Supplemental Material and Method:

Genome wide mRNA microarray analysis and miRNA qPCR: To perform a genome wide analysis for mRNA and miRNA changes caused by Drosha and Dicer knockdown, we cultured eight 10 cm plates (approximate $8X10^6$ cells) of *Dicer*- and *Drosha* -knockdown as well as the control cells on matrigel plates. These cells were harvested after two washes in PBS. Six plates were lysed with Trizol for miRNA profiling and the other two were extracted by use of the RNeasy kit (Qiagen) for mRNA profiling. Each qPCR assay was validated against synthetic target molecules, allowing confident assessment of expression levels across the entire dynamic range of expression observed in biological samples (i.e. copy numbers from <1 to >1,000,000/10 pg total RNA). miRNAs were converted to cDNA in the context of total cellular RNA; modest amounts (~5 ug) of total RNA are required to obtain accurate measurements of ~200 miRNA species. Real-time qPCR data was regressed against standard curves and copies/10pg RNA (copies/cell) were calculated.

For statistical analysis of the data, Eisen Cluster and Tree view-programs were used. The ratios of the net expression levels between differentiated and undifferentiated ES cells were calculated for each pair after background subtraction (Ratio=D/UD). Because we are interested in miRNAs that are expressed abundantly in undifferentiated cells (UD), whenever the D value is 0 or below 0, we assigned it a value of 0.01. If the miRNA was downregulated during differentiation, the ratio should be less than 1.0. The following criteria were set: average of ratio ≤ 0.75, at least four out of five pairs of the ratios are less than 1.0 (consistency) and the average net expression level in UD group ≥ 10.0.

Microarray profiling of RNA extracted from the *Drosha* and *Dicer*-knockdown lines along with RNA from cells transduced with a control vector was carried out as described previously (Jackson et al., 2003).

Hierarchical clustering of miRNA expression was done using Spotfire DecisionSite 9.1 software (Tibco Software Inc), with the following calculation options: clustering method: single linkage (minimum), similarity measure: city block distance, ordering function: average value.

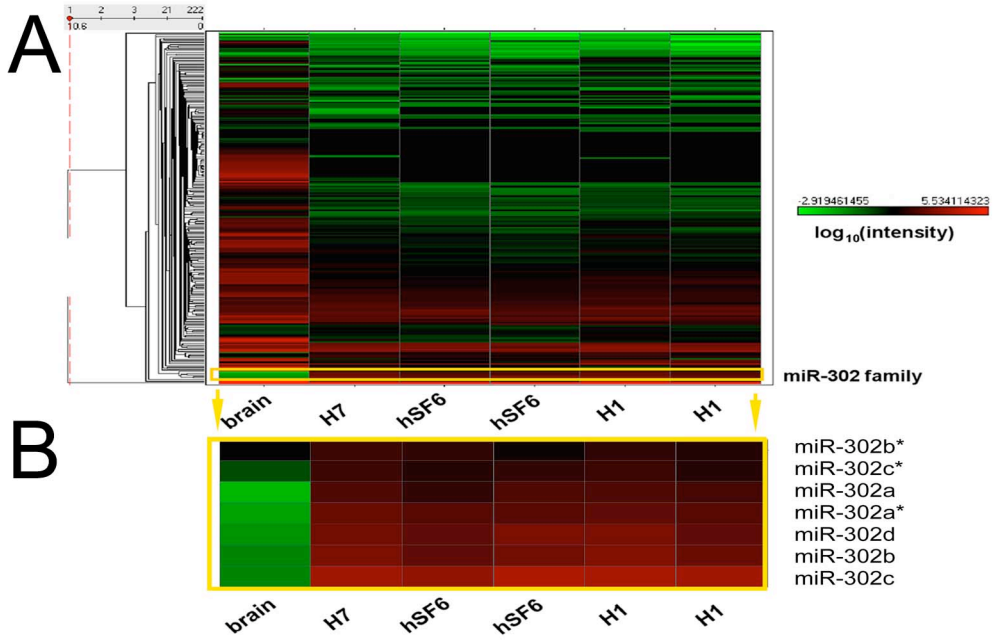
Karyotyping of the *Dicer* and ***Drosha*** *Dicer*-knockdown ***and control lines***: Karyotype analyses for the hESC lines were processed in the Cell Line Genetics (Madison, Wisconsin) and Cytogenetics lab at UW Seattle. To avoid selection for extra chromosomes, we kept the lines on feeders during growth and expansion periods. However, for three passages prior to experiments, we freed the cultures of feeders to avoid feeder cell contaminations in the analysis. The knockdown and control lines were karyotyped at multiple stages of these experiments. Many of the lines showed normal karyotype in early passage stages. One control and *Dicer*-knockdown pair showed trisomy of Chr 12 and chr 17 already at early stages. However the phenotypic differences between the control and KD lines were still observed.

