

Supplementary Information

Multiplexed miRNA northern blots via hybridization chain reaction

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S1 Target and probe sequences

Sequences for miRNA targets were obtained from miRBase [1] and sequences for other small RNAs were obtained from the National Center for Biotechnology Information (NCBI) [2]. RNA marker sequences were obtained from New England Biolabs (NEB). All target, probe, and initiator sequences are listed 5' to 3'. Target sequences are RNA. For probes, all HCR initiators and spacers are DNA (black) and the target recognition site is either 2'OMe-RNA (green), DNA (black), or a proprietary mixture of LNA and DNA nucleotides (blue; Exiquon). The HCR amplifiers and fluorophores used for each figure are listed in Table S1.

Table S1: HCR amplifiers and fluorophores.

Target	# (nt)	HCR amplifier	Fluorophore	Figures
miR-16	22	B1	Alexa488	4, S7, S8, S9
miR-16	22	B1	Alexa647	5, 6, 7, S10, S11, S12, S13, S14, S15, S16, S17
R1	22	B1	Alexa647	7, S14, S15, S16, S17
R2	22	B1	Alexa647	7, S14, S15, S16, S17
miR-195	21	B1	Alexa647	7, S14, S15, S16, S17
miR-15a	22	B1	Alexa647	7, S14, S15, S16, S17
R3	22	B1	Alexa647	7, S14, S15, S16, S17
miR-18a	23	B2	Alexa647	2, 3, 6, 7, S3, S4, S5, S11, S12, S13, S14, S15, S16, S17, S18
miR-18a	23	B2	Alexa546	4, S7, S8, S9
miR-18a	23	B2	Alexa488	S6
miR-18b	23	B2	Alexa647	7, S14, S15, S16, S17
R4	23	B2	Alexa647	7, S14, S15, S16, S17
R5	23	B2	Alexa647	7, S14, S15, S16, S17
miR-20a	23	B2	Alexa647	7, S14, S15, S16, S17
R6	23	B2	Alexa647	7, S14, S15, S16, S17
pre-miR-18a	71	B3	Alexa647	S6
miR-30a	22	B3	Alexa647	4, 6, 7, S7, S8, S9, S11, S12, S13, S14, S15, S16, S17
miR-30d	22	B3	Alexa647	7, S14, S15, S16, S17
R7	22	B3	Alexa647	7, S14, S15, S16, S17
R8	22	B3	Alexa647	7, S14, S15, S16, S17
R9	22	B3	Alexa647	7, S14, S15, S16, S17
R10	22	B3	Alexa647	7, S14, S15, S16, S17
miR-30b	22	B3	Alexa647	7, S14, S15, S16, S17
RNU48	63	B3	Alexa546	2, 3, S2, S4, S5
U6	106	B5	Alexa488	2, 3, S1, S4, S5
microRNA marker	17, 21, 25	B4	Alexa488	2
low range ssRNA ladder	50, 80, 150, 300	B4	Alexa488	2

S1.1 miR-16 and related targets

Target: **hsa-miR-16-5p (miR-16)**

Accession: **MIMAT0000069**

Sequence: UAGCAGCAGGUAUUUUUGGCG

Target: **hsa-miR-195-5p (miR-195)**

Accession: **MIMAT0000461**

Sequence: UAGCAGCACAGAAAUAUUGGC

Target: **hsa-miR-15a-5p (miR-15a)**

Accession: **MIMAT0000068**

Sequence: UAGCAGCACAUAAUUGUUUGUG

Target: **R1**

Sequence: UAGCAGCACAUAAUAUUGGCG

Target: **R2**

Sequence: UAGCAGCACAAAAUUGGCG

Target: **R3**

Sequence: UAGCAGCACAAUUGUUUGUG

HCR amplifier B1: Initiator I1	Spacer	miR-16 Probe Sequence	Spacer	HCR amplifier B1: Initiator I2
GAGGAGGCAGCAAACGGGAAGAGTCTTCCTTTACG	ATATT	CGCCAAUAUUUACGGUCUGCUA	ATATA	GCAATTCITTTCTTGAGGGGGCAGCAAACGGGAAGAG

HCR amplifier B1: Initiator I1	Spacer	Exiqon miR-16 Probe*	Spacer	HCR amplifier B1: Initiator I2
GAGGAGGCAGCAAACGGGAAGAGTCTTCCTTTACG	ATATT	CGCCAAATTTACGGTGTGCTA	ATATA	GCAATTCITTTCTTGAGGGGGCAGCAAACGGGAAGAG

*Exiqon synthesis batch #: **620562**

S1.2 miR-18a and related targets

Target: **hsa-miR-18a-5p (miR-18a)**

Accession: **MIMAT0000072**

Sequence: UAAAGGUGCAUCUAGUGGCAGAUAG

Target: **hsa-miR-18b-5p (miR-18b)**

Accession: **MIMAT0001412**

Sequence: UAAAGGUGCAUCUAGUGGCAGUUAG

Target: **hsa-miR-20a-5p (miR-20a)**

Accession: **MIMAT0000075**

Sequence: UAAAGGUCUUUAUAGUGGCAAGGUAG

Target: **R4**

Sequence: UAAAGUGCAUCUAGUGGCAGUUAG

Target: **R5**

Sequence: UAAAGGUCUUUAUAGUGGCAAGGUAG

Target: **R6**

Sequence: UAAAGGUCUUUAUAGUGGCAGUUAG

HCR amplifier B2: Initiator I1	Spacer	miR-18a Probe Sequence	Spacer	HCR amplifier B2: Initiator I2
CCTCGTAAATCCTCATCAATCATCCAGTAAACGGCC	AAAAA	CUAUCUGCACUAGAUGCACCUUA	AAAAA	AGCTCAGTCCATCCTCGTAAATCCTCATCAATCATC

HCR amplifier B2: Initiator I1	Spacer	Exiqon miR-18a Probe*	Spacer	HCR amplifier B2: Initiator I2
CCTCGTAAATCCTCATCAATCATCCAGTAAACGGCC	AAAAA	CTATCTGCACTAGATGACCCITTA	AAAAA	AGCTCAGTCCATCCTCGTAAATCCTCATCAATCATC

* Exiqon synthesis batch #: **620561**

Target: **hsa-miR-18a (pre-miR-18a)**

Accession: **MIMAT0000072**

Sequence: UGUUCUAAAGGUGCAUCUAGUGGCAGAUAGUGAAGUAGAUUAGCAUCACUGCCCUAAGUCUCCUUCUGGCA

HCR amplifier B3: Initiator I1	Spacer	pre-miR-18aProbe Sequence	Spacer	HCR amplifier B3: Initiator I2
GTCCCTGCCTCTATATCTCCCACTCAACTTTAAACCG	TACAA	AGUAGAUGCUAAUCUACUUCACUUA	TAAAA	AAAAGTCTFAATCCGTCCCTGCCTCTATATCTCCACTC

S1.3 miR-30a and related targets

Target: **hsa-miR-30a-5p (miR-30a)**

Accession: **MIMAT0000087**

Sequence: UGUAAACAUCCUCGACUGGAAG

Target: **hsa-miR-30d-5p (miR-30d)**

Accession: **MIMAT0000245**

Sequence: UGUAAACAUCCCGACUGGAAG

Target: **R7**

Sequence: UGUAAACAUCCUACACUGGAAG

Target: **R8**

Sequence: UGUAAACAUCCACACUGGAAG

Target: **R9**

Sequence: UGUAAACAUCCACACUGGAAG

Target: **R10**

Sequence: UGUAAACAUCCACACUCAAAG

Target: **hsa-miR-30b-5p (miR-30b)**

Accession: **MIMAT0000420**

Sequence: UGUAAACAUCCACACUCAGCU

HCR amplifier B3: Initiator I1	Spacer	miR-30a Probe Sequence	Spacer	HCR amplifier B3: Initiator I2
GTCCCTG CCTCTATATCTCCACTCACTTTAAACCG	TACAA	CUUCCAGUCGAGGAUGUUUACA	TAAAA	AAAGTCTAAATCCGTCCCTGCCTCTATATCTCCACTC
HCR amplifier B3: Initiator I1	Spacer	Exiqon miR-30a Probe*	Spacer	HCR amplifier B3: Initiator I2
GTCCCTG CCTCTATATCTCCACTCACTTTAAACCG	TACAA	CTTCCAGTCGAGGATGTTTACA	TAAAA	AAAGTCTAAATCCGTCCCTGCCTCTATATCTCCACTC

