**FISH Tag™ RNA Kit**

**Table 1. Contents and Storage Information.**

<table>
<thead>
<tr>
<th>Material *</th>
<th>Amount</th>
<th>Concentration</th>
<th>Storage †</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagents for Labeling Reaction ‡</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor® reactive dye (Component A)</td>
<td>5 vials in each of 2 pouches</td>
<td>NA</td>
<td>≤–20°C • desiccate • protect from light</td>
<td></td>
</tr>
<tr>
<td>Dimethylsulfoxide (DMSO) (Component J)</td>
<td>200 μL</td>
<td>NA</td>
<td>≤–20°C • desiccate</td>
<td></td>
</tr>
<tr>
<td>Dithiothreitol (DTT) (Component H)</td>
<td>100 μL</td>
<td>0.1 M in nuclease-free water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycogen, ultrapure, nuclease free (Component I)</td>
<td>20 μL</td>
<td>20 μg/μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium bicarbonate (Component K)</td>
<td>84 mg</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water, nuclease free (Component L)</td>
<td>4.0 mL</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA nucleotide mix (Component M)</td>
<td>20 μL</td>
<td>10X</td>
<td>≤–20°C</td>
<td></td>
</tr>
<tr>
<td>T7 RNA polymerase (Component N)</td>
<td>10 μL</td>
<td>50 units/μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3 RNA polymerase (Component O)</td>
<td>10 μL</td>
<td>50 units/μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP6 RNA polymerase (Component P)</td>
<td>10 μL</td>
<td>15 units/μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNase I (Component Q)</td>
<td>15 μL</td>
<td>1 unit/μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3/T7 transcription buffer (Component R)</td>
<td>80 μL</td>
<td>5X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP6 transcription buffer (Component S)</td>
<td>40 μL</td>
<td>5X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNaseOUT™ ribonuclease inhibitor (recombinant) (Component T)</td>
<td>10 μL</td>
<td>40 units/μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Antifade Reagent ‡</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SlowFade® Gold antifade reagent (Component G)</td>
<td>2 mL</td>
<td>NA</td>
<td>≤–20°C • protect from light</td>
<td></td>
</tr>
<tr>
<td><strong>Reagents for Purifying Labeled Nucleic Acid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Binding buffer (Component B)</td>
<td>6.0 mL</td>
<td>NA</td>
<td>≤25°C</td>
<td></td>
</tr>
<tr>
<td>Wash buffer (Component C)</td>
<td>3.2 mL</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elution Buffer (Component D)</td>
<td>6.0 mL</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spin columns and collection tubes (Component E)</td>
<td>20 columns with tubes</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collection tubes (Component F)</td>
<td>20 tubes</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*All reagents are nuclease free. †The FISH Tag™ RNA kits are 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.

When stored as directed, the kit is stable for at least 6 months.
## Contents

### Before you Begin
- Materials Required but Not Supplied .................................................. 5
- Fluorescence Handling of Amine-Reactive Fluorescent Dyes ...................... 5
- Pre-Protocol Reading .................................................................................. 6

### Synthesis of Amine-Modified RNA ........................................................... 7
- In Vitro Transcription .................................................................................. 7
- Purifying the Amine-Modified RNA ........................................................... 8
- Ethanol Precipitation of the Amine-Modified RNA ..................................... 8

### Labeling the Amine-Modified RNA with Fluorescent Dye .......................... 9
- Purifying the Fluorescent Dye–Labeled RNA ............................................ 9
- Ethanol Precipitation of the Fluorescent Dye–Labeled RNA ....................... 10
- RNA Probe Fragmentation (Optional) .......................................................... 11

### Suggested Hybridization Protocols ........................................................... 11
- Pre-Hybridization ....................................................................................... 12
- Hybridization .............................................................................................. 13
- Post-Hybridization ...................................................................................... 13

### Tips for Success
- Sensitivity ..................................................................................................... 14
- Length of Probe ........................................................................................... 14
- Specimen Integrity ......................................................................................... 14
- Imaging ........................................................................................................ 14

### Troubleshooting ........................................................................................ 14
- Yield ............................................................................................................ 14
- Degree of Labeling ....................................................................................... 15
- Hybridization ............................................................................................... 15

### Calculating the Labeling Efficiency and Concentration of Nucleic Acid .... 15
- Measuring the Base:Dye Ratio .................................................................... 16
- Measuring the Concentration of Nucleic Acid ............................................ 16

### References .................................................................................................. 17

### Product List ................................................................................................. 17

### Additional Products .................................................................................... 17

### Contact Information .................................................................................... 18
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. 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 NH₂ 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 Kits are supplied with one of four spectrally distinct Alexa Fluor® fluorescent dyes (Table 2) or all four of these Alexa Fluor® dyes in the multicolor kit format (Cat. no. F32956). 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, 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 FISH Tag™ RNA Kits.