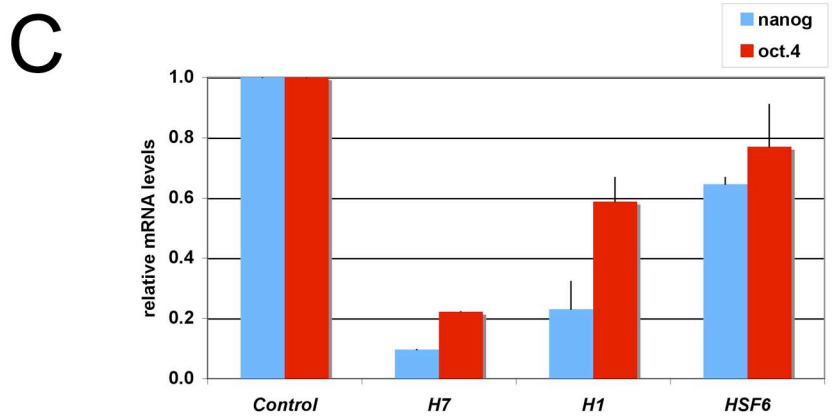
miRNA transfection efficiency*:* To reveal the transfection efficiencies of our protocol with miRNAs, we quantified the concentrations of miR-195 and -372 two days after transfection with and without electroporation. miR-195 and miR-372 TaqMan assays (ABI) were used to perform miRNA qPCR on a ABI 7300 real-time PCR machine.

Irradiation of hESCs*:* H1 hESCs were seeded on 6cm plates. On day 3, plates were irradiated at 5 Gy or kept non-irradiated for the control. After seven hours these cells were treated with TrypLE Express enzyme (Gibco) at 37 C for three minutes and re-suspended in PBS+ 0.1% BSA (wash buffer). After washing twice with wash buffer cells were fixed with 70% ethanol at 4 C for 1 hour. After washing two more times with the wash buffer, the cells were then stained with PI staining buffer (1XPBS, 2%FBS,

50ug/ml PI, 200ug/ml RNAse A, 0.1% Igepal) for three hours and run on BD FACS Canto II machine. Data were analyzed using Watson Pragmatic model (FlowJo software, Tree Star, Inc).

