Print

HNPP Fluorescent Detection Set

For sensitive fluorescent detection of non-radioactively labeled nucleic acids in fluorescence *in situ* hybridization (FISH) and membrane hybridization.

Cat. No. 11 758 888 001

Set for 500 FISH detection reactions (100 µl each) or 10 membrane detection reactions (100 cm^2 each).

Version 08
Content version: April 2016

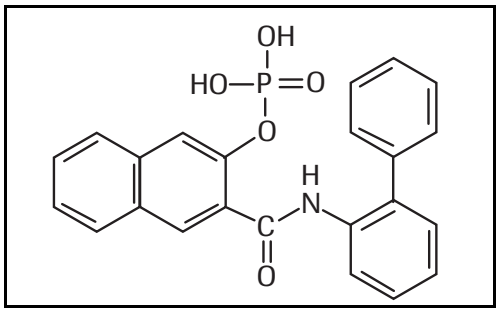
Store at +2 to +8°C

Set contents

Vial 1	**HNPP** 0.5 ml HNPP (10 mg/ml; 23.8 mM) in dimethylformamide (DMF)
Vial 2	**Fast Red TR** 100 mg Fast Red TR, powder
Storage and stability	Stable at +2 to +8°C until the expiration date printed on the label. ***Note:*** Protect from light.

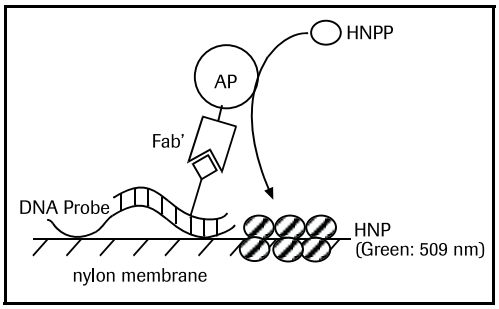
HNPP (2-hydroxy-3-naphtoic acid-2'-phenylanilide phosphate)

Formula	$C_{23}H_{18}NO_5P$
Molecular weight	419.37
Purity	> 98%
Toxicity	The toxicological properties of HNPP have not been investigated. Avoid contact and handle with care. Wear proper personal protection, including gloves, eye protection, and laboratory coat.

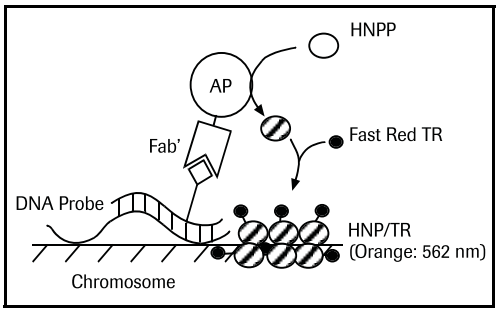


Fast Red TR (4-chloro-2-methylbenzenediazonium hemi-zinc chloride salt)

Formula	$C_7H_6Cl_2N_2 \times {}^1/_2\ ZnCl_2$
Molecular weight	257.2
Purity	> 90%
Toxicity	The toxicological properties have not been investigated. Avoid contact and handle with care. Wear proper personal protection, including gloves, eye protection, and laboratory coat.
Product description	HNPP is a fluorescent substrate for alkaline phosphatase that enables sensitive detection of biomolecules. Upon dephosphorylation, HNPP produces a strongly fluorescent precipitate on membranes with a maximum of absorbance at 290 nm and a maximum of fluorescence emission at 509 nm.



For *in situ* applications Fast Red TR is added to enhance the affinity to tissues and chromosome preparations and to ensure precipitation of the dephosphorylated form (HNP) *in situ*. The absorbance maximum of combined HNP/Fast Red TR precipitate is shifted towards 553 nm and a broad fluorescence emmission (between 565 – 620 nm) with a maximum at 584 nm is generated.



Application

HNPP can be applied for the detection of alkaline phosphatase and alkaline phosphatase conjugates either *in situ* or on membranes. It is especially suited for highly sensitive detection of nonradioactively labeled nucleic acids in fluorescence *in situ* hybridizations (FISH), Southern, Northern, colony or plaque hybridizations and in nonradioactive DNA sequencing blots.

