FISH Tag™ DNA Multicolor Kit

Table 1. Contents and Storage Information.

Material	Amount	Concentration	Storage *	Stability
Reagents for Labeling Reaction †				When stored as directed, the kit is stable for 6 months.
Alexa Fluor® reactive dyes (Components A, B, C, and D)	5 vials in each of 4 pouches	NA	• ≤–20˚C • desiccate • protect from light	
Dimethylsulfoxide (DMSO) (Component M)	200 µL	NA	• ≤–20˚C • desiccate	
Dithiothreitol (DTT) (Component K)	100 µL	0.1 M in nuclease-free water	• ≤–20˚C	
Glycogen, ultrapure, nulcease free (Component L)	20 µL	20 µg/µL		
Sodium bicarbonate (Component N)	84 mg	NA		
Water, nuclease free (Component O)	4.0 mL	NA		
DNA nucleotide mix (Component P)	80 µL	10X		
DNA polymerase I (Component S)	25 µL	10 units/µL		
DNase I (Component R)	15 µL	1 unit/µL		
Nick translation buffer (Component Q)	250 µL	10X		
Antifade Reagent †				
SlowFade® Gold antifade reagent (Component J)	2 mL	NA	• ≤–20˚C • protect from light	
Reagents for Purifying Labeled Nucleic Acid				
Binding buffer (Component E)	6.0 mL	NA	• ≤25˚C	
Wash buffer (Component F)	3.2 mL	NA		
Elution buffer (Component G)	6.0 mL	NA		
Spin columns and collection tubes (Component H)	20 columns with tubes	NA		
Collection tubes (Component I)	20 tubes	NA		

***** The FISH Tag™ DNA Multicolor Kit is shipped on dry ice. **†** The labeling reaction reagents and the antifade reagent components must be stored at ≤–20°C in a non–frost-free freezer. Avoid freeze-thaw cycles. NA = Not applicable.

Number of Labelings: 10 reactions.

Spectral Data: See Table 2.

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Introduction

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) technology permits detection of specific nucleic acid targets within a biological specimen, or *in situ* meaning where it lies. RNA and DNA targets such as mRNAs expressed in a tissue or genes present on a chromosome can be localized using this technology. Detection of a nucleic acid target *in situ* is achieved through hybridization of complementary sequence, fluorescent dye–labeled nucleic acid "probe" to the specimen. Once the hybridization assay is complete, the specimen is viewed under a fluorescence microscope to visualize the hybridized fluorescent probe. Fluorescent dyes, or fluorophores, having different excitation and emission spectra generate fluorescence of different colors when viewed under a fluorescence microscope. Different fluorophores can be used to label different nucleic acid probes for detection of multiple targets simultaneously. Multiplex FISH (MFISH) refers to the simultaneous localization of multiple sequence-specific nucleic acid targets using spectrally distinct fluorescent dye labels.

The labeling technology provided in the FISH Tag™ DNA Kits uses a two step approach.[1] In the first step, nick translation is used to enzymatically incorporate an amine-modified nucleotide into the probe template. The modified nucleotide is dUTP having an NH_2 group attached through a linker to the C5 position of the base. In the second step, dye labeling of the purified amine-modified DNA is achieved by incubation with amine-reactive dyes. These active ester compounds react with the primary amines incorporated into the probe template, covalently conjugating the dye to the modified nucleotide base. The purified probe is then ready for hybridization to the specimen.

The FISH Tag™ DNA Multicolor Kit is supplied with four spectrally distinct Alexa Fluor® fluorescent dyes (Table 2). We have also developed FISH Tag™ DNA Kits in single-dye versions (F32947, F32948, F32949, and F32950). It is important to know the filter sets available on your fluorescence microscope prior to choosing a fluorophore for labeling and detection (Table 2 and Figure 1). The dyes available in the FISH Tag™ DNA Kits are compatible with standard filter sets found on most fluorescence microscopes. Our proprietary Alexa Fluor® dyes are brighter and more photostable than traditional fluorescent labels,[2] providing higher resolution and improved signal to noise ratios compared to conventional dyes. The Alexa Fluor® 488 dye is spectrally similar to fluorescein and has green emission when viewed with the appropriate filter set. The Alexa Fluor® 555 dye is spectrally similar to Cy3 dye and has orange emission, whereas the Alexa Fluor® 594 dye is spectrally similar to Texas Red® dye and has red emission. The Alexa Fluor® 647 dye is spectrally similar to Cy5 dye and has far-red emission not visible to the human eye. The Alexa Fluor® 647 dye must be viewed using a fluorescence microscope equipped with a CCD camera.

Table 2. Alexa Fluor® dyes supplied with the FISH Tag™ DNA Multicolor Kit.

