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In-house production of Fluorescence in Situ Hybridization probes for alpha-satellite centromeric region for detection of chromosomal aneuploidy

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Abstract: Fluorescence in situ hybridization (FISH) depends on the complementary nature of the labeled probe and its targeted chromosomal segment. FISH technique allows the detection of numerical chromosomal aneuploidy by using the centromeric probes. Numerical aberrations represent a significant percentage of chromosomal abnormalities and is a main cause of pregnancy loses and cancer. The present study aimed to produce in-house FISH centromeric probes that help in the identification of numerical chromosomal aberrations (copy number variants) and can be used as a control probe. In this study, the probes were produced from human genomic DNA using Polymerase Chain Reaction (PCR) with specific primers. PCR was used for amplification and labeling of alpha satellite centromeric probes of chromosomes 1 and 7. The alpha satellite centromeric probes allow us to identify the numerical abnormalities of its corresponding chromosome. All the produced probes were sensitive and specific for the detection of chromosomal copy number variants. To our knowledge this is the first time to produce FISH probes in Egypt; our next plan is to produce double color probes for specific locus.

Keywords: Fluorescence In Situ Hybridization (FISH), alpha satellite centromeric probe, aneuploidy, copy number variants (CNV), probe production.

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1. INTRODUCTION

Fluorescence in situ hybridization is a powerful technique that identify a known chromosome sequence with labeled DNA probes, thus enabling visualization of specific regions of chromosomes by fluorescent microscopy (1,2). FISH probe are sets of short segments of DNA labeled by fluorescent nucleotides, which matches to its corresponding sequence in the target nucleic acid (3,4). Most of the probes used are commercial and are expensive and only available for well-known genetic diseases (3). Scientists are interested to improve the design of the probes, to augment the fluorescent signal intensity and to inhibit the presence of cross-hybrid (5). Probe production depends on the availability of the source of DNA template source as DNA clones or the suitable DNA sequences for PCR amplification (6).These DNA fragments could be directly labeled by nick translation, random primed labeling, and PCR to incorporate nucleotides coupled with different fluorophores (1,7). The length of probe fragments should be between 200 and 500bp to allow effective sample penetration and hybridization to the target region (3).

Fish probes can be classified into three groups according to the targeted regions: locus-specific probes, repetitive sequence probes and whole chromosome paint probe (1).

The choice of the FISH probe takes into consideration the diseases and the chromosomal abnormality of the region of interest (7). Whole chromosome probes or centromeric and telomeric probes are present commercially for every human

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chromosome. Thought, the variety of locus-specific probes available is significantly more restricted (8).

Repetitive DNA probes (e.g., alpha-satellite) hybridize to particular sequences in the centromere are used in chromosome enumeration (9). Alpha satellite is a tandem repetitive DNA sequence which includes 5% of the genome. It consists of a DNA replicates built of a 171 bp monomer. Monomers sequences are 50%–70% similar and are located in all centromeres. A well-known repeats of monomer are arranged in chromosome specific higher order repeats (HORs) which are repeated numerous times. The size of the HOR (i.e., repeats of monomer) causes the chromosome specificity (10). α-Satellite DNA probes anneal to numerous replicas of the repetitive sequence in the centromeres producing two very bright fluorescent signals in the studied metaphase or interphase of a diploid cells, which makes centromere specific probes mainly appropriate for the detection of aneuploidies (11). Also it can be used as a control for locus specific probes on the same chromosome using double color probes (12).

Aneuploidy, the numerical chromosomal aberration, is a very common cause of most human cancers and the most important cause of miscarriage and neonatal birth defects. The increased level of aneuploidy affects many factors of aggressiveness in cancers, as well as poor prognosis, metastatic spread, and resistance to therapy (13,14). It is also the main cause of inherited birth defects and sterility. Therefore, it is required to monitor these birth defects and chromosomal abnormalities by identifying numerical chromosomal aberration using centromeric probes (15).

The present study aimed to produce some in-house FISH probes to help in the detection of chromosomal abnormalities, and to overcome the high price of some commercially available probes.

2. METHODS

The centromere-specific probes were produced for chromosomes 1 and 7, which are used to detect copy number variation of chromosomes and as a control probe. The PCR technique was applied for both amplification and labeling of alpha-satellite sequences using human genomic DNA as a template.

2.1. Extraction of Human genomic DNA:

Peripheral blood samples were taken from healthy donors. The Human genomic DNA was extracted using QIAamp genomic DNA Kit (Qiagen, according to the manufacturer's recommendations).

