Stellaris RNA FISH

Protocol for fresh frozen mouse brain tissue

General protocol and storage

Product description

A set of Stellaris™ RNA FISH Probes, from LGC, Biosearch Technologies™, is comprised of up to 48 singly labelled oligonucleotides designed to selectively bind to targeted transcripts. Stellaris RNA FISH Probes bound to target RNA produce fluorescent signals that permit detection of single RNA molecules as diffraction-limited spots by conventional fluorescence microscopy.

Storage guidelines

Stellaris RNA FISH Probes

Stellaris RNA FISH Probes are shipped dry and can be stored at +2 to +8 °C in this state. Dissolved probe mix should be subjected to a minimum number of freeze-thaw cycles. For daily and short-term use of dissolved probe mix, storage at +2 to +8 °C in the dark for up to a month is recommended. For storage lasting longer than a month, we recommend aliquoting and freezing probes in the dark at -15 to -30 °C.

Stellaris RNA FISH Hybridization Buffer

Stellaris RNA FISH Hybridization Buffer should be stored at +2 to +8 °C for short-term and long-term use.

Stellaris RNA FISH Wash Buffer A and Wash Buffer B

Stellaris RNA FISH Wash Buffers A and B should be stored at room temperature for short-term and long-term use.

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Reagents and equipment

Reagents and consumables:

a) TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
b) 20% Electron Microscopy Grade Formaldehyde
c) 10X Phosphate Buffered Saline (PBS), RNase-free
d) Nuclease-free water
e) Ethanol for molecular biology
f) Triethanolamine
g) Acetic Anhydride
h) Chloroform
i) Deionised formamide
j) Stellaris RNA FISH Hybridization Buffer (Biosearch Technologies Cat# SMF-HB1-10; SMF-HB1-100)
k) Stellaris RNA FISH Wash Buffer A (Biosearch Technologies Cat# SMF-WA1-60; SMF-WA1-600)
l) Stellaris RNA FISH Wash Buffer B (Biosearch Technologies Cat# SMF-WB1-20; SMF-WB1-200)
m) 4′,6-diamidino-2-phenylindole (DAPI)
n) Prolong® Gold Antifade Mountant (ThermoFisher™ Scientific Cat #P36930)
o) CoverGrip™ Coverslip Sealant (Biotium Cat# 23005) or clear nail polish
p) 24 × 60 mm rectangular coverglass
q) RNase free consumables such as pipette tips
r) Humidified chamber (or equivalent): 150 mm tissue culture plate; a single water-saturated paper towel placed alongside the inner chamber edge
s) 37 °C laboratory oven

Microscope:

a) Wide-field fluorescence microscope (e.g. Nikon Eclipse Ti or equivalent). We provide limited support for confocal applications.
b) A high numerical aperture (>1.3) and 60-100x oil-immersion objective.
c) Strong light source, such as a mercury or metal-halide lamp (newer LED-based light sources may also be sufficient).
d) Filter sets appropriate for the fluorophores.
e) Standard cooled CCD camera, ideally optimised for low-light level imaging rather than speed (13 µm pixel size or less is ideal).

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Preparation of reagents

NOTE: When performing Stellaris RNA FISH, it is imperative to limit RNA degradation. Please ensure that all consumables and reagents are RNase-free. Recipes below are for two set volumes (1X and 10X). Please adjust accordingly.

Reconstituting the dried probe stock:

ShipReady Probe Set (1 nmol):

- A ShipReady probe set can provide up to 80 hybridisations. Re-dissolve the dried oligonucleotide probe blend in 80 µL of TE buffer to create a probe stock of 12.5 µM. *Mix well by pipetting up and down,* and then vortex and centrifuge briefly.

DesignReady or Custom Probe Set (5 nmol):

- A DesignReady or custom probe set can provide up to 400 hybridisations. Re-dissolve the dried oligonucleotide probe blend in 400 µL of TE buffer to create a probe stock of 12.5 µM. *Mix well by pipetting up and down,* then vortex and centrifuge briefly.

Fixation Buffer:

Final composition is 3.7% (vol./vol.) formaldehyde in 1X PBS

For a final volume of 50 mL (500 mL), mix:

- 1 mL (10 mL) 20% formaldehyde solution
- 5 mL (50 mL) 10X Phosphate Buffered Saline (PBS)
- 44 mL (440 mL) nuclease-free water

WARNING! This buffer is light sensitive. It is suggested to wrap reagent bottle in foil.

TEA Buffer (10X):

NOTE: This buffer is light sensitive. It is suggested to wrap reagent bottle in foil.

For a final volume of 100 mL (1 L), mix:

- 13.3 mL (133 mL) Triethanolamine
- 60 mL (600 mL) nuclease-free water H2O

Then bring to pH 8.0 using HCl (~5-10 mL (50-100 mL) of HCl). Once at pH 8.0, add water to 100 mL (1 L) final volume.

