Long Noncoding RNAs

New and Emerging Research Frontier

Noncoding RNAs represent a new frontier in molecular genetic, molecular biological, physiological, and cell biological research—and have tremendous potential for advancing our comprehensive understanding of biological processes in human health and disease.

Recent data suggest that there are thousands of long noncoding RNAs that are expressed in a developmentally regulated manner in mammals [1,2]. Growing evidence also suggests that these noncoding RNA transcripts are functional, especially in the regulation of epigenetic processes, and are emerging as important regulators of diverse functions [3,4]. While much of the protein-coding portion of the genome has been the main focus of studies over the past 50 years, the noncoding RNAs clearly represent a vastly undiscovered area in biology. The leading indications suggest that these transcripts contain an additional layer of genomic programming in humans and other complex organisms, and demand thorough experimental evaluation and functional validation (Figure 1).

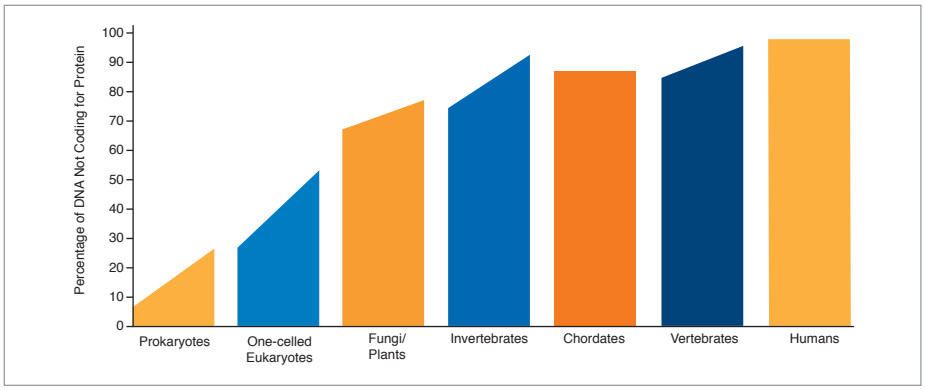

Figure 1. Non–Protein-Coding Sequences Make Up Only a Small Fraction of the DNA of Prokaryotes. Among eukaryotes, as their complexity increases, so does the proportion of their DNA that does not code for protein. The noncoding sequences have been considered "junk", but perhaps they actually help to explain organisms' complexity.

Discovery of Long Noncoding RNAs

Most of the noncoding RNAs are either predicted by computational methods or found by large-scale transcriptome sequencing and analysis or genome-wide microarrays [5-8]. Evidence from genome-wide sequencing and microarrays of several mammalian and other complex organisms has shown that only a very small percentage of the genome codes for proteins are being transcribed. Some estimates indicate that up to half of all transcription in human and mouse genomes may result in noncoding RNA [4,9].

Biology of Noncoding RNAs

Origin

It is becoming evident that the vast majority of noncoding RNAs overlap with, or are transcribed antisense to, protein-coding genes or are expressed as intergenic or intronic regions of the genome [1,4] and are transcribed in a developmentally regulated manner. More recently, conserved large intervening noncoding RNAs (lincRNAs) have been also reported [10].

Subcellular Localization

In mammalian cells, many small noncoding RNAs are localized to the cell nucleus, where they play important roles in various steps of gene expression. Unlike RNAs coding for nuclear proteins, which exit the nucleus when used as templates for protein synthesis, long noncoding nuclear RNAs may exist solely within the nucleus throughout their lifetime. Intronic ncRNA expression seems to be predominantly nuclear, although some subsets are primarily detected in the cytoplasm, and only a few seem to be equally expressed in both compartments [11,12]. For example, some of the bona fide nuclear-localized noncoding RNAs include Neat1, MALAT-1 (Neat2), and Xist. RNA FISH analyses have confirmed the nuclear enrichment of both NEAT1 and NEAT2/MALAT-1 in a broad range of human and mouse cell lines [13]. Other noncoding RNAs that function as chromatin modulators in nuclear scaffolds depend on their localization to the nucleus. Subsets of small nuclear RNAs (snRNAs) constitute a large ribonucleoprotein complex

called the spliceosome that excises introns from pre-mRNA [14].

Classification Based on Function

Based on the functionalities, noncoding RNAs can be divided into two main groups: regulatory noncoding RNAs (e.g., miRNAs, piRNA, and Xist) and housekeeping/structural noncoding RNAs (e.g., tRNA, rRNA). The regulatory noncoding RNA group can be further subdivided based on size. Long noncoding RNAs are transcripts larger than 200 nucleotides. This group represents the largest portion of the newly discovered noncoding RNAs implicated in diverse biological functions.

