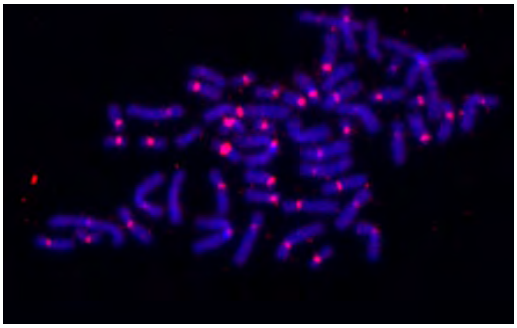
PRODUCT INFORMATION SHEET



OiGlo™

FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH) KIT

Product M1620 Series

MARKER GENE TECHNOLOGIES, INC.
1850 MILLRACE DRIVE
EUGENE, OREGON 97403-1992
1-888-218-4062
EMAIL: TECHSERVICE@MARKERGENE.COM

OliGlo™ Fluorescence *in situ* Hybridization (FISH) kit
(Product M1620 series)

NOTE: Products in this kit series provide an optimized method for the preparation and hybridization of fluorescently labeled DNA probes to targeted interphase/metaphase chromosome spreads. The use of Biotin probes and subsequent detection using any of a variety of avidin, streptavidin or anti-biotin antibody fluorophore conjugates can offer multiplexing options with a combination of fluorescence probes. For more information about these techniques, please visit our website at www.markergene.com or contact our technical assistance department at techservice@markergene.com.

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I. OVERVIEW

Fluorescence *in situ* hybridization (FISH) is a cytogenetic technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes. FISH uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence similarity. Fluorescence microscopy can be used to find out where the fluorescent probe is bound to the chromosome. FISH is often employed for discovery of specific features in DNA and is useful in genetic analysis, medicine, or species identification. FISH can also be used to detect and localize specific mRNAs within tissue samples. In this context, it can help define the spatial-temporal patterns of gene expression within cells and tissues.

The probes used in these analyses are typically short complimentary sequences to the target DNA which can be fluorescently labeled using this kit. In a typical protocol, metaphase chromosome spreads are prepared on glass slides, RNase treated, dehydrated and denatured. The slides are then hybridized with the fluorescently labeled DNA probes for 16-24 hours. Post hybridization, the slides are washed, counterstained with a 4',6-diamidino-2-phenylindole (DAPI) / antifade mixture, and analyzed using fluorescence microscopy.

A number of methods have been described for labeling nucleotide probes to facilitate detection of target DNA or RNA sequences. It is essential that the labeling method not perturb base-pairing hybridization. Commonly used enzyme-based labeling incorporates modified nucleotides with fluorescent or aminoallyl labels attached to their base (purine, pyrimidine) portion. In addition, enzymatic labeling can be sequence dependent. To remedy this, methods of

direct labeling have been developed such as platinum-based ("ULYSIS") or guanosine reactive (Mirus) direct labeling compounds. These direct labeling methods utilize chemical modification through the base portion of DNA/RNA sequences. Our OliGlo™ kits directly label nucleic acid through reaction with the phosphate groups (terminal and backbone) of the probe nucleotide providing cleaner hybridization analysis.

In our OliGlo™ FISH kit, the active labeling reagents are prepared *in situ* from stable precursor molecules derived from a variety of highly fluorescent dyes and other detection labels. This allows the highly reactive labels to function at optimum efficiency for each sample. The supplied standard labeling protocol will yield labeling efficiency of approximately 10 to 100 labels per kilobase of nucleic acid depending on the properties of the different labeling dyes and purity of the sample (Table 1). We found that this labeling density is sufficient for most FISH applications. Should there be a need for adjusting labeling efficiency, the user can simply modify the ratio of labeling dye to nucleic acid as well as incubation times for the labeling reaction as necessary.

Table 1: Excitation and emission wavelength for fluorescent OliGlo™ labeling reagents.

Fluorophore	EX (nm)	EM (nm)
TAMRA	552	582
Cy3	548	567
Cy5	649	670
Fluorescein	488	520

Probes that are commonly used for FISH analysis on chromosomes are:

- Alpha-satellite centromeric probes – generated from repetitive sequences found at the centromeres of specific chromosomes.

- Whole chromosome PAINT probes – collection of small probe sequences that hybridize to different regions along the length of the same chromosome.