*Exiqon synthesis batch #: **620563**

S1.4 Other RNA targets

Target: **Homo sapiens small nucleolar RNA, C/D box 48 (SNORD48), small nucleolar RNA (RNU48)**

Accession: **NR_002745.1**

Sequence: AGUGAUGAUGACCCAGGUAACUCUGAGUGUGUGUGCAUCACCGCGCUCUGACC

HCR amplifier B3: Initiator I1	Spacer	RNU48 Probe Sequence	Spacer	HCR amplifier B3: Initiator I2
GTCCCCTCTATATCTCCCACTCAACTTTAACCCG	TACAA	TGCGGTGATGGCATCAGCGACACACTCAGAGTTACCTGGGTCAATCA	TAAAA	AAAGTCTAATCCCGTCCCTGCCTCTATATCTCCACTC

Target: **Homo sapiens RNA, U6 small nuclear 1 (RNU6-1), small nuclear RNA (U6)**

Accession: **NR_004394.1**

Sequence: GUGCUCGUCUGCGGACACAUAUCUAAAAUUGGAAACGAGUACAGAGAAUAUAGCAUGGCCCGCGCAAGGAUGACACGCAAAUUGGAAUUCUUAUUUU

HCR amplifier B5: Initiator I1	Spacer	U6 Probe Sequence	Spacer	HCR amplifier B5: Initiator I2
CTCACTCCCAATCTCTATCTACCCACAAATCGAAT	AAAAA	TCTTCTGTATCGTTCCAAATTTTAGTATATAGTGTGCTGCCGAAAGCGAGCAC	ATTTT	CAC TTCA TATCACTCACTCCCAATCTCTATCTACCC

Target: **microRNA Marker**

NEB catalog number: **N2102S**

Subsequence: CAGUGGUCUGGUUGAGAU (common to markers of length 17, 21, 25 nt)

HCR amplifier B4: Initiator I1	Spacer	microRNA Marker Probe Sequence	Spacer	HCR amplifier B4: Initiator I2
CCTCAACCTACCTCCAACCTCTACCATATTCGCTTC	TAAAA	AUCUCAACCCAGCCACUG	ATTTT	CACATTTACAGACTCAACCTACCTCCAACCTCTCAC

Target: **Low Range ssRNA Ladder**

NEB catalog number: **N0364S**

Subsequence: GGGAGUCUUCGUCGAG (common to markers of length 50, 80, 150, 300 nt)

HCR amplifier B4: Initiator I1	Spacer	Low Range ssRNA Ladder Probe Sequence	Spacer	HCR amplifier B4: Initiator I2
CCTCAACCTACCTCCAACCTCTACCATATTCGCTTC	TAAAA	CTCGAGAGACTCCC	ATTTT	CACATTTACAGACTCAACCTACCTCCAACCTCTCAC

S2 Relative quantitation

S2.1 Replicates for Figure 3

Relative quantitation replicates for Figure 3 were performed on different days on different batches of total RNA. Each replicate was a 3-channel multiplexed experiment, with individual channels displayed as follows:

- Channel 1: U6 in Figure S1,
- Channel 2: RNU48 in Figure S2,
- Channel 3: miR18a in Figure S3.

Signal values are normalized for each channel for each replicate. Linear regression is performed using ordinary least squares, corresponding to the assumption that there are negligible errors in the independent variable (total RNA in each sample). The coefficient of determination (R^2) is displayed for each channel for each replicate, with values of R^2 close to 1 indicating a good degree of fit.

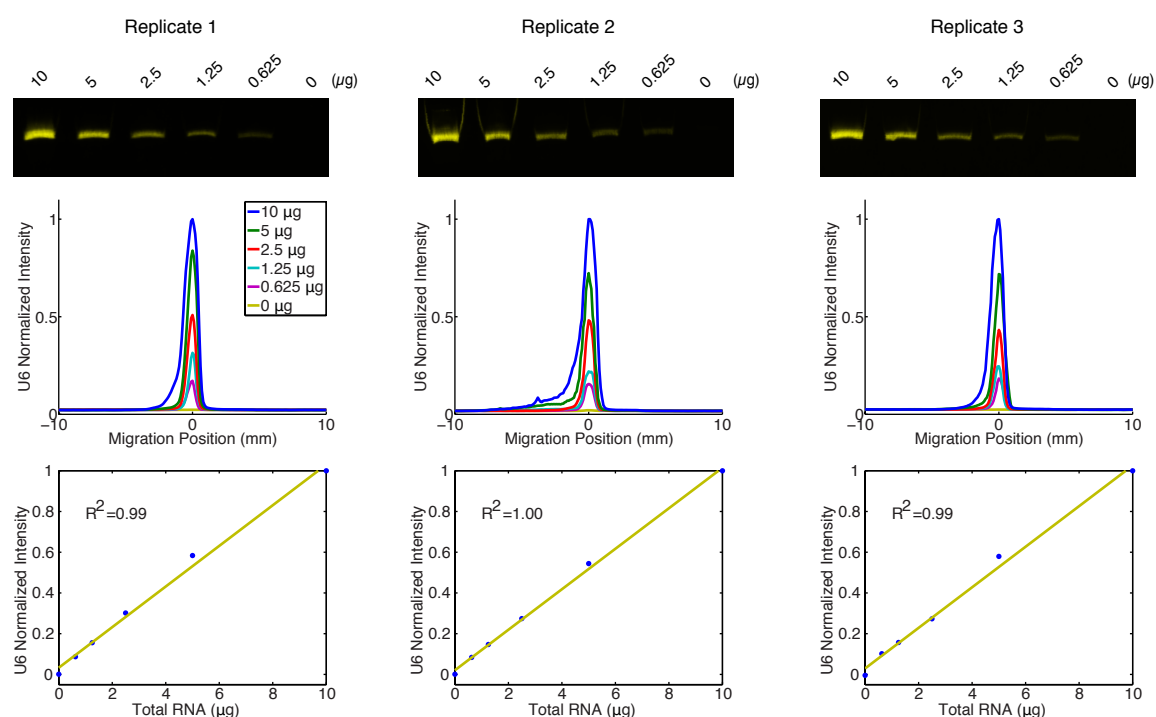


Figure S1: Relative quantitation of RNA via HCR northern blot (Channel 1: U6). Total RNA extracted from 293T cells.

