

Supplementary Materials

Assessment of NanoString technology as a tool for profiling circulating miRNA in maternal blood during pregnancy

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Supplementary Table 1. Post-hybridisation quality control metrics

Group	Sample	Imaging QC	Binding density QC	Positive Control Linearity QC	Limit of detection QC of RNA	Ligation QC	FOV count	FOV counted
Control	R5	0.99	0.16	0.98	29.03	29.03	555	551
	R8	0.99	0.14	0.99	27.43	27.43	555	550
	R20	0.99	0.15	0.99	23.88	23.88	555	548
	R22	0.99	0.14	0.98	25.03	25.03	555	547
	R23	0.97	0.13	0.99	22.2	22.2	555	538
	R24	0.98	0.15	0.99	22.83	22.83	555	545
Pregnant	R9	0.99	0.15	0.99	38.69	38.69	555	549
	R11	0.99	0.16	0.99	27.32	27.32	555	550
	R12	0.99	0.15	0.99	27.07	27.07	555	550
	R14	0.99	0.14	0.99	24.41	24.41	555	551
	R18	0.99	0.17	0.99	26.48	26.48	555	547
	R19	0.99	0.16	0.99	30.58	30.58	555	547

Supplementary Table 2. Negative control probe data and background level calculation

	Control						Pregnant					
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19
NEG_A	19	11	13	10	16	16	12	18	18	10	21	16
NEG_B	18	10	10	3	11	11	6	7	10	8	13	16
NEG_C	8	11	10	16	3	9	9	11	14	5	16	11
NEG_D	8	10	13	11	7	8	13	12	13	6	10	7
NEG_E	11	19	13	17	10	17	20	15	13	18	16	16
NEG_F	21	19	21	11	12	12	31	20	19	16	18	25

NEG_G	10	6	12	4	3	7	9	12	5	5	10	10
NEG_H	10	4	15	9	6	14	6	6	7	7	11	13

Mean	13.13	11.25	13.38	10.13	8.50	11.75	13.25	12.63	12.38	9.38	14.38	14.25
SD	5.30	5.39	3.50	4.97	4.57	3.69	8.48	4.90	4.90	5.01	4.03	5.44
Mean + 1SD	18.43	16.64	16.88	15.09	13.07	15.44	21.73	17.52	17.27	14.39	18.41	19.69
Mean + 2SD	23.73	22.03	20.38	20.06	17.63	19.14	30.21	22.42	22.17	19.40	22.44	25.14

The table shows the raw count data for the 8 synthetic negative control probes included in the nCounter probeset (Neg_A to Neg_H). The level of background for each test sample is calculated from the mean of these values. In some calculations, a higher level of stringency is applied by adding one or two standard deviations to the mean.

Supplementary Table 3. Positive control probe data and calculation of normalisation factor

	Control						Pregnant					
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19
POS_A	4761	4344	4780	4206	3445	4666	5044	5390	4676	3939	4455	4466
	7	1	0	7	5	8	7	6	4	0	4	9
POS_B	1624	1433	1572	1403	1169	1491	1638	1761	1534	1336	1475	1465
	0	6	4	6	7	0	5	9	5	0	7	0
POS_C	4399	3890	4193	3840	3248	4192	4439	4794	4230	3623	3932	3937
POS_D	873	798	932	842	736	891	909	1078	864	757	846	909
POS_E	117	122	128	106	106	145	127	148	123	107	118	125
POS_F	115	104	130	104	72	100	117	123	91	93	133	129

GeoMean	1849	1704	1912	1661	1394	1831	1916	2114	1756	1559	1803	1832
Arithmetic mean of GeoMeans	1778											
Positive Control Normalisation	1.04	0.96	1.08	0.93	0.78	1.03	1.08	1.19	0.99	0.88	1.01	1.03

Factor												
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The table shows the raw count data for the 6 synthetic positive control probes included in the nCounter probeset (Pos_A to Pos_H). nSolver calculates the geometric mean (GeoMean) for each lane, followed by the arithmetic mean of all samples. The positive control normalisation factor is then calculated for each lane by dividing the lane-specific GeoMean by the arithmetic mean.

Supplementary Table 4. Set of 36 miRNAs used for nCounter sample normalisation with the Total miRNA method.

Probe Name	Accession #	% Samples above Threshold	Count after subtraction			%CV
			Avg	Min	Max	
rno-miR-122	MIMAT0000827	100	1786	49	5697	111
rno-miR-22	MIMAT0000791	100	679	58	2167	96
rno-miR-451	MIMAT0001633	100	600	30	2511	115
rno-miR-150	MIMAT0000853	100	551	30	1858	113
rno-miR-20a+rno-miR-20b-5p	MIMAT0000602	100	292	20	811	83
rno-miR-27b	MIMAT0000798	100	262	9	1157	127
rno-miR-23a	MIMAT0000792	100	203	14	985	133
rno-let-7b	MIMAT0000775	100	194	12	633	94
rno-miR-25	MIMAT0000795	100	192	3	665	107
rno-miR-27a	MIMAT0000799	100	170	17	635	107
rno-miR-449c-3p	MIMAT0017804	100	132	107	157	11
rno-miR-130a	MIMAT0000836	100	129	10	486	98
rno-miR-3594-5p	MIMAT0017898	100	92	71	119	13
rno-miR-3593-5p	MIMAT0017896	100	79	57	109	19
rno-miR-450a	MIMAT0001547	100	74	30	243	93
rno-miR-3590-5p	MIMAT0017889	100	71	51	117	29
rno-miR-421	MIMAT0001320	100	69	41	95	21