II. MATERIALS

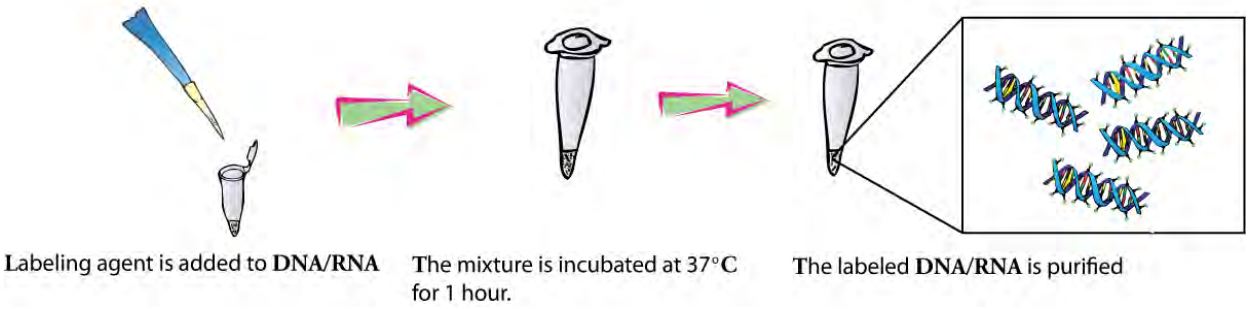
- OliGlo™ labeling dye precursor (one vial)
- Anhydrous DMF (for reconstituting the labeling reagent precursor)
- Activating Resin
- Denaturation buffer D
- Renaturation buffer R
- Hybridization buffer H
- 3M NaOAc, pH5.2 (for DNA/RNA precipitation)
- TE buffer (for reconstituting the labeled DNA/RNA samples).

Materials required but not provided:

- DNase-free RNase A
- Ultrapure deionized formamide (molecular biology grade)
- Absolute ethanol (for precipitating DNA or RNA samples)
- Sheared salmon sperm DNA and species-specific Cot-1 DNA (Sigma D-9156, Roche #11581074001 or equivalent)
- 20xSSC; Tween-20; 1M HCl
- Antifade mounting medium with 4',6-diamidino-2-Phenylindole (DAPI)
- Additional biotin detection reagents, including conjugated antibodies, conjugated streptavidin or chemiluminescent substrates can be obtained from Marker Gene Technologies, Inc. or from a variety of commercial sources.
- Mounting medium (Clear Nail polish)

Storage and Handling. Store the OliGlo™ labeling dye precursor and activating resin frozen (-20°C) until use. Once reconstituted in DMF or activated with activating resin, the reagent should be capped tightly and stored under anhydrous conditions at -20°C. Avoid exposure to moisture and light. In case of contact with skin or eyes, wash thoroughly with soap and cold water. Un-reconstituted and un-converted labeling precursors are stable for at least 6 months upon receipt. Once reconstituted and converted, the activated labeling reagent should be used within 20 days.

III. PROBE LABELING PROTOCOL



Overview of labeling protocol

Probe preparation:

1. To ensure efficient labeling, the probe should be dissolved in RNase and DNase free water. Avoid EDTA, phosphate or acetate-containing buffers, since these will cause side reactions with the labeling reagent. The A260/A280 of the purified probe should be in the range of 1.8-2.2.

NOTE: If your probe is in another buffer, purify first by ethanol precipitation followed by reconstitution in RNase and DNase free water. Protein impurities affecting the A260/A280 ratio can be removed by phenol:chloroform extraction prior to ethanol precipitation.

Preparation of active labeling dye:

2. Prior to each use, warm the vials containing the labeling dye precursor, resin and DMF to room temperature. Centrifuge briefly before opening.

3. Add 150µL of DMF to the dried pellet to resuspend at 5 mM concentration. If using a trial size kit, add 40 µL of DMF. To ensure complete resuspension, you can add one-half volume (½V) of DMF to the pellet, vortex briefly and transfer to the tube containing the activating resin. Add another ½V of DMF to the labeling dye tube, vortex briefly and transfer again to the resin tube.

4. Mix resin and resuspended dye precursor thoroughly by vortexing. Then store at room temperature in the dark for at least 16 hours before labeling nucleic acids.

Labeling reaction:

Note: Spin the tube containing resin and dye mixture briefly to completely pellet the resin. Avoid pipetting up the resin when aspirating the active labeling dye.

