TaqMan® Non-coding RNA Assays help characterize hidden RNA regulatory networks

Long non-coding RNAs represent a large new class of gene expression regulators

Advances in genomic analysis technologies have recently uncovered new classes of regulatory non-coding RNAs (ncRNAs) in mammalian and many other complex organisms' genomes [1–3]. Unlike messenger RNAs (mRNAs), which may participate in regulation of gene expression through protein intermediaries, ncRNAs are postulated to represent a vast regulatory layer that functions more directly. This network includes both microRNAs (miRNAs), a large group of small regulatory RNAs that have been characterized extensively, and long ncRNAs (>200 nt), which have emerged as a new focus of biological research.

Long ncRNAs were first discovered by large-scale sequencing of full-length cDNA libraries and expression profiling using high-resolution genome tiling arrays [4,5]. They vary from several hundred to thousands of nucleotides in length, have little or no protein-coding capacity, and are abundantly expressed in a developmentally regulated manner. Large numbers of mouse ncRNAs are specifically expressed during embryonic stem cell differentiation [6], and they exhibit precise subcellular localization in the brain [7], suggesting specific regulatory roles in development. While functional characterization of ncRNAs is still in its infancy, early discoveries pertaining to certain groups of ncRNAs imply that they are involved in a surprisingly wide array of cellular functions, including epigenetic silencing, transcriptional regulation, and RNA processing and modification [8]. In addition, ncRNAs have been associated with human diseases such as cancer [9], Alzheimer's disease [10], and heart disease [11]. Better understanding of ncRNA functional roles has tremendous potential to advance our understanding of cell regulatory and disease mechanisms.

Quantification of ncRNA using TaqMan® Assay technology

To meet the specific needs of researchers investigating ncRNAs, we have developed a comprehensive set of pre-designed TaqMan® Non-coding RNA Assays for reliable and accurate quantification of long ncRNA expression levels. Based on proven TaqMan® probe–based technology and developed with a state-of-the-art assay design pipeline, TaqMan® Non-coding RNA Assays provide the confidence and familiar workflow of TaqMan® Assay technology to accelerate discoveries in the ncRNA field.

life technologies

TaqMan® Non-coding RNA Assay design

TaqMan® Non-coding RNA Assays are designed using proprietary and functionally validated design algorithms similar to those developed for TaqMan® Gene Expression Assays (read "The design process for a new generation of quantitative gene expression analysis tools," Pub. No. CO011189, for details). This sophisticated assay design pipeline integrates an extensive array of bioinformatics tools, including comprehensive target sequence analysis, computational and experimentally tested assay design rules, assay quality control strategies, and robust assay selection criteria (Figure 1). Briefly, ncRNA target sequences are retrieved from NCBI and other ncRNA databases and mapped to the genome. Single nucleotide polymorphisms (SNPs), repeats, and areas of sequence discrepancy are masked, and suitable locations for assay design (i.e., exon–exon junctions) are identified. Next, the most robust primer/probe sets are designed based on both thermodynamic and chemical properties, including optimal T_m requirements, GC content, secondary structure, optimal amplicon size, and primer-dimer minimization.