Biological Significance of Noncoding RNAs in Health and Disease

Noncoding RNAs Have Diverse Functions

Noncoding RNAs are implicated in diverse functions in mammals, other animals, plants, and fungi. Some of these functions include roles in developmental processes, epigenetic regulation, regulation of chromosomal architecture and dynamics, transcriptional regulation, posttranscriptional processing, RNA editing, RNA trafficking, protein degradation and translocation, organelle biogenesis, stress response, and assembly of macromolecular complexes [1,15,16]. One major emergent function of long noncoding RNAs is their ability to modulate the epigenetic status of nearby protein-coding genes by recruiting chromatin activators or repressor complexes [17].

Noncoding RNAs in Disease

It is not surprising, considering that noncoding RNAs have such diverse functions, that the dysregulation of noncoding RNAs appears to be a primary feature of many complex human diseases. Some of these include leukemia, other cancers such as breast cancer, hepatocellular carcinoma, colon cancer, and prostate cancer, psoriasis, ischaemic heart disease, and Alzheimer's disease (Table 1) [19-23].

For a Comprehensive Understanding of Biology, Study Long Noncoding RNAs

The fundamental roles of noncoding RNAs in development and epigenetic regulation—and its involvement in several diseases—suggest that these classes of molecules need to be studied in detail to understand their biological implications. Noncoding RNAs call into question the long-standing assumption that most genes encode proteins (with cis-acting regulatory elements) and that proteins transact most genetic information. Annotating functions for these noncoding RNAs is not going to be a trivial task, but seminal discoveries could come out of these studies.

Complexity and Challenges with Noncoding RNAs

While we now know that noncoding RNAs are emerging as important biological regulators with diverse functions, it is also important to realize there are more "unknowns" with noncoding RNAs compared to coding RNAs.

The fact that the number of noncoding RNAs could be significantly higher than mRNAs illustrates the magnitude of the task involved. In fact, it may be an equal or greater challenge than what we already face working out the biochemical functions and biological roles of all of the known and predicted proteins and their isoforms.

However, unlike the mRNAs, newer noncoding RNAs are still being discovered. The information is still being catalogued—and is changing rapidly. And there is currently no central location for the collection of information from all these studies (Table 2).

There are also several interesting challenges with noncoding RNAs, related to biology, that are not encountered with mRNA targets. They include:

- Tissue-, organ-, or developmentally specific differential expression
- Nuclear localization of some of the noncoding RNA

Table 1. Examples of ncRNAs Associated With Human Diseases.

ncRNA	Accession Number	TaqMan® Non-coding RNA Assay ID	Function	Disease	Reference
p15AS (CCKN2BAS)	NR_015342	Hs03309852_g1	Epigenetically silencing p15 (CCKN2B) tumor suppressor gene	Cancer	Yu W, Gius D, Onyango P et al. (2208) *Nature* 451(7175):202–206.
FMR4	NR_024499.1	Hs03680972_mH	Anti-apoptotic function	Fragile X syndrome	Khalil AM, Faghihi MA, Modarresi F et al. (2008) *PLoS One* 3(1):e1486.
ATXN8OS (SCA8)	NR_002717.2	Hs01382089_m1	Hypothesized to deregulate pre-mRNA splicing	Spinocerebellar ataxia	Chen WL, Lin JW, Huang HJ et al. (2008) *Brain Res* 1233:176–184.
MALAT-1	NR_002819.2	Hs00273907_s1	Predicts metastasis and survival in early-stage non–small cell lung cancer	Cancer	Ji P, Diederichs S, Wang W et al. (2003) *Oncogene* 22(39):8031–8041.
aHIF	U85044.1	Hs03454328_s1	Biomarker for breast cancer prognosis	Cancer	Cayre A, Rossignol F, Clottes E et al. (2003) *Breast Cancer Res* 5(6):R223–230.
MIAT	NR_003491.1	Hs00402814_m1	SNP in the gene increases risk of the disease	Cardiovascular disease	Ishii N, Ozaki K, Sato H et al. (2006) *J Hum Genet* 51(12):1087–1099.
PCA3 (DD3)	NR_015342	Hs03462121_m1	Overexpressed in prostate cancers	Prostate cancer	de Kok JB, Verhaegh GW, Roelofs RW (2002) *Cancer Res* 62(9):2695–2698.
HOTAIR	NR_003716.2	Hs003296680_s1	Epigenetically represses HOXD genes, a known predictor of breast cancer metastasis	Breast cancer	Rinn JL, Kertesz M, Wang JK et al. (2007) *Cell* 129(7):1311–1323.
LIT1 (KCNQ1OT1)	NR_002728	Hs03665990_s1	Imprinting	Beckwith-Wiedemann syndrome	Arima T, Kamikihara T, Hayashida T et al. (2005) *Nucleic Acids Res* 33(8):2650–2660.
H19	NR_002196.1	Hs00262142_g1	Imprinting	Cancer	Yoshimizu T, Miroglio A, Ripoche MA et al. (2008) *Proc Natl Acad Sci U S A* 105(34):12417–12422.

