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## Full Karyotype Interphase Cell Analysis

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## **Abstract**

Aneuploidy seems to play not only a decisive role in embryonal development but also in tumorigenesis where chromosomal and genomic instability reflects a universal feature of malignant tumors. The cost of whole genome sequencing has fallen significantly, but it is still prohibitive for many institutions and clinical settings. No applied, cost-effective and efficient technique has been introduced yet aiming at research to assess the ploidy status of all 24 different human chromosomes in interphases simultaneously, especially in single cells. Here we present the selection of human probe DNA and a technique using multi-step FISH employing four sets of six labelled FISH probes able to delineate all 24 human chromosomes in interphase cells. This full karyotype analysis approach will provide additional diagnostic potential for single cell analysis. The use of spectral imaging (SI) has enabled the use of up to eight different fluorochrome labels simultaneously. Thus, scoring can be easily assessed by visual inspection, since SI permits computer-assigned and distinguishable pseudo-colors to each probe during image processing. This enables full karyotype analysis by FISH of single cell interphase nuclei.

## Introduction

Evaluating numerical abnormalities and structural aberrations of chromosomes by fluorescence *in situ* hybridization (FISH) has been extensively carried out on cell metaphases and interphases since this technique has been introduced in the mid-1980s.<sup>1</sup> Methodologies such as spectral karyotyping (SKY)<sup>2</sup> or multiplex FISH<sup>3</sup> and also comparative genomic hybridization (CGH)<sup>4</sup> were able to effectively evaluate cytogenetic damage on metaphases across the whole genome. Nowadays, modern techniques like high-resolution chip-based CGH arrays and next-generation sequencing (NGS), in particular massive parallel sequencing (MPS), are capable of evaluating a plethora of cytogenetic changes. For array CGH, segmental DNA copy number variations at kilobase-pair resolution can be detected;<sup>5</sup> while MPS is capable to analyze large parts of the genome by using shallow or low-pass whole genome sequencing when no coverage of the full genome is required, e.g. for preimplantation genetic diagnosis (PGD).<sup>6</sup>

In the early 2000s implantation rates had improved due to PGD aneuploidy screening by using commercially available chromosomal probe sets for single-cell analysis allowing the enumeration of up to ten chromosomes with fluorescence filter-based evaluation. Thereby, more than half of the numerical abnormalities seen in abnormal embryos originating from non-disjunction of chromosomes during cell division were covered.<sup>7-10</sup> Without a doubt, aneuploidy is the most common cause of chromosomal abnormalities in humans leading to pregnancy loss.<sup>11, 12</sup> Hence, shifting from partial karyotype FISH analysis to genome sequencing in the last decade allowed for simultaneous testing of numerous genetic aberrations and abnormalities. This was also evident when looking at the biopsied specimens, shifting away from polar bodies or blastomeres towards the trophectoderm.<sup>13</sup> In human embryos, multiple molecular mechanisms that may also be involved in cancer formation can lead to aneuploidy and chromosomal mosaicism, thus to negative pregnancy outcomes; however, low-level mosaicism in human development may be a normal feature after all.<sup>14</sup>

Aneuploidy seems to also play a crucial role in cells of the extra-embryonal tissue that are important in implantation during early pregnancy and the formation of the placenta. These so-called invasive cytotrophoblasts (iCTBs) showed different aneuploidy levels on the basis

of their invasive behavior when assessed by using spectral imaging targeting six different chromosomes.<sup>15</sup>

Aneuploidy seems to play not only a decisive role in embryonal development but also in tumorigenesis where chromosomal and genomic instability reflects a universal feature of malignant tumors.<sup>16</sup> It seems that the primary cause of pre-neoplastic / neoplastic genomic instability is the progression from stable diploid cells to unstable aneuploid cell species<sup>17</sup> making aneuploidy a useful marker of malignant transformation.<sup>18</sup>

Although the cost for sequencing the whole genome has fallen to around \$1,000 per analyzed genome<sup>19</sup> the price tag for equipment and material is quite high.<sup>20</sup> Even though larger hospital trusts, major universities and private biotech companies may have the funds to carry out high-throughput array chip methods and NGS on a daily basis, no applied, cost-effective and efficient technique has been introduced yet aiming at research to assess the ploidy status of all 24 different human chromosomes in interphases simultaneously, especially in single cells. Improving the coverage of all the chromosomes and devising sophisticated, fast and reliable methods to evaluate these single cells is favorable as not all of the possible numerical abnormalities can be currently assessed due to the limited number of available chromosome-specific probes and the limited number of suitable fluorochromes.<sup>21, 22</sup>

Here we present the selection of human probe DNA and a technique using multi-step FISH employing four sets of six labelled FISH probes able to delineate all 24 human chromosomes in interphase cells. This full karyotype analysis approach will provide additional diagnostic potential for single cell analysis.

