Preface

The new techniques of molecular cytogenetics, mainly fluorescence *in situ* hybridization (FISH) of DNA probes to metaphase chromosomes or interphase nuclei, have been developed in the past two decades. Many FISH techniques have been implemented for diagnostic services, whereas some others are mainly used for investigational purposes. Several hundreds of FISH probes and hybridization kits are now commercially available, and the list is growing rapidly. FISH has been widely used as a powerful diagnostic tool in many areas of medicine including pediatrics, medical genetics, maternal–fetal medicine, reproductive medicine, pathology, hematology, and oncology.

Frequently, a physician may be puzzled by the variety of FISH techniques and wonder what test to order. It is not uncommon that a sample is referred to a laboratory for FISH without indicating a specific test. On the other hand, a cytogeneticist or a technologist in a laboratory needs, from case to case, to determine which procedure to perform and which probe to use for an informative result. To obtain the best results, one must use the right DNA probes and have reliable protocols and measures of quality assurance in place. Also, one must have sufficient knowledge in both traditional and molecular cytogenetics, as well as the particular areas of medicine for which the test is used in order to appropriately interpret the FISH results, and to correlate them with clinical diagnosis, treatment, and prognosis.

Molecular Cytogenetics: Protocols and Applications provides reliable protocols for most of the FISH techniques in a step-by-step and easy-to-follow style for laboratory physicians and scientists who offer diagnostic services in genetics and oncology. Notes for best results and troubleshooting are offered based on the authors' first-hand experience. In many chapters, the authors have provided an extensive review on the applications of the technology and its sensitivity, limits, and pitfalls. Several review chapters on some particular topics have also been included. Although the major focus is on diagnosis, the state-of-the-art protocols detailed in depth here can be very useful for all scientists who are interested in genomic research in the areas of human development and the molecular biology of human cancer.

It is hoped that *Molecular Cytogenetics: Protocols and Applications* would serve as a major source of guidance and reference for the providers of

diagnostic services, including cytogeneticists, pathologists, technologists, trainees, and students in cytogenetics, hematology, and molecular pathology. The authors have written here with particular considerations for the needs of clinicians, as well as other health care professionals, who utilize molecular cytogenetic tests for the diagnosis and clinical management of patients with developmental disorders, reproductive problems, or oncologic diseases.

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Labeling Fluorescence *In Situ* Hybridization Probes for Genomic Targets

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1. Introduction

Fluorescence *in situ* hybridization (FISH) requires nucleic acid probes, including deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or nucleic acid analogs, labeled directly with fluorophores, or capable of indirect association with fluorophores. The nucleic acid provides the FISH assay with its specificity through complementary pairing of the probe nucleotides with nucleotides of the target nucleic acid. The appended fluorophores provide the ability to visually detect the homologous regions within the cellular structure using a fluorescence microscope. Photographic or electronic cameras can also be used to provide permanent images of the fluorescence staining patterns, and the latter can be used to provide quantitative measurements of the probe fluorescence.

This chapter describes a variety of methods by which DNA can be coupled to fluorophores to form FISH probes directed toward genomic targets. Following a brief discussion of labeling methodologies, fluorophore selection, and sources of probe DNA, a number of detailed protocols are provided that describe both enzymatic and chemical labeling of FISH probes.

1.1. Direct and Indirect Fluorophore Labeling

Fluorophores can be associated with nucleic acid probes by chemical conjugation to the nucleic acid, or by chemical conjugation of the nucleic acid with a nonfluorescent molecule that can bind fluorescent material after hybridization. The former method is called "direct labeling" and the latter method is called "indirect labeling." In indirect labeling, the molecule directly attached to the nucleic acid probe is typically either biotin or a hapten, such as dinitrophenol (DNP) or digoxigenin. The *in situ* hybridization is performed with the hapten- or biotin-labeled probe, after which the specimen is incubated with fluorophore-labeled antibody or avidin. Because a number of

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fluorophores can be attached to each antibody or avidin molecule, the indirect method allows for the association of multiple fluorophores with each directly attached binding moiety. Furthermore, additional rounds of antibody binding, sometimes referred to as "sandwiching," can be utilized to further increase the number of bound fluorophores. For example, if goat IgG anti-DNP was used to bind to DNP-labeled probes, then fluorophore-labeled anti-goat IgG can be used to amplify the signal in a second round of indirect labeling.

In addition to binding multiple rounds of avidin and/or antibody secondary reagents, the amount of fluorescence staining can be increased using enzyme conjugates of avidin or antibodies. For enzyme conjugates to be effective in FISH, fluorescent products of the enzymatic reaction must remain localized near the site of probe binding. Two approaches to dye localization include the generation of a precipitating fluorescent product (ELF reagent, Molecular Probes, Inc., Eugene, OR) ***(1–3)***, and generation of highly reactive fluorescent compounds that covalently attach to neighboring cellular material (CARD/TSA system, NEN, Boston, MA) ***(4–6)***.

While indirect labeling has the potential for generating greater fluorescence signal, it also has the disadvantage of requiring additional incubation steps to bind the antibody and avidin reagents. The introduction of fluorescent antibodies also can increase the background fluorescence owing to nonspecific binding of the antibodies and avidin proteins to extraneous cellular material on the microscope slide, and the slide surface itself. Furthermore, when multicolor FISH is utilized to simultaneously identify several different genomic targets, a different, spectrally distinct fluorophore must be used to unambiguously identify each of the targets. For direct-labeled probes, this means finding *N* spectrally distinct fluorescent labels to identify *N* different targets. For indirect-labeled probes this means not only selecting *N* different labels, but also finding *N* different binding pairs (hapten-antibody or biotin-avidin pairs) for binding each of the *N* fluorescent labels. For very small genomic targets, for example, targets less than 70 kilobases (kb), indirect labeling may be required to achieve visually interpretable staining. However, larger targets are usually detectable using direct labeling alone. For research applications where probes or particular targets may be used infrequently or are under initial investigation, individual laboratories may opt for small target probes, such as plasmid or cosmid clones, for which indirect labeling may be a necessity. However, with the availability of bacterial artificial chromosome (BAC) libraries generated in connection with the Human Genome Project, large target probes can be easily generated and discerned with little difficulty when directly labeled.

1.2. Survey of Nucleic Acid Labeling Chemistry

For either direct- or indirect-labeling, the probe nucleic acid must be modified to attach a fluorophore, biotin, or hapten. Both chemical and enzymatic reactions have been used for this purpose. Early fluorescence *in situ* hybridization was performed with a chemically modified probe, using periodate oxidation of a 3'-terminal ribonucleotide to form the dialdehyde, coupled in turn with a hydrazine derivative of fluorescein ***(7)***. Biotin or a hapten could presumably be added by this same chemistry,

however, the chemistry is restricted to RNA probes or DNA probes to which a 3'-terminal ribonucleotide has been added, using terminal transferase, for example. Other chemical modifications reported for *in situ* hybridization probes include a reaction to introduce the hapten aminoacetylfluorene (AAF) ***(8–12)***, and mercuration ***(13)***. Mercurated probes are reacted post hybridization with a bifunctional molecule containing the detection moiety and a thiol group ***(14,15)***.

