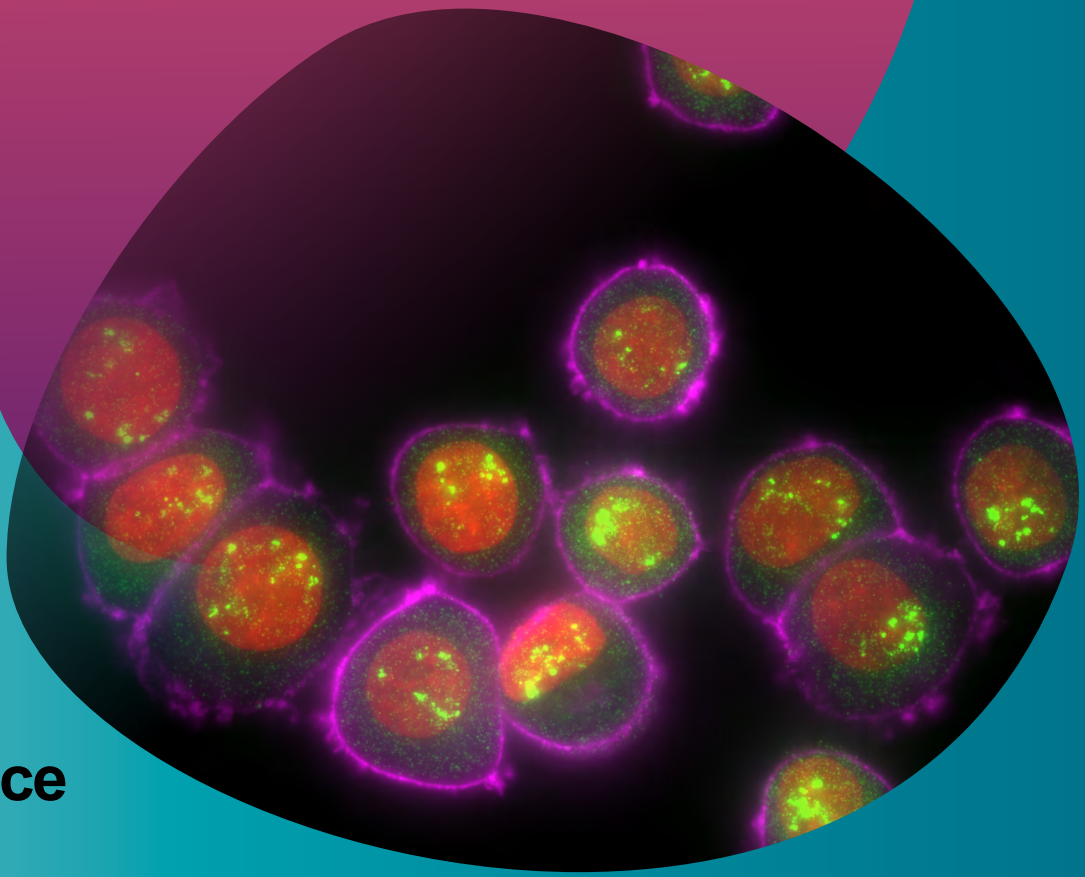
Guide to getting started Stellaris RNA FISH



Fluorescence *in situ* hybridisation

BIOSEARCH™ TECHNOLOGIES

GENOMIC ANALYSIS BY LGC

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

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.