## Material and Methods

### **Clone selection and DNA preparation**

BAC clones<sup>23</sup> from the RP11 library (Invitrogen, Gaithersburg, MD) were chosen based on information available from the UC Santa Cruz (UCSC) genome sequence database (<http://genome.ucsc.edu/cgi-bin/hgGateway>) and the U.S. National Institute of Health, National Center for Biotechnology Information (NIH/NCBI) ([http://www.ncbi.nlm.nih.gov/mapview/map\\_search.cgi?taxid=9606](http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9606)). Comprehensive DNA sequence information as well as structural organization of these BACs can be found in the above-mentioned databases. The preparation of DNA from BAC clones has been described in detail before.<sup>24</sup> Briefly, clones were cultured overnight in 10 ml Luria-Bertani (LB) medium containing 12.5 µg/ml chloramphenicol (Sigma, St. Louis, MO) and DNA was isolated using an alkaline lysis DNA extraction protocol.<sup>25</sup> In addition to BAC clones (see Table 1 for a complete overview) bacterial plasmid clones from the Weier lab at the LBL have been used. For the isolation of plasmids from clones RMC16L006<sup>26</sup>, pBS444/7, pBS864, pBS1131, pBS8B/9, pBS239'-5', pBS609/51, 680TA-4 and W21R2-TA13 a commercial Kit from Qiagen was used on an overnight LB culture containing 30 µg/ml ampicillin (RMC and pBS clones) or kanamycin (TA clones), respectively. Except for the RMC16L006 plasmid DNA, all other plasmid DNA has been employed as templates in PCR reactions. The final probe sets can be seen in Table 2.

For PCR, 100 ng genomic DNA (Sigma), BAC or plasmid DNA was used as template for DNA amplification. PCR reactions (50 µl) were performed using 0.02 U/ml Taq Polymerase (Invitrogen) or JumpStart Taq polymerase (Sigma) in 1x PCR buffer (Invitrogen), 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, and 0.6 mM of the forward and reverse primers (Qiagen; Alameda, CA). The chromosomes 1 and 6 alpha-satellite primers have been used as described previously.<sup>27</sup> The generation of chromosome 17 and 18 specific probes has also been previously published.<sup>28</sup> BlueScript primers WBS2 (ctc gga att aac cct cac taa agg) and WBS4 (gaa ttg taa tac gac tca cta tag) for the DNA amplification of alpha-satellite repeats were employed for chromosomes 8, 10, and 12. For chromosome 7 and 11, primers M13F / M13R<sup>29</sup> and WA8 (gat ggt agt agg ca[a/t] [c/g]t[c/a] aca gag) / WA9 (gat ggt agt agg cat c[a/c]c [a/c]aa g[a/t/c]a), respectively, have been used to amplify chromosome-specific DNA. For the amplification of chromosome 9 and 21 specific probe DNA a single primer was

in use for both, the satellite III primer W21R2 (caa acg tgc tca aag taa ggg aat g) and Jun15 (ccc aag ctt gca tgc gaa ttc), respectively. After an initial denaturation step of 2 min at 95 °C, 35 PCR cycles followed: denaturation at 95 °C for 40 sec, primer annealing at 54 °C for 1 min, and primer extension at 72 °C for 2.5 min. Ramp time was set to 30 sec for the first step followed by 1 min for the next two steps. A final step at 72 °C for 10 min concluded the PCR. PCR products were confirmed on a 2% agarose gel by applying 5 µl of the PCR reaction mixed with 1 µl of 0.4 g/ml sucrose solution.

### **DNA labeling and FISH**

Using a commercially available kit (BioPrime Kit, Invitrogen, Gaithersburg, MD), random priming was employed to label all the BAC, plasmid and PCR-derived probe DNA. After initial testing employing indirect labels;<sup>30-32</sup> the random priming process was slightly modified to incorporate various fluorochrome dUTPs into the probe DNA: Cy5-dUTP and Cy5.5-dCTP (Amersham, Arlington Heights, IN), DEAC-dUTP (PerkinElmer, Waltham, MA) as well as SpectrumGreen-dUTP, SpectrumRed-dUTP and SpectrumOrange-dUTP (Vysis, Abbott Molecular Inc, Des Plaines, IL). Regarding the four sets of chromosome-specific probes, Table 2 gives also an overview of their direct fluorescent labels.

For FISH, labeled probe DNA is mixed with blocking DNA and concentrated via precipitation in ethanol. Salmon sperm DNA is added to block non-specific binding of the probe, and human COT1 DNA is added to block repetitive DNA sequences in the probes from binding to sites spread throughout several chromosomes/loci. The desired combination of labeled probe is mixed using 2-5 µl of each individual probe, depending on intensity of signal, as previously described.<sup>15</sup>