5. Prepare the labeling reaction according to the example shown below. Use DNase and RNase-free water, and add Labeling reagent last.

DNase and RNase-free H_2O	34µL
1mg/ml probe	1µL
Activated labeling reagent soln. (5 mM in DMF)	5µL
Total volume:	40µL

For less water-soluble labeling dyes, such as for Biotin-X labeling, more DMF is included so that the reaction takes place in 1:1 of H_2O:DMF.

DNase and RNase-free H_2O	19µL
1mg/ml probe	1µL
DMF	15µL
Activated labeling reagent soln. (5 mM in DMF)	5µL
Total volume:	40µL

6. Incubate the reaction at 37^{o}C for 1 hour. Do a quick spin at the end of labeling reaction.

Purify the labeled probe by ethanol precipitation:

7. Bring the total volume to 200µl with DNase and RNase-free water.

8. Add 20µL (1/10 of the total volume) 3M NaOAc, pH5.2 and 400 µL (2V) ice cold 100% ethanol.

9. Mix well and place at -20^{o}C or lower temperature for at least 30 minutes.

10. Centrifuge for 15 minutes to pellet the labeled nucleic acid. Aspirate the ethanol, careful not to disturb the pellet.

11. Gently wash the pellet once with 500-1000µl 70% ethanol prepared with DNase and RNase-free water. Then centrifuge for an additional 10 minutes.

12. Remove all traces of ethanol with a micropipetter. Do not allow to air dry extensively, as the pellet may become difficult to resuspend.

13. Resuspend the pellet in 10µL of TE buffer. Quantify using a spectrophotomerter and store protected from light at -20^{o}C or colder.

IV. TREATMENT OF SLIDES WITH METAPHASE SPREAD

Note: Warm slides to room temperature prior to pre-treatment. Use fresh slides if possible, as slides 6 months or older may not hybridize well. It is recommended to process only 4 slides per coplin jar in order to maintain proper temperature.

RNase pre-treatment of slides

1. Incubate metaphase spread slides with 150µL per slide of RNase A digestion solution (100µg/ml in 2xSSC, freshly prepared) under a 22x60mm coverslip for 1hr at 37^{o}C in a humid chamber.

2. Wash slides in 2xSSC until coverslips slide off. Slightly agitate if necessary.

3. Dehydrate slides in a series of ethanol solutions: 70% ethanol for 2 minutes, 85% ethanol for 2 minutes followed by 100% ethanol for 2 minutes. Then air dry. The treated slides can be stored at room temperature overnight.

Denature and dehydrate metaphase spread slides

4. Prepare denaturation solution: 70% formamide in 2xSSC, adjust pH to 7-7.5 with 1M HCl. This prepared solution can be used up to 1 week. Store in a sealed coplin jar at 4^{o}C.

5. Warm up denaturation solution in a coplin jar to 72^{o}C.

Note: It is essential that the temperature of denaturation solution does not fall below 70^{o}C. Otherwise, hybridization will be impaired. Keep in mind that coplin jars tend to be 3^{o}C lower than the water bath temperature and each slide will cause a 1^{o}C drop.

6. Warm up slides to 42^{o}C and then denature the slides for 2 to 4 minutes in denaturation solution.

Note: The denaturation time needs to be optimized. If too long, chromosomes will appear puffy and distorted. If too short, probe will appear as a haze over the spreads. In general, older slides need longer denaturation time.

7. Dehydrate the slides in a series of ethanol solutions as described above.

V. HYBRIDIZATION OF THE TREATED SLIDES

Preparation of probes for hybridization

1. Depending on the type of probes for chromosome FISH:
   - PAINT probes: combine 300ng of OliGlo™ labeled probe with 15µg species-specific Cot-1 DNA and 22µg sheared salmon sperm DNA
   - Alpha-satellite centromeric probes: combine 60ng of OliGlo™ labeled probe with 2.4µg species-specific Cot-1 DNA and 36µg sheared salmon sperm DNA

   Bring volume to 20µl with nuclease-free water.

2. Add 1/10 volume Denaturation buffer D to the probe mix. Mix well and incubate at room temperature for 5 minutes.

3. Add 1/10 volume Renaturation buffer R to the denatured probe mix. Mix well and incubate on ice for 10 minutes.

4. Bring volume to 100µl with nuclease-free water.

5. Add 1/10 volume of 3M Sodium Acetate (pH5.2) and then 2 V of absolute ethanol to precipitate the labeled probe as described previously.