2.2. Optimization of each chromosome 1 and 7 amplification with different DNA concentrations and different annealing temperature:

PCR was performed with chromosome-specific alphoid primers for chromosome 1& 7 centromeres according to Dunham et al.(1992) with some modification (16).

To produce chromosome 1 centromeric probe optimization was done using different concentrations of human genomic DNA (100 & 200 ng) with forward primer: 5'GGCCTATGGCAGCAGAGGATATAACTGCC 3' and reverse primer: 5'GTGAGTTTTCTCCCGTATCCAACGAAATCC 3'. In a total volume of 25µl containing 2.5 units of Recombinant DNA polymerase, 1 µM of each primer pair, 2.5mM $MgC1_2$, 200 µM of each dATP, dCTP, dGTP, and 100 µM of each dTTP with spectrum Red 598 dUTP (Enzo Life Sciences). Initial denaturation at 95˚C for 5 min, 35 cycles: denaturation at 95˚C for 30 sec, different annealing temperature at (66, 67 and 68°C) according to Tm of primer sequence for 30 sec and extension at 72˚C for 30 sec followed by final extension at 72°C for 5 min.

Regarding to chromosome 7 centromeric probe production, optimization was done using different concentrations of human genomic DNA (100 & 200 ng) with forward primer: 5'TTCATTGGAATCGCGAATAC3' and reverse primer: 5'CAAGAAGGCTTCAAAGCACC3'. In total volume 25µl containing 1 µM of each primer pair, 2.5mM MgC12, 200 µM of each dATP, dCTP, dGTP, 100 µM of each dTTP, spectrum Red 598 dUTP (Enzo Life Sciences), and 2 units of Recombinant DNA polymerase. Initial denaturation at 95˚C for 5 min, 35 cycles: denaturation 95˚C for 30 sec, different annealing temperature at (62, 63 and 64°C) according to Tm of primer sequence for 30 sec and extension at 72˚C for 30 sec followed by final extension at 72°C for 5 min.

2.3. Precipitating the Probe

The probe was precipitated using 0.1 volumes (2µl) of 3 M sodium acetate (pH 5.2) and 2.5 volumes (52.5µl) absolute ethanol, 10 µg (10µl) Cot-1 DNA, and 200 ng (10µl) of probe placed in ice box for 15 min. The mixture was centrifuged for 30 min at 12000 rpm at 4˚C. In order to evaporate all the solution Concentrator plus (Eppendorf, Germany) was used at 30°C for 2 hours. It was left in an open eppendorf tube overnight to insure complete evaporation.

The precipitated probe pellet was dissolved by 8µl hybridization buffer and 2µl distilled water and centrifuged well then stored at -20˚C until used.

2.4. **Performing FISH with the labeled probe**

Routine method was used to yield metaphase chromosome spreads from phytohemagglutinin-stimulated normal human peripheral blood lymphocyte cultures (17). Slides were aged in 2X SSC, pH 7.0, at 37°C for 20 min, dehydrated through an ethanol series (70%, 80%, and 95%) at room temperature for 2 min each, air-dried, then 10 µl of probe were added to the marked region and covered with coverslip. For hybridization, the slides with the normal metaphase and the labeled probe were denatured together at 75°C for 10 minutes then at 37 °C for 16 hour in ThermoBrite Denaturation/ Hybridization unit (Abbott). Afterwards, the coverslip was removed, and then the slides were immersed in pre-warmed 0.4X SSC at 75 °C in a water bath for 5 minutes. Then, they were immersed in 2X SSC solutions with 15 µl NP-40 for 2 minutes, followed by 2X SSC solutions for another 2 minutes, and finally in PBS solution for another 2 minutes and allowed to air dry. DNA was counterstained with 10 µl of DAPI antifade solution (Cytocell).The produced probes were examined using the fluorescence microscope (Zeiss Axio Imager Z2) and Isis Fluorescence Imaging System(18).

Tests for specificity and sensitivity were done on normal metaphases for all the produced probes and the results were comparing to those of the commercial probes was done.

3. RESULTS

Two centromeric probes for chromosomes 1 and 7 were produced using PCR technique for both amplification and labeling of human genomic DNA and labeled with spectrum Red 598 dUTP.

PCR amplification results of specific alpha satellite centromeric regions of chromosome 1 at different annealing temperatures which gave a weak amplification with a band size of 201bp at annealing temp 66°C, strong amplification band at annealing temp 67°C and no band at annealing temp 68°C was detected. It produced a weak amplification with a band size of 201 bp when using DNA concentration of 100 ng and strong amplification band when using DNA concentration of 200 ng.

