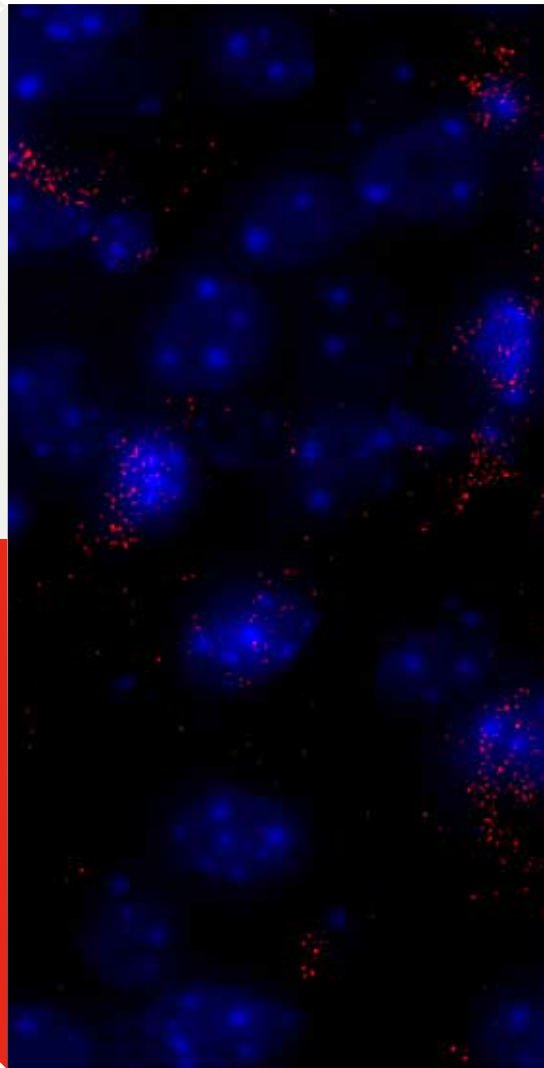
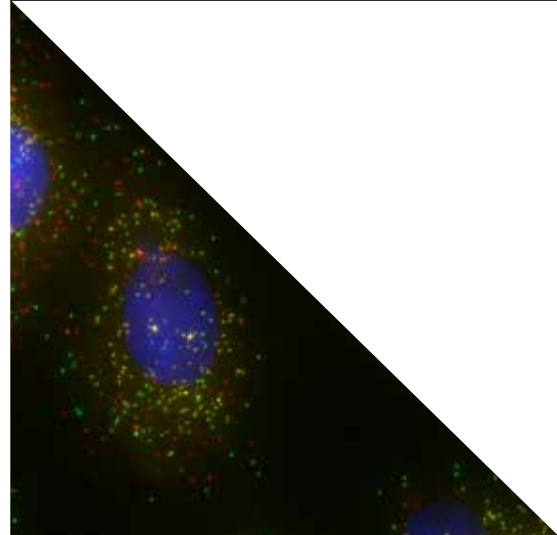




Guide to Getting Started

STELLARIS[®] RNA FISH

(fluorescence in situ hybridization)

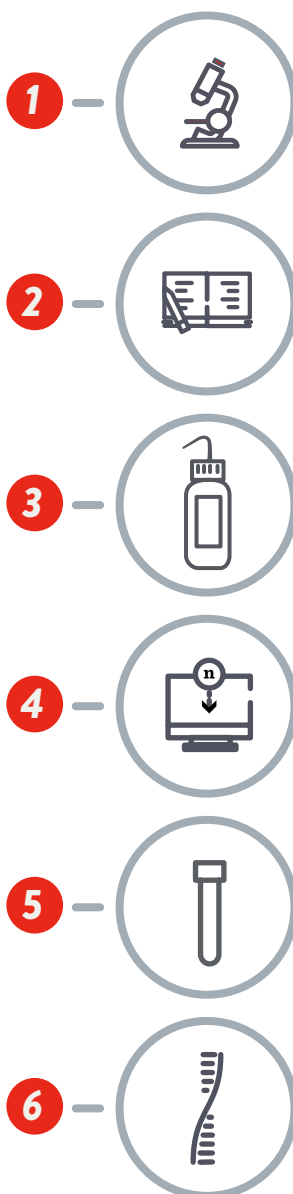


*Stellaris RNA FISH showing Olig2 expression in
mouse brain tissue.*

STELLARIS RNA FISH is a method for the detection of single or clustered RNAs in cultured cells, tissue sections, or whole-mount early embryos. Stellaris RNA FISH is a quantitative gene expression method because spatially separated RNAs can be enumerated.