6. Resuspend the labeled probe pellet with 30µl hybridization buffer (warmed to 37^{o}C). Vortex probe mixtures and incubate at room temperature for 10 minutes to aid in dissolving the DNA pellet.

7. Denature the probe by incubating at 75^{o}C for 10 minutes.

8. Warm slides to 42^{o}C. Pipet the 30µl probe mixture onto slides. Cover with a 22x50mm coverslip and eliminate any visible bubbles by gently pressing coverslip to the slide.

9. Place the slides in humidified chamber and hybridize at 42^{o}C overnight, protected from light.

VI. POST-HYBRIDIZATION WASH AND DETECTION

Post hybridization wash

Note: DO NOT let the slides dry out during the following washes or chromosomes will appear morphologically deformed with a high fluorescence background.

1. Remove coverslips by immersing in 2xSSC in a coplin jar. Gentle agitation will be required.

2. Prepare the formamide washing solution: 50% formamide in 2xSSC, adjust pH to 7-7.5 with 1M HCl. This prepared solution can be used up to 1 week. Store in a sealed coplin jar at 4^{o}C.

3. Depending on the type of the probes used, the washing steps should be as following:

PAINTs:

a) Pre-warm formamide/SSC washing solution to 45^{o}C in three separate coplin jars. Pre-warm 2xSSC to 45^{o}C in 3 separate coplin jars as well.

b) Wash 3 times, 5 minutes each in formamide/SSC solution at 45^{o}C.

c) Wash 3 times, 5 minutes each in 2xSSC solution at 45^{o}C.

d) Wash slides in 4xSSC/0.1% Tween-20 (freshly prepared) for 1 minute at room temperature.

e) Wash slides in molecular biology grade water for 1 minute at room temperature.

Alpha-satellites:

a) Pre-warm formamide/SSC washing solution to 45^{o}C in three separate coplin jars. Pre-warm 0.1xSSC to 45^{o}C in 3 separate coplin jars as well.

b) Wash 3 times, 5 minutes each in formamide/SSC solution at 45^{o}C.

c) Wash 3 times, 5 minutes each in 0.1xSSC solution at 45^{o}C.

d) Wash slides in 4xSSC/0.1% Tween-20 (freshly prepared) for 1 minute at room temperature.

e) Wash slides in molecular biology grade water for 1 minute at room temperature.

Detection of Biotin

Note: For biotin-labeled probes, further detection with the streptavidin-fluorescent conjugates will be required. If necessary, several rounds of amplification with Biotin/streptavidin conjugates will facilitate the detection of biotin-labeled probes hybridized to the chromosomes.

4. Rinse slides in PBS or suitable detection buffer for 1 minute.

5. Make an appropriate dilution of biotin detection reagent in PBS/blocking solution. Incubate with slides (200µl/slide) at room temperature for 1 hour in a humid chamber.

6. Wash 3 times, 5 minutes each in PBS at room temperature.

Mounting slides

7. Prepare antifade mounting medium with 500ng/ml DAPI.

8. For every 22x50mm coverslip area, add 30-50µl of mounting medium with DAPI. Cover with glass coverslip.

9. Push gently on coverslip with lab tissue to remove any air bubbles and excess mounting medium. Seal edges of coverslip with nail polish or rubber cement.

10. View slide under fluorescent microscope with proper filters.

M1620 Series Kit Contents			
Kit component	**Trial size**	**Standard kit**	**Cap Color**
Reagents			
OliGlo™ labeling dye precursor	dried pellet	dried pellet	amber glass vial
DMF	50µL	200 µL	black
Activating Resin	dried powder	dried powder	white
Hybridization buffer H	150 µL	750 µL	red
Denaturation buffer D	50 µL	200 µL	blue
Renaturation buffer R	50 µL	200 µL	yellow
3M NaOAc, pH5.2	100 µL	500 µL	orange
TE buffer	100 µL	500 µL	green
Documentation			
MSDS	2	2	