PCR amplification results of specific alpha satellite centromeric regions of chromosome 7 at different annealing temperature which gave no amplification at annealing temp 62°C and 63°C but it showed a weak amplification with band size of 348 bp at annealing temp 64°C. It produced a weak amplification with a band size of 348 bp when using DNA concentration of 100 ng and strong amplification band when using DNA concentration of 200 ng.

FISH was done on normal metaphases to confirm that the probe produced two centromeric signals corresponding to the two copies of its chromosome. The FISH results of the in-house produced probes were compared to FISH results of commercial probes and they gave similar results. The produced probes were tested several timed to confirm for it sensitivity and specificity. Each of chromosomes 1and 7 centromeric probes hybridized specifically to the centromere of chromosome 1 and 7 respectively which proved it specificity, and produced detectable signals in each time the test was repeated.

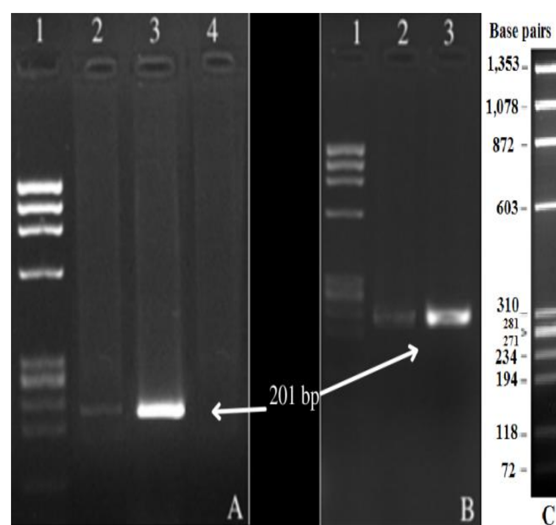

Figure 1: PCR amplification results of specific alpha satellite centromeric regions of chromosome 1. (A) PCR amplification result of specific alpha satellite centromeric regions of chromosome 1 at different annealing temperature. Lane 1 represents ΦX174 DNA- Hae III Digest marker, lane 2 represents weak amplification with band size 201 bp at annealing temp 66°C, lane 3 represents strong amplification band at annealing temp 67°C and lane 4 represents no band at annealing temp 68°C. (B) PCR amplification result of specific alpha satellite centromeric regions of chromosome 1 at different DNA concentration. Lane 1 represents ΦX174 DNA- Hae III Digest marker (Thermo Scientific, Cat. No.**:** SM0251), lane 2 represents weak amplification with band size of 201 bp when using DNA concentration of 100 ng and lane 3 represents strong amplification band when using DNA concentration of 200 ng. (C) ΦX174 DNA- Hae III Digest marker with its bands size (Thermo Scientific, Cat. No.**:** SM0251).

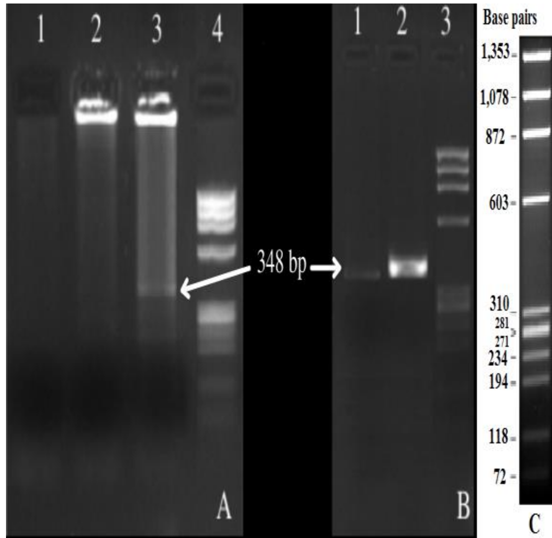

Figure 2: PCR amplification results of specific alpha satellite centromeric regions of chromosome 7. (A) PCR amplification result of specific alpha satellite centromeric regions of chromosome 7 at different annealing temperature. Lane 1 represents no amplification band at annealing temp 62°C, lane 2 represent no amplification band at annealing temp 63°C represents, lane 3 represents weak amplification with band size 348 bp at annealing temp 64°C and lane 4 represents ΦX174 DNA- Hae III Digest marker. (B) PCR amplification result of specific alpha satellite centromeric regions of chromosome 7 at different DNA concentration. Lane 1 represents weak amplification with band size of 348 bp when using DNA concentration of 100 ng, lane 2 represents strong amplification band when using DNA concentration of 200 ng and lane 3 represents ΦX174 DNA- Hae III Digest marker. (C) ΦX174 DNA- Hae III Digest marker with its bands size (Thermo Scientific, Cat. No.**:** SM0251).