- Relatively low levels of expression of noncoding RNAs compared to mRNAs
- Several noncoding RNAs are antisense transcripts or have significant overlap with coding transcripts (Figure 2)

siRNAs for Noncoding RNA Research

First, the assignment of function for noncoding RNAs needs to be accomplished in a relevant biological context to generate interest for further studies. Ascribing function to these noncoding RNAs is an enormous effort and may require both *in vitro* and *in vivo* assays.

Moreover, the continued discovery of several thousand noncoding RNAs necessitates developing strategies for rapidly moving from discovery to functional annotation. So as one begins thinking about functional validation experiments, it is extremely important to use the right set of tools or reagents specific for detection and knockdown of noncoding RNAs so that meaningful interpretation of results can be inferred and functions annotated to the noncoding RNAs.

RNA interference (RNAi) using siRNAs offers a unique way to rapidly analyze the function of any gene in a given biological context by perturbing the expression of the function. The noncoding RNAs similar to the mRNAs are perfect for such siRNA applications. The siRNAs are well-suited for large-scale functional screening of noncoding RNAs. Many researchers have used RNAi to explore noncoding RNA function. A recent review article by Mattick summarizes and lists different studies in which the manipulation of noncoding RNA levels includes the application of RNAi to study noncoding RNA function [17]. However, most of these studies used chemically unmodified siRNAs. Now that we know that a significant number of noncoding RNAs are antisense and contain sequence complementarities to conventional coding (sense) or noncoding RNAs [23], the chemically unmodified siRNAs will not be able to distinguish between the two overlapping transcripts. It is likely the siRNA will be working against both transcripts since both the guide and passenger strands loaded onto the RISC complex can find matching targets. One way to mitigate the issue is by using chemically modified siRNAs such that only the intended noncoding RNA is targeted. This is very important because now the functional readout that is obtained can be related to the effect of the specific knockdown tested.

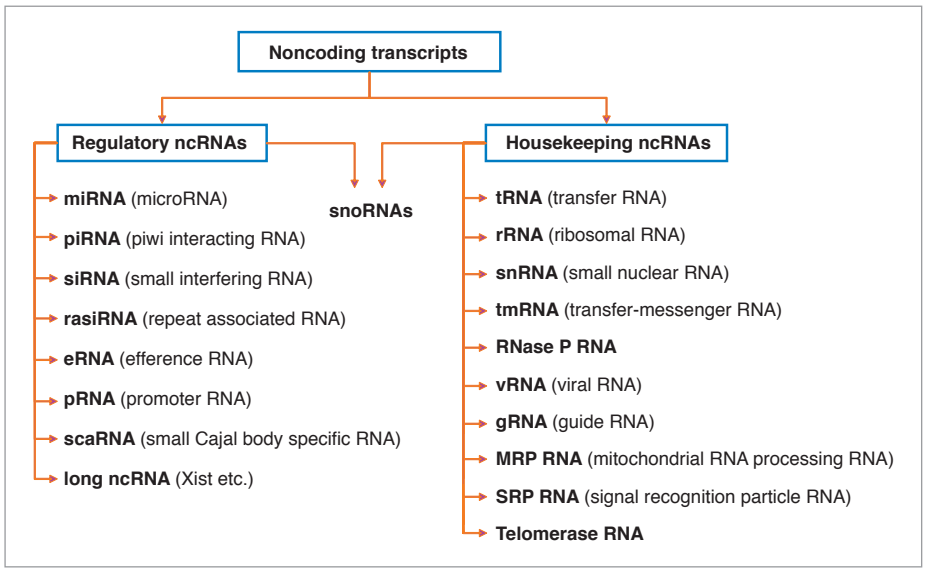

Figure 2. Examples of Noncoding Transcripts for Regulatory Function and Housekeeping Functions Within the Cell.