As HNP (dephosphorylated form of HNPP) alone only possesses weak affinity to tissues and chromosome preparations, for *in situ* applications HNPP is coupled with the diazonium salt Fast Red TR.
The reaction product generated after dephosphorylation (HNP/Fast Red TR) is a highly fluorescent precipitate that accumulates during the reaction. The accumulation can be triggered by repeated addition of fresh substrate solution.

Thus the HNPP-azo dye method enables enhanced detection sensitivity compared to conventional non-enzymatic fluorescence *in situ* detection methods employing fluorescein- or rhodamine-labeled antibody or avidin. Single-copy sequences down to 1 kb in length can be detected on metaphase chromosomes. For counterstaining of metaphase chromosomes we recommend to use DAPI in conjunction with the HNPP-azo dye. Signal detection by fluorescence microscopy can be conducted using either filter sets for fluorescein or rhodamine. This is due to the wide fluorescence emission range of 540–590 nm of the HNP/Fast Red TR precipitate.

In membrane applications, HNPP replaces the color substrates NBT/BCIP, following the same detection protocol. The fluorescent precipitate on the membrane is detected by UV illumination with hand-held UV lamps or by placing the membrane on a UV transilluminator. UV lamps of 302 nm are recommended (254 nm or 366 nm give slightly reduced signal intensity).
The fluorescent image can be captured on film using the same aparatus and procedures used to photograph ethidium bromide-stained gels (same camera, film, and optical filters). Alternatively, CCD or video cameras established for gel documentation can be used. Blots detected by HNPP can, in contrast to blots detected with NBT/BCIP, easily be stripped and reprobed.

Detection of DIG-labeled nucleic acids with HNPP

Nucleic acid probes can be labeled very efficiently with digoxigenin (DIG) and be used as hybridization probes in various *in situ* and membrane applications. Detailed protocols for DIG labeling and hybridization are available in the product descriptions of various DIG labeling and detection reagents (see ordering informations at the end of the pack insert).

Detection procedure for *in situ* applications

Required reagents and solutions

- Anti-digoxigenin-AP, Fab fragments*
- Blocking reagent*
- Tween 20*
- DAPI*
- Anti-fading reagent, e.g. 2% DABCO (w/v) in 50% glycerol/PBS

Buffer 1:

100 mM Tris-HCl, 150 mM NaCl, pH 7.5 (+20°C)

Buffer 2: Blocking solution

0.5% Blocking reagent in buffer 1

Buffer 3: Washing buffer

0.05% Tween 20 in buffer 1

Buffer 4: Detection buffer

100 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, pH 8.0 (+20° C)

Fast Red TR solution

25 mg/ml Fast Red TR in redist. H_2O.
Note: Store at +2 to +8°C in the dark. Do not use solution longer than 4 weeks as progressive precipitation occurs. We recommend to dissolve aliquots of 5 mg Fast Red TR in 200 µl redist. H_2O. This is sufficient for 20 HNPP/Fast Red TR mix preparations (see below).

Perform the preparation of chromosomal spreads, denaturation, hybridization and washing steps according to established methods.

Immunological detection

1. Dilute **anti-digoxigenin-AP conjugate** 1:500 in **buffer 2 (blocking solution)**. Stable for only 12 h at +2 to +8°C.
2. Apply 20–100 µl of diluted anti-digoxigenin-AP to the slide, cover with coverslip.
3. Incubate for 1 h at +37°C in a moist chamber.
4. Wash the slide three times with **buffer 3 (washing buffer)** for 10 min each at +15 to +25°C.
5. Wash the slide twice with **buffer 4 (detection buffer)** for 10 min at +15 to +25°C.

Preparation of HNPP/Fast Red TR mix

Mix the following components in a sterile tube:

10 µl **HNPP** (10 mg/ml in dimethylformamide)

10 µl **Fast Red TR solution.**

Note: Do not use the solution longer than 4 weeks as progressive precipitation occurs.

1 ml **buffer 4 (detection buffer)**

Pass the **HNPP/Fast Red TR mix** through a 0.2 µm nylon syringe filter before use.