Supplementary Figures





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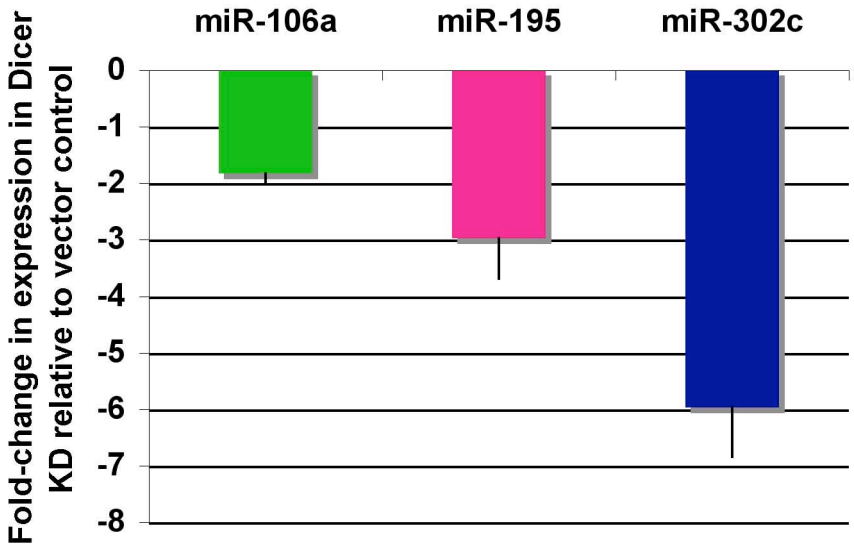
hESC microRNAs that are down-regulated after 4 day differentiation:

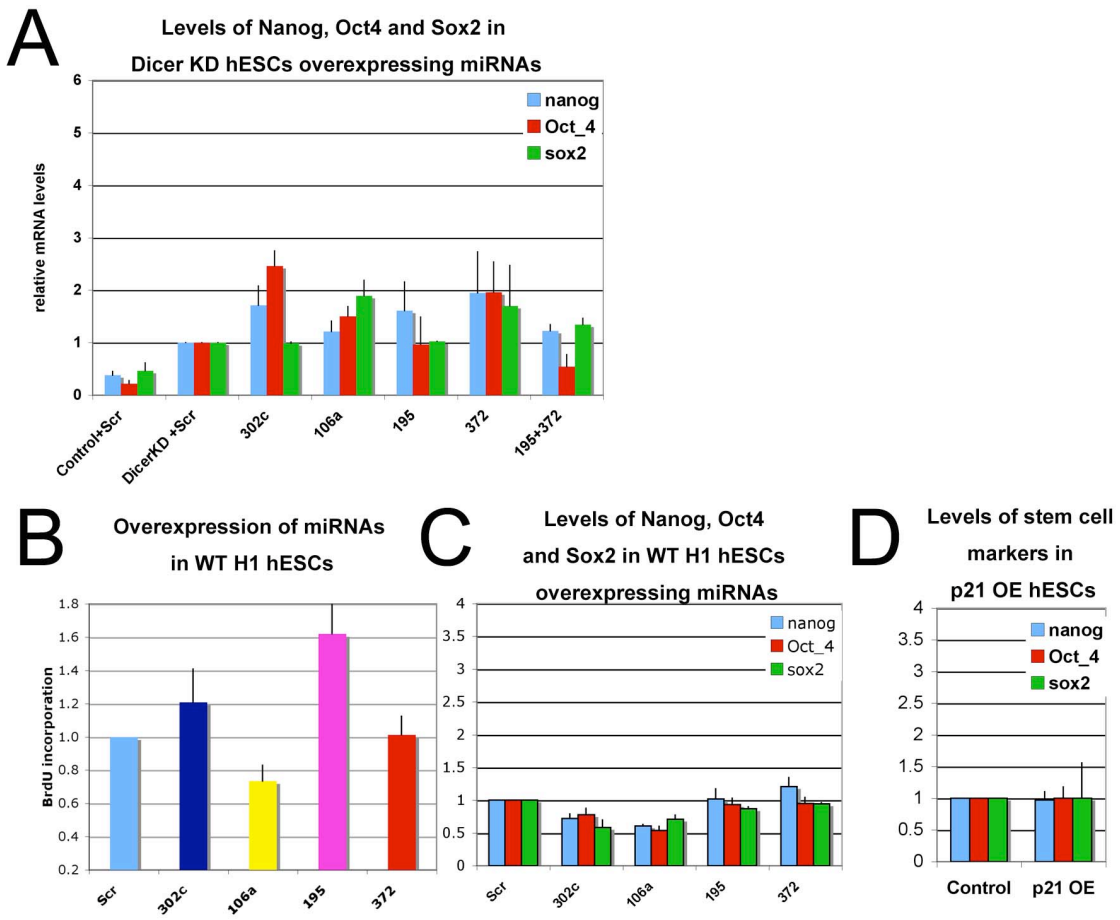
miR-18	miR-195	miR-302a*	miR-302d
miR-19a	miR-200a	miR-302b	miR-367
miR-19b	miR-200b	miR-302b*	miR-372
miR-106a	miR-204	miR-302c	miR-422b
miR-187	miR-302a	miR-302c*	