<table>
<thead>
<tr>
<th>Product</th>
<th>Cat. no.</th>
<th>Dye Supplied</th>
<th>Ex/Em* (fluorescent color)</th>
<th>Filters†</th>
</tr>
</thead>
<tbody>
<tr>
<td>FISH Tag™ RNA Green Kit</td>
<td>F32952</td>
<td>Alexa Fluor® 488</td>
<td>492/520 (green)</td>
<td>Alexa Fluor® 488 Filter Set</td>
</tr>
<tr>
<td>FISH Tag™ RNA Orange Kit</td>
<td>F32953</td>
<td>Alexa Fluor® 555</td>
<td>555/565 (orange)</td>
<td>Alexa Fluor® 555 Filter Set</td>
</tr>
<tr>
<td>FISH Tag™ RNA Red Kit</td>
<td>F32954</td>
<td>Alexa Fluor® 594</td>
<td>590/615 (red)</td>
<td>Alexa Fluor® 594 Filter Set</td>
</tr>
<tr>
<td>FISH Tag™ RNA Far Red Kit</td>
<td>F32955</td>
<td>Alexa Fluor® 647</td>
<td>650/670 ‡</td>
<td>Alexa Fluor® 647 Filter Set</td>
</tr>
</tbody>
</table>

*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.
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. 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 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.

![Diagram of plasmid construct](image)

**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).

### Before you Begin

**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 J) 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 B) 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 C) add 11.8 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 K) add 1 mL of nuclease-free water (Component L)

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. Note that the dye pair Alexa Fluor® 555 and Alexa Fluor® 594 are spectrally inseparable using most standard epifluorescence microscope filter sets.
**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 L)
- 5X transcription buffer of choice (T3/T7 transcription buffer, Component R or SP6 transcription buffer, Component S)
- 0.1 M DTT (Component H)
- 10X RNA nucleotide mix (Component M)

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 N or T3 RNA polymerase, Component O, or SP6 RNA polymerase, Component P)
- RNaseOUT™ ribonuclease inhibitor (Component T)

**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 0.1 M DTT (Component H) into 9 μL nuclease-free water (Component L). **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.

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

*If the reaction uses T3/T7 RNA polymerase, add 1 μL 0.1 M DTT (Component H) in this reaction. If the reaction uses SP6 RNA polymerase, add 2 μL 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 DNase I (Component Q) 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 nuclease-free water to the sample (Component L) 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 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 E).

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 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 F).

5.8 Apply 55 μL elution buffer (Component D) 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 3 M sodium acetate (pH 5.2)
- 1 μL glycogen (Component I)
- 39 μL nuclease-free water (Component L)

6.2 Add 300 μL 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 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 nuclease-free water (Component L) 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 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 (Component A) to better see the dye pellet.

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

7.7 Transfer the 2 μL 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 water to the sample.

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

---

**Purifying the Fluorescent Dye–Labeled RNA**

8.1 Add 400 μL 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 E).

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 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 F).

8.8 Apply 55 μL elution buffer (Component D) 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 the Fluorescent Dye–Labeled RNA**

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

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

9.2 Add 300 μL 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 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 **nuclide-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 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 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.


**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 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 three times in PBT for 5 minutes each with gentle rocking.

13.8 Incubate with 70% glycerol/30% PBT for 10 minutes with gentle rocking. At this stage, the sample can be stored at ≤–20°C.

13.9 When you are ready to image the embryo, let it settle and pipette off the glycerol/PBT mixture. It is important to pipette off as much glycerol–PBT as possible without damaging the embryo.

13.10 Add two drops (80–100 μL) SlowFade® Gold antifade reagent (Component G).

13.11 To minimize damage to the embryo, remove the embryo and all of the antifade reagent from the tube with a pipette tip that has had the bottom cut off (~3 mm).

13.12 Deposit the embryo with the antifade reagent onto a slide and cover with 22 × 22 mm coverslip.

13.13 Seal the coverslip with Parafilm 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. The filter sets for each channel should accommodate accurately the spectral characteristics of the dye (see Figure 1 and Table 2). Note that the dye pair Alexa Fluor® 555 and Alexa Fluor® 594 are spectrally inseparable using most standard epifluorescence microscope filter sets. 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**

**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
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. 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 ($\lambda_{\text{max}}$). The calculations are based on the Beer-Lambert law:

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

where $\varepsilon$ 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 $\lambda_{\text{max}}$ for the dye ($\lambda_{\text{dye}}$). Measure the background absorbance at 260 nm and $\lambda_{\text{max}}$ using buffer alone, and subtract these numbers from the raw absorbance values for the sample. The $\lambda_{\text{max}}$ 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.

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 $\lambda_{\text{max}}$. 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:

$$A_{\text{base}} = A_{260} - (A_{\text{dye}} \times \text{CF}_{260})$$
14.3 Calculate number of dyes per 100 bases.

