FISH Tag™ RNA 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		
RNA nucleotide mix (Component P)	20 µL	10X		
T7 RNA polymerase (Component Q)	10 µL	50 units/µL		
T3 RNA polymerase (Component R)	10 µL	50 units/µL		
SP6 RNA polymerase (Component S)	10 µL	15 units/µL		
DNase I (Component T)	15 µL	1 unit/µL		
T3/T7 transcription buffer (Component U)	80 µL	5X		
SP6 transcription buffer (Component V)	40 µL	5X		
RNaseOUT™ ribonuclease inhibitor (recombinant) (Component W)	10 µL	40 units/µL		
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™ RNA 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.

Contents

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™ RNA Kits uses a two step approach.[1] In the first step, *in vitro* transcription is used to enzymatically incorporate an amine-modified nucleotide into the probe template. The modified nucleotide is UTP 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 RNA 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™ RNA Multicolor Kit is supplied with four spectrally distinct Alexa Fluor® fluorescent dyes (Table 2). We have also developed FISH Tag™ RNA Kits in single-dye versions (F32952, F32953, F32954, and F32955). It is important to know the filter sets available on your fluorescence microscope prior to choosing a fluorophore for labeling and detection (Table 1 and Figure 1). The dyes available in the FISH Tag™ RNA 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™ RNA Multicolor Kit.

Product	Catalog number	Dye Supplied	Ex/Em* (fluorescent color)	Filters †
FISH Tag™ RNA Multicolor Kit	F32956	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

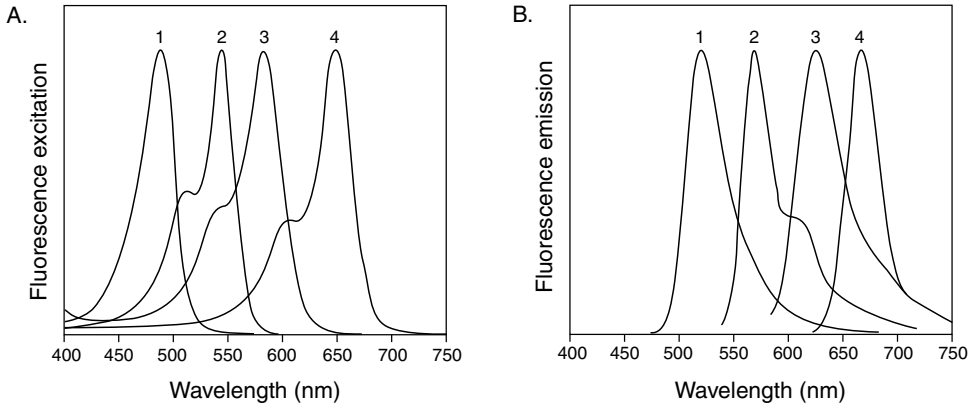

Figure 1. Fluorescence excitation (A) and emission (B) of the dyes supplied with the FISH Tag™ RNA Kits. 1. Alexa Fluor® 488 dye. 2. Alexa Fluor® 555 dye. 3. Alexa Fluor® 594 dye. 4. Alexa Fluor® 647 dye.