Product	Catalog number	Dye Supplied	Ex/Em* (fluorescent color)	Filters †
FISH Tag™ DNA Multicolor Kit	F32951	Alexa Fluor® 488	492/520 (green)	Alexa Fluor® 488 Filter Set
		Alexa Fluor® 555	555/565 (orange)	Alexa Fluor® 555 Filter Set
		Alexa Fluor® 594	590/615 (red)	Alexa Fluor® 594 Filter Set
		Alexa Fluor® 647	650/670 ‡	Alexa Fluor® 647 Filter Set

***** Approximate fluorescence excitation and emission maxima, in nm. **†** Molecular Probes offers a selection of Semrock BrightLine® filter sets ideal for our Alexa Fluor® dyes. See probes.invitrogen.com for ordering information. **‡** Alexa Fluor® 647 dye has far red emission that is not detectable by eye and requires a CCD camera for imaging

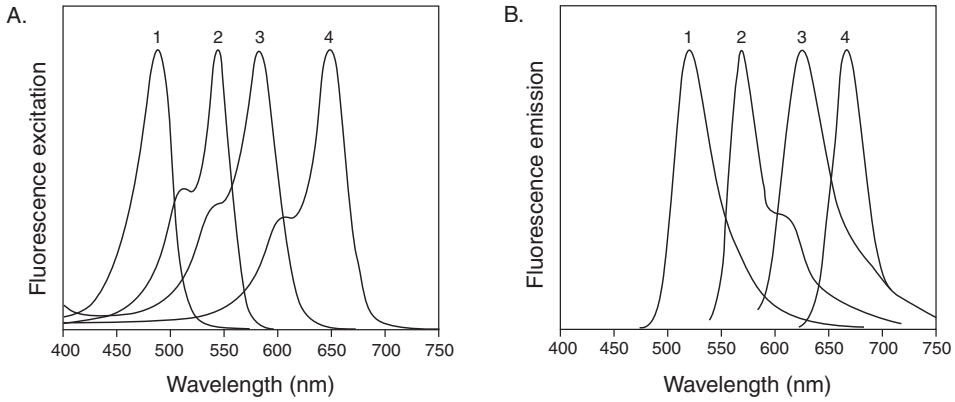

Figure 1. Fluorescence excitation (A) and emission (B) of the dyes supplied with the FISH Tag™ DNA Kits. 1. Alexa Fluor® 488 dye. 2. Alexa Fluor® 555 dye. 3. Alexa Fluor® 594 dye. 4. Alexa Fluor® 647 dye.

FISH Tag™ Kits

The FISH Tag™ DNA Kits are based on traditional nick translation protocols but use a two-step labeling approach to provide improved dye incorporation.[1] The probe synthesis protocol consists of four basic processes: 1) DNA synthesis, 2) purification, 3) dye coupling, and 4) purification.

For DNA probe labeling, the process of nick translation enzymatically incorporates an amine-modified nucleotide during DNA synthesis, which is subsequently labeled using an amine-reactive fluorescent dye compound. Nick translation uses DNase I and DNA polymerase I in concert to modify a given DNA template. DNase I randomly generates single-stranded nicks in the DNA template that are recognized by DNA polymerase I. The DNA polymerase replaces the DNA strand in the 5′ → 3′ direction, substituting amine-modified dUTP (aminoallyl dUTP) for some of the thymidine bases in the DNA. The 10X DNA nucleotide mix provided in the kit contains an optimal ratio of aminoallyl dUTP:dTTP to generate a degree of dye labeling that provides optimal S/N in hybridization. Importantly, the amount of DNase I in the nick translation reaction should result in the size of the DNA template being reduced to an average length of ~500 base pairs for optimal hybridization. The length of the DNA provided by the user will determine exactly how much DNase I is needed to generate a 500 base pair average length (300 bp to 700 bp range). DNase I recommendations are provided in the nick translation protocol based on 7–10 kb DNA templates and 100 kb BAC DNA templates. Thus, it is important to evaluate the size of the DNA produced by nick translation by gel electrophoresis prior to dye labeling (see *Before You Begin*).

After purification of the amine-modified DNA, coupling of the fluorescent dye is performed. The amine-reactive ester dye compound will react with the primary amines incorporated into the DNA and covalently attach the fluorophore to the base. The amount of amine-reactive dye ester compound provided in each vial is optimal for labeling 1 µg or less of amine-modified DNA. Following this coupling reaction, the labeled DNA is purified as before.

