



Bioinformatics Tools Allow Targeted Selection of Chromosome Enumeration Probes and Aneuploidy Detection

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Summary

Accurate determination of cellular chromosome complements is a highly relevant issue beyond prenatal/pre-implantation genetic analyses or stem cell research, because aneuploidy may be an important mechanism by which organisms control the rate of fetal cellular proliferation and the fate of regenerating tissues. Typically, small amounts of individual cells or nuclei are assayed by *in situ* hybridization using chromosome-specific DNA probes. Careful probe selection is fundamental to successful hybridization experiments. Numerous DNA probes for chromosome enumeration studies are commercially available, but their use in multiplexed hybridization assays is hampered due to differing probe-specific hybridization conditions or a lack of a sufficiently large number of different reporter molecules. Progress in the International Human Genome Project has equipped the scientific community with a wealth of unique resources, among them recombinant DNA libraries, physical maps, and data-mining tools. Here, we demonstrate how bioinformatics tools can become an integral part of simple, yet powerful approaches to devise diagnostic strategies for detection of aneuploidy in interphase cells. Our strategy involving initial *in silico* optimization steps offers remarkable savings in time and costs during probe generation, while at the same time significantly increasing the assay's specificity, sensitivity, and reproducibility. (*J Histochem Cytochem* 61:134–147, 2013)

Keywords

molecular cytogenetics, fluorescence *in situ* hybridization, DNA probes, bioinformatics, data mining, interphase cells, aneuploidy, regenerating tissues

Nowadays, fluorescence *in situ* hybridization (FISH) plays a significant role in many research algorithms and became indispensable for routine clinical cytogenetic diagnostics (Tkachuk et al. 1991; Ried et al. 1992; Bednarz et al. 2010; Anguiano et al. 2012; Wallander et al. 2012). For example, in prenatal/pre-implantation genetic analyses (Cassel et al. 1997; Bucksch et al. 2012) or stem cell research (Hessel et al. 1996; Weier et al. 2004; Kwon et al. 2010), as a means of controlling the rate of fetal cellular proliferation at the fetal-maternal interface (Weier et al. 2005; Weier et al. 2010) or determining the fate of regenerating tissues, assessment of aneuploidy may be of substantial importance (Pujol et al. 2003; Kodama et al. 2009; Kwon et al. 2010).

However, whereas other methods, such as chorionic villus sampling (CVS) with *in vitro* culture-based karyotyping of metaphase spreads, have presented difficulties in detecting aneuploidies because abnormal cells can arise in any portion of the placenta or fetal-maternal interphase but might not be

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proliferating (Weier et al. 2005), we rely on chromosome-specific FISH assay to accurately determine the cellular chromosome complements in interphase cells.

Robust cytogenetic diagnosis depends on the availability of bright and specific FISH probes with carefully chosen fluorescent labels for multi-color, multi-target experiments (O'Brien et al. 2010; Avens et al. 2011; Bucksch et al. 2012; Hovhannisyan et al. 2012). Numerous commercially available probes presently serve the basic needs of the biomedical research community. But in order to be commercially viable, these probes have been restricted to high yield targets and typically are limited to a narrow choice of fluorochrome labels (Avens et al. 2011).

In many cases, seeking a conclusive answer to a well-defined, clinical question requires a custom nucleic acid probe that has to be specific and appropriately labeled, yet it may not be available commercially. Therefore, these studies would require additional expertise in preparing target-specific DNA and subsequent labeling with the hapten of choice (Baumgartner et al. 2006). Briefly, such tailored FISH experiments can be divided into several crucial steps or milestones: 1) choosing the appropriate genomic DNA target sequence, 2) sourcing or designing a suitable DNA probe, 3) devising a rational probe labeling/detection strategy, 4) optimizing wet lab procedures necessary to achieve optimal hybridization between the probe and the DNA target, 5) documenting the hybridization images, and finally, 6) interpreting the results and putting them in context with clinical and histopathological findings (Wallander et al. 2012).

There is no doubt that information technology has led to advances in most of the aforementioned steps. For example, composite image capture of several monochrome fluorescence images recorded at different wavelengths found its way into the routine clinical laboratory after multi-spectral digital imaging became affordable (Farkas et al. 1998; Levenson 2006; Wells et al. 2007). However, it has been difficult, time consuming, and expensive to design and produce high quality DNA probes suitable for rapid, multiplexed chromosome enumeration or detection of chromosomal translocations (Weier et al. 1991b; Fung et al. 1998; Fung et al. 2000; Greulich et al. 2000; Lu et al. 2009a). Over the years, the research community has invested many man-hours in target-specific probe design, often duplicating each other's efforts and being unable to benefit from other groups' efforts with identical DNA targets (Willard et al. 1983; Wolfe et al. 1985; Vorsanova et al. 1986; Waye et al. 1987; Yurov et al. 1987; Devilee et al. 1988; Kievits et al. 1990; Lengauer et al. 1990; Weier et al. 1990b; Weier and Gray 1992; Vooijs et al. 1993; Guan et al. 1996).

Tailored bioinformatics-guided probe selection strategies provide powerful advantages in the first two fundamental steps of FISH-based chromosome counting. This database aided method is commonly referred to as "data

mining" and replaces time consuming and costly in vitro target acquisition experiments with a speedy and efficient in silico optimization, avoiding much of the time and resources spent in the wet lab. Laboratories experienced in FISH assays are therefore able to streamline the onerous process of optimizing the target DNA/probe match. The use of publicly available, web-based bioinformatics tools (Kent et al. 2002) will also help expand the use of FISH into a technique that can be accessed by non-specialized and less established laboratories or groups with more constrained resources (Zeng et al. 2011).

In this communication, we report the development of a chromosome enumeration assay for use in reproductive studies that demonstrates the bioinformatics-guided FISH approach. In several distinct examples, we show how data mining strategies were used for target identification and probe selection. Probes were optimized to identify copy numbers of chromosome 10, as well as gonosomal genotyping (chromosomes X and Y). This assay development highlights the necessity of optimization and quality control steps throughout the process. Poor specificity complicates and limits the information that can be deduced from the hybridization result. We demonstrate how DNA sequence analysis can be used to predict probe performance. After selection and quality control experiments, the best BAC probes were applied in diagnostic scenarios including interphase and metaphase preparations from healthy volunteers, placental tissues, as well as pathological samples such as a human cancer cell line. These typical diagnostic implementations demonstrate how the strategic introduction of bioinformatics tools into routine hybridization algorithms can save time and cost and improve the signal-to-noise ratios, sensitivity, specificity, and reproducibility of FISH experiments.

Materials & Methods

All procedures involving human subjects have been conducted under Human Subjects Use Protocols reviewed and approved by the University of California, Berkeley Institutional Review Board.

Probe Development

Retrieval of X and Y Probe Target DNA Sequences for PCR Amplifications. The nucleic acid sequence used for the Y chromosome-specific probe was defined in our previous studies using in vitro DNA amplification from the 3.6-kb pentanucleotide DNA repeat described by Nakahori et al. (DYZ1, Genbank accession number X06228) (Nakahori et al. 1986; Gray and Weier 1998). The annealing sites of primers WYR2 and WYR4 (Table 1) were chosen to have a maximum of sequence deviation from the human satellite III DNA pentameric repeat consensus motif TTCCA and to yield a 124-bp DNA fragment by PCR

Table 1. Oligonucleotide Sequences and PCR Primer Pairs Used for DNA Probe Preparation

Name	Forward 5'–3'	Reverse 5'–3'	Reference
WXR1	tcgaaacgggtatgctcagctaaa	WXR2	unpublished
WXR2	WXR1	aagacagttcaaaactgctccatcaa	unpublished
WXR3	attccgtacgattccattcctttgaa	WXR4	Weier (1990b)
WXR4	WXR2	gaatgtattagaatgtaatgaacttta	Weier (1990)

(Nakahori et al. 1986; Weier et al. 1990b; Gray and Weier 1998, 1999). Blood samples from six normal human volunteers were used to validate the Y chromosome-specific PCR assay (Weier et al. 1990a; Gray and Weier 1999).

The oligonucleotide primers used to generate an X chromosome-specific DNA probe by PCR from genomic DNA are WXR1 and WXR2 (Table 1). They were defined based on the published DNA sequence of chromosome X-specific alpha satellite DNA (Willard et al. 1983; Waye and Willard 1985) and designed to yield a 124-bp DNA fragment. The PCR conditions and hybridization modalities have been described previously (Wyrobek et al. 1994; Robbins et al. 1995; Baumgartner et al. 2001).

DNA Amplification and Probe Biotinylation

Detailed methods have been described in our previous publications (Weier et al. 1990b; Weier et al. 1991a; Weier et al. 1994a). Briefly, genomic DNA was extracted from the capillary blood of a male donor and DNA amplification was performed on the automated thermal cycling system designed and built in our laboratory. Unbound deoxynucleoside triphosphates were removed from the PCR solution by spinning the sample through a 1-mL Sephadex G-50 column (Pharmacia; Pleasant Hill, CA). Then, 5- μ L aliquots of the PCR solution were re-suspended in 275 μ L of biotinylation buffer (10 mM Tris-HCl, pH8.4, 1.5 mM MgCl₂, 50 mM KCl, dATP, dCTP, and dGTP, 0.2 mM each, 0.33 mM biotin-11-dUTP [Sigma-Aldrich; St. Louis, MO], 1.2 mM each primer) and 20 units of Taq polymerase (Perkin Elmer; Waltham, MA). The DNA solutions were then amplified for an additional 20 cycles to generate biotin-labeled DNA fragments, which were stored unpurified at -18C in the freezer until used for FISH.

Retrieval of Chromosome-Specific Probe Information for the Pericentromeric Region of the Target Chromosomes Using Data Mining and BAC-FISH Methods

Database Searches for the Y Chromosome-Specific DNA Probe. We screened the National Center for Biotechnology Information human genome nucleotide (nt) DNA database for homologous sequences. Execution of the Basic

Local Alignment Search Tool (BLAST) (Altschul et al. 1997) querying the human genome database with the 27-nt sequence "ATTCCGTACGATTCCATTCTTTTGAA" from position 3089-3115 of the human Y-specific 3564-bp repeat (Genbank accession number X06228) retrieved multiple hits. Parameters were chosen to identify clones with a range of nucleic acid homology (setting: "Optimize for somewhat similar sequences" [BLASTn]).

Retrieval of Probe Information for the Pericentromeric Region of the X Chromosome

We used the University of California, Santa Cruz (UCSC) Genome Browser GRCh37/hg19, built February 2009, at the University of California, Santa Cruz, accessible at <http://genome.ucsc.edu/>, to identify bacterial artificial chromosome (BAC) clones with high satellite DNA content. The graphic user interface was set to display BAC end pairs and repeat DNA elements in the pericentromeric region of the human X chromosome, that is, from position 58,232,531 to 61,922,800 bp.

Retrieval of Information for Probes in the Pericentromeric Region of Chromosome 10

Similar to the X chromosome, we used UCSC Genome Browser GRCh37/hg19 (Kent et al. 2002), built February 2009, to identify BAC clones with high satellite DNA content around the pericentromeric region of human chromosome 10.

DNA Probe Preparation

The BAC DNAs were extracted from overnight cultures following an alkaline lysis protocol (Birnboim and Doly 1979) or using a ZR BAC DNA Miniprep Kit (Zymo Research; Irvine, CA). The isolation of high molecular weight BAC DNAs was confirmed on 1% agarose gels and quantitated by Hoechst fluorometry using a Hoefer TK 100 instrument (Hoefer; South San Francisco, CA). Probe DNAs were labeled with biotin-14-dCTP (Invitrogen; Carlsbad, CA), digoxigenin-11-dUTP (Roche Molecular Systems; Indianapolis, IN), Spectrum Orange-dUTP (Abbott; Abbott Park, IL), Spectrum Green-dUTP (Abbott), or Cy-5-dUTP (GE Healthcare; Piscataway Township, NJ)

by random priming using a commercial kit (BioPrime Kit; Invitrogen) (Weier et al. 1995a; O'Brien et al. 2010). When incorporating fluorochrome-labeled deoxynucleoside triphosphates, the dTTP-to-dUTP ratio in the reactions was adjusted to 2:1 (Weier et al. 1994a; Weier et al. 1994b; Fung et al. 1998).

Cell Culture and Preparation of Metaphase Spreads.

Control metaphase spreads were made from phytohemagglutinin stimulated short-term cultures of normal male lymphocytes according to the procedure described by Harper and Saunders (1981). Fixed lymphocytes were dropped on ethanol-cleaned slides in a CDS-5 Cytogenetic Drying Chamber (Theratron Industries, Inc.; Holland, MI) at 25°C and 45% to 50% relative humidity (Munné et al. 1996).

The S48TK cultures were established as described by Zitzelsberger et al. (1999). All procedures followed protocols approved by the Ethics Committee of the Bavarian Board of Physicians and the LBNL/UC Berkeley Committee on Human Research concerning use of surplus surgical tissues for research. In essence, S48TK lines were obtained from the tumor tissue of a 14-year-old patient (7 years old at time of exposure to elevated levels of radiation) undergoing surgery at the Center for Thyroid Tumors in Minsk, Belarus, following the diagnosis of Hashimoto's thyroiditis and papillary thyroid cancer (PTC). Initial chromosome preparations were carried out after an *in vitro* culture of S48TK cells for 8 to 21 days. Later on, clones were isolated by limiting dilution and cultured for more than 20 passages. The clone number is indicated following the name of the primary line, S48TK. For example, S48TK6 and S48TK18 refer to clone 6 and clone 18 of cell line S48TK. After G-banding with Wright's staining solution, karyotypes were recorded according to the International System for Human Cytogenetic Nomenclature (ISCN, 2005). To further reduce heterogeneity, a second round of cloning by limiting dilution was performed on 96-well microtiter plates. Resulting clones were identified by adding the plate position to the parental clone name, that is, line S48TK18C3 has been derived from line S48TK18.

Spectral Karyotyping (SKY) Analysis

The SKY analysis was performed as described previously (Garini et al. 1996; Schroeck et al. 1996; Fung et al. 1998; Zitzelsberger et al. 1999; Fung et al. 2000; Zitzelsberger et al. 2001; Zitzelsberger et al. 2002; Weier et al. 2011). Briefly, acetic acid:methanol (1:3, vol.:vol.) fixed metaphase spreads were pretreated with RNase A and pepsin, then fixed in freshly prepared 1% paraformaldehyde (PFA), hybridized with a probe mix composed of 24 chromosome-specific painting probes and detected following the probe manufacturer's instructions (Applied Spectral Imaging; Carlsbad, CA).

Tissue Section Preparation

Deparaffinization, slide pretreatment, and FISH hybridization procedures followed the published protocol (Rautenstrauss and Liehr 2002). Briefly, the mounted section was deparaffinized in 50 ml xylene in Coplin jars (two times 5 min each) and rehydrated in an ethanol series (100%, 90%, 70%, 50%; 3 min each) and 0.9% NaCl solution (2 min × 2). If necessary, undesirable parts of the tissue and excess paraffin could be removed at this point by scratching it off the slide with a scalpel.

As in a conventional FISH approach, a pretreatment of the slides with RNase and pepsin followed by postfixation with formalin-buffer was required to reduce the background (Liehr et al. 1995). Slides were then soaked in 2X saline-sodium citrate (SSC) buffer for 5 min at 21°C (in a 50-ml Coplin jar on a shaker) (20X SSC is 3 M sodium chloride and 300 mM trisodium citrate, pH 7.0). Slides were removed from the Coplin jar, and 100 µl of RNase solution was added per slide and covered with a suitably sized coverslip (RNase solution: 50 µg/ml in 2X SSC). The slides were incubated in a humid chamber for 15 min at 37°C, then washed with 2X SSC for 3 min on a shaker, before a final wash with 1X PBS for 5 min. Slides were then pretreated with pepsin-buffer at 37°C for 10 min (without agitation) (pepsin buffer: freshly prepared 50–100 µg/ml pepsin in 0.01 M HCl, prewarmed to 37°C). This was replaced with 1X PBS/MgCl₂ [5%(v/v) 1 M MgCl₂ in 1X PBS] and incubated at 21°C for 5 min with gentle agitation. Nuclei were post-fixed on the slide surfaces by replacing 1X PBS/MgCl₂ with formalin-buffer (1.5 ml acid free formaldehyde in 50 ml 1X PBS) for 10 min (21°C, with gentle agitation). Formalin-buffer was replaced by 1X PBS for 2 min (21°C, with gentle agitation). Finally, slides were dehydrated by an ethanol series (70%, 85%, 100%; 3 min each) and air dried.

Fluorescence In Situ Hybridization (FISH)

A 100-µl aliquot of denaturation buffer was added to the slides (metaphase or interphase cell slides or deparaffinized tissue section slides) and covered with appropriately sized coverslips. Denaturation buffer is 70%(v/v) deionized formamide, 10% (vol./vol.) filtered double distilled water, 10% (vol./vol.) 20X SSC, 10% (vol./vol.) phosphate buffer. Phosphate buffer is 1:1 mixture of 0.5 M Na₂HPO₄ and 0.5 M NaH₂PO₄, pH 7.0, and stored as aliquots at -20°C. Slides were incubated on a hot plate for 2 to 5 min at 75°C. The coverslips were removed immediately with forceps and the slides were post-fixed in 70% ethanol at 4°C to conserve target DNA as single strands. Slides were dehydrated in ethanol (70%, 85%, 100%; 4°C, 3 min each) and air dried.

For hybridization, 1 µl of either labeled DNA probe, salmon sperm DNA (10 mg/ml, 5 Prime-3 Prime Inc.; Boulder, CO), or water, and 7 µl of the hybridization master mix (78.6% formamide, 14.3% dextran sulfate in 1.43X

SSC, pH 7.0) were thoroughly mixed and denatured at 76°C for 10 min. The final concentration in the hybridization mixture is 55% formamide (FA; Invitrogen), 10% dextran sulfate, and 1X SSC, pH 7.0. About 2.5 µl of hybridization mixture was placed on the denatured samples, covered with a 12-mm circular coverslip, sealed with rubber cement, and incubated at 37°C in a humid chamber (for metaphase and interphase slides: incubated for 16–20 hrs; for SKY analysis: incubated for 48 hrs; for deparaffinized tissue section slides: incubated for 72 hrs).

Post-Hybridization Washes and Detection of Bound Probes

After the rubber cement was carefully removed from the slides, the coverslips were allowed to slide off in 2X SSC at 20°C. Post-hybridization washes were performed as described and included two washes in 50% FA/2X SSC buffer at 43°C for 15 min each followed by two washes in 2X SSC at 20°C, each for 10 min (Kwan et al. 2009; Lu et al. 2009b). If labeled indirectly, after unspecific binding sites were blocked with PNM buffer (5% nonfat dry milk, 0.1% NaN₃ in PN buffer, that is, 0.1 M sodium phosphate buffer, pH 8.0, plus 0.05% NP-40) for 10 min, non-fluorescent probes were detected with either fluorescein-conjugated avidin DCS (Vector; Burlingame, CA) or anti-digoxigenin rhodamine (Roche), and two 2X SSC washes were used to wash away unbound antibodies. Finally, the slides were mounted with 4,6-diamino-2-phenylindole (DAPI) (0.1 µg/ml) in antifade solution (0.1% p-phenylenediamine dihydrochloride [Sigma-Aldrich], 0.1X PBS [Invitrogen], 45 mM NaHCO₃, 82% glycerol [Sigma-Aldrich], pH 8.0) and coverslipped.