Use the following equation:

\[
\frac{100}{(A_{\text{base}} \times \varepsilon_{\text{dye}}) / (A_{\text{dye}} \times \varepsilon_{\text{base}})}
\]

where \( \varepsilon_{\text{dye}} \) is the extinction coefficient for the fluorescent dye (found in Table 3) and \( \varepsilon_{\text{base}} \) is the average extinction coefficient for a base in RNA (\( \varepsilon_{\text{base}} \) 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 \( A_{\text{dye}} \) 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 (\( A_{\text{base}} \)) 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 (\( MW_{\text{base}} \) for RNA is 340 g/mol):

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

---

### References

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

<table>
<thead>
<tr>
<th>Cat. no.</th>
<th>Product Name</th>
<th>Description</th>
<th>Unit Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>F32947</td>
<td>FISH Tag™ DNA Green Kit <em>with Alexa Fluor® 488 dye</em></td>
<td><em>10 reactions</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>F32948</td>
<td>FISH Tag™ DNA Orange Kit <em>with Alexa Fluor® 555 dye</em></td>
<td><em>10 reactions</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>F32949</td>
<td>FISH Tag™ DNA Red Kit <em>with Alexa Fluor® 594 dye</em></td>
<td><em>10 reactions</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>F32950</td>
<td>FISH Tag™ DNA Far Red Kit <em>with Alexa Fluor® 647 dye</em></td>
<td><em>10 reactions</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>F32951</td>
<td>FISH Tag™ DNA Multicolor Kit <em>Alexa Fluor® dye combination</em></td>
<td><em>10 reactions</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>F32952</td>
<td>FISH Tag™ RNA Green Kit <em>with Alexa Fluor® 488 dye</em></td>
<td><em>10 reactions</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>F32953</td>
<td>FISH Tag™ RNA Orange Kit <em>with Alexa Fluor® 555 dye</em></td>
<td><em>10 reactions</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>F32954</td>
<td>FISH Tag™ RNA Far Red Kit <em>with Alexa Fluor® 594 dye</em></td>
<td><em>10 reactions</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>F32955</td>
<td>FISH Tag™ RNA Multicolor Kit <em>Alexa Fluor® dye combination</em></td>
<td><em>10 reactions</em></td>
<td>1 kit</td>
</tr>
<tr>
<td>F32956</td>
<td>FISH Tag™ RNA Green Kit <em>with Alexa Fluor® 488 dye</em></td>
<td><em>10 reactions</em></td>
<td>1 kit</td>
</tr>
</tbody>
</table>

Related Products

<table>
<thead>
<tr>
<th>Product</th>
<th>Description</th>
<th>Unit Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>P36930</td>
<td>ProLong® Gold antifade reagent</td>
<td>10 mL</td>
</tr>
<tr>
<td>P36931</td>
<td>ProLong® Gold antifade reagent with DAPI</td>
<td>10 mL</td>
</tr>
<tr>
<td>S36937</td>
<td>SlowFade® Gold antifade reagent</td>
<td>5 x 2 mL</td>
</tr>
<tr>
<td>S36938</td>
<td>SlowFade® Gold antifade reagent with DAPI</td>
<td>5 x 2 mL</td>
</tr>
<tr>
<td>K3100-01</td>
<td>PureLink™ PCR Purification Kit</td>
<td>50 rxns</td>
</tr>
<tr>
<td>K3100-02</td>
<td>PureLink™ PCR Purification Kit</td>
<td>250 rxns</td>
</tr>
</tbody>
</table>

Contact Information

Molecular Probes, Inc.
29851 Willow Creek Road
Eugene, OR 97402
Phone: (541) 465-8300
Fax: (541) 335-0504

Customer Service:
6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338
Fax: (541) 335-0305
probesorder@invitrogen.com

Toll-Free Ordering for USA:
Order Phone: (800) 438-2209
Order Fax: (800) 438-0228

Technical Service:
8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353
Toll-Free (800) 438-2209
Fax: (541) 335-0328
probestech@invitrogen.com

Invitrogen European Headquarters
Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100
Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

For country-specific contact information, visit www.invitrogen.com.

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Paisley, United Kingdom. All others should contact our Technical Service Department in Eugene, Oregon.

Molecular Probes products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.

Limited Use Label License No. 223: Labeling and Detection Technology

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Molecular Probes, Inc., Business Development, 29851 Willow Creek Road, Eugene, OR 97402, Tel: (541) 465-8300. Fax: (541) 335-0354.

Several Molecular Probes products and product applications are covered by U.S. and foreign patents and patents pending. All names containing the designation “®” are registered with the U.S. Patent and Trademark Office.

Copyright 2009, Molecular Probes, Inc. All rights reserved. This information is subject to change without notice.