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FISH CODETECTION

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Protocol status: Working
We use this protocol and it's working

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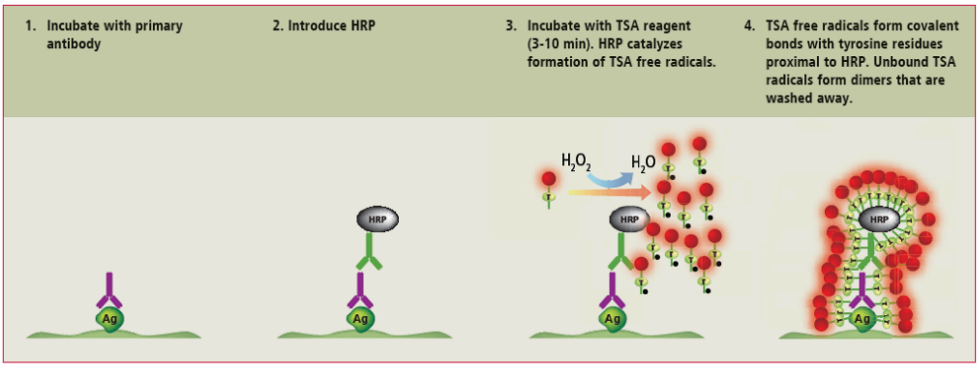
Abstract

DIG and Fluorescein labeled
riboprobes
Fluorescent *in situ*
hybridization
Frozen brain sections

Image Attribution



Troubleshooting

PCR production by plasmid of interest

1 PCR reagents/plasmid

- In ice, place in a PCR tube :

H2O: 21µl
Mix primers S et AS (10 µM each) : 3 µl
DNA plasmid (10ng/µl): 6 µl
REDEXTRACT PCR Mix : 30µl

Dispatch 2 times 19,8uL of PCR mix in two other PCR tubes
(Note that the optimal vol for this PCR is 20 µl).

2 PCR Cycles

	94ºC	5 min
	94ºC	45sec
x 35	55ºC	45 sec
	72ºC	1 min
	72ºC	5 min
	4ºC	...

3 PCR gel control:
Make a 1% agarose gel
-1 µl 1kb+DNA ladder+ lµl of10X Blue juice Gel loading buffer + 8 µl H_20
-5 µl of PCR product/ mRNA

4 PCR purification, (QIAquick PCR Purification kit)

- Pool the 3 PCR tubes in an 1,5 ml tube
- Add 5 volume PB buffer to 1 volume of the PCR reaction (roughly 250 µl de PB) and mix
- Place a QIAquick column in a provided 2 ml collection tube
- To bind DNA, apply the sample to the QIAck column.
- Centrifuge

1 min at 13 000 rpm

- Discard flow-through
- Place the QIAquick column back in the same tube
- To wash, add 750 ul of PE Buffer to the QIAquick column

- o Centrifuge 1 min à 13 000 rpm .
- o Discard flow-through
- o Place the QIAquick column back in the same tube
- o Centrifuge 1 min à 13 000 rpm to remove residual wash buffer.
- o Discard flow-through
- o Place the QIAquick column in a clean 1,5 ml RNAse free tube
- o To elute DNA, add 50 µl Buffer EB to the center of the QIAquick membrane and wait for 3 min.
- o Centrifuge 1 min à 13 000 rpm.

5 Dosage : Nano drop

Riboprobe synthesis

6 Transcription reagents/riboprobe

In ice, place in a 1,5 ml tube :

5X transcription buffer	4µl
10X Dig-RNA labelling mix **or** 10X Fluorescein-RNA labelling mix	2µl
100mM DTT	1µl
Rnasin (20U/µl)	0.5µl
T3/T7 RNA polymerase (20U/µl)	2.0µl
PCR-product	150 ng
H2O	up to 20 µl

7 Transcription reaction

Incubate 2h at **37°C**

8 Riboprobe purification (illustra ProbeQuant G-50 Micro Columns)

Column preparation

- Re-suspend the resin in the column by vortexing
- Loosen the cap one-quarter turn and twist off the bottom closure

- Place the G-50 column in the supplied Collection tube
- Centrifuge 1 minute 735 × g
- Place the G-50 column in a clean 1,5 ml RNAse free tube

Sample application

- Add to the riboprobe sample 30 µl Probe Buffer type 1.
- apply the sample in the middle of the column.
- Centrifuge 2 min à 750 g at 4°C
- Place tube in ice

9 Dosage (Nanodrop)

10 Storage
- Add 100 µl Hyb Buffer (50% « Hybe–F » + 50% Formamide).
- Aliquot in 100 µl tube (50 µl/tube)
- Keep at -80°C.

Tissue Preparation

11 Adult mice are sacrificed by cervical dislocation, and brains are rapidly dissected out and snap frozen in cold (-30°C to -35°C) 2-methylbutane (≥99%, Honeywell, M32631) on dry ice. Brains are sealed with parafilm and aluminum foil and stored at -80°C until use. Embryonic brains, which are less rich in myelin and fat, require more gentle freezing in 2-methylbutane (-20°C to -25°C) immediately after extraction from the euthanized and sacrificed mother.