Image Acquisition and Analysis

Fluorescence microscopy was performed on a Zeiss Axioskop microscope (Zeiss; New York, NY) equipped with filter sets for observation of Cy5/Cy5.5, Texas red/rhodamine, FITC, or DAPI (84000v2 Quad; Chroma Technology, Brattleboro, VT). Images were collected using a CCD camera (VHS Vosskuehler; Osnabrueck, Germany) and processed using Adobe Photoshop software (Adobe Inc.; Mountain View, CA) (Weier et al. 2011).

Results

Chromosome X- and Y-specific Probe Selection by PCR Assay and Validation by FISH

The PCR product resulting from the PCR (primers WYR2 and WYR4) was approximately 124 bp (gel picture not shown), as expected. The PCR product resulting from the PCR (primers WXR1 and WXR2) was 124 bp. Both PCR products were labeled with biotin and used for in situ

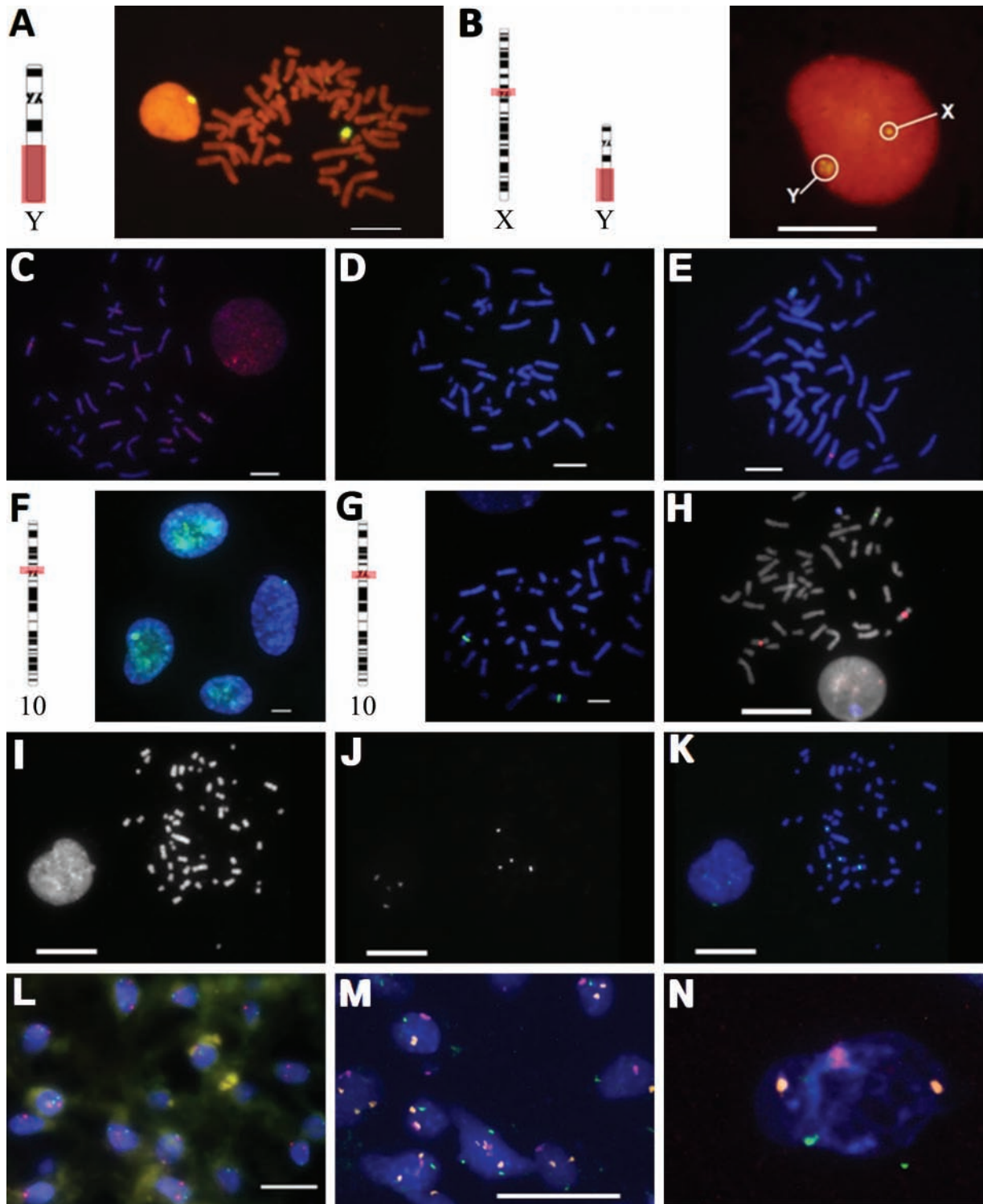
hybridization without further purification. Figure 1A shows the results of hybridization of the probe to the long arm of the Y chromosome. Figure 1B shows the combination of biotinylated probes for both Y and X chromosomes. The signals can be distinguished by size. Biotin-labeled probe detection was accomplished using avidin-FITC as described (Weier et al. 1990b). The chromosomes and cells were counterstained with propidium iodide (PI). Fluorescence from the hybridized probe appears yellow (resulting from the green of the avidin-FITC overlaid onto the red PI staining).

Even with similar probe concentrations, the two labeled DNAs showed drastically different hybridization specificities (Figs. 1C and 1D). The digoxigenin-labeled probe prepared from BAC clone RP11-348g24 containing the interspersed repeats gave a strong signal on the X chromosome but also unacceptably high levels of cross-hybridization on the autosomes (Fig. 1C). In interphase nuclei, such high levels of cross-hybridization prevented identification of the X chromosome target. In contrast, hybridization of a biotinylated DNA probe prepared from BAC clone RP11-294c12 resulted in signals that localized exclusively to the pericentromeric region of the human X chromosome (Fig. 1D). Figure 1E demonstrates that the highly specific DNA probes for chromosomes X and Y can be combined in dual-color multiplex hybridization experiments (Jossart et al. 1996; Fung et al. 2001; Bednarz et al. 2010). We labeled the Y chromosome-specific BAC clone RP11-242e13 directly with Spectrum Green-dUTP (green fluorescence) and the X chromosome-specific BAC clone RP11-294c12 with Spectrum Orange-dUTP (red fluorescence). Hybridization of this probe mixture gave strong, specific signals in metaphase as well as interphase cells (Fig. 1E).

