NuRNA™ Human Small RNA Biogenesis Proteins PCR Array

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Instruction Manual Version 1.0

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I. Introduction

A. Overview

Small RNAs are non-coding RNA molecules < 200 nt in length, which include microRNAs (miRNA), PIWI-interacting RNAs (piRNA), short-interfering RNAs (siRNA), small nucleolar RNAs (snoRNA), small nuclear RNAs (snRNA), transfer RNAs (tRNA), tRNA-related fragments (tRF) and tRNA halves (tiRNA). These small RNA classes have distinctive characteristics and diverse biological functions. While some small RNA types have well known canonical functions, for example, tRNA in protein translation, many others are classified as small regulatory RNAs to regulate gene expression at many levels by, for example, controlling the stability and translation of mRNA, or targeting epigenetic modifications to specific regions of the genome. Biogenesis pathways of these small RNAs are important factors in the dynamic regulation of small RNA functions. Disorder of these pathways are commonly associated with diseases such as cancer. For rapid and convenient expression profiling of protein and enzymatic components involved in small RNA biogenesis, Arraystar has developed the first commercial small RNA biogenesis PCR panel. NuRNA™ Human Small RNA Biogenesis Proteins PCR Array profiles the mRNA expression of 185 protein coding genes categorized in the small RNA biogenesis gene ontological (GO) pathways.

B. Small Regulatory RNAs

Small regulatory RNAs are non-coding RNA molecules that play important roles in activation or inhibition of cellular processes. They are 20-31 nt in length and interact with Argonaute family proteins to form effector ribonucleoprotein complexes. Three major classes of small regulatory RNAs have been identified to date: microRNAs (miRNA), PIWI-interacting RNAs (piRNA) and short-interfering RNAs (siRNA) (**Table 1**). Different kinds of small regulatory RNAs interact with distinct Argonaute proteins. Based on amino acid sequence similarities, Argonaute family proteins can be divided into two categories: AGO, named after its founding member in Arabidopsis thaliana, and PIWI, named after the Drosophila protein PIWI (P-element induced wimpy testis)(Carmell et al., 2002). AGO proteins are ubiquitously expressed in all the tissues and complex with miRNAs or siRNAs typically 20–23 nt in length (Bartel, 2004; Farazi et al., 2008; Ghildiyal and Zamore, 2009; Kim et al., 2009; Liu et al., 2008), whereas PIWI proteins are specifically expressed in germline cells.

Table 1 | Small Regulatory RNAs and Argonaute Family Proteins (Suzuki et al., 2012)

	AGO		***PIWI***
Expression	All tissues		Germline and cancer
Homologs			
Human	AGO1, AGO2, AGO3, AGO4		HIWI, HILI, PIWIL3, HIWI2
Mouse	AGO1, AGO2, AGO3, AGO4		MIWI, MILI, MIWI2 PIWI,
Drosophila	AGO1, AGO2		AUB, AGO3
Bound small RNA	miRNA	siRNA	piRNA
Length (nt)	20–23	20–23	25–31
Precursor	Hairpin-structured RNA	dsRNA	ssRNA
Biogenesis	Drosha, Dicer	Dicer	Dicer-independent
3' End	OH	2'-O-methyl	2'-O-methyl
Mechanism of action	• Translational Repression • mRNA degradation	• RNA cleavage	• Translational or post-transcriptional repression of transposons • Multigenerational epigenetic phenomena in worms
Function	Regulation of protein-coding genes	Regulation of transposon, protein -coding genes, antiviral defense	Regulation of transposon, unknown function

miRNAs and their biogenesis

miRNAs regulate post-transcriptional gene expression by RNA silencing in a wide range of eukaryotic organisms and viruses (Ambros, 2004; Bartel, 2004). While majority of miRNAs are located within the cell, some miRNAs, commonly known as circulating miRNA or extracellular miRNA, have also been found in extracellular environment, including various biological fluids and cell culture media.

miRNAs can origin from the introns or exons of protein-coding genes (about 30%) or the intergenic regions (70%) (Lin and Gregory, 2015). miRNAs are mainly transcribed by RNA polymerase II (Pol II) in the nucleus, and the primary miRNAs (pri-miRNAs) are capped, polyadenylated and spliced. The pri-miRNAs are several kilobases long and are processed in the nucleus by microprocessor, which includes DROSHA and DGCR8, to produce the 60-70 nucleotide precursor miRNAs (pre-miRNAs). The pre-miRNAs are then exported from the nucleus to the cytoplasm by XPO5 and its partner Ran-GTP. In cytoplasm, the pre-miRNAs are recognized by RNase III and DICER1 and further generate the mature miRNA duplexes. One strand of the mature miRNAs binds to miRNA-induced silencing complex (miRISC), which contains DICER1, AGO and miRNAs. The miRISC targets mRNAs by sequence base pairing and mediates mRNA degradation (**Figure 1**) (Lin and Gregory, 2015).

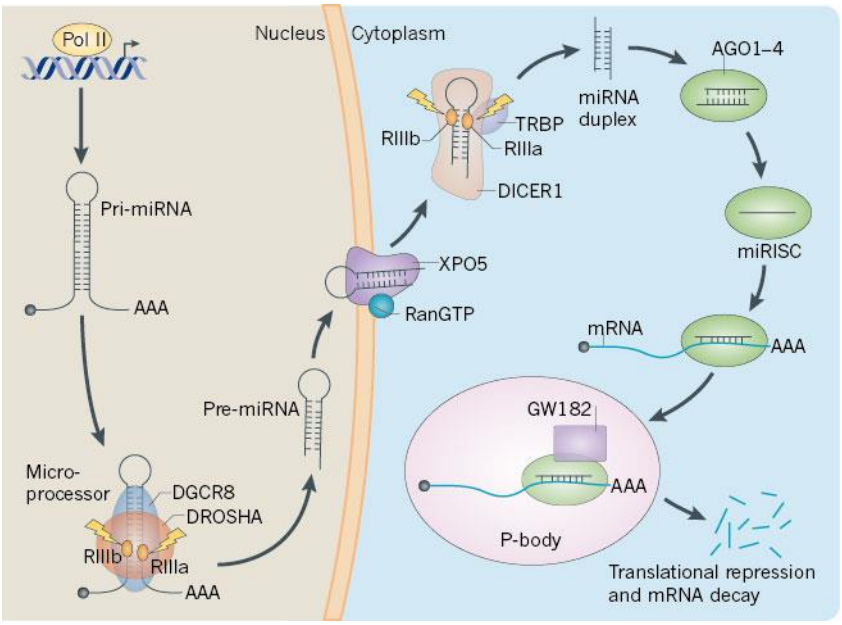


Figure 1 | Schematic representation of miRNA biogenesis (Lin and Gregory, 2015).

As described above, DROSHA and DICER1 play vital roles in miRNA biogenesis pathway. However, they have lower-expression levels in some cancers, such as lung cancer, ovarian cancer and neuroblastoma. As a result, miRNA expression is globally suppressed in cancer cells compared with normal tissues (Lin and Gregory, 2015). Furthermore, DROSHA and DICER1 expression levels change along with the stage of tumor and can be used as a potential biomarker for neuroblastoma prognosis (Lin et al., 2010). Apart from microprocessor, other proteins involved in miRNA biogenesis are dysregulated in kinds of certain cancers (**Table 2**). In addition, mutational analysis revealed that DROSHA is frequently mutated in Wilms tumours samples and ovarian cancers without affecting its expression levels. The alternatively spliced DROSHA transcripts are also found in melanoma and teratocarcinoma cells (Lin and Gregory, 2015). AGO proteins and their associated miRNAs are downregulated in activated CD4+ T cells (Bronevetsky et al., 2013). The lack of DICER1 in the proximal epididymis causes dedifferentiation of the epithelium, leading to unbalanced sex steroid receptor expression, defects in epithelial lipid homeostasis, and subsequent male infertility (Bjorkgren and Sipila, 2015). Taken together, dysregulation of miRNAs biogenesis pathway not only alters miRNA production, but also causes diseases.

Table 2 | Dysregulation of miRNA biogenesis in cancers (Lin and Gregory, 2015).

Protein	Dysregulation	Cancer type	Clinical correlation
DROSHA	Upregulation	Cervical SCC	Altered miRNA profile; associated with neoplastic progression
		Oesophageal cancer	Regulates cell proliferation; associated with poor patient survival
		BCC	Not determined
		SCC	Not determined
		Triple-negative breast cancer	No clinical correlation
		Smooth muscle tumours	Associated with tumour progression
		Gastric cancer	Associated with pathological characteristics and patient survival
		Serous ovarian carcinoma	Associated with advanced tumour stages
		Non-small cell lung cancer	Associated with poor prognosis
	Downregulation	Bladder cancer	Altered miRNA profile
		Ovarian cancer	Associated with poor patient survival
		Endometrial cancer	Correlated with histological grade
		Nasopharyngeal carcinoma	Correlated with shorter patient survival
		Breast cancer	Not determined
		Gallbladder adenocarcinoma	Correlated with metastasis, invasion and poor prognosis
		Neuroblastoma	Correlated with global downregulation of miRNAs and poor outcome
		Cutaneous melanoma	Associated with cancer progression and poor survival
DGCR8	Upregulation	Oesophageal cancer	Associated with poor patient survival
		Bladder cancer	Altered miRNA profile
		SCC and BCC	Not determined
		Prostate cancer	Associated with dysregulated miRNA
		Colorectal carcinoma	Not associated with any clinical parameters
		Ovarian cancer	Required for cell proliferation, migration and invasion
DICER1	Upregulation	Smooth muscle tumours	Associated with high-grade disease and tumour progression
		Gastric cancer	Correlated with gastric tumour subtype
		Serous ovarian carcinoma	Associated with advanced tumour stages
		Prostate cancer	Dysregulated miRNA expression; correlated with tumour stage
		Oral cancer	Required for proliferation
		Colorectal cancer	Correlated with tumour stage and associated with poor survival
		Precursor lesions of lung adenocarcinoma	Associated with histological subtypes and stages
		Cutaneous melanoma	Correlated with clinical stage
	Downregulation	Triple-negative breast cancer	No clinical correlation
		Bladder cancer	Altered miRNA profile
		BCC	Not determined
		Ovarian cancer	Associated with advanced tumour stage and poor patient survival
		Endometrial cancer	No association with histological grade detected
		Nasopharyngeal carcinoma	Correlated with shorter patient survival
		Breast cancer	Associated with cancer progression and recurrence
		Neuroblastoma	Associated with global downregulation of miRNAs and poor outcome
		Gallbladder adenocarcinoma	Correlated with metastasis, invasion and poor prognosis
		Non-small cell lung cancer	Low levels of *DICER1* expression correlate with shortened survival
		Hepatocellular carcinoma	Not associated with clinical characteristics
		Chronic lymphocytic leukemia	Associated with progression and prognosis
		Colorectal cancer	Associated with tumour stage and shorter survival
PACT	Upregulation	AK, SCC and BCC	Not determined
XPO5	Downregulation	Bladder cancer	Associated with altered miRNA profile
AGO1	Upregulation	AK, SCC and BCC	Not determined
		Serous ovarian carcinoma	Associated with advanced tumour stages
AGO2	Upregulation	AK, SCC and BCC	Not determined
		Serous ovarian carcinoma	Correlated with advanced tumour stages and associated with shorter survival

piRNAs and their biogenesis

PIWI-interacting RNAs (piRNAs) are a class of small RNAs that are 25-31 nucleotides in length. piRNAs associate with PIWI proteins, which are specifically expressed in germline, to form piRNA-induced silencing complexes. These complexes repress transposons via transcriptional or post-transcriptional mechanisms. piRNAs play vital roles in maintaining germline genome integrity.