A convenient method of chemical labeling that is described in more detail below uses platinum complexes ***(16)***. In this method, the detection moiety is derivatized to form a coordinating ligand of a platinum complex. The labeled complex is further reacted with nucleic acid resulting in the formation of a coordinate covalent bond between the platinum and primarily guanine residues of the nucleic acid. Other chemistries employed in labeling hybridization probes include, bisulfite mediated transamination of cytosine ***(17,18)***, photochemical reaction with photobiotin ***(19)***, bromination of thymine, guanine, and cytosine with *N*-bromosuccinimide; followed by reaction with amine-containing detection moieties ***(20)***, and condensation of terminal phosphate groups with diamines, followed by coupling with amine reactive detection moieties ***(21,22)***.

Enzymatic reactions, especially those using polymerases to incorporate labeled nucleoside triphosphates, have been the most popular means of labeling nucleic acids by far. Of these, nick translation to incorporate biotinylated nucleoside triphosphates is the oldest and most frequently used method ***(23–25)***. Other haptens incorporated by this method include dinitrophenol ***(26)***, digoxigenin ***(27)***, and fluorescein ***(28)***. In addition to being used as an indirect label with anti-fluorescein antibodies, fluorescein incorporated by nick translation has been used for directly detected probes ***(26,28)***. A variety of fluorophores are now commercially available that can be incorporated by polymerases for directly detected FISH (e.g., from Molecular Probes Inc., Eugene, OR; New England Nuclear, Boston, MA; or Vysis, Inc., Downers Grove, IL).

In addition to nick translation, DNA polymerases have been used to incorporate labeled nucleoside triphosphates into FISH probes by PCR. This has included PCR with flanking primers that amplify DNA inserts within plasmids ***(29)***, as well as PCR with random and degenerate ***(30)*** primers. Examples of these important enzymatic labeling protocols are provided below. (*See* **Subheadings 3.1.–3.3.**).

Note that *in situ* hybridization probes perform best when the probe lengths are <1 kb. Published procedures often call for probe lengths in the range of 200–600 base pairs (bp). Methods for fragmenting probes have included sonication, alkali treatment, heat, or enzymatic degradation. Smaller probe lengths can also be generated as a consequence of certain enzymatic labeling methods, such as the polymerase chain reaction (PCR).

1.3. Fluorophore Selection

A wide variety of fluorophores are available for labeling *in situ* hybridization probes, with emission extending from the ultraviolet end of the spectrum to the near infrared. The most frequently used fluorophores belong to several common chemical classes—the coumarins, fluoresceins, rhodamines, and cyanines. The structures of these compounds are shown in **Fig. 1**, together with two frequently used indirect-

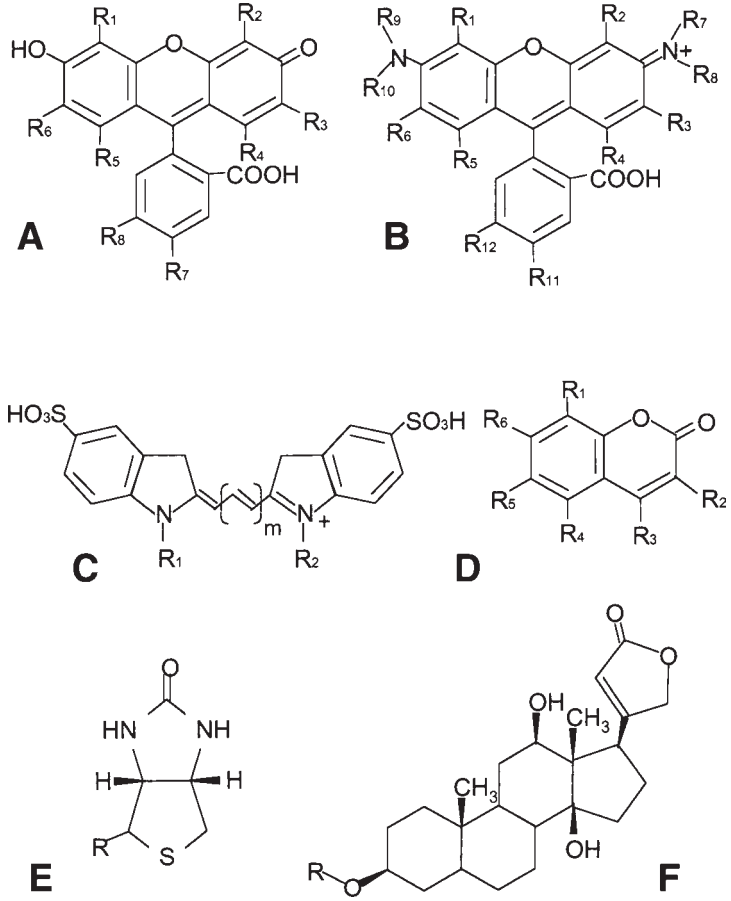

Fig. 1. Chemical structures of four common fluorophore classes **(A–D)** and two common indirect labels (**E** and **F**). **A**. fluoresceins, **B**. rhodamines, **C**. cyanines (Cy 3, Cy 5, and Cy 7 only), **D**. coumarins, **E**. biotin, **F**. digoxigenin. Specific compounds in each class differ by their chemical substituents, indicated as R's in the chemical structures.

labels. Changing the substituents (R_n) on the basic structures modifies the chemical and spectral properties, including the extinction coefficient for absorption of light, the fluorescence quantum yield, the fluorescence lifetime, and the fluorescence excitation and emission spectra. For example, 7-amino-4-methylcoumarin-3-acetic acid (AMCA; R_6 = amino, R_3 = methyl, R_2 = acetate, in Structure **D**, **Fig. 1**) has an excitation max at 354 nm and an emission max of 441 nm. Changing the substituents to form 7-diethylaminocoumarin-3-carboxylic acid (R_6 = diethylamino, R_2 = carboxylate, in structure D, **Fig. 1**) shifts the excitation max to 432 nm and the emission max to 472 nm. In the case of rhodamines, Rhodamine Green™ (R_7 = R_8 = R_9 = R_{10} = hydrogen, R_{11} or R_{12} = carboxylate, in Structure B., **Fig. 1**) has an excitation max at 504 nm and

an emission max of 532 nm, while tetramethylrhodamine isothiocyanate (TRITC; $R_7 = R_8 = R_9 = R_{10}$ = methyl, R_{11} or R_{12} = isothiocyanate) has an excitation max at 544 nm and an emission max of 572 nm. The spectral characteristics of the cyanines are strongly affected by changing the number of carbons separating the two indole rings. For example, Cy 3 (*m* = 1 in structure C, **Fig. 1**) has an excitation maximum at 550 nm and an emission maximum of 570 nm, while Cy 5 (*m* = 2 in Structure C., **Fig. 1**) has an excitation max at 649 nm and an emission max of 670 nm. The excitation and emission maxima for a number of fluorescent labels used on *in situ* hybridization probes are listed in **Table 1**. Also included are two common nucleic acid counterstains, 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI).

