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# Fluorescence In Situ DNA and nuclear RNA Hybridization using Tyramide Signal Amplification

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## I. Introduction

Fluorescence In Situ Hybridization (FISH) is a very powerful technique for precisely localizing specific nucleic acid sequences in prepared cell samples. While FISH has most commonly been applied for gene mapping purposes (localizing a specific gene to a chromosomal region), it increasingly is being used as a tool for studying organization of specific sequences within the nucleus, and for detecting discrete nuclear RNAs. Hybridization to nuclear RNA is a sensitive means of directly determining whether a gene is transcribed, by visualizing the actual site of transcription. Low abundance mRNAs that may not be detectable in the cytoplasm may be detectable as more localized nuclear RNA, thereby increasing the ability to accurately assess expression state.

NEN Life Science Product's Tyramide Signal Amplification (TSA) System is an ideal method for detecting nuclear RNA and DNA. It offers high sensitivity and high resolution detection, and is usable both for in situ nucleic acid hybridization and for immunofluorescence. Up to the point of signal detection, no changes in typical in situ or immunofluorescence procedures are required at all. The following protocols, adapted from those traditionally used with non-amplification detection strategies, details procedures for using TSA detection for fluorescence in situ hybridization to mammalian cells grown in vitro. Cell culture and fixation protocols, procedures for detection of DNA, nuclear RNA, and nuclear antigens, and methods for simultaneous visualization of multiple targets are all discussed and described.

## II. Cell culture, fixation and preparation for hybridization.

Two cell fixation protocols are provided that differ in the order of extraction and chemical fixation. Both have been successfully used with TSA. In-house results suggest that Protocol I, fixation followed by detergent extraction, results in lower levels of non-specific signal. Protocol II, detergent extraction followed by fixation, may be beneficial if probe penetration is believed to present a problem. Successful retention of nuclear RNA has been demonstrated with both procedures, whereas cytoplasmic RNA is better retained with the Protocol I.

### Cell Culture

Common in vitro cell culture procedures are used. For ease of subsequent steps, cells are grown on 22mm<sup>2</sup> glass coverslips, which easily fit into small coplin jars (Thomas Scientific), or into appropriate stainless steel racks (Shandon Lipshaw) for subsequent washes.

Depending of cell type used, treatment of the coverslip with attachment factors may be necessary.

1. Place 22mm<sup>2</sup> glass coverslips (prepared by autoclaving in a 0.5% gelatin solution, and maintained under sterile conditions) into an appropriate culture dish. For 100mm diameter cell culture dishes, 7 coverslips can fit arrayed 2-3-2.
2. Wet coverslips using 5-10 ml of sterile Hanks Balanced Salt Solution (HBSS). If coverslips begin to float, tap them down using sterile tweezers. The wetting step will prevent surface-tension wicking of the applied cell suspension under the coverslip, thereby decreasing the number of cells that grow under the coverslip.
3. Trypsinize or mechanically dissociate cells from the culture flask.
4. Using a cell density appropriate for the particular cell type used, pipet 5-10 ml of cell suspension in the appropriate media into the dish. Culture under appropriate conditions until a suitable cell density is obtained. Confluent cell cultures tend to produce higher levels of hybridization background. Therefore we recommend that sub-confluent cultures be used.

**DRAFT COPY****Protocol I. Post-fixation extraction**

1. Using HBSS, rinse cells 2x2 min. at room temperature (rt).
2. Chemically fix cells in 4% paraformaldehyde (PAF) 10 min, at rt.
3. Store cells until use in 70% EtOH at either 4°C or -20°C. We have successfully detected single-copy genes in cells stored in excess of 3 months. For optimal RNA retention, use freshly prepared cells.
4. Prior to hybridization, rinse cells in 1xPBS 2x2min at rt.
5. Detergent permeabilize cells in 1xPBS/0.5% Triton X-100 for 10 min. at rt. If retention of RNA is desired, include 5% v/v vanadyl ribonucleoside complex (VRC; NE Biolabs, Inc.), and perform permeabilization at 4°C.
6. Rinse cells 2x1 min. at 4°C in 1xPBS. For DNA hybridization, go to step 8.
7. For RNA hybridization, dehydrate cells for 5 min. each, at -20°C, in 70% EtOH followed by absolute EtOH. Air dry cells. Proceed to Section IV.
8. For DNA hybridization, denature cells in 70% formamide/2xSSC at 70°C for 2 minutes. Rapidly heat the denaturing solution using a microwave oven to minimize pH changes.
9. Quickly remove the coverslips into 70% EtOH at -20°C for 5 min., followed by absolute EtOH at -20°C for 5 min. Air dry cells. Proceed to Section IV.

**Protocol II. Pre-fixation extraction**

1. Using HBSS, rinse cells 2x2 min. at room temperature (rt).
2. Detergent permeabilize cells by extraction in CSK/0.5% Triton X-100, at 4°C, for between 1 min. and 5 min., depending on cell type. If retention of nuclear RNA is desired, include 5% v/v vanadyl ribonucleoside complex (VRC; NE Biolabs, Inc.) in the extraction solution.
3. Without rinsing cells, chemically crosslink using 4% PAF at rt for 10 min.
4. Store cells until use in 70% EtOH at either 4°C or -20°C. We have successfully detected single-copy genes in cells stored in excess of 3 months. For optimal RNA retention, use freshly prepared cells.
5. For RNA hybridization, dehydrate cells for 5 min. each, at -20°C, in 70%EtOH followed by absolute EtOH. Air dry cells. Proceed to Section IV.
6. For DNA hybridization, denature cells in 70% formamide/2xSSC at 70°C for 2 minutes. Rapidly heat the denaturing solution using a microwave oven to minimize pH changes.
7. Quickly remove the coverslips into 70% EtOH at -20°C for 5 min., followed by absolute EtOH at -20°C for 5 min. Air dry cells. Proceed to Section IV.

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**DRAFT COPY****Protocol Addendum**

Hybridization to DNA vs RNA. It is generally accepted that non-denaturing hybridization allows for discrete detection of RNA. The obverse is not true, though. Target denaturation allows for detection of denatured DNA, as well as RNA. If discrete DNA detection is required, two protocol variations are available that degrade the majority of RNA. These are base-denaturation, and RNase treatment.

**Base Denaturation**

1. In place of the DNA denaturation steps described above, simultaneously hydrolyze RNA and denature DNA by immersing the coverslip in freshly prepared 0.07N NaOH in 70% EtOH at rt for 5 min.
2. Remove the coverslips into 70% EtOH at -20°C for 5 min. Repeat once, followed by absolute EtOH at -20°C for 5 min. Air dry cells. Proceed to Section IV.

**RNase Treatment**

1. Remove the cells from 70% EtOH storage into 2xSSC. Rinse 2x2 min. at rt.
2. Prepare 1ml of DNase-free RNase (BMB) by diluting 1:1000 in 50mM Tris-HCl, pH 8.0/5mM MgCl<sub>2</sub>.
3. Pipet an aliquot of RNase solution onto Parafilm™. Place the coverslip face-down on the solution. Incubate at rt for 30 min.
4. Rinse 2x5 min. in 2xSSC at rt.
5. Denature as described above.

NOTE: It is critical to prevent RNase contamination of glassware and forceps if RNA hybridization is also to be performed in the same setting. Either use segregated instruments, or eliminate RNase using RNase-Zap™ (Ambion Corp.) or similar.

**Critical Points**

Use of VRC. Cellular RNAs are labile to varying degrees. While successful detection of cytoplasmic and nuclear RNAs has been accomplished without inclusion of VRC in the extraction buffers, its use has correlated with an increase in the rate of success of detection of RNA in situ. In the protocols described, we have not found its use detrimental to any step of the hybridization or detection procedure.

Proteolytic digestion for probe penetration. For detection of many different genes and RNAs in situ, we have found it unnecessary to use proteolytic digestion for successful hybridization. Obviously, this may vary between cell types, and by gene or RNA. Due to the possible adverse effects on cell morphology, we recommend its use only if necessary. Using small hybridization probe fragments (~200bp as verified by agarose electrophoresis) facilitates probe penetration. Section III, Probe Labeling describes nick translation generation of small fragments.

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**DRAFT COPY****III. Probe labeling**

Most probes used for fluorescence in situ hybridization are labeled with a reporter nucleotide using nick translation. NEN Life Science Products Inc. supplies a wide variety of labeling kits and formats, as well as different labels, such as biotin, fluorescein, other fluorochromes, and digoxigenin. The following protocol is adapted for use with the NEN™ Biotin Nick Translation Kit (NEL-814), which uses N6-biotin-dATP as the label. The same adaptations described are applicable to all other reporter deoxynucleotides as well.

1. Set-up reaction tubes and kit components as detailed in the kit manual. Store all components on ice while in use.
2. Add all components as described in the kit protocol, with the exception of the DNA polymerase I and DNase I enzyme mixes.
3. Add 1.5µl each of the DNA PolI and DNaseI enzyme mixes to the reaction tube containing all other components.
4. Incubate at 14°C - 16°C for 2.5 hours. This longer incubation time generates a fragment population biased towards sizes between 100-200 bp in length, which facilitates probe entry for in situ hybridization.
5. Inactivate the nick translation enzymes by addition of 5µl 0.5M EDTA, pH 8.0, followed by thermal denaturation at 70°C.
6. Add 2µl of sheared salmon sperm DNA, 1µl of 10mg/ml yeast tRNA, 12µl of ddH<sub>2</sub>O, and 5µl of 3M NaOAc, pH 5.5.
7. Ethanol precipitate by addition of 125µl of ice-cold absolute EtOH. Precipitate overnight at -20°C, pellet DNA at 14,000 RPM, remove supernatant, rinse once with cold 70% EtOH, pellet and decant. Vacuum dry the DNA.
8. Resuspend the dried DNA in 100µl of TE, pH8.0. Store at -20°C. The labeled probe concentration is 10ng/µl, and the probe is stable indefinitely.

Note: G-50 spin-column purification, as well as ultrafiltration are also appropriate purification methods. We find ethanol precipitation the most cost-effective method, though.

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**DRAFT COPY****IV. In Situ RNA and DNA Hybridization****Hybridization probe solution**

The increased sensitivity provided by TSA generally requires a decrease in the amount of DNA probe typically used for hybridization. This is primarily to prevent non-specific signal. Whereas ~100ng of labeled probe (based on a ~10 kb plasmid probe) would typically be used, we recommend between 25ng - 50ng for TSA use.

1. Combine 2.5 $\mu$ l to 5 $\mu$ l of labeled probe, 10 $\mu$ l (1 $\mu$ g) of CotI DNA (Gibco/BRL), 1 $\mu$ l of 10mg/ml sheared salmon sperm DNA, and 1 $\mu$ l of 10mg/ml yeast tRNA in a microcentrifuge tube. Vacuum centrifuge to dryness.
2. Thoroughly resuspend the dried probe solution in 10 $\mu$ l of formamide.
3. Denature the probe solution at 95°C for 10 min. Quickly chill on ice for 5 min.
4. For RNA hybridization, add 8 $\mu$ l RNA hybridization buffer and 2 $\mu$ l of VRC to the chilled probe solution (see appendix).
5. For DNA hybridization, add 10 $\mu$ l of DNA hybridization buffer to the chilled probe solution (see appendix).
6. Apply the entire probe solution to a square of Parafilm™. Place the dried coverslip (from Section II) cell surface down onto the drop of probe solution. Using forceps, flatten the coverslip to evenly distribute the probe solution over the surface of the coverslip, and to remove bubbles. If desired, the coverslip can be covered by another square of Parafilm™, sealed around the edges.
7. Hybridize overnight in a humidified chamber, at 37°C. Do not use a CO<sub>2</sub> incubator, which will adversely affect the pH of the probe solution.

**Post-Hybridization washes.**

For typical RNA and DNA hybridizations using nick translated probes, washes are performed using the following conditions. Note that we have generally not found formulas for calculating T<sub>m</sub> to accurately determine proper wash conditions. If the following conditions are not acceptable, empirical determination of proper conditions is advised.

1. Using forceps, remove the coverslips from the Parafilm™ square and place into a jar containing 2xSSC/50% formamide preheated to 37°C. Incubate at 37°C while shaking for 30 min.
2. Decant and replace with 1xSSC. Incubate as above.
3. Decant and wash with 1xSSC at rt. for 30min.

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**DRAFT COPY****Application of TSA detection system.**

TSA has been found to be completely compatible with typical in situ hybridization protocols, as well as with immunofluorescence protocols. As with any detection system, some parameters may require optimization, depending on cell type and probe type used. Counter intuitively, optimization using TSA detection usually requires a decrease in reagent concentration for optimal results. Most commonly, this will entail decreasing the concentration of the applied probe, or of the HRP-conjugated streptavidin used for TSA. In certain specific cases where very high resolution is desired, concomitant with high sensitivity and very low background (noise), a further dilution of the tyramide solution is suggested. The following protocol is specifically designed for use with all TSA-Direct kits, employing fluorochrome-conjugated tyramides. This protocol has to date not been used with the TSA-Indirect kits, employing biotinyl-tyramides.

1. Dilute the streptavidin-HRP (SA-HRP) solution 1:100 in 4xSSC/1% BSA immediately before use. Approximately 100 $\mu$ l per 22mm<sup>2</sup> coverslip is suggested. Incubate at room temperature for 30 min.
2. Wash 3x15 min. at rt. in 4xSSC/0.1% Triton X-100.
3. Wash 1x5 min. in 4xSSC at room temp.
4. It is suggested that between 100 $\mu$ l and 300 $\mu$ l of diluted tyramide solution per 22mm<sup>2</sup> coverslip be used. Prepare the working tyramide solution by dilution of the stock tyramide solution 1:50 with the supplied amplification diluent. In cases where high resolution, lowest background signal is desired, the tyramide stock solution can be diluted 1:100 in amplification diluent. Note that sensitivity will be somewhat decreased.
5. Pipet the tyramide solution onto a Parafilm™ square, and place the coverslip face-down. Incubate at rt, protected from light, for between 5-10 minutes.
6. Wash 3x15 min. at rt. in 4xSSC/0.1% Triton X-100.
7. If desired, counterstain total DNA using either DAPI, Hoechst 33258, or propidium iodide (dependent on tyramide used) at typical concentrations and times.
8. Wash once in 1xPBS, and mount onto a microscope slide. A variety of mounting media are available. We are not aware of any commonly used mounting media that are incompatible with TSA.

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**DRAFT COPY****V. Simultaneous Visualization of multiple probes or haptens.**

Unlike typical *in situ* hybridization detection methods, TSA covalently deposits the fluorochrome at the site of probe hybridization. This makes TSA an ideal detection method for multiple probe or hapten visualization. While current TSA reagents do not allow for simultaneous distinct detection of multiple targets, sequential TSA application does, and further provides all the benefits of signal amplification. Typical non-TSA multi-target applications employ the use of signal fixation between steps, commonly a paraformaldehyde crosslinking. This may interfere with subsequent steps by altering target accessibility and/or antigenicity. TSA detection specifically covalently deposits only the desired signal, and does not interfere with subsequent steps. For multi-target detection, it is advisable that the most labile target be hybridized and/or detected first. Typically, this will be RNA. It is noted that some exceptions to this have been encountered. The researcher will have to adapt to the specific attributes of their individual system. Using protocols based on prior publications, TSA has been successfully used to simultaneously visualize specific nuclear RNAs, their cognate genes, and nuclear splicing-related antigens. The researcher can freely adapt this protocol for their individual needs.

1. Follow cell permeabilization steps as previously described (Section II). Use VRC in the appropriate solutions to facilitate retention of RNA.
2. Hybridize to nuclear RNA as described above (Section IV). Follow all steps up to application of total DNA counterstain.
3. Degrade residual RNA by treatment with DNase-free RNase (BMB) diluted 1:1000 in 50mM Tris-HCl, pH 8.0/5mM MgCl<sub>2</sub>. A 30 min. treatment at rt. has been found to be adequate for most applications.
4. Rinse 2x5 min. in 2xSSC at rt.
5. Denature target DNA as described in Section IV. Dehydrate as usual.
6. Apply DNA hybridization probe. We have successfully employed the same probe for detection of both the nuclear RNA and cognate gene, sequentially. RNase treatment prevents subsequent hybridization to the RNA, and the denaturation step inactivates the HRP previously used to deposit tyramide signal at the site of hybridization to the RNA.
7. Following DNA hybridization, wash and detect the DNA hybridization signal as described above. Use a tyramide conjugate that will allow clear distinction between RNA and DNA signals (see below).
7. If a third target is desired, follow appropriate procedures dependent on detection of nucleic acids or immunofluorescence detection of protein. For detection of DNA, a further denaturation step is advised. For immunofluorescence, apply the appropriate primary antibody. Follow typical subsequent detection steps. After the final detection, mount the coverslip as described above.

**Critical Points**

**Choice of Tyramide.** It is advised to carefully choose the tyramide used for each application. In general, the Cyanine-3 tyramide is the most sensitive, followed by tetramethylrhodamine, fluorescein, and coumarin. It is suggested that the most abundant signal be detected using the least sensitive tyramide. In the above application, RNA detection is using Cyanine-3 tyramide, the gene using fluorescein, and the nuclear splicing antigen coumarin. These fluorochromes can be freely changed depending on use. TSA amplification has been demonstrated to result in simultaneous visualization of low or single-copy genes, nuclear RNAs from endogenous genes, and nuclear antigens, all with sub-micron resolution. Comparison of TSA generated results using hybridization targets identical to those previously published using conventional detection strategies show similar levels of spatial resolution. Signal intensity was consistently higher with TSA, evidence of the applicability of TSA in the above procedures.