Supplementary Figure 1. miRNAs enriched in hESCs. (A-B) miRNA expression levels were profiled by miRNA qPCR analysis in five undifferentiated hESC samples from three different hESC lines (H1, H7 and HSF6) and from brain tissue, which serves as a differentiated control. Shown is a dendrogram (a tree graph) generated based on the

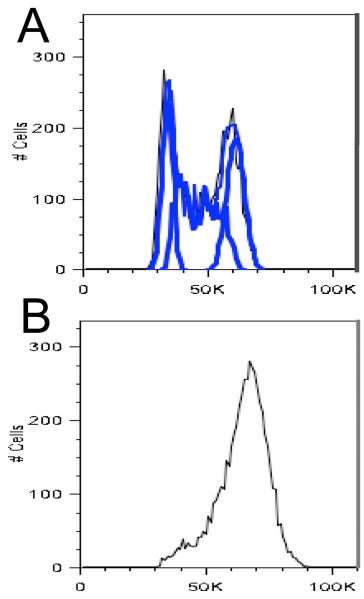
similarity between the samples or the expression values. miRNA levels are represented as $\log_{10}$ (intensity). Values range from shared minimum (green) to shared maximum (red). Gold rectangle highlights the miR-302 family, which is also shown as a close-up in panel B. (C) Nanog and Oct4 mRNA levels after 4 day differentiation in three different hESC lines H7, H1 and HSF6 were analyzed by quantitative RT-PCR. (D) List of identified hESC-enriched miRNAs that are down-regulated after 4 day serum forced undirected differentiation.



Supplementary Figure 2. The KD level of candidate miRNAs (miR-106a, 195 and 302c) in *Dicer* KD line. Fold changes in mature miRNA expression levels are shown for miR-106, 195 and 302c in Dicer KD line comparing to the control line. Mean+SD was calculated from three independent experiment based on qPCR assays.