For *in situ* hybridization, the most desirable properties are high absorption extinction coefficient (preferably greater than 10,000/*M*·cm) and high fluorescence quantum yield (preferably >0.2). The excitation and emission spectra are also very important and must be selected with regard to the spectral distribution of the excitation source, the microscope optical system, the fluorescence detector, and the filter sets available. In the case of multitarget hybridization, the spectral distributions of each fluorophore present in the assay must be chosen carefully to allow the fluorescence of each to be individually distinguished (for a review of multitarget hybridization, *see* ***ref. 31***).

Spectral properties are not all that must be considered in label selection. In particular, the different chemical structures of the fluorescent labels lead to different levels of interaction with various cellular components, cellular debris, extracellular matrix, and the slide surface. Background staining of a specimen is highly dependent, therefore, upon the chemical structure of the fluorescent label. While highly hydrophobic labels can reduce probe solubility and increase adsorption to some cellular components, often the only way to determine how a label will perform in an *in situ* hybridization is by actually preparing the labeled probe and hybridizing it to the target tissue.

1.4. Sources of Probe DNA

For most FISH applications, probes are prepared by culturing bacteria or yeast that contains the desired cloned sequence. The cells are harvested, lysed, and the clone DNA is purified from the host chromosomal DNA and cellular material. Bacterial cells containing the clone of interest are typically grown in media that selects for the clone by use of an antibiotic, or in the case of yeast, in media which lacks a particular nutrient. Bacterial clone DNA can be isolated by several common methods such as alkaline lysis or boiling ***(32)***.*,** Alternatively, extraction kits are available commercially from several suppliers, including Qiagen, Inc. (Chatsworth, CA), Stratagene (La Jolla, CA), and Gentra Systems, Inc. (Minneapolis, MN). The preferred method for generating FISH probes from YACs involves amplification of the insert by Alu-PCR**.

Vectors which maintain large inserts such as cosmids, P1s, PACs, or BACs, are best suited for generating FISH probes homologous to unique sequence DNA, as the

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**See* various volumes in the series *Current Protocols in Molecular Biology*, Janssen, K. (series editor), John Wiley and Sons, New York.

***See* various volumes in the series *Current Protocols in Human Genetics*, Boyle, A. L.. (series editor), John Wiley and Sons, New York.

Table 1
Fluorescent Probe Labels and Counterstains
Used in FISH (NHS = *N*-Hydroxysuccinimidyl Ester)
Spectral Information is from Manufacturer Specification Sheets

		Peak Wavelengths (nm)	
Fluorophore	Abs. Extinction Coeff.	Excitation	Emission
Labeling Compounds:			
Coumarins			
7-Amino-4-methylcoumarin-3-acetic acid, NHS	17,000	354	441
7-Diethylaminocoumarin-3-carboxylic acid, NHS	56,000	432	472
Pacific Blue™, NHS	36,000	416	451
Fluoresceins			
5- or 6-carboxyfluorescein, NHS	83,000	496	516
Fluorescein-5- and/or -6-isothiocyanate	80,000	494	519
Oregon Green® 488 isothiocyanate	78,000	493	520
Rhodamines			
Rhodamine Green™ carboxylic acid, NHS	78,000	504	532
Alexa Fluor® 488	71,000	494	519
Alexa Fluor® 532	81,000	530	555
Alexa Fluor® 546	104,000	554	570
Alexa Fluor® 568	91,000	577	602
Alexa Fluor® 594	85,000	590	615
Tetramethylrhodamine-5 and/or -6-isothiocyanate	84,000	544	572
Texas Red® sulfonyl chloride	85,000	587	602
Cyanines			
Cy2™	150,000	489	506
Cy3™	150,000	550	570
Cy3.5™	150,000	581	596
Cy5™	250,000	649	670
Cy5.5™	250,000	675	694
Cy7™	200,000	743	767
Commercial labeled probe preparations:			
SpectrumBlue™		400	450
SpectrumAqua™		433	480
SpectrumGreen™		497	524
SpectrumGold™		530	555
SpectrumOrange™		559	588
SpectrumRed™		592	612
SpectrumFRed™		655	675
DNA counterstains:			
4',6-Diamidino-2-phenylindole (DAPI)		367	452
Propidium iodide (PI)		543	614

probe should span at least 40 kb of contiguous sequence. Probes that represent highly repetitive sequences, such as that found near centromeres or telomeres, can be made from a single plasmid containing an insert of approximately 300–10,000 bp.

Two of the most common means for identifying a clone containing the desired target sequence are: (1) screening the appropriate clone libraries, or (2) searching online databases, such as Genbank at the National Center for Biotechnology Information, with a known sequence such as mRNA from a particular gene, or anonymous sequence from the end of a clone insert or a Sequence Tagged Site (STS).

Whole chromosome painting probes, which contain sequences spread across the breadth of a specific chromosome, were originally prepared from whole chromosome phage and bacterial libraries ***(33,34)***. These libraries were ultimately obtained from chromosomes that were isolated by flow sorting. More recently, whole chromosome probes are prepared from flow sorted chromosomes ***(35,36)*** or microdissected chromosomes ***(37)*** that are amplified by DOP-PCR, without the intervening steps required to make bacterial libraries ***(30,38)***.

2. Materials

2.1. Nick-Translation

1. Sample DNA, typically 1 µg/50 µL labeling reaction.
2. Fluorophore labeled dUTP at a working concentration of 0.2 m*M*.
3. 0.3 m*M* dATP.
4. 0.3 m*M* dCTP.
5. 0.3 m*M* dGTP.
6. 0.3 m*M* dTTP.
7. Nick-translation enzyme: mix of DNA polymerase I (10,000 U/mL, DNase I (3 U/mL, such as Promega (Madison, WI) enzyme mix) .
8. 10X Nick-translation buffer: 500 m*M* Tris-HCl, pH 7.2, 100 m*M* $MgSO_4$, 1 m*M* dithiothreitol (DTT).
9. Nuclease-free water.
10. 15°C incubator.
11. Stop solution: 0.25 m*M* EDTA.
12. 3*M* sodium acetate, pH 5.5.
13. 70% ethanol.
14. 100% ethanol.
15. TE solution: 10 m*M* Tris-HCl, pH 7.5–8, 1 m*M* EDTA.
16. Sephadex G-50 type spin column (e.g., ProbeQuant™ G-50 spin columns, Amersham Pharmacia, Piscataway, NJ). Other methods can be used for removal of unincorporated nucleotides.
17. Microcentrifuge tubes.
18. Microcentrifuge.
19. Speed-vac lyophilizer.
20. Pipetors.
21. Pipet tips, preferably sterilized.
22. Tris-acetate buffer (TAE): 40 m*M* Tris-acetate, pH 7.5–7.8, 1 m*M* EDTA, or Tris-borate buffer (TBE): 45 m*M* Tris-borate, pH 8.0, 1 m*M* EDTA.
23. Agarose.