FISH Tag™ Kits

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

For RNA probe labeling, *in vitro* transcription is used to enzymatically incorporate an amine-modified nucleotide during RNA synthesis, which is later labeled using an amine-reactive fluorescent dye compound. RNA synthesis is driven from an RNA promoter sequence (T3, T7, or SP6) present near the 5′ end of the DNA strand to be transcribed. The RNA polymerase synthesizes new RNA substituting amine-modified UTP (aminoallyl UTP) for UTP. The 10X RNA nucleotide mix provided in the kit contains an optimized ratio of aminoallyl UTP:UTP to generate a degree of labeling that provides optimal S/N in hybridization. Importantly, the DNA construct for transcription requires a T3, T7, or SP6 RNA promoter sequence near the 5′ end of the DNA strand to be transcribed. The kit provides all three of these RNA polymerases for optimal flexibility. Also the construct should be linearized using restriction enzymes to cut the plasmid at the 3′ end of the DNA sequence of interest to prevent transcription of the DNA vector sequence. Ideally, the plasmid construct will have the DNA of interest between different RNA promoter sequences on opposite strands and opposite ends of the cloning cassette (Figure 2). Linearizing the plasmid near the 3′ end of the DNA in the anti-sense direction allows one RNA polymerase to generate anti-sense RNA probe, which has sequence complementary to the target and will hybridize *in situ* to the specimen. Conversely, linearization of the plasmid near the 3′ end of the DNA in the sense direction allows the user to use a different RNA polymerase to generate sense RNA probe (in a separate reaction), which has sequence identical to the target and serves as a negative control as it will not hybridize to the specimen. Knowing the sequence and orientation of the DNA insert will determine which promoter to use for sense and anti-sense probes.

After purification of the amine-modified RNA, coupling of the fluorescent dye is performed. The amine-reactive ester dye compound will react with the primary amines incorporated into the RNA, covalently attaching 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 RNA. Following this coupling reaction, the labeled RNA is purified as before.

RNA purification is accomplished using the spin columns provided (technology based on PureLink™ PCR Purification Kits from Invitrogen). These columns are used for both the purification of the amine-modified RNA following the *in vitro* transcription reaction and the purification of the dye-labeled RNA following the dye coupling reaction. 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 RNA hybridization protocol provided in this manual for *in situ* hybridization is based on RNA hybridization to *Drosophila* (fruit fly) embryos[3] and should be generally applicable to tissues. It is provided as an example. Depending on your model system or specimen requirements, optimization of this protocol may be required.

Before you Begin

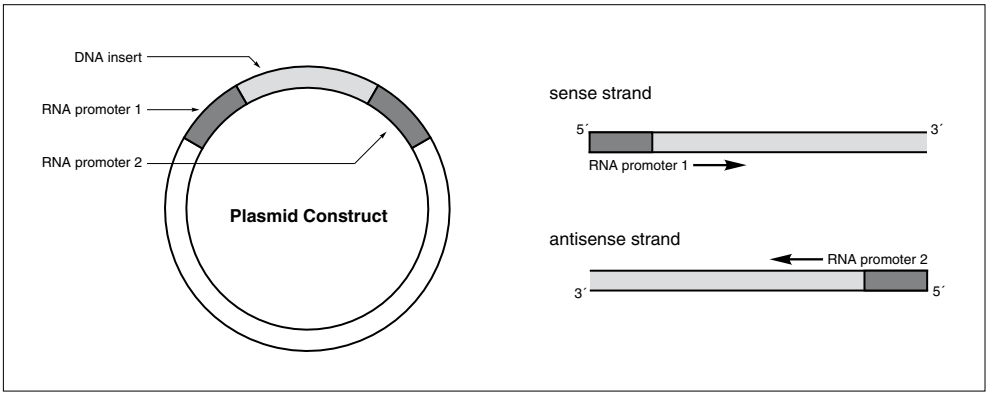

Figure 2. Plasmid construct scheme for in vitro transcription using the FISH Tag™ RNA Kits. The DNA insert should ideally be contained between different RNA promoter sequences (RNA promoter 1 and RNA promoter 2; either T3, T7, or SP6 promoters) on opposite strands and opposite ends of the cloning cassette. If the plasmid is linearized near the 3´ end of the DNA insert in the antisense direction, RNA polymerase will generate an antisense RNA probe complementary to the target that will hybridize to the specimen. If the template for RNA polymerase is the plasmid linearized at the 3´ end in the sense direction, RNA polymerase will generate a sense RNA that will not hybridize to the specimen (serves as a negative control).

Materials Required but Not Supplied

- DNA template for transcription
- 100% isopropanol
- 100% ethanol
- 70% ethanol
- 3M sodium acetate, pH 5.2
- incubator at 37°C
- heat block at 65°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.