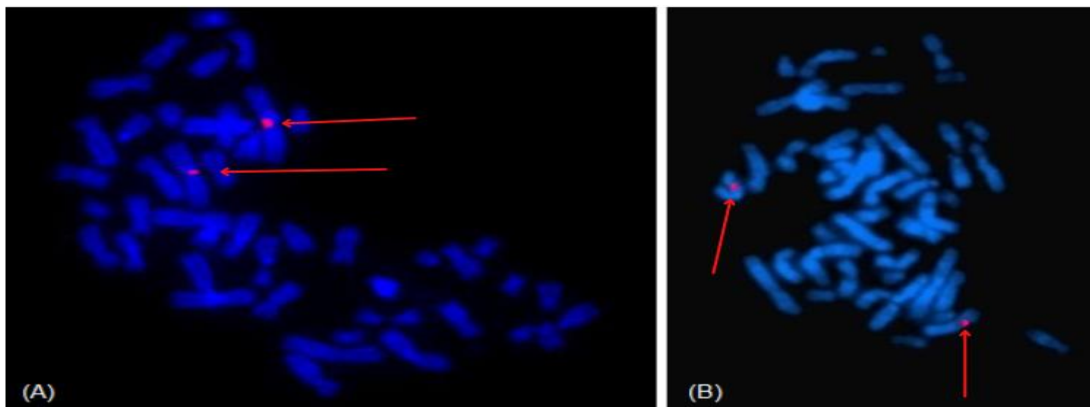

Figure 3: FISH results of in-house produced centromeric probes of chromosome 1& 7 (red arrows) on normal metaphases. (A) FISH result showing two red signals representing chromosome 1 centromeres which confirmed the presence of two copies of chromosome 1 as it is a normal metaphase. (B) FISH results showing two red signals representing chromosome 7 centromeres which confirmed the presence of two copies of chromosome 7 as it is a normal metaphase.

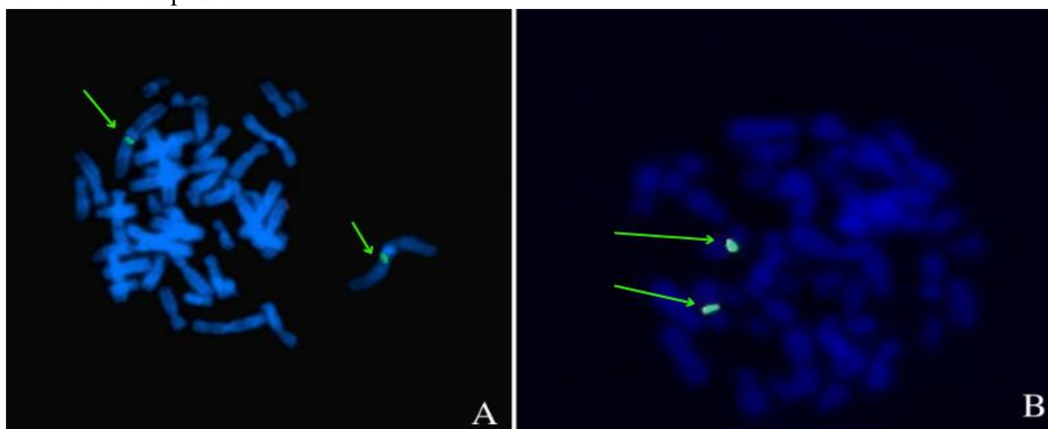

Figure 4: FISH results of commercial centromeric probes of chromosome 1&7 (green arrows) on normal metaphases. (A) FISH results of commercial probe (Cytocell) showing two green signals representing the two copies of chromosome 1 centromeres. (B) FISH results of commercial probe (Cytocell) showing two green signals representing the two copies of chromosome 7 centromeres.

4. DISCUSSION

Fluorescence in situ hybridization probes can be produced from different sources of template DNA such as human genomic DNA, bacterial artificial chromosomes, fosmids (19). The amount of the starting DNA depends on the labeling technique as nick translation needs micrograms of initial DNA while PCR needs nanograms of it (20).

Polymerase chain reaction is an effective technique for probes production as it has the ability to amplify and label at the same time (21). It provides numerous advantages as it is rapid, versatile, efficient, and the fact that most labs are now performing PCR routinely (22). Locus-specific PCR is a popular and powerful technique for generating locus-specific FISH probes. This technique especially amplifies a short DNA segment and needs that the target region is either tandem repetitive sequence (e.g. alpha satellite repeat sequences) or long enough to produce a signal that is exceeding the detection threshold of a FISH technique. By the careful selection of the primers and due to the minor sequence differences in the repeats, the probe will be the length of the repeat or it's multiples (23).