Note

It is recommended to use the **HNPP/Fast Red TR mix** freshly prepared. It may be stored up to a few days at +2 to +8°C protected from light. Always pass the **HNPP/Fast Red TR mix** through a 0.2 µm nylon syringe filter immediately before use.

Staining

1. Apply 20–100 µl of the HNPP/Fast Red TR mix to the slide, cover with a coverslip, and incubate for 30 min at +15 to +25°C.
2. For detection of single or low copy sequences, repeat this step 3 times. At each intervals, wash the slide with buffer 3 once. For detection of multiple copy sequences, skip this step.
3. Wash the slide with redist. H_2O for 10 min.

Counterstaining

We recommend to use DAPI for counterstaining of HNPP-detected probes.

1. Incubate slides for 5 min in the dark at +15 to +25°C in 50 ml PBS containing 1 µl DAPI solution (5 mg/ml).
2. Wash 2–3 min under running water.
3. Allow to air-dry in the dark.

Mounting

1. Apply 20 µl anti-fading solution per coverslip (24 × 24 mm).
2. Cover with a coverslip.
3. For longer storage (in the dark at −15 to −25°C) and to prevent the coverslip from sliding, the edges of the coverslip can be sealed with nail polish.

   Note: We do not recommend to store the stained slides longer than a few days as crystal-like structures might be formed inhibiting fluorescence signal detection.

Fluorescent detection

The signals are detected by fluorescence microscopy. Appropriate filters must be used for detection: Since HNP/Fast Red TR emits fluorescence in a wide range between 540–590 nm with a maximum at 562 nm, filter sets for fluorescein or rhodamine can be used.

Trouble shooting *in situ* applications

I. High background

1. **Anti-digoxigenin-AP concentration**
   Decrease concentration of the anti-digoxigenin-AP conjugate. The conjugate can be diluted down to 1:10,000 in buffer 2.
2. **Immunological detection**
   Reduce incubation time with diluted anti-digoxigenin-AP down to 20 min.
3. **Staining**
   Reduce incubation time or incubation steps.

II. Crystal-like structures

Storage of slide preparations
When storing the stained slide preparations for several weeks, a crystal-like structure might be formed, coating tissues and chromosomes. Thus storage of stained preparations over long periods is not recommended. For repeated analysis we recommend to carry out a new detection reaction using fresh slide preparations.

III. Faint signals

Staining Increase incubation time and/or incubation steps. The precipitate accumulates over a period of approx. 16 hours.

Detection procedure for membrane applications

Required reagents and solutions

- Anti-digoxigenin-AP, Fab-fragments
- Blocking reagent
- Maleic acid
- SDS
- Tween 20

or alternatively:

- DIG Wash and Block Buffer Set (complete set of concentrated washing, blocking and detection solutions)

Buffer 1: Maleic acid buffer

0.1 M maleic acid, 0.15 M NaCl, adjusted to pH 7,5 (+20°C) with solid NaOH.

Washing buffer

Maleic acid buffer with 0.3% Tween 20 (v/v).

Blocking stock solution (10 ×)

10% (w/v) Blocking reagent in maleic acid buffer. Dissolve blocking reagent by constantly stirring on a heating block (+65°C) or heat in a microwave oven, autoclave and store at +2 to +8°C. The solution remains opaque.

Buffer 2: Blocking solution

Prepare the 1 × conc. working solution by diluting the stock solution 1:10 in buffer 1.

Buffer 3: AP-buffer, detection buffer

0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (+20°C).

Detection procedure

1. After hybridization and stringency washes, rinse membrane briefly (1-5 min) in **washing buffer.**
2. Incubate for 30 min in 100 ml **blocking solution (buffer 2).**
3. Dilute **anti-digoxigenin-AP conjugate** to 75 mU/ml (1:10,000) in **buffer 2.**
4. Incubate membrane for 30 min in 20 ml **antibody** solution.
5. Wash 2 × 15 min in 100 ml **washing buffer.**
6. Equilibrate 2–5 min in 20 ml **AP-buffer (buffer 3)**.
7. Dilute **HNPP** (10 mg/ml) 1:100 in 3–5 ml **AP-buffer (buffer 3)**.
8. Incubate membrane sealed in a hybridization bag for 1 h up to overnight in 3–5 ml **HNPP-solution** at +37°C, protected from light.
9. Remove the membrane from the bag and place it under a UV lamp or on a UV transilluminator (302 nm) with the DNA side facing the UV source (the fluorescence transmits the membrane).
10. Record the fluorescent signals with a Polaroid film (type 667 b/w; yellow filter Y48; 1 s exposure time; f-stop 11–16).