Chromosome X and Y BAC Clone Selection and Validation

Chromosome X and Y BAC clone selection and validation was previously described by Zeng et al. (2011, 2012). A chromosome-specific 3.5-kb satellite III DNA repeat had been identified (Nakahori et al. 1986) in the q12 band of chromosome Y, for which we designed and tested various PCR primer sets (Zeng et al. 2011). The BAC clone RP11-242e13 (Genbank accession number AC068123) had the highest homology score when compared with the best performing primer (a 27-nt primer). Clone RP11-242e13 actually contained 28 copies of a sequence with 85% homology to this primer within its 98295-bp insert.

For the X chromosome, we observed that chromosome-specific alpha satellite DNA repeats could be identified in BAC clones through database searches (Baumgartner et al. 2006). Based on this prior observation, we identified two BAC clones with large inserts that targeted the tandem DNA repeats in the heterochromatic pericentromeric region



of the human X chromosome. As shown in a screen capture from the interface of the UCSC Genome Browser (Fig. 2) (Zeng et al. 2011), one clone (RP11-294c12) was essentially free of interspersed DNA repeats, such as short or

long interspersed elements (SINEs, LINEs, respectively), and maps to X q11.1, and a second clone (RP11-348g24), which contains a few interspersed DNA repeats, mapped to chromosome X, band p11.1.

BAC Clones for the Pericentromeric Region of Chromosome 10

Similar to the X chromosome probe data mining search, we used the graphical user interface of the UCSC Genome Browser and distinguished two BAC clones (Fig. 3A) located on the pericentromeric region of chromosome 10, band 10p11.1. One was RP11-96f8, containing several interspersed DNA repeats, which, when hybridized, showed multiple signals and a high level of cross-specificity in the interphase nuclei of normal cells (Fig. 1F). However, the other clone CTD-3241j23 chosen contained no interspersed repeat DNA (Fig. 3A) but showed weak-to-little hybridization signals on chromosome 10, likely due to its smaller size/coverage. In our search for a better candidate target, we turned to the other side of the pericentromeric region of chromosome 10, band 10q11.21, and found a BAC clone RP11-168p20, which is composed of alpha satellite repeat DNA (of a pure single repeat sequence) and contains no LINEs or SINEs (Fig. 3B). A biotinylated probe prepared from BAC clone RP11-168p20 gave a very strong and specific signal localized to the centromeric region of chromosome 10, with no observable cross-hybridization in metaphases of normal cells (Fig. 1G).

Hybridization of Probe RP11-168p20 to Tumor Cells Reveals Polyploidy

The same probe (prepared from BAC clone RP11-168p20) was hybridized to PTC cell line S48TK18, clone A6 (Zitzelsberger et al. 1999; Weier et al. 2006; Weier et al. 2011). The majority of the cells showed four signals (Fig.

4A). Three independent analysts scored the signals and chromosomes in each of 40 metaphase spreads. The results were grouped into two genotypes (Fig. 5, Table 2). A large proportion of the metaphase spreads had on average four signals for chromosome 10 (out of a total of about 50 chromosomes per cell), whereas another smaller group had about eight signals (out of a total of 100 chromosomes per cell, or double the chromosome count). S48TK6, clone C3 carries three copies of chromosome 10. A small Y chromosome was identified with SKY and confirmed by hybridizing a commercially available Y chromosome probe in interphase cells.

Hybridization of Combined X,Y, and Chromosome 10 Probes onto Deparaffinized 12-Week Placenta Tissue Section

When a combination of three differentially labeled probes was hybridized onto deparaffinized placenta (12-week-old) tissue sections on glass slides, individual signals could be distinguished. In slides made with female placental tissue (Fig. 1L), only chromosome X and 10 signals were visible. Slides made from male placental tissue (Figs. 1M–N) additionally showed signals from the Y chromosome-specific probe.

Discussion

About four decades after its inception, *in situ* hybridization has established itself firmly as an indispensable tool in the genetic characterization of cells in health and disease. Whereas the initial, groundbreaking studies used isotopically labeled nucleic acid probes (Pardue

Figure 1. *In situ* hybridization results for selected DNA probes. Red shaded areas on the idiograms in (A–B) and (F–G) indicate the hybridization target sites. (A) *In situ* hybridization using fluorescent probes for the highly repeated satellite III DNA segment (flanked by the PCR primers WYR2 and WYR4) shows bright signals on the long arm of the Y chromosome. (B) Combining biotinylated *in situ* hybridization probes for the Y chromosome-specific DNA segment (large signal) with probes for X chromosome-specific DNA flanked by the PCR primers WXR1 and WXR2 (small signal) allows chromosome identification based on the size of the signal. (C) A digoxigenin-labeled DNA probe prepared from BAC RP11-348g24 resulted in specific signals on the X chromosome in the presence of a significant amount of cross-hybridization to other chromosomes in metaphase spreads. High level of cross-hybridization was also evident in interphase nuclei. (D) In contrast to the result shown in (C), a probe prepared from BAC RP11-294c12 bound almost exclusively to the centromeric heterochromatin of the human X chromosome. (E) Combining differentially labeled DNA probes in a single hybridization experiment, the Y chromosomal target appears in green, whereas the X chromosomal centromeric repeat DNA is shown in red. (F) *In situ* hybridization of an autosome-targeting DNA probe prepared from BAC clone RP11-96f8 showed multiple signals and a high level of cross-hybridization in the interphase cell nuclei. (G) The biotinylated probe prepared from BAC RP11-168p20 exhibited strong signals on both homologues of chromosome 10. (H) A combination of three differently labeled probes was hybridized simultaneously. Chromosome 10 signals are shown in red; X chromosomal and Y chromosomal signals are shown in green and blue, respectively. (I–K) The same probe as in (G) (BAC RP11-168p20) was hybridized to the cell line S48TK18A6. Multiple signals indicate chromosomal abnormalities. (I) shows DAPI picture, (J) shows green avidin-FITC signal, and (K) shows the superposition of both. (L–N) A chromosome X probe (BAC clone RP11-294c12) labeled with biotin/avidin-FITC, a Cy-5 labeled chromosome Y probe (RP11-242e13), and a chromosome 10-specific probe prepared from BAC RP11-168p20 (labeled with Spectral Orange-dUTP) were hybridized onto a deparaffinized 12-week placenta tissue section. (L) The chromosome 10 probe (red signals) and bound chromosome X probe (green signals) in this female placental tissue section. (M–N) In our triple probe FISH experiment, the chromosome 10 probe is represented by orange signals, the chromosome X probe by green signals, and the chromosome Y probe by red signals in the male cell nuclei. (Size marker bars indicate 10 μ m.)