24. Hot plate or microwave oven.
25. 55°C water bath.
26. Mini horizontal gel electrophoresis apparatus with casting tray and combs, power supply.
27. DNA molecular weight markers, size range should cover 50–1000 bp.
28. 10 mg/mL ethidium bromide (EtBr).
29. 10X gel loading buffer: 50% (v/v) glycerol, 100 m*M* EDTA, 0.25% bromphenol blue.
30. UV transilluminator and UV protective visor.
31. Polaroid camera with Kodak Wratten red filter and type 667 Polaroid film.

2.2. Random Priming

1. Sample DNA, typically 10 ng to 3 µg/50 µL labeling reaction.
2. Fluorophore labeled dUTP at a working concentration of 1 m*M*.
3. 10 m*M* dATP.
4. 10 m*M* dCTP.
5. 10 m*M* dGTP.
6. 10 m*M* dTTP.
7. 40 U/mL Klenow fragment, (such as Life Technologies (Gaithersburg, MD) enzyme) .
8. 2.5X Random primer/buffer solution: 125 m*M* Tris-HCl, pH 6.8, 12.5 m*M* $MgCl_2$, 25 m*M* 2-mercaptoethanol, 750 µg/mL random octamer primers (Life Technologies).
9. Nuclease-free water.
10. 37°C incubator.
11. Boiling water bath.
12. Ice.
13. Stop solution, 0.25 m*M* EDTA.
14. 3*M* sodium acetate, pH 5.5.
15. 70% ethanol.
16. 100% ethanol.
17. Sephadex G-50 type spin column (optional, depending on method preferred for removal of unincorporated nucleotides).
18. Microcentrifuge tubes.
19. Microcentrifuge.
20. Speed-vac lyophilizer.
21. Pipetors.
22. Pipet tips, preferably sterilized.

2.3. DOP-PCR

1. Flow sorted chromosomes (500–1000/PCR)
2. 10X PCR buffer I: Applied Biosystems (Foster City, CA).
3. PCR assay buffer: 10 µL of 10X PCR buffer I, 2 µL dNTPs (10 m*M* each), 1 µL of primer (100 ng), 1 µL Taq polymerase, nuclease-free water to 100 µL.
4. PCR labeling buffer: Same as PCR assay buffer, with addition of 1 µL of SpectrumOrange™ or SpectrumGreen™ dUTP, except with final vol of 99 µL.
5. 100 ng/µL primer.
6. 50 nmol SpectrumOrange™, SpectrumGreen™ 2'-deoxyuridine- 5'-triphosphate (Vysis, Inc., Downers Grove, IL) or other labeled nucleoside triphosphate–Reconstitute with 50-µL of nuclease-free water to give a 1 m*M* solution. Stable for up to 3 mo if stored at –20°C.
7. Taq polymerase: any commercial variety available can be used, but optimal concentration should be first determined by titrating with appropriate substrate.

8. Nuclease-free water.
9. Mineral oil (if necessary), light white (Sigma Chemical Co., St. Louis, MO).
10. NuSieve GTG agarose (FMC BioProducts, Rockland, ME).
11. 1X TBE buffer for gel electrophoresis (BioRad, Hercules, CA).
12. Primers: The primer used was designated 6 MW ***(30)***, with sequence 5' CGA CTC GAG NNN NNN ATG TGG 3'.

2.4. Labeling Probes with Aliphatic Amines

1. Amine-modified DNA to be labeled.
2. A suitable reaction buffer (50 m*M* sodium tetraborate at pH 9.3 for isothiocyanates and sulfonic acid chlorides, 0.2 *M* 3-[*N*-morpholino]-propanesulfonic acid (MOPS; Sigma Chemical Co.) at pH 7.4 or 50 m*M* sodium tetraborate at pH 8.5 for *N*-hydroxysuccinimidyl esters).
3. Amine-reactive derivative of the desired label.
4. Dimethyl sulfoxide, dimethyl formamide, or acetone (whichever is capable of dissolving the desired amine-reactive label in a 10–20 m*M* solution).
5. A prepacked Sephadex G-25 column (Amersham Pharmacia, Piscataway, NJ).
6. TE solution: 10 m*M* Tris-HCl, pH 7.5–8, 1 m*M* EDTA).

2.5. ULS Labeling

1. TE buffer 10 m*M* Tris-HCl, 0.3 m*M* EDTA, pH 8.0.
2. DNA for labeling.
3. DNase I (Roche Biochemicals, Nutley, New Jersey) cat. no. 104 159, approx 2000 Kunitz U/mg.
4. 5 m*M* sodium acetate, 1 m*M* $CaCl_2$, 50% glycerol, pH 5.2
5. 10X nicking buffer: 50 m*M* Tris-HCl, pH 7.5, 10 m*M* $CaCl_2$, 10 m*M* $MgCl_2$.
6. 10 *M* ammonium acetate.
7. 100% ethanol.
8. Kreatech kit components: Vial 1: code LK1101 (60 µL rhodamine-ULS®, 0.5 U/µL), or code LK1301 (60 µL dGreen-ULS®, 0.5 U/µL), Vial 2: code LK006, Labeling Solution, 2 mL.
9. Spin columns: e.g., ProbeQuant™ G-50 spin columns (Amersham Pharmacia Biotech, Piscataway, NJ) or QIAquick™ spin columns (Qiagen, Valencia, CA).
10. Ultrasonic disruptor (e.g., Branson Ultrasonics, Danbury, CT).

2.6. Labeling Proteins for Indirect Detection

1. *N*-hydroxysuccinimidyl derivative of desired fluorophore.
2. Dimethyl sulfoxide, dimethyl formamide, or acetone (whichever is capable of dissolving the desired fluorophore in a 20 m*M* solution).
3. A prepacked Sephadex G-25 column (Amersham Pharmacia).
4. 50 m*M* boric acid, pH 8.5 to 9.3.
5. TBS: 25 m*M* Tris-HCl, 140 m*M* NaCl, 51 m*M* KCl, pH 7.4.
6. PBS: 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, 140 m*M* NaCl, 2.7 m*M* KCl, pH 7.4.

