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Comparative analysis of three methods to produce diagnostic FISH Probes: Nick translation, PCR amplification and chromosome microdissection

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Introduction

Fluorescence in situ hybridization (FISH) probes are vital diagnostic tools used to identify genetic disorders and malignancies. These probes enable precise localization of specific DNA sequences within chromosomes, making them crucial for both research and clinical diagnostics.

Aim

The aim of this study is to evaluate and compare the effectiveness of three distinct methods for generating FISH probes by assessing the quality, advantages, and limitations of each. We aimed to provide insights into their suitability for producing locus-specific identifier and whole chromosome paint probes for diagnostic applications.

Methods

In all used methods, we followed the guidelines established by the American College of Medical Genetics (ACMG) for FISH probe production. We employed three methods to generate diagnostic probes: 1- Nick Translation to incorporate the labeled dyes into DNA segments from bacterial artificial chromosome (BAC) clones or chromosome-specific DNA as templates. 2- PCR Amplification and Labeling using genomic DNA as a template and specific primers 3- Chromosome Microdissection using laser dissection microscope (LDM) to dissect and isolate specific chromosomes for further amplification.

Results

Using BAC clones and nick translation, we developed locus-specific probes for chromosomes 6p, 6q, 1p36, and PAR1. Furthermore, by using chromosome-specific DNA templates and nick translation, we successfully produced WCPs for chromosomes 1, 4, 6, 14, 22, and X. PCR amplification and Labeling Using specific primers, we successfully produced alpha-satellite probes for several chromosome centromeres which were published before. Degenerate oligonucleotide-primed PCR (DOP-PCR) was employed to amplify a whole chromosome paint (WCP) for chromosome 13. Chromosome microdissection enabled the production of chromosome 6 centromere; however, amplifying small DNA quantities remains a challenge.

Conclusion

Nick translation is the most effective and generative method for producing locus-specific probes and WCPs due to its reproducibility, accuracy, and stability. PCR-based methods are efficient for short unique repetitive sequences of chromosomes' centromere. DOP-PCR is effective for amplification of entire chromosomes. While chromosome microdissection is promising, optimizing DNA amplification from very small DNA quantity is critical. The use of BAC clones ensures a sustainable resource for developing multiple locus-specific and whole chromosome paint probes, significantly addressing our research needs.

Keywords: Fluorescence in situ hybridization, bacterial artificial chromosome (BAC), whole chromosome paint, locus specific identifier, laser dissecting microscope.

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Introduction

Fluorescence in situ hybridization (FISH) is a powerful cytogenetic technique in medical diagnosis. The FISH probes hybridize to their complementary DNA sequence within the interphases or metaphases. FISH probes can identify numerical and structural abnormalities in autosomes and sex chromosomes, identify microdeletion syndromes and have wide applications in the diagnosis of malignancies. FISH is considered an intermediate in the resolution between molecular analysis and cytogenetics [1].

One of the most used methods for FISH probe production is the use of bacterial artificial chromosome (BAC). BAC clones can be selected from the BAC library ((https://www.ncbi.nlm.nih.gov/genome/cyto/hbrc.shtml). These clones are specific fragments of DNA (e.g. Human) that are inserted and propagated inside the bacterial plasmid. BAC clones include DNA sequences in the size from 100 to 200 Kilobases (Kb). The targeted regions are locus-specific genes, part of the chromosome or the entire chromosome. BAC clones can be isolated, labeled, and used to visualize specific sequences within the interphases or metaphases [2,3]). BAC libraries are very useful resources for FISH probes, they can provide large contiguous DNA segments that can be isolated and labeled by different methods like nick translation or DOP-PCR.

Unique sequence FISH probes are important in the detection of DNA segments, genes and alpha satellite of chromosome centromeres [4]. The primers for unique sequence FISH probes can be designed de novo or obtained from databases or previously reported probe sequence [5-7].

Chromosome microdissection using laser microdissection microscopy is a highly precise and efficient method for producing whole chromosome paint probes. Laser capture microdissection (LCM) microscopes can dissect individual chromosomes. DNA from several copies of an individual chromosome, can be amplified by DOP-PCR, labeled and used as FISH probes [8,9]. These probes are invaluable tools in cytogenetics for studying chromosomal abnormalities, understanding genetic disorders, and advancing cancer research. Despite the technical challenges, the integration of laser microdissection with downstream molecular techniques has significantly enhanced the resolution and accuracy of chromosomal analysis [10,11]. Laser microdissection microscopy (LMM) has emerged as a transformative technology in cytogenetics, enabling the precise isolation of chromosomes or chromosomal regions to generate WCP probes. These probes are essential for visualizing and analyzing chromosomal abnormalities in human genetics, cancer research and other genomic studies [10-12].