DNA purification is accomplished using the spin columns provided (technology based on PureLink™ PCR Purification Kits from Invitrogen). These columns are used both for the purification of the amine-modified DNA following nick translation and the purification of the dye-labeled DNA following dye coupling. The purification is based on selective binding of nucleic acids to a silica-based membrane in the presence of chaotropic salts. The nucleic acid is mixed with binding buffer for binding to the column. Impurities, salts, and excess dye are removed by the wash buffer while the nucleic acid is bound to the silica membrane. The nucleic acid is then recovered by the addition of elution buffer.

The DNA hybridization protocol provided in this manual for *in situ* hybridization is based on classical cytogenetic FISH using centromeric (alpha satellite) probes to human metaphase chromosome spreads. It is provided as an example. Depending on your model system or specimen requirements, optimization of this protocol may be required.

Before you Begin

Materials Required but Not Supplied

- DNA template for nick translation reaction
- 100% isopropanol
- 100% ethanol
- 70% ethanol
- 3M sodium acetate, pH 5.2
- incubator at 15°C
- heat block at 96°C
- microcentrifuge

Handling of Amine-Reactive Fluorescent Dyes

Amine-reactive fluorescent dyes are sensitive to light and moisture. Ensure that the amine-reactive fluorescent dyes remain desiccated. Minimize the exposure of the labeled probe (both during the labeling reaction and during your experiments) to light.

Storage of DMSO

The DMSO used for dissolving the amine-reactive dye compounds (Component M) is hygroscopic. Store at ≤–20°C or room temperature, tightly sealed.

Preparing Binding Buffer with Isopropanol

1.1 To the binding buffer concentrate supplied in the kit (6 mL, Component E) add 4 mL of **100% isopropanol** to make a final volume of 10 mL of binding buffer.

1.2 Mix well.

1.3 Mark the checkbox on the bottle label to indicate that isopropanol has been added. The working solution of binding buffer is stable for 6 months at room temperature.

Preparing Wash Buffer with Ethanol

2.1 To the wash buffer concentrate supplied in the kit (3.2 mL, Component F) add 12.8 mL of **100% ethanol** to make a final volume of 15 mL of wash buffer.

2.2 Mix well.

2.3 Mark the checkbox on the bottle label to indicate that ethanol has been added. The working solution of wash buffer is stable for 6 months at room temperature.

Preparing the Sodium Bicarbonate Solution

3.1 To the tube containing the sodium bicarbonate powder (Component N) add 1 mL of nuclease-free water (Component O)

3.2 Vortex until solid material is no longer visible in the tube.

3.3 Store at ≤–20˚C when not in use. This solution of sodium bicarbonate will be stable for 6 months.

Calculating the Amount of DNase I to Use

For each nick translation reaction you perform, a working solution of DNAse I will be prepared from the stock solution provided in this kit. It is important to determine beforehand how much DNAse I working solution will generate DNA fragments that have an average size of ~500 bp. (We recommend probe length of between 300 bp and 700 bp for optimal results.) Based on the following guidelines, estimate the amount of DNAse I working solution required for your plasmid DNA:

For 1 µg of a 5–7 kb plasmid template we recommend 4 µL of DNase I working solution.

For 1 µg of a 100 kb BAC template we recommend 6 µL of DNase I working solution.

Perform a test digestion of your DNA sample with DNase I by setting up a reaction as described in step 4.4. Following the incubation period (step 4.6), ethanol precipitate the nick translation reaction (purification is not required) and examine the products by gel electrophoresis. Based on the average fragment size as revealed by gel electrophoresis, adjust the amount of DNase I working solution used for the reaction.

Pre-Protocol Reading

At the end of this instruction manual are two sections entitled *Tips for Success* and *Troubleshooting*. It may be beneficial to read through these topics before you start your experiment, especially if you are relatively new to the preparation and use of FISH probes.

Synthesis of Amine-Modified DNA

Nick Translation Reaction

4.1 Remove the following components from the freezer, thaw to room temperature, and mix by vortexing:

- water, nuclease free (Component O)
- 10X nick translation buffer (Component Q)
- 0.1M DTT (Component K)
- 10X DNA nucleotide mix (Component P)

4.2 Remove the following components from the freezer, and place them on ice or in a –20°C bench top cooler. **Do not vortex.**

- DNase I (Component R)
- DNA polymerase I (Component S)

Note: When in use, ensure these enzymes remain in the –20°C bench top cooler or on ice and return them to the non–frost-free freezer as soon as possible after use.

4.3 Make a working solution of DNase I **on ice** as described below.

Component	Volume
water, nuclease free	89 µL
10X nick translation buffer	10 µL

Leave the mixture **on ice** for 10 minutes and **then add**:

DNase I (Component R)	1 µL
Final Volume	100 µL

Mix the DNase I working solution **gently** by slowly pipetting the mixture up and down three times **(do not vortex)** and then leave the tube on ice.