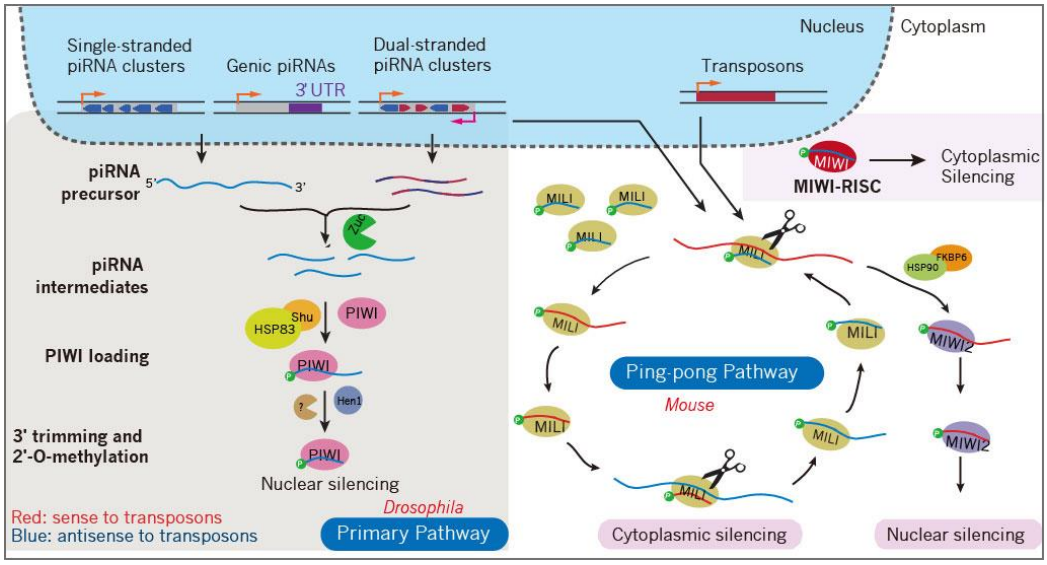


Figure 2 | Biogenesis of piRNAs: the primary processing pathway in Drosophila (left) and the ping-pong pathway in mice (right).

In *Drosophila*, nascent transcripts transcribed from piRNA clusters are processed into piRNA intermediates, which are then loaded onto PIWI proteins by Zucchini (**Figure 2**, left). During this step, the size of the bound PIWI proteins determines the length of mature piRNA. However, the factors involved in the transcription of piRNA clusters and its regulation remain elusive. Associated with PIWIs, the 3' ends of piRNA intermediates are trimmed and subsequently 2'-O-methylated by Hen1/Pimet.

In mice, primary piRNAs are subjected to ping-pong pathway to enforce high levels of piRNA production in the germline cells (**Figure 2**, right). In the pathway, MILI associates with the primary piRNA, cleaves it with its endonuclease activity, and form the 5' ends of secondary piRNAs. The cleavage products are then transferred onto MIWI2 and trimmed from the 3' end to give rise to mature piRNAs. MIWI2 associated with the secondary piRNAs is localized to the nucleus upon piRNA loading. Therefore, MIWI2 does not contribute to the synthesis of secondary piRNAs via the ping-pong pathway. Like MIWI2, MIWI associated with pachytene piRNAs, which are expressed starting at the pachytene stage of meiosis in mouse spermatogenesis, is also barely involved in the ping-pong pathway. Instead, Aub and AGO3 play crucial roles in the secondary piRNAs synthesis. Like the primary piRNAs, secondary piRNAs are 2'-O-methylated by Hen1/Pimet (Ishizu et al., 2012; Iwasaki et al., 2015).

Functionally, PIWIs associate with piRNAs to regulate transposon activity by RNA silencing or epigenetic regulation. It is reported that MILI and MIWI2 are needed for the methylation of genomic regions encoding transposons (Aravin et al., 2007; Kawaoka et al., 2008). PIWI physically binds heterochromatin protein 1 (HP1a) both in vivo and in vitro, leading to loss of heterochromatin. One common feature of defects in PIWI proteins is the increased DNA damage as measured by the foci of γ-H2Av, a histone H2A variant present at sites of dsDNA damage (Klattenhoff et al., 2007). MIWI and piRNAs are associated with polysomes, translation initiation factor eIF4E, and microRNA processor DICER, indicating multiple roles in regulation of protein translation and mRNAs stability (Grivna et al., 2006; Thomson and Lin, 2009).

PIWI proteins play crucial roles in meiosis. PIWIs mutations cause meiosis disorders. For example, MIWI2 mutants show predominant meiosis arrest at the leptotene stage (Thomson and Lin, 2009).

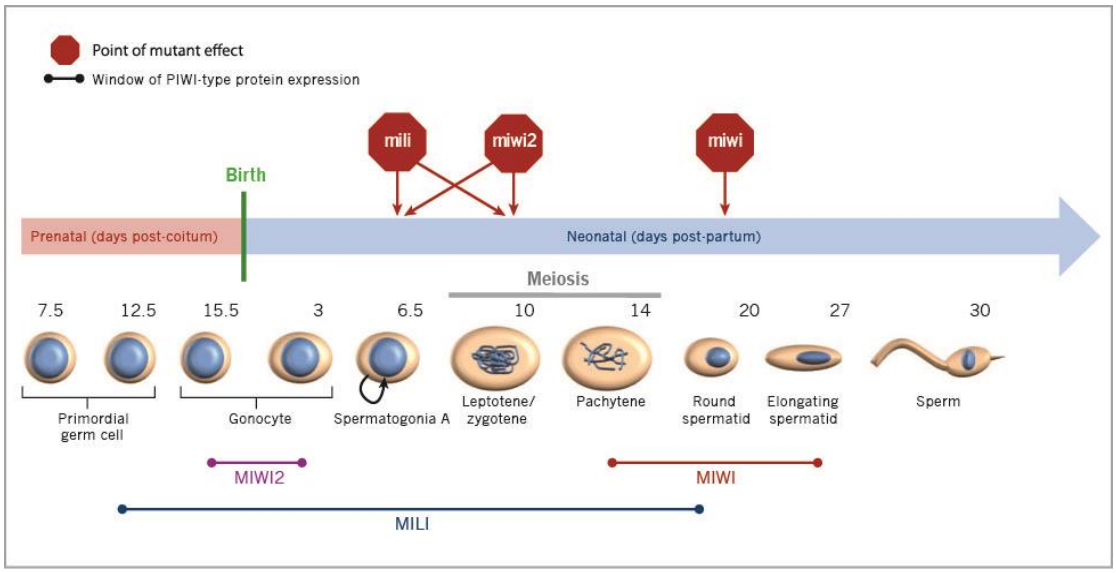


Figure 3 | The expression time course of PIWI proteins during mammalian spermatogenesis. (Thomson and Lin, 2009).

PIWI protein expression is mostly restricted to the germline cells in different stages of the germline cycle (**Figure 3**). All three murine PIWI proteins, MIWI, MILI, and MIWI2, are expressed during spermatogenesis. More specifically, MIWI2 is expressed from 15.5 dpc to 3 dpp in mitotically arrested prenatal germline stem cells (GSC), although also in Sertoli cells not necessary for a germline function. MILI expression is detected from 3 dpp in mitotically arrested prenatal GSCs to round spermatids. MIWI is merely expressed from meiotic spermatocytes to elongating spermatids.

Furthermore, PIWIs is not only expressed in germline, but also detected in somatic tissues. In *Drosophila* somatic tissues such as salivary glands and eyes, PIWIs bind to chromosomes and are associated with epigenetic effects at the binding sites. In planaria, PIWI proteins are expressed in neoblasts that are capable of tissue regeneration. In human, HIWI is detected in hematopoietic stem cells but not in the downstream progenitors and mature immune cells. Particularly, PIWIs are expressed in a wide variety of human cancers (**Table 3**)(Suzuki et al., 2012). For example, the expression of HIWI is positively correlated with glioma grade: high HIWI expression has poorer clinical outcomes (Sun et al., 2011), although it is unclear whether or how the piRNAs are involved in this process.