Methods

Ethical approval: The project has ethical approval from the Medical Research Ethics Committee. National Research Centre. Approval No. 02421223

Source of used DNA

1- DNA from bacterial artificial chromosomes (BAC) clones. (https://genome.ucsc.edu/cgi-bin/hgGateway). Different Bac clones were selected for the different probes: 6p25 (RP11 661C14), 6q27 (RP11 168J11) ,1p36 (RPC1-11 703E10) and pseudo autosomal region 1 on Xp22.33/Yp11 (RP11-91D5).

2- DNA for individual chromosomes

3- We used Genomic DNA (Promega) to be used as a template for the selected primers for alpha satellite probes.

4- DNA from the micro-dissected chromosomes.

Methods of probe amplification and labeling

1. BAC clone and nick translation methods

We selected our BAC clones from: **(https://www.ncbi.nlm.nih.gov/genome/cyto/hbrc.shtml),** and selected some BAC clones inside the plasmid of the bacteria and others as unlabeled DNA of BAC clones to generate our FISH probes [13].

Bacterial propagation and Plasmid DNA Isolation

For BAC clones inside the plasmid of the bacteria, we used appropriate media for bacterial propagation. Plasmid DNA was extracted using the Bacterial Plasmids Isolation Kit, by Kamal M. A. Khalil (2011) Method for Bacterial Plasmids Isolation Using Kit by Alkaline and Heat. Patent No: 24997. Egyptian Patent Office, Academy of Scientific Research and Technology.

Bacterial propagation and Plasmid DNA Isolation

Luria broth Medium (LB)

This medium was used for growth at 37°C: Tryptone 10 g, Yeast extract 5 g, NaCl 5 g, Agar 15 g, distilled water up to 1000 ml, and pH adjusted to 7.0 before sterilization. Media was supplemented with 12.5 ug/ml

Plasmid isolation and sample preparation

Plasmid DNA was extracted using Plasmid DNA was extracted using Bacterial Plasmids Isolation Kit, Khalil (2010). In an Eppendorf, 1.5 ml from the overnight culture was taken, and centrifuged at 8,000xg for 1 min, the pellet was kept and 25 µl of solution A was added, mixed by automatic pipette up and down. Then 250 µl of solution B was added and mixed by moving up and down three times. Then 250 µl of solution C was added and centrifuged at 13,000 x g for 5 min. Finally, the upper phase was removed into a new Eppendorf. After extraction of the DNA samples, an appropriate amount (about 25 µl) of each sample

was transferred to a fresh Eppendorf, and 5µl of loading buffer was added.
DNA precipitation with isopropanol: about 600 µl of isopropanol was added to Eppendorf and mixed gently by inverting 4 to 6 times. It was kept at -20°C for 20 min. Eppendorf centrifuged at 12000 rpm for 15 min and the supernatant was discarded. The DNA will be precipitated in the pellet and air-dried. Pellet Resuspend in 25 to 50 µl TE (pH 8.0) containing 20 µg/ml RNase A. vortex and incubate at 37 °C for 20 to 30 minutes to digest remaining RNA.

Nick translation method
Nick translation for BAC clones was performed according to the manufacturer's instructions. We used the nick translation reagent kit (Abbott) and we used dUTP red or green dyes (Abbott) for labeling.

Preparing the reagents
1-0.2mM dUTP red and green, reconstitute the vial in 50 microliter nuclease free water then take 1 partd UTP red or green to 4-part nuclease water and add 2.5 µl /reaction
2- 0.1mM dTTP prepare by 1partdTTP to 2parts nuclease free water mix well then add 5 µl /reaction
3- 0.1mM dNTPs mix prepared by taking equal volumes from dATP, dCTP, dGTP mix well then take 10 µl /reaction. All steps are taken in the dark.
Add the following component to the 1.5 ml microfuge tube:15.5 µL nuclease free water, 2.5 µL dUTP, 5 µl dTTP, 10 µl dNTPs mix, 5 µl nick buffer, 2 µl target DNA,10 µl nick enzyme, giving total volume 50 µl. vortex briefly then incubate at 15 ºC cooling incubator for 3 hrs. Then stop the reaction by putting the tubes in 70 ºC water bath for 10min, then chill in ice and keep in -20◦C.

Probe precipitation method
The probes precipitated using the Human cot 1 DNA (Invitrogen). In the 1.5 ml Eppendorf tube put: 10 ul labeled DNA, 10 ul Human cot 1 DNA,

2.1 ml 3 M sodium acetate (pH 5.2), 52.5 ml absolute ethyl alcohol, placed in icebox for 15 min. The mixture was centrifugated for 30 min at 12000 rpm at 4 ºC. To evaporate all the solutions. We used Concentrator plus (Eppendorf, Germany) at 30 ºC for 2 h. It was left in an open Eppendorf tube overnight to insure complete evaporation. The precipitated probe pellet was dissolved by 8 µl hybridization buffer and 2 µl distilled water and centrifuged well then stored at 20˚C until used.