This is then combined with 1 µl of human Cot-1 DNA® (1 mg/ml, Invitrogen), 1 µl of salmon sperm DNA (10 mg/ml; Invitrogen), and 7 µl of the hybridization master mix (78.6% formamide, 14.3% dextran sulfate in 1.43x SSC, pH 7.0; 20x SSC is 3 M sodium chloride, 300 mM tri-sodium citrate) and thoroughly mixed and denatured at 76 °C for 10 min. The hybridization mixture was then pre-annealed by incubating at 37 °C for 30 min (allowing the Cot-1 DNA® to anneal to non-chromosome specific DNA repeats on the probes). In parallel, the metaphase slides prepared from phytohemagglutinin (PHA)-stimulated peripheral blood

lymphocytes from a karyotypically normal male<sup>31</sup> were denatured for 3 min at 76 °C in 70% formamide/2x SSC, pH 7.0, dehydrated in 70%, 85%, and 100% ethanol for 2 min each, and allowed to air-dry. The hybridization mixture was then carefully applied to the slides, covered with a 22x22 mm<sup>2</sup> cover-slip and sealed with rubber cement. Slides were incubated overnight in a moist chamber at 37 °C. After removing rubber cement and the cover-slips, the slides were washed in 0.1x SSC at 43°C for 2 min, then, when biotin or digoxigenin labels have been used, incubated in PNM blocking reagent (5% nonfat dry milk powder (NESTLÉ Carnation, Wilkes-Barre, PA), 1% Nonidet-P40 (Sigma), 1% sodium azide (Sigma), 0.1 M sodium phosphate buffer, pH 8.0) for 10 min at room temperature. Bound probes were detected with fluorescein-conjugated avidin (avidin DCS, Vector labs, Burlingame, CA) and rhodamine-labeled anti-digoxigenin antibodies (Roche Diagnostics, Indianapolis, IN). In the case of direct-labeled probes, no immuno-detection step was necessary. Finally, after a last wash in PN or 2xSSC the slides were mounted with 4',6-diamidino-2-phenylindole (DAPI, 0.5 µg/ml; Calbiochem, La Jolla, CA) in antifade solution.<sup>32, 33</sup>

### ***Image acquisition and analysis***

Fluorescence microscopy was performed on a Zeiss Axioskop microscope equipped with a SKY filter set (ChromaTechnology, Brattleboro, VT) for simultaneous observation of SKY suitable fluorochromes and also a DAPI filter (ChromaTechnology, Brattleboro, VT) for the detection of the counter stain. Images were collected using a cooled CCD camera (CCD-1300DS, VDS Vosskuehler, Osnabrück, Germany).<sup>24</sup> Further processing and printing of the images were done using the image processing software Adobe Photoshop (Adobe Systems Inc., San Jose, CA).



**Results**

Our probe sets have been constructed by choosing the most suitable probe DNA and fluorochrome so that each chromosome-specific probe within its set provides specificity and similar high efficiency. The probes used (see Tables 1 and 2 for a complete overview) are either locus-specific or repeat-specific (targeting alpha-satellites or satellite III). They are labeled with the following fluorescent labels: DEAC (excitation wavelength: 432 nm / emission wavelength: 472 nm, light blue), SpectrumGreen (497 nm / 524 nm, greens), SpectrumOrange (559 nm / 588 nm, orange), SpectrumRed (592 nm / 612 nm, red), Cy5 (649 nm / 666 & 670 nm, infrared), and Cy5.5 (675 nm / 694 nm, infrared).

Sets I and II detect chromosomes 13, 14, 16, 20, 21, and 22 as well as of chromosomes 15, 17, 18, 19, X, and Y, respectively (Figure 1) according to their fluorochrome labels (Figure 2), whereas sets III and IV are able to evaluate the rest of the chromosomes for the full karyotype by detecting chromosomes 2, 3, 4, 5, 9, and 12 as well as chromosomes 1, 6, 7, 8, 10, and 11, respectively (Figure 3). All the signals produced in metaphases and interphases by FISH are unambiguous, strong and do not cross-hybridize to other chromosomes (Figures 1 and 3). The exception was chromosome 9 in set III (Figure 3) which did produce a strong signal when tested for itself but was rather dim when co-hybridized with the rest of set III probes.

## Discussion

Detection techniques for assessing numerical abnormalities and other cytogenetic aberrations often utilizes cost-effective and rapid classical staining methods such as Giemsa staining for karyotyping, but also molecular diagnostic tools like FISH and its multiple variations for a quick, robust and reliable detection of genetic damage. During the last decade, methodology development has been progressing towards a full karyotype analysis;<sup>34</sup> however, adjusting probe sets, i.e. probe target and fluorochrome label, rapidly to particular needs in the laboratory is still quite difficult. Using BAC clones as a source of probe DNA for FISH is cheap and effective<sup>28, 35, 36</sup> on the other hand allows the analysis of the whole genome<sup>37, 38</sup> for cytogenetic diagnoses. However, this metaphase-specific approach cannot be used for analysis of a single cells *per se*, as they are likely to be in an interphase stage.

While several bright chromosome-specific DNA repeat probes have been prepared and cloned by our labs in previous years,<sup>30, 39</sup> the approach does not work for all human chromosomes. The acrocentric chromosomes 13 and 21, for example, share a high level homologous of sequences which is found heteromorphic in some individuals.<sup>40</sup> The BAC approach<sup>41</sup> has advantages for complete chromosome enumeration in interphase cells. During preparation of this manuscript, Ioannou *et al.* demonstrated this using BAC clones from Roswell Park Cancer Institute RP-11 library. We decided to combine preexisting DNA repeat probes with optimized BAC contigs which identified using bioinformatics<sup>22, 42</sup> to obtain optimal specificity and brightness (Table 2).

