Selection and Optimization of Custom Probes for GeoMx® RNA Assays
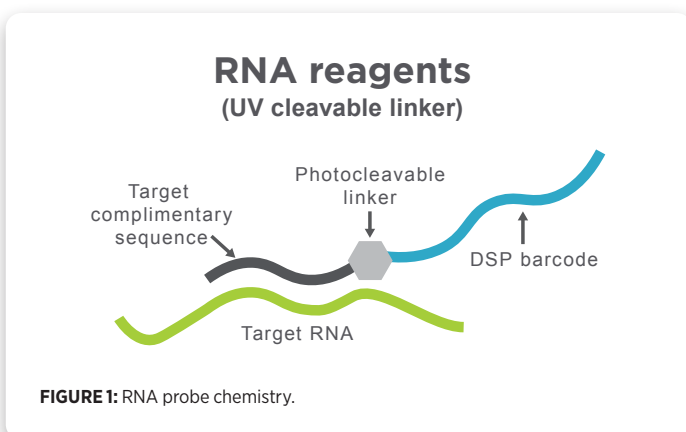
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To profile the biology of a tissue, it is critical to explore a broad set of targets. The GeoMx Digital Spatial Profiler (DSP) RNA assays allow you to perform high-plex, spatially resolved gene expression analysis with a single tissue slide. Each probe in the GeoMx RNA assay contains a unique oligonucleotide barcode that can be quantified on the nCounter Analysis System or an Illumina Next Generation Sequencer (NGS).

Profiling your targets of interest is key to achieving meaningful results within a spatial experiment. As each research project has its own requirements, the GeoMx RNA Barcoding Service (RBS) allows you to add your targets of interest to our off-the-shelf assays. GeoMx RNA assays can be customized with up to 10 targets using the nCounter readout and with up to two 200 target assays using NGS readout (each assay containing it's own unique internal controls in addition to the custom targets). Additionally, design of fully custom assays with up to 400 targets is also possible using NGS readout by combining up to two 200 target custom assays. Customization requires conjugation of our unique DSP barcodes to the target complementary sequence for quantification. This customization can be performed with the help of NanoString's Bioinformatics team.

Ordering GeoMx RNA Assay custom probes from NanoString

How to place an order

The first step is to communicate with your NanoString Sales Representative, to obtain a Target Submission Form for your list of targets, and to generate a quote to initiate the design process by our Bioinformatics team. The targets listed in the submission form should contain the following information:

RNA reagents
(UV cleavable linker)

Target complimentary sequence

Photocleavable linker

DSP barcode

Target RNA

FIGURE 1: RNA probe chemistry.

• **Assay design** The first critical decision in custom assay design is whether the probes will be used as a spike-in to an Atlas assay or as a standalone assay. For spike-in use, it is not necessary to include additional probes to targets of interest that are already in the Atlas assay you will use, unless you want to target a specific transcript region or splice isoform(s). Please contact geomxsupport@nanostring.com for a list of targets included in each Atlas product. Compatibility of multiple assays together is best determined at the time of design; if later you decide to use this custom assay with any other assay, please contact NanoString's Bioinformatics. For standalone use, we recommend including targets with a range of expression levels in your tissue, if this information is available. In addition to your targets of interest, custom assays must also include appropriate positive and negative control probes. NanoString negative control probe sequences are based on ERCC sequences and will be added to your panel automatically. By contrast, NanoString requires that you designate at least two targets as positive controls. These targets should be known to be constitutively expressed in your sample or tissue type. Inclusion of positive control targets serves two functions. First, detection of these targets confirms that the custom probe pool was correctly added to the in situ hybridization reaction. Second, positive control targets can be used for housekeeper normalization during data analysis. If you plan to use housekeeper normalization, we recommend including at least five positive control targets so that the best target(s) for normalization can be chosen based on consistency across samples using an algorithm such as geNorm.

• **Target Identifier** Please provide the formal gene name from NCBI, or the HUGO gene symbol as preferred Target Identifier. If you are requesting a transcript-specific probe or are designing to a sequence that is not part of the reference transcriptome, please provide specific accession number(s) for the nucleic acid sequence(s) of interest. You can also provide sequences, preferably in FASTA format, directly to NanoString's team.

• **Name** Please provide your preferred name for each target of interest (gene name, serial number, etc). If this field is used, this information will be associated with your target in the data output.

• **Comments** Specific information about your target of interest: RefSeq accession version information, splice variant information, gene IDs from other platforms, etc. can be included here. This field is optional but is for your reference and may provide useful information for the design.

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- **Other Considerations** Other information you may consider adding includes the species you intend to profile with your custom assay.

Probe Design by NanoString's Bioinformatics team

After submitting the Target Submission Form, NanoString's team will start with the probe design and will evaluate the feasibility of the selected targets. Each sequence is profiled for intrinsic properties – non-canonical bases, GC content, secondary structure, and runs of polynucleotides. Probes may range in size from 35-50nt. Further, the position within the target sequence is considered with respect to coding sequence and exon junctions. Next, BLAST is used to identify potential off-target interactions within the transcriptome. In addition to a cross-hybridization score, a splice isoform coverage score is generated. Probes are selected by employing optimal splice form coverage, the cross-hybridization score, and the most optimal thermodynamic profile as scoring metrics. The transcriptome used for design is sourced from the latest version of NCBI RefSeq unless a separate transcriptome is requested.

To ensure that there are no potential probe-probe interactions, a stringent intramolecular screen is run on every collection of probes assembled. This screen calculates and considers both the Tm and the free energy potential of every probe compared to every other probe in the project. If a probe is identified having either Tm or free energy profile above a conservative threshold, an alternative probe is selected for that target and the screening is re-run until all requirements are met.

Note: When possible, three or ten probes (for NGS or nCounter readout, respectively) will be designed across each target transcript. For NGS readout, having multiple probes per target will increase the efficiency and reproducibility of the data by allowing any under-performing probes to be removed as outliers. For nCounter readout, the higher number of probes will increase the sensitivity of the assay.

A probe kit configuration (PKC) file is generated by the Bioinformatics design pipeline. Based on numerous experiments with NanoString's probe chemistry, probes with 95% identity to a target in question function equally as well as those with 100% identity. Thus, NanoString provides all genes, accessions, and mappable genome coordinates targeted down to this level of identity.

Note: The same custom probes can be spiked into both the Whole Transcriptome Atlas (WTA) and the Cancer Transcriptome Atlas (CTA), or into a previous Custom Assay,

if this is requested up front. The stringent probe-probe hybridization screen relies on having this information on hand for design.

Important: NanoString's probe sets (both nCounter and NGS readout) include an internal negative control set formed by probes targeting sequences from the External RNA Controls Consortium (ERCC). Please make sure that the custom probe set does not include any of the ERCC transcript. If this is not possible, please contact the Bioinformatics team to understand the risks to your assay.

Non-coding RNA targets

Many lncRNA targets can be targeted by GeoMx probes, but there are considerations in probe design that may make it difficult (or impossible) to get a good design. These considerations include length or sequence features. For example, a probe from the design pipeline may not be specific enough to a particular target due to similarities to alternate targets. Targeting miRNA is not feasible due to their short length. For circular RNA, it would be possible to design a single probe tile targeting the back-splice junction. However, NanoString has not validated these types of designs. If you wish to add targets that are covering non-coding areas, contact our Bioinformatics team via geomxsupport@nanostring.com to get more information on your target request.

Shipment and Storage

The custom assay will be shipped in 16 aliquots, each with sufficient material to process 12 slides. They should be stored at -20˚C. Once thawed, **do not re-freeze probes**. Refrigerate at 4°C for up to 3 months.

Testing functionality of RNA Assay custom probes using the GeoMx DSP

Custom probes undergo quality control testing for length using electrospray ionization (ESI) MS and concentration. NanoString's service does not include a validation process to test the functionality of custom probes. Since there are three or ten probes (for NGS or nCounter readout, respectively) per target in custom RNA assays, it is unlikely that a missing or non-functional probe will affect performance, since that probe should be removed as an outlier when collapsing probe counts to target counts during data analysis QC. Still, it is recommended to run appropriate positive/negative control tissues with known expression profiles in a pilot experiment to test functionality of the custom probes and to determine

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nonspecific binding. For all steps, use the signal to noise ratio (SNR) with nCounter readout, the limit of quantification (LOQ) with NGS readout or normalized counts for comparison.

Note: Cell pellet arrays from cell lines known to express the targets of interest or previously characterized samples (e.g. positive and negative samples analyzed by nCounter or RNAseq) can be used to validate and test performance of the custom probes (possible vendor: Acepixbio). Please note, tissues with high protein levels may not be good positive controls as high protein levels may not always correlate with high RNA expression.

When using the custom assay as a spike-in, follow the protocol in the Slide Preparation manual up to *Step 7: In Situ Hybridization (overnight)*. Add the Custom RNA spike-in assay to the master mix as indicated. For custom standalone assays, refer to the following guidance document - GeoMx® Custom RNA Assays for NGS Readout: Guideline for Use as a Standalone Assay.

Note: Use of RNA spike-ins at different concentrations has not been validated. In our experience, changing probe concentration changes counts but not the SNR.

Optimally, target counts will be strongly above the noise counts which will result in a high SNR. If a target is not detected in a control sample known to express that gene, check that the positive control targets were detected. Also check the probe-level counts to see if some probes of the target are performing better than others. If the background counts are higher than in other GeoMx experiments with similar tissue it could result in higher LOQ and lower SNR. If the noise is above standard, check the washing, blocking and the target retrieval steps to minimize non-specific binding of the controls. Low target counts may also result in low SNR. In the next section, we have described a few steps to evaluate the target counts.

Low counts in GeoMx assay

If the target counts are low and close to background counts, it is recommended to look at the counts for the positive control targets, since those should be detectable if the experiment was successful.

If the positive control target counts are close to background, follow the steps below to find the root cause:

• Make sure to use the validated GeoMx protocol (including the recommended target retrieval buffer with the correct pH).

• If using non-validated tissue types, adjust proteinase K (concentration and/or incubation time) and target retrieval (temperature and/or incubation time) if needed. Refer to our RNA slide prep manual for adjustments of proteinase K concentrations or contact geomxsupport@nanostring.com for guidance if the tissue type has not been validated by NanoString.

  • Excess fixation and proteinase K digestion reduces assay counts

  • Insufficient proteinase K digestion and target retrieval reduces assay counts and sensitivity

  • It's strongly recommended to use the same target retrieval conditions for all samples within one project

Note: Proteinase K concentration is not only related to RNA counts but can also affect morphology marker staining. Please include morphology marker staining in the test run, to make sure appropriate staining is obtained to meet the desired ROI selection strategy.

If positive target counts are strongly above background but binding of your custom target(s) is compromised, contact geomxsupport@nanostring.com for further assistance with troubleshooting.

Custom assays contain their own negative probes, so any concentration differences between the custom assay or the Atlas assay that it is spiked into can be handled by using negative probe normalization. The DSP data analysis suite (DSPDA) would calculate a separate LOQ value for the Atlas assay and the custom assay using their negative probes. In order to balance the results between segments within an analysis, normalization with a specific set of probes (e.g. housekeepers) can be performed. Find more information and instructions on normalization strategies in NanoString's data analysis manuals for **nCounter** or **NGS** readouts.

References

1 GeoMx Slide Preparation user manuals for **nCounter** and **NGS** readouts

For more information, please visit nanostring.com

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