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OBM Genetics

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Technical Note

Validation of Fluorescence in Situ hybridization (FISH) Assay Using An Analyte-Specific Reagent in Detecting Aneuploidies of Chromosomes 13, 18, 21, X, and Y in Prenatal Diagnosis

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Academic Editor: Thomas Liehr

Special Issue: Applications of Fluorescence in Situ Hybridization

OBM Genetics
2023, volume 7, issue 1
doi:10.21926/obm.genet.2301177

Received: October 01, 2022
Accepted: January 30, 2023
Published: February 06, 2023

Abstract

Fluorescence *In-Situ* hybridization (FISH) is a sensitive and highly efficient technique commonly used in routine diagnostics. Most of these tests that use analyte-specific reagents are not approved by US Food and Drug Administration (FDA) but are developed by individual test laboratories. There is an emerging demand for prenatal diagnosis of aneuploidies by FISH. Since most of these assays are laboratory-developed tests, it is essential to validate them prior to their use in diagnosis. However, validation procedures of these assays are oversight despite the presence of several validation guidelines. To validate FISH assay using analyte-specific reagents in detecting aneuploidies of chromosomes 13, 18, 21, X, and Y as per American College of Medical Genetics (ACMG) guidelines in 2016. Analyte-specific reagents supplied by

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Oxford Gene Technologies were used in the validation process using blood and amniotic fluid samples obtained from healthy male and female adults and fetuses respectively. The validation process includes probe localization, evaluation of assay specificity, and establishment of lower cut-off and reportable reference ranges. Probe localization indicated a 100% specificity for all probes tested. Interphase FISH on uncultured amniotic fluid demonstrated significantly high (≥95%) overall disomic signal patterns for all autosomes and sex chromosomes tested. The reportable 95% confidence interval was 94.84, 94.84, 95.24, 94.54, and 94.54 for chromosomes 13, 18, 21, X, and Y respectively. The present study illustrates an experimental design in validating laboratory-developed FISH assay using analyte-specific reagents in detecting aneuploidies of chromosomes 13, 18, 21, X, and Y as per ACMG guidelines. Test probes used in the present study are consistent with probe localization characteristics, assay specificity, and reportable reference ranges recommended by ACMG. Therefore, the FISH assay used in the present study could be recommended as a supplementary prenatal diagnostic test that can be carried out along with standard chromosomal analysis.

Keywords
Amniotic fluid; FISH probe localization; lower cut-off; assay specificity

1. Introduction

Fluorescence *in-situ* hybridization (FISH) is a powerful molecular cytogenetic technique that allows the detection of aneuploidies associated with interphase and metaphase cells [1]. The American College of Medical Genetics (ACMG) recommends FISH to diagnosis cryptic and complex chromosomal aberrations, as it is more sensitive than routine karyotype analysis and has a lower threshold for detecting a small population of abnormal cells [2]. The use of FISH has integrated with routine prenatal diagnosis targeting the most frequently occurring chromosomal aberrations. It analyses many cell populations, giving rise to early, accurate, and reproducible test outcomes [3].

Fetal chromosomal abnormalities constitute one of the leading causes of early pregnancy loss, fetal abnormalities, global developmental retardation, and sexual anomalies [4]. Errors in meiotic segregation primarily contribute to these anomalies during gametes' formation and parental inheritance [5]. Advanced maternal age, subfertility, and family histories of chromosomal anomalies are some of the well-identified associated risks for reported anomalies [6].

Early detection of these fetal anomalies is beneficial for both patient and the clinician and such detections are in current clinical practice by using different detection approaches [7]. Nuchal translucency combined with maternal biochemical markers is frequently applied screening tools during the first trimester of pregnancy [8]. Increased nuchal translucency in the fetus is closely associated with an increased risk of chromosomal abnormalities and other diseases. Maternal serum alpha-fetoprotein (MSAFP), unconjugated estriol, human chorionic gonadotropin (hCG), and inhibin A are considered useful biochemical markers during the second trimester of pregnancy [9, 10]. Using anomaly scans at 18-20 weeks of gestation also facilitates the detection of major fetal anatomic abnormalities during late pregnancy [4].