Choosing the Appropriate DNA Template for *In Vitro* Transcription

The DNA template should be a plasmid construct that has T7, T3, or SP6 RNA polymerase promoter sequences at opposite ends and on opposite strands of the DNA insert of interest. This type of construct allows one to generate sense (negative control) and anti-sense hybridization probes. The vector should be linearized by restriction digest at one end of the insert or the other in order to utilize the respective RNA promoter sequence to initiate RNA synthesis. The nucleotide sequence of the DNA insert and proximity to the RNA polymerase promoter will determine which polymerase to use for each linearized form of the plasmid (Figure 2).

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 RNA

In Vitro Transcription

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

- water, nuclease free (Component O)
- 5X transcription buffer of choice (T3/T7 transcription buffer, Component U **or** SP6 transcription buffer, Component V)
- 0.1M DTT (Component K)
- 10X RNA nucleotide mix (Component P)
- RNaseOUT™ ribonuclease inhibitor (Component W)

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.**

- RNA polymerase of choice (T7 RNA polymerase, Component Q **or** T3 RNA polymerse, Component R, **or** SP6 RNA polymerase, 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 If you plan to use SP6 RNA polymerase, first make a 0.01 M DTT solution by diluting 1 µL of 0.1 M DTT (Component K) into 9 µL of nuclease-free water (Component O). **Use this diluted solution of DTT if the *in vitro* transcription reaction (step 4.4) uses the SP6 RNA polymerase.**

4.4 Prepare *in vitro* transcription reactions on ice as described below.

Component	Volume
water, nuclease free	to final 20 µL
5X transcription buffer (either T3/T7 or SP6)	4 µL
DTT	1–2 µL*
10X RNA nucleotide mix	2 µL
linearized DNA template (user supplied)	1 µg
RNaseOUT™ inhibitor	1 µL
RNA polymerase (T7, T3, or SP6)	1 µL
Final Volume	20 µL

* If the reaction uses T3/T7 RNA polymerase, add 1 µL of **0.1 M DTT** (Component K) in this reaction. If the reaction uses SP6 RNA polymerase, add 2 µL of **0.01 M DTT** (prepared in step 4.3).

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

4.6 Incubate at 37°C for 1 hour.

4.7 Add 1 µl of DNase I (Component T) and mix **gently** by slowly pipetting the mixture up and down three times (**do not vortex**).

4.8 Incubate at 37°C for 15 minutes.

4.9 Add 79 µl of nuclease-free water to the sample (Component O) and vortex the reaction at maximum speed for 10 seconds. The vortexing is important to inactivate the DNase I.

4.10 Proceed immediately to *Purifying the Amine-Modified RNA*.

Purifying the Amine-Modified RNA

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 RNA 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 collection tube contains your purified amine-modified RNA. Discard the column and proceed to *Ethanol Precipitation of the Amine-Modified RNA*.

Ethanol Precipitation of the Amine-Modified RNA

6.1 To the eluted RNA 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 300 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 RNA 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 RNA and place the sample on ice.

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 *in vitro* transcription reaction often generates 1–4 µg of RNA depending on DNA template. The dye conjugation reaction (below) is optimized for 1 µg of amine-modified RNA. Using more RNA per reaction will result in lowered labeling efficiency.

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 RNA with Fluorescent Dye*.

Labeling the Amine-Modified RNA with Fluorescent Dye

7.1 Denature 1 µg (5 µL) of the RNA by incubating it at 65°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).

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 RNA sample at room temperature.

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 RNA*.

Purifiying the Fluorescent Dye–Labeled RNA

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 RNA 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 collection tube contains your purified fluorescent dye–labeled RNA. Discard the column.

Ethanol Precipitation of th Fluorescent Dye–Labeled RNA

9.1 To the eluted dye-labeled RNA 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 300 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 L) 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 RNA and store on ice.

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

9.12 The dye–labeled RNA is now ready for hybridization buffer. Alternatively, store the dye-labeled RNA at ≤–70˚C until ready for use. It is stable when protected from light for up to 2 weeks when stored at ≤–70˚C.