3. Methods

3.1. Nick-Translation

Nick translation is a method for incorporating labeled nucleotides into DNA such as an isolated fragment or an intact clone ***(39,40)***. The method uses a combination of

two enzymes, deoxyribonuclease I (DNase I) which nicks the DNA creating free 3' hydroxyls, and DNA polymerase I, which processively adds nucleotides to the 3' terminal hydroxyl. The 5' to 3' exonuclease activity of the DNA polymerase removes nucleotides from the 5' terminus of the nick as the polymerization proceeds. Both labeled and unlabeled nucleotides are substituted during the reaction and varying sized fragments are generated; however, there is no net synthesis of DNA. The resultant double-stranded fragments must be denatured prior to hybridization.

1. Prepare 0.1 m*M* dTTP by adding 100 µL of 0.3 m*M* dTTP to 200 µL nuclease-free water.
2. Prepare 0.1 m*M* dNTP mix by combining 100 µL each of 0.3 m*M* dATP, 0.3 m*M* dCTP, 0.3 m*M* dGTP. Excess nucleotide mixtures can be stored at –20°or –80°C.
3. For each labeling reaction, on ice, prepare a tube containing 1 µg DNA , 2.5 µL of 0.2 m*M* fluorophore-labeled dUTP, 5 µL of 0.1 m*M* dTTP, 10 µL of 0.1 m*M* dNTP mix, 5 µL of 10X nick translation buffer and make up to a final vol of 40 µL with nuclease-free water (*see* **Note 1**).
4. To each tube add 10 µL nick-translation enzyme mix.
5. Mix and briefly centrifuge.
6. Incubate at 15°C for 8–16 h.
7. Add 5 µL stop solution.
8. To remove unincorporated nucleotides, add 5 µL 3 *M* sodium acetate, 125 µL 100% ethanol; centrifuge at 12K for 20–30 min. Carefully pour off supernatant, or draw off with a pipettor. Add 100 µL 70% ethanol per tube, briefly vortex pellet; centrifuge at 15,000*g* for 5 min. Carefully pour off supernatant, or draw off with pipettor (*see* **Note 2**).
9. Dry pellet under vacuum 10–20 min. Resuspend in 10 µL TE, which yields an approx 100 ng/µL final concentration (*see* **Notes 3** and **4**). Alternatively, unincorporated nucleotides can be removed using a Sephadex G-50 type spin column according to the manufacturer's instructions. Labeled DNA will be in 50–100 µL after the column and will need to be concentrated by ethanol precipitation as described in **step 8**.
10. To determine size of the labeled DNA fragments, add 0.5 g agarose to 50 mL of TAE buffer or TBE buffer, carefully heat to boiling in microwave, or on a hot plate. After all agarose is melted, cool to 55°C in a water bath.
11. Add 2.5 µL EtBr to the agarose and mix. Pour molten agarose into casting tray using a 12 or 16 well comb.
12. For each labeled DNA, mix 2 µL DNA (~200 ng) plus 7 µL of water, or 1X TAE or TBE, plus 1 µL loading buffer.
13. Run labeled DNAs plus molecular weight marker at 70–100 V until leading dye has migrated approx 5 cm into the gel.
14. View DNA with UV transilluminator and take a Polaroid picture. Majority of fragments should be in the range of 200–600 bp.

3.2. Random Priming

Random priming is a means of labeling DNA fragments whereby, a mixture of all possible combinations of hexamers, octamers, or nonamers are annealed to denatured DNA ***(41,42)***. These small oligonucleotides then act as primers that allow for synthesis of the complementary DNA strand by the Klenow enzyme and incorporation of both labeled and unlabeled nucleotides. The labeled material will be a combination of both double- and single stranded fragments that must be denatured prior to hybridization.

1. Prepare a 10X dNTP mixture such that final concentrations are 1 m*M* dATP, 1 m*M* dCTP, 1 m*M* dGTP, 0.3 m*M* fluorophore-labeled dUTP, 0.7 m*M* dTTP. Excess mixture can be stored at –20°or –80°.
2. Dissolve DNA (400–500 ng preferred) in 20 µL nuclease-free water (*see* **Notes 5** and **6**).
3. Add 20 µL 2.5X random primer/buffer solution.
4. Heat denature for 5 min in boiling water; rapidly cool on ice.
5. On ice, add 5 µL 10X dNTP mix and 4 µL nuclease-free water to a final vol of 49 µL.
6. Mix and briefly centrifuge.
7. Add 1 µL Klenow enzyme and mix. Briefly centrifuge.
8. Incubate at 37°C for 1–6 h. Longer incubation times usually increase product yield (*see* **Note 7**).
9. Add 5 µL stop solution.
10. Unincorporated nucleotides and primers can be removed by adding 5 µL of 3 *M* sodium acetate, 125 µL of 100% ethanol; centrifuge at 15,000*g* for 20–30 min. Carefully pour off supernatant, or draw off with a pipetor. Add 100 µL of 70% ethanol per tube, briefly vortex pellet; centrifuge at 15,000*g* for 5 min. Carefully pour off supernatant, or draw off with pipetor.
11. Dry pellet under vacuum 10–20 min. Resuspend in 20 µL TE, which yields an approx 100 ng/µL final concentration. Alternatively, unincorporated nucleotides can be removed using a Sephadex G-50 type spin column according to the manufacturer's instructions. Labeled DNA will be in 50–100 µL after the column, and will need to be concentrated by ethanol precipitation as described in **step 10**.
12. Optimally, fragments should range from 200–600 bp, and can be visualized by gel electrophoresis. Refer to **steps 10–14** (*see* **Subheading 3.1.**) for electrophoresis protocol.

3.3. DOP-PCR

PCR generated whole chromosome DNA, either from microdissected or flow sorted chromosomes, is currently viewed as the preferred route to high quality probes suitable for whole chromosome staining of individual chromosomes. This is true for staining single chromosomes as well as for staining all 24 human chromosomes combinatorially in a multiplex FISH (M-FISH) ***(43)*** or spectral karyotyping ***(44)*** assay. The procedure provided below ***(45)*** is a modification of the original Degenerative-Oligonucleotide-Primed-PCR (DOP-PCR) protocol ***(38)***, and is optimized to amplify chromosome DNA in the presence of SpectrumOrange™ or SpectrumGreen™ dUTP (Vysis, Inc.). The protocol also should permit labeling with a variety of other labeled dUTP's, with little or no modification. The reaction involves the use of an oligonucleotide with an Xho-I restriction endonuclease site at its 5' end, a defined six-nucleotide sequence at the 3' end, and a set of degenerate nucleotides (a random mix of all 4 nucleotides) in between. Theoretical calculations indicate that the defined 3' sequence occurs every 4 kb along the genome. Under suitable conditions, an amplification using this primer could be primed off the specific 3' sequence, probably stabilized by annealing of one or more of the degenerate nucleotides ***(38)***. The specific 5' sequence permits the annealing of this primer at a higher temperature to previously amplified DNA. In practice, the initial cycles in the DOP-PCR protocol include a low temperature annealing step (low fidelity PCR), followed by multiple cycles at a higher annealing temperature (higher fidelity PCR), resulting in a population of randomly amplified DNA.