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Hybridization Buffer (1 mL/10 mL):

Final composition is 10% (vol./vol.) formamide in Hybridization Buffer

Hybridization Buffer should be mixed fresh for each experiment:

- Due to viscosity of the solution, we recommend accounting for a 10% final volume excess in order to have enough Hybridization Buffer for all of your samples.

For a final volume of 1 mL (10 mL), mix:

- 900 µL (9 mL) Stellaris RNA FISH Hybridization Buffer
- 100 µL (1 mL) deionizsed formamide. Mix thoroughly by vortexing and pipetting up and down.

NOTE: Do not freeze Hybridization Buffer.

WARNING! Formamide is a teratogen that is easily absorbed through the skin and should be used in a chemical fume hood. Please consult the appropriate SDS (safety data sheet) prior to use.

WARNING! Be sure to let the formamide warm to room temperature before opening the bottle.

WARNING! Formaldehyde is a known human carcinogen and should be use in a chemical fume hood. Please consult the appropriate SDS (safety data sheet) prior to use.

Wash Buffer A (50 mL/500 mL):

Final composition is 10% (vol./vol.) formamide in 1X Wash Buffer A

Mix and dilute Wash Buffer A fresh for each experiment:

For a final volume of 50 mL (500 mL), mix:

- 10 mL (100 mL) Stellaris RNA FISH 5X Wash Buffer A
- Add 5 mL (50 mL) deionised formamide.
- Add 35 mL (350 mL) nuclease-free water.

Mix well by vortexing gently.

Wash Buffer B:

Add nuclease-free water to Wash Buffer B bottle upon first use.

- Add 88 mL (880 mL) of nuclease-free water to bottle before use. Mix thoroughly.

Nuclear stain for use after hybridisation:

- 4′,6-diamidino-2-phenylindole (DAPI) prepared in Wash Buffer A (see above) at 5 ng/mL.

This solution is to be used in step 21 below.

Mounting media:

- Prolong Gold Antifade Mountant (ThermoFisher Scientific Cat #P36930).

NOTE: Samples mounted with Prolong Gold should be allowed to cure overnight at room temperature and then imaged the following day.

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Day 1 - Slide preparation and hybridisation

1. Thaw the slide-mounted tissue section to room temperature.
2. Immerse the slide in cold 4% E.M. grade formaldehyde in 1X PBS for 15 minutes.
3. Wash twice with 1X PBS for 5 minutes.
4. Dip the slide in nuclease-free water.
5. Dip the slide in 1X TEA buffer.
6. Immerse the slide in 1X TEA + Acetic Anhydride for 10 minutes (Stirring!)

   WARNING! Acetic Anhydride is an irritant and should be used in a chemical fume hood. Please consult the appropriate SDS (safety data sheet) prior to use.

   Note: Acetic Anhydride must be added fresh each time. Add Acetic Anhydride to fresh 1X TEA buffer after the second 1X PBS wash in step 3, about 1 minute before dipping slides in DEPC water. For 50 mL of 1X TEA, add 63 µL of Acetic Anhydride.

7. Immerse the slide in 2X SSC for 3 minutes.
8. Immerse the slide in 70% ethanol for 3 minutes.
9. Immerse the slide in 95% ethanol for 3 minutes.
10. Immerse the slide in 100% ethanol for 3 minutes.
11. Immerse the slide in Chloroform for 5 minutes.

    WARNING! Chloroform is an irritant and carcinogen and should be used in a chemical fume hood. Please consult the appropriate SDS (safety data sheet) prior to use.

12. Immerse the slide in 100% ethanol for 3 minutes.
13. Immerse the slide in 95% ethanol for 3 minutes.
14. Let air dry for 90+ minutes (but no longer than 4 hours).

Hybridisation in frozen tissue sections

If frozen before using, warm the reconstituted probe solution to room temperature. Mix well by vortexing, then centrifuge briefly.

To prepare the hybridisation solution, add 4.0 µL of probe stock solution to 200 µL of Hybridization Buffer, and then vortex and centrifuge. This creates a working probe solution of 250 nM. This solution will be used on step 16.

15. Assemble a humidified chamber: 150 mm tissue culture plate; a single water-saturated paper towel placed alongside the inner chamber edge. This chamber will help prevent evaporation of the probe solution from the tissue section.
16. After slide has dried for 90+ minutes, dispense 200 µL of Hybridization Buffer containing probe onto the tissue sections of the slide.

17. Carefully place a clean 24 × 60 mm rectangular cover glass over the hybridisation solution to completely cover the tissue sections and allow for even distribution of the hybridisation solution. Place the slide in the humidified chamber, cover with the tissue culture lid, and seal chamber with parafilm.
18. Incubate in the dark at 37 °C for at least 4 hours (incubation can be continued up to 16 hours).