Now, combining the versatility of BACs and preexisting repeat probes with a wide repertoire of different fluorochromes together with the use of the SKY system resulted in a cost-effective, flexible and reliable methodology to detect four sets of six probes in interphase nuclei (see Figures 1 and 3). Incorporating consecutive hybridization steps (cell recycling) considerably increased the diagnostic range of existing FISH technologies.<sup>43</sup>

Sequential multi-locus interphase FISH is one strategy where chromosome specific (locus-specific and alpha-centromeric) FISH probes have been hybridized sequentially to the same cell, initially done in formalin fixed and paraffin embedded histological sections from tumor tissues.<sup>44</sup> The use of spectral imaging (SI) has enabled the use of up to eight different

fluorochrome labels (with emission spectra from 450-1000 nm) simultaneously.<sup>45</sup> The scoring can be easily assessed by visual inspection, since SIm permits computer-assigned and distinguishable pseudo-colors to each probe during image processing.<sup>2</sup> This led to the full karyotype analysis by FISH of single cell interphase nuclei like those of iCTBs in placental cell analysis (data not shown in this publication).

The benefits of combing sets of single copy DNA probes with separate sets of DNA repeat probes that contain super-bright signals in sequential hybridizations may raise concerns of DNA loss in repeated cycles of denaturation, hybridization and wash steps. The approach presented here does not eliminate potential problems associated with DNA losses, but optimized BAC contigs and plasmids targeting highly reiterated DNA repeat sequences consistently leads to brighter signals, thus reducing the negative effect of said losses.

As seen in Figure 3, the flexibility of choosing probe DNA and fluorochromes may have an unforeseen negative consequence, chromosome 9 showed a very dim signal which was below the threshold of detection for the SKY system, even though the same probe resulted in good strong signals when tested individually. Hence, either cell-type or donor differences as well as the interaction of DNA probes with each other within a set could lead to a less prominent or even a very dim and almost not visible signal. Thus, quality control when tailoring such probe sets to work in particular cell types is imperative.

In this publication, we presented the development of a probe collection made up of four sets of six labeled chromosome-specific FISH probes which can easily be modified towards three sets of eight probes using far-infrared fluorochromes like Cy7. These have been arranged so that the first two sets detect the most prevalent numerical abnormalities observed in human embryos, and the last two sets fill in the gap towards a full karyotype analysis. The generation of these probe sets shows the full potential of BAC/plasmid clones, which can be rapidly selected and tailored for specific genetic screening applications. The strong signal intensities from these repeat-rich probes and the labeling methodology employed, allows reduction in the costs for a single hybridization event by approximately 10-fold over conventional FISH.

In a clinical PGD setting, there is no apparent reason to do further analysis using multiple FISH probe sets after determining an abnormal ploidy status such as trisomy 13; however, if information on all 24 different chromosomes for individual embryos, i.e. the discarded chromosomally abnormal embryos, can be collected, this could be very important information for early embryo development, and this information may be useful for future clinical diagnosis. Also, with regard to array and NGS analysis in a clinical PGD setting, 250-500 ng of DNA, an equivalent of about 35,000-70,000 cells, would be required for analysis. This entails that if there is only a limited number of cells (i.e. blastocytes) whole genome amplification will have to be applied increasing time and cost but also errors.<sup>46</sup> As our 24-probe set was originally developed for and applied to score all 24 chromosomes in placental invasive cytotrophoblasts (CTBs), we have found that at least 75% of the male invasive CTBs have gained extra copies of chromosomes with the most common gains being acrocentric chromosomes. Also, most of the invasive CTBs did not have the same karyotype (unpublished data). The probes that have been developed and tested are typically very useful to do full karyotyping on the few interphase cells, such as cancer stem cells, fetal cells in maternal blood, or heterogeneous cells with different karyotypes.

The set of 4x6 chromosome enumerating FISH probes has been initially developed in-house to study invasive placental cells and to aid in PGD due to the fact that only some commercial probes are available as enumeration sets with a maximum of four different colors. There are many clinical applications for DNA probe sets like the ones described here, i.e. prenatal analysis of aneuploidy. But for example, if one wishes to study malignant mesothelioma, the panel would include a probe for *ERBB2*.<sup>47</sup> Similarly, overexpression due to potential amplifications of the epidermal growth factor receptor (*EGFR*) gene in squamous carcinoma cell lines might include an EGF receptor-specific DNA probe.<sup>48</sup> Hence, it is very conceivable that our set of probes, original or modified, could find a use in other studies, such as in tumor cytogenetic evaluations or chromosome analysis in genotoxicology and mutagenesis, or whenever quick chromosome analysis of numerical abnormalities in interphase cells is required.

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### **Competing Interest Statement**

The authors declare they have no competing interests.