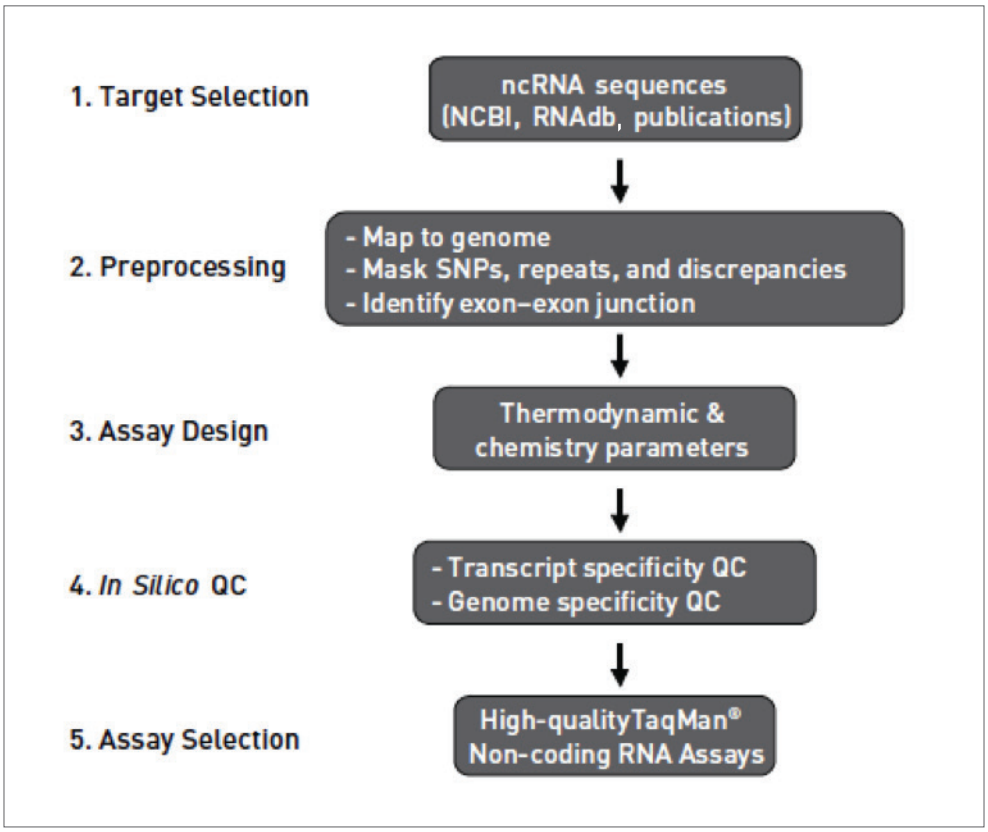


Figure 1. TaqMan® Non-coding RNA Assay design pipeline.

Because ncRNAs are expressed pervasively throughout the genome, the specificity of TaqMan® Non-coding RNA Assays at both transcript and genomic levels is critically important. The *in silico* QC process scores potential ncRNA assays for transcript specificity using a process similar to that used for TaqMan® Gene Expression Assays. For genome specificity, however, the *in silico* QC includes a position-specific, genome-wide alignment matrix to identify assays with minimal potential cross-reactivity with nontarget transcripts or genomic sequences. This extensively validated assay design and QC pipeline ensures that TaqMan® Non-coding RNA Assays are highly specific to their target and discriminative between highly homologous genes and gene families.

In addition, all TaqMan® Non-coding RNA Assays are carefully screened to detect only the targeted noncoding transcript, and not coding NCBI annotated transcripts—even when they colocate within the same genomic locus (Figure 2).