Day 2 - *In situ* washes

19. Immerse the slide in wash buffer A, and allow the submerged cover glass to slide off the tissue section. Gentle agitation may be required to remove the cover glass.
20. Incubate in the dark at 37 °C for 30 minutes.
21. Decant wash buffer A, and then add DAPI nuclear stain (wash buffer consisting of 5 ng/mL DAPI) to counterstain the nuclei.
22. Incubate in the dark at 37 °C for 30 minutes.
23. Decant DAPI staining buffer, and then immerse slide in Wash Buffer B for 3 minutes.
24. Immerse slide in 50% ethanol for 3 minutes.
25. Immerse slide in 85% ethanol for 3 minutes.
26. Immerse slide in 100% ethanol for 3 minutes.
27. Let air dry for 5-10 minutes.
28. Add a drop or two (approximately 50-100 µL) of Prolong Gold Antifade Mountant onto the tissue sections. Cover with a clean 24 × 60 mm cover glass, allowing the antifade to spread evenly across the tissue sections.
29. Allow Prolong Gold to cure overnight, in the dark, at room temperature.
30. Seal the cover glass perimeter with CoverGrip (or clear nail polish), and allow to dry in the dark.
31. Proceed to imaging.

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References

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2. Femino, A.M., Fay, F.S., Fogarty, K., and Singer, R.H. Visualization of single RNA transcripts in situ. Science 1998; 280, 585-90. doi: 10.1126/science.280.5363.585
3. Raj A, Tyagi S. Detection of individual endogenous RNA transcripts in situ using multiple singly labeled probes. Methods in Enzymology 2010; 472, 365-86. doi: 10.1016/S0076-6879(10)72004-8
4. Kleaveland, B., Shi, C.Y., Stefano, J., and Bartel, D.P. A Network of noncoding regulatory RNAs acts in the mammalian brain. Cell. 2018; 174, 350-362. doi: 10.1016/j.cell.2018.05.022

Guidelines citing the use of Stellaris RNA FISH Probes and methods in scientific publications

Please acknowledge the use of Stellaris RNA FISH Probes and/or protocols in the experimental **Materials and Methods** or **Methods** section of your manuscript. Refer to the following examples as guidelines for proper citation of the Stellaris RNA FISH Probe sets and/or protocols:

Citing catalogued probe sets:

"Stellaris™ RNA FISH Probes recognising <catalogued gene set name> and labelled with Quasar™ 570 dye (Catalog #, LGC, Biosearch Technologies, Petaluma, CA) were hybridised to <samples>, following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocols. Briefly, <describe any deviations from the published protocol or a short summary of what was actually performed>."

Citing Custom Probe sets designed with the Stellaris FISH Probe Designer:

"Custom Stellaris™ RNA FISH Probes were designed against <your RNA of interest (include accession number and nucleotides covered if relevant)> by utilising the Stellaris RNA FISH Probe Designer (LGC, Biosearch Technologies, Petaluma, CA) available online at www.biosearchtech.com/stellarisdesigner (version #). The <samples> were hybridised with the <your RNA of interest> Stellaris RNA FISH Probe set labelled with <your dye of choice> (Biosearch Technologies), following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocols. Briefly, <describe any deviations from the published protocol or a short summary of what was actually performed>."

Citing Custom Probe sets utilising previously published sequences:

"Custom Stellaris™ RNA FISH Probes recognising <your RNA of interest (include accession number and nucleotides covered if relevant)> and labelled with <your dye of choice>, were purchased from LGC, Biosearch Technologies (Petaluma, CA). Probe set sequences utilised in the experiments have been previously described <cite published manuscript>. The <samples> were hybridised with the <your RNA of interest> Stellaris RNA FISH Probe set, following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocols. Briefly, <describe any deviations from the published protocol or a short summary of what was actually performed>."

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Citing 3′ Amine Oligos in plates used for Stellaris RNA FISH designed with the Stellaris FISH Probe Designer:

"Custom 3′ amine oligos in plates were designed against <your RNA of interest (include accession number and nucleotides covered if relevant)> by utilising the Stellaris™ RNA FISH Probe Designer (LGC, Biosearch Technologies, Petaluma, CA) available online at www.biosearchtech.com/stellarisdesigner (version #). Probes were labelled with <your dye of choice> using <insert your labellling protocol or citation of previously published labelling protocol>. The <samples> were hybridised with the <your RNA of interest> oligonucleotides (Biosearch Technologies), following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocols. Briefly, <describe any deviations from the published protocol or a short summary of what was actually performed>."

Citing 3′ Amine Oligos in plates used for Stellaris RNA FISH using previously published sequences:

"Custom 3′ amine oligos in plates recognising <your RNA of interest (include accession number and nucleotides covered if relevant)> were purchased from LGC, Biosearch Technologies (Petaluma, CA). Probe set sequences utilised in the experiments have been previously described <cite published manuscript>. Probes were labelled with <your dye of choice> using <insert your labelling protocol or citation of previously published labelling protocol>. The <samples> were hybridised with the <your RNA of interest> oligonucleotides (Biosearch Technologies), following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocols. Briefly, <describe any deviations from the published protocol or a short summary of what was actually performed>."

Technical support

If you require additional information or technical assistance, please feel free to email our Technical Support Team at: techsupport@lgcgroup.com.

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