If this is your first time, we strongly recommend testing your method and probe set first using cultured, adherent cells to maximize your odds for success. Consider using a catalogued probe set as a control.

This guide was designed to provide a beginning user with the basics for getting started with Stellaris RNA FISH. Please read through the guide, consider appropriate positive and negative controls, and ensure that proper imaging equipment is available prior to your first experiments.



Microscope and Hardware
pg. 2



Protocols and Controls
pg. 3



Reagents and Consumables
pg. 4



Probe Design
pg. 7



Order Custom and
Catalogued Probe Sets
pg. 9



Start your experiments!

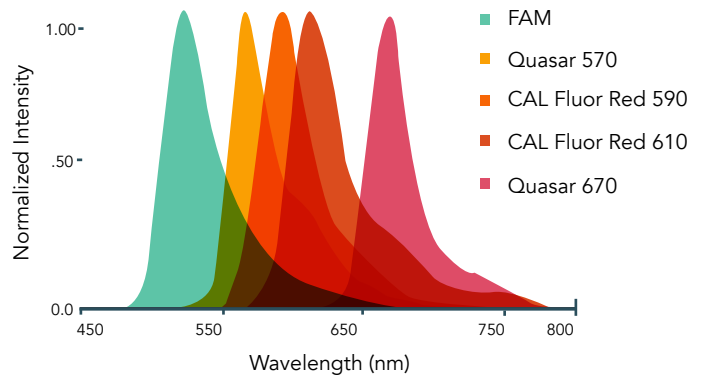


MICROSCOPE REQUIREMENTS

For successful implementation of the Stellaris technology, ensure the proper imaging equipment and imaging capabilities as outlined below.

WIDEFIELD FLUORESCENCE MICROSCOPE

APPROPRIATE FILTERS TO MATCH DYES



60-100X OIL IMMERSION OBJECTIVE (>1.3 NA)

EYEPIECE

COOLED CCD CAMERA

MOTORIZED XYZ STAGE

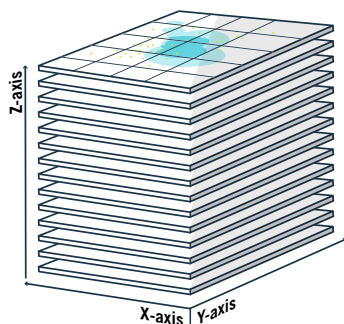
MERCURY OR METAL HALIDE LAMP

Quasar® 570 (Cy3™ replacement), CAL Fluor Red® 610 (Alexa Fluor® 594 replacement), and Quasar 670 (Cy5 replacement) dyes are common dyes used for labeling Stellaris probe sets. We do not recommend using Fluorescein (FAM) due to autofluorescence common to cells and tissue.

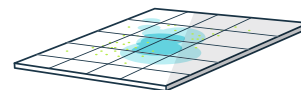
You will not be able to view most fluorescent RNA spots using the microscope eye-piece, which is why the proper light source and camera are essential.

PLEASE NOTE: While highly experienced RNA FISH and confocal microscopy users have experienced successful Stellaris RNA FISH imaging, we do not recommend the use of confocal microscopy as an entry point. This type of microscope uses point illumination to limit the focal plane for imaging and restricts out-of-focus light, but it also diminishes the sensitivity of low-light imaging. We can only provide minimal support for confocal applications.

Z STACK



Acquire images using a 1-2 second exposure time per plane with 0.25 μm between z-sections.



Perform maximum intensity projection using software such as ImageJ.



PROTOCOLS AND CONTROLS

To prepare reagents for Stellaris RNA FISH assays, please refer to our protocols webpage:

www.biosearchtech.com/stellarisprotocols.

The success of a Stellaris experiment is also dependent on the RNA integrity of your samples and the careful consideration of controls for your experiment.

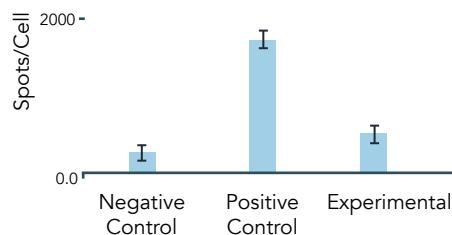
RNA degrades rapidly in the cell when not preserved properly. Interrogating tissue or cells with questionable preservation of RNA may give poor results. Please prepare your samples in a manner where the RNA is preserved and avoid RNase contamination at all times.