In the present study, specific centromeric probes were generated by PCR using human genomic DNA which is considered a rapid and easy technique for their production. As the production of these probes by cloning technique takes prolonged time and cannot simply be done in all cytogenetic laboratories (24). PCR was used for the labeling of the probe by direct labeling method which depends on the addition of a fluorophore attached to nucleotides during the amplification process (7). The direct labeling method used avoids the need for additional steps and reagents of the indirect labeling method (25). It also reduces background fluorescence noise (signal-to-noise ratios) of the indirect labeling method (26). α-Satellite DNA probes hybridize to several replicas of the repeat sequences existing at the chromosomal centromeres, which result in two very bright fluorescent signals in both metaphase and interphase diploid cells (27). The sensitivity and specificity of our probes were evaluated by repeating the FISH test several times on several normal metaphases. Two detectable signals were produced each time which confirms the sensitivity of the probe, as no false negative results were observed. The specificity of the produced probes was confirmed as each time the probe produce two signals on the centromere of its corresponding chromosome only and there was no observation of false positive results. The sensitivity and specificity of our in-house produced probes were the same as those produced from the commercial probes by producing two bright signals of the centromeres in normal metaphase indicating the presence of two copies of the corresponding chromosome. So the production of in-house centromeric probes enables us to overcome the expensive cost of the commercial probes and gives us reliable results.

Dunham et al.(1992) and Baumgartner et al.(2006) produced centromeric probes using either BAC clones or human genomic DNA as a source of DNA template and used nick translation or PCR technique as indirect labeling method (16,28).

Chromosomal aneuploidy is the most common feature of human cancers (29). Many types of cancer carry at least one structural or numerical aberration, which is detected more regularly than any other oncogenic or tumor-suppressor mutation (30). The centromeric probes can detect the aneuploidy, which is considered as genetic biomarkers, help in the diagnosis and prediction of the extent of disease progression of several cancers. Chromosome 1 centromeric probe can be used in the diagnosis of cervical and endometrial cancers (31). Chromosome 7 centromeric probe can be used in the detection of renal cell carcinoma and myeloid disorders (32,33). Chromosomes 1 and 7 centromeric probes can be used to detect prostate cancer, breast cancer and myeloproliferative neoplasms (27,31,34). A previous study indicated that the recognition of aneuploidy in chromosomes 1 and 7 by FISH appears to be more sensitive than DNA ploidy for the assessment of abnormal DNA (27).

In addition to cancer, centromeric probes can be used as a control probes such as in diseases like 1p36 deletion syndrome chromosome 1 centromeric probe can be used as a control probe. Moreover, in Williams syndrome due to 7q deletion, the chromosome 7 centromeric probe can be used as a control.

5. CONCLUSIONS

We can conclude that centromeric probes can be produced using human genomic DNA and PCR with specific primers for the alpha-satellite region of each chromosome. The centromeric probes enable the detection of chromosomal numerical aneuploidy as a genetic biomarker and can be used as a control probe in the diagnosis of other structural abnormalities on the same chromosome. Chromosomes 1 and 7 centromeric probes are examples for all chromosomes centromeric probes, and by adjusting the PCR primers and conditions we can proceed and produce more probes.

We recommend probe production for research purposes and also to compensate for the expensive commercial probes. We encourage the production of FISH probes that fulfill the research and diagnostic needs as

they are very sensitive and specific. Also we recommend the production of double color probes.

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Ethical Statement: The study was approved by the ethical committee of Faculty of Pharmacy (Girls), Al-Azhar University (code: 179; session 18 at 18-12-2017).

Author Contribution: Shymaa H. Hussein: PCR amplification, probe production and wrote the manuscript; Abeer I. Abd El Fattah: supervised plane of the work and reviewed the manuscript; Khalda S. Amr: revised the molecular bases of the work and reviewed the manuscript; Amal M. Mohamed: design the scientific plane of the probe production, supervised the quality of the produced probes and reviewed the manuscript.

List of Abbreviations: BAC: Bacterial artificial chromosome; CNV: Copy number variants; DAPI: 4,6 diamidime phenyl indole dihydrochloride; dATP: deoxyadenosine triphosphate; dCTP: deoxycytidine triphosphate; dGTP: deoxyguanosine triphosphate; dTTP: deoxythymidine triphosphate; dUTP: deoxyuridine triphosphate; FISH: Fluorescence in situ hybridization; HOR: Higher order repeats; NP: nonyl phenoxypolyethoxylethanol; PBS: phosphate-buffered saline; PCR: Polymerase chain reaction; SSC: saline-sodium citrate.

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