4.4 Prepare nick translation reactions **on ice** as described below.

Component	Volume
water, nuclease free	to final 50 µL
10X nick translation buffer	5 µL
0.1M DTT	5 µL
10X DNA nucleotide mix	5 µL
DNA (user supplied)	1 µg
DNA polymerase I (Component S)	1.7 µL
DNase I working solution (prepared in step 4.2)	4–6 µL*
Final Volume	50 µL

* For 1 µg of a 5–7 kb plasmid template we recommend 4 µL of DNase I working solution. For 1 µg of a 100 kb BAC template we recommend 6 µL of DNase I working solution. The optimal size of a DNA hybridization probe should average 500 base pairs in length. See *Before You Begin* for instructions on *Calculating the Amount of DNase I to Use*.

4.5 Mix the nick translation reaction **gently** by slowly pipetting the mixture up and down three times (**do not vortex**).

4.6 Incubate the reaction at 15°C for 2 hours.

4.7 Add 50 µL nuclease-free water (Component O) and vortex the reaction at maximum speed for 10 seconds. The vortexing is important to inactivate the DNase I.

4.8 Proceed immediately to *Purifying the Amine-Modified DNA*.

Purifying the Amine-Modified DNA

5.1 Add 400 µL of binding buffer with isopropanol (see *Before You Begin*, above) to the synthesis reaction and mix well.

5.2 Add the entire volume (500 µL) to a spin column seated inside a collection tube (Component H).

5.3 Centrifuge the column at >10,000 × g for 1 minute. The DNA is bound to the column. Discard the flow-through.

5.4 Wash the column with 650 µL of wash buffer with ethanol (see *Before You Begin*, above).

5.5 Centrifuge the column at >10,000 × g for 1 minute. Discard the flow-through.

5.6 Centrifuge the column >10,000 × g for 1 minute to remove any residual wash buffer.

5.7 Place the spin column in a clean 1.7 mL collection tube (Component I).

5.8 Apply 55 µL of elution buffer (Component G) to the center of the column.

5.9 Allow the column to stand at room temperature for 1 minute.

5.10 Centrifuge the column > 10,000 × g for 1 minute.

5.11 The elution tube contains your purified amine-modified DNA. Discard the column and proceed to *Ethanol Precipitation of the Amine-Modified DNA*.

Ethanol Precipitation of the Amine-Modified DNA

6.1 To the eluted DNA from step 5.11, add:

- 10 µL of 3M sodium acetate (pH 5.2)
- 1 µL of glycogen (Component L)
- 39 µL of nuclease-free water (Component O)

6.2 Add 250 µL of 100% ethanol.

6.3 Store sample at –20˚C for 30 minutes.

6.4 Centrifuge the sample at >10,000 × g for 10 minutes.

6.5 Remove the supernatant. Be careful not to lose the pellet.

6.6 Carefully rinse the pellet with 400 µL of 70% ethanol. Remove the supernatant and repeat this rinse step.

Note: Free amines carried over with the DNA will inhibit the efficiency of the dye coupling reaction. These rinse steps with 70% ethanol are important to eliminate any trace amines.

6.7 With a pipet, remove as much of the residual 70% ethanol as possible without disturbing the pellet and then allow the sample to air dry (about 5–10 minutes).

6.8 Add 5 µL of **nuclease-free water** (Component O) to the pellet (buffer should not be used in order to avoid introduction of free amines).

6.9 Incubate the sample at 37°C for 5 minutes.

6.10 Vortex the sample to fully resuspend the DNA.

Note: The sample can be stored at this stage for up to 2 weeks.

6.11 Determine the concentration of the sample (see *Calculating the Labeling Efficiency and Concentration of Nucleic Acid*).

Note: The concentration of the nick translated DNA is usually close to 0.2 µg/µL so the sample may normally be taken immediately to the fluorescent labeling step without further alteration of the concentration.

6.12 Adjust the concentration of the sample with water to a final concentration of 0.2 µg/µL.

6.13 Proceed to *Labeling the Amine-Modified DNA with Fluorescent Dye.*

Labeling the Amine-Modified DNA with Fluorescent Dye

7.1 Denature 1 µg (5 µL) of the DNA by incubating it at 96°C for 5 minutes.

7.2 Place the sample on ice for 3 minutes.

7.3 Centrifuge the sample at >10,000 × g for 3 minutes.

7.4 Add 3 µL of sodium bicarbonate solution to the sample (prepared in step 3.3 and frozen).

Note: The thawed sodium bicarbonate solution may precipitate. Vortex thoroughly before using.