Table 3 | PIWI expression in human cancers (Suzuki et al., 2012)

Disease	**Material**	**PIWI**	**Method**
Breast cancer	Tissue, MDA-MB-231	HILI	RT-PCR, RNA array, WB, IC
Breast cancer	Tissue	HILI	IHC
Breast, cervical, and other cancers	MDA-MB-231, MDA-MB-468, MCF-7, HeLa, THP-1, CCRF, Jurkat, H9, Raji, Daudi, HEL, Dami, HL-60, K562, PBL985, HCT-8, 3B11, CaoV3, CaCo, HT-29, SW480, Huh7, CT26CL25, Hey1B, SW872, H1299, C8161, CT26CL25, Hey1B, SW872, H1299, C8161, HepG2, INS-1, LL2, N2a	HILI, PL2L50, PL2L60, PL2L80	RT-PCR, WB, IHC
Cervical cancer	Tissue	HIWI	IHC
Cervical cancer	Tissue	HILI	IHC

Cervical cancer	HeLa	HILI	WB
Colon cancer	Tissue	HIWI	IHC
Colorectal and other cancers	Human tissue, 823, AGS, N87, GES1, E30, E70, E140, E180, E410, HepG2, 7402, 7721, YES2, T12, LoVo, CL187, HT-29, RKO, SW480, HCT116, PG, GLC82, H446, H460, H1299, A549	HIWI	WB, IHC
Endometrial cancer	Tissue	HIWI	IHC
Esophageal cancer	Tissue, KYSE70, KYSE140, KYSE450	HIWI	WB, IC, IHC
Gastric cancer	Tissue	HIWI, HILI, PIWIL3, HIWI2	IHC
Gastric cancer	Tissue, AGS, NCI-N87, SNU-1, SNU-5, SNU-16	HIWI	RT-PCR, IHC, WB
Glioma	Tissue, U251, U87, LN229	HIWI	RT-PCR, WB, IHC
Liver cancer	Tissue, HepG2, SMMC7721, MHCC97L, MHCC97H, HCCLM3	HIWI	qRT-PCR, WB, IHC
Ovarian cancer	A2780, CP70, CDDP, MCP2, MCP3, MCP8, 2008, 2008C13	HILI	WB
Pancreatic cancer	Tissue	HIWI	qRT-PCR, IHC
Sarcoma	Tissue	HIWI	qRT-PCR
Sarcoma	Tissue, MFH	HIWI	IHC
Seminoma	Tissue	HIWI	qRT-PCR
Seminoma and other cancers	Tissue, MDA-MB-231	HILI	RT-PCR, IC, IHC

■ siRNAs and their biogenesis

Short interfering RNAs (siRNAs), also known as small interfering RNAs or silencing RNAs, are a class of double-stranded RNA molecules, 20-23 nt in length. Like miRNAs, siRNAs cleave and degrade target mRNAs guided by base pairing in the RNA interference (RNAi) pathway (Agrawal et al., 2003). Moreover, some siRNAs can base pair with DNA and induce DNA methylation to regulate gene expression (Kawasaki and Taira, 2004). Furthermore, siRNAs also play crucial roles in virus defense and chromatin remodeling.

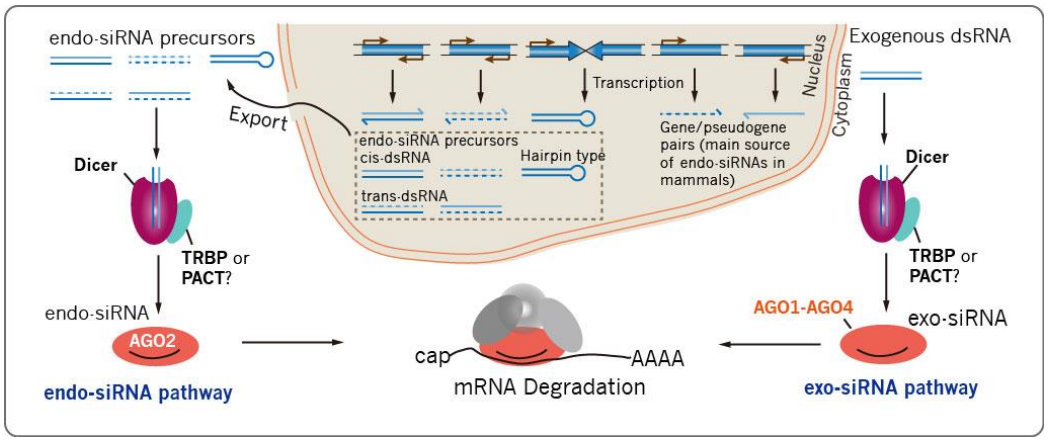


Figure 4 | Biogenesis of siRNAs in mammals.

Exogenous siRNAs (exo-siRNAs) are originated from foreign dsRNAs taken up from outside the cells, for example, virus infection and therapeutic dsRNAs. Endogenous siRNAs (endo-siRNAs), on the other hand, arise from genomic loci, such as centromeres, transposons, and inverted repetitive sequences that produce transcripts capable of forming dsRNA structures. Bidirectional transcription, antisense transcripts, or pseudogenes at the same (cis-nat-siRNA clusters) or different loci (trans-nat-siRNA clusters) are also the

sources of the dsRNAs (Carthew and Sontheimer, 2009; Watanabe et al., 2008). Although both endo-siRNAs and miRNAs function as RISC, miRNAs arise from the ~60-70 nt precursors of intramolecular stem-loop structures that lack perfect Watson-Crick base pairing, whereas siRNAs can be processed from duplex structures that are perfectly base paired (Golden et al., 2008). The other significant difference is that siRNAs carry 2'-O-methyl modifications at their 3' termini, whereas miRNAs do not (**Table 1**).

Exo-siRNAs derived from exogenous dsRNAs are processed by Dicer and Dicer binding proteins (Dicer-TRBP or Dicer–PACT). The processing of endo-siRNAs also requires Dicer, however the role of TRBP and PACT remains undetermined. Mature endo-siRNAs are loaded onto Argonaute 2 (AGO2). Whether endo-siRNAs are loaded onto other AGO members, such as AGO1, AGO3 and AGO4, remains to be determined. In mammals, exo-siRNAs are loaded onto AGO1, AGO2, AGO3 and AGO4; however, only the AGO2–siRNA complex functions in RNA interference, as other AGO members lack Slicer activity (Siomi et al., 2011)(**Figure 4**).

The main function of siRNAs is gene silencing via mRNA cleavage by RNA-induced Silencing Complex (RISC). siRNAs involved in DNA methylation also play a crucial role in chromatin remodeling. Biologically, endo-siRNAs keep tabs on domestic miscreants such as transposons, whereas exogenous siRNAs are called upon to defend against foreign opponents such as viruses (Golden et al., 2008).

In *Drosophila*, mutants of key protein components in the siRNA silencing pathway are viable and fertile, but highly susceptible to virus infection (Marques and Carthew, 2007). Additionally, transposon mRNAs are increased 2 ~ 10-fold in the heads and ovaries of AGO2 mutants, in the heads of DICER2, and in the S2 cells with DICER2 or AGO2 knockdown. However, no significant increase in transposon transcripts was apparent after DICER1 knockdown (Marques and Carthew, 2007). The levels of the 3' overlapping transcripts Pdzd11 and Kif4 in the cis-nat-siRNA cluster increased modestly in DICER mutant mice (Watanabe et al., 2008).

C. Other Types of Small RNAs

Apart from small regulatory RNAs, there are diverse small RNAs playing vital roles in cellular activities and human diseases. Transfer RNA (tRNA), small nucleolar RNA (snoRNA) and small nuclear RNA (snRNA) have well established canonical functions and have received increasing attention for their new non-canonical biological activities recently. As a new class of small RNAs, tRNA-related fragments (tRF) and tRNA halves (tiRNA) are generated from tRNAs via precise biogenesis mechanism, having functions different from the parent tRNAs.

■ tRNAs

The principal function of tRNAs is decoding mRNAs and protein translation. tRNAs are composed of 73~95 nucleotides and made up of the D-loop, T-loop, anticodon loop and variable loop in the cloverleaf structure representation. Alteration of tRNA repertoire affects mRNA translation efficiency (Gorochowski et al., 2015) and mRNA stability (Gingold et al., 2014). tRNA-related fragments (tRF) and tRNA halves (tiRNA) are derived from tRNAs by angiogenin (ANG), Dicer, and other nuclease cleavages. This new class of small RNAs has recently gained discovered for their surprising roles in biological processes and human diseases (Fu et al., 2015; Kirchner and Ignatova, 2015; Raina and Ibba, 2014).

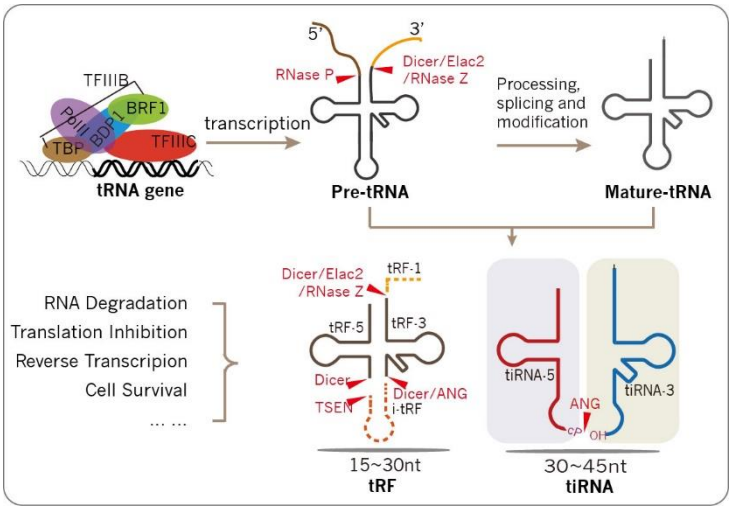


Figure 5 | Biogenesis and functions of tRNA, tRF and tiRNA.

tRNA biogenesis comprises multiple processes of transcription, processing, splicing, chemical group modification, CCA addition, nuclear-to-cytoplasmic transportation, and aminoacylation. tRNA precursors (pre-tRNA) are transcribed from individual tRNA genes by RNA polymerase III (Pol III), transcription factor TFIIIC and TFIIIB (consist of BDP1, BRF1 and TBP) (**Figure 5**). The pre-tRNAs are removed of the 50- and 30-nt trailers by endonucleases RNase P and RNase Z, modified with chemical groups, and added with the terminal CCA by nucleotidyl transferase. Finally, the mature tRNAs are exported to the cytoplasm by exportin-T (XPOT) to participate in protein synthesis (Phizicky and Hopper, 2010). Accurate amino acid charging by aminoacylation is catalyzed by aminoacyl tRNA synthetases (aaRS).