The flow sorted chromosomes are normally amplified through two rounds of DOP-PCR, and the resulting product is then labeled by a third round of amplification in the presence of fluorescently labeled nucleoside triphosphates. Although nick-translation protocols could be used to label probes for chromosomal analysis, this additional step is usually unnecessary to obtain high quality probes.

3.3.1. Amplification of DNA

Typically, flow sorted chromosomes are resuspended and amplified in PCR assay buffer, using the following conditions: 95°C for 5 min, followed by 9 cycles of 94°C for 1 min, 30°C for 1.5 min, and 72°C for 3 min, with a ramp time of 2 min, 5 s, followed by 35 cycles at 94°C for 15 s, 62°C for 15 s, and 72°C for 15 s (*see* **Notes 8–10**). This is followed by a single extension at 72°C for 10 min, and then holding the temperature at 4°C. Using the same procedure, 1 µL of the PCR product is then reamplified in a final vol of 100 µL. 10 µL of this reaction should then be electrophoresed on a 1% agarose gel, resulting in a smear, ranging in size from approx 300–1000 bp. The yield of DNA from this reaction varies from 1.5–3.0 µg (*see* **Note 11**).

3.3.2. Labeling of DNA

1 µL from the previous round of PCR is labeled in PCR labeling buffer, using the same conditions described for DNA amplification. 1–2 µL of the labeled DNA can then be used directly for hybridization without purification.

3.4. Labeling of DNA Probes with Aliphatic Amines

In addition to the one-step labeling of probes using polymerases to incorporate labeled nucleotides, a two-step labeling procedure can be utilized involving: (1) incorporating nucleoside triphosphates with aliphatic amine substituents into the probe DNA using the polymerase-based labeling protocols described above (*see* **Subheading 3.1.–3.3.**), and (2) reacting the modified DNA with amine reactive fluorophores. The two-step procedure offers the advantage that any amine reactive fluorophore can be attached to the modified DNA, instead of only fluorophores that are commercially available already attached to nucleoside triphosphates. Additionally, all polymerase-based labeling reactions can use the same nucleoside triphosphate–allylamine-dUTP. Therefore, a single set of polymerase reaction conditions can be used for all fluorescent labels, instead of having to optimize for each different fluorescent nucleoside triphosphate. Also, incorporation of allylamine-dUTP may be more efficient than incorporation of other labeled nucleoside triphosphates. Labeling kits for two-step label incorporation via allylamine-dUTP are commercially available (ARES labeling kits, Molecular Probes, Inc., Eugene, OR).

Aliphatic amines introduced into DNA enzymatically, using allylamine-dUTP in place of labeled nucleoside triphosphates, can be conjugated with amine-reactive fluorophores, biotins, or haptens by the following procedure. The procedure can also be used to label probes containing aliphatic amines introduced by a number of other chemistries ***(22,46)***. This includes synthetic DNA, RNA, and PNA oligomers containing aliphatic amine modified bases, or 3'- or 5'-amine-modified termini, introduced via phosphoramidite chemistry.

1. Dissolve amine-modified DNA in a suitable reaction buffer at a concentration of 10 nmol aliphatic amines/0.6–1.0 mL reaction buffer.
2. Dissolve the amine reactive labeling compound in a suitable solvent to a final concentration of 10–20 m*M*.
3. Add a 50-fold (isothiocyanates) or 100- to 200-fold (*N*-hydroxysuccinimidyl esters or sulfonic acid chlorides) molar excess of the amine-reactive dye to the amine modified DNA (*see* **Note 12**).
4. Allow the reaction solution to stir at room temperature overnight.
5. Separate the probe from unconjugated label by gel permeation chromatography using a Sephadex G-25 column equilibrated and eluted with water or TE buffer. The labeled probe will elute in the excluded volume (*see* **Note 13**).
6. Store the labeled probe at 4°C or lower until ready for use.

3.5. ULS Labeling

The Universal Linkage System (ULS®; KREATECH Biotechnology BV, Amsterdam, The Netherlands) is a labeling methodology that uses a platinum dye complex to react with the N7 position of the guanine nucleotides. This reaction results in the formation of a stable bond between the nucleic acid and the platinum fluorophore complex. Depending on reaction conditions the ULS compound, to a lesser extent, will also form a complex with the adenine bases. This methodology has been used to label DNA (including plasmids, cosmids, molecular weight markers, DNA in low melting point agarose, BACs, PACs, YACs, whole chromosome libraries, DOP-PCR products and highly repetitive sequences such as satellite, centromeric, and telomeric DNA), RNA, PNA, oligonucleotides, and amplified nucleic acid products ***(16,47)***. ULS reagents and kits are also offered by Molecular Probes (Eugene, OR).

Prior to any labeling with the ULS compounds the template to be labeled must be of a size range <1000 bp. Template larger than 1000 bp will result in a substantial amount of spotted background. PCR products are usually <1000 bp and therefore can be labeled directly. The two methods of size reduction described will work with both the Kreatech and the Molecular Probes labeling protocols–sonication and enzymatic cleavage. Molecular Probes recommends an alternative DNase I protocol for use with the Alexa-ULS reagents. This protocol and the corresponding reagents are part of the ULS labeling kits provided by Molecular Probes.

3.5.1. DNA Fragmentation

3.5.1.1. Sonication

1. Prepare a DNA solution at a concentration of 20 ng/μL in TE buffer. Using a minimum volume of 100 μL for sonication is best.
2. Sonicate the DNA solution in a small conical bottom plastic tube for 3 cycles of 1 min each, while keeping the sample on ice. Select ultrasonic disruptor power level and duty cycle to deliver the highest power possible while minimizing cavitation. Allow the DNA solution to cool on the ice for 1 min prior to the start of sonication, and after each cycle. In a microcentrifuge, centrifuge the DNA solution for 5 s at max speed before the second and third sonication steps to force all of the solution to the bottom of the tube (*see* **Note 14**).
3. Confirm adequate DNA size by electrophoresis on an aliquot of the DNA solution using a 1% agarose gel. Refer to **steps 10–14** of **Subheading 3.1.** for electrophoresis protocol.
4. Proceed to labeling protocols.