RNA Probe Fragmentation (Optional)

For RNA probes greater than 500 bases, the following optional fragmentation protocol can be used to reduce the size of the labeled RNA probe to an average length of 500 bases. We recommend the user test fragmented and non-fragmented probes in hybridization to determine which gives the best signal to noise ratio.

10.1 Prepare 2X carbonate buffer

- 127.2 mg sodium carbonate
- 67.2 mg sodium bicarbonate
- add water to 10 mL
- pH to 10.2 with NaOH
- store in small aliquots at ≤–20˚C.

10.2 Prepare stop solution

- 164.1 mg sodium acetate
- add water to 10 mL
- pH to 6.0 with acetic acid
- store in small aliquots at ≤–20˚C.

10.3 Mix 10 µL of labeled RNA with 10 µl 2X carbonate buffer.

10.4 Incubate at 42˚C for 20–30 minutes.

10.5 Add 20 µL stop solution and precipitate with ethanol (as described above).

10.6 Verify the length by gel electrophoresis. Decrease or increase the time of fragmentation accordingly.

Suggested Hybridization Protocols

The RNA hybridization protocol for *in situ* hybridization is based on RNA hybridization to *Drosophila* (fruit fly) embryos[3] and should be generally applicable to tissues. 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).

Note: perform these steps at room temperature unless specified otherwise and never let the specimen become dry at any point as this will increase autofluorescence.

Pre-Hybridization

11.1 Use routine fixation procedures to prepare tissue specimens (see introductory paragraphs under *Suggested Hybridization Protocols* for references).

11.2 Incubate the specimen in 100% ethanol for 5 minutes with gentle rocking.

11.3 Remove most of the ethanol and incubate the specimen in xylene for 1–2 hours with gentle rocking.

11.4 Rinse the specimen twice with 100% ethanol.

11.5 Incubate the specimen in 100% ethanol for 5 minutes with gentle rocking.

11.6 Rinse the specimen twice with 100 % methanol.

11.7 Incubate the specimen in 100% methanol for 5 minutes with gentle rocking.

11.8 Incubate the specimen in 50% methanol/5% formaldehyde/PBT for 5 minutes with gentle rocking. (PBT = PBS/0.1% Tween 20).

11.9 Rinse the specimen with 5% formaldehyde/PBT.

11.10 Incubate the specimen in 5% formaldehyde/PBT for 25 minutes with gentle rocking.

11.11 Wash the specimen in PBT four times for 5 minutes each with gentle rocking.

11.12 Incubate the specimen in PBT/proteinase K for 5 minutes with gentle rocking.

Note: Prepare proteinase K stock solution in water at a final concentration of 10 mg/mL and store in small aliquots at –20°C. The concentration needed in treatment is 5–50 µg/mL final concentration and should be determined in separate, parallel 5 minute incubations at different concentrations. The optimal amount is determined by optimal signals. Too little proteinase K will result in lowered signals whereas too much proteinase K will result in loss of tissue integrity.

11.13 Rinse the specimen twice in PBT.

11.14 Wash the specimen in PBT for 5 minutes with gentle rocking.

11.15 Wash the specimen in PBT/5% formaldehyde for 25 minutes with gentle rocking.

11.16 Wash the specimen in PBT four times for 5 minutes each with gentle rocking.

11.17 Wash the specimen in PBT/50% hybridization buffer for 10 minutes with gentle rocking. (Hybridization buffer is 50% formamide, 5X SSC, 100 µg/mL fragmented salmon testes DNA, 50 µg/ mL heparin, 0.1% Tween 20.)

11.18 Wash the specimen in hybridization buffer for 5 minutes at 55°C with gentle rocking.

11.19 Exchange with fresh hybridization buffer and incubate for 30 minutes at 55°C with gentle rocking.

11.20 Exchange with fresh hybridization buffer and incubate for 30 minutes at 55°C with gentle rocking.

The specimen can now be stored at –20°C in hybridization buffer for 1–2 weeks, although morphology will slowly degrade over time.