POSITIVE CONTROL

- 1 Utilize one of our catalogued probe sets. These have been functionally tested and if the experiment is performed properly and the gene is expressed in your sample type, a specific signal will be produced.
- 2 If using a catalogued probe set is not an option, design a custom probe set to a gene target that has relatively medium to high abundance in your sample type, in addition to designing a custom probe set to your target gene.

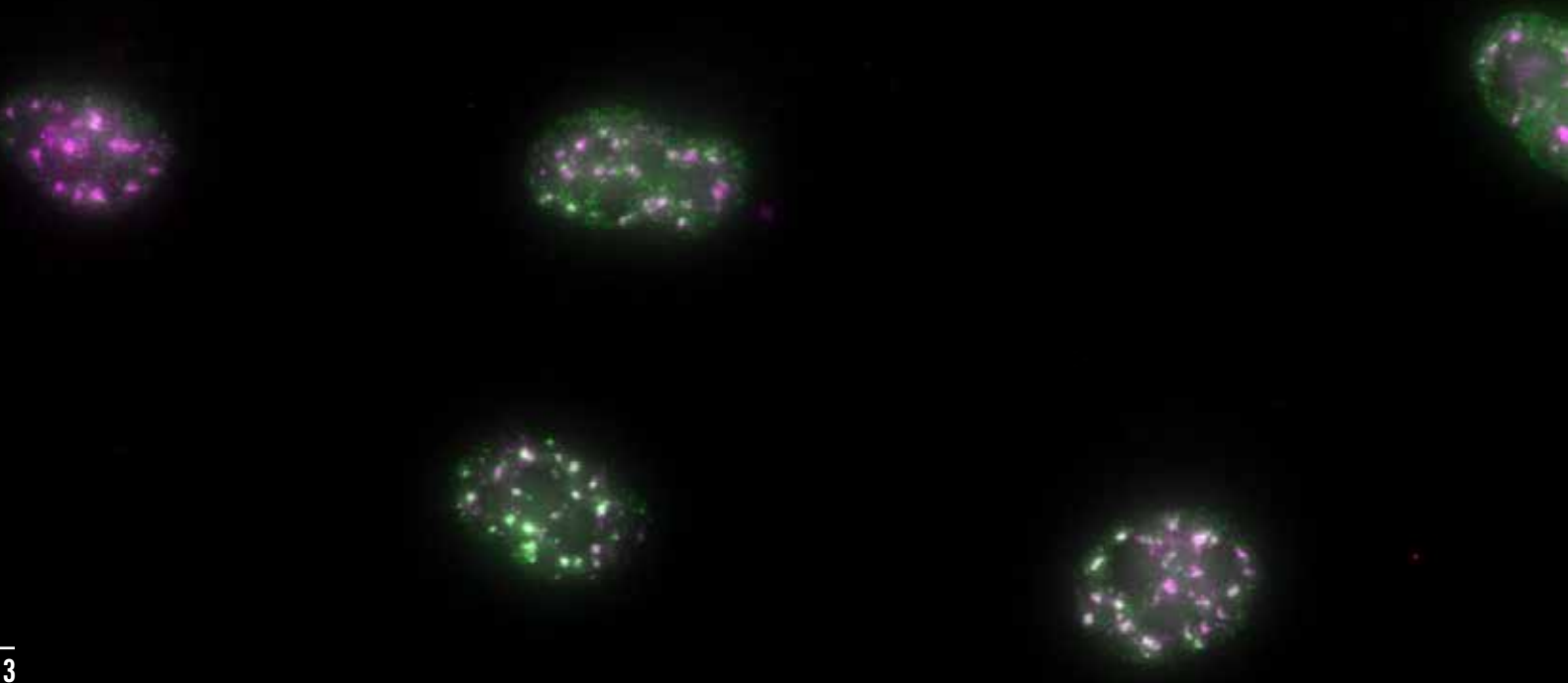
NEGATIVE CONTROL

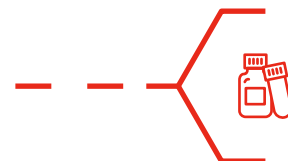
- 1 When performing your experiments, it is imperative to include a no-probe control sample. This sample should be treated the same as the rest of your samples, but probed with hybridization buffer only. This will reveal potential auto-fluorescent features in your sample.
- 2 To determine the specificity of your signal from the gene specific probe set, an ideal negative control is to test the probe set in a cell line/tissue void of the transcript.