### **Author Contribution Statement**

UW & BO designed the study, AB & UW created and optimized the DNA probes using BACs, plasmids and PCR; AB, CH, AP & JW optimized fluorescent labels and carried out fluorescence in situ hybridization and spectral imaging as well as analysis of single probes and set of probes, AB, BO, UW & JW drafted the manuscript. All authors have read and approved the final manuscript.

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## **Table legends**

### **Table 1 legend**

Table 1 shows a complete overview of BAC clones and bacterial plasmid clones used.

### **Table 2 legend**

Table 2 shows the final probe set used.

It also gives an overview of the direct fluorescent labels for the four sets of chromosome-specific probes.

**Table 1**

| Locus                | Probe name  | Clones/DNA  | End sequence 1 | End sequence 2 | Full sequence ID |
|----------------------|-------------|-------------|----------------|----------------|------------------|
| 13q21.31             | OR7E156P    | RP11-527N12 | -              | -              | AL354810         |
|                      |             | RP11-282D7  | -              | -              | AL355609         |
|                      |             | RP11-320N6  | -              | -              | AL359208         |
|                      |             | RP11-67L17  | -              | -              | AL354774         |
|                      |             | RP11-473M10 | -              | -              | AL445989         |
|                      |             | RP11-394A14 | -              | -              | AL445238         |
|                      |             | RP11-520F9  | -              | -              | AL355879         |
|                      |             | RP11-205B18 | -              | -              | AL354736         |
| 14q13.3              | PAX9        | RP11-12H15  | B75808         | B75809         | -                |
|                      |             | RP11-150O18 | AQ378665       | AQ378667       | -                |
|                      |             | RP11-452H6  | AQ583099       | AQ583102       | -                |
|                      |             | RP11-381L10 | AQ532441       | AQ554647       | -                |
|                      |             | RP11-73H19  | AQ266602       | AQ266604       | -                |
|                      |             | RP11-49P15  | AQ051953       | AQ051955       | -                |
|                      |             | RP11-151J2  | AQ379285       | AQ379286       | -                |
|                      |             | RP11-410J4  | AQ549717       | -              | -                |
| 16qh, sat II         | RMC16L006   | RMC16L006   | -              | -              | X06138           |
| 20p11.1-11.2         | SRCext      | RP11-298O1  | AQ507400       | AQ507403       | -                |
|                      |             | RP11-465M13 | AQ636482       | -              | -                |
|                      |             | RP11-192N1  | AQ412321       | AQ412322       | -                |
|                      |             | RP11-151C5  | AQ376308       | AQ376305       | -                |
|                      |             | RP11-451G10 | AQ583252       | AQ583256       | -                |
|                      |             | RP11-103B19 | AQ313159       | AQ313162       | -                |
|                      |             | RP11-467A7  | AQ637700       | AZ516714       | -                |
|                      |             | RP11-99B19  | AQ318386       | AQ318387       | -                |
| RP11-76O8            | AQ266982    | AQ266948    | -              |                |                  |
| 21q22                | D21S167     | YAC 141G6   | -              | -              | X52289           |
| 22q11.22-q11.23      | BCR         | RP11-357H16 | AZ518881       | AQ553050       | -                |
|                      |             | RP11-62K15  | AQ199674       | AQ199676       | -                |
|                      |             | RP11-164N13 | AQ380113       | AQ380117       | -                |
|                      |             | RP11-116G21 | AQ348695       | AQ348692       | -                |
| 15q25.3              | NTRK3ext    | RP11-113C11 | AQ344985       | AQ344986       | -                |
|                      |             | RP11-96B23  | AQ313684       | AQ313681       | -                |
|                      |             | RP11-114I9  | AQ344858       | AQ344856       | -                |
|                      |             | RP11-285I14 | -              | -              | AC011966         |
|                      |             | RP11-427O16 | -              | -              | AC023844         |
|                      |             | RP11-356B18 | -              | -              | AC009711         |
|                      |             | RP11-247E14 | -              | -              | AC087593         |
|                      |             | RP11-97O12  | -              | -              | AC013489         |
| 17cen, $\alpha$ -sat | 17cen       | RP11-285M22 | -              | -              | AC131274         |
| 18cen, $\alpha$ -sat | 18cen       | genomic DNA | -              | -              | M65181           |
| 19q13.2              | AXL         | CTD-2052L21 | AQ270406       | AQ235108       | -                |
|                      |             | CTD-2288H11 | B98832         | -              | -                |
|                      |             | CTD-2195C23 | -              | -              | -                |
|                      |             | CTD-2017C04 | -              | -              | -                |
|                      |             | CTD-2195B23 | -              | -              | AC011510         |
|                      |             | CTD-2218D21 | AQ032786       | -              | -                |
|                      |             | CTD-2218D21 | AQ032786       | -              | -                |
| Xq21                 | CYCL1       | RP11-422C9  | AQ553389       | AQ553391       | -                |
|                      |             | RP11-475A12 | AQ635998       | AQ636000       | -                |
|                      |             | RP11-14A18  | B76225         | B82244         | -                |
| Yq12                 | RP11-242E13 | RP11-242E13 | -              | -              | AC068123         |
| 1cen, $\alpha$ -sat  | 1cen        | genomic DNA | -              | -              | -                |
| 2p16.1-15            | RELext      | RP11-65A9   | AQ237129       | AQ237131       | -                |
|                      |             | RP11-139C21 | AQ382574       | AQ382578       | -                |
|                      |             | RP11-71D7   | AQ236987       | AQ267891       | -                |
|                      |             | RP11-373L24 | -              | -              | AC010733         |