Hybridization

12.1 Prepare probe in hybridization buffer (prepared in step 11.17) at a final concentration of 1 µg/mL.

12.2 Denature probe by incubation at 80°C for 2 minutes and then place on ice.

12.3 Heat specimen in hybridization buffer at 55°C for 5 minutes.

12.4 Remove hybridization buffer from specimen and replace with probe/hybridization buffer mixture.

12.5 Incubate the specimen in probe/hybridization buffer at 55°C in a water bath for 16–20 hours.

Post-Hybridization

13.1 Remove probe/hybridization buffer and replace with fresh hybridization buffer without probe.

Note: In most cases, the probe/hybridization buffer may be used more than once without significant loss of signal. Store used probe/hybridization buffer at –20°C.

13.2 Incubate the specimen in hybridization buffer at 55°C in a water bath for 5 minutes with gentle rocking.

13.3 Exchange with fresh hybridization buffer and incubate the specimen in hybridization buffer at 55°C in a water bath for 30 minutes with gentle rocking.

13.4 Repeat step 13.3.

13.5 Incubate the specimen in 50% PBT/50% hybridization buffer at room temperature for 10 minutes with gentle rocking.

13.6 Rinse the specimen in PBT.

13.7 Wash the specimen in PBT three times for 5 minutes each with gentle rocking.

13.8 Mount the specimen with 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 (between 300 bp and 700 bp). Thus, the experimental design should include a reliable positive control, such as an abundantly expressed gene for mRNA FISH.

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). For labeled RNA probes used on tissues, the fragmentation protocol provided can be used to reduce the average probe length. We recommend testing unfragmented and fragmented probes in hybridization assays to determine the benefit of (or need for) fragmentation.

Specimen Integrity

Proper fixation of the specimen is critical to successful hybridization of the probe to the target. Specimens for RNA hybridization should be treated to maintain the integrity of the target RNA and obviate RNA degradation by RNase activity. We recommend consulting the *in situ* hybridization text books above for proper fixation technique.

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 *in vitro* transcription protocol is optimized for use with 1 µg of linearized template DNA. Typical yield expected with SP6 RNA polymerase is 1–2 µg and with T3 or T7 RNA polymerase is 1–4 µg. RNase contamination can degrade RNA and reduce yield so good molecular biology technique is important to success. It is important to evaluate the size of the RNA template by gel electrophoresis in order to be sure there is not RNase contamination. The optional fragmentation protocol should be optimized to result in an average size of 500 bases. The DNase I step in the *in vitro* transcription protocol is important to eliminate the DNA template, which could otherwise lead to overestimation of RNA yield as well as be misinterpreted as RNA in gel electrophoresis, especially if the RNA has been degraded in the process by RNase contamination.

Degree of Labeling

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 RNA 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 RNA 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. Moderate to strongly expressed marker genes work well as a positive control in RNA FISH. RNA FISH can fail for multiple reasons but it is important to be able to verify that the RNA in the specimen is intact and has not been degraded by RNase. 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 (λ_{max}). The calculations are based on the Beer-Lambert law:

$A = \varepsilon \times$ path length (cm) $\times$ 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

14.1 Measure the absorbance of the nucleic acid–dye conjugate at 260 nm (A_{260}) and the λ_{max} for the dye (A_{dye}). Measure the background absorbance at 260 nm and λ_{max}, using buffer alone, and subtract these numbers from the raw absorbance values for the sample. The λ_{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.

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

Fluorescent Dye	λ_{max} (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.

14.2 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 λ_{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:

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

14.3 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 RNA (ε_{base} for RNA is 8250 cm^{-1} M^{-1}). Note that since the calculation is a ratio, the path length has canceled out of the equation.

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 14.2. 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 14.1 and 14.2 above and then use the following equation (MW_{base} for RNA is 340 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); **3.** Science 305, 846 (2004).

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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