- 3 In addition to the above recommendations, you may consider using a probe set targeting a gene from an unrelated organism. For example, if your cells/tissue do not express GFP, a probe set targeting GFP can be used.

*MALAT1 (green) and SC35 protein (magenta)
using RNA FISH and immunofluorescence in
human cells.*





REAGENTS AND CONSUMABLES

Below is a list of common reagents and consumables necessary to perform a Stellaris FISH experiment. Note that consumable type may vary with sample types.

REAGENTS



PHOSPHATE-BUFFERED SALINE
(1X)



FORMAMIDE,
DEIONIZED



DAPI FLUORESCENT NUCLEAR
STAIN



FORMALDEHYDE



ETHANOL (95%)
MOLECULAR GRADE

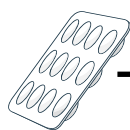


TRIS EDTA BUFFER SOLUTION,
PH 8.0



VECTASHIELD MOUNTING
MEDIA (WITHOUT DAPI) VECTOR
LABORATORIES H-1000

EXAMPLE CONSUMABLES



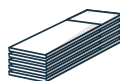
12- WELL TISSUE
CULTURE PLATES



STELLARIS RNA FISH
HYBRIDIZATION BUFFER



MICROCOVER GLASSES
ROUND NO. 1



SUPERFROST PLUS
MICRO SLIDE



STELLARIS RNA FISH
WASH BUFFER A



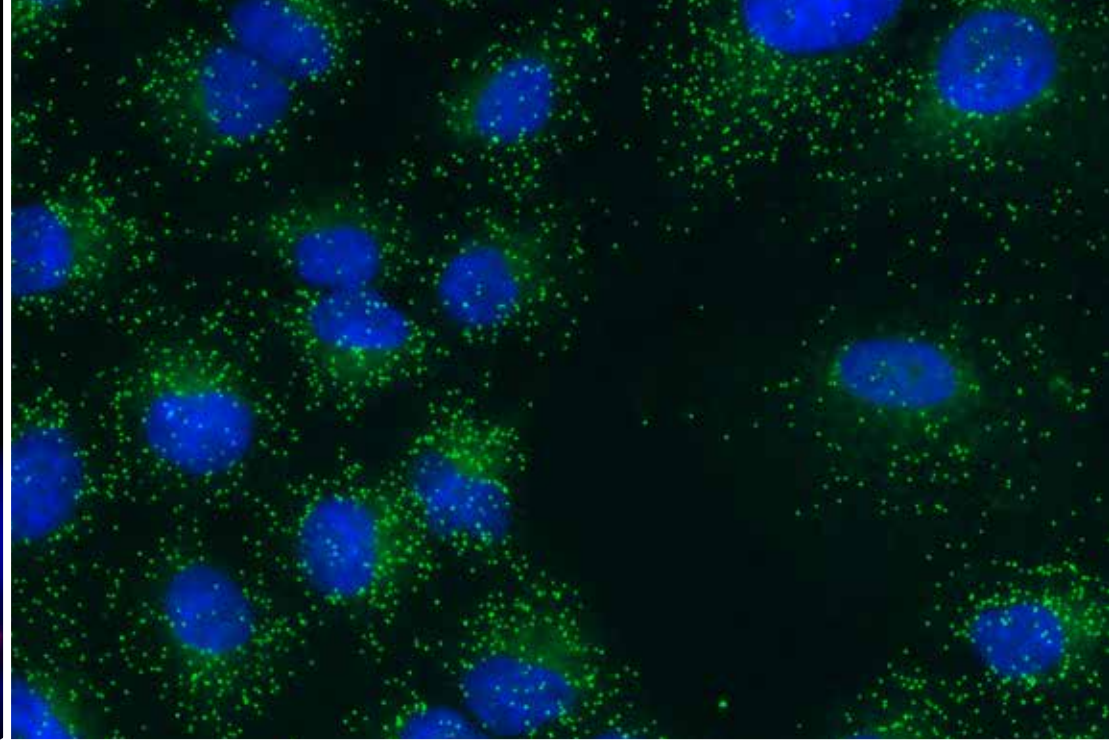
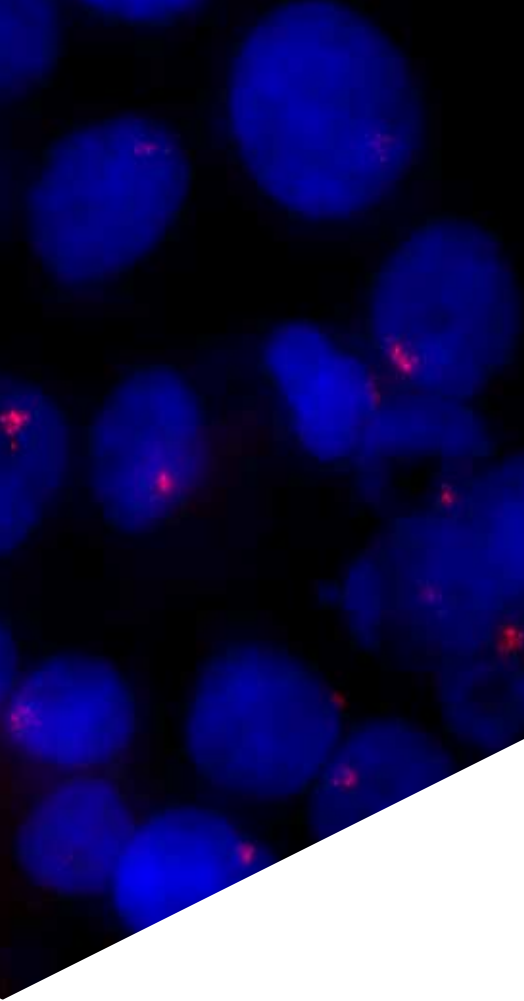
STELLARIS RNA FISH
WASH BUFFER B