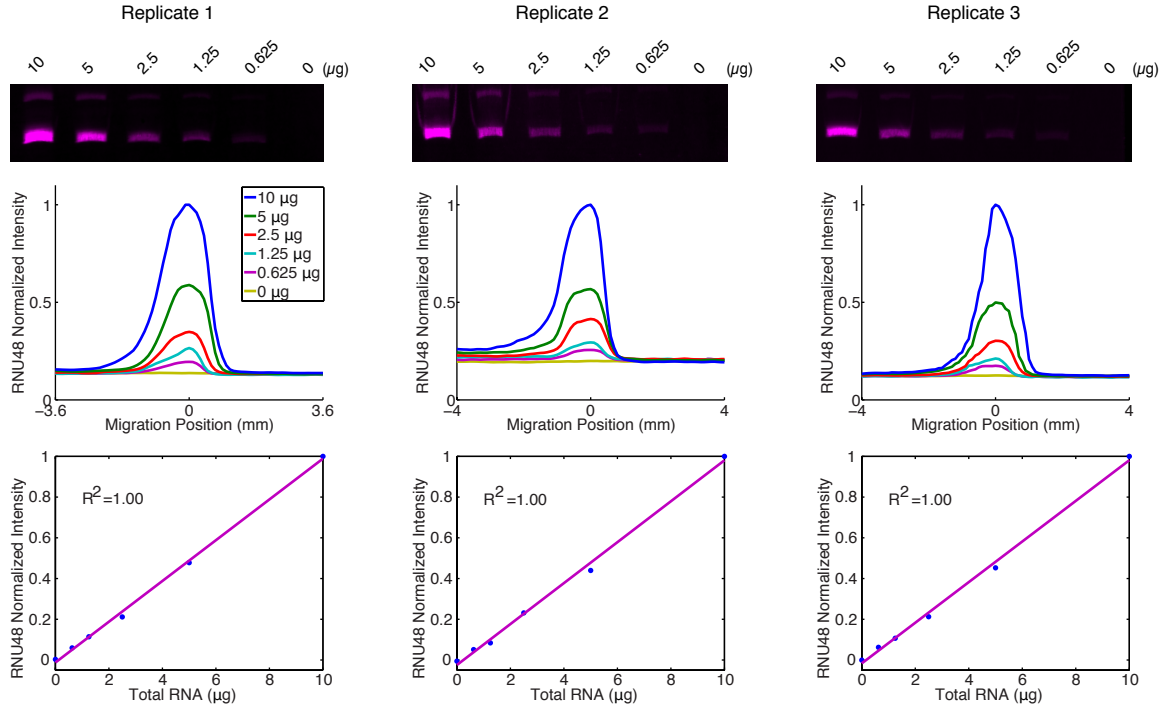


Figure S2: Relative quantitation of RNA via HCR northern blot (Channel 2: RNU48). Total RNA extracted from 293T cells.

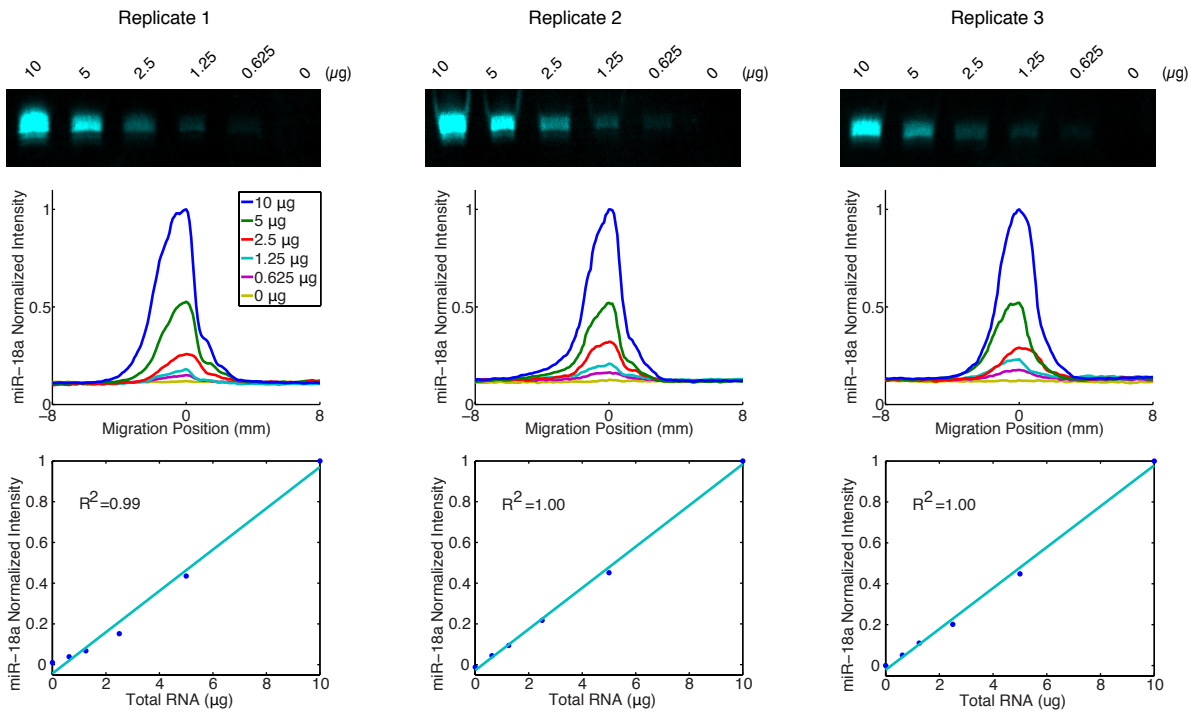


Figure S3: Relative quantitation of RNA via HCR northern blot (Channel 3: miR-18a). Total RNA extracted from 293T cells.

S2.2 Checking for spectral crosstalk and miRNA precursors

Examination of the individual channels for the multiplexed HCR northern blot of Figure 3 reveals weak upper bands in Channel 2 (Figure S4b) and Channel 3 (Figure S4c) that could result from either spectral crosstalk between fluorescent channels, or from probes hybridizing to additional targets.

To discriminate between these two possibilities, we performed blots using only one of the three probe/amplifier pairs at a time, and scanned these blots in all three channels (Figure S5). Since only one type of fluorophore is present in each blot, if the target band from one channel is faintly visible in another channel, this corresponds to spectral crosstalk. This is the case in Figure S5a, where the U6 band in Channel 1 also shows up faintly in Channel 2. Likewise, since only one probe/amplifier pair is present in each blot, if the target band in one channel is accompanied by another fainter band in the same channel, this corresponds to detection of an alternate target. This is the case in Figure S5c, where the miR-18a band in Channel 3 is accompanied by a faint higher band also in Channel 3.

Based on these results, we conclude that the higher bands observed in Figure S4b are due to spectral crosstalk from U6 signal in Channel 1, and the higher bands observed in Figure S4c are additional targets detected by the miR-18a probe (e.g., the precursor miRNA, pre-miR-18a). To explore whether pre-miR-18a is being detected, we performed a 2-channel multiplexed study using both the miR-18a probe and a new probe complementary to the loop and a portion of the stem of pre-miR-18a (Figure S6). This new probe detects the putative pre-miR-18a and also an additional longer target of unknown identity. While definitive identification of these additional targets is beyond the scope of the present work, the ability to characterize target size (e.g., discriminating miRNA from pre-miRNA) is a key advantage of northern blots relative to other RNA detection technologies.

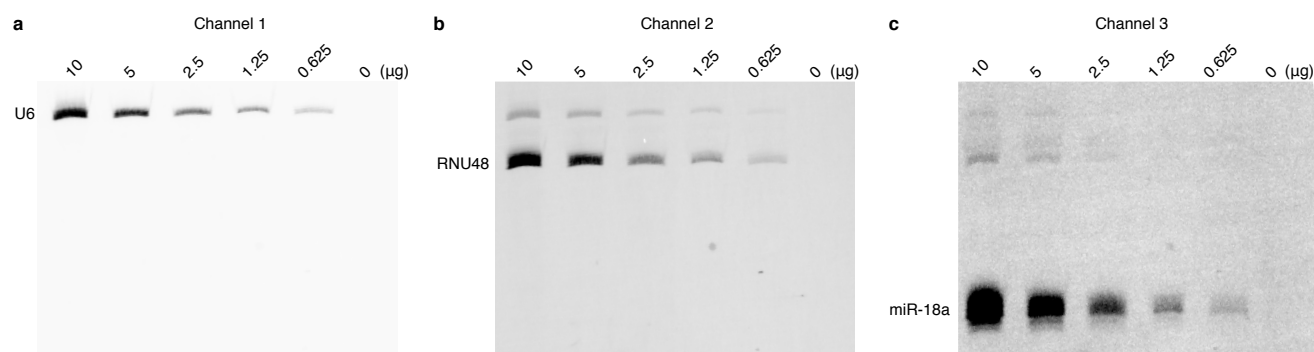


Figure S4: Individual channels for the multiplexed HCR northern blot of Figure 3. (a) Channel 1: U6. (b) Channel 2: RNU48 plus upper bands. (c) Channel 3: miR-18a plus upper bands. Total RNA extracted from 293T cells.