7.5 Remove the label from a vial of reactive dye (Components A, B, C, **or** D) in order to better see the dye pellet.

7.6. Resuspend the reactive dye in 2 µl of DMSO (Component M). Vortex well (10 seconds at high speed) in order to fully resuspend the dye.

7.7 Transfer the 2 µl of reactive dye in DMSO to the DNA sample.

7.8 Vortex the mixture at maximum speed for **at least** 15 seconds.

Note: Sufficient mixing of the labeling reaction is critical.

7.9 Centrifuge the sample briefly in order to collect the labeling reaction in the bottom of the tube.

7.10 Incubate the labeling reaction at room temperature in the dark for 1 hour.

7.11 Add 90 µL of water to the sample.

7.12 Proceed immediately to *Purifying the Fluorescent Dye–Labeled DNA*.

Purifiying the Fluorescent Dye–Labeled DNA

8.1 Add 400 µL of binding buffer with isopropanol (see *Before You Begin*, above) to the labeling reaction and mix well.

8.2 Add the entire volume (500 µL) to a spin column seated inside a collection tube (Component H).

8.3 Centrifuge the column at >10,000 × g for 1 minute. The labeled DNA is bound to the column. Discard the flow-through.

8.4 Wash the column with 650 µL of wash buffer with ethanol (see *Before You Begin*, above).

8.5 Centrifuge the column at >10,000 × g for 1 minute. Discard the flow-through.

8.6 Centrifuge the column >10,000 × g for 1 minute to remove any residual wash buffer.

8.7 Place the spin column in a clean 1.7 mL collection tube (Component I).

8.8 Apply 55 µL of elution buffer (Component G) to the center of the column.

8.9 Allow the column to stand at room temperature for 1 minute.

8.10 Centrifuge the column >10,000 × g for 1 minute.

8.11 The elution tube contains your purified fluorescent dye–labeled DNA. Discard the column.

Ethanol Precipitation of the Fluorescent Dye–Labeled DNA

9.1 To the eluted dye-labeled DNA from step 8.11 add:

- 10 µL of 3M sodium acetate (pH 5.2)
- 1 µL of glycogen (Component L)
- 39 µL of nuclease-free water (Component O)

9.2 Add 250 µL of 100% ethanol.

9.3 Store sample at –20˚C for 30 minutes.

9.4 Centrifuge the sample at > 10,000 × g for 10 minutes.

9.5 Remove the supernatant. Be careful not to lose the pellet.

9.6 Carefully rinse the pellet with 400 µL of 70% ethanol. Remove the supernatant and repeat this rinse.

9.7 With a pipet, remove as much of the residual 70% ethanol as possible without disturbing the pellet and then allow the sample to air dry (about 5–10 minutes).

9.8 Add 10 µL of **nuclease-free water** (Component O) to the pellet.

9.9 Incubate the sample at 37˚C for 5 minutes.

9.10 Vortex the sample to fully resuspend the dye-labeled DNA.

9.11 Determine the concentration of the sample (see *Calculating the Labeling Efficiency and Concentration of Nucleic Acid*).

9.12 The dye–labeled DNA is now ready for hybridization buffer. Alternatively, store the dye-labeled DNA at ≤–20˚C until ready for use. It is stable when protected from light for up to 2 weeks when stored at ≤–20˚C.

Suggested Hybridization Protocol

The DNA hybridization protocol for *in situ* hybridization is based on classical cytogenetic FISH using centromeric (alpha satellite) probes to human metaphase chromosome spreads. It is provided as an example. Depending on your model system or specimen requirements, optimization of this protocol may be required.

Useful protocols for various *in situ* hybridization applications can be found in In Situ *Hybridization: A Practical Approach* by D.G. Wilkinson (Ed.) Oxford University Press; 2nd edition (1999), In Situ *Hybridization Protocols (Methods in Molecular Biology)* by I. A. Darby (Ed.) Humana Press; 2nd edition (2000), *Practical* In Situ *Hybridization* by T. Schwarchzacher and P. Heslop-Harrison, BIOS Scientific Publishers (1999), and *Introduction to Fluorescence* In Situ *Hybridization: Principles and Clinical Applications* by M. Andreeff (Ed.) and D. Pinkel, Wiley-Liss; 1st edition (1999).

10.1 Use routine procedures to produce metaphase chromosome spreads from phytohemagglutinin-stimulated normal human peripheral blood lymphocyte cultures. (ACT Cytogenetics Laboratory Manual, 2nd edition (ed. M.J. Barch, Raven Press, New York, 1991)).