We recommend preparing Hybridization Buffer fresh for each experiment by adding deionized formamide as outlined in our protocols.

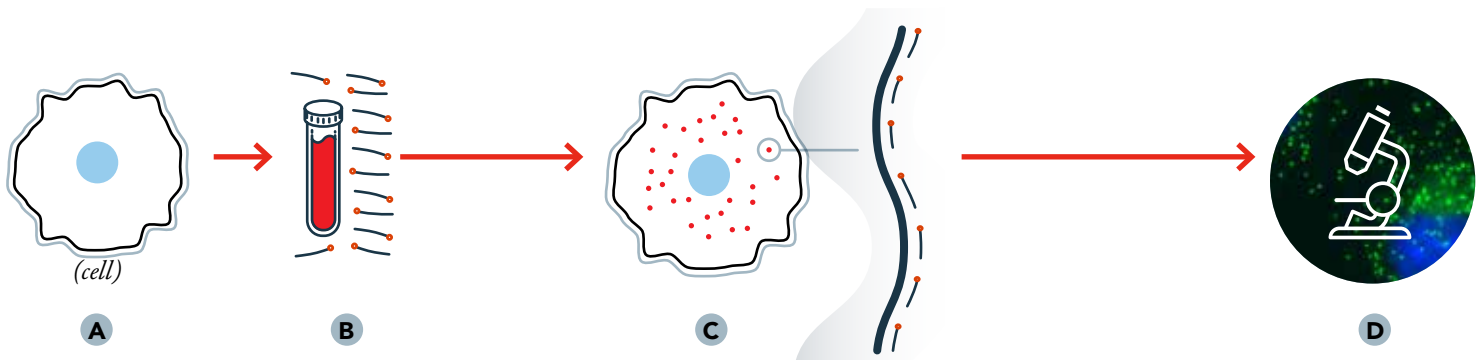
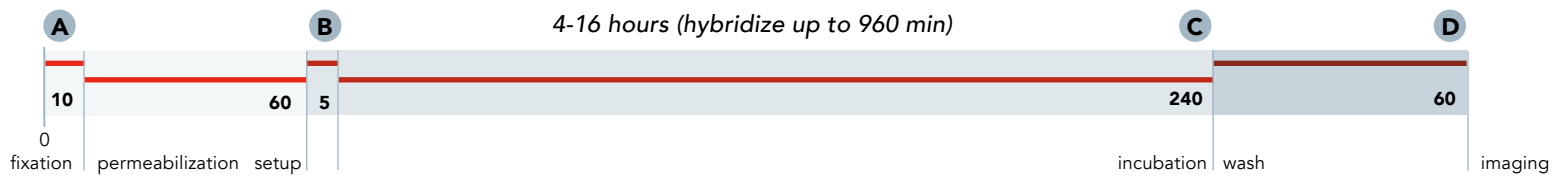
We suggest diluting Wash Buffer A and adding deionized formamide fresh for EACH experiment. Use the formamide within 3 months, stored at 4 °C. The formamide breakdown products can adversely affect the quality of the target RNA.