3.5.1.2. DNase Treatment

1. Prepare a stock solution of DNase I by dissolving 1 mg of DNase I (Roche cat. no. 104 159, approx 2000 Kunitz U/mg) in 1 mL of 5 m*M* sodium acetate, 1 m*M* CaCl2, 50% glycerol, pH 5.2. Keep the buffer on ice prior to and during the addition of the lyophilized DNase. Invert this solution until completely mixed. Do not vortex. The stock solution should be stored at –20°C avoiding freeze thaw cycles.
2. Dilute the DNase I stock 1:5000 in 1X nicking buffer (*see* **Note 15**).
3. Add the following components to a microcentrifuge tube on ice: 1 µg template DNA, 2.5 µL 10X nicking buffer, 3–5 µL diluted DNase I, H_2O to 25 µL.
4. Incubate this reaction at 37°C for 10 min.
5. Stop the reaction by placing on ice.
6. Precipitate the reaction mixture with 1/4 vol of 10 *M* ammonium acetate and 2.5 vol of 100% ethanol.
7. Resuspend the pellet in the appropriate amount of labeling solution (*see* **Subheading 3.5.2.**).
8. Confirm adequate DNA size by electrophoresis on an aliquot of the DNA solution using a 1% agarose gel.

3.5.2. Labeling

3.5.2.1 Protocol Recommended by KREATECH

Optimal labeling efficiencies are achieved when the ULS reagent and nucleic acid are combined at a 1:1 ratio (i.e. 1 U ULS reagent:1 µg DNA). For labeling amounts of nucleic acid other than the standard 1 µg, the ratio of nucleic acid to ULS reagent should be kept at 1:1. When labeling small amounts, a minimum of 100 ng of nucleic acid in a 20 µL vol should be used. Alternatively, larger amounts should not exceed 10 µg of nucleic acid in a 20 µL vol. Labeling in a larger vol is possible as long as the template concentration is not lower than 5 ng/µL, and the amount of ULS reagent added is adjusted to the amount of input template. In the case of very dilute template, the labeling solution can be omitted.

1. Add 1 U (2 µL) ULS reagent to 1 µg of input template (*see* **Notes 16** and **17**).
2. Adjust volume with labeling solution to 20 µL and mix well.
3. Incubate for 15 min at 65°C.
4. Centrifuge briefly.
5. Purify probe on a spin column.

3.5.2.2. Protocol Recommended by Molecular Probes

Molecular Probes provides a kit that utilizes the ULS labeling system coupled to a variety of their dyes. The kit contains reagents both for fragmentation of the DNA and labeling protocols. The labeling protocols and precautions are very similar with exception of the following:

1. Molecular Probes ULS dye reagents are dissolved in different buffers prior to labeling, depending upon the dye selected. A chart is provided with all specific information relating to each available dye.
2. Immediately prior to labeling, DNA is denatured at 95°C for 5 min, and then snap cooled on ice. It is noted that denaturation is not a necessity but can improve the labeling efficiency by 20–40%.

3. The labeling reaction total volume is 25 µL.
4. Incubation is at 80°C for 15 min.
5. Molecular Probes states that the modifications to the previously stated Kreatech protocols have been made owing to the specifications of their own dyes that are coupled to the ULS reagents.

3.6. Labeling Proteins for Indirect Detection

Secondary detection reagents for indirectly labeled FISH probes are prepared by conjugating fluorophores to proteins such as avidin, streptavidin, or antibodies. A large variety of fluorophore labeled avidins and antibodies are commercially available (e.g., Accurate Chemical and Scientific Co., Westbury, NY; Calbiochem, San Diego, CA; Cappel Organon Teknika, Durham, NC; DAKO Corp., Carpinteria, CA; Jackson ImmunoResearch Laboratories, West Grove, PA; Kirkegaard and Perry Laboratories, West Grove, PA; Molecular Probes, Inc., Eugene, OR; Pierce Chemical Co., Rockford, IL; Sigma Chemical Co., St. Louis, MO). Labeled proteins can also be prepared fairly easily in the laboratory. Unlabeled avidins and antibodies can be obtained from the same suppliers listed above.

Streptavidin is often preferred over avidin as a secondary reagent because of its near-neutral pH isoelectric point, which is believed to reduce nonspecific binding. Similarly, while whole antibodies can be used in FISH, the more hydrophobic F_c region is often removed to reduce nonspecific binding. The F_c region can be cleaved from the antigen-binding regions of antibodies by digestion with papain, to produce Fab particles (single binding arms), or pepsin, to produce $F(ab')_2$ with the two binding arms still connected. Fab' particles can be prepared from $F(ab')_2$ preparations by selective reduction of disulfide bonds that joint the two binding arms. Methods for preparing $F(ab')_2$, Fab, and Fab' antibody fragments can be found in the literature ***(48,49)***.

1. Dissolve the protein to a final concentration of 1–10 mg/mL in 50 m*M* boric acid at pH 8.5–9.3.
2. Dissolve the *N*-hydroxysuccinimidyl derivative of the desired fluorophore in a solvent such as dimethyl sulfoxide, dimethyl formamide, or acetone, to a final concentration of 20 m*M*.
3. Add a sufficient volume of the fluorophore solution to the protein solution to provide a 1- to 20-fold molar excess of fluorophore to protein *(see* **Notes 18** and **19**).
4. Allow the reaction to proceed with gentle stirring at room temperature for 2 h.
5. Separate the protein from unconjugated fluorophore by gel permeation chromatography using a Sephadex G-25 column equilibrated and eluted with TBS or PBS. The labeled protein will elute in the excluded volume (*see* **Note 20**).
6. Store the labeled protein at 4°C until ready for use (*see* **Note 21**).

3.7. Characterization of Labeled Probes

To calculate accurate probe concentrations, enough DNA must be available to generate an absorbance value of at least approx 0.05 in the minimal volume of liquid required to fill the spectrophotometer cuvet. For conventional absorbance spectrophotometer cuvets with 1 cm pathlengths, the minimal volumes are several hundred microliters (semimicro cuvets). Spectrophotometers specifically designed for DNA work can utilize cuvets with volumes near 10 µL. The following equation can be used to

calculate the nucleic acid concentration, [nucl], from the measured absorbance at 260 nm, A_{260}:

$$[\text{nucl}] = [A_{260} - (\varepsilon_{F,260}/\varepsilon_{F,MAX})A_{F,MAX}]/\varepsilon_{\text{nucl},260}$$

where $\varepsilon_{\text{nucl},260}$ is the absorbance extinction coefficient of the nucleic acid at 260 nm, and $\varepsilon_{F,260}/\varepsilon_{F,MAX}$ is the ratio of the absorbance extinction coefficients of the label at 260 nm, $\varepsilon_{F,260}$, and at the peak wavelength of the longest wavelength absorbance band, $\varepsilon_{F,MAX}$. This ratio for the labeled probe is approximated by the ratio of absorbance values of the unconjugated label at these two wavelengths. For single-stranded DNA, $\varepsilon_{\text{nucl},260}$ = 10,000 $M^{-1}\cdot cm^{-1}$, or 0.0286 (μg/mL) mL·$ng^{-1}\cdot cm^{-1}$. The former value gives the concentration in nucleotide molarity, while the latter value gives the concentration in micrograms nucleic acid per milliliter. The label concentration, [F], is calculated from the probe absorbance at the long wavelength absorbance maximum of the label:

$$[F] = A_{F,MAX}/\varepsilon_{F,MAX}$$

at which the nucleic acid absorbance is assumed to be negligible. Values of $\varepsilon_{F,MAX}$ can be obtained from the suppliers of the labeling reagents. The percentage of nucleotides labeled is then equal to 100[F]/[nucl].