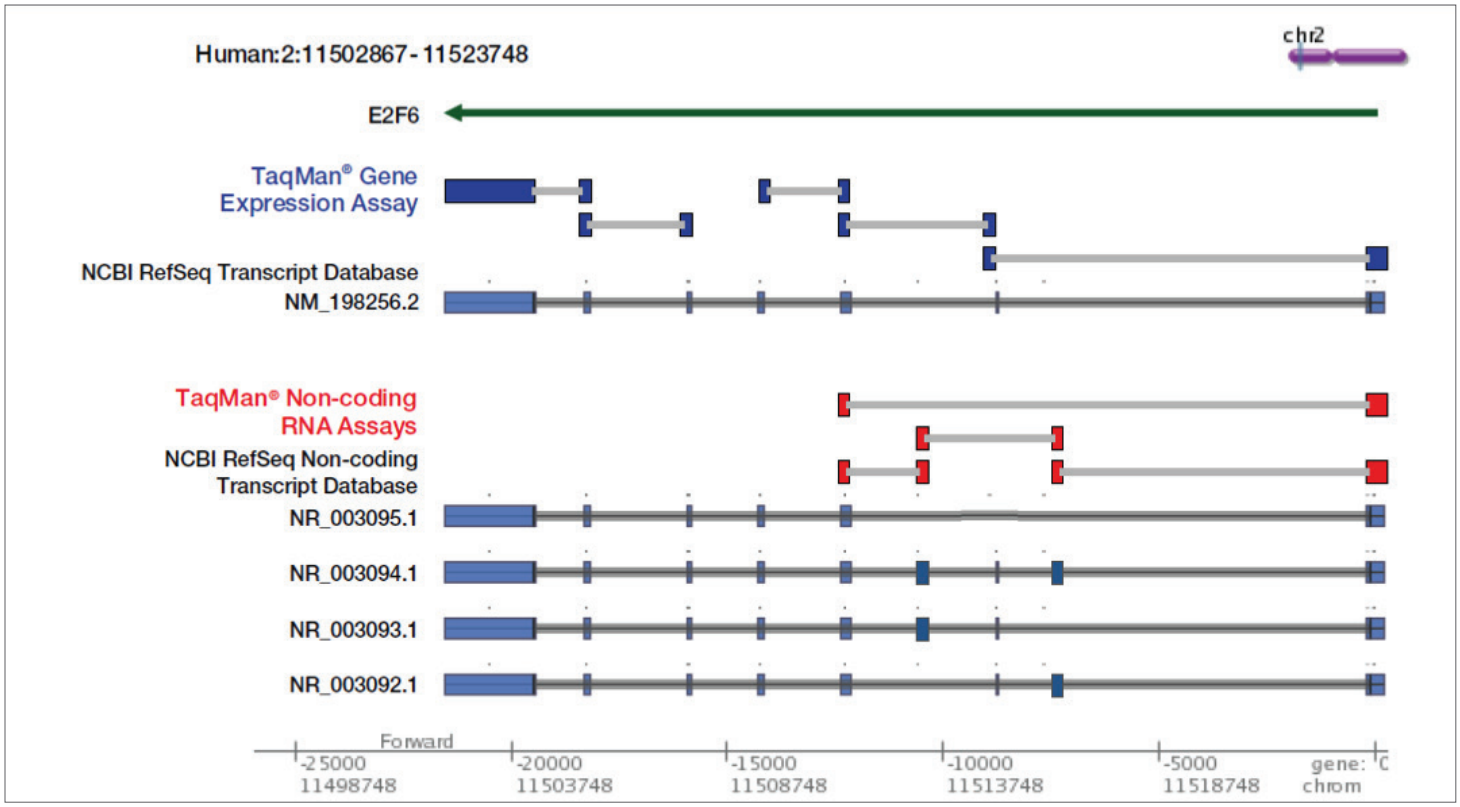


Figure 2. TaqMan® Non-coding RNA Assays are specific to ncRNA targets. An example of TaqMan® Non-coding RNA Assay specificity is shown on the E2F6 gene locus, which is associated with one coding transcript (prefix NM_) and 4 noncoding transcript variants (prefix NR_). Whereas some TaqMan® Gene Expression Assays can detect both coding and noncoding transcripts, all TaqMan® Non-coding RNA Assays are carefully screened to specifically detect only ncRNA transcripts. Transcript exons are shown as light blue boxes, introns as gray bars. For TaqMan® Gene Expression Assays, dark blue boxes indicate the exons spanned by the probe; for TaqMan® Non-coding RNA Assays, red boxes indicate the exons spanned by the probe.

Pre-designed TaqMan® Assays for >27,000 ncRNAs

Currently, there is a comprehensive set of over 27,242 pre-designed TaqMan® Non-coding RNA Assays available for ncRNAs from human, mouse, and rat species (Table 1). This initial set of ncRNA targets was selected based on biological relevance and quality of sequence curation. Examples of assay coverage for known disease-associated ncRNA targets are shown in Table 2.

Table 1. Available pre-designed TaqMan® Non-coding RNA Assays.

	Human	Mouse	Rat	Total
Number of assays	15,369	11,510	363	**27,242**

Table 2. 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. (2008) *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.

Comprehensive mapping and annotation information facilitates assay selection

The Life Technologies™ website (lifetechnologies.com/ncRNA) includes an alignment map and comprehensive annotation with direct links to the NCBI GenBank database for each TaqMan® Non-coding RNA Assay.