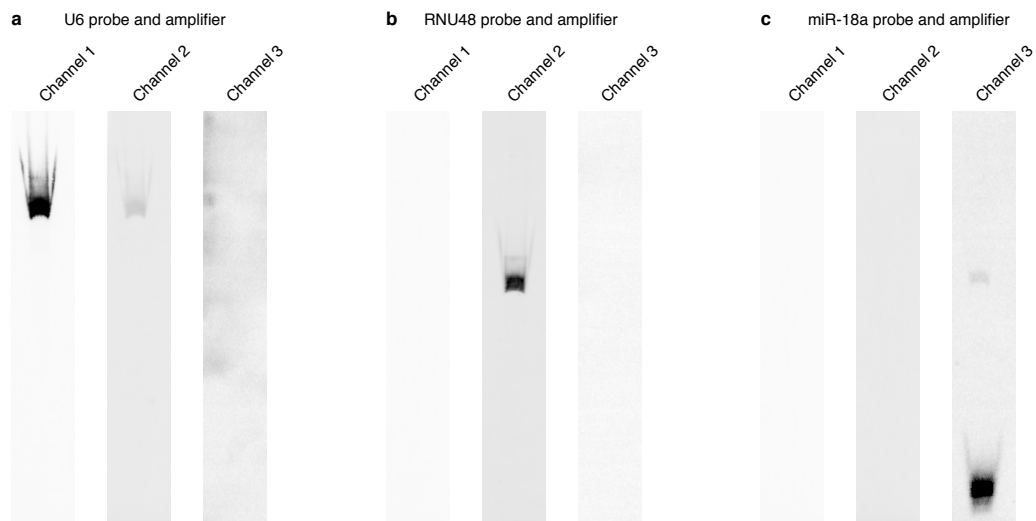


Figure S5: Blots using a probe and HCR amplifier for only one target. (a) U6 probe and amplifier. (b) RNU48 probe and amplifier. (c) miR-18a probe and amplifier. Each blot is scanned in all three channels. 20 μg of total RNA extracted from 293T cells.

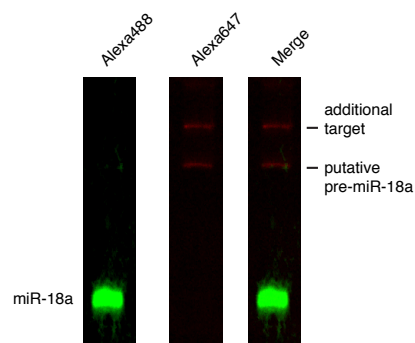


Figure S6: Detection of putative pre-miRNA-18a. miR-18a probe and amplifier (Alexa 488; green). pre-miR-18a probe and amplifier (Alexa647; red). Spectral crosstalk is negligible between Alexa488 and Alexa647. 10 μg of total RNA extracted from 293T cells.

S3 miRNA multiplexing and relative quantitation

S3.1 Replicates for Figure 4

Replicates for the multiplexed miRNA study of Figure 4 were performed simultaneously using aliquots from the same total RNA samples. Each replicate was a 3-channel multiplexed experiment (Figure S7). Signal values are normalized for each channel for each replicate.

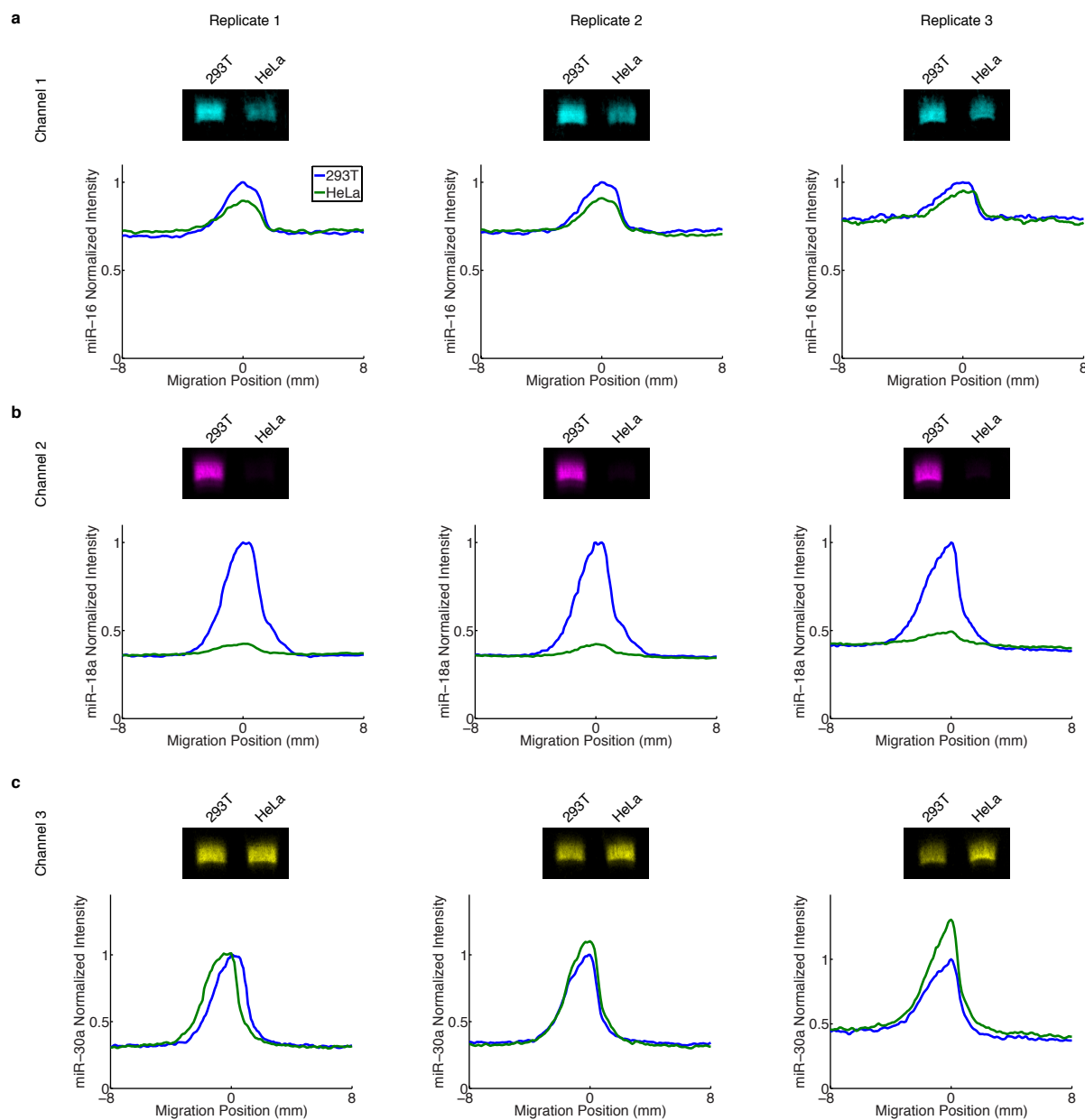


Figure S7: miRNA multiplexing and relative quantitation. (a) Channel 1: miR-16 signal is 1.3 ± 0.4 for 293T relative to HeLa. (b) Channel 2: miR-18a signal is 8 ± 2 for 293T relative to HeLa. (c) Channel 3: miR-30a signal is 0.8 ± 0.1 for 293T relative to HeLa. Mean \pm standard deviation for $N = 3$ blots. $10 \mu\text{g}$ of total RNA extracted from either 293T or HeLa cells.

S3.2 Checking for spectral crosstalk between HCR amplifiers for co-localized targets

Because all three miRNA targets are located in the same place on the blot, it is important to check for possible spectral crosstalk that could interfere with relative quantitation. For these miRNA targets, we observe no spectral crosstalk between channels (Figure S8).

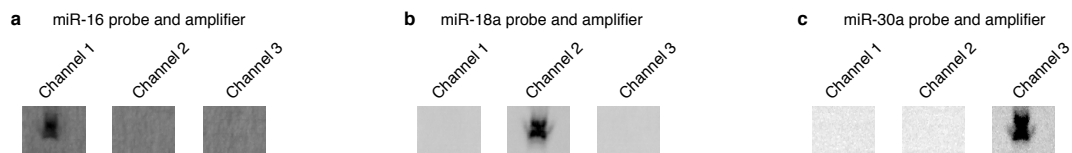


Figure S8: Blots using a probe and HCR amplifier for only one target. (a) miR-16 probe and amplifier. (b) miR-18a probe and amplifier. (c) miR-30a probe and amplifier. Each blot is scanned in all three channels. 10 μ g of total RNA extracted from 293T cells.