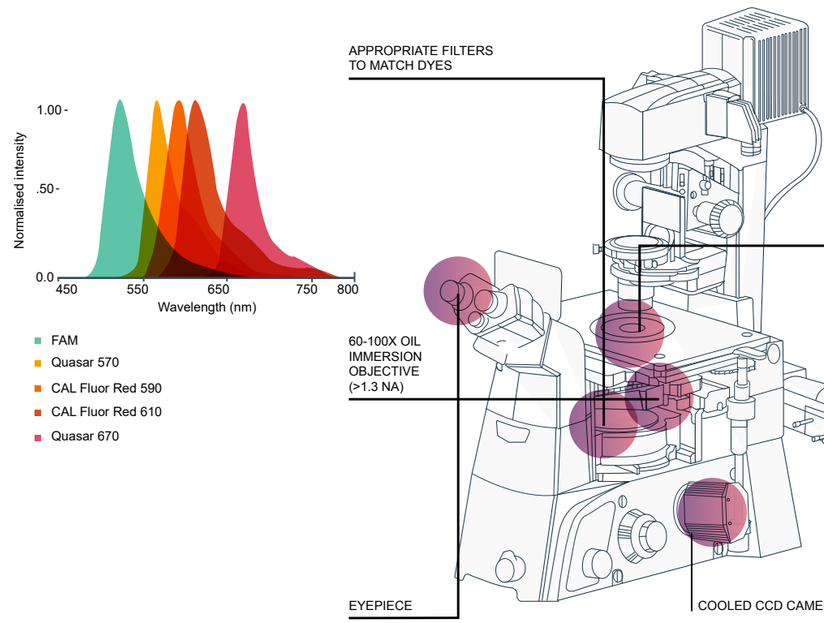
If this is your first time, we strongly recommend testing your method and probe set first using cultured, adherent cells to maximise your odds for success. Consider using a catalogued ShipReady probe set as a control.

EGFR mRNA (yellow), TOP1 mRNA (green), and POLR2A mRNA (red) in human cells

Microscope requirements

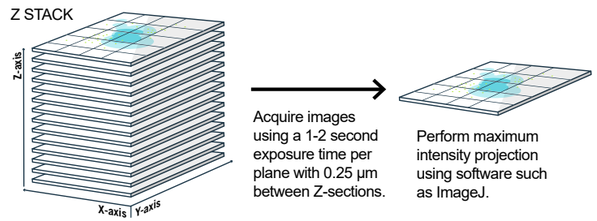
For successful implementation of the Stellaris technology, ensure the proper imaging equipment and imaging capabilities as outlined below.

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.



Widefield fluorescence microscope
Quasar™ 570 (Cy3™ equivalent), CAL Fluor™ Red 610 (Alexa Fluor® 594 equivalent), and Quasar 670 (Cy5 equivalent) 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.

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.



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

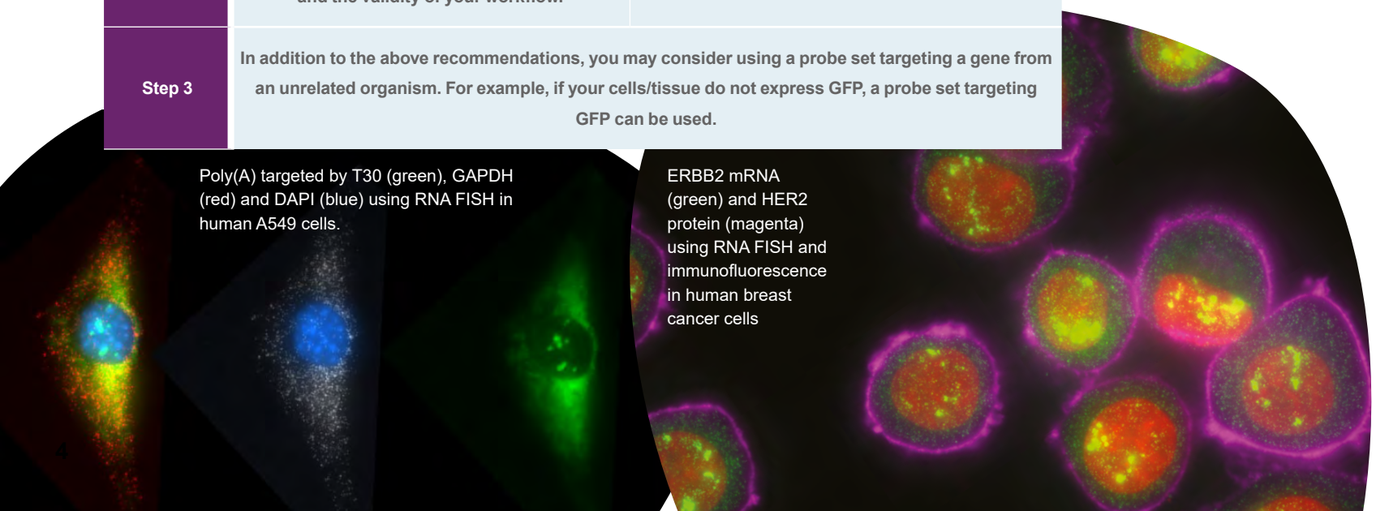
Perform maximum intensity projection using software such as ImageJ.