Annotation on adjacent or overlapping coding genes for each TaqMan® Non-coding RNA Assay is also provided to illustrate the genomic context of each target ncRNA so that researchers can develop initial hypotheses for functional studies (Table 3).

Table 3. An example of annotation of genomic context (adjacent/overlapping coding genes) for TaqMan® Non-coding RNA Assays.

	Gene name	Gene symbol	Alias	Distance	Relative orientation	NCBI chromosome location
Upstream	Homeobox C12	*HOXC12*	HOC3F, HOX3, HOX3F	–10.7 kb	Antisense	Chr. 12: 52634981–52636617
Downstream	Homeobox C11	*HOXC11*	HOX3H, MGC4906	4.4 kb	Antisense	Chr. 12: 52653177–52656470
Overlapping	Hox transcript antisense RNA (non–protein coding)	*HOTAIR*	FLJ41747, HOXAS, NCRNA00072	NA	Sense	Chr. 12: 52642496–52648727

TaqMan® Non-coding RNA Assay workflow and performance

Like current TaqMan® Gene Expression Assays, all TaqMan® Non-coding RNA Assays are designed for real-time PCR using standardized qPCR conditions with universal cycling and buffer concentrations. In addition, they are also compatible with preamplification, which expands small samples to enable analysis with hundreds of TaqMan® Non-coding RNA Assays using only nanogram amounts of input RNA.

We experimentally validated the performance of TaqMan® Non-coding RNA Assays extensively. To evaluate sensitivity and dynamic range of input nucleic acid, 10-fold serial dilutions of genomic DNA were amplified using a panel of 96 TaqMan® Non-coding RNA Assays targeting sequences within single exons (thus, assays can detect the targets from either genomic DNA or cDNA). Our results showed that TaqMan® Non-coding RNA Assays are highly sensitive: 72% of the assays tested with genomic DNA showed detectable signals (C_t value <35) with an estimated 15 copies of target, while 97% of tested assays detected 150 copies of target. The TaqMan® Non-coding RNA Assays also showed a wide dynamic range: greater than 5 orders of magnitude, from an estimated 10 to 10^6 copies of input target (Figure 3A), with a PCR amplification efficiency of 100% ±10% (Figure 3B).