- The volumes are calculated for a membrane size of 100 cm^2.
- All incubations should be performed at +15 to +25°C with gentle agitation.
- UV sources of around 302 nm are recommended; lamps with 254 nm or 366 nm yield a reduced signal (factor 3–10).
- Do not allow membrane to completely dry during any steps of the procedure.

Flow diagram of detection steps

washing	2 min	washing buffer
blocking	30 min	blocking solution, buffer 2
antibody binding	30 min	75 mU/ml anti-digoxigenin-AP in blocking solution, buffer 2
washing	2 × 15 min	washing buffer
equilibration	2 min	AP-buffer, buffer 3
fluorescence reaction	1 h–overnight.	HNPP, 1:100 in AP-buffer, buffer 3
Polaroid photo	1 s exposure	damp membrane under UV lamp
total time	2.5 h up to overnight	

Stripping and reprobing of DNA blots

If DNA probes are labeled with digoxigenin (DIG)-11-dUTP, alkali-labile, blots can be stripped efficiently and reprobed, provided that the membranes never dried to completion at any time during the procedure. Southern blots hybridized with DIG-labeled RNA probes can be stripped following the same procedure.

1. Rinse membrane briefly in redist. water.
2. Wash for 10 min 2 × in ethanol at +15 to +25°C.
3. Rinse briefly in redist. water
4. Wash for 2 × 15 min at +37° C in 0.2 M NaOH, 0.1% SDS to remove the DIG-labeled probe.
5. Rinse thoroughly 5 min in 2 × SSC.
6. Prehybridize and hybridize with next probe.

Alternative stripping protocols are described in the "DIG System User's Guide for Filter Hybridization" (available on request).

Sensitivity and specificity

A single copy gene (tissue plasminogen activator, tPA) is detected in a Southern blot in 1 μg human placental DNA with a DIG-labeled DNA-probe and HNPP after 16 h substrate reaction by UV-transillumination on a Polaroid photo. Using DIG labeled RNA-probes, similar sensitivity is obtained.

Trouble shooting membrane applications

I. Low sensitivity

1. Labeling	Check the efficiency of your DIG DNA or RNA labeling by comparison to a labeled control DNA or RNA*.
2. Membrane	The membrane quality influences sensitivity and speed of detection. We recommend nylon membranes, positively charged from Roche Diagnostics. Other types of nylon membranes [*e.g.*, Biodyne A (Pall), Magnagraph (MSI)] are also suitable. Some membranes may cause strong background formation. When using nitrocellulose membranes a reduction in sensitivity must be accepted.
3. Hybridization	Increase the concentration of DIG-labeled DNA or RNA probe in the hybridization solution, but only to a concentration where background is still low. Reduce the stringency of the washing steps, e.g., perform the last stringency wash with 0.5 × SSC, 0.1% SDS instead of 0.1 × SSC, 0.1% SDS at +68°C.
4. Detection	Increase the concentration of the anti-digoxigenin-AP conjugate to a 1:5,000 dilution (150 mU/ml).
5. Substrate incubation	Incubation at +37° C of the sealed membrane in HNPP solution yields stronger signals compared to +15 to +25° C and should be performed in the dark.
6. Documentation	Use UV lamps of 302 nm; for optimal results adjust 2 lamps in a position of highest irradiation intensity closely above the membrane (Polaroid camera situated between the UV lamps.) Commonly used transilluminators are also suitable; note that different wavelengths cause signal reduction (254 nm about factor 10; 366 nm about factor 3). The DNA side of the membrane must always face the UV source. Remove the membrane from the hybridization bag. Some types of plastic can partially block the UV light. The type of film may also influence the sensitivity. We have tested and can recommend the Polaroid film 667 b/w.