S3.3 Checking for steric inhibition between HCR amplifiers for co-localized targets

For multiplexed miRNA blotting, the targets are all co-localized on the blot. If growth of HCR amplification polymers for one target interferes with HCR polymer growth for other targets, this would interfere with relative quantitation. To check for this effect, we compare the signal when one or all three miRNAs are present (Figure S9). The signal is approximately the same in either case, suggesting that any interference between the amplifiers is negligible for these miRNA targets.

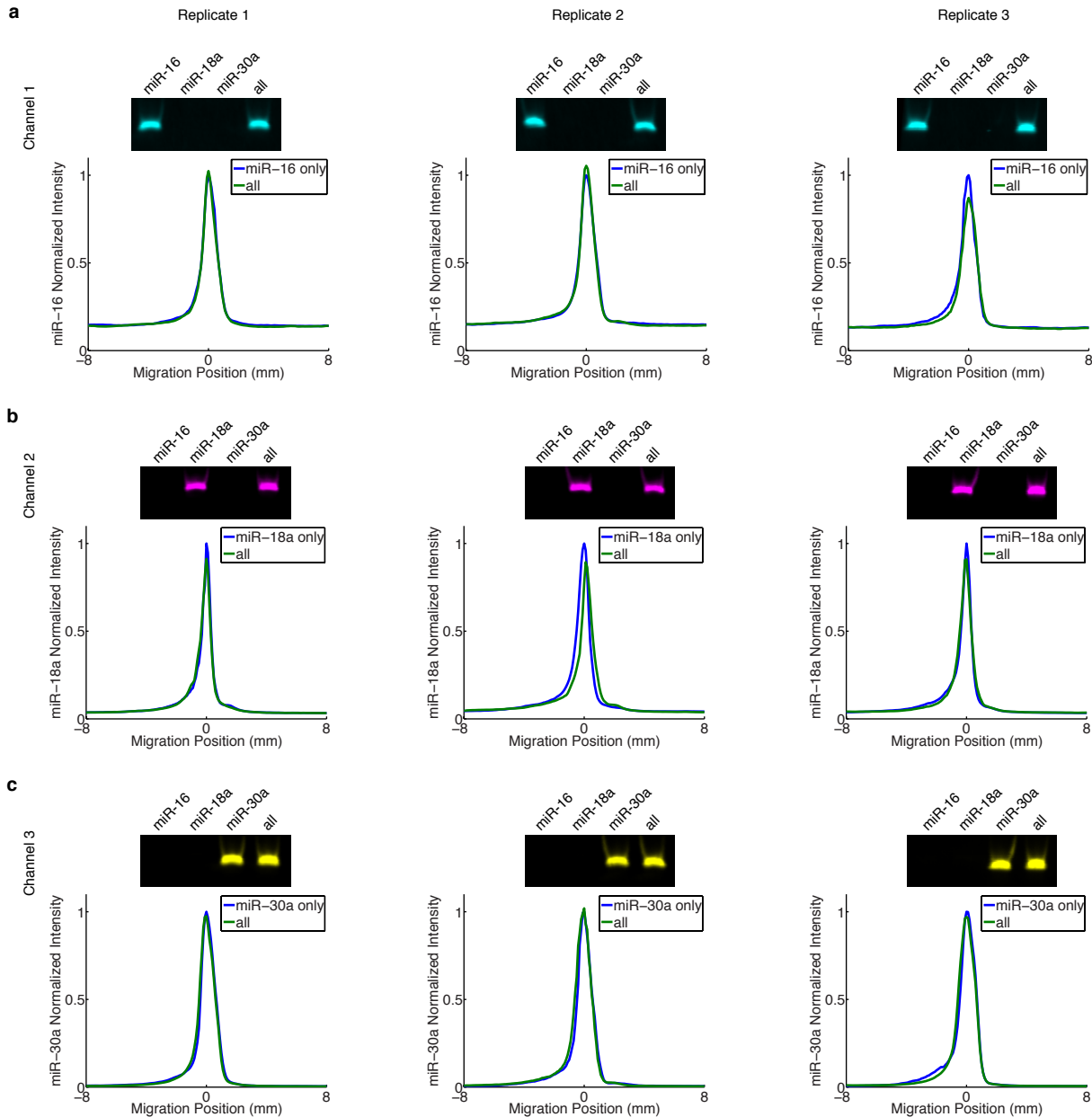


Figure S9: Checking for interference between amplifiers for co-localized targets. (a) Channel 1: miR-16 signal is 0.95 ± 0.08 for all three targets relative to miR-16 alone. (b) Channel 2: miR-18a signal is 0.94 ± 0.05 for all three targets relative to miR-18a alone. (c) Channel 3: miR-30a signal is 1.01 ± 0.05 relative to miR-30a alone. Mean \pm standard deviation for $N = 3$ blots. 20 fmol per synthetic miRNA target.

S4 miRNA absolute quantitation

To perform absolute quantitation using HCR northern blots, some lanes within a blot are allocated to synthetic samples (each with a known abundance of a target RNA of interest) and others are allocated to biological samples containing unknown quantities of endogenous target. The measured band intensities from the dilution series are used to create a standard curve, enabling deduction of endogenous target quantities via comparison of measured band intensities from the biological samples. Figure S10 demonstrates absolute quantitation of miR-16 in total RNA extracted from either 293T or HeLa cells.

The standard curve is obtained via linear regression using ordinary least squares, corresponding to the assumption that there are negligible errors in the independent variable (relative quantities of total RNA in the dilution series of synthetic miR-16). The coefficient of determination (R^2) is displayed for each replicate, with values of R^2 close to 1 indicating a good degree of fit.

Using the calculated absolute quantities of endogenous miR-16, the calculated ratio for 293T to HeLa is 1.1 for each of the two replicates (Figure S10), consistent with the previously measured ratio of 1.3 ± 0.4 ($N = 3$ replicates) for the relative quantitation studies of Figures 4 and S7.

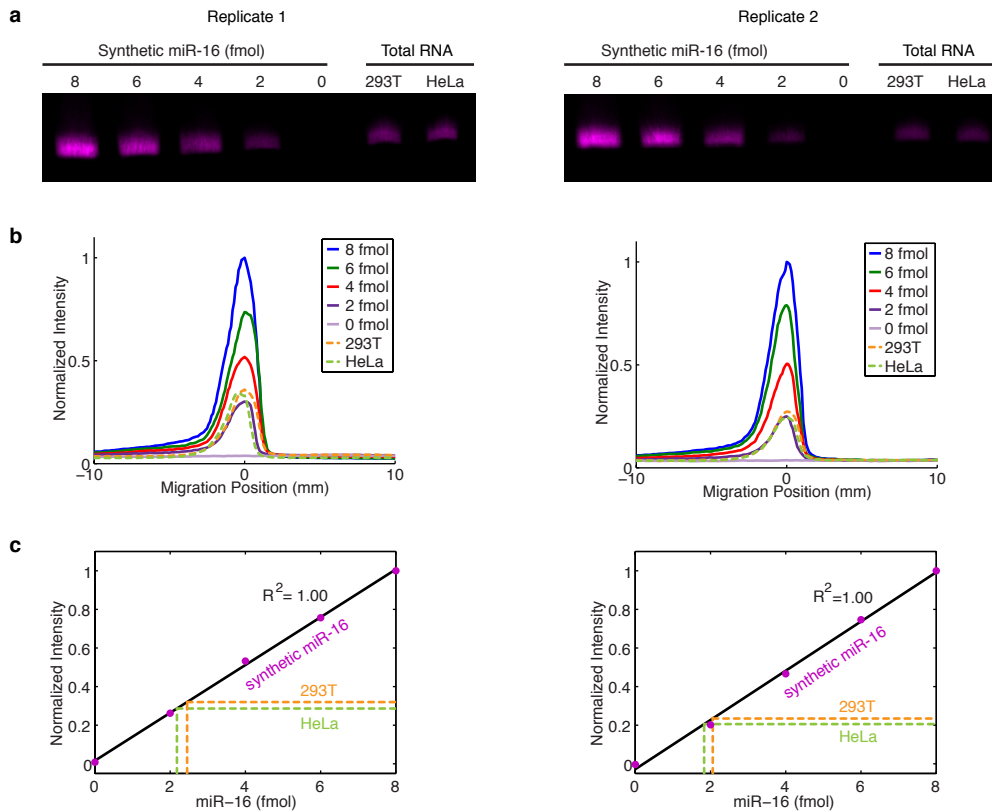


Figure S10: miRNA absolute quantitation via HCR northern blot. Standard curve samples: synthetic 5'-phosphorylated miR-16. Biological samples: 10 μg of total RNA extracted from either 293T or HeLa cells. (a) HCR northern blot for miR-16. (b) Normalized band intensity profiles. (c) Absolute quantitation using a standard curve. The 293T and HeLa band intensities enable deduction of absolute quantities from the standard curve (black line). Replicate 1: 2.45 fmol (293T) and 2.18 fmol (HeLa). Replicate 2: 2.06 fmol (293T) and 1.83 fmol (HeLa).

S5 miRNA sensitivity

We compare sensitivity in detecting three miRNAs using 2'OMe-RNA probes at 37 °C (Figure S11), 2'OMe-RNA probes at 60 °C (Figure S12), or LNA probes at 60 °C (Figure S13). Each replicate was a 1-channel experiment. For a given target, a new serial dilution was performed for each replicate blot. For these comparison studies using 2'OMe-RNA or LNA probes at 60 °C, the protocol is identical to that described in Materials and Methods, except that the probe pre-hybridization, hybridization, and wash steps are performed at 60 °C instead of 37 °C.

S5.1 Replicates for Figure 6: miRNA sensitivity using 2'OMe-RNA probes at 37 °C

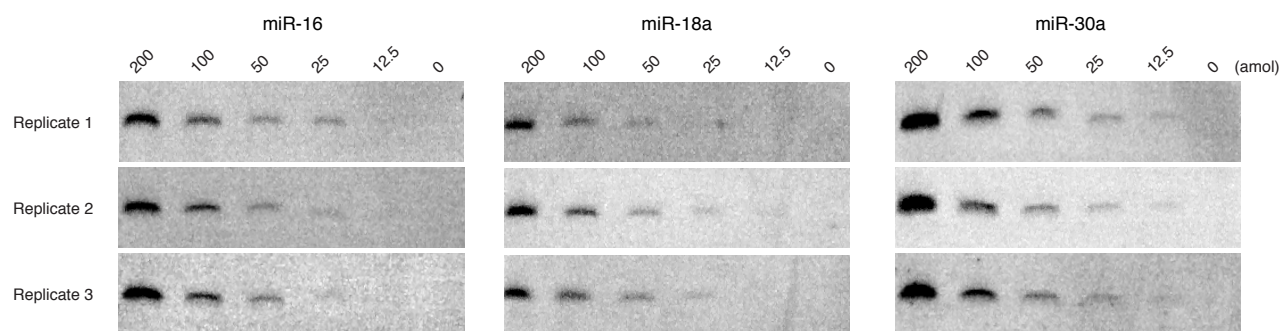


Figure S11: miRNA sensitivity for HCR northern blots using 2'OMe-RNA probes at 37 °C. Synthetic 5'-phosphorylated miR-16, miR-18a, and miR-30a targets in a background of 5 μg of poly-ACGT DNA. See Figure S15 for corresponding selectivity study.

S5.2 miRNA sensitivity using 2'OMe-RNA probes at 60 °C

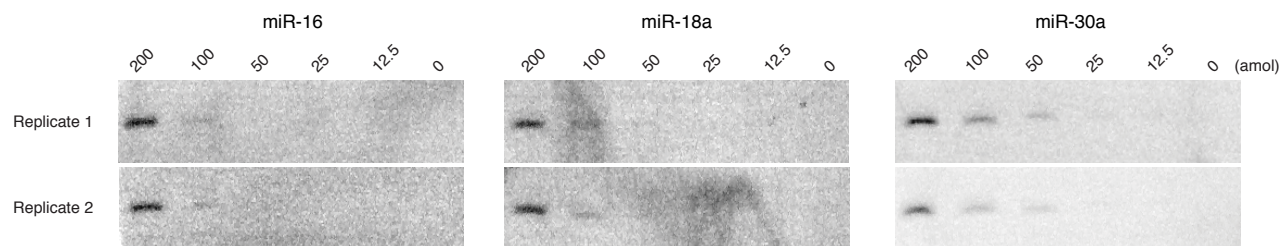


Figure S12: miRNA sensitivity for HCR northern blots using 2'OMe-RNA probes at 60 °C. Synthetic 5'-phosphorylated miR-16, miR-18a, and miR-30a targets in a background of 5 μg of poly-ACGT DNA. See Figure S16 for corresponding selectivity study.

S5.3 miRNA sensitivity using LNA probes at 60 °C

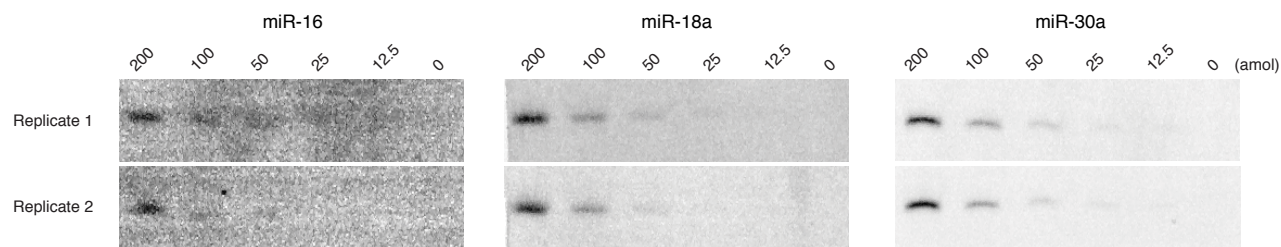


Figure S13: miRNA sensitivity for HCR northern blots using LNA probes at 60 °C. Synthetic 5'-phosphorylated miR-16, miR-18a, and miR-30a targets in a background of 5 μg of poly-ACGT DNA. See Figure S17 for corresponding selectivity study.

S6 miRNA selectivity

To characterize selectivity for three miRNAs, we assembled panels of the most closely related human miRNAs supplemented by other synthetic off-targets as needed. Selectivity is compared for 2'OMe-RNA probes at 37 °C, 2'OMe-RNA probes at 60 °C, and LNA probes at 60 °C in Figure S14. Replicate blots and band profiles for the three methods are shown in Figures S15-S17. For these comparison studies using 2'OMe-RNA or LNA probes at 60 °C, the protocol is identical to that described in Materials and Methods, except that the probe pre-hybridization, hybridization, and wash steps are performed at 60 °C instead of 37 °C.

S6.1 Comparison of probe materials and hybridization temperatures

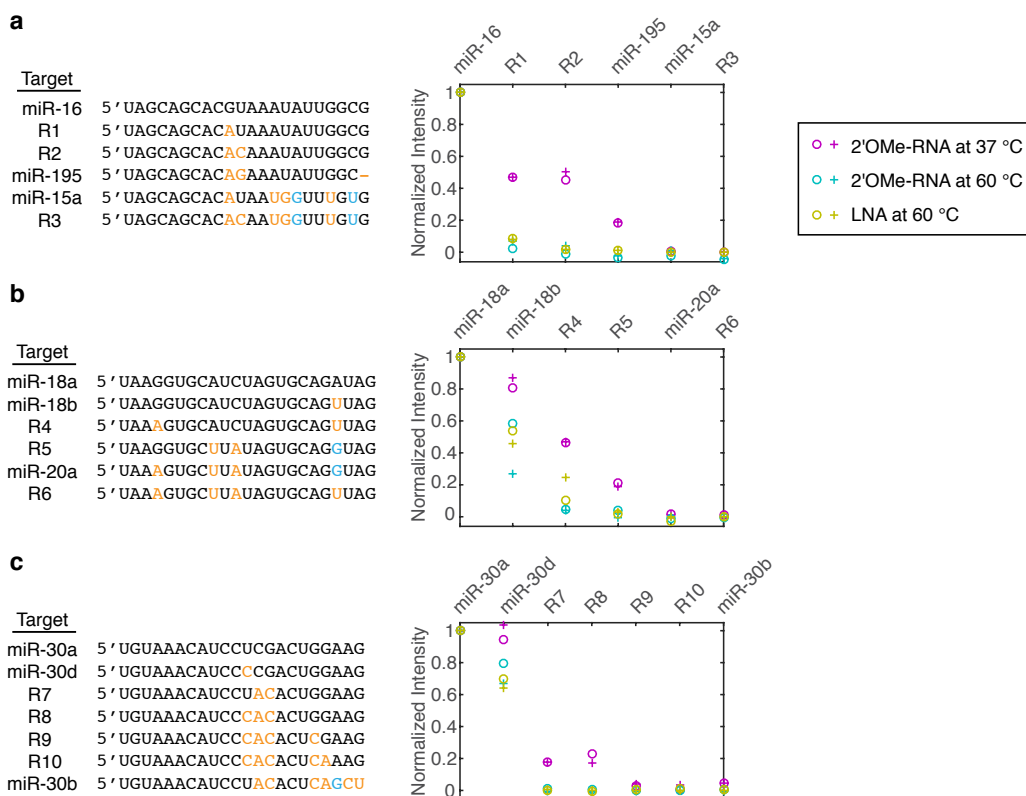


Figure S14: miRNA selectivity for HCR northern blots. (a) 2'OMe-RNA probes at 37 °C. (b) 2'OMe-RNA probes at 60 °C. (b) LNA probes at 60 °C. Off-target sequences depict mismatches in orange and wobble pairs in blue. Each sample is 10 fmol of a 5'-phosphorylated synthetic RNA target. Normalized signal (symbols denote $N = 2$ replicate blots). See Figures S15–S17 for corresponding blots and Figures S11–S13 for corresponding sensitivity studies.

S6.2 Replicates for Figure 7: miRNA selectivity using 2'OMe-RNA probes at 37 °C

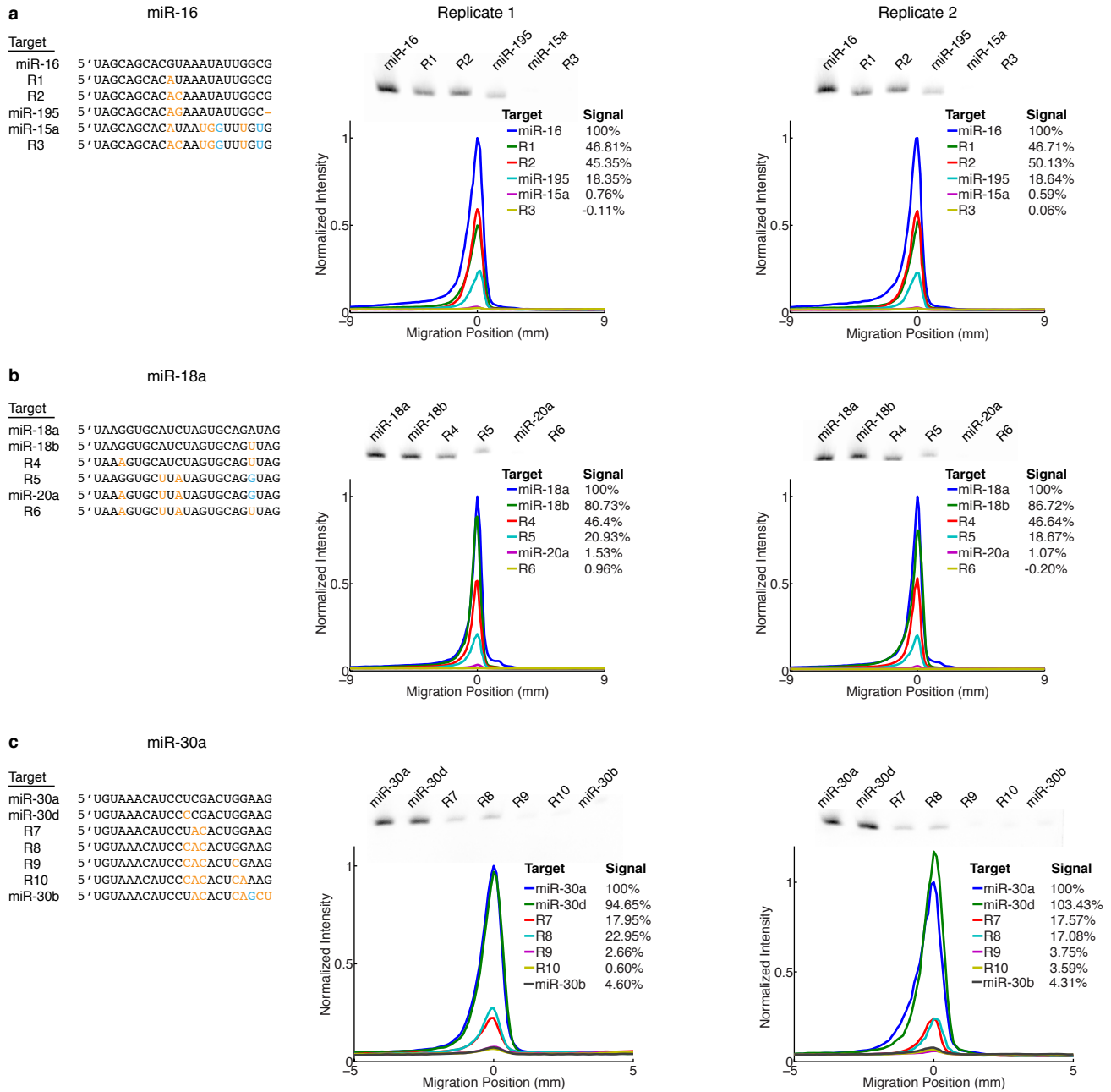


Figure S15: miRNA selectivity for HCR northern blots using 2'OMe-RNA probes at 37 °C. (a) miR-16 and related off-targets. (b) miR-18a and related off-targets. (c) miR-30a and related off-targets. Off-target sequences depict mismatches in orange and wobble pairs in blue. Each sample is 10 fmol of a 5'-phosphorylated synthetic RNA target. Two replicate blots.