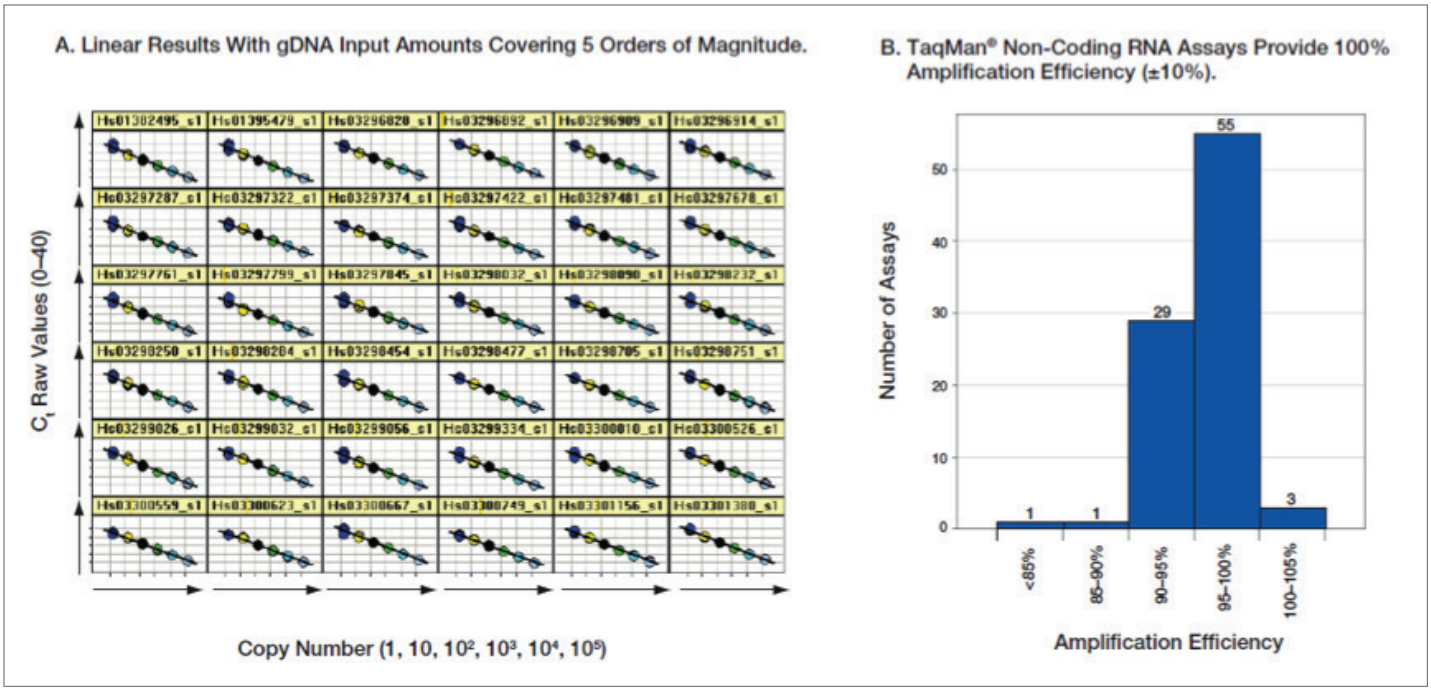


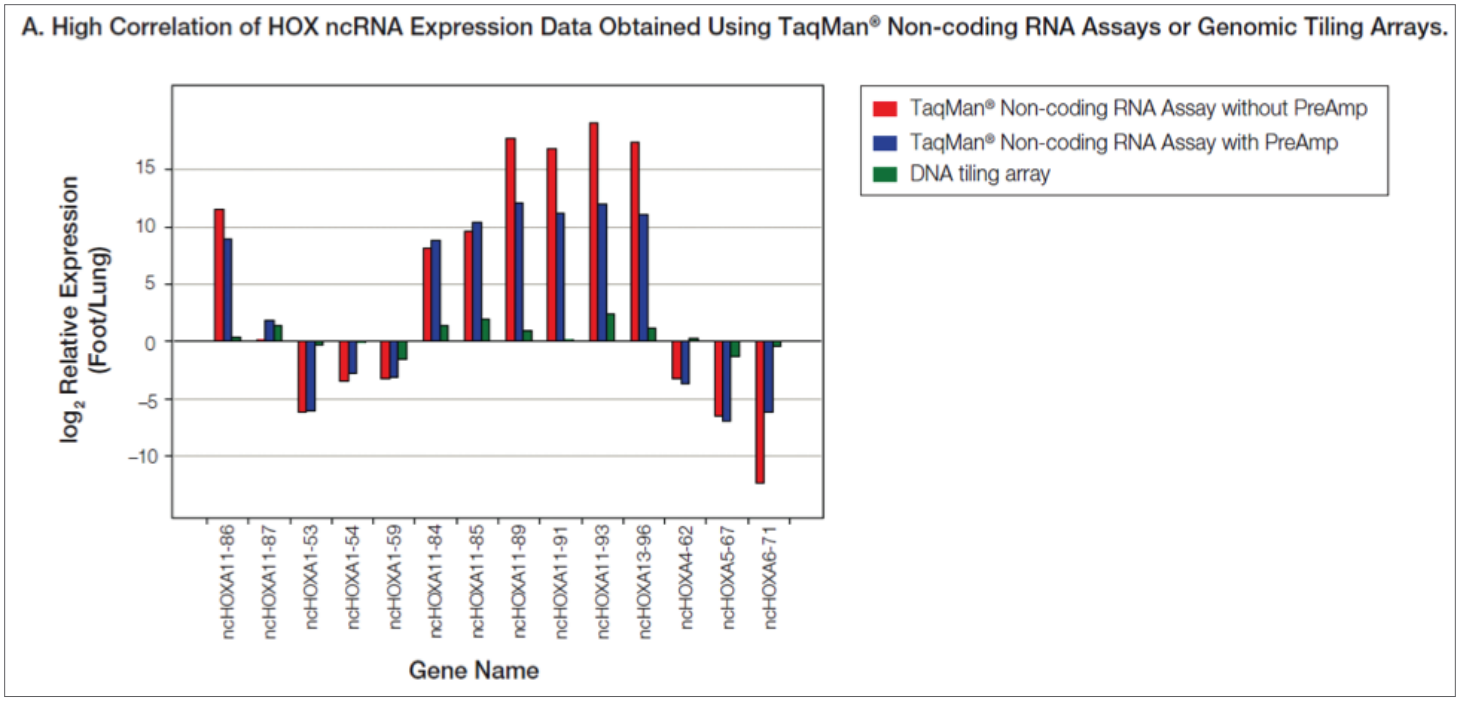
Figure 3. TaqMan® Non-coding RNA Assays provide >5-log dynamic range and 100% amplification efficiency. (A) 10-fold dilution series using gDNA. Copy number of gDNA was estimated using spectrophotometry, and 10-fold serial dilutions were prepared for the estimated copy number shown (covering 5 logs). Each dilution was combined with the TaqMan® Non-coding RNA Assay and TaqMan® Gene Expression Master Mix in 4 replicate 10 µL reactions and run on an Applied Biosystems® 7900HT Fast Real-Time PCR System using universal cycling conditions. **(B)** Distribution of amplification efficiencies measured across at least 5 logs for 89 TaqMan® Non-coding RNA Assays. Amplification efficiency was determined from the slope of the gDNA dilution series using the equation, Amplification efficiency (E) = $10^{(-1/\text{slope})}$ – 1, multiplied by 100. Data show that TaqMan® Non-coding RNA Assays provide 100% amplification efficiency (±10%).