2- Sequence-based design

Sequence-based design of single-copy genomic DNA probes for fluorescence in situ hybridization [14,15], we selected the proper primers for PCR amplification for short alpha satellite repetitive probes, data previously published.

Degenerate oligonucleotide-primed PCR (DOP-PCR)
DOP-PCR [16] is used for the general amplification of target DNA, DOP-PCR is a rapid, efficient technique for general DNA amplification. It can amplify the whole chromosome. The Dop-PCR depends on the repetitive sequences through the genome which allow the amplification of DNA segments [17].
In total volume of 50 µl containing: 5 µl of 15 µM DOP 1, 2, 3 primer mix, 5 µl of 10× PCR buffer without MgCl2, which is specially designed for use with Platinum® Taq DNA polymerase and 5 µl 50 mM MgCl2. For the dNTP's we used 1 µl 10 mM dATP, dCTP, dGTP each, 0.7 µl 10 mM dTTP, 1 µl of 1 mM Spectrum Green™-, or Spectrum Red™-dUTP, As DNA polymerase we used 0.5 µl Platinum® Taq DNA polymerase and finally added H2O to 48 µl and 2 µl of the DOP amplified BAC DNA.
After initial denaturation at 95°C for 10 minutes, the reaction was as follows: 35 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min and a final extension step of 72°C for 10 min.
The quality of the PCR products can be analyzed on a 2% agarose-ethidium-stained gel. The smear of bands from 200 to 2000 base pairs should be visible. (Backx et al., 2008)

3- Chromosome dissection by Laser capture microdissection microscope

Laser Capture Microdissection of a metaphase chromosome: We used Arcturus (XT) microdissecting microscope with UV cutting and infrared capture (Dual IR and UV LCM System). We used ArcturusXT™ LCM Instrument, CapSure® Macro LCM Caps, Glass Membrane Slides, UV - Laser Cutting and IR - Laser Capture Chromosome culture and preparation were performed [18], the metaphase chromosomes were spread on the PEN membrane slides (PEN Membrane Glass Slides Catalog number: LCM0522). The membrane slides are glass slides covered with a membrane. Using the micro dissecting microscope, we selected the appropriate power and diameter for single chromosome microdissection.
Laser cutting: We set the laser cutting power to optimal setting that allows full cutting through the PEN membrane. The CapSure Macro LCM cap (CapSure™ Macro LCM Caps Catalog number: LCM0211) was loaded into the cap loader in the microscope, we identified the needed chromosome and drew around it. The laser cutting process was activated while LCM cap on top of selected metaphase, we microdissected 10-15 copies of the selected chromosome from different fields (Fig. 3 a and 1b). The CapSure was placed on top of 1,5 ml

Eppendorf tube containing the PicoPure DNA Extraction Kit.

We extracted the DNA from the micro dissected chromosomes on the CapSure Macro LCM cap by PicoPure DNA Extraction Kit. following the kit protocol [19].

For micro dissected chromosomes, we used sequenase 7 for amplification of the very small amount of DNA then we used DOP-PCR for further amplification and labeling of the probes (Supplement 6). The probes were precipitated using the Human cot 1 DNA (Invitrogen). All produced probes were labeled, precipitated and dissolved and ready for FISH technique. We adjust the denaturation temperature and time for every produced probe [20]). In all our produced probes we followed the American College of Medical Genetics technical standards and guidelines: Fluorescence in situ hybridization [21,22]. We tested all the produced probes for accuracy by ensuring the specific bind of the test probe to its target without cross reacting with other chromosomal segments. We tested the probe for efficiency and be sure that the probe hybridizes effectively with the target DNA and produces a clear and bright signal. We optimize the denaturation time, temperature and hybridization conditions for every probe. We tested the produced probes for the reproducibility by repeating the hybridization multiple times to assess whether the probe gives consistent results in terms of signal intensity and localization. We evaluate the probes for their stability by storing the probe for extended periods (3-6 months) and re-testing their performance to evaluate how well they maintain their efficiency and accuracy over time.

Probe sensitivity: We hybridized each probe to a minimum of 200 cells, including at least 20 metaphases. To ensure the high sensitivity of the produced probes to the target sequence and to prevent cross-hybridization with non-target sequences, we optimized the methods for probe amplification and labeling. We used high-quality metaphase and interphase samples and ensured optimal hybridization conditions. We considered a sensitivity of at least 95% acceptable.

Probe specificity: We involved 100 metaphases for each probe. Our accepted specificity threshold is 98 %.