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	Negative control
Step 1	Utilise one of our catalogued ShipReady probe sets. These have been functionally tested upon development. So, if the experiment is performed properly and the gene is expressed in your sample type, a specific signal will be produced.	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 hybridisation buffer only. This will reveal potential auto-fluorescent features in your sample.
Step 2	If using a catalogued probe set is not an option, then use the T30 control that binds to the poly(A) tail of RNAs, in addition to designing a custom probe set binding to the target. The T30 controls enable you to check your microscope and filter sets, your experimental technique and the validity of your workflow.	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.
Step 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.	



Poly(A) targeted by T30 (green), GAPDH (red) and DAPI (blue) using RNA FISH in human A549 cells.

ERBB2 mRNA (green) and HER2 protein (magenta) using RNA FISH and immunofluorescence in human breast cancer cells

Reagents and consumables

Reagents

- Phosphate-buffered saline (1X)
- Formamide, deionised
- DAPI fluorescent nuclear stain
- Formaldehyde
- Tris EDTA buffer solution, pH 8.0
- Vectashield® Mounting Media (without DAPI) Vector Laboratories H-1000
- Ethanol (95%) molecular grade

Example consumables

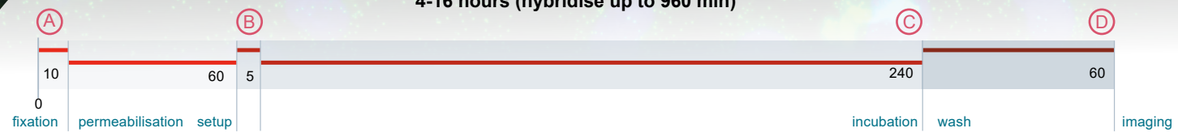
- 12- well tissue culture plates
- Stellaris RNA FISH Hybridisation Buffer
- Microcover glasses round no. 1
- Superfrost plus micro slide
- Stellaris RNA FISH wash Buffer A
- Stellaris RNA FISH wash Buffer B

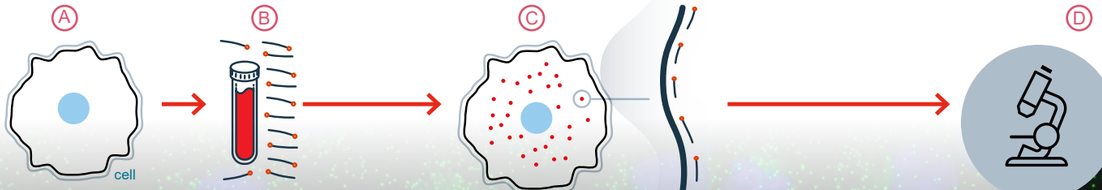
We recommend preparing hybridisation mRNA Buffer fresh for each experiment by adding deionised formamide as outlined in our protocols.

We suggest diluting Wash Buffer A and adding deionised 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

Time frames (minutes)