10.2 Dry the slide at room temperature.

10.3 Incubate the slide in 2X SSC for 2 minutes at 73°C.

10.4 Add 500 µL of 5% pepsin to 50 mL of 10 mM HCL in a Coplin jar at 37°C.

Note: The 5% w/v pepsin should be made in water and stored in aliquots before use. Step 10.4 describes pepsin treatment of slides using a Coplin jar. Other formats using different volumes may be acceptable as long as the final concentration of pepsin is 0.05% w/v.

10.5 Incubate the slide in the 10 mM HCL/pepsin solution for 10 minutes at 37°C.

10.6 Wash the slide twice for 5 minutes each in 1X PBS at room temperature.

10.7 Incubate the slide in 1% formaldehyde/1X PBS solution for 5 minutes at room temperature.

10.8 Wash the slide twice for 5 minutes each in 1X PBS at room temperature.

10.9 Dehydrate the slide for 1 minute each at in 70%, 85%, and 100% EtOH at room temperature and allow the slide to air dry.

10.10 Incubate the slide in 70% formamide/2X SSC (pH 7.0) at 72°C for 2 minutes.

10.11 Dehydrate the slide in 70%, 80%, and 95% cold ethanol (–20°C) for 2 minutes each and then allow the slide to air dry at room temperature.

10.12 Resuspend the DNA probe in TE buffer at a final concentration of 4 ng/µL and vortex thoroughly to ensure complete resuspension of the labeled DNA.

10.13 Prepare the DNA probe/hybridization buffer by mixing 2.5 µL of DNA probe, 1 µL 20X SSC, and 6.5 µL formamide in a microfuge tube.

10.14 Denature the DNA probe/hybridization buffer by incubation at 72˚C for 5 minutes and then place the tube on ice.

10.15 Apply 10 µL of the DNA probe/hybridization buffer to one half of the specimen slide, cover with a 22 × 22 mm coverslip, and seal the edge of the coverslip with a bead of rubber cement.

10.16 Incubate the slide in a humidified chamber at 37˚C overnight.

10.17 Soak the coverslip off the slide by soaking for 5 minutes in 2X SSC/0.1% NP-40 at 37°C.

10.18 Agitate the slide for 1–3 seconds in 0.4X SSC/0.3% NP-40 at 73°C and then allow the slide to incubate in this solution for 2 minutes.

10.19 Agitate the slide for 1–3 seconds in 2X SSC/0.1% NP-40 at room temperature and then allow the slide to soak in this solution for 1 minute.

10.20 Dip the slide quickly in H_2O and allow to air dry in the dark.

10.21 Counterstain the specimen by incubation in 20 ng/mL DAPI for five minutes at room temperature.

10.22 Apply one drop of *SlowFade*® Gold antifade reagent (Component J), cover with a 22 × 22 mm coverslip and proceed to imaging.

Tips for Success

Sensitivity

Limits of detection for dye-labeled nucleic probes can be related to several parameters including length of labeled probe, labeling density or degree of labeling (DOL), and the abundance of target molecule. Longer probes can harbor more dyes per probe molecule and thus provide better sensitivity, but can limit penetration if not reduced to an average of 500 base pairs. For instance, the method described in this instruction manual is useful for detecting single-copy genes in chromosome FISH using a probe 10 kb in length, whereas it is not sensitive enough for a 1 kb probe (W. Gregory Cox, personal communication). Thus, the experimental design should include a reliable positive control, such as a centromeric probe for chromosome FISH.

Stringency

Stringency of the post-hybridization washes can have a great effect on the specificity of the hybridization signal. Stringency of post-hybridization washes is increased by lowering the salt conditions (for example, 0.5X SSC to 0.4X SSC) and/or by increasing the wash temperature. The best approach to reduce nonspecific or cross-hybridization artifacts following hybridization is to reduce the salt concentration of the wash. Hybridization temperature can also be increased to reduce these artifacts.

Length of Probe

Central molecular biology dogma dictates that optimal penetration and hybridization of labeled nucleic acid probes is achieved with probes of 500 bases average length (between 300 bp and 700 bp). Product size in nick translation is determined by the number of DNase I units, which can be adjusted per DNA template to achieve the optimal size range. It is important to target the optimal size range for every template for consistency and reproducibility.

Specimen Integrity

The integrity of the specimen is critical to successful hybridization of the probe to the target. Specimens for DNA hybridization should be treated to denature the target DNA so that it is available for hybridization to the probe. The hybridization protocols provided in this instruction manual serve as a general guide to standard procedures; optimization may be required for your system.