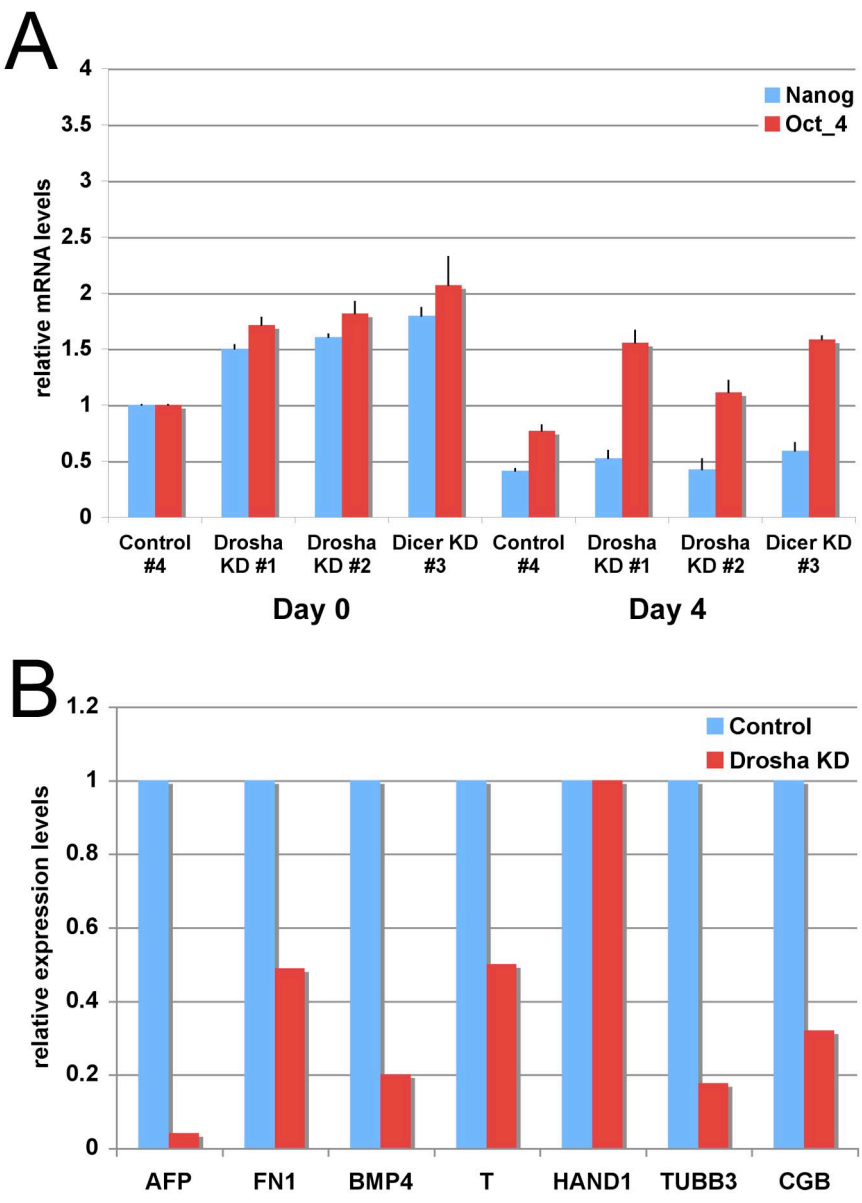
Supplementary Figure 3. Stem cell markers in hESCs overexpressing miRNAs. (A) Overexpression of miRNAs in *Dicer*-knockdown hESCs does not reduce stem cell markers *Nanog*, *Oct4* and Sox2 to the control level. (B) BrdU incorporation in wild-type H1 hESCs overexpressing miR-302c, 106a, 195 or 372 (two separate RNA duplexes used for 106a, IDT and Sigma.) (C) After overexpression of miR-302c, 106a, 195 or 372 in wildtype H1 hESCs, mRNA levels of the stem cell markers *Nanog*, *Oct4* and *Sox2* measured by qPCR (D) Overexpression of p21 in wildtype H1 hESCs does not dramatically change stem cell fate as mRNA levels measured by qPCR of the stem cell markers *Nanog*, *Oct4* and *Sox2* are not dramatically reduced.



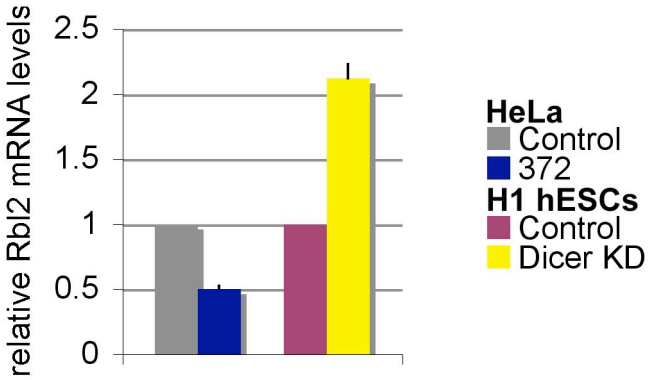
H1 hESCs
No irradiation

H1 hESCs
7 hours after irradiation

Supplementary Figure 4. Irradiation-induced G1 checkpoint is not observed in hESCs. Wild type H1 hESCs.were irradiated at 5 Gy. Compared to non-irradiated control (A) which showed typical ES cell cycle distribution with a large percent of cells in the S phase and peaks in both G1 and G2, H1 hESCs did not show G1 arrest after irradiation (B), showing a single peak at G2. This figure shows the result of a representative of four experiments.



Supplementary Figure 5. Delayed differentiation in Dicer or Drosha KD hESCs. (A) Nanog and Oct4 levels in *Dicer*- or *Drosha*-knockdown hESCs after 4 day serum-forced differentiation compared to the control line. Oct4 mRNA levels remain higher in *Drosha* (#1, #2) and *Dicer* (#3) knockdown H1 lines 4 day after differentiation than control line (#4). (B) Relative expression levels of differentiation markers in *Drosha*-knockdown line #2 hESCs compared with the control line after an 11 day serum-forced differentiation, except for T (4 day differentiation) and TUBB3 (20 day differentiation) based on qPCR assays. All the differentiation markers tested showed delayed induction in *Drosha*-knockdown hESCs compared with the control cells except HAND1. The time point was chosen based on when the particular marker was upregulated in the serum forced differentiation protocol used in this experiment.



Supplementary Figure 6. Rbl2 is regulated by miR-372 in HeLa and H1 hESCs. RbL2 mRNA levels were reduced in HeLa cells overexpressing miR-372 and increased in *Dicer*-knockdown H1 hESCs.

Supplementary table 1. Dicer and Drosha KD hESCs do not express differentiation markers.

Accession Number	Gene Name	Marker for which germlayer	Day 11 Diff vs UD in wildtype hESCs	Dicer KD Vs Control	Drosha KD #1 Vs Control	Drosha KD #2 Vs Control
NM_001134	AFP	Endoderm	250	0.9 ± 0.3	1.2 ± 0.2	0.8 ± 0.4
NM_002026	FN1	Endoderm	50	0.9 ± 0.6	1.1 ± 0.3	0.7 ± 0.5
NM_130851	BMP4	Mesoderm	14	0.7 ± 0.3	0.9 ± 0.1	0.8 ± 0.4
NM_004821	HAND1	Mesoderm	13	0.8 ± 0.3	0.8 ± 0.3	0.6 ± 0.6
NM_006086	TUBB3	Ectoderm	2	1.0 ± 0.0	1.3 ± 0.4	1.3 ± 0.4
NM_000737	CGB	Ectoderm	25	1.1 ± 0.0	1.2 ± 0.2	1.2 ± 0.3

Note: Mean ± range are shown from two independent microarray analysis for Dicer and Drosha KD hESCs comparing to the control. Day 11 Diff vs UD values are given for comparison. The wildtype H1 hESCs were grown under serum-induced differentiation for 11 days and compared with undifferentiated H1 hESCs for the expression levels of the genes listed above by qPCR. Results are shown in fold changes.

Supplementary table 2. Fold change of FGF5/REX1/GBX2 genes in Dicer and Drosha knockdown hESCs.

Accession Number	Gene Name	Dicer KD Vs Control	Drosha KD #1 Vs Control	Drosha KD #2 Vs Control
NM_004464	FGF5	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.6
NM_020695	REXO1	1.4 ± 0.3	1.2 ± 0.8	1.2
NM_001485	GBX2	1.0 ± 0.0	1.0 ± 0.5	1.2 ± 0.2

Supplementary table 3. The "Top20" miRNAs expressed in hESCs. 9 out of these 20 miRNAs were previously identified by Suh et al. through cloning efforts and 15 by Bar et al. through sequencing efforts.