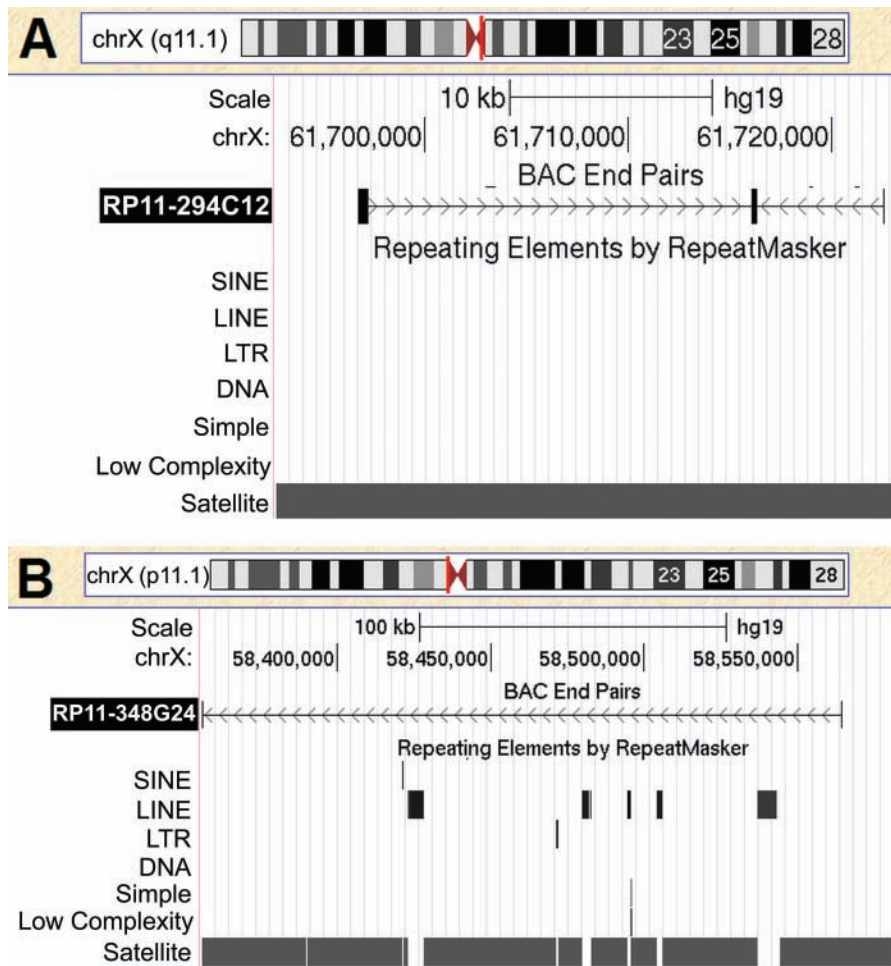


Figure 2. Data mining selection of FISH probes for chromosome X. Screenshots of the UCSC Genome Browser query for BAC clones targeting repeat-rich regions of chromosome X. (A) Clone RP11-294c12 has a target sequence within the pericentromeric heterochromatin of the q arm that is rich in satellite repeats, whereas (B) a clone (RP11-348g24) selected from the pericentromeric region of the p arm contains both satellite and non-chromosome-specific repeats (LINEs, SINEs, and LTRs).

and Gall 1969, 1970), these techniques have mostly been replaced by assays involving non-isotopically labeled probes and detection by fluorescence microscopy (Manuelidis 1978).

Recent advances in bioinformatics have significantly facilitated the development of new algorithms to investigate genotypes for research or diagnostics. This contributes to our assessment that cytogenetic techniques and especially Fluorescence In Situ Hybridization, if combined with bioinformatic support tools, will be able to reach a new level of efficacy and user-friendliness.

When interrogating sample DNA for numerical and structural chromosomal abnormalities, it is of fundamental importance to make use of chromosome- or gene-specific nucleic acid probes that, once bound to their targets, produce specific, unambiguous signals. BAC probes covering a majority of the human genome, and indeed DNA loci of interest in several other species, are now commercially available. These probes are tested, are quality controlled, and can easily be used in FISH hybridizations with a high success rate.

However, purchasing commercially available DNA probes comes at a huge expense when performing larger multi-target screening studies. Compounding this, probe composition remains almost always proprietary, thereby restricting the end user to applications specified by the probe manufacturer. Undisclosed addition of blocking reagents, a customary procedure in the preparation and “copy protection” of single copy DNA probes, may further interfere with multiplex assays.

Furthermore, commercial FISH probes for specific loci or centromeres are available with a limited array of distinct fluorochromes and cannot satisfy all of the possible label combinations required in specific multiplexed assays.

This shortcoming of commercially available probes increases with the ever-expanding number of regions of interest and fluorochrome/detection modalities. Strategies for efficient in-house target-specific probe development are therefore a critical component of most de novo multiplexed FISH assays.