Imaging

Prior to imaging the labeled specimen, it is important to verify the correct filter sets to match the dye choice are available on the microscope and that they are in good condition. They filter sets for each channel should accommodate accurately the spectral characteristics of the dye (see Figure 1 and Table 2). The filter sets should be inspected for wear that might lead to excitation/emission beyond the filter window specifications. Multicolor experiments should be designed with the available filter sets in mind such that the emission windows accommodate separation of individual dye emissions cleanly without overlap or bleed-through. All fluorescent dyes are subject to photobleaching, so labeled specimens should be protected from light whenever possible. We provide *SlowFade*® Gold antifade reagent for mounting Alexa Fluor® dye–labeled specimens because it is optimized for high photostability of these dyes where other more traditional mounting media fail. The *SlowFade*® Gold antifade reagent is non-gelling. ProLong® Gold mounting media provides the same level of photostability as *SlowFade*® Gold, but slowly gels over time. Both mounting media are available with DAPI counterstain added.

Troubleshooting

In troubleshooting your work, consider the following topics:

Yield

The standard nick translation protocol is optimized for use with 1 µg of template DNA. Nick translation does not generate an overall increase in DNA, so yields following purification can be expected to be 70–100%. It is important to verify the concentration and purity of the template DNA prior to the nick translation reaction. Protein contamination of the DNA template stock can overestimate the true DNA concentration due to contribution to the absorbance at 260 nm of the solution. Poor yields could also reflect over digestion by DNase I

if the size of the product is below 100 base pairs, which will not be retained by the purification column. It is important to verify the size of the nick translation product by gel electrophoresis, which should be 500 base pairs on average.

Degree of Labeling (DOL)

DOL is a measure of the number of dyes per 100 bases of nucleic acid probe, as determined from absorbance readings at 260 nm and at the dye maximal absorbance (Table 3). The calculation is provided below and is available on our website at probes.invitrogen.com. Accurate absorbance readings require the entire sample in the smallest volume possible. Microcuvettes of 1 cm pathlength and 100 µL can be used. Other microscale spectrophotometers are available. It is important to blank the instrument with the diluent prior to measurement and not to dilute the sample too greatly as to fall into the non-linear dynamic range of the instrument. Expected DOLs should be from 1–6 dyes per 100 bases, depending on the dye. It is important to follow the guidelines in the instruction manual in detail in order to obviate the possibility of free amine contamination that will result in low DOLs. We strictly recommend two, large volume 70% washes of the amine-modified nucleic acid pellet in order to eliminate free amines. The amine-modified DNA should be fully resuspended prior to the coupling reaction by vortexing and using low heat (37°C) if resuspension is problematic. The amine-reactive dye is extremely sensitive to moisture and thus, must be stored sealed tightly in its pouch bag with desiccant to prevent loss of activity, which can result in low DOLs. Thorough mixing of the coupling reaction is important to optimal labeling and vortexing the reaction at full speed for a full 15 seconds is highly recommended in order to avoid low DOL.

Hybridization

The hybridization protocols suggested are provided as a general guideline to standard DNA FISH and your model system will require some optimization. Consult published scientific literature and the handbooks above for further details on general *in situ* hybridization technique. By far the most important aspect of the experimental design is a reliable positive control that will verify that the hybridization and detection protocols are working. Centromeric probes work well for this in chromosome FISH applications. DNA FISH can fail for multiple reasons but it is important to be able to verify that DNA targets are denatured and available for hybridization. A reliable positive control is crucial to successfully troubleshooting your model system.

Calculating the Labeling Efficiency and Concentration of Nucleic Acid

The relative efficiency of a labeling reaction can be evaluated by calculating the approximate ratio of bases to dye molecules.[1] This ratio can be determined, as described below, by measuring the absorbance of the nucleic acid at 260 nm and the absorbance of the dye at its absorbance maximum (ε_{max}). The calculations are based on the Beer-Lambert law:

$A = \varepsilon \times \text{path length (cm)} \times \text{concentration (M)}$,

where ε is the extinction coefficient in $cm^{-1}M^{-1}$. The absorbance measurements can also be used to determine the concentration of nucleic acid in the sample. Values needed for these calculations are found in Table 3. Alternatively, the ratio can be determined by using our Base:Dye Ratio Calculator on our website (probes.invitrogen.com) in the *Resources* section.

Measuring the Base:Dye Ratio

11.1 Measure the absorbance of the nucleic acid–dye conjugate at 260 nm (A_{260}) and the ε_{max} for the dye (A_{dye}). Measure the background absorbance at 260 nm and ε_{max}, using buffer alone, and subtract these numbers from the raw absorbance values for the sample. The ε_{max} values for the fluorophores are given in Table 3.

- To perform these measurements, the nucleic acid–dye conjugate should be at a concentration of at least 5 µg/mL. Depending on the dye used and the degree of labeling, a higher concentration may be required.
- For most applications, it will be necessary to measure the absorbance of the entire sample using either a conventional spectrophotometer with a 100 µL cuvette or an absorbance microplate reader with a microplate.
- Use a cuvette or microplate that does not block UV light and that is clean and nuclease-free. Note that most plastic disposable cuvettes and microplates have significant absorption in the UV.