The diverse and extensive post-transcriptional alterations of pre-tRNAs are one of the most striking features of tRNA biogenesis and maturation. RNA editing and alternative splicing are highest in the brain compared with other tissues, which has important implications in the central nervous system (CNS). Polyribonucleotide 5'-hydroxyl-kinase CLP1 is an enzyme in the steps of tRNA intron splicing. CLP1 mutation causes accumulation of toxic pre-tRNA splice intermediate, leading to motor sensory neuropathy and neurological diseases (Hanada et al., 2013; Karaca et al., 2014; Schaffer et al., 2014). Additionally, GCN2, a kinase played special role in tRNA metabolism, has an essential role in supporting tumor cell growth and proliferation (Anderson and Ivanov, 2014).

tRNA modifications are involved in all aspects of tRNA biochemistry, from secondary and tertiary structures, precise recognition by aminoacyl-tRNA synthetases, to mRNA decoding. Aberrant tRNA modifiers and tRNA modifications are linked to human diseases such as cancer, Type 2 diabetes, neurological disorders, and mitochondrial disorders (Torres et al., 2014). For example, tRNA methyltransferase 12 homolog (TRMT12), one of the enzymes that catalyze wybutosine modification at position 37 on $tRNA^{Phe}$, is amplified and over expressed in breast cancer cell lines tumors.

■ tRNA derived fragments (tRF) and tRNA halves (tiRNA)

tRFs are derived from tRNAs by precise enzymatic cleavages by endonucleases angiogenin (ANG) and Dicer to produce tRF-5 and tRF-3 (**Figure 5**). Further, tRNA-splicing endonuclease (TSEN) complex excises the pre-tRNAs and produces i-tRF in nucleus (Anderson and Ivanov, 2014) (**Figure 5**). Additionally, tRF-1 is generated from the 3' tails of pre-tRNAs by Dicer, RNase Z, and zinc phosphodiesterase ELAC protein 2 (ELAC2). tiRNAs are tRNA halves generated by angiogenin cleavage in the anticodon loop of mature tRNAs.

tRFs and tiRNAs are not random degradation products of tRNAs. Rather they are a class of bioactive functional small RNAs. They are known to act as microRNAs in RNA interference; directly inhibit protein

synthesis by displacing eIF4G translation initiation factor eIF4G from mRNA on ribosomes [9-10]; bind protein factors such as CBX1 to regulate target mRNA stability; interact with cytochrome c to modulate apoptosis; assemble stress granules in response to stress conditions; sensitize cells to oxidative-stress-induced p53 activation and p53-dependent cell death; alter transcriptional cascades in intergenerational inheritance as paternal epigenetic factors. Clinically, tRF/tiRNAs are associated with or are causal factors for disease conditions including cancers, neurodegeneration, and metabolic disorders. Due to the high enrichment in biofluids, sometimes more so than microRNAs, tRF/tiRNA populations have many desired properties as biomarkers. For example, the tRF profiles have been shown to discriminate triple-negative, triple positive breast cancer cells from the normal controls.

snRNAs/snoRNAs and their biogenesis

Small nuclear RNAs (snRNA) and small nucleolar RNAs (snoRNAs) are two well-studied classes of ncRNAs in the form of ribonucleoproteins (RNPs). snRNPs form the core of the spliceosome and catalyze the removal of introns from pre-mRNA. snoRNAs are involved in the modification and processing of pre-ribosomal RNAs. Additionally, snoRNAs are essential for major biological processes including protein translation, mRNA splicing and genome stability (Dragon et al., 2006; Matera et al., 2007).

According to the common sequence features and protein cofactors, the snRNAs can be divided into two classes: Sm- and Lsm-class snRNAs (**Figure 6A,B**) (Matera et al., 2007). Sm-class genes are transcribed by a specialized form of RNA polymerase II (Pol II). Integrator complex subunits INT9 and INT11 are essential for the proper cleavage and polyadenylation of the 3' ends of snRNAs. Following transcription and 3' processing in the nucleus, Sm-class snRNAs are transported to the cytoplasm by an export complex that contains phosphorylated adaptor for RNA export (PHAX), exportin 1 (CRM1), cap binding complex protein (CBC), and Ran GTPase (**Figure 7**). The export complex dissociates from the pre-snRNA in the cytoplasm after binding with the assemblyosome SMN complex. The SMN complex recruits a set of seven Sm proteins to form-core RNP. Following assembly of the Sm core, the m^7G cap is hypermethylated by TGS1 to form a 2,2,7-trimethylguanosine (TMG) cap structure, and the 3' end is trimmed by an unknown exonuclease. Triggered by TMG cap, SPN associates with Imp-β to assemble the nuclear import complex. After transported into the nucleus, the Sm-class snRNPs target to Cajal bodies for snRNP maturation. Additional RNP remodeling and assembly steps are thought to take place in Cajal bodies, including RNA-guided modification of the spliceosomal snRNAs and assembly of factors that are specific to a given species of snRNP. Finally, the newly minted snRNPs either participate in splicing at perichromatin fibrils (PFs) or are stored in interchromatin granule clusters (IGCs) for later use. With great difficulty, Lsm-class snRNA genes are transcribed by Pol III using specialized external promoters. The run of uridines that forms the Lsm binding site at the 3' end also doubles as a Pol III transcription terminator. Therefore, there are few parallels between Lsm-class genes and protein-coding genes. Additionally, Lsm-class snRNAs never leave the nucleus during the maturation process(Matera et al., 2007).

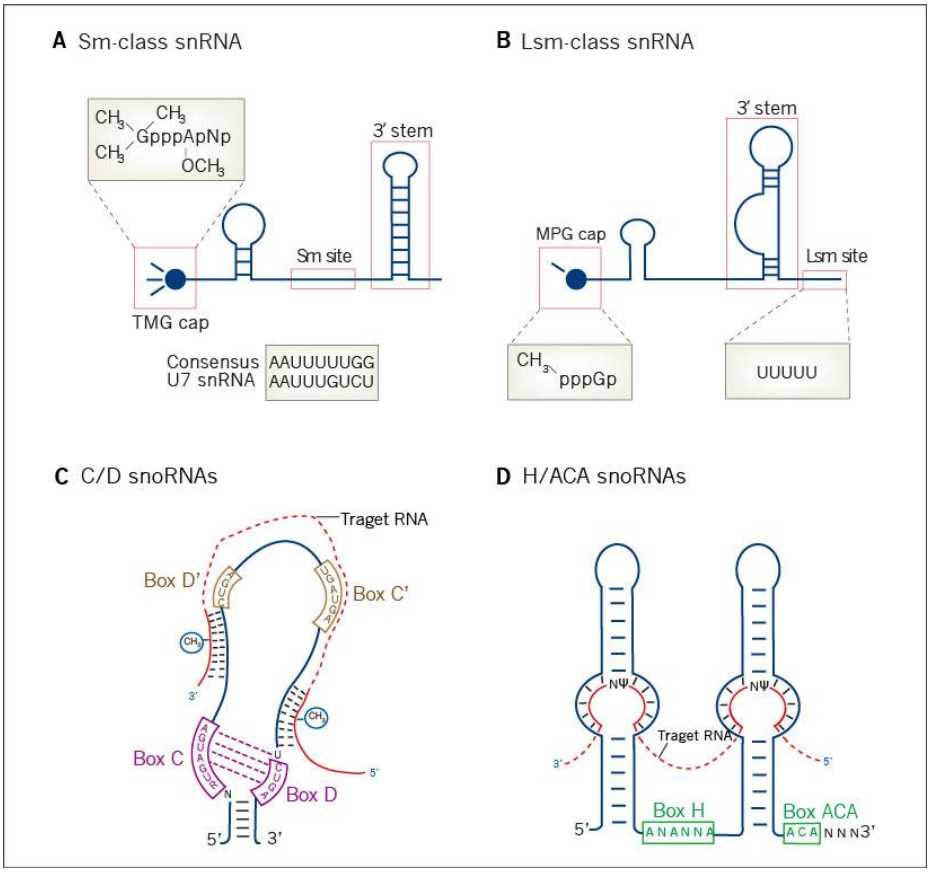


Figure 6 | The features of snRNAs and snoRNAs (Matera et al., 2007). (A) Sm-class RNAs typically contain a 5'-trimethylguanosine cap, a 3' stem-loop and a Sm proteins binding site. (B) Lsm-class RNAs are characterized a monomethylphosphate cap and a 3' stem-loop, terminating in a Lsm proteins binding site that is made up of a stretch of uridines. (C) C/D RNAs are characterized by conserved motifs C and D that form a kink-turn (K-turn) or alternate boxes C' and D' (orange) that could also form a K-turn; 2'-O-ribose methylation is performed on the rRNA residue that is base-paired to the fifth position upstream from box D (or D'). (D) H/ACA RNAs adopt a hairpin-hinge-hairpin-tail structure where box H is found in the hinge region and box ACA is found three nucleotides upstream of the 3'- end; each hairpin usually contains an internal loop called pseudouridylation pocket where C formation in rRNA occurs on the first unpaired U residue upstream from box H or ACA.

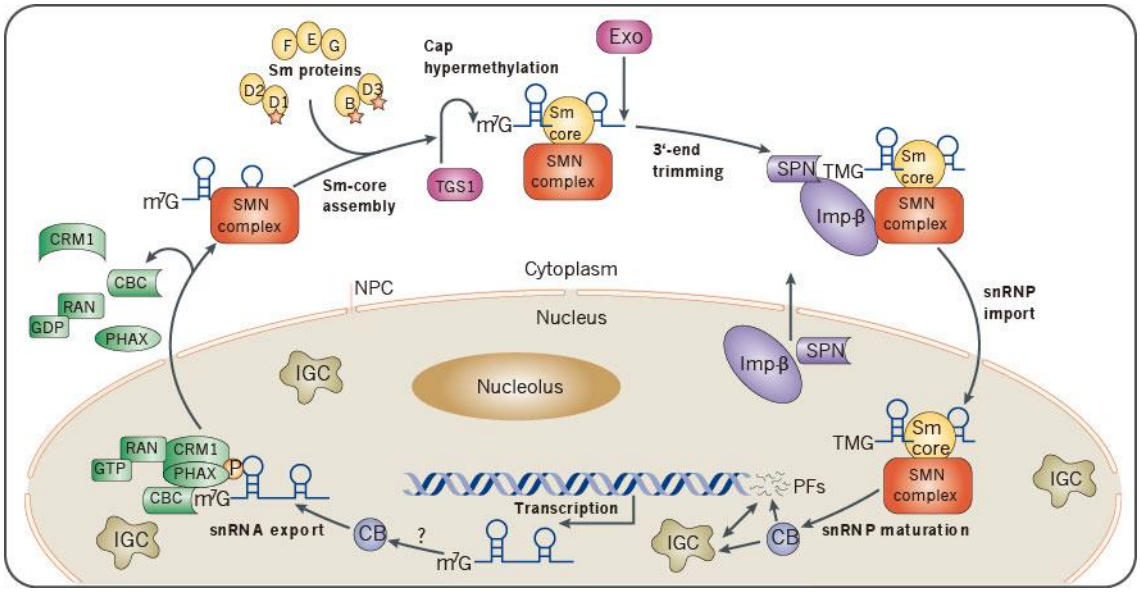


Figure 7 | Biogenesis of Sm-class snRNPs (Matera et al., 2007)

