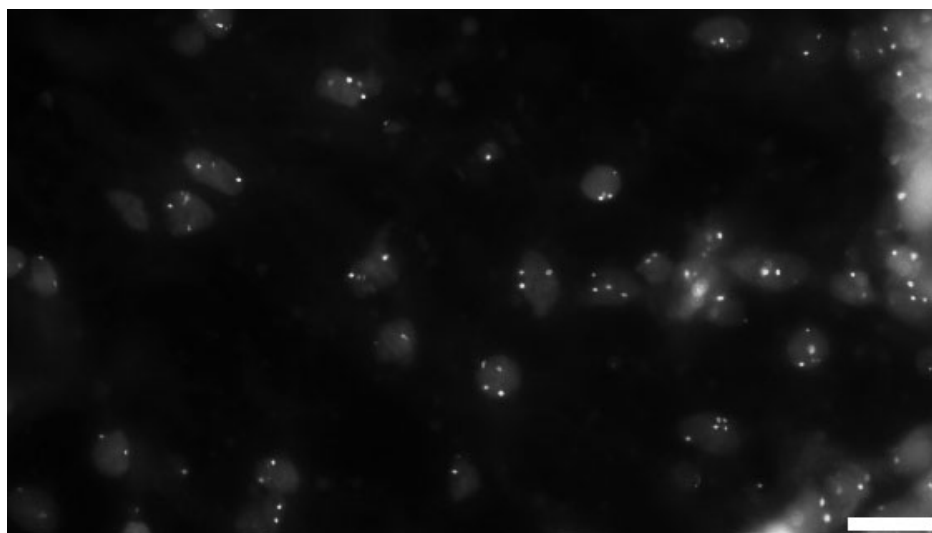


Fig. 6. FISH analysis of placental tissue sections. Probes prepared from BAC clones RP11-294C12 (confirmed target is chromosome X) and RP11-360M1 (supposed to bind to chromosome 4) give compact, easy-to-score hybridization signals on formalin-fixed, paraffin-embedded tissue sections. The red or green signals from the original image together with the DAPI counterstain are overlaid in this gray-scale image. (Bars = 10 mm).

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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ABBREVIATIONS

BAC	=	Bacterial Artificial Chromosome*
CEP	=	Chromosome Enumerator Probe
CPM	=	Confined Placental Mosaicism
DAPI	=	4,6-Diamino-2-phenylindole
iCTB	=	Invasive Cytotrophoblast
LINE	=	Long Interspersed DNA Repeat
LSP	=	Locus-Specific Probe
SAB	=	Spontaneous Abortions
SINE	=	Short Interspersed DNA Repeat
UCSC	=	University of California Santa Cruz
YAC	=	Yeast Artificial Chromosome
PGD	=	Preimplantation Genetic Diagnosis

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