When the amount of probe is too small to obtain accurate absorbance measurements, or contains some contaminating RNA, fluorometry with bisbenzimide, commonly known as Hoescht 33258 dye, can be used to determine nucleic acid concentration. The Hoescht dye has little affinity for RNA but binds to the minor groove of double stranded DNA. Hoescht 33258 dye bound to DNA can be excited at 365 nm, and has peak emission at 458 nm. Fluorometry is very sensitive and can be used for DNA concentrations ranging from 0.01–5 mg/mL, with an optimum DNA concentration range of 0.05–0.3 mg/mL.

The relative fluorescence intensities are measured on the sample DNA solution, F_S, and the same solution minus the DNA (blank solution), F_B. In addition, the fluorescence intensity of a DNA standard solution, F_{STD}, and corresponding blank solution, $F_{STD,B}$ are also measured (typically the sample and standard blank solutions are the same). The DNA standard solution should have a DNA concentration, $[DNA]_{STD}$, close to that expected for the DNA sample solution. The sample DNA concentration, $[DNA]_S$, is then calculated as follows:

$$[DNA]_S = [DNA]_{STD}(F_S - F_B)/(F_{STD} - F_{STD,B})$$

Accurate pipetting and thorough mixing of solutions is critical for reproducible results.

The concentrations and labeling percentages of proteins can be determined by absorbance spectroscopy using the same equations as for nucleic acid probes, except that measurements are recorded at 280 nm instead of 260 nm. The absorbance extinction coefficients for goat IgG and Fab' have been reported to be 198,000/$M^{-1}\cdot cm^{-1}$ and 61,200/$M^{-1}\cdot cm^{-1}$ ***(49)***, respectively, and the extinction coefficient for $F(ab')_2$ should be twice that for Fab'.

4. Notes

1. Fluorophores will photobleach if exposed to light for extended periods of time. Labeled DNAs and dUTPs can be handled for short periods of time in light but should not be exposed any longer than required to set up an experiment.

2. It is easier to assess range of fragment sizes if unincorporated nucleotides are removed prior to gel electrophoresis.
3. After ethanol precipitation, DNA pellets labeled with a red or orange fluorophore are usually readily seen by eye, whereas, those labeled with a green fluorophore may appear white, or very pale green.
4. 50–100 ng of a red or orange labeled unique sequence probe, and 200–400 ng of a green labeled probe per 10 µL FISH mix typically yield sufficiently bright signals on the majority of sample types.
5. For optimal results, template DNA should be linear, preferably by digestion using a restriction enzyme with a six base pair recognition site and purified by phenol/chloroform extraction followed by ethanol precipitation.
6. Starting amounts of DNA can range from 10 ng to 3 µg.
7. Labeling at 37°C can range from 1 h to overnight. Optimal conditions should be determined for individual applications.
8. Contamination is a very serious issue in PCR. To avoid contamination of PCR reactions, use the best quality reagents possible, and use them exclusively for PCR. It is best to routinely aliquot all solutions and store them frozen at –20° C until ready for use. Also, use aerosol-resistant tips for pipetting to minimize cross-contamination. Designate one area in the lab exclusively for PCR work. When possible, prepare DNA samples in a separate room. In addition, it is absolutely essential to run suitable negative controls (i.e., without target sequence) each time an experimental PCR is performed.
9. Titrate the Taq polymerase with the specific target. Excess enzyme can result in nonspecific background.
10. Vary the number of cycles to find the appropriate number of cycles which gives the best signal to noise ratio.
11. The presence of high molecular weight DNA (visible during electrophoresis as a smear extending from the well to approx 2.0 kb size) indicates unacceptable labeled probe. Such probe results in very high nonspecific, noncellular background that can sometimes be reduced by sonication. As mentioned in **Note 9**, titrate the Taq polymerase, and find the appropriate number of PCR cycles.
12. The organic solvent: aqueous buffer ratio should not exceed 1:4 unless the DNA is known to be soluble at a higher ratio.
13. As an alternative to gel permeation chromatography, the unconjugated fluorophore can be separated from the protein by dialyzing in a storage buffer (e.g., TE), changing the buffer solution at several hour intervals until the unconjugated label is completely removed (2 or more buffer changes).
14. When sonicating, the volume of the sample, the type of vessel in which the sample is contained, the length of the sonication period, the number of sonication cycles, the ultrasonic disruptor power level, and the ultrasonic disruptor duty cycle, are all dependent upon the make and model of the ultrasonic disruptor instrument and the sonication probe used. Some experimentation may be required to obtain properly sized nucleic acid fragments.
15. When performing the DNase I digestion, all solutions should be kept on ice. Prepare solutions immediately before use.
16. DNA should be purified to remove proteins, RNA, and free nucleotides before labeling.
17. High tris(hydroxymethyl)aminomethane (Tris) concentrations (>40 m*M*) or EDTA (>5 m*M*), Mg acetate (>100 m*M*), NaCl (>100 m*M*), and restriction enzyme digestion buffers should be avoided because of their rate-limiting effect on the labeling reaction.
18. The molar excess of reactive fluorophore-to-protein, and the protein concentration will determine the extent of protein labeling. The labeling ratio required for optimal perfor-

mance of the labeled protein reagent will depend upon which fluorophore and protein are used, and will need to be determined experimentally. Too low a labeling ratio results in weak fluorescence signals, while too high a ratio can inhibit the specific protein binding reaction and increase nonspecific binding.

19. The amount of 20 m*M* fluorophore solution added to the protein should not result in the organic solvent concentration exceeding 20% of the total reaction volume, necessary to prevent denaturation of the protein.
20. As an alternative to gel permeation chromatography, the unconjugated fluorophore can be separated from the protein by dialyzing in TBS or PBS, changing the buffer solution at several hour intervals until the dye color is no longer imparted to the buffer.
21. Alternative protein labeling protocols abound in the literature and are available from suppliers of labeling reagents (e.g., *see* the "Amine-Reactive Probes" information sheet from Molecular Probes, Inc., or "Procedure for Labeling Proteins with Fluorochromes" by Research Organics, Cleveland, OH).

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