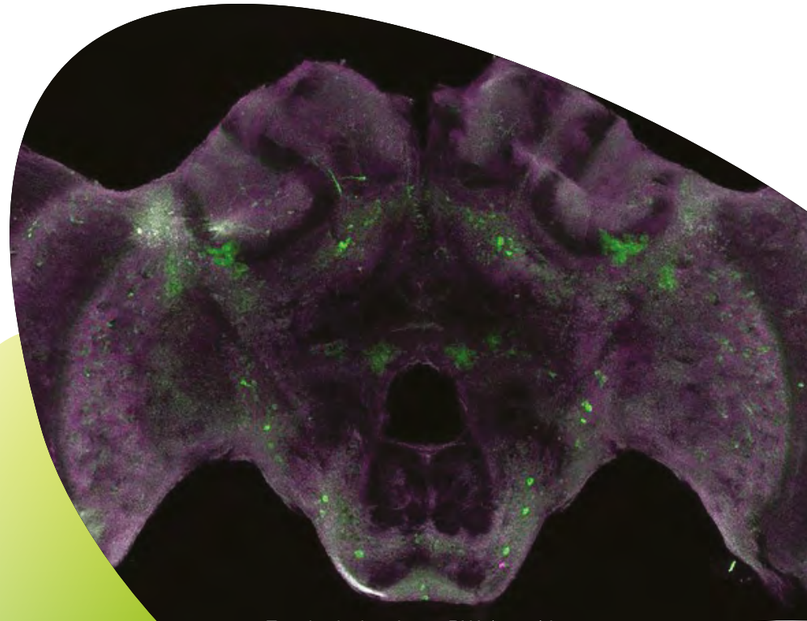
Custom probe design considerations

Custom probe sets can be designed by using our online Stellaris Probe Designer software, (www.biosearchtech.com/stellarisdesigner) 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.

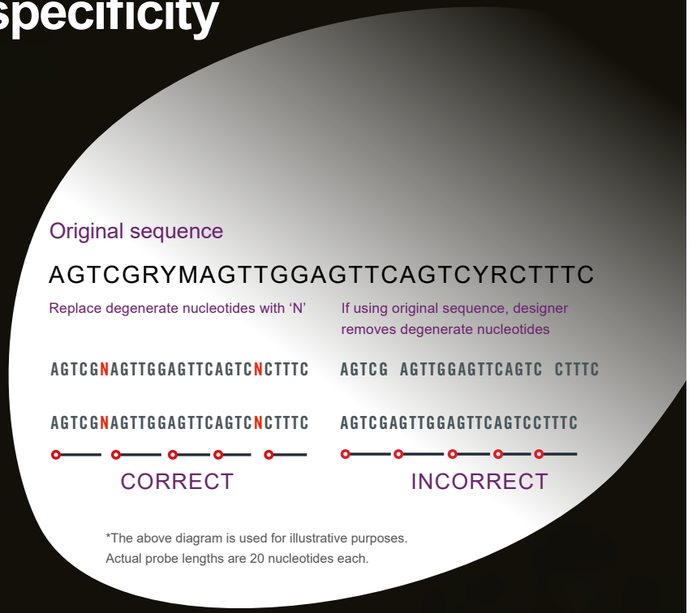
Please note: When entering a target sequence, any degenerate nucleotides must be represented as "n" in the sequence; IUPAC letters other than A, G, C, and T are stripped from the sequence to potentially cause design against a non-existent sequence.



Tyrosine hydroxylase mRNA (green) in honey bee brain Courtesy of Matthew McNeill and Geae Robinson

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 20-mer oligonucleotides with >16 nucleotide matches to other RNA(s) to minimise possible cross-hybridisation, 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.

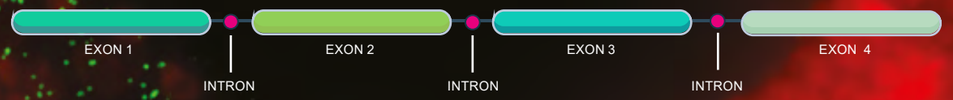


*The above diagram is used for illustrative purposes. Actual probe lengths are 20 nucleotides each.

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.