Invasive prenatal diagnosis using chorionic villus and amniotic fluid samples directly assesses the chromosomal constitution of the fetus. Even though this is considered the gold standard in definitive prenatal diagnosis, such invasive procedures are associated with the risk of miscarriages, and poor patient compliance [4, 11]. Therefore, non-invasive techniques in detecting fetal anomalies has gained renewed interest and prompted using fetal materials obtained either from cervical mucus or maternal blood [12]. Isolation of cell-free fetal DNA (cffDNA) in maternal blood is one of the breakthroughs in non-invasive prenatal testing (NIPT) [13]. However, the appearance of cffDNA in maternal blood significantly varies with the gestational period and can reliably be interpreted only after the 8th week of gestation [4]. Since cffDNA consists of short DNA fragments, it clears rapidly from maternal circulation leading to compromised detection in maternal circulation [14]. As per the given issues associated with NIPT, the test was recommended as a supplementary test along with invasive confirmatory tests [4, 13].

Since these tests have their benefits and drawbacks, it is extremely important to interpret their overall outcomes in arriving at a precise conclusion for a given pregnancy [15]. Further, it should also be noted that test accuracy, efficiency, and reproducibility substantially impact the final clinical judgment.

Fluorescence-tagged DNA probes used in FISH tests are analyte-specific reagents that require comprehensive validation prior to their diagnostic use [16, 17]. This validation process institutes scoring criteria, analyte specificity, sensitivity, accuracy, precision, and reporting normal reference ranges. It is tightly regulated by regulatory bodies such as the College of American Pathologists (CAP), American College of Medical Genetics and Genomic (ACMG), and Clinical Laboratory Improvement Amendments (CLIA) [16-18].

This technical note illustrates a comprehensive test procedure for validating the FISH assay using analyte-specific reagents targeting chromosomes 13, 18, 21, X, and Y supplied by Oxford Gene Technologies.

2. Materials and Methods

2.1 Sample Collection

Probe localization assay was carried out by five (05) peripheral blood samples (2 ml for each sample) obtained from apparently healthy males previously karyotyped and reported as normal male complements. Twenty uncultured amniotic fluid samples (5 ml for each sample) obtained from apparently healthy male and female fetuses (10 samples for each sex) reported having diploid status for chromosomes 13, 18, and 21 were selected to evaluate assay performance characteristics. Ethical clearance for the study was obtained from the institutional ethics review committee, Lanka Hospitals, Sri Lanka.

Probe localization studies focused on probe accuracy, specificity, and sensitivity. Assay performance characteristics were evaluated by test accuracy, specificity, and reportable ranges per ACMG guidelines for the FISH test validation [19, 20].

2.2 Probes

The study was carried out by using FAST FISH prenatal enumeration probe kit supplied by Oxford Gene Technologies, which consisted of three alpha satellite DNA probes for chromosomes 18, X,

and Y (D18Z1, DXZ1, and DYZ3) and two locus-specific probes for 13q14.2 and 21q22.13.

2.3 Analysis of Probe Localization

Probe localization studies were carried out using previously karyotyped peripheral blood samples obtained from five normal male complements as negative controls. All peripheral blood samples were cultured and harvested and five metaphases from each sample were karyotyped using conventional G-banding procedures as originally described by Moorhead *et al.* (1960). Coordinates of chromosomes 13, 18, 21, X, and Y for each metaphase were captured and archived. Subsequently, all captured slides were hybridized with a probe mix containing three alpha satellite DNA probes for chromosomes 18, X, and Y (D18Z1, DXZ1, and DYZ3) and two locus-specific probes for 13q14.2 and 21q22.13. Fluorescence signals of each slide were recorded by evaluating the coordinates of chromosomes 13, 18, 21, X, and Y in the previously archived metaphases. Probe specificity was assessed as follows [21].

$$\text{Specificity} = \frac{\text{Number of FISH signals at the expected chromosomal locus of true negatives}}{\text{Total number of FISH signals}} \times 100$$