Similarly, snoRNAs can be grouped into two major families on the basis of conserved sequence motifs: C/D and H/ACA snoRNAs (**Figure 6C,D**) (Dragon et al., 2006). C/D RNAs direct 2'-O-ribose methylation, whereas H/ACA RNAs guide pseudouridylation. Additionally, other modification targets include snRNAs in eukaryotes, tRNAs in archaea, spliced leader RNAs in trypanosomes and perhaps at least one brain-specific mRNA in mammals. Spliceosome function also depends on the modification of snRNAs by C/D and H/ACA RNAs. Moreover, a H/ACA telomerase RNA is required for telomere synthesis.

snoRNAs are predominantly located in introns of mRNAs and transcribed by pol II (**Figure 8**). During the transcription, three of the four core H/ACA RNP proteins, DKC1, Nop10 and Nhp2, and an assembly factor Naf1 associate with the snoRNAs. However, the core RNP proteins of C/D RNP include fibrillarin, NHPX, NOP56 and NOP58, which are different from H/ACA RNPs'. Naf1 ensures the assembly of a stable H/ACA pre-RNP which is inactive until Naf1 is exchanged for Gar1. Naf1 is required only for the accumulation of all classes of H/ACA RNA, whereas Gar1 is essential for the function of H/ACA RNPs. The assembly of snoRNPs seems to occur co-transcriptionally and to be tightly coupled to pre-mRNA splicing by IBP160 (**Figure 8**). For C/D RNAs, the assembly of C/D RNPs requires a potential exchange factor Bcd1 that acts the same role as Naf1 in H/ACA RNPs assembly. Both C/D and H/ACA RNPs are rapidly targeted to Cajal bodies where their essential maturation steps occur. Additionally, PHAX may help to localize certain C/D and H/ACA RNAs to Cajal bodies for RNA modification and RNP assembly, where TGS1 and SMN complex are two important components. Depending on their functions, the C/D and H/ACA RNAs ultimately localize to nucleoli, Cajal bodies or telomeres (Esteller, 2011; Matera et al., 2007).

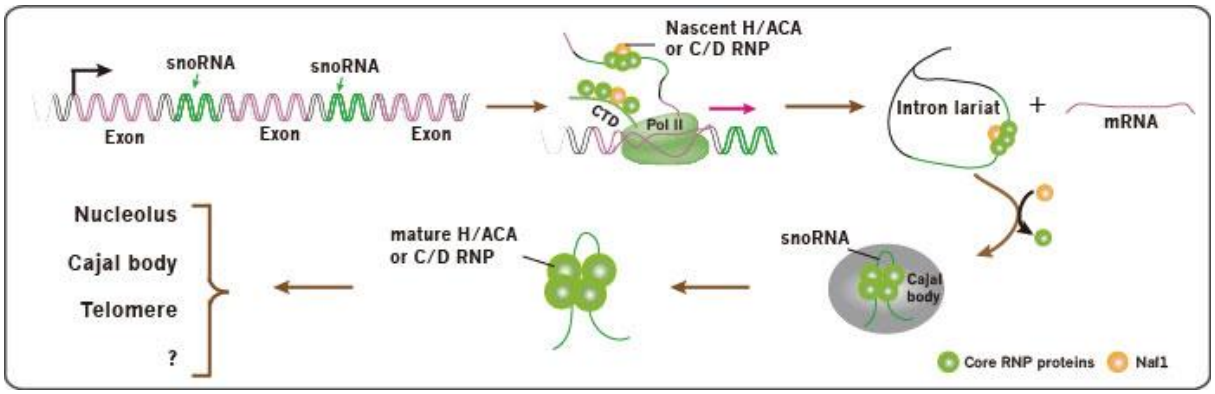


Figure 8 | Biogenesis of snoRNPs.

As described above, the C/D RNPs consist of a core of four proteins — fibrillarin, NOP56, NOP58 and NHP2L1 — whereas the H/ACA RNPs contain DCK1, GAR1, NHP2 and NOP10. Fibrillarin is essential for development, and its depletion is lethal in embryos. Mutations in the human DKC1, NOP10 and NHP2 genes are associated with the X-linked genetic disorder dyskeratosis congenita, which is susceptible to epithelial cancers. Biallelic null mutations in U6 snRNA biogenesis phosphodiesterase 1 (Usb1) cause poikiloderma with neutropenia (PN), which is predisposed to developing myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). In *S. pombe*, loss of Usb1 (Mpn1) function leads to an increase in TERRA telomere transcripts and decrease in telomere length. Disorder of Cajal bodies, a subnuclear structure for snRNA and snoRNA maturation, occurs in human pathologies including cancers, inherited neurodegenerative diseases, aberrant cellular proliferation, cell cycle, stress response, and aging (Cioce and Lamond, 2005; Mroczek and Dziembowski, 2013; Stepanov et al., 2015).

D. Product summary

NuRNA™ Human Small RNA Biogenesis Proteins PCR Array profiles the transcript expressions of 185 protein coding genes for the enzymes and protein factors critically involved in the small RNA biogenesis pathways. The Array simultaneously analyzes the biogenesis pathways for 7 small RNA biotypes: miRNA, piRNA, siRNA, snoRNA, snRNA, tRNA, and tRNA-related fragments (tRF). It is a rapid, accurate and convenient tool to study small RNA biogenesis, regulation and functional/disease implications in small RNA research.

■ Kit Contents

Figure 9 | Array layout for NuRNA™ Human Small RNA Biogenesis Proteins PCR Array.