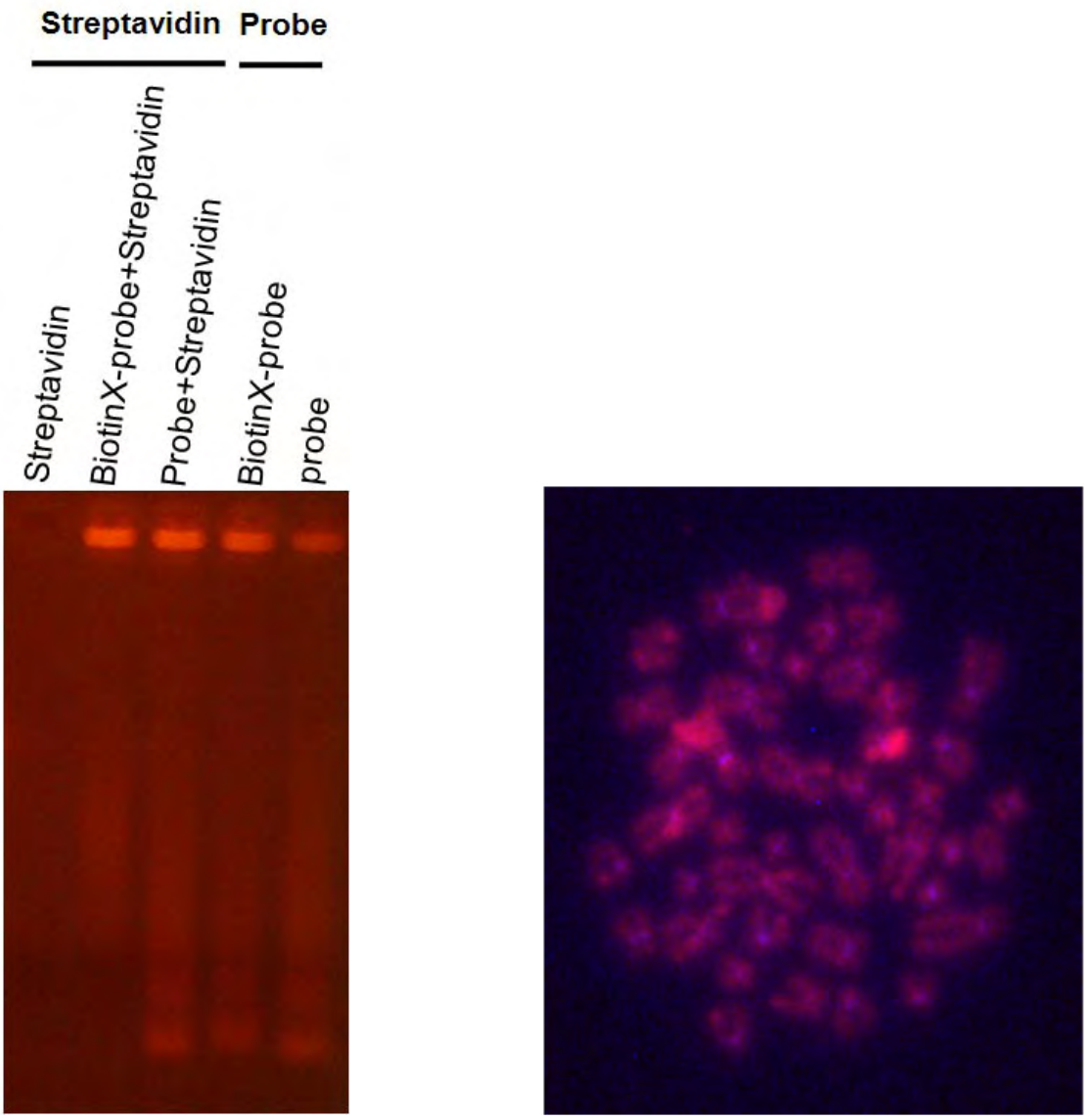


Figure 2: OliGlo™ Biotin FISH Kit (M1624/M1625). Left panel: pan-centromeric probes were labeled with OliGlo™ Biotin labeling dye, purified and then incubated with or without streptavidin (1mg/ml) for 15 minutes before applied to agarose gel electrophoresis. Streptavidin caused a supershift of biotin-labeled probe while it had no impact on the unlabeled. It is also noted that the Biotin labeled probe migrates slightly slower than the unlabeled. Right panel: Biotin labeled pan-centromeric probe was applied to chromosome FISH assay followed with incubation with F405 conjugated streptavidin (Invitrogen) for detection. Blue represents the