|                      |           |             |          |          |          |
|----------------------|-----------|-------------|----------|----------|----------|
|                      |           | RP11-375M18 | AQ533441 | AQ551245 | -        |
|                      |           | RP11-418N22 | AQ550069 | AQ550072 | -        |
|                      |           | RP11-77P21  | AQ284573 | AQ284575 | -        |
|                      |           | RP11-477N2  | AQ637330 | AQ637326 | -        |
| 3q27.3               | BCL6      | RP11-143D11 | -        | -        | AC092103 |
|                      |           | RP11-567G11 | -        | -        | AC104635 |
|                      |           | RP11-88P6   | -        | -        | AC018919 |
|                      |           | RP11-211G3  | -        | -        | AC072022 |
|                      |           | RP11-58M14  | AQ199229 | AQ199231 | -        |
|                      |           | RP11-120O8  | AQ350519 | AQ350515 | -        |
| 4q22.1               | TIGD2ext  | RP11-1E24   | B48349   | AQ312932 | -        |
|                      |           | RP11-502A23 | -        | -        | AC079141 |
|                      |           | RP11-84C13  | -        | -        | AC104785 |
|                      |           | RP11-173C9  | -        | -        | AC105388 |
|                      |           | RP11-549C16 | -        | -        | AC093862 |
|                      |           | RP11-15F14  | B76416   | B76417   | -        |
|                      |           | RP11-115D19 | -        | -        | AC097478 |
|                      |           | RP11-67M1   | -        | -        | AC093759 |
|                      |           | RP11-350B19 | -        | -        | AC105445 |
|                      |           | RP11-176N15 | -        | -        | AC108038 |
| 5q23.1               | 05BP1-S47 | RP11-183D16 | -        | -        | AC093781 |
|                      |           | RP11-23E11  | B86433   | B86434   | -        |
|                      |           | RP11-254M1  | AQ479016 | AQ479018 | -        |
|                      |           | RP11-464H3  | AQ586366 | AZ515952 | -        |
|                      |           | RP11-133L2  | AQ350910 | AQ350911 | -        |
|                      |           | RP11-59G17  | AQ194868 | AQ194864 | -        |
|                      |           | RP11-42O22  | AQ116158 | AQ046673 | -        |
|                      |           | RP11-185N19 | -        | -        | AC021224 |
| 12cen, $\alpha$ -sat | 12cen     | 444/7       | -        | -        | G03348   |
| 6cen, $\alpha$ -sat  | 6cen      | 864         | -        | -        | G04505   |
| 7cen, $\alpha$ -sat  | 7cen      | 680TA-4     | -        | -        | AJ295152 |
| 8cen, $\alpha$ -sat  | 8cen      | 8B/9        | -        | -        | M64779   |
| 9cen, sat III        | 9cen      | W21R2-TA13  | -        | -        | X06137   |
| 10cen, $\alpha$ -sat | 10cen     | 609/51      | -        | -        | X63622   |
| 11cen, $\alpha$ -sat | 11cen     | 238'-5'     | -        | -        | M21452   |