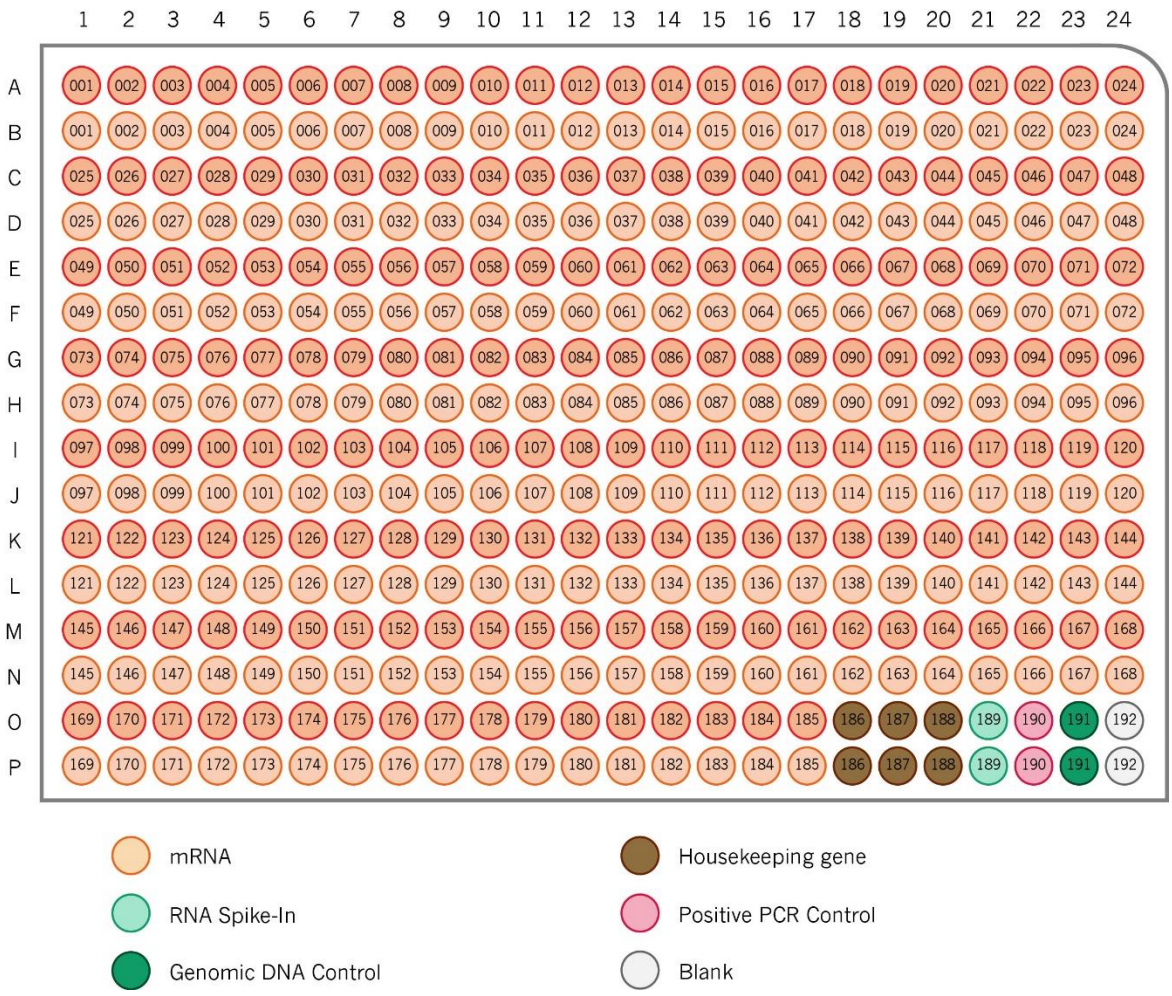


Table 4 | List of Human Small RNA Biogenesis Proteins and the Controls

1	AGO3	33	RELA	65	PIWIL1	97	RUVBL1	129	RPAP2	161	NUPL2
2	AGO4	34	RPL7A	66	PIWIL2	98	RUVBL2	130	SMN1	162	POM121C
3	BCDIN3D	35	RQCD1	67	PLD6	99	SNU13	131	SNAPC1	163	POP1
4	CNOT1	36	SMAD1	68	TDRD1	100	ZNHIT6	132	SNAPC2	164	POP4
5	CNOT10	37	SMAD2	69	TDRD12	101	DKC1	133	SNAPC3	165	POP5
6	CNOT11	38	SMAD3	70	TDRD6	102	EXOSC2	134	SNAPC4	166	POP7
7	CNOT2	39	SNIP1	71	TDRD7	103	EXOSC3	135	SNAPC5	167	PTCD1
8	CNOT3	40	SRRT	72	TDRD9	104	EXOSC4	136	SNUPN	168	RAE1
9	CNOT4	41	SUPV3L1	73	TDRKH	105	EXOSC5	137	TGS1	169	RANBP2
10	CNOT6	42	TNRC6A	74	PIWIL4	106	EXOSC6	138	TUT1	170	RPP14
11	CNOT6L	43	TNRC6B	75	CELF1	107	NOP10	139	USB1	171	RPP21
12	CNOT7	44	TNRC6C	76	CLP1	108	CPSF3L	140	XPO1	172	RPP25
13	CNOT8	45	ZC3H12A	77	MBD2	109	EXOSC7	141	ZPR1	173	RPP30
14	DIS3L2	46	ZCCHC11	78	MBD3	110	EXOSC8	142	ANG	174	RPP38
15	EIF6	47	ADAR	79	MECP2	111	EXOSC9	143	Ybx1	175	RPP40
16	ESR1	48	DROSHA	80	NRDE2	112	INTS1	144	EIF2AK4	176	TPR
17	ETS1	49	MRPL44	81	TERT	113	INTS10	145	HSD17B10	177	TRMT10C
18	FOSL1	50	PRKRA	82	TLR7	114	INTS12	146	KIAA0391	178	TRNT1
19	HNRNPA2B1	51	XPO5	83	TLR9	115	INTS2	147	NUP107	179	TSEN15
20	HRAS	52	AGO1	84	AQR	116	INTS3	148	NUP153	180	TSEN2
21	KHSRP	53	AGO2	85	FBL	117	INTS4	149	NUP155	181	TSEN34
22	LIN28B	54	DICER1	86	FBLL1	118	INTS5	150	NUP160	182	TSEN54
23	METTL14	55	DGCR8	87	GAR1	119	INTS6	151	NUP188	183	XPOT
24	METTL3	56	RAN	88	NAF1	120	INTS7	152	NUP210	184	ELAC1
25	MOV10	57	TARBP2	89	NOP56	121	INTS8	153	NUP214	185	ELAC2
26	NCOR1	58	ASZ1	90	NOP58	122	INTS9	154	NUP35	186	GAPDH
27	NCOR2	59	DDX4	91	NUDT16	123	KPNB1	155	NUP37	187	ACTB
28	NFKB1	60	EXD1	92	PIH1D1	124	MEPCE	156	NUP43	188	B2M
29	PNPT1	61	FKBP6	93	PRPF31	125	NCBP1	157	NUP54	189	RNA Spike-In
30	PPP3CA	62	HENMT1	94	RNF113A	126	NCBP2	158	NUP62	190	PPC
31	PTBP1	63	MAEL	95	RNF113B	127	NHP2	159	NUP93	191	GDC
32	RBM4	64	MOV10L1	96	RPAP3	128	PHAX	160	NUP98	192	Blank

Description of the control assays

NuRNA™ Human Small RNA Biogenesis Proteins PCR Array includes a series of external and internal controls for effective correction and normalization of sample and qPCR variabilities. In addition, Positive PCR Control and Genomic DNA Control are included to monitor the experiment process and the quality of RNA sample. These controls are described below.

- **HK** (Housekeeping genes; Internal Controls): Three stably expressed housekeeping genes GAPDH (HK-1), ACTB (HK-2), and B2M (HK-3) are included in the array as the internal qPCR normalization references. Arraystar PCR system provides multiple reference genes selected among commonly used reference genes by using a stringent bioinformatic algorithm, which offers the flexibility of choosing the most valid reference gene(s) for qPCR normalization for your sample types.

- **RNA Spike-In** (External Control): One External RNA Spike-In Mix is added in the RNA sample prior to the first strand cDNA synthesis. The RNA Spike-In control assay indicates the overall success and the efficiency of the reaction beginning from the cDNA synthesis to the final qPCR. Any problem(s) in these steps will result in a failed or compromised RNA Spike-In outcome. RNA spike-in assay results for samples are compared and outliers or failed reactions may be identified and excluded from further data analysis.

- **PPC** (Positive PCR control)**:** The artificial DNAs and the PCR primer pairs are applied to indicate the qPCR amplification efficiency. A Ct value greater than 25 is an indication of low qPCR amplification efficiency. More importantly, the PPC are used as inter-plate calibrator (IPC) between PCR plate runs to give the same Ct value for the calibrator, thereby reducing run-to-run variance. Inter-plate calibration (IPC) can easily be performed with the data analysis software avaliable on our website (www.arraystar.com).

- **GDC** (Genomic DNA Control): The control assay consists of PCR primers for an untranscribed genomic region. Non-RT sample or RNA sample are added during the qPCR Process. The Ct values should be greater than 35. A positive GDC signal indicates the array result may have been compromised with genomic DNA contamination.

Shipping and Storage

Arraystar PCR Arrays are shipped at ambient temperature, on ice, or on dry ice depending on the destination and accompanying products. Store at –20°C upon receipt. The contents are stable for at least 6 months.

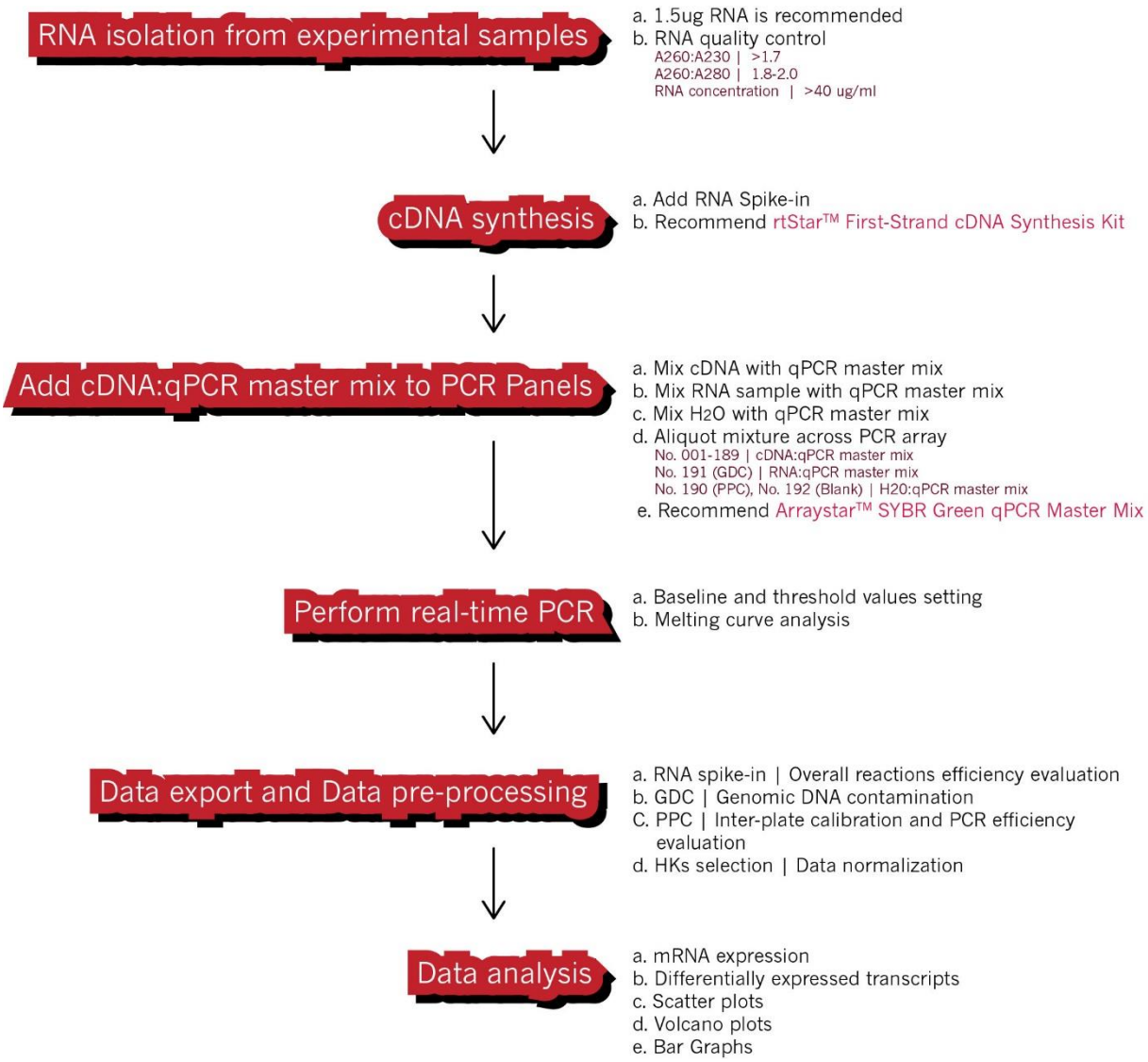
Additional Required Equipment

- Thermal cycler
- Real time qPCR instrument, compatible with 384-well format

Additional Required Reagents

- rtStar™ First-Strand cDNA Synthesis Kit (Cat# AS-FS-001)
- Arraystar SYBR® Green qPCR Master Mix(ROX+) (AS-MR-006-5)
- Nuclease-free PCR-grade water

E. Protocol overview



II. Protocol

IMPORTANT: Please read through this entire protocol before your first experiment. Prepare a workspace and use labwares free of DNA, RNA, and nuclease contamination. Wear gloves while performing this protocol.

A. RNA sample preparation and quality control

For best results from the PCR array, RNA samples must meet the QC standards of integrity and purity from protein, organics, genomic DNA contamination and excessive RNA degradation. You can measure the RNA concentration and purity by UV spectrophotometry. You can also check 18S and 28S ribosomal RNA as an indicator of RNA integrity by denaturing gel electrophoresis or by using an Agilent BioAnalyzer.

- A260:A230 ratio greater than 1.7.
- A260:A280 ratio between 1.8 and 2.0.

- Total RNA concentration greater than 40 ng/μl

Eliminating genomic DNA contamination is essential for accurate gene expression profiling by qPCR, which is particularly important for genes at low expression levels. Due to the presence of pseudogenes, even cross-intron primers are not a guarantee of avoiding amplification from contaminating genomic DNA. The Genomic DNA Control (GDC) in the PCR Array specifically detects potential genomic DNA contamination. A Ct for GDC less than 35 indicates the presence of genomic DNA contamination that may compromise the qPCR results.