Case study: characterization of ncRNAs in human HOX loci

The HOX gene family includes 39 HOX transcription factors clustered on 4 genomic loci (HOXA through HOXD); it is essential for defining the positional identities of cells during embryonic development. Such embryonic patterns of HOX expression are faithfully retained by adult human fibroblast cells to maintain regional identities via complex epigenetic regulation. Previous transcriptional surveys conducted using high-resolution tiling arrays of the 4 HOX loci across 11 anatomic sites identified 231 HOX ncRNAs [12]. These HOX ncRNAs appeared to be spatially expressed along developmental axes and were hypothesized to play an important role in epigenetic regulation of HOX genes [12]. In collaboration with Dr. Howard Chang's group at Stanford, we used the HOX gene family as a model system and investigated the role of HOX ncRNAs in gene regulation during development.

A panel of 96 TaqMan® Non-coding RNA Assays was developed targeting 43 HOX ncRNAs and 39 HOX transcription factors across the 4 HOX loci. Human fibroblast cells from lung and foot, representing two distinct anatomic origins, were profiled with this HOX assay panel. The resulting transcription profile of the HOX ncRNAs showed positional expression patterns consistent with published results generated using genomic tiling arrays [12].

These experiments highlighted some of the advantages of TaqMan® Non-coding RNA Assays over tiling arrays; the real-time PCR approach provided better sensitivity and dynamic range, and the data were both reproducible and less ambiguous (Figure 4A). More interestingly, the position-specific expression patterns of HOX coding and noncoding RNAs were also highly correlated with the diametric chromatin domains previously defined by ChIP-chip experiments [12]. For example, in the HOXA locus, a switch of expression patterns between foot and lung fibroblast cells occurred between HOXA7 and HOXA9 (Figure 4B, upper panel). This expression switch precisely correlated with the previously defined chromatin domains that undergo diametrically opposing modification (H3K27 trimethylation) in an anatomically specific manner (Figure 4B, lower panel). These results provided further evidence that transcription from HOX loci in adult fibroblasts is regulated by opposing epigenetic modifications. The coordinated expression pattern of HOX ncRNAs also suggests that they may play an important role in establishing this epigenetic regulation.