Bioinformatic platforms now allow the research community to share and summarize knowledge on target DNA

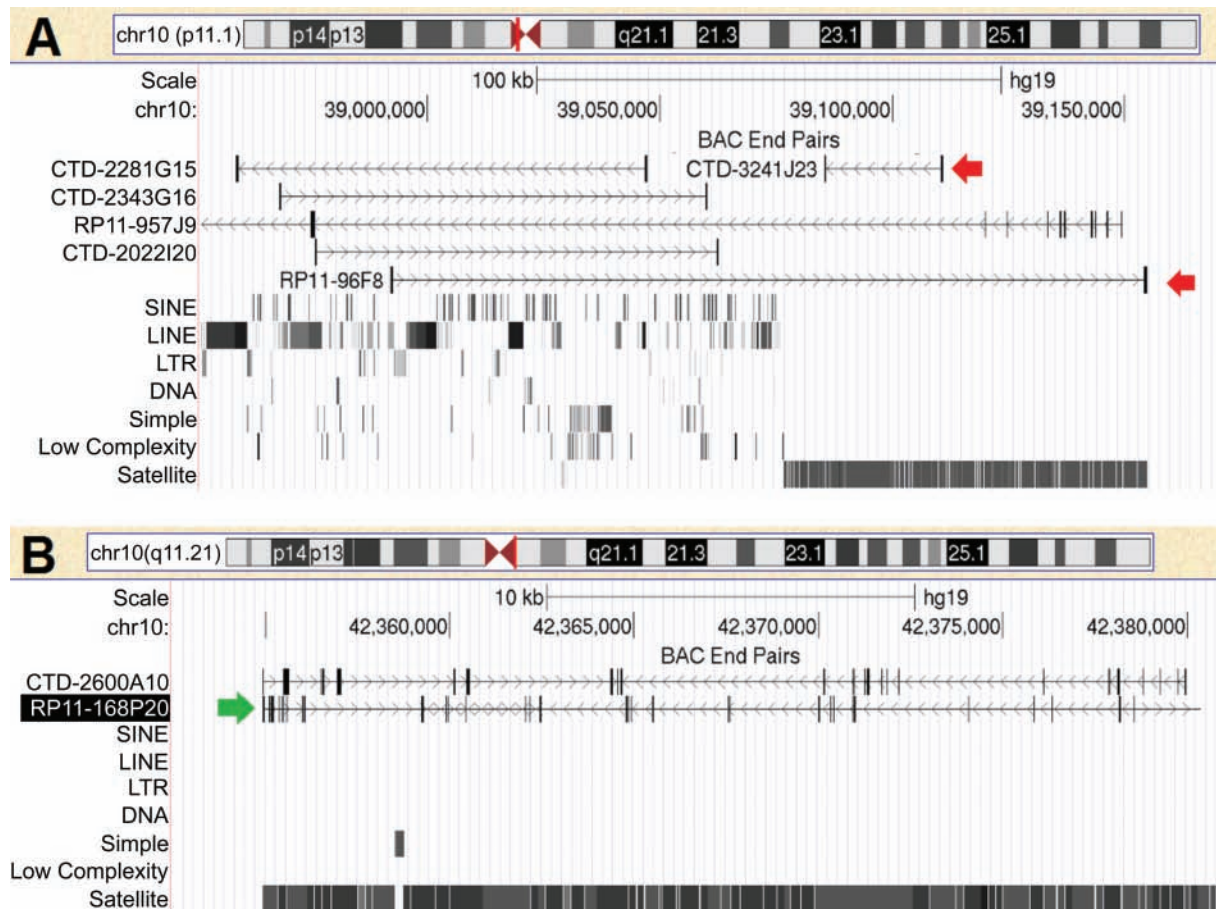


Figure 3. Data mining for probes specific for chromosome 10. (A) Two probes selected for chromosome 10 (red arrows) contained both non-chromosome-specific repeat regions (i.e., interspersed repeats such as SINE, LINE) and chromosome-specific (satellite) DNA repeats and produced poor hybridization results. (B) A search in another repeat-rich region of chromosome 10 (q11.21) resulted in an alternate probe (green arrow) that produced the desired strong and specific signals in the presence of background signals.

sequences and probes. Running a publicly available bioinformatics search tool, such as the Basic Local Alignment Search Tool, against a database such as the National Center for Biotechnology Information human genome nucleotide DNA database, allows *in silico* identification of appropriate target DNA sequences. Many hours of optimization experiments *in vitro* can be bypassed by spending a fraction of the time researching the optimal target/probe combination *in silico*.

We describe how the use of bioinformatics tools for chromosome X and Y BAC clone selection and validation convincingly demonstrates the power of this modified approach. From the onset we had a clearer understanding of the DNA target/probe interaction we could expect. For example, it could be forecast that clone RP11-294c12 would yield better specificity for chromosome X than clone RP11-348g24, due to the lack of non-chromosome-specific repeat elements in its genomic target sequence (LINEs, SINEs, and long terminal repeats [LTRs]). The lack of specificity of the latter clone greatly reduced the

ability to reliably count chromosomes in interphase cells. Prior *in silico* screening/optimization in this case would fast-track the identification of a reliable chromosome X enumeration probe. This optimization step would also greatly shorten the efforts in developing multiplexed assays. If we had had the advantage of bioinformatic pre-screening at our disposal when we designed our Chromosomal Rainbows (O'Brien et al. 2010), we could have saved vast amounts of time and expense during probe selection, validation, and optimization. Analogous to the example for chromosome X probe selection, we used *in silico* analysis to help predict the *in vitro* behavior of candidate probes for chromosome 10. As expected, the probe predicted with the greatest specificity and the largest target area/brightest signal (RP11-168p20) yielded near-perfect *in vitro* hybridization results compared to a probe suspected to be suboptimal (RP11-96f8) (Baumgartner et al. 2006). In future selection strategies, such suboptimal probes could be omitted from any further *in vitro* testing.