2.4 Assay Performance Analysis

2.4.1 Sample Processing

An amniotic fluid of 5 ml from each sample was centrifuged at 1000 rpm for 8 minutes in a conical centrifuge tube. It was re-suspended in 5 ml of trypsin/EDTA followed by 1-hour incubation at 37°C. The cell suspension was again centrifuged, and the cell pellet was added with 5 ml of 0.075 M KCl and incubated for another 20 minutes at 37°C. Subsequently, the pellet was added with 2 ml of 3:1 fixative (methanol: glacial acetic acid) dropwise; the cell suspension was centrifuged. To the pellet, 5ml of fixative was added and refrigerated for 30 minutes. The cell suspension was centrifuged and re-suspended in 50 µl of fixative. The cell suspension was dropped on two clean glass slides. Air-dried slides were treated with saline sodium citrate (2xSSC) for 20 minutes, followed by an ethanol gradient of 70%, 80%, and 100%, for 2 minutes each. Slides were pre-warmed on a 37°C hotplate for 5 minutes and a probe mixture was added, covered with a cover slip, and sealed with rubber cement. Denaturation was carried out at 75°C for 5 minutes and slides were kept in the dark humidified chamber at 37°C overnight.

Post-hybridization wash was performed by incubating slides in 0.4 X SSC at 72°C for 2 minutes followed by a 2 X SSC wash with tween 20 and distilled water. Air-dried slides were mounted in a fluorescence antifade medium containing DAPI (4, 6- diamino -2-phenylindole) as counterstain. The fluorescence signals were analyzed using Metasystems Fluorescence Imaging System- *ISIS* software with Cyan, FITC, SPO, and DAPI fluorescence filters.

2.5 Statistical Analysis

All slides were first evaluated for adequacy and consistency of signal strength (brightness), lack of background, and/or cross-hybridization signals. Once the probe localization outcome on peripheral blood samples was satisfactorily achieved, an analysis of assay performance was carried out. Fifty inter-phase cells from each uncultured amniotic fluid sample were analyzed to calculate

test accuracy and assay specificity. The normal cutoff value for each probe used in the current study was calculated using a 95% confidence interval [21]. Further, 95% of the Bootstrap confidence limit was also calculated using R/RStudio software to minimize the impact of assumptions made during binomial distribution.

3. Results

The present study elaborates the experimental design in validating laboratory-developed FISH assay using analyte-specific reagents supplied by Oxford Gene Technologies in detecting aneuploidies of chromosomes 13, 18, 21, X, and Y as per ACMG guidelines. This process involves the assessment of (a) probe localization characteristics, and (b) assay performance characteristics.

3.1 Probe Localization Characteristics

Probe localization characteristics were evaluated by analyzing a hundred metaphases from five peripheral blood samples from healthy males (20 metaphases from each sample). Overall results indicated an accurate probe hybridization and 100% probe specificity for chromosomes 13, 18, 21, X, and Y [Figure 1], [Table 1].

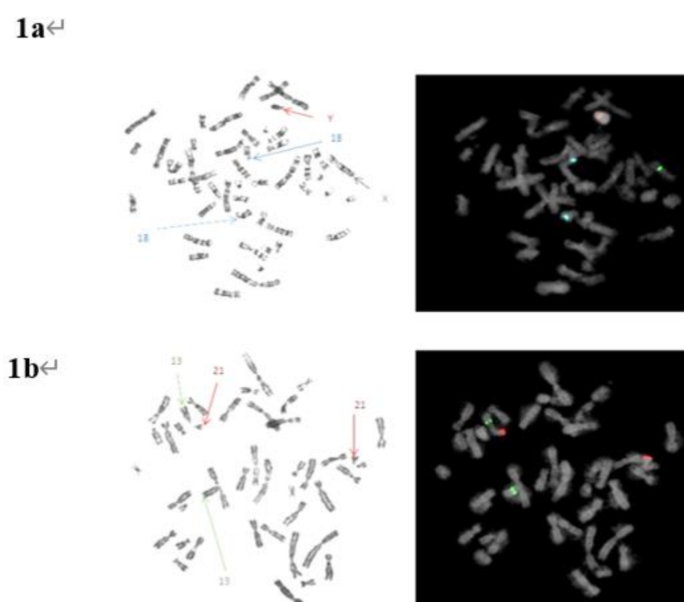

Figure 1 FISH probe colocalization in peripheral blood samples obtained from previously karyotyped apparently healthy males; (1a) chromosome 18, X, and Y (1b) chromosome 13 and 21.

Table 1 FISH probe specificity on detecting aneuploidies of chromosomes 13, 18, 21, X, and Y based on twenty metaphases analyzed per sample using five peripheral blood samples obtained from known normal male complement.