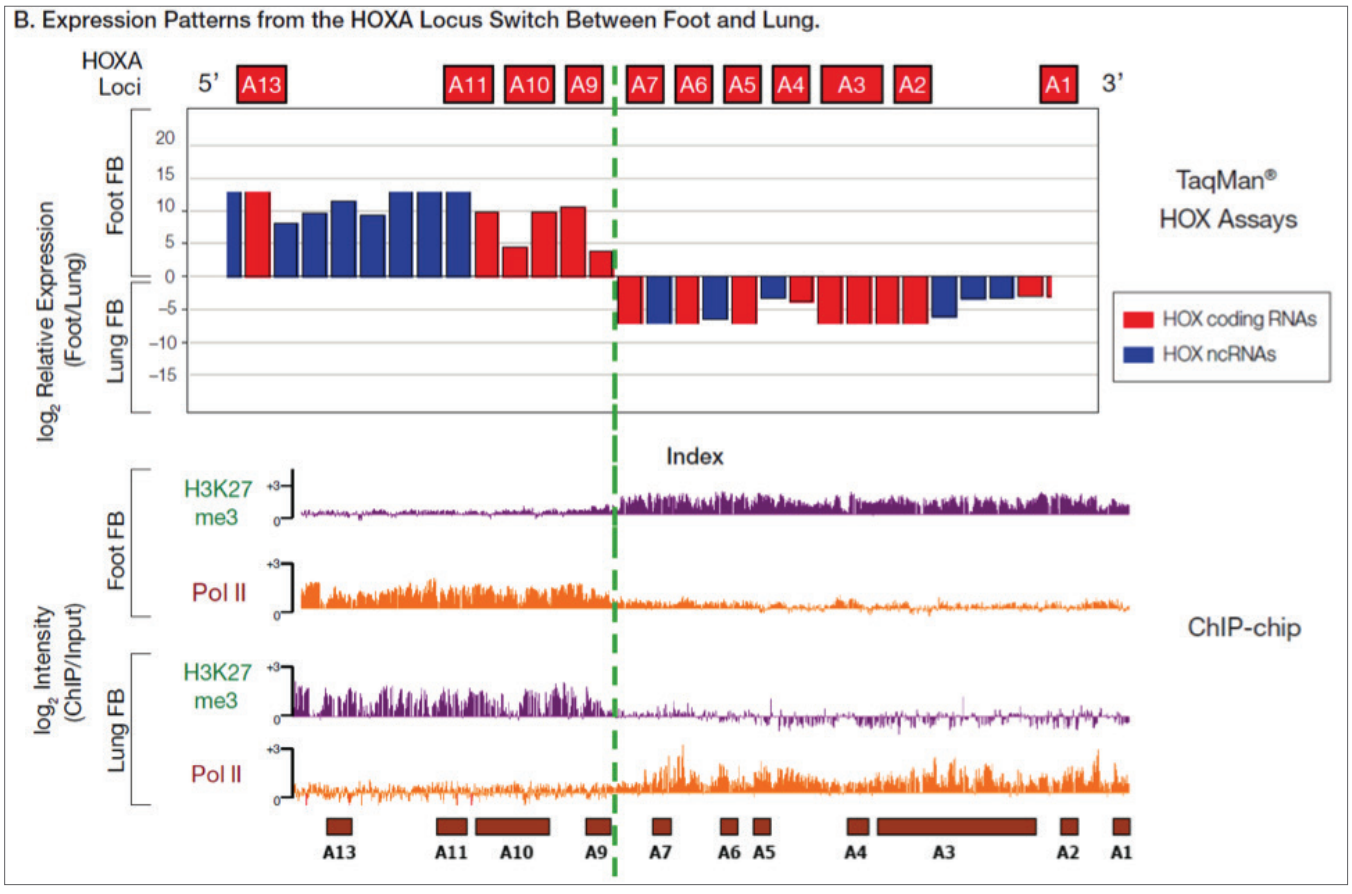


Figure 4. HOX ncRNA showed position-specific expression patterns colinear with coding HOX genes. (A) In general, results from real-time PCR using HOX TaqMan® Non-coding RNA Assays correlated well with published tiling array results. The TaqMan® Assay approach, however, showed significantly higher relative expression changes (therefore less ambiguous) than tiling arrays, presumably due to their wider dynamic range and higher signal-to-noise ratio. **(B)** Upper panel: TaqMan® Assay expression profiling showed a position-specific, colinear expression pattern of coding and noncoding HOX transcripts within the HOXA locus, with a switch of expression patterns between foot and lung fibroblasts and HOXA7 and HOXA9. Lower panel: This expression switch precisely correlated with the previously defined boundary of diametrically opposed chromatin modifications and transcriptional accessibility in the HOXA locus based on a tiling array study [12].

TaqMan® Non-coding RNA Assays put you at the forefront of ncRNA studies

Noncoding RNAs represent a new frontier of biological research and molecular medicine. Our comprehensive set of pre-designed TaqMan® Non-coding RNA Assays make quantification of the expression of long ncRNA transcripts reliable and accurate. With sophisticated assay design and reliable TaqMan® Assay performance, TaqMan® Non-coding RNA Assays provide a powerful tool to help uncover the hidden RNA regulatory network in gene regulation.

Acknowledgments

We would like to thank Yulei Wang, Benjamin Kong, Pius Brzoska, Fangqi Hu, Caifu Chen, Madhu Augustine, Kathy Lee, and Allen Nguyen, Thermo Fisher Scientific Inc., for their contributions to this application note.

The HOX ncRNA study was conducted in collaboration with Dr. Rajnish Gupta and Dr. Howard Chang from Stanford University.

References

1. Birney E, Stamatoyannopoulos JA, Dutta A et al. (2007) Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* 447(7146):799–816.