miR	average in undifferentiated	# of sequencing reads in Bar et al., 2008	cloning frequency in Suh et al., 2004
miR-22	4770	0	-
miR-302c	4701	10425	10
miR-365	1746	30	-
miR-107	1325	14	-
miR-21	1104	64204	4
miR-19	953	109	8
miR-302b	729	34782	45
miR-302d	672	37506	13
miR-191	660	434	-
miR-106a	658	Not listed	-
miR-29b	525	12	-
miR-367	402	Not listed	-
miR-30e-3p	362	0	-
miR-302a*	338	498	6
miR-92	326	3346	2
miR-17-5p	307	7262	1
miR-130a	289	119	1
miR-20	265	1456	-
miR-148a	264	0	-
miR-143	214	10	-

Note: copy numbers per cell are shown for each miRNA in this table. This data came from qPCR based large-scale miRNA profiling including 220 human miRNA assays. The average was calculated from 5 samples from three undifferentiated hESC lines, H1, H7 and HSF6.

Supplementary table 4. Primers for qPCR

name	Length	Sequence 5' - 3'
gapdh-SS	25	TGATGACATCAAGAAGCTGGTGAAG
gapdh-AS	23	TCCTTGGAGGCCATGTGGGCCAT
Nanog-SS	20	GCTTGCCTTGCTTTGAAGCA
Nanog-AS	20	TTCTTGACTGGGACCTTGTC
Oct4-SS	19	GAGCAAAACCCGGAGGAGT
Oct4-AS	19	TTCTCTTTCGGGCCTGCAC
Sox2-SS	21	CACCTACAGCATGTCCTACTC
Sox2-AS	22	CATGCTGTTTCTTACTCTCCTC
Drosha-SS	21	AGGAGTACGCCATAACCAACG
Drosha-AS	22	CAATCGTGGAAAGAAGCAGACA
Dicer-SS	21	TTCCTCACCAATGGGTCCTTT
Dicer-AS	22	GCTTCAAGCAGTTCAACCTGAT
Wee1-SS	19	CCCGCCACACAAGACCTTC
Wee1-AS	21	CCCGGAGTTTAACAGAGCTGG

Supplementary table 5. miRNA duplex sequences

name	Length	Sequence 5' - 3'
Scrambled control-SS	21	rArCrUrUrArCrGrArGrUrGrArCrArGrUrArGrArUrU
Scrambled control -AS	21	rUrCrUrArCrUrGrUrCrArCrUrCrGrUrArArGrUrUrU
miR-106a (IDT)	26	rArArArArGrUrGrCrUrUrArCrArGrUrGrCrArGrGrUrArGrCrUrU
miR-106a-AS (IDT)	26	rGrCrUrArCrCrUrGrCrArCrUrGrUrArArGrCrArCrUrUrUrUrArC
miR-106a (Sigma)	22	rArArArArGrUrGrCrUrUrArCrArGrUrGrCrArGrGrUrA
miR-106a-AS (Sigma)	22	rCrCrUrGrCrArCrUrGrUrArArGrCrArCrUrUrUrUrAU
miR-195	21	rU*rArG*rCrArGrCrArCrArGrArArArUrArUrUrGrGrC
miR-195-AS	21	rCrArArUrArUrUrUrCrUrGrUrGrCrUrGrCrArArArU
miR-195-mismatch-ss	21	rU*rGrA*rCrArGrCrArCrArGrArArArUrArUrUrGrGrC
miR-195-mismatch-AS	21	rCrArArUrArUrUrUrCrUrGrUrGrCrUrGrUrGrArArU
miR-302C*	22	rUrUrUrArArCrArUrGrGrGrGrGrUrArCrCrUrGrCrUrG
mir-302C	23	rUrArArGrUrGrCrUrUrCrCrArUrGrUrUrUrCrArGrUrGrG
miR-372	23	rArArArGrUrGrCrUrGrCrGrArCrArUrUrUrGrArGrCrGrU
mir-372-as	23	rGrCrUrCrArArArUrGrUrCrGrCrArGrCrArCrUrUrUrCrU