B. First-strand cDNA synthesis

Use the same amount of total RNA in this reaction for every sample. High quality cDNA synthesis is vital for the following qPCR performance. We highly recommend using rtStar™ First-Strand cDNA Synthesis Kit (Cat# AS-FS-001), which is specifically optimized for and fully compatible with the Arraystar PCR Arrays.

NOTE: The recommended amount of starting material is 1 μg of total RNA. Lower amounts may reduce the assay sensitivity particularly for genes at lower expression levels.

1. Dilute Template Total RNA

   Adjust the volume of template RNA sample to 11 μl using Nuclease-free Water.

2. Prepare reagents

   Gently thaw all of the kit components except for Reverse Transcriptase, and immediately place on ice. Mix by vortexing. Spin down all reagents.

   NOTE: The first time to use this kit, please reconstitute the RNA Spike-in by adding 20 μl Nuclease-free Water to the tube. Mix by vortexing and spin down. Leave on ice for 20 ~ 30 min to fully dissolve the RNA Spike-in. Vortex again, then spin down.

3. Combine Annealing Mix according to Table

   If performing first-strand cDNA synthesis on multiple RNA samples, it is recommended to prepare an Annealing Mix of the Primers, dNTP Mix and RNA Spike-in (in the proportion indicated in Table). 10% excess volume for pipetting losses is recommended.

Oligo(dT)$_{18}$, or Random Primers	1.0 μl
dNTP Mix	1.5 μl
RNA Spike-in	1.0 μl
Template Total RNA	10.5 μl
Total volume	14.0 μl

4. Incubate in a thermal cycler at 65°C for 5 min, and then immediately place on ice for at least 1 min. Collect the contents of the tube by brief centrifugation.

5. Combine cDNA Synthesis Mix

cDNA Synthesis Mix is recommended to prepare for multiple RNA samples. It includes the components in the following table. 10% excess volume for pipetting losses is recommended.

5 × RT Reaction buffer	4.0 µl
0.1 M DTT	1.0 µl
RNase Inhibitor	0.6 µl
Reverse Transcriptase	0.4 µl
Total volume	6.0 µl

6. Add cDNA Synthesis Mix to the tube from STEP 4. Vortex the sample briefly to mix, and collect by brief centrifugation. Incubate at 50°C for 50 min.

7. Terminate the reactions at 85°C for 5 min. Chill on ice.

NOTE: Store the cDNA synthesis reaction at -20°C, or proceed directly to PCR. Reconstitute the RNA Spike-in qPCR Primer Mix by adding 100 µl Nuclease-free Water to the tube. Use 1 µl RNA Spike-in qPCR Primer Mix in 10 µl qPCR reaction system.

C. Perform qPCR for the PCR array

NOTE: The fellow operations are designed for one sample. If repetitive experiments are planned, the volume of the reagent should be accordingly increased.

1. Dilute the cDNA in Nuclease-free Water. If 1 µg input RNA is used with rtStar™ First-Strand cDNA Synthesis Kit (Cat#AS-FS-001), the dilution factor is 1:40. Mix well and spin down. The diluted cDNA is used as the qPCR template in the wells for Small RNAs Biogenesis Transcript Assays, Housekeeping gene Internal Controls, and Spike-in External Control.

2. For GDC Controls, combine 1.5 µl NRT (mock cDNA synthesis reaction without the reverse transcriptase) sample or 1.5 µl RNA sample (without cDNA synthesis), 7.5 µl SYBR Green Master Mix, and 6 µl Nuclease-free Water. Mix well and spin down.

3. For Blank Controls, combine 20 µL SYBR Green Master Mix and 20 µL Nuclease-free Water. Mix well and spin down.

4. Prepare the qPCR Mix according to the Table below. There are total of 192 wells of PCR reaction. Some extra amount is included for consumption by the liquid dispensing operation.

SYBR Green Master Mix	1000 µl
diluted cDNA template	800 µl
ddH_2O	200 µl
Total volume	2000 µl

5. Loading the PCR Array plate.

NOTE: The reagents should load to the related well number corroding to **Figure 9** and **Table 4**.

a. CAREFULLY remove the plate seal from the PCR Array;

b. Add 10 μl of the cocktail from STEP 4 to each PCR Array plate well (except No.190-No.192);

c. Add10 μl GDC Mixture aliquot from STEP 2 into the No.191 to detect genomic DNA contamination.

d. Add 10 μl Blank Mixture aliquot from STEP 3 into the No.190 and No.192.

e. CAREFULLY but tightly seal the PCR Array plate with the optical adhesive cover. Be sure that no bubbles appear in any of the wells. To remove bubbles, tap the plate gently on the bench top or centrifuge the plate briefly.

f. Keep the plate on ice while setting up the PCR program described in "Running Real-Time PCR Detection" below.

6. Running Real-Time PCR Detection

Cycles	Temperature	Time
1	95 ℃	10 minutes
40	95 ℃	15 seconds
	60 ℃	60 seconds
Melting curve analysis		

D. Data pre-processing and data analysis

Pre-process the qPCR run data according to the qPCR instrument manufacturer's instructions. If using baseline and threshold method for Ct calculations, such as with ABI 7900HT, the optimal baseline and threshold manual settings applied consistently across all assays on the plate are preferred over the software automatic settings for better reliability and accuracy.

Inspect the melting curve analysis of the post-PCR products to verify the amplification specificity. If the melting curve has multiple peaks or poor peak morphology deviated from that of the known single amplicon product, it may indicate non-specific off-target amplification or primer dimer formation, which will compromise the quantification. In such cases, gel electrophoresis or DNA fragment analysis can be performed to verify whether the product is from a single correct amplicon.

Export the raw Ct values to Excel. Free, easy-to-use data analysis software is available online, please refer to www.arraystar.com for detailed instruction. The data analysis procedures include:

■ Data pre-processing

1. Set all Ct values ≥ 35 or N/A (not detected) to 35. From this point on, any Ct value equal to 35 is considered as negative result.

2. Examine the Ct values of the Genomic DNA Control (GDC) wells. If the value is greater than 35, no genomic DNA contamination is detected and no action is needed. If the value is less than 35, genomic DNA contamination is evident and the result may be compromised.

3. Before initiating the data analysis, the RNA spike-in wells are compared. Outlier samples (Ct >25) may be identified and considered for exclusion in the further data analysis.

4. Inter-plate calibration (IPC) can be performed using the PPC assay replicates either with the data analysis software or manually. For each plate, verify that the replicates have Ct standard deviation ≤0.5. If this is not the case, exclude the outlier if it can be identified. Calculate the average of the replicates for each plate, the overall average (average of IPC values from all plates). The calibration factor for each plate is calculated as the difference between the plate average and the overall average:

$$calibration\ factor = IPC(plate\ n) - IPC(overall)$$

The Ct value is corrected with the calibration factor as

$$Ct_{RNA} = Ct_{RNA}(Raw\ value, plate\ n) - IPC(plate\ n) + IPC(overall)$$

or

$$Ct_{RNA} = Ct_{RNA}(Raw\ value, plate\ n) - calibration\ factor$$

	Plate 1	Plate 2	Plate 3
SNAPC2	20.26	20.83	20.44
IPC Plate average	16.70	16.70	16.40
IPC overall average	16.60	16.60	16.60
Calibration factor	0.10	0.10	-0.20
SNAPC2 (Calibrated)	20.16	20.73	20.64

5. Calculate the ΔCt for each mRNA in the plate.

$$\Delta Ct_{RNA} = Ct_{RNA} - average(Ct_{HKs})$$

Where average (Ct_{HKs}) is the average of the Ct values derived from the multiple reference genes. Three most stably expressed housekeeping genes were selected from abroad range of samples by our stringent algorithm that evaluates the optimal properties and the number for endogenous controls.

Data analysis

1. Calculate the ΔΔCt for each mRNA

$$\Delta\Delta Ct = \Delta Ct(sample\ 1) - \Delta Ct(sample\ 2), \text{ between samples}$$

or

$$\Delta\Delta Ct = \Delta Ct(group\ 1) - \Delta Ct(group\ 2), \text{ between groups}$$

2. Calculate the fold changes for each gene from sample 1 to sample 2 or group 1 to group 2 as following:

$$Fold\ Change = 2^{-\Delta\Delta Ct}$$

NOTE: By convention, if the fold change is greater than 1, the result is reported as a fold up-regulation. If the fold change is less than 1, its negative inverse is reported as a fold down-regulation.

3. When comparing profiling differences between two groups (such as disease versus control) with biological replicates, the statistical significance of the difference can be estimated as p-value by *t*-test. mRNAs having fold changes ≥ 2 and *p*-values ≤ 0.05 are selected as the significantly differentially expressed mRNAs.

NOTE: Fold change is related to biological effect size. Ranking by fold change is preferred over *p*-value. qPCR as commonly used in confirmation has a limit of quantification of 0.5 ΔCt, which is equivalent to approximately 2 fold change.

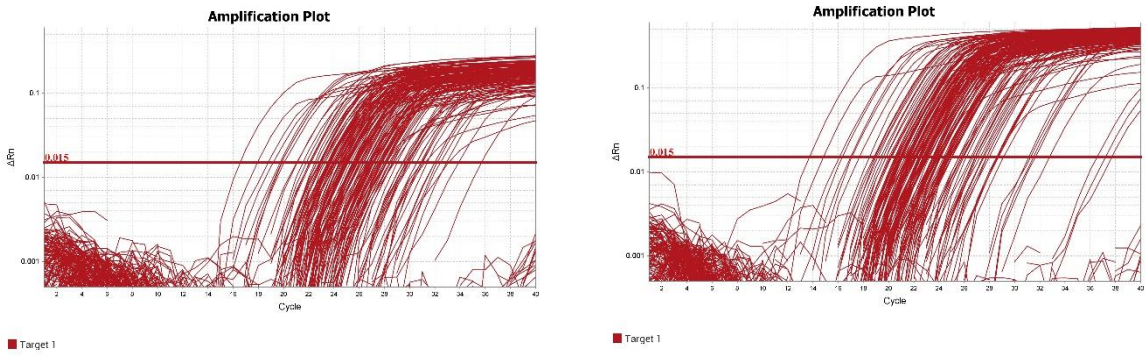
4. Other analyses such as scatter plots, volcano plots, list of differentially expressed genes and bar graph of expression differences for the mRNAs are performed and included in the standard analysis package.