Cut-off values: We determined the negative and positive borderline, for all produced probes we used a cut-off value of 10% for all the produced probes.

Results

By using different BAC clones and labeled them with nick translation methods, we could produce different locus specific probes, we followed the guidelines of the American College of Medical Genetics (ACMG) technical standards and guidelines for fluorescence in situ hybridization (21,22). We could produce locus specific identifier for chromosome 6p, and 6q, 1p36, PAR1 (Figures 1: a, b, c, d).

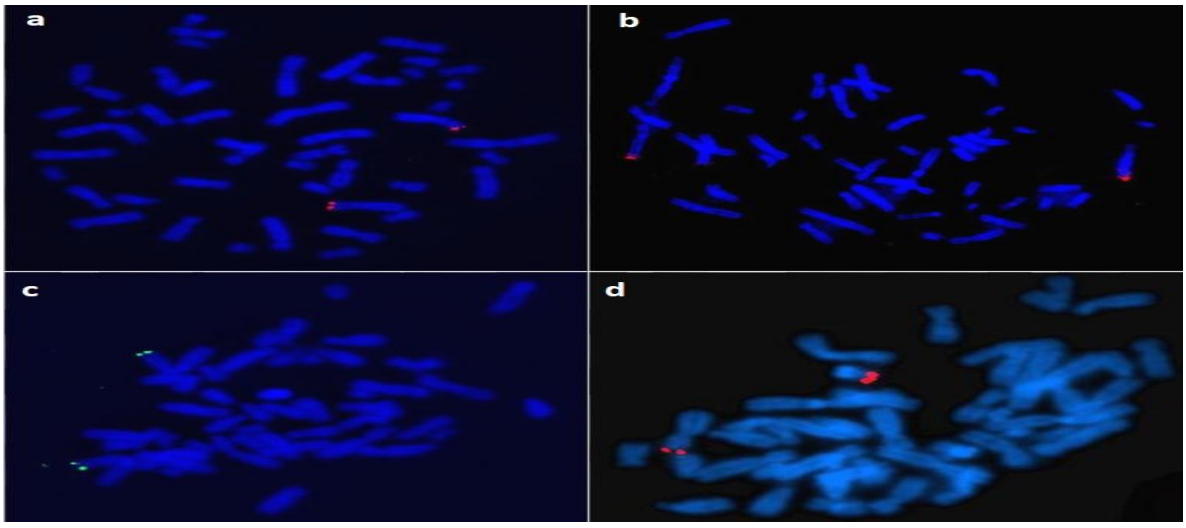

Fig. 1 Locus specific identifiers where the probe hybridizes to its specific locus a- 6p (Red color) b-6q (Red color), c-1p36 (Green color), d-PAR1 (Red color).

We tested the ability of nick translation to amplify the length of the whole chromosome and by use of chromosome specific DNA as a template we could produce WCP for chromosomes 1,4,6,14, 22 and X (Fig. 2:a, b, c, d, e, f).