Table 2. Comparing mRNA to Long Noncoding RNA.

	mRNA	Long noncoding RNA
Size	200 bases to several kb	200-several Kb
Number	Total 20–24,000 mRNAs	Predicted 3–100-fold of mRNA
Origin	Defined by ORF	Diverse—overlap mRNA from both strands
Localization	Cytosolic	Nuclear and/or cytosolic
Expression patterns	Complex, differential, known	Complex, differential, unknown
Expression levels	Low to high	Very low to moderate
Function	Protein-coding	Regulatory/unknown
Role in diseases	Yes	Yes
Role in development	Yes	Yes
Databases	Several, well maintained	Emerging
Annotation	Standardized, well supported	No system in place

Silencer® Select siRNAs for Noncoding RNA Offer the Easiest and Most Efficient Way to Knock Down Noncoding RNA

Silencer® Select siRNAs for noncoding RNAs are specifically designed and chemically modified to dramatically enhance specificity. The predesigned *Silencer*® Select siRNAs for noncoding RNAs are designed for all the noncoding RNAs (>100 nt) in the NCBI RefSeq database RNAdb for human, mouse, and rat targets. Off-target checks were performed against all the RefSeq contents, including both NR and NM accession numbers of respective species. On top of the additional filtrations that are part of *Silencer*® Select siRNAs, the selected sequences are made to include carefully selected LNA chemical modifications that make the siRNAs even more specific and enable strand specificity. Since such a vast number of sense and antisense transcripts are known to use chemically modified siRNAs that are specific and effective only against the intended target, this is a much-desired feature.

The *Silencer*® Select siRNAs for noncoding RNAs address some of the challenges that are associated with noncoding RNAs and offer solutions in terms of siRNA design, off-target check, specificity, and performance.

- Ambion® *Silencer*® Select siRNAs now enable researchers to find and obtain predesigned siRNAs for noncoding RNAs with ease of application in their experimental settings
- These siRNAs are designed using industry-leading *Silencer*® Select siRNA to incorporate updated off-target checks and predict algorithms
- The siRNAs incorporate advanced LNA chemical modifications to greatly and consistently enhance strand specificity, consistency, and potency
- The siRNAs are matched to corresponding

TaqMan® Assays for noncoding RNAs, so ordering and testing is easy

Strong guide strand bias, where the guide strand of the siRNA is selectively taken up into the RISC over the passenger strand, is important both for maximizing siRNA silencing potency and for decreasing passenger strand–related, off-target effects. Although incorporating the right siRNA design parameters can help, siRNA design alone is not sufficient to ensure strong guide strand bias. The chemical modifications in *Silencer*® Select siRNAs (Figure 3):

- Consistently enhance guide strand bias, which has been shown to correlate strongly with knockdown efficiency
- Prevent the passenger strand from inducing silencing, which serves to reduce off-target effects
- Result in no loss in siRNA silencing potency

Workflow Recommendations for Application of *Silencer*® Select siRNAs for Noncoding RNA

Step 1. Validate Noncoding RNA Expression and Obtain Effective siRNAs

Recent studies have revealed that most transcribed RNA is noncoding—and noncoding RNA (ncRNA) has been shown to perform a variety of functions and to participate in numerous biological processes. It remains a challenge to identify and validate ncRNA in biological experiments, particularly those ncRNAs expressed at low levels, in specific tissue types or subcellular compartments or under exact conditions. In addition, ncRNA detection and quantification studies must take into account the existence of transcript variants in the same target.

To meet the needs of researchers investigating ncRNAs, a comprehensive set of predesigned TaqMan® Non-coding RNA Assays have been developed to enable reliable and reproducible quantitation of ncRNA expression levels. Using a sophisticated assay design pipeline integrating bioinformatics tools with experimentally tested assay design rules, quality control strategies, and selection criteria, TaqMan® Non-coding RNA Assays are highly specific and detect only a single ncRNA target. Further, the assays are highly sensitive and exhibit a wide dynamic range of detection, providing linear results from input amounts covering more than five orders of magnitude.

Since it is possible that many noncoding RNA are expressed at low levels, it is important to test the expression levels in the cell lines of interest first. TaqMan® Non-coding RNA Assays provide a sensitive, accurate method to experimentally detect and quantitate ncRNA expression and further investigate their involvement in biological pathways. Further information about TaqMan® Non-coding RNA Assay applications can be obtained at www.appliedbiosystems.com/absite/us/en/home/applications-technologies/real-time-pcr/long-non-coding-rna-analysis.html.