11.2 Correct for the contribution of the dye to the A_{260} reading. Most fluorescent dyes absorb light at 260 nm as well as at their ε_{max}. To obtain an accurate absorbance measurement for the nucleic acid, it is therefore necessary to account for the dye absorbance using a correction factor (CF_{260}). Use the CF_{260} values given in Table 3 in the following equation:

$$A_{base} = A_{260} - (A_{dye} \times CF_{260})$$

11.3 Calculate number of dyes per 100 bases.

Use the following equation:

$$\text{\# dyes/100 bases} = \frac{100}{(A_{base} \times \varepsilon_{dye}) / (A_{dye} \times \varepsilon_{base})}$$

where ε_{dye} is the extinction coefficient for the fluorescent dye (found in Table 3) and ε_{base} is the average extinction coefficient for a base in double stranded DNA (dsDNA) (ε_{base} for dsDNA is 6600 $cm^{-1}\ M^{-1}$). Note that since the calculation is a ratio, the path length has canceled out of the equation.

Table 3. Spectral characteristics for dyes supplied with the FISH Tag™ DNA Kits.

Fluorescent Dye	λmax (nm)*	ε_{dye} ($cm^{-1}\ M^{-1}$) †	CF_{260} §
Alexa Fluor® 488	492	62,000	0.30
Alexa Fluor® 555	555	150,000	0.04
Alexa Fluor® 594	588	80,400	0.43
Alexa Fluor® 647	650	239,000	0.00

* Absorbance maximum for the fluorophore. † Extinction coefficient for the dye. § Correction factor = A_{260} for the free dye / A_{max} for the free dye.

Measuring the Concentration of Nucleic Acid

The absorbance values A_{260} and A_{dye} may also be used to measure the concentration of nucleic acid in the sample ([N.A.]). In order to obtain an accurate measurement for a dye-labeled nucleic acid, a dye-corrected absorbance value (A_{base}) must be used, as explained in step 11.2. In addition, for concentration measurements, the path length (in cm) is required. If the path

length of the cuvette or of the solution in a microplate well is unknown, consult the manufacturer. Follow steps 11.1 and 11.2 above and then use the following equation (MW_{base} for dsDNA is 330 g/mol):

$$[\text{N.A.}]\ (\text{mg/mL}) = (A_{base} \times MW_{base}) / (\varepsilon_{base} \times \text{path length})$$

References

1. Biotechniques 36, 114 (2004); **2.** J Histochem Cytochem 47, 1179 (1999).

Product List

Current prices may be obtained from our website or from our Customer Service Department.

Cat #	Product Name	Unit Size
F32947	FISH Tag™ DNA Green Kit *with Alexa Fluor® 488 dye* *10 reactions*	1 kit
F32948	FISH Tag™ DNA Orange Kit *with Alexa Fluor® 555 dye* *10 reactions*	1 kit
F32949	FISH Tag™ DNA Red Kit *with Alexa Fluor® 594 dye* *10 reactions*	1 kit
F32950	FISH Tag™ DNA Far Red Kit *with Alexa Fluor® 647 dye* *10 reactions*	1 kit
F32951	FISH Tag™ DNA Multicolor Kit *Alexa Fluor® dye combination* *10 reactions*	1 kit
F32952	FISH Tag™ RNA Green Kit *with Alexa Fluor® 488 dye* *10 reactions*	1 kit
F32953	FISH Tag™ RNA Orange Kit *with Alexa Fluor® 555 dye* *10 reactions*	1 kit
F32954	FISH Tag™ RNA Red Kit *with Alexa Fluor® 594 dye* *10 reactions*	1 kit
F32955	FISH Tag™ RNA Far Red Kit *with Alexa Fluor® 647 dye* *10 reactions*	1 kit
F32956	FISH Tag™ RNA Multicolor Kit *Alexa Fluor® dye combination* *10 reactions*	1 kit

Additional Products

Invitrogen offers additional reagents that may be useful in FISH studies.

Cat #	Product Name	Unit Size
P36930	ProLong® Gold antifade reagent	10 mL
P36931	ProLong® Gold antifade reagent with DAPI	10 mL
S36937	*SlowFade*® Gold antifade reagent	5 x 2 mL
S36939	*SlowFade*® Gold antifade reagent with DAPI	5 x 2 mL
K3100-01	PureLink™ PCR Purification Kit	50 rxns
K3100-02	PureLink™ PCR Purification Kit	250 rxns.

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