Case No	Normal at 13q (2G)	Normal at 18q (2B)	Normal at 21q (2O)	Normal at Cen X (1G)	Normal at Cen Y (1O)	Other
1	20	20	20	20	20	0

2	20	20	20	20	20	0
3	20	20	20	20	20	0
4	20	20	20	20	20	0
5	20	20	20	20	20	0

Abbreviations: G; Green, O; Orange, B; Blue

3.2 Assay Performance Characteristics

Assay performance characteristics were evaluated by analyzing 1000 interphases obtained from 20 uncultured amniotic fluid samples of both sexes (50 interphases from each sample) that have previously been reported to have a diploid status for chromosomes 13, 18, and 21. Assay accuracy, sensitivity, and reportable reference range were the main outcome measures of the current study. The overall hybridization outcome for each chromosome is summarized in Table 2.

Table 2 Summary of FISH analysis for chromosomes 13, 18, 21, X, and Y performed on uncultured amniotic fluid samples obtained from fetuses of both sexes previously reported as healthy (M = 10, F = 10).

Chromosome complement	**Chromosome 13**	**Chromosome 18**	**Chromosome 21**	**Female (XX) complement**	**Male (XY) complement**
Number of samples	20	20	20	10	10
Percentage of signals reported as true negatives	96.8%	96.8%	97.2%	96.5%	96.5%
Standard deviation	0.037	0.040	0.027	0.031	0.045
Standard error	0.008	0.008	0.006	0.009	0.014
Margin of error	0.016	0.017	0.012	0.019	0.027
Lower cut-off at 95% of Lower Confidence Limit	94.84	94.84	95.24	94.54	94.54
Lower cut-off at 95% Bootstrap Confidence Limit	95.10	95.10	95.90	94.24	94.86

Assay sensitivity was reported as 96.8%, 96.8%, 97.2%, 96.5%, and 96.5% for chromosomes 13, 18, 21, X, and Y respectively which was consistent with the required sensitivity specified by ACMG guidelines.

As per ACMG guidelines, any sample considered normal should consist of many cells with diploid status within the 95% confidence interval in a normal hybridization database [22]. The normal hybridization database should consist of an adequate number of cells from normal individuals who are not having abnormalities of targeted chromosomes. The lower cut-off which is 95% of the lower confidence interval is the determinant factor in deciding the status of the sample.

Accordingly, it was reported that the lower cut-off for all probes tested was 95% which is an acceptable norm per ACMG guidelines. [Table 2]. It was observed that there was a marginal improvement in the lower cut-off at 95% Bootstrap confidence limit for all probes tested [Table 2].

However, the lower cut-off arrived from either a 95% confidence interval or Bootstrap confidence does not show any impact on the final sample interpretation in the current study. Further, the reported deviation of interphase hybridization signals for all chromosomes tested was insignificant, and most cells indicated normal hybridization signal patterns [Table 3].

Table 3 Reported deviation of interphase hybridization signals for chromosomes 13, 18, 21, X, and Y observed on uncultured amniotic fluid samples (n = 20).

CHROMOSOME	**1 SIGNAL**		**2 SIGNALS**	**3 SIGNALS**	
13	2.80%		96.80%	0.30%	
18	2.50%		96.80%	0.70%	
21	2.30%		97.20%	0.50%	
CHROMOSOME X & Y	**XO**	**XX**	**XY**	**XXY**	**OTHER**
FEMALE	3.10%	96.50%	0.00%	0.20%	0.20%
MALE	1.60%	0.00%	96.50%	0.90%	0.60%

4. Discussion

Fluorescence *in-situ* hybridization is an extensively used technique in clinical diagnosis and employs fluorescence-tagged (FISH) DNA binding probes as analyte-specific reagents. However, many commercially available FISH probes are not validated by U.S. Food and Drug Administration. It is mandatory to undergo an extensive probe and assay validation prior to their use in the diagnostics as per guidelines laid down by any recognized accreditation body such as American College of Medical Genetics and Genomic (ACMG) or College of American Pathologists (CAP) [16, 18]. This process involves the assessment of a probe's technical specifications, establishment of standard operating procedure (SOP), determination of clinical sensitivity and specificity, calculation of cut-off, baseline, and normal reference ranges, gathering of analytics, confirmation of applicability to a specific research or clinical setting, testing of samples with or without the abnormalities that the probe is meant to detect, staff training, and report building [16]. The workflow is carried out at pre-clinical, clinical, and post-clinical evaluation stages.