**Table 2**

| Set | Locus                | Position (Mbp)  | Probe type          | Label     | Color  | Clone/Contig ID |
|-----|----------------------|-----------------|---------------------|-----------|--------|-----------------|
| I   | 13q21.31             | 62.520-63.638   | BAC pool of 8 BACs  | Cy5       | IR1    | OR7E156P        |
| I   | 14q13.3              | 35.678-36.975   | BAC pool of 8 BACs  | DEAC      | aqua   | PAX9            |
| I   | 16qh, sat II         | -               | Plasmid             | Cy5.5     | IR2    | RMC16L006       |
| I   | 20q11.1-11.2         | 34.950-36.152   | BAC pool of 9 BACs  | Sp.Red    | red    | SRCext          |
| I   | 21q22                | around 37.770   | PCR product         | Sp.Green  | green  | D21S167         |
| I   | 22q11.22/23          | 21.545-22.085   | BAC pool of 3 BACs  | Sp.Orange | orange | BCR             |
| II  | 15q25.3-26.1         | 85.745-86.975   | BAC pool of 7 BACs  | Sp.Orange | orange | NTRK3ext        |
| II  | 17cen, $\alpha$ -sat | -               | PCR product         | Sp.Red    | red    | 17cen           |
| II  | 18cen, $\alpha$ -sat | -               | PCR product         | Cy5.5     | IR2    | 18cen           |
| II  | 19q13.2              | around 46.500   | BAC pool of 6 BACs  | Sp.Green  | green  | AXL             |
| II  | Xq21                 | 82.447-82.915   | BAC pool of 3 BACs  | Cy5       | IR1    | CYCL1           |
| II  | Yq12                 | 57.158-57.256   | Single BAC          | DEAC      | aqua   | RP11-242E13     |
| III | 2p16.1-15            | 60.525-61-831   | BAC pool of 9 BACs  | Sp.Red    | red    | RELext          |
| III | 3q27.3               | 188.590-188.976 | BAC pool of 6 BACs  | Sp.Green  | green  | BCL6            |
| III | 4q22.1               | 90.168-91.675   | BAC pool of 10 BACs | Sp.Orange | orange | TIGD2ext        |
| III | 5q23.1               | 116.279-117.541 | BAC pool of 7 BACs  | DEAC      | aqua   | 05BP1-S47       |
| III | 9cen, sat III        | -               | PCR product         | Cy5.5     | IR2    | 9cen            |
| III | 12cen, $\alpha$ -sat | -               | PCR product         | Cy5       | IR1    | 12cen           |
| IV  | 1cen, $\alpha$ -sat  | -               | PCR product         | Cy5.5     | IR2    | 1cen            |
| IV  | 6cen, $\alpha$ -sat  | -               | PCR product         | Sp.Green  | green  | 6cen            |
| IV  | 7cen, $\alpha$ -sat  | -               | PCR product         | Sp.Red    | red    | 7cen            |
| IV  | 8cen, $\alpha$ -sat  | -               | PCR product         | DEAC      | aqua   | 8cen            |
| IV  | 10cen, $\alpha$ -sat | -               | PCR product         | Sp.Orange | red    | 10cen           |
| IV  | 11cen, $\alpha$ -sat | -               | PCR product         | Cy5       | IR1    | 11cen           |

## Figure legends

### Figure 1

(A) FISH probe set I (see Table 2) hybridized on one metaphase spread from normal male lymphocyte and (B) one interphase nucleus. Computer assigned pseudo-colors can be seen showing two copies each of chromosomes 13, 14, 16, 20, 21, and 22. (C) FISH probe set II (see Table 2) hybridized on one metaphase spread from normal male lymphocyte and (D) one interphase nucleus. It showed two copies each of chromosomes 15, 17, 18, 19, and one copy of chromosome X and Y. The size bars on panels A and C indicate 5  $\mu\text{m}$  while those on panels B and D indicate 2.5  $\mu\text{m}$ .

### Figure 2

(A) The emission spectra of DEAC, Spectrum Green, Spectrum Orange, Spectrum Red, Cy5, and Cy5.5 can be seen in this graph. (B) By using the distinguished emission spectra of these fluorochromes saved in a classified file the SKY system can easily identify individual chromosomes and create a karyotype. This is an example of an abnormal female cell hybridized with probe set II showing four copies each of chromosomes 15 and 19, three copies of chromosome X, and two copies each of chromosomes 17 and 18.

### Figure 3

(A) FISH probe set III (see Table 2) hybridized on one normal male interphase nucleus (RGB colors). (B) The corresponding classified image from SKY system (pseudo-colors) showed two copies each of chromosomes 2, 3, 4, 5, and 12. The chromosome 9-Cy5.5 probe developed in-house showed weak hybridization signals and was not detected by the SKY system. (C) FISH probe set IV (see Table 2) hybridized on one normal male interphase nucleus (RGB colors). (D) The corresponding classified image from SKY system (pseudo-colors) showed two copies each of chromosomes 1, 6, 7, 8, 10, and 11. The size bar on panel A indicates 2.5  $\mu\text{m}$  and is representative of all panels.