II. High background

1. Labeling	Purify DNA/RNA by phenol/chloroform extraction and/or ethanol precipitation before labeling. Make sure that the probe does not contain cross hybridizing vector sequences.
2. Membrane	Although the protocol is optimized for the use of the nylon membranes, positively charged, some types which are very highly charged can cause background. Lot-to-lot variations in some membranes may also cause problems. When using the recommended function-tested Roche Diagnostics membrane, these problems are avoided.
3. Hybridization	**Note:** It can be necessary to decrease the concentration of the DIG labeled DNA or RNA probe.The critical probe concentration limit is the maximal concentration of probe recommended for hybridization, without the incident of background. It can be unique to a membrane lot and brand, and can be determined by hybridizing a blank piece of membrane with increasing probe concentrations. Homologous DNA may also be spotted on this piece of membrane for a test hybridization. Care should be taken not to permit the membranes to dry throughout the whole procedure.
4. Detection	Decrease concentration of the anti-digoxigenin-AP conjugate. Increase volumes of the washing and blocking solution and duration of the washing and blocking steps. Spotty background may be caused by precipitates in the anti-digoxigenin-AP conjugate: remove by a quick centrifugation step. (Note: Several centrifugation steps can cause a certain loss of material, which must be compensated for by use of larger amounts).
5. Documentation	Adjust the camera set up. Alter exposure time.

Quality control

Using DIG-labeled control-DNA (pBR328/BamHI digested) as hybridization probe, 0.1 pg homologous DNA diluted with 50 ng heterologous DNA are detected in a dot blot after overnight incubation with HNPP, following the standard detection protocol.

Ordering Information for DIG labeling and detection

Product	Cat. No.
DIG High Prime	11 585 606 910
DIG RNA Labeling Kit (SP6/T7)	11 175 025 910
DIG Easy Hyb, hybridization solution	11 603 558 001
Nylon membranes, positively charged	11 209 272 001
DIG Wash and Block Buffer Set	11 585 762 001
Anti-digoxigenin-AP conjugate, Fab Fragments	11 093 274 910
Blocking reagent	11 096 176 001
DIG-Nick Translation Mix for *in situ* probes	11 745 816 910
DAPI	10 236 276 001

References

1 Kagiyama, N. et al. (1992) *Acta Histochem. Cytochem.* **25**, 467–471.
2 Kagiyama, N. et al. (1993) *Acta Histochem. Cytochem.* **26,** 441–445.
3 Diamandis, E.P. et al. (1993) *Journal of Clinical Laboratory Analysis* **7**, 174–179.
4 Furuichi, M. et al. (1994) *Genomics* **24**, 485-490
5 Kasahara, N. et al (1995) *Mammalian Genome* **6**, 52-54.
6 Kondoh, Y et al. (1995) *Cytogenet. Cell Genet.* **71**, 96-98.
7 Sawada, J. et al. (1995) *Jpn. J. Cancer Res.* **86.** 10-12.
8 Yamada, K. et al. (1995) *Genomics* **29**, 552-553.
9 Yamaguchi, N. (1996) *Applied and Environment Microbiology* **62,** 275-278.
10 Kusakabe, A. et al (2002) The Neural Differentiation Gene Mash-1 has a Distinct Pattern of Expression from the Taste Reception-related Genes gustducin and T1R2 in the Taste Buds. *Chem. Senses* **27**: 445-451.
11 Lee, S.K. & Hollenbeck P.J. (2003) Organization and translation of mRNA in sympathetic Axons. *Journal of Cell Science* **116,** 4467-4478.

Please refer to our website for the following informations
http://lifescience.roche.com/DIG/
12 DIG Product Selection Guide
13 DIG Application Manual for Filter Hybridization
14 Non-radioactive In situ Hybridization Manual
15 Lab FAQS

Changes to previous version	Editorial changes
Trademarks	DIG EASY HYB is a trademark of Roche. All other product names and trademarks are the property of their respective owners.
Regulatory Disclaimer	For life science research only. Not for use in diagnostic procedures.
Disclaimer of License	For patent license limitations for individual products please refer to: List of biochemical reagent products

Contact and Support

To ask questions, solve problems, suggest enhancements and report new applications, please visit our **Online Technical Support Site**.

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany