

## nCounter<sup>®</sup>

# Expression CodeSet Design Manual

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Molecules That Count<sup>®</sup>

Translational Research • Gene Expression • miRNA Expression • Copy Number Variation

MAN-C0002-03

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## PREFACE

### Conventions Used

The following conventions are used throughout this manual and are described below for your reference:

#### Fonts

Special font formatting is used in this manual. Such formatting conventions are used in specific instances as described below:

- |                |   |   |
|----------------|---|---|
| <b>TIP</b>     |   | Information contained in a Tip may offer helpful suggestions, alternative procedures, methods and/or shortcuts.   |
| <b>NOTE</b>    |   | This note type emphasizes general information.  |
| <b>CAUTION</b> |  | This note type presents essential content indicating that the potential exists for assay failure, diminished data quality, and/or a loss of data if the information presented is ignored.   |
| <b>WARNING</b> |  | This note type indicates that a potential hazard to your personal safety, or the potential for equipment damage exists.   |
| <b>BOLD</b>    |   | When appearing in text or in a procedure, the bold text serves to highlight a specific button, key stroke, or menu option available. <ul style="list-style-type: none"><li>• <b>Bold</b> text may appear elsewhere to highlight important text or terms.</li><li>• <b>Green</b> text is used to help the reader identify active hyperlinks.</li></ul> |
| <b>ITALICS</b> |   | Used to emphasize an important word or expression within the text. <ul style="list-style-type: none"><li>• Formatting of a book title, journal, or other documentation.</li><li>• Used to indicate the special or unusual meaning of a word or phrase.</li></ul>  |

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## Introduction

Nanostring Technologies nCounter expression assays are designed to provide a single-tube, ultra-sensitive, reproducible, and highly multiplexed method for detecting nucleic-acid targets across all levels of biological expression. These assays provide a method for direct detection of targets with molecular barcodes without the use of reverse transcription or amplification. The collection of barcodes specific to a set of identified targets is called a CodeSet. Nanostring customers can request “made-to-order” custom CodeSets due to the unique and flexible technology of the nCounter Analysis System.

Customers can order custom CodeSets in two configurations. These configurations are described below.

- **Gene Expression CodeSets:** CodeSets which can measure up to 800 made-to-order mRNA targets from any species in less than 15-minutes hands on time.
- **miRGE Codesets:** CodeSets which can measure subsets of both miRNA and mRNA targets together in a single tube in less than 1 hour hands on time.

This manual describes in detail the steps of the design process for both custom nCounter Gene Expression and miRGE CodeSets. This manual also provides instructions for the submission of CodeSet Design Forms for the products.

## NanoString® Technology Principles and Procedures

NanoString technology is based on digital detection and direct molecular barcoding of target molecules through the use of a color coded probe pair. The probe pair consists of a Reporter Probe, which carries the signal on its 5' end, and a Capture Probe which carries a biotin moiety on the 3' end. The color codes carry six positions and each position can be one of four colors, thus allowing for a large diversity of tags that can be mixed together in a single well for direct hybridization to targets and yet still be individually resolved and identified during data collection.

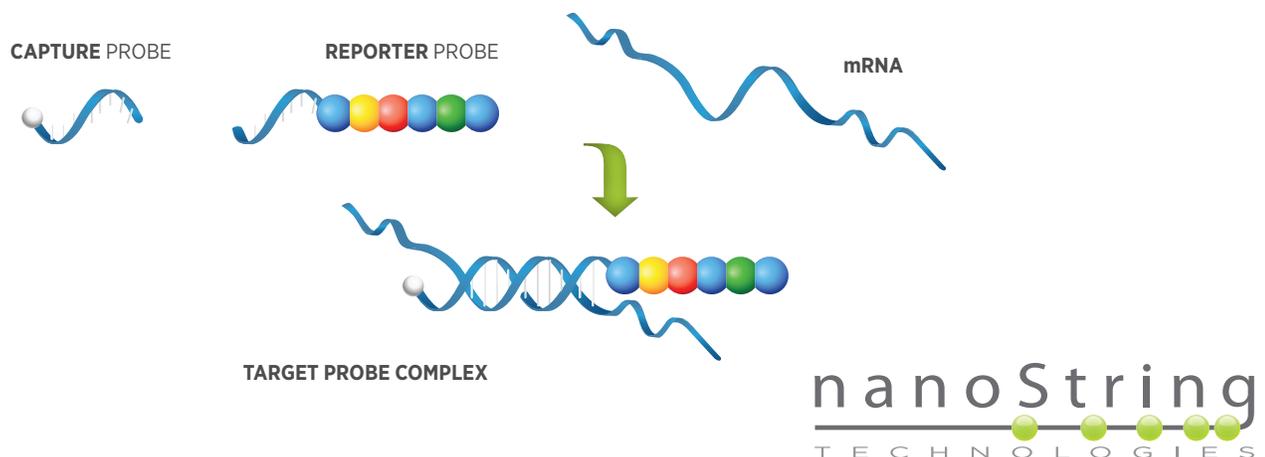
Probe pairs are placed into a reaction in massive excess relative to targets to ensure that each target finds a probe pair. After hybridization, excess probes are washed away using a two step magnetic bead-based purification on the nCounter® Prep Station. Magnetic beads derivatized with short nucleic acid sequences that are complementary to all Capture Probe or all Reporter Probes are used respectively. First, the hybridization mixture is allowed to bind to the magnetic beads by the Capture Probe. Wash steps are performed and excess Reporter Probes and non-target cellular transcripts and other cell debris are removed during wash steps. After washing, the Capture Probes and Target/Probe Complexes are eluted off of the beads and are then hybridized to magnetic beads complementary to the Reporter Probe. Wash steps are performed and excess Capture Probes are washed away. Finally, the purified Target/Probe Complexes are eluted off and are immobilized in the cartridge for data collection.

Data collection is carried out in the nCounter® Digital Analyzer. Fields of view (FOV) are collected per sample using a microscope objective and a CCD camera yielding data of hundreds of thousands of target molecule counts. Digital images are processed on the Digital Analyzer and the barcode counts are then tabulated and displayed.

For more information on specific assay protocols please see:

- **nCounter Gene Expression Assay Manual**
- **nCounter miRGE Assay Manual**

**FIGURE A:** Capture and Reporter Probes and Probe pair bound to an mRNA (right)

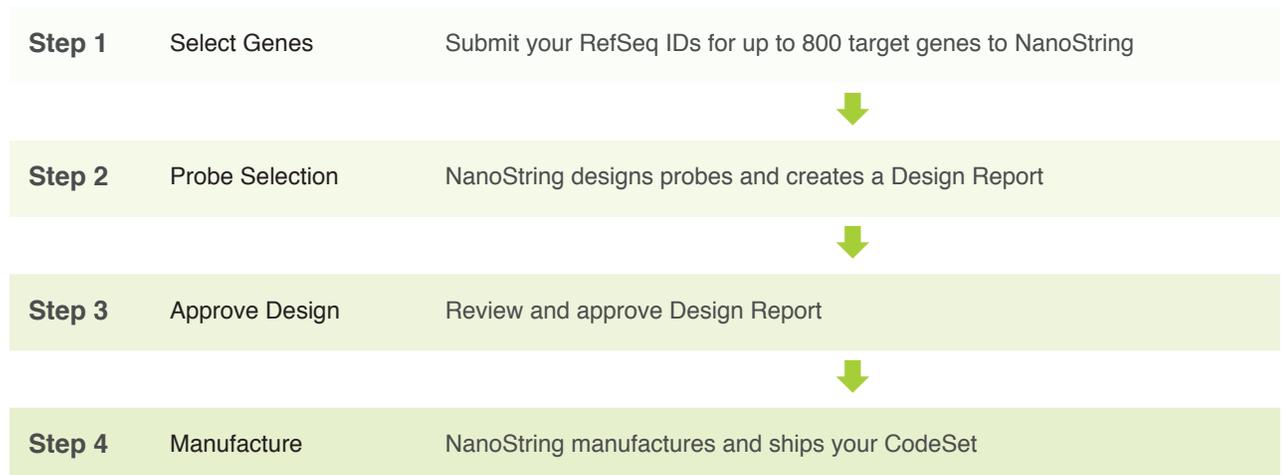


## 1

# nCounter® Gene Expression CodeSet Design Process Overview

The design process for custom Gene Expression CodeSets is completed in four simple steps. First select your list of target genes, and then NanoString selects probes and sends you a design report. After you review and approve the design report, your CodeSet is manufactured and sent to you.

**FIGURE 1.1:** nCounter CodeSet Design Process



## Step 1. Target Sequence Selection

The custom Gene Expression CodeSet design process begins by selecting a list of targets and submitting them to NanoString using the CodeSet Design Form. NanoString prefers the Reference Sequence (RefSeq) database as the source for both the target sequences and the species database. Therefore, to ensure the best possible design, all target sequences should be identified using a RefSeq Accession number. Our design process leverages full length sequences for the RNA molecules that you are interested in detecting. Additionally, to prevent cross hybridization to non-target molecules in the sample, the full non-redundant dataset that represents the sequence information for a species is required. If the target sequences can't be submitted using a RefSeq ID, please contact your Field Application Scientist (FAS) for information on using alternative sequence databases.

## Gene Expression CodeSet Design Form

The **CodeSet Design Form** (CDF) is used to submit your targets. At the top of the form please provide a name for the CodeSet in the field provided. It is important that you chose this name carefully. The CodeSet name that you select will be used in the labels of the CodeSet vials shipped to you. This name will also be used to identify the **Reporter Library File** (RLF). The RLF will be used during image processing to assign target identities to the barcodes.

The CodeSet name should be no longer than eight characters and may only contain alphanumeric characters [A-Z, a-z, 0-9] or underscore characters. Below the **CodeSet Name** field on the Design Form are additional fields for your **Quote Number**, model **Organism** and a field for providing any **Additional Notes** about the design. Following these fields is a table for entering the target identity information. Currently, an nCounter Gene Expression CodeSet can accommodate up to 826 transcripts. Twenty-six transcripts are reserved by the system for positive, negative, binding and purification controls. Please select up to 800 targets and submit them in the table at the bottom of the nCounter CodeSet Design Form (Figure 1.2).

The Table asks you to provide the following information:

- **Target Identifier:** The RefSeq accession number for the nucleic acid sequence of interest. If you do not have a RefSeq accession number for the target sequences please contact your Field Application Scientist.
- **Name:** A common name for your 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 file of tabulated reporter code counts. Names must be less than 16 characters long and contain no spaces. If no name is provided NanoString will use the gene name found in the RefSeq record for that target sequence.
- **Comments:** Any additional information concerning specific transcript variants, gene IDs from other platforms, etc. that may help in the design may be included here. The Comments section is information for the bioinformaticist and will NOT be a part of the experimental data output.
- **FASTA Format:** We can process custom targets submitted in FASTA file format. Please refer to the FAQ section at the end of this guide for instructions.
- **HK Gene?:** Indicate [Y/N] if the gene will be used as a housekeeping or normalization gene.

FIGURE 1.2: nCounter CodeSet Design Form

**nanoString TECHNOLOGIES**  
**nCounter™ CodeSet Design Form**  
 Rev 062911

**Instructions**  
 To submit your CodeSet design, please fill out and email this form to "orders@nanostring.com". Once the form has been received, your Field Application Scientist may contact you for more information about your design and can provide you with a status on your request. The *nCounter CodeSet Design Manual* contains additional information about the ordering process and the criteria used for the design.

For each target of interest (up to 800 targets) provide a row with the following information:

**Target Identifier:** The RefSeq accession number for the nucleic acid sequence of interest. If you do not have a RefSeq accession number for the target, please refer to the *nCounter CodeSet Design Manual* and contact your FAS for further information.

**Name:** A common name for your 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 file of tabulated raw code counts. Names must be less than 16 characters long and contain no spaces.

**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.

**HK Gene?:** When possible, please indicate if this gene will be used as a "housekeeping" or normalization gene. Please see note below about the importance of including reference genes in your CodeSet.

Questions? Please contact your Field Application Scientist or call NanoString at 1-888-358-6266.

**Quality Control**  
 Each CodeSet undergoes extensive customized Quality Control to evaluate reporter quality and background. For an additional charge, further testing is available in which your probes are hybridized against synthetic control targets. In designing and testing over 60,000 probes, NanoString Technologies has demonstrated that >99.96% of probe designs generate positive results for this test. If you are interested in this additional testing, please contact your Field Sales Representative, Field Application Scientist or call NanoString at 1-888-378-6266.

\*Required Information

*CodeSet Name:	
*Quote Number:	
*Organism:	
Additional Notes:	

*Target Identifier	Name	Comments	HK Gene?
Example NM_002871	ITGB2	Please target exon regions 5-7	Y
1			



**CAUTION:** Nanostring will label your CodeSet with the CodeSet name you provided followed by the lot number. If you re-order the CodeSet, your new shipment will be labeled with the same CodeSet name, but a different lot number. We will always ship a USB drive containing a new RLF with each new CodeSet order. It is important that you only use the RLF shipped with the CodeSet. The RLF will have the same name and lot number as the CodeSet vials.

NanoString will, by default, select probe pairs that recognize the maximum possible number of transcript variants of a particular gene. It is possible to design probes that distinguish different isoforms in some cases; in general the analysis of specific transcript variants can become complicated. For more information, see Frequently Asked Questions. If you have any other questions, please contact your Field Application Scientist by calling NanoString at **1-888-358-6266** or [support@nanosttring.com](mailto:support@nanosttring.com).

Please send your completed CodeSet Design Form to [orders@nanosttring.com](mailto:orders@nanosttring.com). Upon receipt of a completed purchase order from your company/institution and the completed nCounter CodeSet Design Form, NanoString will begin the design work.



**CAUTION:** The nCounter Analysis System may include transcripts from the External RNA Controls Consortium (ERCC) as part of an internal control set. Please make sure that your experiments do not include any of the ERCC transcripts specified in the nCounter CodeSet Design Form.

**NOTE:** If the CodeSet Design Form is not completed correctly it may result in a delay in finalizing your design.

## Step 2. Probe Design

Upon receiving the nCounter CodeSet Design Form, NanoString designs probes specific to your target sequences. NanoString selects two target-specific probes for each target sequence; one probe to capture the target and a second Reporter Probe that contains the molecular barcode. Probes are screened for hybridization efficiency, potential for cross hybridization, GC content and predicted secondary structure. After the best scoring probes for your targets are selected, a CodeSet Design Report is sent to you for review. In general, the probe design process takes approximately less than one week from the receipt of your nCounter CodeSet Design Form and PO, but may vary depending on the complexity of the design.

## Step 3. Review and Approve Design Report

Once the design of your CodeSet is complete, you will receive a **CodeSet Design Report** (CDR). The CDR is an Excel workbook containing four worksheets; Definitions, Design Data, Version History and CodeSet Design Approval.

## DESIGN REPORT WORKSHEETS

The first worksheet in the CodeSet Design Report is the Definitions worksheet (Figure 1.3) which contains definitions for each column found in the Design Data worksheet. See *Design Report Content on page 13* for additional information.

FIGURE 1.3: Definitions Worksheet in the CodeSet Design Report

Section 1: Customer Order Info [CUSTOMER_INFO]	
Section 2: Combined CodeSet summary [CODESET_SUMMARY]	
Organism	Name of CodeSet
CodeSet Name:	Version of CodeSet
CodeSet Version:	Quote Number for CodeSet request
CodeSet Quote Number:	Purchase Order number for CodeSet
CodeSet PO Number:	Nanostring Part Number for this version of this CodeSet
CodeSet Part Number:	Number of requested targets
CodeSet Requested Probe Count:	Number of unique sequence targets
CodeSet Confirmed Probe Count:	CodeSet Discrepancy Notes:
CodeSet Ideal Probe Count:	Number of probe pairs passing ideal quality cutoffs
CodeSet cutoff Exceptions:	Summary of non-ideal exceptions in this library:
Xhyb: Tm: GC: Structural:	Xhyb = Cross hybridization potential
(some probes may be in multiple categorie	Tm = out of range melting temp
	GC = GC content ratio out of range
	Structural = secondary structure interference potential
Section 3: Transcript Variant Coverage columns: [TRANSCRIPT_VARIANT_DATA]	
1 - Target Accession:	Accession identifier for the mRNA target sequence.
2 - Gene:	Common name for mRNA target sequence, as defined by RefSeq.
3 - Number of Transcript Variants Covered:	Count of all known transcript variants that the probe will hit.
4 - Number of Known Transcript Variants:	Count of all known transcript variants for this gene.
5 - Accession for Non-Target TV's being hit:	List of accessions of transcript variants of requested target, excluding target accession.
Section 4: Detailed probe information columns: [CODESET_DETAILS]	
1 - Customer Identifiers:	Default is RefSeq Gene name, otherwise specified by customer
2 - Accession number:	Accession number of requested target sequence.
3 - Target Region in accession:	Region in the target mRNA being probed.
4 - Target Sequence:	Sequence of target region.
5 - Tm of capture probe IF below or above c	For probes with a Tm out of range, calculated Tm's are posted.
6 - Tm of reporter probe IF below or above c	For probes with a Tm out of range, calculated Tm's are posted.
7 - Exception Flags	
[T]	Tm out of range
[X]	Cross hyb signal expected
[V]	Variant Specific
[S]	Structural issues (GC%, hairpins, poly-G etc)
[C]	Custom Sequence
8 - Gene:	Common name for mRNA target sequence, as defined by RefSeq.
9 - Part Numbers:	NanoString part number for this probe pair.
10 - Nanostring Internal Identifiers	
11 - Comments	

The second worksheet in the Design Report is the Design Data worksheet (Figure 1.4) which contains data specific to your CodeSet design. Please review your design information carefully. It is critical that the sequences indicated on the form correspond to the sequences you wish to target. If you have any questions please contact your Field Application Scientist or e-mail [support@nanosttring.com](mailto:support@nanosttring.com). Once you have reviewed and accept your CodeSet design, you will be asked to sign the CodeSet Design Approval form (Figure 1.5), the third worksheet in the Design Report, and e-mail or fax your approval to NanoString (e-mail to [CDR@nanosttring.com](mailto:CDR@nanosttring.com) or fax to **1-206-378-6288**). Your approval authorizes NanoString to proceed with the synthesis of your CodeSet. The CodeSet will be invoiced at the time of shipment. Upon receipt of your signed approval by NanoString, changes cannot be accepted to the CodeSet design.

**NOTE:** It is very important that you review and approve the nCounter CodeSet Design Report (CDR) in a timely manner. A delay in the receipt of this authorization may lead to delays in the final design, manufacturing and shipping of your CodeSet.

FIGURE 1.4: Example Design Data Worksheet in the CodeSet Design Report

 nCounter™ CodeSet Design Report - Data										
<b>CUSTOMER INFORMATION</b>										
Customer Name:	Virtual Panels Team									
Customer Institute:	NanoString									
<b>CODESET SUMMARY</b>										
CodeSet Name:	NS_inf_m									
CodeSet Report Version:	1									
CodeSet Design Report Date:	2/16/10									
CodeSet Quote #:										
CodeSet PO:										
CodeSet requested probe count:	185									
CodeSet confirmed probe count:	185									
CodeSet notes:										
CodeSet Tier1 pass:	179									
CodeSet Probe Exceptions:	Xhyb	TM	GC	Structure						
Exception Count:	6	1	0	0						
<b>TRANSCRIPT VARIANT DATA</b>										
Target Accession	Gene	TVs Hit	Other TVs giving signal							
NM_001025093.1.3995	Atf2	2	NM_009715.2							
NM_144938.2.2490	C1s	2	NM_001097617.1							
XM_356827.6.215	C7	2	XM_904680.2							
NM_011124.4.170	Ccl21b	2	NM_011335.1							
NM_013654.2.215	Ccl7	2	NM_021878.2							
NM_011611.2.1425	Cd40	6	NM_001013812.1;NM_170701.2;NM_170703.2;NM_170704.2;NM_170702.2							
NM_008198.2.1685	C1b	2	NM_001142706.1							
<b>Customer Identifier</b>	<b>Accession</b>	<b>Targeted Ref</b>	<b>Target Seg</b>	<b>Tm</b>	<b>RP</b>	<b>CP</b>	<b>Flags</b>	<b>Gene</b>	<b>PN(CP:RP)</b>	<b>NSID</b>
Atf2	NM_001025093.1	3995-4095	GCAATTGT	83	81			Atf2	325616;225616	NM_001025093.1.3995
Bcl6	NM_009744.3	185-285	ACGTTGTC	82	83			Bcl6	308690;208690	NM_009744.3.185
C1qa	NM_007572.2	566-666	CAGTGCC	82	82			C1qa	314196;214196	NM_007572.2.566
C1qb	NM_009777.2	865-965	GTGCCAAC	81	82			C1qb	325637;225637	NM_009777.2.865
C1r	NM_023143.3	64-164	CCCTCCAC	85	86			C1r	325663;225663	NM_023143.3.64
C1s	NM_144938.2	2490-2590	GGCAGGG	79	80	X		C1s	325684;225684	NM_144938.2.2490
C2	NM_013484.2	2359-2459	ATGGTGTC	79	81			C2	315139;215139	NM_013484.2.2359

**NOTE:** On rare occasions, an approved probe sequence may need to be redesigned. You may authorize NanoString to alter probe sequences as necessary for a specific target to facilitate CodeSet synthesis. If you opt to review any probe sequence changes (see *CodeSet Authorization, below*), it may lengthen the time line for CodeSet delivery.

