Stellaris RNA FISH

Protocol for adherent cells in 96-well glass bottom plates

General protocol and storage

This protocol is specifically designed for high-throughput applications of Stellaris™ RNA FISH Probes in 96-well glass bottom plates.

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) TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
b) Methanol
c) Glacial Acetic Acid
d) 37% Formaldehyde Solution
e) Ethanol for molecular biology
f) 10X Phosphate Buffered Saline (PBS), RNase-free
g) Nuclease-free water
h) Deionised Formamide
i) Stellaris RNA FISH Hybridisation Buffer (LGC, Biosearch Technologies Cat# SMF-HB1-10, SMF-HB1-100)
j) Stellaris RNA FISH Wash Buffer A (Biosearch Technologies Cat# SMF-WA1-60, SMF-WA1-600)
k) Stellaris RNA FISH Wash Buffer B (Biosearch Technologies Cat# SMF-WB1-20, SMF-WB1-200)
l) 4',6-diamidino-2-phenylindole (DAPI)
m) Vectashield® Mounting Medium (Vector Laboratories Cat #H-1000)
n) 96-well glass bottom cell culture plates with #0 or #1 coverglass*
o) Mineral Oil
p) RNase free consumables such as pipette tips
q) 37 °C laboratory oven

*Cell culture plate must be resistant to the fixation, wash buffers, and microscope objective immersion oil used in this protocol

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 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 two set volumes (1X and 10X). Please adjust accordingly.*

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Reconstituting the dried probe stock:

ShipReady Probe Set (1 nmol):

- A ShipReady probe set can provide up to 50 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 or Custom Probe Set (5 nmol):

- A DesignReady or custom probe set can provide up to 250 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.

Standard fixation solution:

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

For a final volume of 10 mL (100 mL), mix:

- 1 mL (10 mL) 37% Formaldehyde solution
- 1 mL (10 mL) 10X Phosphate Buffered Saline (PBS), RNase-free
- 8 mL (80 mL) Nuclease-free water

WARNING! *Formaldehyde is a known carcinogen and should be used in a chemical fume hood. Please refer to the appropriate SDS (Safety Data Sheet) prior to use.*

Alternative fixation solution:

Final composition is 3:1 Methanol-Glacial Acetic Acid

For a final volume of 10 mL (100 mL), mix:

- 7.5 mL (75 mL) Methanol
- 2.5 mL (25 mL) Glacial Acetic Acid

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 (10 mL), mix:

- 900 µL (9 mL) Stellaris RNA FISH Hybridisation Buffer
- 100 µL (1 mL) Deionised Formamide

NOTE: *Do not freeze Hybridisation 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.*

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Wash Buffer A (10 mL/100 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 (100 mL), mix:

- 2 mL (20 mL) Stellaris RNA FISH Wash Buffer A
- Add 7 mL (70 mL) Nuclease-free water
- Add 1 mL (10 mL) Deionised Formamide
- 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) dissolved in Wash Buffer A (see above) at 5 ng/mL. This solution is to be used in Step J below.

Mounting media:

- Vectashield Mounting Medium from Vector Laboratories.

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

Protocol for adherent cells

NOTE: *This protocol has been adapted for a 96-well glass bottom system. To adapt this protocol for your preferred system, volumes should be adjusted accordingly.*

Fixation of adherent cell lines (choose one)

Standard fixation

a) Grow cells in a 96-well glass bottom cell culture plate.
b) Decant growth medium, and wash with 200 µL of 1X PBS.
c) To fix cells, add 200 µL of 3.7% Formaldehyde fixation solution.
d) Incubate at room temperature for 10 minutes.
e) Wash twice with 200 µL of 1X PBS.
f) To permeabilise, immerse cells in 200 µL of 70% (vol./vol.) ethanol for at least 1 hour at +2 to +8 °C up to a week before hybridisation.

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Alternative fixation

a) Grow cells in a 96-well glass bottom cell culture plate.
b) Decant growth media, and wash with 200 µL of 1X PBS.
c) To fix and permeabilise cells, add 200 µL of methanol-acetic acid (MeOH-AcOH) fixation solution.
d) Incubate at room temperature for 10 minutes.
e) Cells can be stored at +2 to +8 °C in MeOH-AcOH up to 48 hours before hybridisation. Do not use a well if the MeOH-AcOH has completely evaporated.

Hybridisation in adherent cells

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

To prepare the Hybridisation Buffer containing probe, add 1.5 µL of probe stock solution to 75 µL of Hybridisation Buffer, and then vortex and centrifuge (enough for one well). This creates a working probe solution of 250 nM. This solution will be used on step D.

a) Decant MeOH-AcOH or 70% ethanol from wells containing adherent cells.
b) Add 200 µL of Wash Buffer A (see recipe above), and incubate at room temperature for 2–5 minutes.
c) Decant Wash Buffer A.
d) Add 75 µL of Hybridisation Buffer containing Probe into each well, seal plate to prevent evaporation.
e) Incubate in the dark at 37 °C for 4 to 16 hours.
f) Incubation is recommended for 16 hours using the Standard Fixation Method.
g) Incubation is recommended for 2 hours using the Alternative Fixation Method.
h) Aspirate the Hybridisation Buffer containing Probe, and add 200 µL of Wash Buffer A.
i) Incubate in the dark at 37 °C for 30 minutes.
j) Decant Wash Buffer A, and then add 200 µL of DAPI nuclear stain (Wash Buffer A consisting of 5 ng/mL DAPI) to counterstain the nuclei.
k) Incubate in the dark at 37 °C for 30 minutes.
l) Decant DAPI staining buffer, and then add 200 µL of Wash Buffer B. Incubate at room temperature for 2-5 minutes. Decant the wash buffer.
m) Add 30 µL of VectaShield Mounting Medium to the well and top with 30 µL of Mineral Oil.

Proceed to imaging.

NOTE: *You may find it necessary to oil the bottom of the glass bottom plate for easier imaging. This can be done after step J with the empty wells by inverting the plate. A small drop of oil can be placed underneath each well to be imaged.*

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References

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3. Raj, A., van den Bogaard, P., Rifkin, S.A., van Oudenaarden, A., and Tyagi, S. Imaging individual mRNA molecules using multiple singly labeled probes. Nat. Methods 2008; 5, 877-879. doi: 10.1038/nmeth.1253
4. Femino, A.M., Fay, F.S., Fogarty, K., and Singer, R.H. Visualisation of single RNA transcripts *in situ*. Science 1998; 280, 585-590. doi: 10.1126/science.280.5363.585

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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