Figures

Figure 1

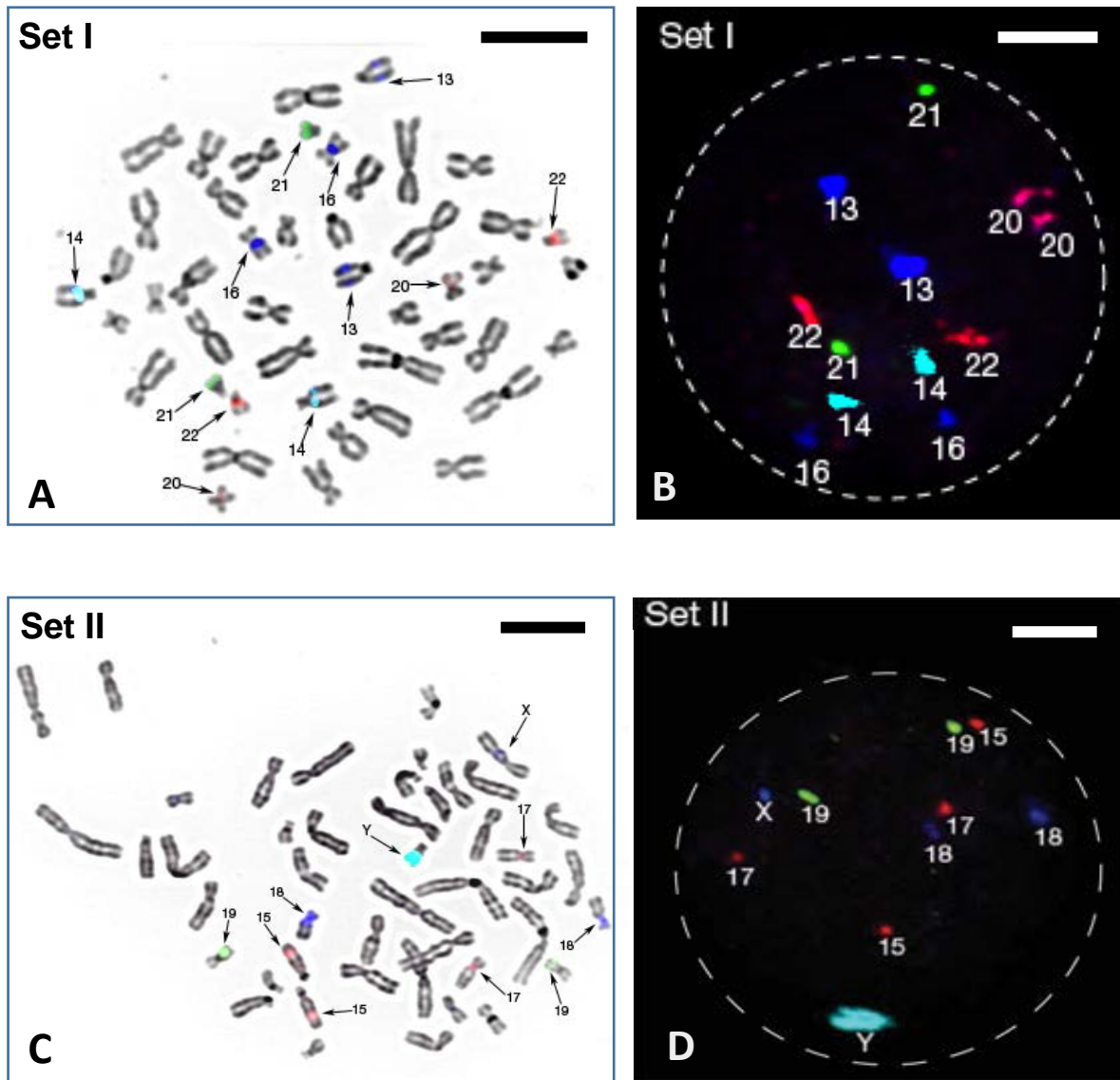




Figure 2

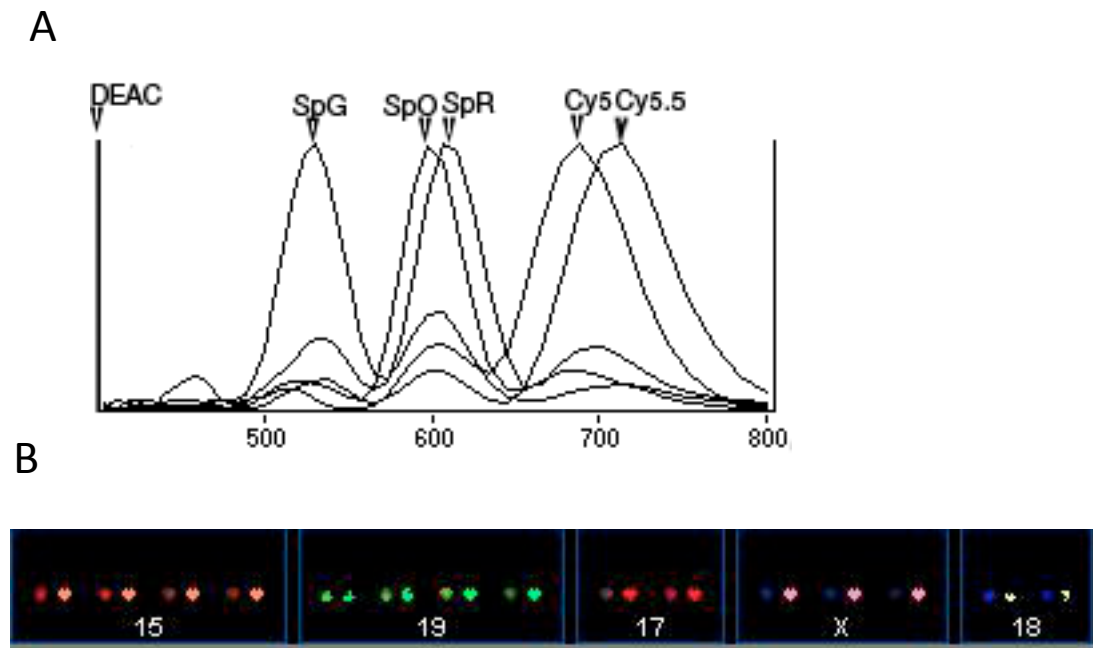


Figure 3