**Order of target detection.** Many factors influence which target should be detected first, which second, and so on. In general, it should be noted that many antibody preparations contain significant levels of nucleases. Therefore if possible, immunofluorescence application should

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be performed after nucleic acid hybridization and detection, especially if nuclear RNA is to be detected. Conversely, hybridization conditions have been shown to eliminate antigenicity of certain antibodies, requiring immunofluorescence applications first. The particular order of applications to be performed will have to be optimized for best results.

Compatibility with other detection methods. These protocols have all been used at NEN Life Science Products Inc. for the above applications, and have been used to detect RNAs from endogenous genes as well as integrated viral sequences. We are not aware of any applications of these types that are incompatible with the use of TSA as the detection mechanism. Furthermore, TSA detection has been used simultaneously with detection of a distinct hapten (i.e. a digoxigenin labeled probe followed by conjugated anti-digoxigen detection) without loss of sensitivity or impact on either procedure. If simultaneous incubation of SA-HRP and anti-digoxigenin is being used, it is advisable to be performed at room temperature to decrease non-specific binding of streptavidin. We have not noted any adverse effect of the subsequent tyramide amplification on the anti-digoxigenin conjugate. Similarly, if TSA is employed to detect a biotinylated probe simultaneously hybridized with a direct fluorochrome-labeled probe, no loss of direct-label detectability has been noted.



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**DRAFT COPY****Appendix****Reagents**

## 10X PBS (1l)

80 gm NaCl  
2 gm KCl  
11.5 gm  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$   
2 gm  $\text{KH}_2\text{PO}_4$

## 20x SSC

3M NaCl  
0.3M  $\text{Na}_3$  citrate  $\cdot 2\text{H}_2\text{O}$   
pH to 7.0 with HCl

## CSK buffer

10mM Pipes pH 7.8  
100mM NaCl  
0.3M Sucrose  
3mM  $\text{MgCl}_2$   
Filter and store at 4°C.

## 4% Paraformaldehyde in PBS

Add 4 gm paraformaldehyde powder (Sigma; store at 4°C) to 75ml  $\text{dH}_2\text{O}$  while stirring. Solution will be cloudy.

Add 1.5ml 10N NaOH. Solution clears after several minutes.

Add 10ml 10xPBS and 0.5ml 1M  $\text{MgCl}_2$ . Solution will become cloudy.

Add conc. HCl dropwise until the pH is 7.5. Solution will be clear.

Filter and store at 4°C.

NOTE: Do not heat solution to dissolve PAF.

## DNA Hybridization Buffer

2ml 50% Dextran Sulfate (Pharmacia/LKB)  
1ml 20xSSC  
1ml 20mg/ml BSA, ultrapure (BMB)  
1ml  $\text{ddH}_2\text{O}$   
Store at 4°C.

## RNA Hybridization Buffer

2ml 50% Dextran Sulfate (Pharmacia/LKB)  
1ml 20xSSC  
1ml 20mg/ml BSA, ultrapure (BMB)  
Store at 4°C.

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DRAFT COPY**References**

## Tyramide Signal Amplification

## NEN™ TSA Kit Manuals

Bobrow, M.N., T.D. Harris, K.S. Shaughnessy and G.J. Litt. 1989. Catalyzed Reporter Deposition: A novel method of signal amplification: applications to immunoassays. *J. Immunol. Methods* 125:279-285.

Raap, A.K., M.P. van de Corput, R.A.W. Vervenne, R.P.M. van Gijlswijk, H.J. Tanke and J. Wiegant. 1995. Ultra-sensitive FISH using peroxidase mediated deposition of biotin and fluorochrome tyramides. *Hum. Mol. Genet.* 4:529-534.

## RNA and/or DNA fluorescence in situ hybridization

Villnave-Johnson, C., R.H. Singer and J.B. Lawrence. Fluorescent detection of nuclear RNA and DNA: Implications for genome organization. 1991. *Meth. Cell Biol.* 35:73-99.

Xing, Y., C.V. Johnson, P.T. Moen, Jr., J.A. McNeil and J.B. Lawrence. 1995. Nonrandom gene organization: Structural arrangements of specific pre-mRNA transcription and splicing with SC-35 domains. *J. Cell Biol.* 131:1635-1647.

Gerdes, M.G., K.C. Carter, P.T. Moen, Jr. and J.B. Lawrence. 1994. Dynamic changes in the higher-level chromatin organization of specific sequences revealed by in situ hybridization to nuclear halos. *J. Cell Biol.* 126:289-304.