Stellaris RNA FISH

Protocol for *Arabidopsis thaliana* root meristem

General protocol and storage

Product description

A set of Stellaris™ RNA FISH Probes 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 Hybridisation Buffer

Stellaris RNA FISH Hybridisation 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) MS media minus glucose (Sigma-Aldrich Cat. # M5519, or equivalent)
b) TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
c) Liquid Nitrogen
d) 16% Methanol-free Formaldehyde Solution (ThermoFisher Scientific Cat# 28906 or equivalent)
e) 10X Phosphate Buffered Saline (PBS), RNase-free
f) Nuclease-free water
g) 1 M HCl
h) 5 % w/v sodium hypochlorite
i) Deionised Formamide
j) Ethanol for molecular biology
k) Stellaris RNA FISH Hybridisation Buffer (LGC, Biosearch Technologies Cat# SMF-HB1-10)
l) Stellaris RNA FISH Wash Buffer A (Biosearch Technologies Cat# SMF-WA1-60)
m) Stellaris RNA FISH Wash Buffer B (Biosearch Technologies Cat# SMF-WB1-20)
n) 4',6-diamidino-2-phenylindole (DAPI) (ThermoFisher Scientific Cat# 62248 or equivalent)
o) Vectashield® Mounting Medium (Vector Laboratories Cat #H-1000 or equivalent)
p) Poly-L-Lysine slides (Sigma, Cat# PO425 or equivalent)

NOTE: These are not essential, but root samples adhere better to these than to untreated slides.

q) Low stender-form preparation dishes (VWR, Cat# 470144-866 or equivalent)
r) HybriSlip Hybridisation covers (Grace Bio-Labs Cat # 712222)
s) 22 x 22 mm square #1 coverglass
t) Coplin jar (Sigma, Cat# S6016 or similar)
u) CoverGrip™ Coverslip Sealant (Biotium Cat# 23005) or clear nail polish
v) RNase free consumables such as pipette tips and razor blades
w) Parafilm®
x) Humidified chamber (or equivalent): 150 mm tissue culture plate; bottom lined evenly with a flat water-saturated paper towel and a single layer of Parafilm placed on top of the paper towel.
y) Plant growth cabinet (Sanyo MLR-351H, or equivalent)
z) Orbital shaker
aa) 37 °C laboratory oven

Microscope:

a) Wide-field fluorescence microscope (*e.g.*, Nikon Eclipse Ti or equivalent).
b) We provide limited support for confocal applications.
c) A high numerical aperture (>1.3) and 60–100x oil-immersion objective.
d) Strong light source, such as a mercury or metal-halide lamp (newer LED-based light sources may also be sufficient).

e) Filter sets appropriate for the fluorophores.
f) Standard cooled CCD or sCMOS camera, ideally optimised for low-light level imaging rather than speed (13 µm pixel size or less is ideal).

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 set volumes. 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 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to create a probe stock of 12.5 µM.
- Mix well by pipetting up and down, and then vortex and centrifuge briefly.

DesignReady and 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 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to create a probe stock of 12.5 µM.
- *Mix well by pipetting up and down*, and then vortex and centrifuge briefly.

Fixation Buffer:

Final composition is 4% (vol. /vol.) formaldehyde in 1X PBS

For a final volume of 4 mL, mix:
- 1 mL 16% Formaldehyde solution, Methanol Free
- 3 mL 1X Phosphate Buffered Saline (PBS), RNase-free

Hybridisation Buffer:

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

Hybridisation 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 Hybridisation Buffer for all of your samples.

For a final volume of 1 mL, mix:
- 900 µL Stellaris RNA FISH Hybridisation Buffer
- 100 µL Deionised Formamide

NOTE: Do not freeze Hybridisation Buffer.

WARNING! Formamide is a teratogen that is easily absorbed through the skin and should be used.

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

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Wash Buffer A (10 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 10 mL, mix:

- 2 mL Stellaris RNA FISH Wash Buffer A
- Add 7 mL Nuclease-free water
- Add 1 mL Deionised Formamide
- Mix thoroughly by vortexing and pipetting up and down.

Wash Buffer B:

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

- Add 88 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 1 µg/mL. This solution is to be used in Step h of the Hybridisation for *Arabidopsis thaliana* root meristem.

Mounting media:

- Vectashield Mounting Medium

NOTE: For best results, samples mounted with Vectashield Mounting Medium should be imaged the same day.