The current study validated the FISH assay detecting aneuploidies of chromosomes 13, 18, 21, X, and Y using non-FDA-approved analyte-specific reagents supplied by Oxford Gene Technologies (OGT) as per ACMG guidelines.

The probe hybridization adequacy was initially evaluated and found to have an acceptable probe signal intensity in the background of DAPI as the counterstain. Any probe that fails in hybridization adequacy is reported as uninformative and will not be subjected to proceed with the validation process. Subsequently, probe colocalization studies were carried out using peripheral blood samples from five known healthy males to demonstrate that the test probes only hybridize to the intended target with no reported cross-hybridization [16, 17, 23]. Twenty metaphases from each blood samples were karyotyped along with sequential fluorescence staining (FISH on G-Banded slides) per ACMG guidelines. The overall outcome of the colocalization studies indicated 100% specificity for all probes tested. Probe specificity assures that the probe binds only to a specific locus of the intended chromosome, but not to any other chromosomal locations, and is determined by assessing at least twenty metaphases from five known negative controls. The specificity reported in the

present study assures the test probes' accuracy in reporting true negatives.

Clinical validation of a FISH assay involves the establishment of clinical sensitivity, specificity, reference range, and upper and lower cut-off values for each test probe [16]. Reporting of abnormal and normal signal patterns can be anticipated when the test probes are used in normal and abnormal samples respectively. Such incidences are acceptable to a lesser extent, rarely reported, and defined as either false positives or false negatives. However, in distinguishing true and false positives and negatives, it is essential to establish normal cut-off values for each test probe. As per Clinical and Laboratory Standard Institute (CLSI) guidelines, evaluating at least 20 healthy individuals accommodating 50 interphase cells per individual is mandatory to establish a normal reference range and lower cut-off values for each test probe [16]. In the present study, 20 uncultured amniotic fluid samples equally representing both sex that have previously been confirmed with diploid status for chromosomes 13, 18, and 21 were hybridized with test probes and assessed their fluorescence signal outcome by accommodating fifty interphase cells per sample. Our findings indicated a greater agreement between the outcome of the current FISH assay and the previously confirmed observation on the aneuploidy status of chromosomes 13, 18, 21, X, and Y with an average accurate signal percentage of more than 95%. Gaussian distributions, inverse beta functions, and binomial distributions are among the most frequently used methods of calculating normal cutoff values [19-21, 24]. There are certain prerequisites in the test protocol such as a higher number of trials, classification of trial outcomes, independent observations, one-sided confidence limit, etc. to be satisfied for each of the methods mentioned above. In the present study, the lower cut-off was calculated by the two-standard deviation technique at the 95th percentile interval from the mean accurate signal outcome for each tested probes. The lower cut-off for all the tested probes was approximately 95% and was well compatible with the cut-off recommended by ACMG. Any impact due to assumptions made during binomial distribution can be minimized by considering 95% of the Bootstrap confidence limit [25]. In the present study, the lower cut-off was marginally improved for all chromosomes tested once the 95% of the Bootstrap confidence limit was considered [26]. Further, it was observed that the lower cur-off of the present study complies with the previous remarks of diploid status for chromosomes 13, 18, and 21 for all the amniotic fluid samples tested.

5. Conclusion

The present study demonstrates the test procedure involved in validating the FISH assay using analyte-specific reagents targeting chromosomes 13, 18, 21, X, and Y supplied by Oxford Gene Technologies as per ACMG guidelines. The overall outcome of the study validates the use of the above FISH assay in detecting aneuploidies of chromosomes 13, 18, 21, X, and Y as it fulfills the required probe localization and assay performance characteristics specified by ACMG.

Author Contributions

BMS: Executed experimental design and data analysis. NK: Assist in research design, supervision of experiments, data analysis, and assist in manuscript. MR: Supervision of experiments, data analysis, and assist in manuscript. MJ: Assist in laboratory work, and data interpretation. RN: Assist in laboratory work, and data interpretation. SW: Assist in statistical analysis, and manuscript writing. BPG: Experimental design, supervision of laboratory work, data analysis, and manuscript writing.

Competing Interests

The authors have declared that no competing interests exist.

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