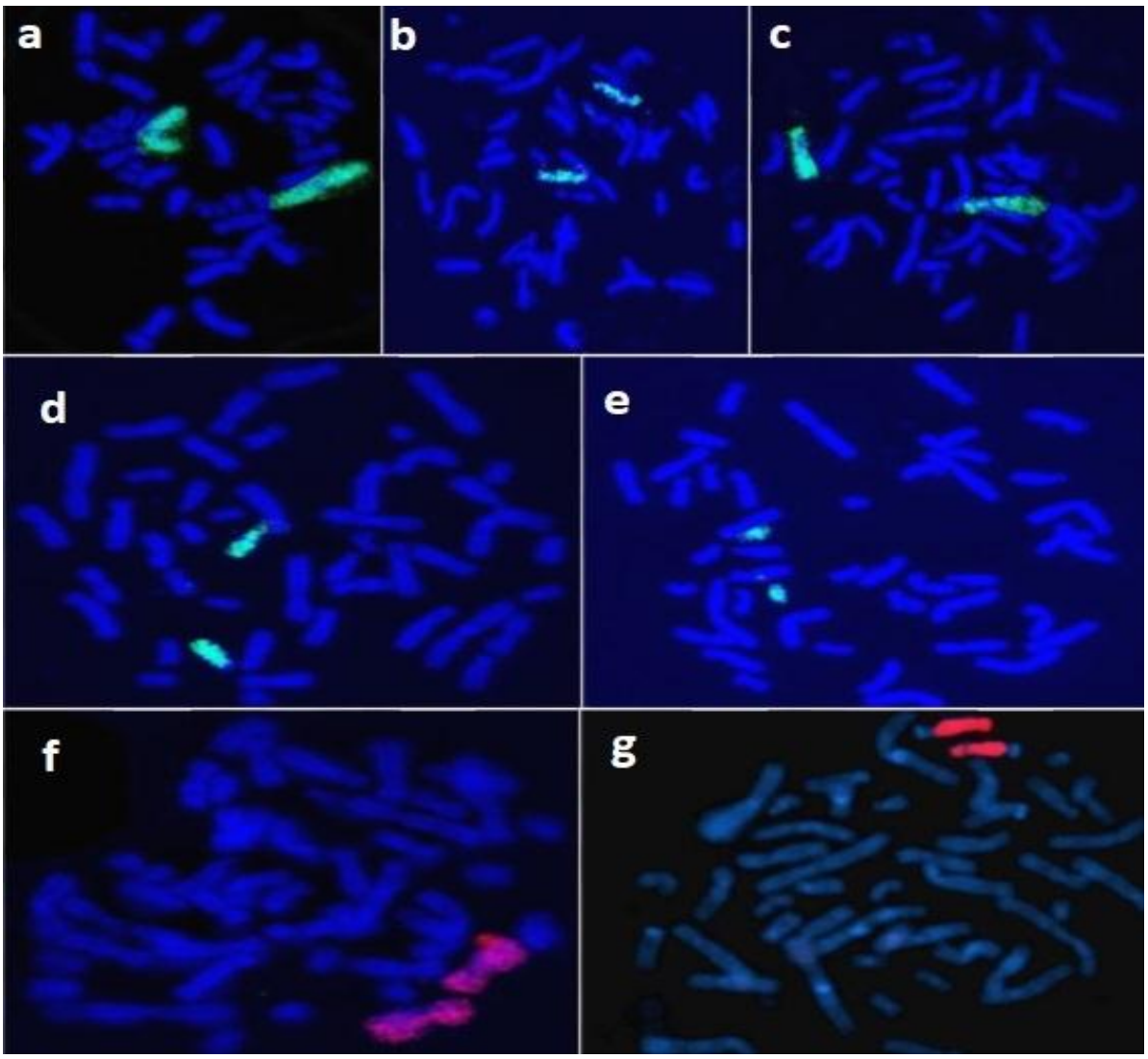

Fig. 2 Whole chromosome paint for specific chromosomes a- Chromosome 1 WCP (Green color), b-WCP chromosome 4 (Green color), c- WCP chromosome 6 (green color). d- WCP 14 (Green color), e- WCP 22 (Green color), f- WCP X (red color), g- WCP 13 (Red color, By DOP-PCR).

Polymerase chain reaction (PCR): In previous studies and using primers of unique sequence we could produce alpha satellite probes for chromosomes 1 and 7, and centromeres 17,18 and X. In this study, we used DOP-PCR as universal primers we could amplify the whole chromosome 13 Fig. 2 g

Laser dissecting microscope results

Using the LDM, we dissected several copies of the large chromosomes like 1,3,6 and 7. We used primary amplification with sequenase 7 and DOP-PCR, then we used the DOP-PCR for the second whole chromosome amplification and labeling. The trials could produce only a chromosome 6 centromere, which can be used to identify the copy number of chromosome 6. Fig. 3: a) showing the metaphase before laser cutting, b) metaphase after ultraviolet laser beam cut and infrared remove of the dissected chromosome to the cap c) the amplified segments from chromosome6.