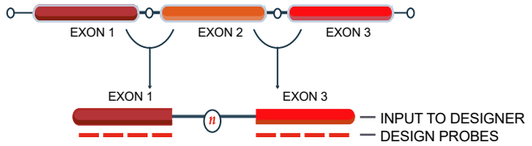
Design to detect exons

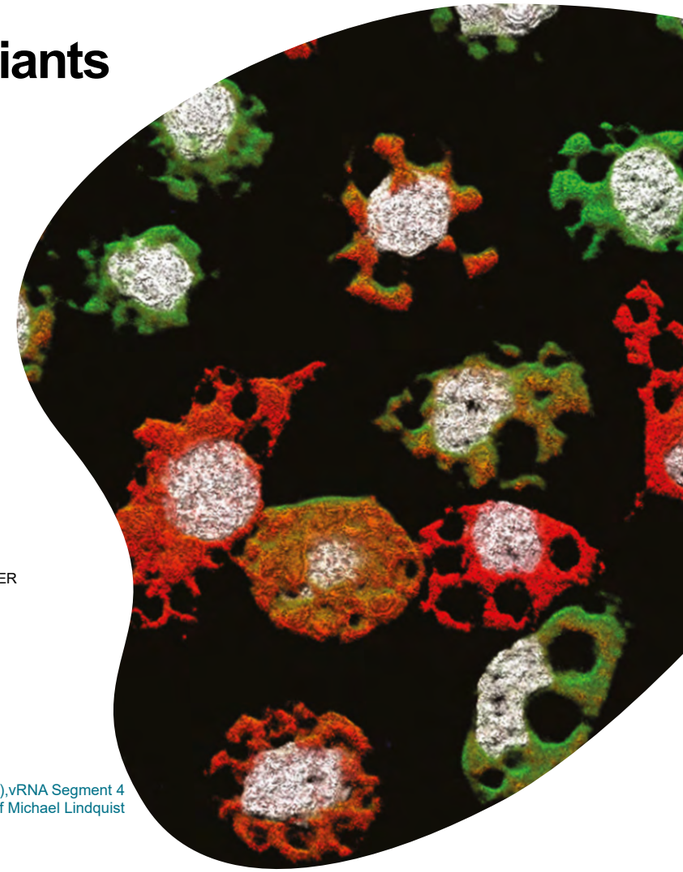
If detection of mature RNA is the goal, we recommend designing against the coding sequence if possible. It is also important to consider whether your probes should recognise single or multiple transcript variants. In this case, additional design considerations will be required. For detection of multiple 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 (see below).



Designing against variants

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). Example of a design against exons 1 and 3 to recognise 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.





Guaico Culex Virus (GCXV)vRNA Segment 3 (green),vRNA Segment 4 (red), vRNA Segment 5 (blue) Courtesy of Michael Lindquist

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 hybridised 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.

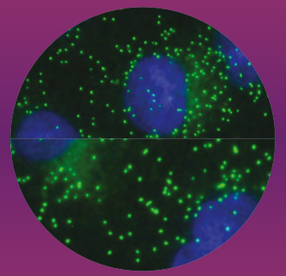
This avoids the possibility of all your probes binding to an intron only transcribed from an alternative transcription start site. In addition, we recommend avoiding the last intron, as its splicing may be delayed and occur away from the site of transcription.

Summary

For the successful implementation of Stellaris RNA FISH:

1. Ensure you have access to the proper microscope equipped with the correct filters, light source, and camera.

2. Read the protocols and order the consumables and reagents you will need to conduct your experiments.



3. Ensure you have appropriate negative and positive controls. Consider using a ShipReady probe set as a control.

4. Carefully consider the sequence of the target you want to interrogate and whether the probe set should be inclusive or exclusive. Design custom probe sets using the Stellaris probe designer.

5. Visit www.biosearchtech.com to order Stellaris products online.

6. Visit the BiosearchTech blog (blog.biosearchtech.com) for the latest tips and pointers for running a Stellaris experiment.

Publications for further reference

Over 1/3 of publications citing Stellaris RNA FISH publish in *Nature*, *Cell*, or *Science* journals. Visit our Citation Center to browse hundreds of publications at www.biosearchtech.com/stellaris-citation-center

Products and services

Products

Black Hole Quencher™ Probes

- Dual-Labeled BHQ™ Probes
- BHQplus™ Probes
- BHQnova™ Probes

ValuMix assays

- ValuMix for gene expression and qPCR
- ValuMix for SNP genotyping

Molecular beacons

- Scorpions™ primers

Custom oligonucleotides

- Research-grade
- GMP

Stellaris RNA FISH

- Custom probe sets
- DesignReady probe sets
- ShipReady probe sets
- RNA FISH buffers

Services

GMP and commercial services

- GMP-compliant oligos for IVD and molecular diagnostics
- Custom analyte specific reagents

Pre-clinical and therapeutics OEM and kit manufacturing

Online design software

- Stellaris RNA FISH Probe Designer
- RealTimeDesign qPCR assay design software
- ChIRP Designer

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

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