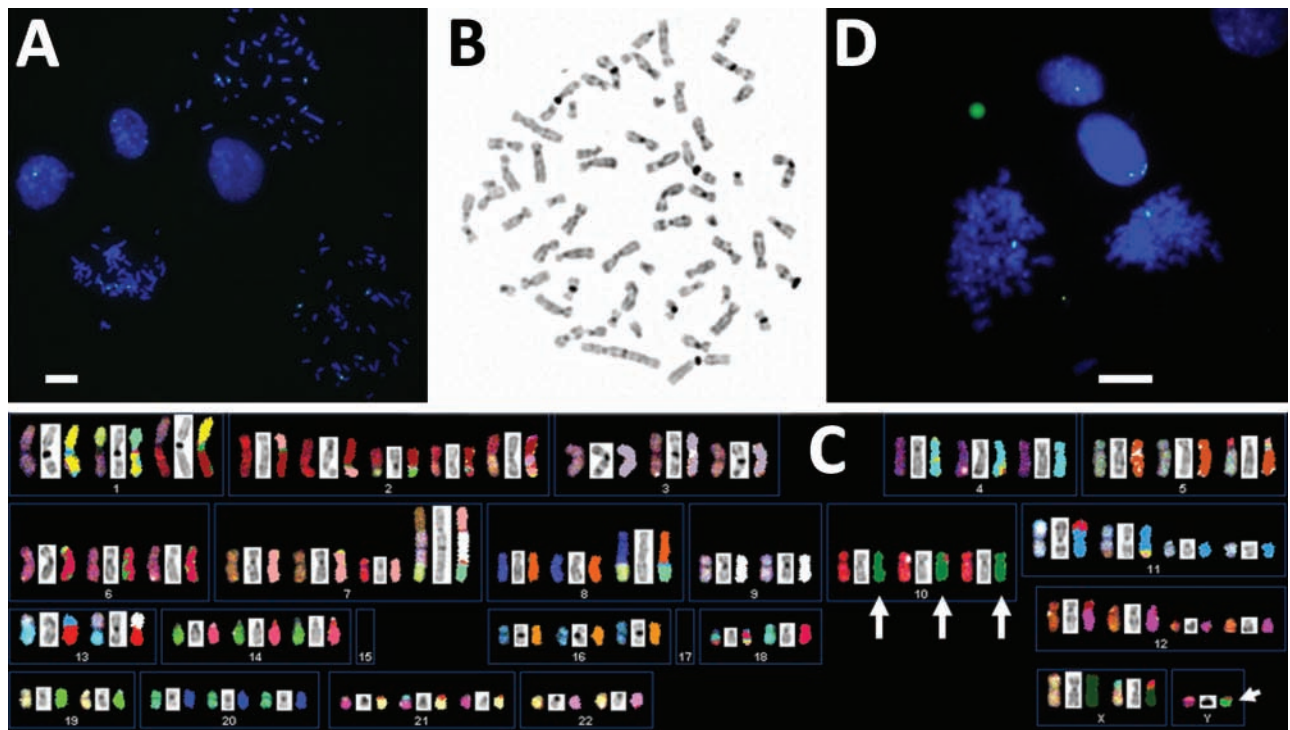


Figure 4. Hybridization of probe RP11-168P20 to tumor cells reveals polyploidy. (A) Hybridization of a biotinylated probe to cells from the PTC cell line S48TK18, clone A6 shows cells with four signals. Please note the bright signals and absence of cross-hybridization on metaphase spreads. Some signals in interphase cells are not visible, likely due to being out of the focal plane of the image. (B–C) Spectral Karyotype analysis of cell line S48TK6, clone C3. (B) shows the inverted DAPI image of a metaphase spread. (C) shows the SKY karyogram. The three large arrows point to the copies of chromosome 10 identified in this metaphase spread. The small arrow points to a small chromosome identified by SKY as the Y chromosome. (D) Confirmation of the presence of Y chromosomal material via hybridization of a commercially available Y chromosome-specific DNA probe labeled with Spectrum Green (Abbott). (Size marker bars indicate 10 μm.)

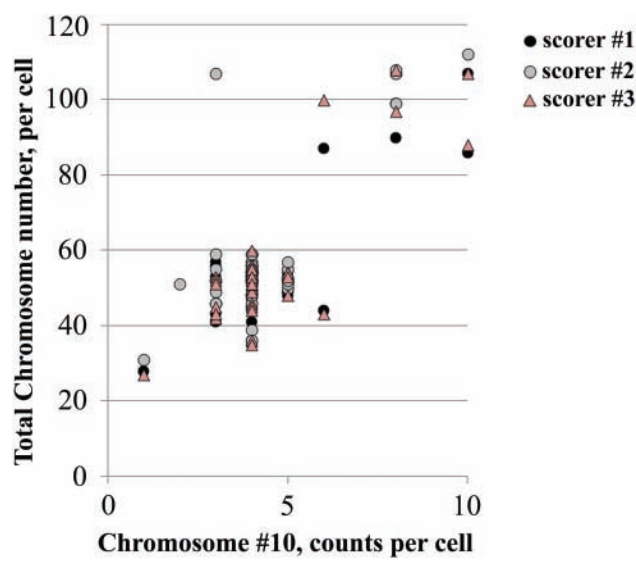


Figure 5. Culture genotype of chromosome 10 in a PTC cell line. Chromosome numbers and signal counts of the chromosome 10-specific DNA probe prepared from BAC RP11-168p20 hybridized to cell line S48TK18A6 (scored by three independent scorers).

The optimized chromosome 10 targeting probe (RP11-168p20) was then used to assay both normal and a cancer derived cell line (S48TK18, a PTC cell line that has been used extensively in our laboratories). Independent scoring by three different investigators gave highly corroborated results. This demonstrates the high quality of chromosome enumeration probe selection that can be accomplished with the aid of bioinformatics tools.

We additionally used all three chromosome-specific probes, chosen with the help of in silico screening and in vitro testing, in a typical diagnostic setting relevant to a clinical application in pre-natal diagnostics. The resultant tissue hybridizations provided clear, easily scorable chromosome enumeration images. A laboratory interested in investigating chromosomal status of placental tissue, or indeed any other tissue of interest, could make full use of the now publicly available bioinformatics resources and arrive at an applicable FISH-based chromosome enumeration assay for their own purposes.

Through the combination of modern web-based tools, such as the UCSC Genome Browser (<http://genome.ucsc.edu/>), the informatics resources at the Wellcome Trust

Table 2. Scoring RPII-168p20 (labeling chromosome 10) Signals and Total Chromosome Counts in Thyroid Cancer Cell Line S48TK18A6

	Group 1		Group 2	
	Average No. of Chromosomes	Average No. of Chr#10 Signals	Average No. of Chromosomes	Average No. of Chr#10 Signals
Scorer #1	5'–3'	4	94	8
Scorer #2	51	4	107	7
Scorer #3	50	4	100	8

Sanger Institute, Hinxton, United Kingdom, as well as the Resources for Molecular Cytogenetics, University of Bari, Italy, and the databases of commercial biotech companies offering specific high quality BAC probes such as Empire Genomics of Rochester, New York, it is now well within the capability of a basic cytogenetic laboratory to perform genotype investigations hitherto reserved for well-funded and well-staffed laboratories at the forefront of probe design and development.

In summary, bioinformatics techniques, including data mining, have not changed the fundamental principles of cytogenetic experiments. But they have provided a great tool for advancing the efficiency of such investigations, thereby making the technique more accessible to laboratories with less in-house expertise and funding, while simultaneously improving the process and outcome for all users.

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Authors' Note

The results have been presented in part in poster format at the 2011 and 2012 Annual Meetings of the Histochemical Society held at the Marine Biological Laboratory, Woods Hole, MA.

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