CDK4 mRNA (green)
in human cells

XIST lncRNA (magenta) in
human cells



TIME FRAMES (MINUTES)





CUSTOM PROBE DESIGN CONSIDERATIONS

WWW.BIOSEARCHTECH.COM/STELLARISDESIGNER

CUSTOM PROBE sets can be designed by using our Stellaris Probe Designer application, which evaluates the target sequence and generates candidate probes. The probe designer lists in a table, from 5' to 3', the sequence for each individual oligonucleotide, the corresponding position on the transcript and the GC content. The probe designer employs algorithms that screen various common and organism specific RNA sequences that can adversely affect probe performance, such as long or short repeat sequences.

If such sequences are of concern for organisms not listed in the designer, please refer to other resources such as repeatmasker.org.

The designer does not discriminate between true genes and other transcribed genes, such as paralogs or active pseudogenes with high sequence similarity. **This design concern can be mitigated by paying careful attention to the specificity of the sequence entered into the designer and by using the highest masking level (5) during the design process.** Further, significant variability is often found in the untranslated regions of mature mRNAs. For example, alternative transcription start sites may affect the sequence and signal if the design is against this region. Probe sets designed against unique coding sequences, therefore, are most likely to allow for single molecule detection with minimal background signal.

IMPORTANT NOTE: FOR SINGLE-MOLECULE DETECTION, we recommend designing at least 25 individual oligonucleotide probes against the RNA of interest, while staying within the maximum limit of 48 sequences per probe set.

DESIGN FOR PROBE SET SPECIFICITY

FOR SHORT MRNA SEQUENCES OR RNAs FROM GENES WITH VERY SIMILAR HOMOLOGS in the organism to be tested, we strongly recommend careful upfront bioinformatic analysis. BLAST and multiple sequence alignments can be used to better determine if 5' and/or 3' UTRs are suitable for inclusion in the design. We also recommend a manual BLAST of individual probes or batch-BLAST of the probe set, designed at masking levels 3 and below against the expressed genome of the organism to be tested. In this case, you may wish to exclude oligonucleotides with >16 nucleotide matches to other RNA(s) to minimize possible cross-hybridization, especially if multiple probes may bind off-target. These recommendations can be relaxed if the expression of given genes are well-known in the sample to be tested.

Please note: When entering a target sequence, any degenerate nucleotides must be represented as "n" in the sequence; IUPAC letters other than A, G, C, & T are stripped from the sequence to potentially cause design against a non-existent sequence.

Original sequence

AGTCGRYMAGTTGGAGTTCAGTCYRCTTTC

Replace degenerate nucleotides with 'N'

AGTCGNAGTTGGAGTTCAGTCNCTTTC

Avoids designing across "N"

AGTCGNAGTTGGAGTTCAGTCNCTTTC



CORRECT

If using original sequence, designer removes degenerate nucleotides

AGTCG AGTTGGAGTTCAGTC CTTTC

Designs against non-existent sequence

AGTCGAGTTGGAGTTCAGTCCTTTC



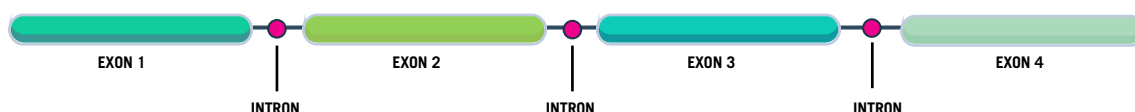
INCORRECT

*The above diagram is used for illustrative purposes. Actual probe lengths are 20 bases each.