Table 1 Comparison of the three methods used for generation of FISH probes

Feature	**Nick translation**	**PCR amplification**	**Chromosome microdissection**
Source of DNA	BAC clones or chromosome-specific DNA	Genomic DNA	Chromosome-specific DNA from dissected chromosomes
Method	Uses a DNase enzyme to introduce nicks and a polymerase to incorporate labeled nucleotides into DNA.	Amplifies specific DNA regions using PCR and incorporates labeled nucleotides during amplification.	Dissect specific chromosomes using laser microdissection, followed by amplification.
Labeling Technique	Incorporates labeled nucleotides (e.g., fluorescein, biotin) during nick translation.	Labeled dNTPs are incorporated during PCR amplification.	No direct labeling: labeling is done during subsequent amplification of isolated chromosomes.
Target DNA	BAC or specific chromosome sequences.	Specific genomic regions based on primer design.	Entire chromosomes or specific regions of chromosomes isolated through LDM.
Speed	Moderate – requires multiple steps (nick translation, labeling, purification).	Fast – can be completed in a few hours depending on the length of the target.	Time-consuming – involves chromosome isolation, amplification, and labeling.
Resolution	Good for large segments e.g., BAC clones.	High resolution especially for alpha satellite repetitive sequences	High resolution in presence of good quantity of chromosome DNA
Sensitivity	Moderate to high – dependent on the quality of the BAC clones or DNA templates.	High – PCR amplification can produce large quantities of target DNA.	High – as it isolates specific chromosomes.
Complexity	Moderate – requires specialized enzymes and optimization of conditions for labeling.	Moderate – requires design of primers, optimization of PCR, and labeling techniques.	High – requires specialized equipment (laser microdissection microscope), handling of chromosomes.
Reproducibility	Moderate – Can be variable depending on the quality of the BAC clones or chromosome-specific DNA.	High – PCR is highly reproducible, and labeled probes are consistent across reactions.	Moderate to Low – Sensitive to handling and contamination, reproducibility can vary based on isolation precision.
Application	Used for generating probes for large genomic regions e.g., LSI from BAC clones or WCP from chromosome-specific DNA	Suitable for generating probes for smaller regions or genes.	Ideal for generating probes from whole chromosome or specific chromosome regions.
Advantage	- Suitable for generating probes for large genomic regions (e.g., BAC clones). - Can generate probes for chromosome paints. - Can label large quantities of DNA for use in FISH.	- High throughput and can generate labeled probes for specific small genomic regions. - Fast process, can be completed in a few hours. - High sensitivity due to PCR amplification.	- Provides high specificity for isolated chromosomes or regions, with minimal contamination. - Ideal for isolating specific chromosomes or chromosome regions.
Limitations	- Limited by the size of the fragment (usually 100-300 kb). - Requires large quantities of high-quality DNA (BAC clones or chromosome-specific DNA).	- Limited by the size of the target DNA (~10-15 kb) and the need for specific primers. - Primer design can be challenging and requires specificity.	- Requires careful isolation of chromosomes. - Labor-intensive and requires specialized equipment (LDM). - Time-consuming and can be prone to contamination if not handled properly.

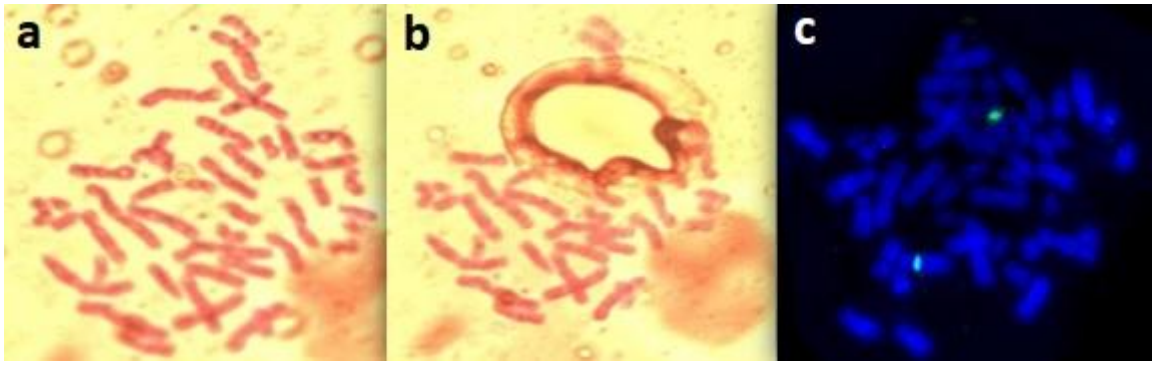

Fig. 3 (a) Image of metaphase chromosomes fixed onto a PEN membrane prior to laser dissection (objective: 100× oil). (b) Same metaphase spread after laser microdissection, showing the absence of the excised chromosome (objective: 100× oil). (c) Amplification results from the micro dissected chromosome; the green signal indicates the region successfully amplified from chromosome 6.

Discussion

FISH probes are essential tools in the diagnosis of genetic disorders and cancers. However, commercially available FISH probes can be expensive, limited in genetic coverage, and may not meet all research needs. To address this challenge and better support our research, we proceed with the development of our own FISH probes. In our effort to produce reliable and cost-effective probes, we explored three different production methods; first, BAC clones and nick translation, second, unique sequence and PCR amplification and labeling and third, a laser dissecting microscope to micro dissect the chromosomes. We carefully evaluate each method for its accuracy, efficiency, and reproducibility for the produced FISH probes. By comparing these methods, we aimed to identify the most reliable approach for generating high-quality FISH probes. Table 1 displays the comparison of the three methods used for the generation of FISH probes.

Traditional FISH probes, often derived from BAC clones containing repetitive DNA sequences, can generate high background signals, necessitating the use of blocking DNA. In our study, for probes derived from BAC clones, we used Human Cot-1 DNA to eliminate these repetitive elements. We followed the method described by Swennenhuis et al. [27], who developed a protocol for generating repeat-free FISH probes. Their approach involves using Cot-1 DNA to remove repetitive sequences, followed by re-amplification of the purified, repeat-free fragments to produce the final probes.