Hybridization

12 -1- Thaw slides (around 5 min)
-2- Fix slides in 4% PFA in PBS, 10 min at RT.
-3- Wash 3×10 min inPBS
-4- Acetylation treatment:
- Equilibrate slides in TEA 5 min
- Prepare extemporaneously the acetylated buffer in the following ratio:

acetic anhydride 25 µl in 10 ml TEA
- Acetylation treatment, 10 min at RT
- Wash 3×5 min in PBS

-5- Hybridization:
- Make the Hyb Buffer by mixing Hyb Buffer without Formamide + Formamide (50/50). Keep it on ice.
- For each riboprobe. Add to 20 µl Hyb Buffer the 2 riboprobes. Amount in accordance with the number of slides that will be hybridized:
  - o Dig-probe (50-75ng/100µl)
  - o Fluoresceinprobe (75-100 ng/100 µl)
- Denature 10 min at 85 °C
- Put in ice immediately 1 min
- Briefly centrifuge tubes at 2000g
- Put in ice
- Add XXX µl of cold Hyb Buffer adapted to the number of slides that will be hybridized.

Keep tubes in ice
- Vortex
- Take slide one by one from the PBS, remove the PBS by shaking the slide, dry the bottom of the slide with a klennex. Place slide in 3 slides hybridization box.
- Put 150 µl of (Hyb Buffer+ denaturated riboprobe) /slide
- Cover with a RNAse free coverslip (Hybrislip)
- Place the 3 slides hybridization box in a humidified chamber (5xSSC)

Note: no need to put Formamide here!
- Hybridize at 65°C between 16h and 18h.
- Prewarm X5 SSC and 0,2X SSC wash solutions at 65°C

Day 2: washes and immunological staining

13
- Place slide one by one in prewarmed X5 SSC and remove the coverslip
- Transfer slides in prewarmed X5 SSC and wash 5 min at 65°C
- Wash 3×20 min in prewarmed 0.2X SSC at 65°C
- Wash 10 min in 0.2X SSC at RT
- Wash 3 × 10min in MABT (RT)

14 **From now, all steps at RT**

Fluorescein-riboprobe revelation.
- Place slides in humidified chamber (H_20)
- Add 500 µl of BR/slide for 20 min
- Replace solution by 1/5000 anti-Fluorescein-POD in BR (500µl/lame) for 1h30
- Wash 3× 10 min in MABT

- Wash 3 × 10 min PBST

During the last wash, prepare a H2O2 0,001% solution from a 30% H2O2 by cascade dilution:

- 10µL H2O2 30% + 90µL PBST ◊ H2O2 3%
- 10µL H2O2 3% + 590µL PBST ◊ H2O2 0.05%
- 10µL H2O2 0.05% + 490µL PBST ◊ H2O2 0.001%

- From now everything must be done away from light.
- Place slides in humidified chamber (H_20)
- Place the solution 1/250 Fluorescein-tyramide in H2O2 0.001% (500 µl/slide)
- When all slides are covered, gently move the slide for a uniform distribution of the solution at the slide surface
- Incubation 1h at RT
- Wash 3 × 10 min PBST

15 **POD- inhibition:**

- Wash 20 min in Glycine buffer 0,1M pH= 2.1
- Quick wash in PBS
- Wash 20 min in 3% H_2O_2 in PBS
- Wash 3 ×10 min in PBST
- Keep the slides in the last wash at 4°C overnight

Day3: washes and immunological staining

16 **Dig-riboprobe revelation:**

- Wash 1 ×10 min in PBST
- Wash 3 × 10 min MABT
- Place slides in humidified chamber (H_20)
- Add 450 µl of BR/slide for 20 min
- Replace solution by 1/1500 anti-DIG-POD in BR (450µl/lame) for 1h
- Wash 3× 10 min in MABT
- Wash 3 × 10 min PBST

During the last wash, prepare a H2O2 0,001% solution

- Prepare 0.001% H2O2 from 30% H2O2 by cascade dilution:

- 10µL H2O2 30% + 90µL PBST ◊ H2O2 3%
- 10µL H2O2 3% + 590µL PBST ◊ H2O2 0.05%
- 10µL H2O2 0.05% + 490µL PBST ◊ H2O2 0.001%

- Place slides in humidified chamber (H_20)
- Place the solution 1/250 Tamra-tyramide in H2O2 0.001% (500 µl/slide)
- When all slides are covered, gently move the slide for a uniform distribution of the solution at the slide surface
-

Incubation 1h at RT

- Wash 5 × 10 min PBST
- Place slides in humidified chamber (H_20)
- Add 450 µl/slide of DAPI for 20 min
- Wash 3× 5min PBST
- Mount in fluoromount.
- Quick microscope slide analysis

Give slide to be scanned