Ambion® *Silencer*® Select siRNAs for noncoding RNAs can be searched and ordered through www5.appliedbiosystems.com/tools/sirna. Currently, the ncRNAs for which predesigned siRNA are available closely match the TaqMan® Non-coding RNA Assays offered. Additional siRNAs can be custom-designed for these targets and other novel ncRNA targets using the custom siRNA design feature. Up to ten predesigned siRNAs are displayed for each ncRNA target.

Table 3. Number of Human, Mouse, and Rat ncRNA Targets for Which Predesigned *Silencer*® Select siRNAs Are Available.

Species	Number of ncRNA targets
Human	14,074
Mouse	10,441
Rat	47
Total	24,562

Step 2. Optimize siRNA delivery

Maximize ncRNA Knockdown

Efficient and reproducible siRNA delivery is essential for successful RNAi experiments. Low transfection efficiency and low cell viability are the most frequent causes of unsuccessful silencing experiments. Because siRNA delivery into a variety of cell types is critical, it is worthwhile to invest time and effort to determine the best siRNA delivery method and conditions for each cell line studied. Transfection optimization eliminates these common causes of failure to achieve maximum siRNA effectiveness. There is no single transfection parameter that by itself ensures efficient siRNA uptake in cell culture. Optimal siRNA delivery is achieved by systematically addressing each of several critical variables. Important parameters in siRNA transfection experiments include:

- Health of cultured cells
- Transfection method
- Transfection reagent and conditions
- Quality and quantity of siRNA

Controls Are Key

In general, the first step when optimizing transfection conditions for a cell type is to test siRNA delivery efficiency using different transfection agents and a positive and negative control siRNA.

Negative controls that do not target any endogenous transcript are needed to control for nonspecific effects. *Silencer*® Select Negative Control siRNAs #1 and #2 include the same modifications for reducing off-target effects found in *Silencer*® Select siRNAs and have been shown by microarray analysis to have minimal effects on gene expression. In addition, these two controls

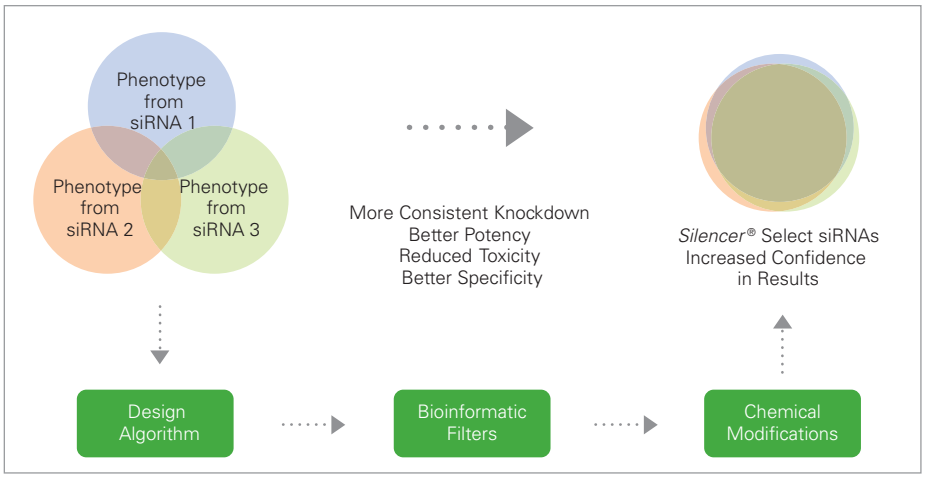

Figure 3. Superior Performance of *Silencer*® Select siRNAs Due to Design Algorithm.

have been tested in multiparametric cell-based assays and are proven to have no significant effect on cell proliferation, viability, or morphology in the cell lines tested.

Easy-to-assay positive controls are needed to optimize transfection conditions, ensure that siRNAs are delivered, and ascertain that a particular downstream assay is working. Ambion® *Silencer*® Select GAPDH (h/m/r) is ideal for developing and optimizing siRNA transfection conditions to confirm that the transfection procedure and cell cultures support gene silencing.

Positive Control siRNA for Noncoding RNAs
For noncoding RNA, human MALAT-1 (also known as NEAT2) long ncRNA is well-suited as an ideal target for a positive control. MALAT-1 is a nuclear-localized 8,708 nt transcript and is expressed ubiquitously at high levels in various mammalian cell lines, including HeLa, A549, HEK293, Jurkat, U2OS, MCF7, and HuH7. This makes MALAT-1 extremely useful for evaluating efficiency of siRNA transfection for ncRNA.

We recommend using MALAT-1 noncoding RNA control siRNA in experiments as a positive control. A TaqMan® Assay for MALAT-1 (Hs00273907_s1) can be used to detect the expression level and knockdown of MALAT-1 in the cell lines of interest. The efficiency with which mammalian cells are transfected with siRNA will vary according to cell type and the transfection agent used. The positive control siRNA helps to determine the optimal transfection conditions that maximize gene silencing while minimizing cytotoxicity. Knockdown of MALAT-1 confirms delivery of MALAT-1 siRNA to the nucleus and provides an indicator of transfection efficiency in noncoding RNA siRNA experiments and consistency between experiments. siRNA titration and time-course experiments are particularly important endeavors to begin to understand the biological impact of reduced target ncRNA expression.

Step 3. Test siRNA Silencing Efficiency
Once siRNAs are obtained, the next step in a gene silencing experiment is to assess the silencing efficiency of the selected siRNAs. siRNAs exert their effect at the transcript level, so the preferred assay for siRNA validation is one that monitors transcript levels. The most sensitive assay for siRNA validation relies on qRT-PCR to measure target transcript levels in gene-specific siRNA–treated cells vs. negative control siRNA-treated cells.

Step 4. Examine the Biological Impact of Silencing
One goal of an RNAi experiment is to understand the biological impact of the reduced target gene expression. Assays that measure the effects of gene silencing are varied and diverse. Morphological, enzymatic, biochemical, and immunological assays can all be useful, depending on the goals of the experiment. In general, it is best to choose the simplest, most reproducible assay that is relevant to the biological process being studied. First, however, it is important to define the time course over which silencing takes place so that this window can be targeted in the biological assays. Because siRNAs exert their effects at the transcript level and ncRNA is not translated into a protein, it is highly recommended to measure ncRNA transcript silencing by qRT-PCR with TaqMan® Non-coding RNA Assays.

Recommendation of Testing 5 siRNAs for Each Noncoding RNA Target
Overall, the functional analysis workflow for noncoding RNAs is going to be very similar to mRNA research. Application of siRNAs to cells to knock down the noncoding RNA doesn't seem to be a major challenge. The challenge, if there is any, will be very similar to the one for coding RNAs and mostly will pertain to the type of cell that is being worked with. It may not be easy to get the desired knockdown in hard-to-transfect cells such as stem cells, primary cells, or suspension cells using lipid-based delivery systems.While lipids like Lipofectamine® RNAiMAX reagent greatly help to deliver siRNAs to a wide variety of cell lines, newer advances such as the Neon™ electroporation system could help overcome delivery problems associated with hard-to-deliver cell lines.

While the workflows for noncoding RNAs are very similar to mRNA, it is important to consider that the study of noncoding RNAs as described above could be much more complex and challenging. We understand very little about the secondary structure, the subcellular localization and distribution, the association of noncoding RNAs with other RNAs, proteins, and DNA, and abundance and differential expression. So in applying siRNA for noncoding RNA research, one or more of these and other unknown factors may influence the accessibility of noncoding RNAs for siRNAs to bind, leading to the cleavage of the targeted noncoding RNA. So the knockdown of noncoding RNAs may not be as robust and/or sufficient as mRNAs or may be long enough to result in the phenotype. Also, while some noncoding RNAs are easy to knock down, the same cannot be said for all. Since the noncoding RNAs are regulatory in function, a small change in their levels may be sufficient to translate to functional readout and result in consistent phenotype across multiple siRNAs [24].

Because of these challenges, we suggest starting testing with five *Silencer*® Select siRNAs for each noncoding RNA target, and identifying the two best ones to move forward with the actual biological experiments. For customer convenience, up to 10 predesigned *Silencer*® Select siRNAs are offered for each noncoding RNA target. This offers flexibility and, if necessary, the option to choose more siRNAs for each target to find the best siRNAs and to ensure cleaner results and success in siRNA experiments. We strive to offer at least two highly effective siRNAs targeting each noncoding RNA in the database.

Silencer® *Select siRNA for Noncoding RNA—siRNA Tube Labels and Product Specification Sheets*
Most mRNAs have smaller names that can fit onto siRNA labels. However, since several of the noncoding RNAs' names are very long, it is necessary to truncate these names to accommodate siRNA tube-label specifications. The full details, including the gene name and siRNA sequence, are provided in the accompanying product specification sheet. Upon receiving the *Silencer*® Select siRNA for noncoding RNAs, we suggest checking the siRNA tube label and the accompanying product sheet to obtain the full gene name.