DESIGN TO DETECT EXONS

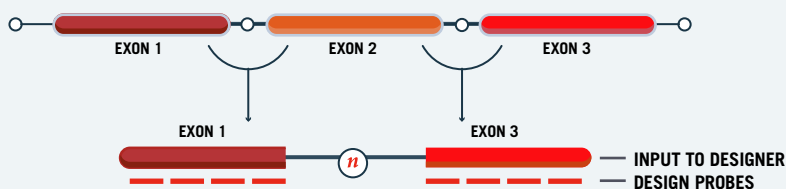
IF DETECTION OF MATURE RNA IS THE GOAL, we recommend designing against the coding sequence if possible. It is also important to consider whether your probes should recognize single or multiple transcript variants. In this case, additional design considerations will be required. For detection of multiple variants (also known as an "inclusive probe set"), the input sequence should include only the sequence that is common to all variants. Conversely, for single-variant detection (also known as an "exclusive probe set"), the input sequence must be void of sequence that is common to other transcript variants.



DESIGNING AGAINST VARIANTS

Example of a design against Exons 1 and 3 to recognize both splice variants (inclusive probe set). The "n" replaces Exon 2 sequence and is used to block the design of probes spanning an alternative splice site. Probes are represented by red dashed lines.

The spliced segments for inclusive or exclusive probe sets should be replaced by a single "n" before being entered into the designer. This step prevents the design of probes spanning the splice site (see below).



DESIGN TO DETECT INTRONS (iceFISH™)

Stellaris RNA FISH can be used to interrogate the transcription status of a particular gene or collection of genes. Because Stellaris RNA FISH probes are hybridized at 37 °C, under mild denaturing conditions, only exposed RNA is accessible, not chromosomal double-stranded DNA. Furthermore, the Stellaris probe designer incorporates several specificity filters, including one against repeat sequences common in introns.