FIGURE 1.5: CodeSet Design Approval Worksheet in the CodeSet Design Report

	
<h2>nCounter™ CodeSet Authorization Form</h2>	
CodeSet Name: CodeSet Version: Quote Number: Customer Institute:	
<b>Instructions:</b>	
Please fax to 206-378-6288 or email a scan of your approval to <a href="mailto:cdr@nanosttring.com">cdr@nanosttring.com</a> . Thank you.	
I acknowledge that I have reviewed and accept the CodeSet design as specified in this CodeSet Design Report, and I am authorized to approve this design. I understand that after this document has been signed and received by NanoString Technologies, I cannot request changes to this CodeSet; this CodeSet will be synthesized as specified and invoiced upon shipment.	
<b>Normalization / Housekeeping Genes:</b>	
<input type="checkbox"/> By checking this box, I confirm that I have included Normalization / Housekeeping targets in my gene list and that these are represented in the CodeSet design contained in this report.	
<b>Optional:</b>	
<input type="checkbox"/> By checking this box, I authorize NanoString Technologies to select an alternate probe sequence in the event that a redesign is necessary due to unforeseen QC issues. NanoString Technologies will select a probe of equal quality as a replacement. Selecting this option will ensure prompt delivery of your CodeSet if a redesign is required.	
<b>Authorization:</b>	
Printed Name	Date
Title	
Signature	
Please fax to 206-378-6288 or email a scan of your approval to <a href="mailto:cdr@nanosttring.com">cdr@nanosttring.com</a> . Thank you.	

## DESIGN REPORT CONTENT

The following information is contained in the Design Report:

### Section 1: Customer Order Information

### Section 2: CodeSet Design Summary

1. **Organism:** Species source for target sequences
2. **CodeSet Name:** Name of CodeSet
3. **CodeSet Version:** Version of CodeSet
4. **CodeSet Quote Number:** Quote Number for CodeSet Request
5. **CodeSet PO Number:** Purchase Order Number for CodeSet
6. **CodeSet Part Number:** NanoString Part Number for this version of the CodeSet
7. **CodeSet Requested Probe Count:** Number of requested targets
8. **CodeSet Confirmed Probe Count:** Number of unique probe pairs generated by NanoString's probe selection software
9. **CodeSet Notes:** Important additional information specific to your CodeSet
10. **CodeSet Ideal Probe Count:** Number of probe pairs passing ideal quality cutoffs
11. **CodeSet Cutoff Exceptions:** Summary of non-ideal exceptions in this CodeSet
  - **Xhyb:** cross hybridization potential
  - **T<sub>m</sub>:** out of range melting temp
  - **GC:** GC content out of range
  - **Structural:** secondary structure interference potential

### Section 3: Transcript Variant Coverage Column

1. **Target Accession:** Accession identifier for the mRNA target sequence
2. **Gene:** Common name for mRNA target sequence, as defined by RefSeq
3. **Number of Transcript Variants Covered:** Count of all known transcript variants to which probe pairs will bind
4. **Number of Known Transcript Variants:** Count of all known transcript variants for that gene
5. **Accession for Non Target Transcript Variants:** List of accession numbers of transcript variants of requested target, excluding target accession

### Section 4: Detailed Probe Information Columns

1. **Customer Identifiers:** Specified by customer, otherwise RefSeq gene name
2. **Accession Number:** Accession number of a requested target sequence
3. **Target Region in accession:** Region in the target mRNA to which probes bind
4. **Target Sequence:** Sequence of target region
5. **T<sub>m</sub> of capture probe,** calculated T<sub>m</sub>(s) are listed
6. **T<sub>m</sub> of reporter probe,** calculated T<sub>m</sub>(s) are listed
7. **Exception Flags**
  - a. **[T]:** T<sub>m</sub> out of range
  - b. **[X]:** Cross hybridization signal expected
  - c. **[V]:** Variant specific
  - d. **[S]:** Structural issues (%GC, hairpins, poly-G, etc.)
  - e. **[C]:** Custom Sequence

- 8. Gene:** Common Name for mRNA target sequence, as defined by RefSeq
- 9. Part Numbers:** NanoString part number for this probe pair
- 10. NanoString internal identifiers:** Identifiers used by NanoString to track and identify target source, target region, and target sequence. The format is:  
**[accession number].[version]:[target\_region coordinate].**
- 11. Comments:** Comments from NanoString to Customer: Technical notes regarding any peculiarities in the design or selection of that probe pair. If you have any questions, contact your Field Applications Scientist. Once you have reviewed and accept your design data, e-mail your approval of the design to [CDR@nanosttring.com](mailto:CDR@nanosttring.com).

## Step 4. NanoString Manufactures Your CodeSet

Once NanoString has received your signed approval on the Design Report, manufacturing of your nCounter CodeSet commences. Nanostring will ship the CodeSet along with a USB drive containing the corresponding RLF once probe synthesis and quality testing is complete. The duration of this process varies depending on the scale (number of assays) ordered and the number of probes in the CodeSet.

### Reference Documents

There are several reference documents available for your use in processing samples and analyzing data once you receive your nCounter CodeSet. The following documents are available online at [www.nanosttring.com](http://www.nanosttring.com) in the Products section.

- nCounter® Prep Station User Manual
- nCounter® Digital Analyzer User Manual
- nCounter® Gene Expression Assay Manual (Total RNA and Cell Lysate Protocols)
- nCounter® Expression Data Analysis Guide

Your feedback is welcome. If you have suggestions about the materials provided or the process for designing and ordering your nCounter CodeSet, please feel free to e-mail us at [support@nanosttring.com](mailto:support@nanosttring.com).

## 2

## nCounter® miRGE CodeSet Design Process Overview

The design process for custom miRGE CodeSets is similar to that of the Gene Expression Codeset but includes an additional step which ensures optimal design of the assay and experiments. First, select your list of target mRNAs and miRNAs. Once this list has been submitted, Nanostring will request a brief design team meeting between you and our design team to ensure optimal CodeSet and experimental design. After this meeting, NanoString will then select probes and send you a design report. After you review and approve the design report, your CodeSet will be manufactured and sent to you.

**FIGURE 2.1:** nCounter miRGE CodeSet Design Process



## Step 1. Target Sequence Selection

The CodeSet design process begins by selecting a list of mRNA and miRNA targets and submitting them to NanoString using the miRGE CodeSet Design Form. The miRGE Assay allow researchers to order both mRNA and miRNA targets, sources for target sequence selection differ slightly between these two targets types and are described in the sections below.

### miRNA Targets

#### Sequence Selection

For miRNA targets, NanoString utilizes miRBase accession numbers as the identifier for miRNA sequences. The list of desired targets should be chosen from the worksheets provided in the miRGE CodeSet Design Form and must be of the same species as your mRNA targets. If you wish to investigate a miRNA target that is not included in this worksheet, please contact your Field Application Scientist for more information on how to pursue this request.

### mRNA Target

#### Sequence Selection

For mRNA targets, NanoString utilizes the Reference Sequence (RefSeq) database as the source for both the target mRNA sequences and the species database. Therefore, to ensure the best possible design all mRNA target sequences should be identified using a RefSeq Accession number. Our design process for mRNA targets leverages full length sequences for the RNA molecules that you are interested in detecting.

Additionally, to prevent or predict any potential cross hybridization to non-target endogenous mRNA molecules in the sample, the full non-redundant dataset that represents the sequence information for a species is required.



**CAUTION:** All miRNA and mRNA targets must be from the same species.

### miRGE CodeSet Design Form

The miRGE CodeSet Design Form is used to submit your targets to NanoString. The following information describes each field of information required for form submission.

**CodeSet Name:** It is important that you chose the name of your CodeSet carefully. The CodeSet name that you select will be used in the labels of the CodeSet vials shipped to you. This name will also be used to identify the Reporter Library File (RLF). The RLF will be used during image processing to assign target identities to the barcodes.

The CodeSet name should be no longer than eight characters and may only contain alphanumeric characters or underscore characters.

**Quote Number:** Please provide the quote number you received from your Regional Account Manager, if available.

**Organism:** Please provide the name of your model organism.

**Additional Notes:** Any additional information that may help in the design may be included here. The Comments section is information for the Nanostring design team and will NOT be a part of the experimental data output.

Following these fields is a table for entering the miRNA target identity information. Currently, an nCounter Combined CodeSet can accommodate between 5 and 30 miRNA transcripts.

Please select up to 30 miRNA targets from any one of the miRNA species worksheets provided and submit them in the miRNA Target table in the nCounter miRGE CodeSet Design Form (Figure 2.2).

**FIGURE 2.2:** Enter requested miRNA targets into the miRNA Target Table.

miRNA Targets			
	*miRNA Accession	Official Symbol	Target Sequence
1	MIMAT0000774	rn0-let-7a	UGAGGUAGUAGGUUGUAUAGUU
2			

The miRNA Target Table asks you to provide the following information:

- **miRNA Accession Number:** The miRBase accession number from the provided miRNA worksheet for either Human, Mouse or Rat. If the miRBase accession number for the target miRNA sequences of interest is not contained in these worksheets please contact your Field Application Scientist.
- **Official Symbol:** The official symbol for your target of interest from the provided miRNA worksheet for either Human, Mouse or Rat.
- **Target Sequence:** The target sequence for your target of interest from the provided miRNA worksheet for either Human, Mouse or Rat.

Following the fields for miRNA targets is a table for entering the mRNA target identity information. Currently, an nCounter miRGE CodeSet can accommodate between 100 and 200 mRNA transcripts. Please enter at least 100 and up to 200 targets and submit them in the mRNA Target table in the nCounter miRGE CodeSet Design Form (Figure 2.3).

**FIGURE 2.3:** Enter requested mRNA targets into the mRNA Target Table.

mRNA Targets				
	*Target Identifier	Name	Comments	HK Gene?
Example	NM_000211	ITGB2	Please target exon regions 5-7	Y
1				



**CAUTION:** All miRNA and mRNA targets must be from the same species.

The mRNA Target table asks you to provide the following information:

- **Target Identifier:** The RefSeq accession number for the nucleic acid sequence of interest. If you do not have a RefSeq accession number for the target sequences please contact your Field Application Scientist.
- **Name:** A common name for your 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 file of tabulated raw code counts. Names must be less than 16 characters long and contain no spaces. If no name is provided NanoString will use the gene name found in the RefSeq record for that target sequence.
- **Comments:** Any additional information concerning specific transcript variants, gene IDs from other platforms, etc. that may help in the design may be included here. The Comments section is information for the bioinformaticist and will NOT be a part of the experimental data output.
- **HK Gene?:** This field ask you to identify if the target selected is going to be used as a housekeeping gene in your experimental design.



**CAUTION:** Nanostring will label your CodeSet with the CodeSet name you provided with the prefix 'miX\_' and followed by the lot number. If you re-order the CodeSet, your new shipment will be labeled with the same CodeSet name, but a different lot number. We will always ship a USB drive containing a new RLF with each new CodeSet order. It is important that you only use the RLF shipped with the CodeSet. The RLF will have the same name and lot number as the CodeSet vials.

## Step 2. Design Team Meeting

Upon receiving your nCounter miRGE CodeSet Design Form, NanoString will schedule a brief design meeting with you to ensure the highest quality experimental and assay design of your miRGE CodeSet. This meeting is intended to be used to discuss any potential technical complexities that may arise out of your selected targets.

## Step 3. Probe Design

Once the design team meeting is complete, NanoString performs the design for your CodeSet. MicroRNA probes are selected from Nanostring's pre-designed miRNA catalog. For mRNA targets, NanoString selects two target-specific probes for each target sequence; one probe to capture the target and a second Reporter Probe that contains the molecular barcode. Probes are screened for hybridization efficiency, potential for cross hybridization, GC content and predicted secondary structure. After the best scoring probes for your mRNA targets are selected, a CodeSet Design Report is sent to you for review and approval. In general, the probe design process takes approximately one week from the receipt of your nCounter miRGE CodeSet Design Form and PO, but may vary depending on the complexity of the design and the schedule availability for the Design Team Meeting.

## Step 4. Review and Approve Design Report

Once the design of your CodeSet is complete, you will receive a CodeSet Design Report. The design report is an Excel workbook containing four worksheets; Definitions, Design Data, Version History and CodeSet Design Approval.

## DESIGN REPORT WORKSHEETS

The first worksheet in the CodeSet Design Report is the Definitions worksheet (*Figure 2.4*) which contains definitions for each column found in the Design Data worksheet. See Design Report Content on page 23 for additional information.

FIGURE 2.4: Definitions Worksheet in the CodeSet Design Report

	
<b>nCounter™ CodeSet Design Report - Definitions</b>	
<b>Section 1: Customer Order Info</b>	
<b>[CUSTOMER_INFO]</b>	
<b>Section 2: Combined CodeSet summary</b>	
<b>[CODESET_SUMMARY]</b>	
Organism	
CodeSet Name:	Name of CodeSet
CodeSet Version:	Version of CodeSet
CodeSet Quote Number:	Quote Number for CodeSet request
CodeSet PO Number:	Purchase Order number for CodeSet
CodeSet Part Number	Nanostring Part Number for this version of this CodeSet
CodeSet Requested Probe Count:	Number of requested targets
CodeSet Confirmed Probe Count:	Number of unique sequence targets
CodeSet Discrepancy Notes:	
CodeSet Ideal Probe Count:	Number of probe pairs passing ideal quality cutoffs
CodeSet cutoff Exceptions	Summary of non-ideal exceptions in this library:
	Xhyb = Cross hybridization potential
	Tm = out of range melting temp
	GC = GC content ratio out of range
	Structural = secondary structure interference potential
Xhyb: Tm: GC: Structural: (some probes may be in multiple categorie	
<b>Section 3: Transcript Variant Coverage columns:</b>	
<b>[TRANSCRIPT_VARIANT_DATA]</b>	
1 - Target Accession:	Accession identifier for the mRNA target sequence.
2 - Gene:	Common name for mRNA target sequence, as defined by RefSeq.
3 - Number of Transcript Variants Covered:	Count of all known transcript variants that the probe will hit.
4 - Number of Known Transcript Variants:	Count of all known transcript variants for this gene.
5 - Accession for Non-Target TV's being hit	List of accessions of transcript variants of requested target, excluding target accession.
<b>Section 4: Detailed probe information columns:</b>	
<b>[CODESET_DETAILS]</b>	
1 - Customer Identifiers:	Default is RefSeq Gene name, otherwise specified by customer
2 - Accession number:	Accession number of requested target sequence.
3 - Target Region in accession:	Region in the target mRNA being probed.
4 - Target Sequence:	Sequence of target region.
5 - Tm of capture probe IF below or above c	For probes with a Tm out of range, calculated Tm's are posted.
6 - Tm of reporter probe IF below or above c	For probes with a Tm out of range, calculated Tm's are posted.
7 - Exception Flags	
[T]	Tm out of range
[X]	Cross hyb signal expected
[V]	Variant Specific
[S]	Structural issues (GC%, hairpins, poly-G etc)
[C]	Custom Sequence
8 - Gene:	Common name for mRNA target sequence, as defined by RefSeq.
9 - Part Numbers:	NanoString part number for this probe pair.
10 - Nanostring Internal Identifiers	
11 - Comments	

The second worksheet in the CodeSet Design Report is the Design Data worksheet (Figure 2.5) which contains data specific to your CodeSet design. Please review your design information carefully. It is critical that the sequences indicated on the form correspond to the sequences you wish to target.

FIGURE 2.5: Example Design Data Worksheet in the CodeSet Design Report

 nCounter™ CodeSet Design Report - Data											
<b>CUSTOMER INFORMATION</b>											
Customer Name:	Virtual Panels Team										
Customer Institute:	NanoString										
<b>CODESET SUMMARY</b>											
CodeSet Name:	NS_Infl_m										
CodeSet Report Version:	1										
CodeSet Design Report Date	2/16/10										
CodeSet Quote #:											
CodeSet PO:											
CodeSet requested probe count:	185										
CodeSet confirmed probe count:	185										
CodeSet notes:											
CodeSet Tier1 pass:	179										
CodeSet Probe Exceptions:	Xhyb	TM	GC	Structure							
Exception Count:	6	1	0	0							
<b>TRANSCRIPT VARIANT DATA</b>											
Target Accession	Gene	TVs Hit	Other TVs giving signal								
NM_001025093.1:3995	Atf2	2	NM_009715.2								
NM_144938.2:2490	C1s	2	NM_001097617.1								
XM_356827.6:215	C7	2	XM_904680.2								
NM_011124.4:170	Ccl21b	2	NM_011335.1								
NM_013654.2:215	Ccl7	2	NM_021878.2								
NM_011611.2:1425	Cd40	6	NM_001013812.1;NM_170701.2;NM_170703.2;NM_170704.2;NM_170702.2								
NM_008198.2:1685	Cfb	2	NM_001142706.1								
<b>Customer Identifier</b>	<b>Accession</b>	<b>Targeted Ref</b>	<b>Target Seq</b>	<b>Tm</b>	<b>CP</b>	<b>Tm</b>	<b>RP</b>	<b>Flags</b>	<b>Gene</b>	<b>PN(CP;RP)</b>	<b>NSID</b>
Atf2	NM_001025093.1	3995-4095	GCAATTGT	83		81			Atf2	325616;225616	NM_001025093.1:3995
Bcl6	NM_009744.3	185-285	ACGTTGTC	82		83			Bcl6	308690;208690	NM_009744.3:185
C1qa	NM_007572.2	566-666	CAGTGCC	82		82			C1qa	314196;214196	NM_007572.2:566
C1qb	NM_009777.2	865-965	GTGCCAA	81		82			C1qb	325637;225637	NM_009777.2:865
C1r	NM_023143.3	64-164	CCCTCCAC	85		86			C1r	325663;225663	NM_023143.3:64
C1s	NM_144938.2	2490-2590	GGCAGGG	79		80	X		C1s	325684;225684	NM_144938.2:2490
C2	NM_013484.2	2359-2459	ATGGTGTC	79		81			C2	315139;215139	NM_013484.2:2359

Once you have reviewed and accept your CodeSet design, you will be asked to sign the CodeSet Design Approval form (Figure 2.6), the third worksheet in the CodeSet Design Report, and e-mail or fax your approval to NanoString (e-mail to [CDR@nanosttring.com](mailto:CDR@nanosttring.com) or fax to **1-206-378-6288**). Your approval authorizes NanoString to proceed with the synthesis of your CodeSet. The CodeSet will be invoiced at the time of shipment. Upon receipt of your signed approval by NanoString, changes cannot be accepted to the CodeSet design.

**NOTE:** It is very important that you review and approve the nCounter CodeSet Design Report (CDR) in a timely manner. A delay in the receipt of this authorization may lead to delays in the final design, manufacturing and shipping of your CodeSet.

**NOTE:** On rare occasions, an approved mRNA probe sequence may need to be redesigned. You may authorize NanoString to alter probe sequences as necessary for a specific target to facilitate CodeSet synthesis. If you opt to review any probe sequence changes (see CodeSet Authorization, below), it may lengthen the time line for CodeSet delivery.

FIGURE 2.5: Design Data Worksheet in the CodeSet Design Report

 nCounter™ CodeSet Design Report - Data											
<b>CUSTOMER INFORMATION</b>											
Customer Name:	Virtual Panels Team										
Customer Institute:	NanoString										
<b>CODESET SUMMARY</b>											
CodeSet Name:	NS_Infl_m										
CodeSet Report Version:	1										
CodeSet Design Report Date	2/16/10										
CodeSet Quote #:											
CodeSet PO:											
CodeSet requested probe count:	185										
CodeSet confirmed probe count:	185										
CodeSet notes:											
CodeSet Tier1 pass:	179										
CodeSet Probe Exceptions:	Xhyb	TM	GC	Structure							
Exception Count:	6	1	0	0							
<b>TRANSCRIPT VARIANT DATA</b>											
Target Accession	Gene	TVs Hit	Other TVs giving signal								
NM_001025093.1:3995	Atf2		2 NM_009715.2								
NM_144938.2:2490	C1s		2 NM_001097617.1								
XM_356827.6:215	C7		2 XM_904680.2								
NM_011124.4:170	Ccl21b		2 NM_011335.1								
NM_013654.2:215	Ccl7		2 NM_021878.2								
NM_011611.2:1425	Cd40		6 NM_001013812.1;NM_170701.2;NM_170703.2;NM_170704.2;NM_170702.2								
NM_008198.2:1685	Cfb		2 NM_001142706.1								
Customer Identifier	Accession	Targeted Ref	Target Seg	Tm	CP	Tm	RP	Flags	Gene	PN(CP;RP)	NSID
Atf2	NM_001025093.1	3995-4095	GCAATTGT	83		81			Atf2	325616;225616	NM_001025093.1:3995
Bcl6	NM_009744.3	185-285	ACGTTGTC	82		83			Bcl6	308690;208690	NM_009744.3:185
C1qa	NM_007572.2	566-666	CAGTGCC	82		82			C1qa	314196;214196	NM_007572.2:566
C1qb	NM_009777.2	865-965	GTGCCAA	81		82			C1qb	325637;225637	NM_009777.2:865
C1r	NM_023143.3	64-164	CCCTCCAC	85		86			C1r	325663;225663	NM_023143.3:64
C1s	NM_144938.2	2490-2590	GGCAGGG	79		80	X;		C1s	325684;225684	NM_144938.2:2490
C2	NM_013484.2	2359-2459	ATGGTGTC	79		81			C2	315139;215139	NM_013484.2:2359

FIGURE 2.6: CodeSet Design Approval Worksheet in the CodeSet Design Report

	
<h2>nCounter™ CodeSet Authorization Form</h2>	
CodeSet Name: CodeSet Version: Quote Number: Customer Institute:	
<b>Instructions:</b>	
Please fax to 206-378-6288 or email a scan of your approval to <a href="mailto:cdr@nanosttring.com">cdr@nanosttring.com</a> . Thank you.	
I acknowledge that I have reviewed and accept the CodeSet design as specified in this CodeSet Design Report, and I am authorized to approve this design. I understand that after this document has been signed and received by NanoString Technologies, I cannot request changes to this CodeSet; this CodeSet will be synthesized as specified and invoiced upon shipment.	
<b>Normalization / Housekeeping Genes:</b>	
<input type="checkbox"/>	By checking this box, I confirm that I have included Normalization / Housekeeping targets in my gene list and that these are represented in the CodeSet design contained in this report.
<b>Design Team Meeting Confirmation:</b>	
<input type="checkbox"/>	By checking this box, I understand that some probes in my design may cause unforeseen saturation and/or background issues. I confirm that these issues have been discussed with me and that I understand Nanostring Technologies's recommended mitigation strategies. I acknowledge that Nanostring Technologies is not responsible should I chose not to follow these recommendations.
<b>Optional:</b>	
<input type="checkbox"/>	By checking this box, I authorize NanoString Technologies to select an alternate probe sequence in the event that a redesign is necessary due to unforeseen QC issues. NanoString Technologies will select a probe of equal quality as a replacement. Selecting this option will ensure prompt delivery of your CodeSet if a redesign is required.
<b>Authorization:</b>	
_____ Printed Name <span style="float: right;">Date</span>	
_____ Title	
_____ Signature	
Please fax to 206-378-6288 or email a scan of your approval to <a href="mailto:cdr@nanosttring.com">cdr@nanosttring.com</a> . Thank you.	

## DESIGN REPORT CONTENT

The following information is contained in the Design Report:

### Section 1: Customer Order Information

### Section 2: CodeSet Design Summary

1. **Organism:** Species source for target sequences
2. **CodeSet Name:** Name of CodeSet
3. **CodeSet Version:** Version of CodeSet
4. **CodeSet Quote Number:** Quote Number for CodeSet Request
5. **CodeSet PO Number:** Purchase Order Number for CodeSet
6. **CodeSet Part Number:** NanoString Part Number for this version of the CodeSet
7. **CodeSet Requested Probe Count:** Number of requested targets
8. **CodeSet Confirmed Probe Count:** Number of unique probe pairs generated by NanoString's probe selection software
9. **CodeSet Notes:** Important additional information specific to your CodeSet
10. **CodeSet Ideal Probe Count:** Number of probe pairs passing ideal quality cutoffs
11. **CodeSet Cutoff Exceptions:** Summary of non-ideal exceptions in this CodeSet
  - **Xhyb:** cross hybridization potential
  - **T<sub>m</sub>:** out of range melting temp
  - **GC:** GC content out of range
  - **Structural:** secondary structure interference potential

### Section 3: Transcript Variant Coverage Column

1. **Target Accession:** Accession identifier for the mRNA target sequence
2. **Gene:** Common name for mRNA target sequence, as defined by RefSeq
3. **Number of Transcript Variants Covered:** Count of all known transcript variants to which probe pairs will bind
4. **Number of Known Transcript Variants:** Count of all known transcript variants for that gene
5. **Accession for Non Target Transcript Variants:** List of accession numbers of transcript variants of requested target, excluding target accession

### Section 4a: Detailed Probe Information miRNA Columns

1. **miRNA Name:** Common Name for miRNA species targeted by probe
2. **Accession:** miRBase accession number for the mature miRNA species targeted by probe
3. **miRNA Sequence:** Sequence of the mature miRNA targeted by probe
4. **NSID:** Internal unique identifier used by NanoString to track and identify probes

### Section 4b: Detailed Probe Information mRNA Columns

1. **Customer Identifiers:** Specified by customer, otherwise RefSeq gene name
2. **Accession Number:** Accession number of a requested target sequence
3. **Target Region in accession:** Region in the target mRNA to which probes bind
4. **Target Sequence:** Sequence of target region
5. **T<sub>m</sub> of capture probe IF below or above cutoff:** Probes with a T<sub>m</sub> out of range, calculated T<sub>m</sub>(s) are listed
6. **T<sub>m</sub> of reporter probe If below or above cutoff:** Probes with a T<sub>m</sub> out of range, calculated T<sub>m</sub>(s) are listed

**7. Exception Flags**

- a. **[T]:**  $T_m$  out of range
- b. **[X]:** Cross hybridization signal expected
- c. **[V]:** Variant specific
- d. **[S]:** Structural issues (%GC, hairpins, poly-G, etc.)
- e. **[C]:** Custom Sequence

**8. Gene:** Common Name for mRNA target sequence, as defined by RefSeq

**9. Part Numbers:** NanoString part number for this probe pair

**10. NanoString internal identifiers:** Identifiers used by NanoString to track and identify target source, target region, and target sequence. The format is:

**[accession number].[version]:[target\_region coordinate].**

**11. Comments:** Comments from NanoString to Customer: Technical notes regarding any peculiarities in the design or selection of that probe pair.

If you have any questions, contact your Field Applications Scientist. Once you have reviewed and accept your design data, e-mail your approval of the design to [CDR@nanosttring.com](mailto:CDR@nanosttring.com).

## Step 5. NanoString Manufactures Your CodeSet

Once NanoString has received your signed approval on the Design Report, manufacturing of your nCounter CodeSet commences. Nanostring will ship the CodeSet along with a USB drive containing the corresponding RLF once probe synthesis and quality testing is complete. The duration of this process varies depending on the complexity of the project and the number of probes in the CodeSet.

## Frequently Asked Questions

### 1. Can I submit GenBank® Accession Numbers instead of RefSeq accession numbers?

We request that you find a RefSeq entry that best represents your GenBank sequence to avoid ambiguities regarding biological context of that sequence. Please contact your FAS if you need help finding the appropriate RefSeq for your sequence.

### 2. What is the difference between the GenBank, UniGene, and RefSeq sequence databases?

GenBank is a general biological sequence repository at NCBI. Although record submission is open to all users, only the submitter is allowed to edit the deposited sequence. Thus, GenBank records do not undergo any organized curation process and vary in quality. Many sequences are redundant, or contain unresolved nucleotide designations, have out of date annotations, or are simply incorrect. Unigene is a database storing clusters of related GenBank sequences. The clusters are generated through an automated process, and contain many types of sequence (cDNA, mRNA, ESTs, etc.) that represent a region predicted to be a gene. For organisms with well annotated genomes such as human and mouse, most Unigene entries will contain a Reference Sequence as a representative for the cluster. This is often used as a tool to find the best RefSeq equivalent for a given GenBank sequence.

The Reference Sequence Database (RefSeq) is a curated, non-redundant database. Sequences in this database are consensus sequences derived from numerous high quality independent sources and are considered standards. The Reference Sequence project standardizes sequence data by merging, correcting, annotating and filtering GenBank data, and using a well defined and consistent method of annotation.

### 3. Why do you require RefSeq identifiers?

NanoString's custom probe design and selection software automatically extracts sequences deposited in the RefSeq database. RefSeq sequences were chosen because it is an authoritative standard that contains useful information in addition to sequence information. This includes information about annotation, transcript variation, gene family, non-redundancy, record tracking across database releases and annotations. Moreover, these sequences are in the public domain and are readily accessible through the NCBI website.

### 4. How do I request probes for my CodeSet that will best represent my microarray probes or probe sets?

NanoString's technology is substantially different from microarray technologies. Therefore, optimal target regions for an nCounter CodeSet probe pair may not correlate with regions that work well for probes on a different platform. To facilitate the design of optimally performing probes, we recommend looking up the corresponding RefSeq for the target sequence of interest from your microarray platform. For example, if you are using the Affymetrix platform, the RefSeq accession can be found on the NetAFFX ([www.netaffx.com](http://www.netaffx.com)) website for your probe sets. Please note, NanoString has no affiliation with third party sources of sequence data and annotation and we will assume the accuracy of any annotation has been confirmed by the customer prior to submission of sequence data.

### 5. What if my target sequence is not in the RefSeq database?

Probes can be designed to sequences that are not in the RefSeq database. In this case, probe selection will involve a significant amount of manual intervention and some of the probe selection rules in our design pipeline may not be applicable. It is best to consult with your FAS or our Design Team before submitting non-RefSeq sequences. If this is the mechanism by which probes will be chosen, these sequences need to be submitted in the FASTA format.

To submit sequences in FASTA format please follow this guidelines:

- The header line must start with the FASTA standard character ">"
- The format of the header line must be consistent from record to record and each entry should include a unique identifier
- Target sequence must be at minimum 100 nucleotides. Longer target regions provide a better chance of designing quality probes

### 6. Do you support organisms that don't have a genome build?

Yes, but you should talk to your FAS about such designs. The quality of the starting sequences is important to obtaining good experimental results and starting with high quality sequences will always yield the best performance possible.

**7. Does your probe selection method screen against genomic DNA?**

No. Internal tests using whole-cell lysates have demonstrated that the presence of small amounts of genomic DNA contamination, relative to the amount of RNA, minimally affects the outcome of a NanoString analysis.

**8. How long are the target-specific probes?**

The two probes each contain 35-50 bases each of sequence that hybridizes to the target molecule. In order to optimize hybridization at a single temperature,  $T_m$ (s) are designed to be in a narrow range and thus probe lengths are varied to accommodate this requirement.

**9. Do the capture and reporter target probes have to be adjacent?**

Yes. Internal experimentation has shown that separating the two hybridizing sequences affects the recovery of useful information from those probes. Fortunately, this observation provides an added level of confidence in the specificity of the nCounter Analysis System. The probability of the two probe simultaneously hybridizing to adjacent non-targeted sequence is minute, the number of false-positive counts that might arise by anomalous annealing of probes is virtually eliminated.

**10. How does your probe selection process address gene families?**

Unless specifically requested otherwise, probes are selected to hybridize to as many members of a gene family as possible. Notably, it might not be possible to design probes that distinguish specific members of highly related genes. Contact your FAS if you have questions about probe selection for your gene family of interest.

**11. Can your system be used to quantify splice variants?**

NanoString will, by default, optimize for maximum coverage across all transcript variants (TV) for every gene that has known TVs associated. This is done by designing the probe-pair matching the requested transcript to a region that is common to the largest number of TVs in that set inclusive of the TV identified as the target. These targets will be reported under the Transcript Variant Coverage table in the CodeSet Design Report as having multiple accessions associated with one probe pair.

The probe design process requires special attention if you require that your CodeSet detect different TVs specifically.

**12. How does your system compare to other platforms when quantifying transcripts?**

There are several fundamental differences between the way NanoString and other platforms such as qPCR systems detect and measure transcript abundance. While these differences will have little effect on measurements of changes in expression levels, they can influence comparisons of absolute quantities of particular transcripts as measured by the two systems. If you plan to compare results across different platforms, please contact your FAS for advice.

**13. Can you design probes that are the same as an array or PCR probe sequence?**

By default, Nanostring does not confine our probe design to a specific region of a gene. Although we will make every effort to satisfy the request, the constraints on sequence choices may result in probes that will not perform optimally. For example, the requested target region could have a significantly different melting temperature, or it could fall in a sequence that binds to more unrelated targets under our hybridization conditions. We therefore recommend against placing constraints on the sequences available for probe designs. Nevertheless, if necessary for your experimental design, please indicate that you want to target specific sequences in the gene in the Comments field of the CodeSet Design Form or by contacting your FAS.

**14. Can I order miRGE CodeSets that have less than 100 mRNA Targets or less than 5miRNA targets?**

Currently nCounter miRGE CodeSets support between 5-30 miRNA targets and 100-200 mRNA targets. If you wish to run assays that fall outside of these standard parameters please contact your sales representative or your Field Application Scientist.

## References

- The NCBI handbook [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2002 Oct. Chapter 17, The Reference Sequence (RefSeq) Project. Available from the NCBI website: ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).
- NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. Pruitt KD, Tatusova T, Maglott DR. *Nucleic Acids Res.* 2007 Jan;35(Database issue):D61-5.
- Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, Fell HP, Ferree S, George RD, Grogan T, James JJ, Maysuria M, Mitton JD, Oliveri P, Osborn JL, Peng T, Ratcliffe AL, Webster PJ, Davidson EH, Hood L, Dimitrov K. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nature Biotechnology* 2008 Mar;26(3):317-25.
- Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. *NAR* 2011 39(Database Issue):D152-D157



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## SALES CONTACTS

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