Application Note
Case Study—Effective Knockdown of MALAT-1 Using *Silencer*® Select Noncoding RNA siRNAs

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1) is a long ncRNA expressed from chromosome 11 and is known to be misregulated in several human carcinomas. MALAT-1 is a bona fide nuclear-localized ncRNA overexpressed in diverse metastatic cancers. MALAT-1 misregulation is implicated in the development and progression of numerous malignant cancers.

1. Validating MALAT-1 Expression and Obtaining Effective siRNAs

Using SOLiD™ System next-generation sequencing technology to perform deep sequencing, MALAT-1 was identified as one of the highly expressed ncRNA transcripts in HeLa cervical cancer cells. High expression levels of MALAT-1 were confirmed in several common human cell lines (HeLa, A549, HEK293, HepG2, Jurkat, U2OS, MCF7, and Huh7) using a MALAT-1 specific TaqMan® Non-coding RNA Assay (Hs00273907_s1) in quantitative real-time PCR (qRT-PCR).

For this study, the Ambion® *Silencer*® Select noncoding RNA siRNA design pipeline was used to design siRNAs targeting human MALAT-1 ncRNA, and five siRNAs were selected and ordered in standard purity.

2. Optimizing *Silencer*® Select MALAT-1 siRNA Delivery in HeLa Cells

Transfection Agent

Transfection conditions in HeLa cells were previously determined, and showed that optimal reverse transfection conditions for siRNA were obtained using 0.2 µL Lipofectamine® RNAiMAX transfection reagent with 4,000–6,000 HeLa cells (96-well plate scale). Lipofectamine® RNAiMAX transfection agent efficiently delivers siRNA into a wide variety of cell types with minimal cytotoxicity and yields highly reproducible results.

siRNA Controls

Silencer® Select Negative Control #1 and GAPDH siRNA were used to optimize transfection conditions. Both of these siRNAs have been extensively validated for use in human, mouse, and rat cells. *Silencer*® Select Negative Control #1 siRNA has been tested in multiple cell lines and found to have minimal effects on cell survival or proliferation. Further, *Silencer*® Select Negative Control #1 siRNA has been demonstrated in array experiments to exhibit no off-target effects. In this study, GAPDH siRNA served as a positive indicator to monitor siRNA transfection efficiency and control for effects of delivering siRNA. MALAT-1 Positive Control siRNA verified the effective siRNA silencing of a nuclear-localized ncRNA target.

3. Verifying Silencer® Select MALAT-1 siRNA Silencing Efficiency

Testing Silencer® Select siRNAs and Results

The efficacy and potency of four distinct *Silencer*® Select siRNAs targeting MALAT-1 were tested by monitoring MALAT-1 ncRNA levels in cells transfected with two concentrations (5 and 30 nM). Each siRNA was reverse transfected into HeLa cells in triplicate using Lipofectamine® RNAiMAX transfection agent. At 48 hours posttransfection, TaqMan® Gene Expression Assays were used to monitor MALAT-1 inhibition caused by the siRNAs compared to *Silencer*® Select Negative #1 Control.

All four of the siRNAs tested gave potent silencing. A low dose of just 5 nM siRNA concentration silenced MALAT-1 80% or more. The use of at least two siRNAs rather than a single validated siRNA is necessary to confirm that phenotype results observed are due to silencing of the intended ncRNA target and not due to an off-target effect. Northern analysis confirmed robust reduction in MALAT-1 transcript levels for all siRNAs, consistent with qRT-PCR data (Figures 4, 5).

Summary

- TaqMan® Non-coding RNA Assays provide reliable and reproducible quantitation of ncRNA expression levels to monitor siRNA efficiency for siRNA validation
- *Silencer*® Select Non-coding siRNA Assays offer industry-leading siRNA design technology targeting thousands of human, mouse, and rat ncRNAs
- The MALAT-1 ncRNA Positive Control siRNA targets the long, nuclear-localized MALAT-1 transcript to confirm delivery of siRNA to the nucleus and provide an estimate of transfection efficiency in ncRNA siRNA experiments
- Transfection optimization experiments are critical to ensure efficient, reproducible siRNA delivery for successful ncRNA RNAi experiments