Backx et al. [17] developed a novel approach using degenerate oligonucleotide-primed PCR (DOP-PCR) to label Bacterial Artificial Chromosome (BAC) clones. Their technique effectively produced high-quality FISH probes with significantly reduced background signals, enabling efficient validation of chromosomal anomalies. In our study, we labeled the selected BAC clone DNA fragments using both the nick translation method and DOP-PCR.

Chromosome-specific BAC-FISH probes have significant applications in human, animal, and plant genetics. Zhang et al. [28] employed this technique in the Pacific abalone, an economically important shellfish. Their study developed and validated these genetic tools, demonstrating their effectiveness in accurately identifying abalone chromosomes and linking them to the genome. The probes were also successfully applied to a related species, highlighting their broader utility. Additionally, using the BAC-FISH technique, we were able to produce locus-specific probes, as well as whole-chromosome paints. This method holds vast potential for genome-level research across a wide range of species, including humans, animals, plants, and others.

Although Preparation of BAC clones and the subsequent labeling processes require considerable time and expertise, BAC clones contain large genomic fragments, making them effective for producing large and highly specific probes. BACs provide a comprehensive representation of the genome, increasing the likelihood of successful hybridization. BACs are stable and can be maintained and replicated, allowing for consistent probe production. The BAC cloning method, combined with nick translation, proved to be highly effective for generating both whole chromosome paint probes and locus-specific identifier probes [23-26]. Using different BAC clones and labeling by the nick translation method, we found it a very producible method. We followed the guidelines of the American College of Medical Genetics (ACMG) technical standards and guidelines for fluorescence in situ hybridization for all the

produced probes [21,22]. This approach enabled the successful production of whole chromosome paint probes for chromosomes 1,4,6,14,22 and X; and locus-specific identifier probes for 1p36,6p,6q and PAR1, demonstrating its versatility. The major advantage of this method is its ability to produce large-scale, high-quality probes that can be used in a wide range of diagnostic applications, particularly for detecting chromosomal abnormalities. However, the BAC cloning approach can be time-consuming and requires a significant amount of work in terms of cloning, quality control, and probe preparation. Additionally, the size of the BAC clones can sometimes lead to lower resolution, which may limit its utility in detecting small chromosomal alterations.

PCR is a relatively straightforward and fast method to generate specific probes. Compared to BAC cloning, PCR-based methods are generally more affordable and require fewer resources. PCR amplification and labeling is a flexible method that allows for the design of probes for any specific genomic region of interest [5-7]. PCR amplification using specific primers offers a more rapid and targeted approach, particularly for producing centromeric probes by using a unique alpha satellite short repetitive sequence [14,15].

Rogan et al. [4] developed smaller, targeted single-copy FISH (scFISH) probes directly from the human genome sequence. These probes were designed through computational analysis of ~100-kb genomic regions. The scFISH probes are generated using long-range PCR, followed by purification, labeling, and hybridization to human chromosomes. Unlike conventional FISH probes, which often contain repetitive DNA and require blocking steps, scFISH probes are specifically designed to lack repetitive elements, thereby streamlining the procedure. This method provides a faster and more customizable approach to probe development directly from genomic DNA, making it a valuable tool analysing various chromosomal rearrangements. Rogan et al. successfully developed single-copy probes for three genomic regions: 1p36, 15q11.2, and 22q11.2. The smallest probe tested measured 2,290 bp in length. Following their methodology in previous studies, and using primers specific to unique sequences, we were able to produce alpha satellite probes for chromosomes 1 and 7, 17, 18, and X [14,15].

In this study, we used Degenerate Oligonucleotide Primed PCR (DOP-PCR) which enabled the amplification of a whole chromosome paint probe for chromosome 13, scFISH is particularly advantageous for its specificity and speed, making it ideal for producing diagnostic probes for well-defined regions or repetitive sequences, such as centromeres. However, a limitation of PCR-based methods is that they rely heavily on the availability of specific primers, and the amplification process may be prone to errors or biases if the primers do not work optimally. Additionally, PCR can be limited in terms of the size of the region that can be amplified, which may prevent the generation of larger probes. PCR amplification is restricted by the size of the DNA fragment; large regions of interest may not be amplified efficiently. The labeling of PCR products can sometimes be less efficient, especially for larger fragments.

Laser microdissection allows for the isolation of specific chromosome regions or individual chromosomes, followed by probe production. Provides high specificity for isolating particular regions or entire chromosomes, ideal for targeted studies. Offers the ability to isolate and work with very specific chromosome regions that may be of particular interest in genetic studies [10, 11]). The method requires specialized equipment and expertise, making it less accessible for routine use. Amplifying small amounts of DNA can result in incomplete or biased amplification, which may affect probe quality.