S6.3 miRNA selectivity using 2'OMe-RNA probes at 60 °C

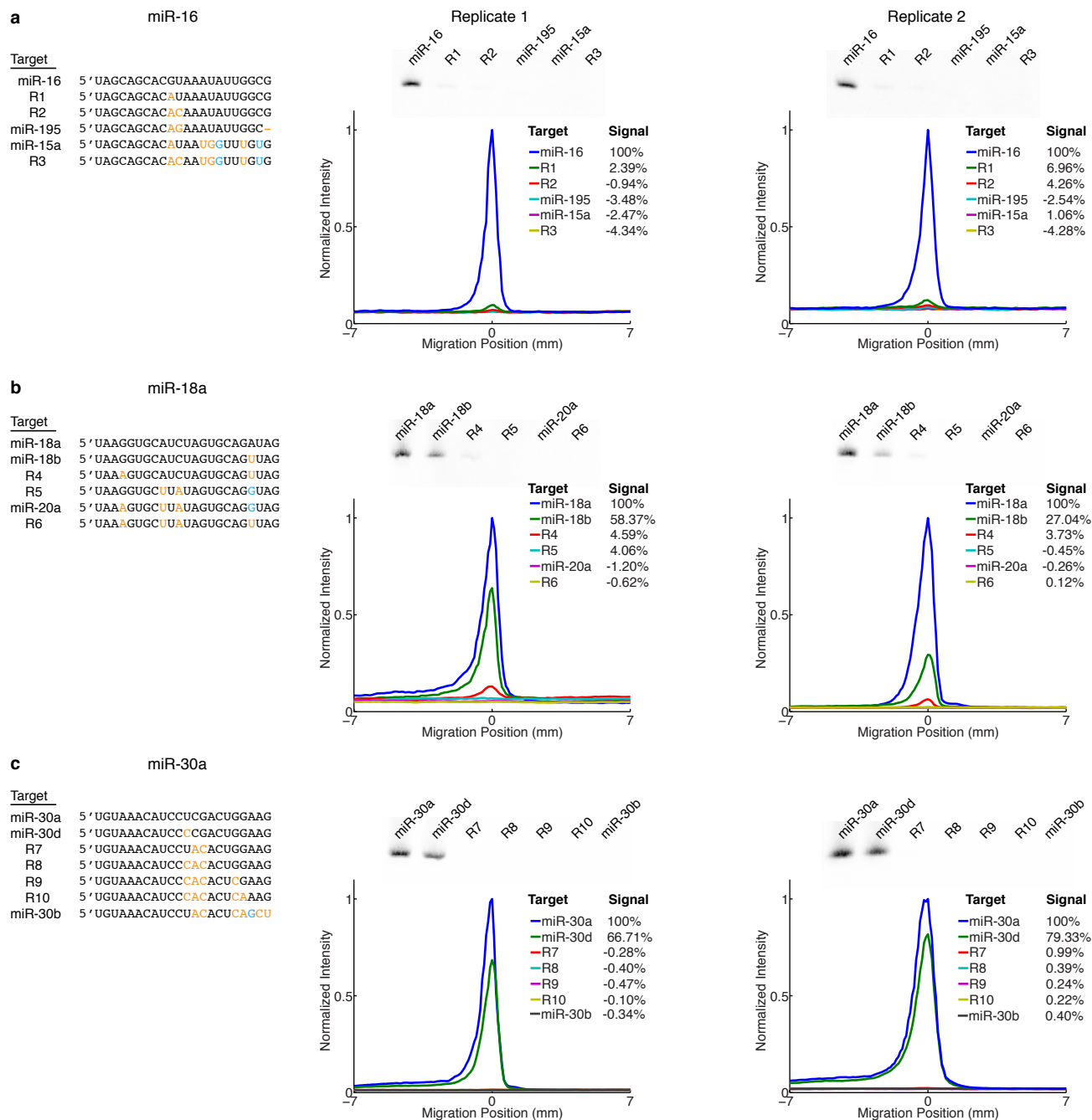


Figure S16: miRNA selectivity for HCR northern blots using 2'OMe-RNA probes at 60 °C. (a) miR-16 and related off-targets. (b) miR-18a and related off-targets. (c) miR-30a and related off-targets. Off-target sequences depict mismatches in orange and wobble pairs in blue. Each sample is 10 fmol of a 5'-phosphorylated synthetic RNA target. Two replicate blots.

S6.4 miRNA selectivity using LNA probes at 60 °C

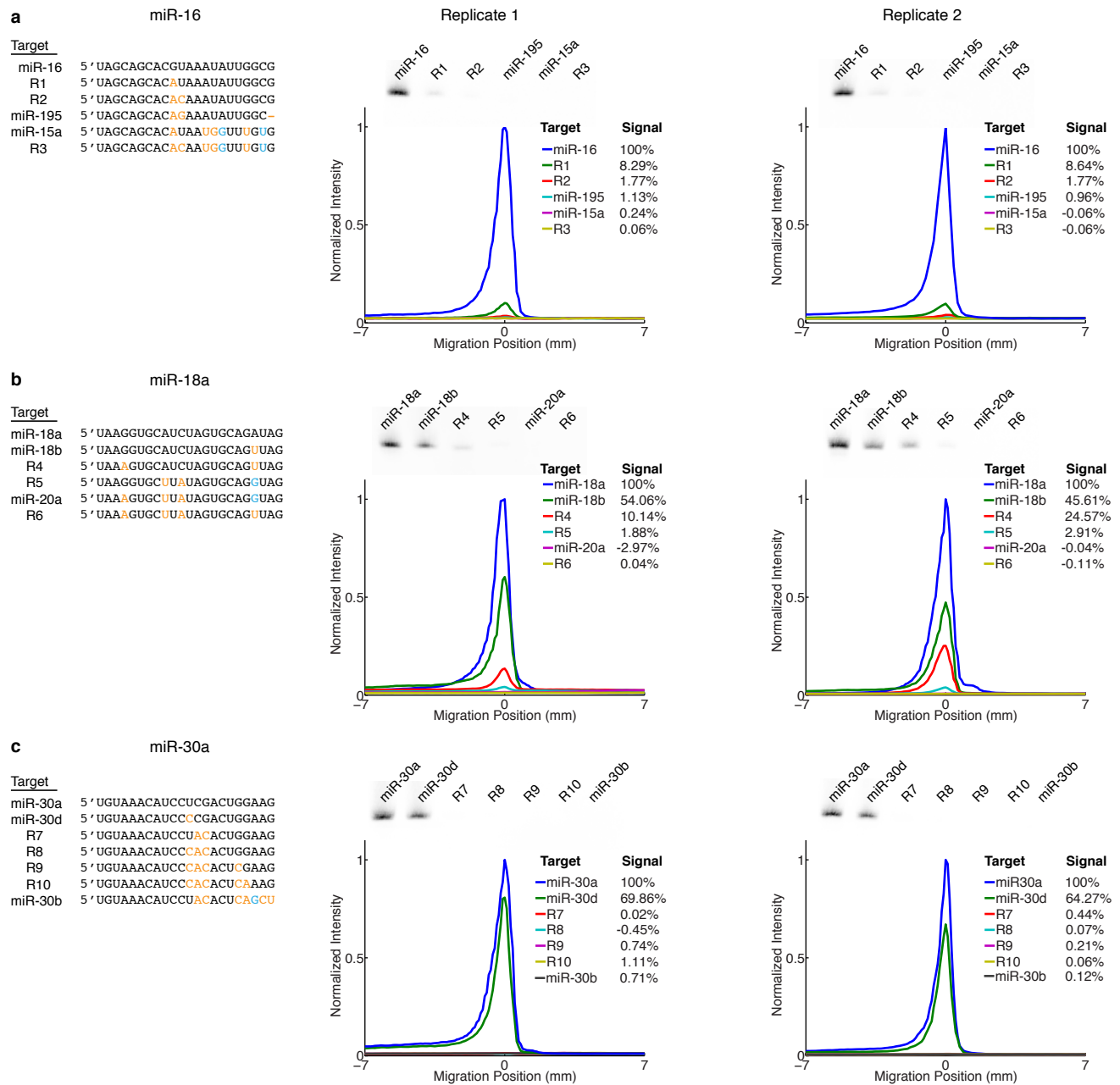


Figure S17: miRNA selectivity for HCR northern blots using LNA probes 60 °C. (a) miR-16 and related off-targets. (b) miR-18a and related off-targets. (c) miR-30a and related off-targets. Off-target sequences depict mismatches in orange and wobble pairs in blue. Each sample is 10 fmol of a 5'-phosphorylated synthetic RNA target. Two replicate blots.

S7 Signal and background as a function of HCR amplification time

Figure S18 demonstrates that the blot background intensity does not depend significantly on HCR amplification time and that the signal does not vary dramatically for amplification times ranging from 1 h to 16 h.

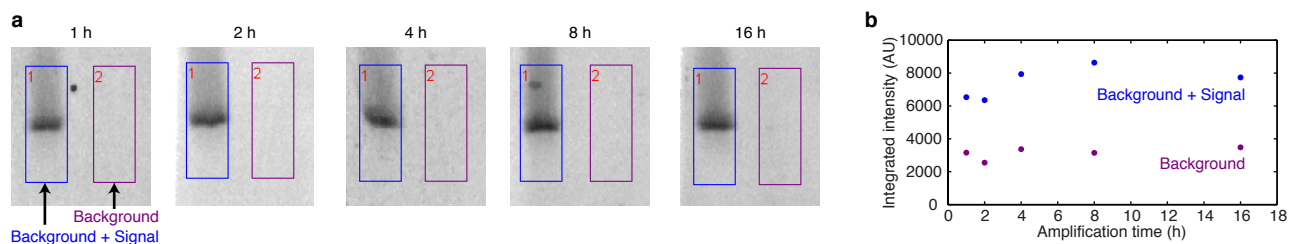


Figure S18: Background and signal as a function of HCR amplification time. (a) HCR northern blots with HCR amplification performed for between 1, 2, 4, 8, or 16 h. Each sample is 5 fmol of synthetic 5'-phosphorylated synthetic miR-18a. (b) Integrated intensity within boxes containing either “Background” or “Background + Signal”.

References

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