Protocol for *Arabidopsis thaliana* root meristem

NOTE: This protocol has been adapted from a protocol published by Drs. Susan Duncan and Stefanie Rosa [1-3] and has not been tested at Biosearch Technologies. Currently, we can only offer limited support for the use of Stellaris probes on A. thaliana.

Fixation and preparation of *Arabidopsis* Roots

a) Grow seeds into seedlings. Surface sterilise seeds in 5% w/v sodium hypochlorite for 5 min and rinse three times in sterile distilled water before sowing on MS media minus glucose. For uniform germination, stratify the seeds for 3 days at 5 °C before transferring to a growth cabinet with the following settings: 16 h light, 100 µmol m^{-2} s^{-1}, 22 °C ± 1 °C.

b) Dissect root tips below the hypocotyl using a razor blade and forceps and immediately fix them for 30 minutes at room temperature in freshly prepared 4% formaldehyde.

c) Remove the roots from the fixative and wash twice with 1x PBS.

d) Arrange 3-4 roots onto a poly L lysine coated slide in the same direction with root tips aligned, cover with a coverslip and manually squash them onto the slide.

e) Briefly submerge the slide (together with the samples and coverslip) in liquid nitrogen for ~5 seconds. After removal flip the coverslip off using a razor blade.

f) Leave samples to dry at room temperature for a minimum of 30 minutes.
g) Immerse the slide into a Coplin jar containing 70% ethanol and shake gently on an orbital shaker for a minimum of one hour. Slides can be stored at +2 to +8 °C in 70% ethanol up to a week before hybridisation (Parafilm can be used to seal the jar and prevent evaporation during this incubation step.)

Hybridisation and mounting for *Arabidopsis thaliana* root meristem

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

To prepare the Hybridisation Buffer containing probe, add 1 µL of each probe stock solution to 100 µL of Hybridisation Buffer, and then vortex and centrifuge (enough for one coverglass). This creates a working probe solution of 125 nM. This solution will be used on step c.

a) Remove slides from the ethanol and allow residual ethanol to evaporate off for 5 minutes at room temperature.
b) Carry out 2 x two-minute washes in a Coplin jar with Wash Buffer A.
c) Add 100 µL Probe Solution to the slide, cover with a HybriSlip to prevent evaporation and incubate in a humid chamber at 37 °C for a minimum of 4 hours (or overnight) in the dark.
d) Remove the HybriSlip and aspirate the Hybridisation Solution.
e) Carefully wash the samples twice with 100-200 µL Wash Buffer A.
f) Carefully remove the HybriSlip from the slide and aspirate the Wash Buffer A.
g) Add 100 µL of DAPI Solution to the slide, cover with a HybriSlip and incubate at 37 °C for 30 minutes in the dark.
h) Aspirate away the DAPI Solution, add 100 µL Wash Buffer B to the slide and allow the samples to equilibrate for 2 minutes.
i) Remove Wash Buffer B.
j) Add a small drop (approximately 10-30 µL) of Vectashield Antifade Mountant onto the root meristems. Place a coverglass over the sample to evenly spread the mounting medium and seal around the edges using Covergrip, or clear nail polish.
k) Image immediately.

Proceed to imaging.

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References

1. Duncan, S., Olsson, T.S.G., Hartley, M., Dean, C., Rosa, S. (2016) A method to detect single molecules of RNA in *Arabidopsis thaliana*. Plant Methods. 12, 1-10. doi: 10.1186/s13007-016-0114-x
2. Rosa S**,** Duncan S, Dean C. (2016) Single-molecule imaging reveals mutually exclusive sense-antisense transcription at individual *FLC* loci*.* Nature Commun. 7, 13031 doi: 10.1038/ncomms13031.
3. Duncan, S., Olsson, T.S.G., Hartley, M., Dean, C., Rosa, S. (2017) Single molecule RNA FISH in *Arabidopsis* root cells. Bio-protocol. 7, e2240. doi: 10.21769/BioProtoc.2240
4. Orjalo, A.V. Jr., and Johansson, H.E. (2016) Stellaris® RNA fluorescence *in situ* hybridisation for the simultaneous detection of immature and mature long noncoding RNAs in adherent cells. Methods Mol. Biol. 1402, 119-34. 10.1007/978-1-4939-3378-5_10

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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