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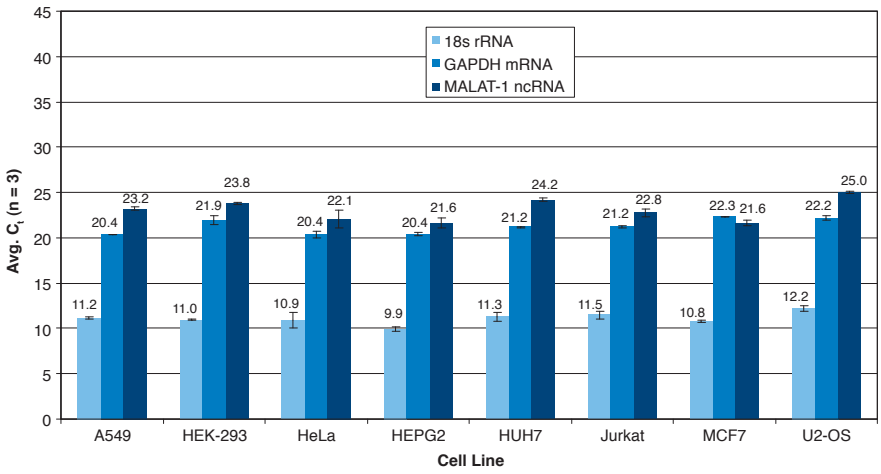

Figure 4. Validation of MALAT-1 Knockdown by Northern Blot Analysis.

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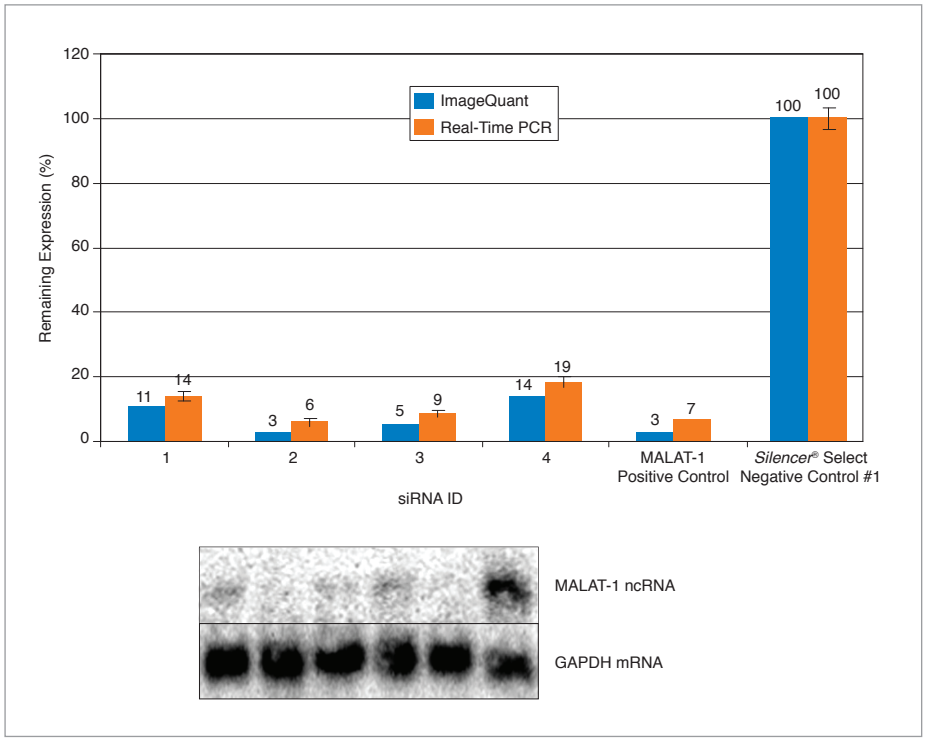

Figure 5. siRNAs Targeting MALAT-1 ncRNA and *Silencer*® Select Negative Control #1 siRNA Were Transfected Into HeLa Cells in a 12-Well Plate at 30 nM Final Concentration. At 48 hours posttransfection, cells were lysed and RNA was isolated using the *mir*Vana™ Total RNA Isolation Kit. RNA was quantitated and assessed for quality and yield by NanoDrop™ and Agilent™. 1 µg of RNA per sample and 2 µg Millennium™ Markers were analyzed on a 1.2 % NorthernMax®-Gly gel and transferred to Bright Star®-Plus Membrane according to recommendations. Probes were designed to the MALAT-1 long ncRNA transcript and GAPDH mRNA and radiolabeled with gamma-^{32}P by IVT using the Ambion® MAXIscript® T7 Promoter Kit. ULTRAhyb™ was used for prehybridization and hybridization. Blots were washed at low and high stringency, exposed to a phosphorimager screen for 2 hours, and bands were quantitated using ImageQuant™ software.

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