Shim et al. [29] employed the laser microbeam microdissection (LMM) technique as an efficient and precise method for generating fluorescence in situ hybridization (FISH) probes. Unlike conventional approaches, LMM enables the non-contact isolation of entire chromosomes or specific chromosomal regions, significantly reducing the risk of contamination and DNA damage. The study demonstrated that probes generated through LMM can be effectively used for applications such as whole chromosome painting. Dissected chromosomes were amplified DOP-PCR, and the resulting FISH probes were labeled with Spectrum Green- or Spectrum Red-dUTP. Remarkably, whole chromosome painting (WCP) probes could be produced from a few copies of a chromosome.

In our study, we used the Arcturus XT microdissection system, which combines UV cutting and infrared capture. We dissected multiple copies of chromosomes 1, 3, 6, and 7; however, we were only able to amplify a segment from chromosome 6. In future work, dissecting a greater number of chromosome copies may be necessary to obtain sufficient DNA for successful amplification.

Sheng et al. [30] described the technique of chromosome microdissection and microcloning in plant research, emphasizing its role as a bridge between cytogenetics and molecular genetics. The authors discussed several challenges associated with applying this technique in plants, including the difficulty of preparing high-quality chromosome spreads and accurately identifying target chromosomes. They also highlighted ongoing developments and future prospects aimed at

enhancing the efficiency and precision of this method.

A key limitation of this procedure is the extremely small quantity of DNA obtained (equivalent to 10–15 chromosomes, less than one cell's worth). Additionally, the process is time-consuming. Although we employed Sequenase 7 and degenerate oligonucleotide-primed PCR (DOP-PCR) for primary amplification, only partial amplification of the dissected chromosomal DNA was achieved. Despite these challenges and the incomplete amplification of chromosomal material, we are actively optimizing the integration of LMM with downstream molecular techniques to enhance the reliability and efficiency of WCP probe production. Chromosome microdissection using a laser dissecting microscope provides a unique approach to generating FISH probes by directly isolating specific regions of chromosomes. This method allows for the targeted collection of chromosomal DNA with high precision, which is particularly useful when working with complex genomes or regions of interest that are difficult to amplify using PCR. In our study, the laser microdissection technique allowed us to isolate specific chromosomal regions, but its primary limitation was the small amount of DNA obtained from the dissection process. This limited the ability to generate sufficient quantities of probe material, which impacted the ability to produce whole chromosome paint probes or probes for larger chromosomal regions. Nonetheless, laser microdissection is valuable and highly specific.

Summary

For the three methods used, we found that BAC clones and nick translation labeling are the most reproductive methods and the probes are stable, sensitive, specific and well visualized by the fluorescence microscope. PCR amplification is successful when the DNA segments are the repetitive alpha satellite probes for centromeres of chromosome [14, 15]. Also, PCR amplification can give a satisfactory result when we use DOP primers as universal primers for DNA amplification and labeling. Chromosome microdissection, we faced several problems starting from the adjustment of the system to just dissect one selected chromosome from inside the metaphase. For the tiny amount of DNA obtained from 10-15 chromosome copies, we dissected the large chromosomes like chromosomes 1,3,6 and 7 to increase the amount of DNA. We tried to adjust the conditions of DOP-PCR and probe amplification. We could only produce a segment on chromosome 6q.

Conclusion

Each of the three methods for generating FISH probes (nick translation of DNA from BCA clones, PCR amplification and laser microdissection with DOP-PCC) has its strengths and limitations.

Nick translation of DNA from BAC clones is a reliable and reproducible method for generating high-quality probes, especially for larger genomic regions, but is more time-consuming and resource intensive.

PCR-based amplification (unique sequence PCR and DOP-PCR) offers a flexible and cost-effective solution but is limited by challenges in amplifying short DNA segments and large regions with unique primers.

Laser microdissection with DOP-PCR allows for precise chromosome selection from small amounts of DNA but suffers from the limitation of low DNA yield, making it less efficient for large-scale FISH experiments.

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Ethical approval

Ethical approval is obtained from the Medical Research Ethics Committee. National Research Centre. Approval No. 02421223.

Authors contribution

AMM: Proposed the idea of the paper, manuscript preparation, manuscript editing and manuscript review; ME: experimental studies; OE: guarantor; SHH: experimental studies data acquisition; WM: experimental studies AMHS: experimental studies, data acquisition, data analysis, FS: experimental studies; KMAK: experimental studies, data acquisition, data analysis, ME: experimental studies, data acquisition and manuscript review; HAH: experimental studies.

Data availability

All data generated through this study are included in the article. Mor details are available from the corresponding author upon request.

Conflicts of interest

There are no conflicts of interest.

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