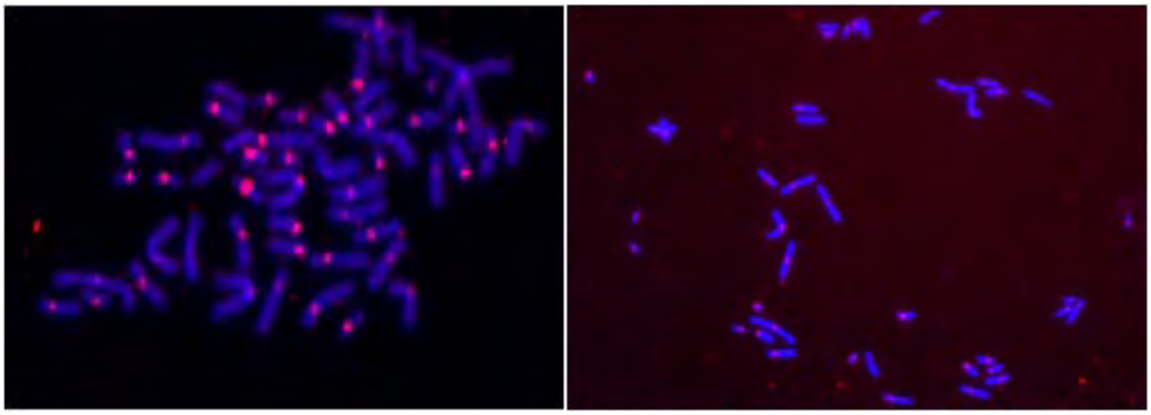


Figure 3: OliGlo™ TAMRA FISH Kit (M1620/M1621) and Cy3 FISH Kit (M1622/1623). Centromeric probes were labeled either with OliGlo™ TAMRA (left panel) or Cy3 (right panel), purified and then applied to chromosome FISH. Red represents the hybridized signals on the centromeres; blue represents the spread chromosomes.

REFERENCES:

1) Durrant I, Brunning S, Eccleston L, Chadwick P, Cunningham M. (1995) "Fluorescein as a label for non-radioactive *in situ* hybridization." Histochem J 27:94-99.
2) Forster AC, McInnes JL, Skingle DC, Symons RH. (1985) "Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin." Nucleic Acids Res 13: 745-791.
3) Hoevel T, and Kubbies M. (2002) "Nonradioactive labeling and detection of mRNAs hybridized onto nucleic acid cDNA arrays." Methods Mol Biol 185: 417-23.
4) Kessler C. (1995) "Methods for nonradioactive labeling of nucleic acids." In *Nonisotopic probing, blotting, and sequencing* (Kricka L J, Ed.) pp 41-109, Academic Press, San Diego.
5) Liu P, Siciliano J, Seong D, Craig J, Zhao Y, Jong P, Siciliano M. (1993) "Dual *Alu* polymerase chain reaction primers and conditions for isolation of human chromosome painting probes from hybrid cells." Cancer Genetics Cytogenetics 65:93-99.
6) Mannot V, Tora C, Lopez S, Menou L, Laayoun A. (2001) "Labeling during cleavage (LDC), a new labeling approach for RNA." Nucleosides, Nucleotides, Nucleic Acids 20: 1177-1179.
7) Olejnik J, Krymanska-Olejnik E, Rothschild KJ. (1998) "Photocleavable aminotag phosphoramidites for 5'-termini DNA/RNA labeling" Nucleic Acids Res 26(15): 3572-3576.
8) Rihn H, Coulais C, Bottin MC, Martinet N. (1995) "Evaluation of non-radioactive labelling and detection of deoxyribonucleic acids: Part one: chemiluminescent methods." Biochem Biophys Methods 30:91-102.
9) Sambrook J, Fritsch EF, Maniatis T (1989) "Molecular Cloning: A Laboratory Manual." 2nd edition.
10) Henegariu O, Bray-Ward P, Artan S, Vance G, Qumsyieh M, Ward D. (2001) **"**Small marker chromosome identification in metaphase and interphase using centromeric multiplex FISH (CM-FISH)." Lab Investig 81(4): 475-481.
11) Weier H, Lucas J, Poggensee M, Segraves R, Pinkel D, Gray J. (1991) "Two-color hybridization with high complexity chromosome-specific probes and a degenerate alpha satellite probe DNA allows unambiguous discrimination between symmetrical and asymmetrical translocations." Chromosoma 100:371-376.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