2. Carninci P, Kasukawa T, Katayama S et al. (2005) The transcriptional landscape of the mammalian genome. *Science* 309(5740):1559–1563.
3. Kapranov P, Willingham AT, Gingeras TR (2007) Genome-wide transcription and the implications for genomic organization. *Nat Rev Genet* 8(6):413–423.
4. Kapranov P, Drenkow J, Cheng J et al. (2005) Examples of the complex architecture of the human transcriptome revealed by RACE and high-density tiling arrays. *Genome Res* 15(7):987–997.
5. Okazaki Y, Furuno M, Kasukawa T et al. (2002) Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. *Nature* 420(6915):563–573.
6. Dinger ME, Amaral PP, Mercer TR et al. (2008) Long noncoding RNAs in mouse embryonic stem cell pluripotency and differentiation. *Genome Res* 18(9):1433–1445.
7. Mercer TR, Dinger ME, Sunkin SM et al. (2008) Specific expression of long noncoding RNAs in the mouse brain. *Proc Natl Acad Sci* U S A 105(2):716–721.
8. Mercer TR, Dinger ME, Mattick JS (2009) Long non-coding RNAs: insights into functions. *Nat Rev Genet* 10(3):155–159.
9. Yu W, Gius D, Onyango P et al. (2008) Epigenetic silencing of tumour suppressor gene p15 by its antisense RNA. *Nature* 451(7175):202–206.
10. Faghihi MA, Modarresi F, Khalil AM et al. (2008) Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of beta-secretase. *Nat Med* 14(7):723–730.
11. Broadbent HM, Peden JF, Lorkowski S et al. (2008) Susceptibility to coronary artery disease and diabetes is encoded by distinct, tightly linked SNPs in the ANRIL locus on chromosome 9p. *Hum Mol Genet* 17(6):806–814.
12. Rinn JL, Kertesz M, Wang JK et al. (2007) Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* 129(7):1311–1323.
13. Shuman S (2007) Transcriptional networking captures the 7SK RNA 5'-gamma-methyltransferase. *Mol Cell* 27(4):517–519.

Find out more at **lifetechnologies.com**

life technologies

A Thermo Fisher Scientific Brand