III. Quality Control and Sample Data

A. NuRNA™ Human Small RNA Biogenesis Proteins PCR Array validation

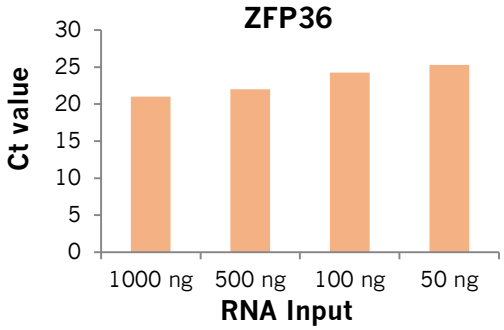
Real-time qPCR Validation

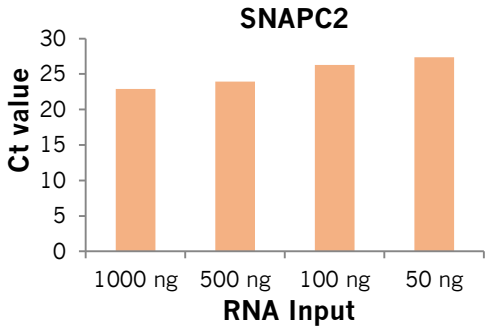
The performance of NuRNA™ Human Small RNAs Biogenesis Proteins PCR Panel was tested using a cohort of pancreatic carcinoma and para-carcinoma tissues. The extracted RNA samples were converted to cDNA using rtStar™ First-Strand cDNA Synthesis Kit (Cat#AS-FS-001). The cDNA were profiled using the PCR array according to aforementioned protocol without modification. The results are charted on the real-time amplification plots for the entire plate for the cell lines.



Sensitivity Test

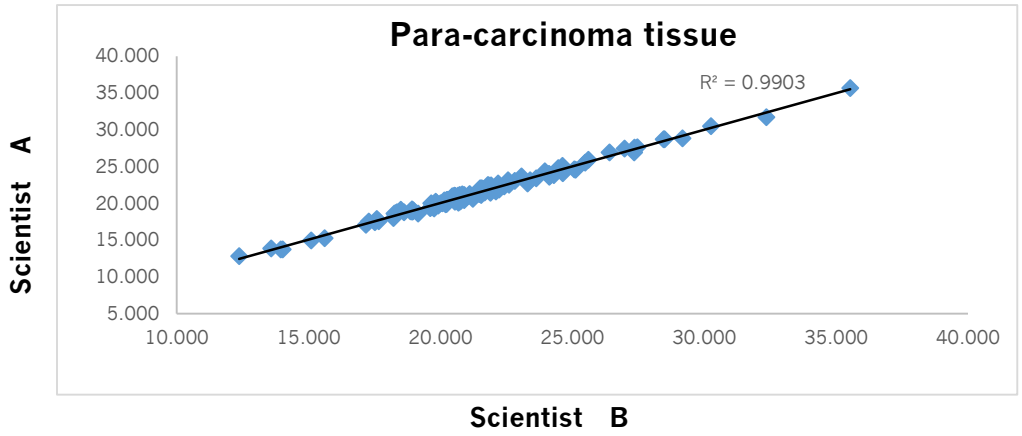
The decreasing input amounts of total RNAs from human glioblastoma cell lines were converted to cDNAs and profiled by the PCR arrays. The Ct values were determined using the software default automatic baseline and Ct settings. ZFP36 and SNAPC2 transcripts were detected at Ct values of 25.2 and 27.0 respectively, with the input RNA amount as low as 50 ng.

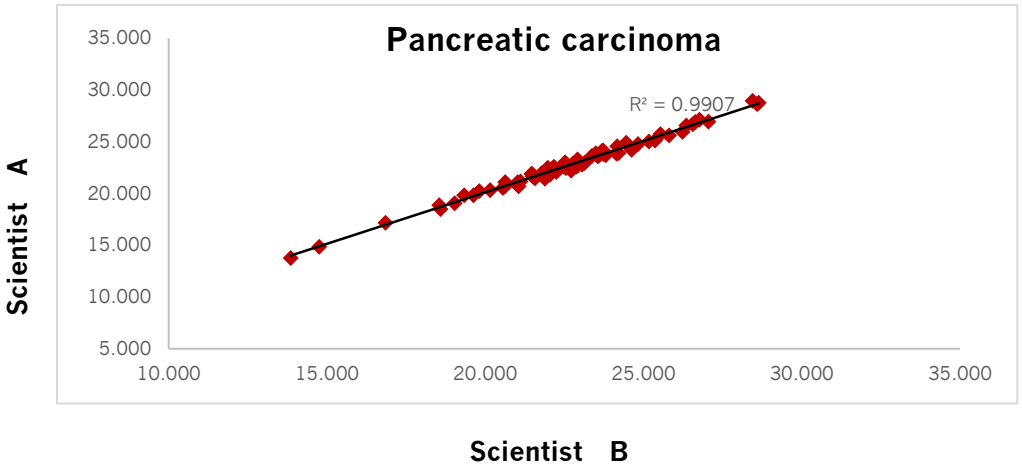




Reproducibility Test

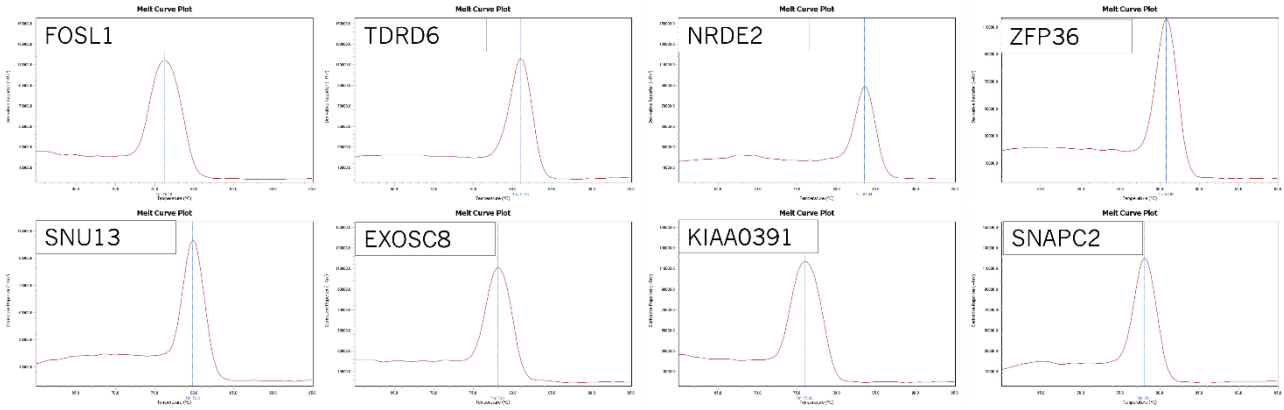
Two independent runs of NuRNA™ Human Small RNA Biogenesis Proteins PCR Array were conducted by two different scientists A and B at two different times using two different tissues. The results demonstrate a high degree of reproducibility with correlation R^2>0.98.





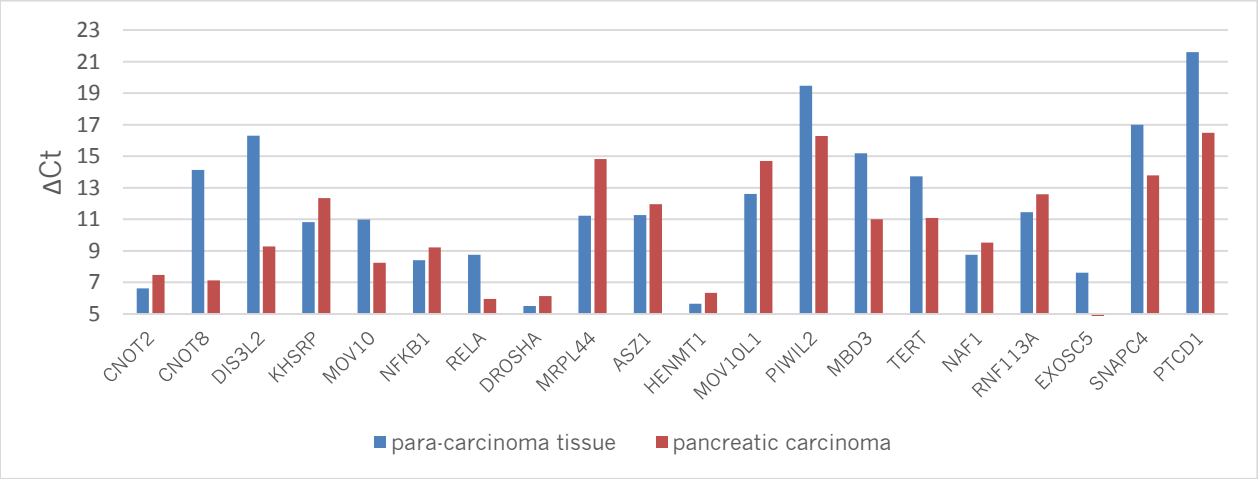
■ Specificity Test

The amplification products of transcripts of FOSL1, TDRD6, NRDE2, ZFP36, SNU13, EXOSC8, KIAA0391 and SNAPC2 were analyzed by melting curves, all of which had single sharp melting peaks. The results demonstrate the high amplification specificities for the transcripts with the assays on the array.



B. Sample data: Analysis of human small RNAs biogenesis proteins levels in tissues

The sample data were generated from RNAs extracted from pancreatic carcinoma and para-carcinoma tissues. The normalization was carried out using the average of the Internal Control Reference. ΔCt for the gene transcripts in the pancreatic carcinoma and para-carcinoma tissues are graphed in the bar chart below.



IV. Troubleshooting

Problem	Possible solution
qPCR background too high	• Reduce the amount of cDNA used in the SYBR® Green Master Mix.
No qPCR signals	• Inspect if the Internal Controls have valid qPCR signal • Set SYBR® Green as the Detector's Reporter Dye • Use more cDNA in the Master Mix • Lower the annealing temperature in Protocol STEP C.6 from 60°C to 50°C.

Baseline and threshold settings	• Follow the instructions of the qPCR system manufacturer. • Contact their technical support as necessary.

V. References

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VI. Technical Support

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