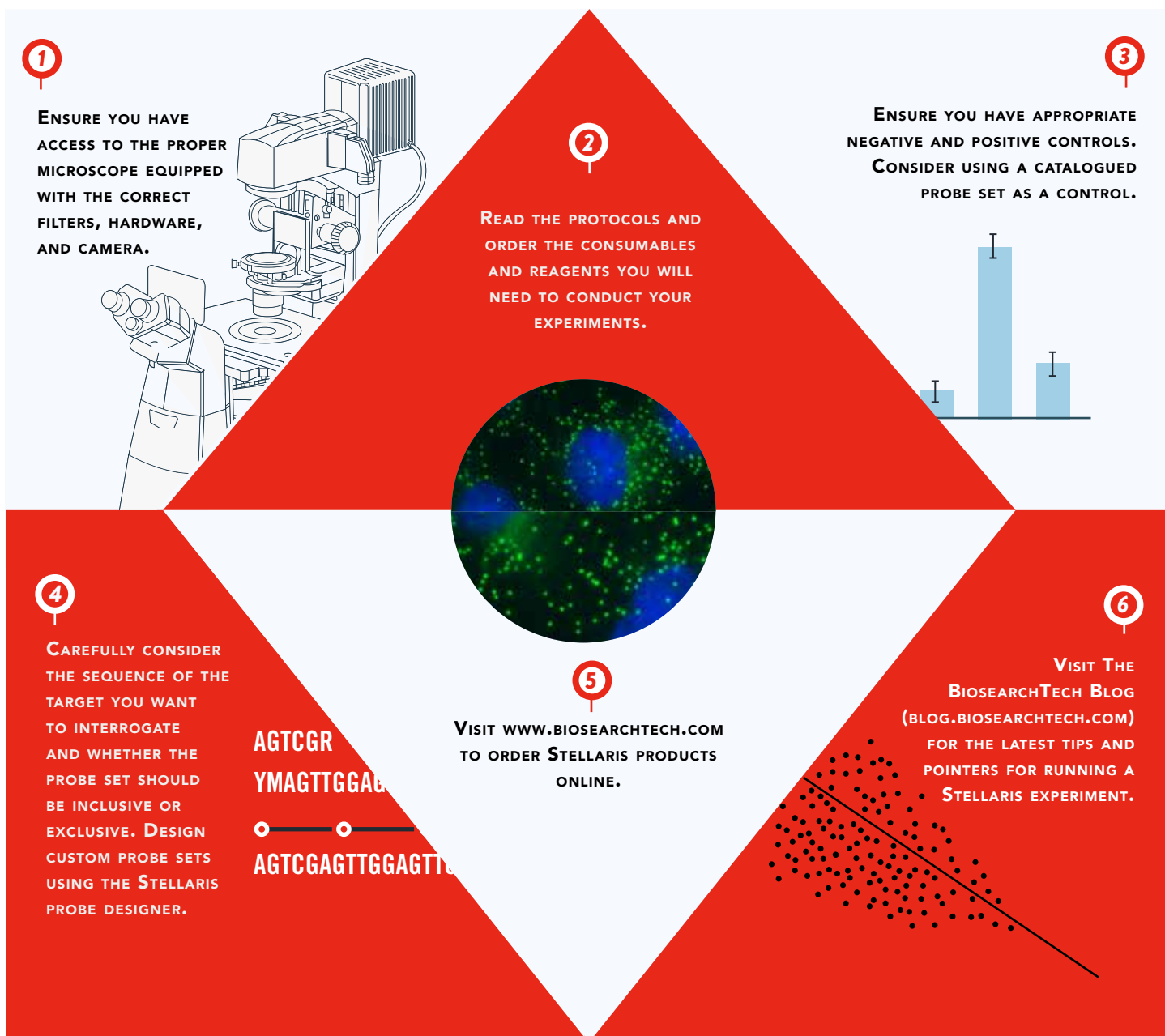


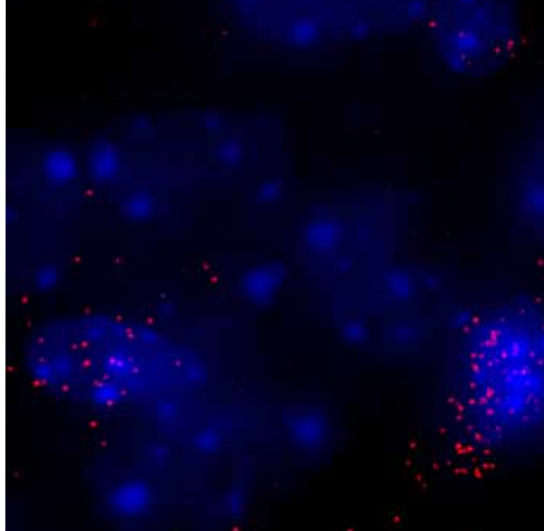
For most genes, there is sufficient intron sequence to design sets with the full 48 oligos. To limit the design to intron regions only, we recommend replacing the exons of the gene sequence with "n" and to use up to 8,000 nucleotides of the common introns for your design.

This avoids the possibility of all your probes binding to an intron only transcribed from an alternative transcription start site. In addition, we recommend avoiding the last intron, as its splicing may be delayed and occurs away from the site of transcription.

SUMMARY

FOR THE SUCCESSFUL IMPLEMENTATION OF STELLARIS RNA FISH:





PUBLICATIONS FOR FURTHER REFERENCE:

Scaffold function of long non-coding RNA HOTAIR in protein ubiquitination.

Yoon, J.H.; Abdelmohsen, K.; Kim, J.; Yang, X.; Martindale, J.L.; Tomimaga-Yamanaka, K.; White, E.J.; Orjalo, A.V.; Rinn, J.L.; Kreft S.G.; Wilson, G.M.; Gorospe, M. *Nat Commun.* 2013;4:2939. doi: 10.1038/ncomms3939.

Protein aggregation behavior regulates cyclin transcript localization and cell-cycle control.

Lee, C.; Zhang, H.; Baker, A.E.; Occhipinti, P.; Borsuk, M.E.; Gladfelter, A.S. *Dev Cell.* 2013 Jun 24;25(6):572-84. doi: 10.1016/j.devcel.2013.05.007.

Single-cell gene expression profiling reveals functional heterogeneity of undifferentiated human epidermal cells.

Tan, D.W.; Jensen, K.B.; Trotter, M.W.; Connelly, J.T.; Broad, S.; Watt, F.M. *Development.* 2013 Apr;140(7):1433-44. doi: 10.1242/dev.087551.

Imaging individual mRNA molecules using multiple singly labeled probes.

Raj, A.; van den Bogaard, P.; Rifkin, S.A.; van Oudenaarden, A.; and Tyagi, S. *Nat. Methods* 2008; 5, 877-9. doi: 10.1038/nmeth.1253.

Visualization of single RNA transcripts in situ.

Femino, A.M.; Fay, F.S.; Fogarty, K.; and Singer, R.H.; *Science* 1998; 280: 585-590. doi: 10.1126/science.280.5363.585.

ORDERING AND TECHNICAL SUPPORT:

Visit **www.biosearchtech.com** to place a Stellaris order or email **info@biosearchtech.com** for order-related questions. If you require further technical assistance, please email our technical support team at **techsupport@biosearchtech.com**.



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