rno-miR-3596c	MIMAT0017877	100	62	47	78	14
rno-miR-466d	MIMAT0017824	100	55	44	76	17
rno-miR-455	MIMAT0005316	100	50	33	68	20
rno-miR-501	MIMAT0003116	100	49	32	71	24
rno-miR-466b	MIMAT0005278	100	38	25	57	22
rno-miR-3589	MIMAT0017888	100	34	25	46	18
rno-miR-539	MIMAT0003176	100	31	20	54	40
rno-miR-3595	MIMAT0017903	100	30	23	34	11
rno-miR-3578	MIMAT0017866	100	26	15	36	30
rno-miR-465	MIMAT0012850	100	24	13	41	29
rno-miR-3571	MIMAT0017851	100	21	15	27	21
rno-miR-3585-3p	MIMAT0017879	100	20	13	27	23
rno-miR-3592	MIMAT0017895	100	20	10	26	25
rno-miR-3581	MIMAT0017870	100	17	10	35	38
rno-miR-3590-3p	MIMAT0017890	100	16	7	31	41
rno-miR-3579	MIMAT0017867	100	13	9	20	23
rno-miR-543	MIMAT0004787	100	9	1	23	64
rno-miR-376b-5p	MIMAT0003195	100	9	1	18	75
rno-miR-448	MIMAT0001534	100	6	1	17	82

These were identified by applying a background subtraction of mean + 1SD of negative control probes without positive control normalisation.

Supplementary Table 5. The most stably expressed miRNAs identified by NormFinder software

miRNA name	NormFinder Stability Value	Count after subtraction		
		Avg	Min	Max
miR-20a+miR-20b-5p	0.076	292	20	811
miR-27b	0.121	262	9	1157

GeoMeans												
NormFinder												
Normalisation Factor	0.69	0.69	0.68	0.81	0.73	0.38	0.33	0.14	0.78	1.11	2.44	3.20

The raw count values of the 5 miRNAs identified by NormFinder were first divided by the positive control normalisation factor [Supplementary Table 3], the geometric mean (GeoMean) was calculated, and the arithmetic mean of these GeoMeans was taken. The NormFinder normalisation factor was then calculated by dividing the corresponding GeoMean by the arithmetic mean.

Supplementary Table 7. *In silico* recalculation: Total RNA content normalisation calculation

	Control						Pregnant					
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19
rno-let-7b	410	225	239	120	99	115	107	30	86	80	369	653
rno-miR-122	682	263 2	127 6	190	129	115	259 9	67	571 4	176 0	107 1	539 7
rno-miR-130a	168	197	132	118	67	52	32	32	108	178	161	506
rno-miR-150	151	178	348	571	591	290	112	48	267	567	181 5	187 8
rno-miR-20a+ rno-miR-20b-5p	204	169	309	366	179	192	105	38	237	324	753	831
rno-miR-22	493	572	437	353	332	132	308	76	102 9	700	173 6	218 7
rno-miR-23a	197	145	110	152	132	89	36	32	94	184	461	100 5
rno-miR-25	134	98	133	204	109	173	69	21	113	187	683	589
rno-miR-27a	120	139	140	167	140	58	43	35	92	209	444	655
rno-miR-27b	197	169	166	179	146	99	65	27	201	198	727	117 7
rno-miR-3571	45	35	43	32	31	30	47	44	43	35	38	37

rno-miR-3578	53	37	34	43	31	44	51	51	32	50	52	37
rno-miR-3579	34	31	30	27	33	27	31	29	29	27	29	30
rno-miR-3581	36	35	34	25	31	35	34	53	30	26	40	35
rno-miR-3585-3p	35	32	42	35	29	28	44	42	36	33	42	47
rno-miR-3589	58	49	42	51	43	61	58	55	49	43	44	58
rno-miR-3590-3p	49	33	36	25	22	27	37	37	35	21	43	37
rno-miR-3590-5p	91	83	96	73	64	120	82	78	82	66	135	83
rno-miR-3592	44	37	43	33	23	33	42	42	37	32	41	33
rno-miR-3593-5p	114	98	95	86	88	108	79	127	92	74	106	88
rno-miR-3594-5p	137	98	119	104	99	106	121	108	105	85	121	110
rno-miR-3595	52	46	45	49	46	48	49	46	48	42	41	52
rno-miR-3596c	84	71	81	68	73	86	83	86	64	73	87	98
rno-miR-376b-5p	36	24	29	21	15	32	23	35	23	17	20	32
rno-miR-421	113	79	102	86	54	75	89	89	70	93	95	87
rno-miR-448	32	19	21	18	15	21	23	35	26	21	20	31
rno-miR-449c-3p	161	146	146	148	120	146	179	147	147	121	161	162
rno-miR-450a	75	54	64	62	48	60	61	48	58	89	208	263
rno-miR-451	408	137	459	723	217	590	168	48	299	520	252	130
											9	4
rno-miR-455	73	64	78	66	46	64	74	60	71	50	86	76
rno-miR-465	38	41	43	40	29	56	47	47	41	27	44	41
rno-miR-466b	56	54	42	58	52	46	53	66	49	53	75	56
rno-miR-466d	80	62	93	74	58	76	78	77	61	71	63	76
rno-miR-501	79	66	61	59	47	86	66	57	76	46	74	81
rno-miR-539	62	37	37	46	43	69	43	42	48	34	71	44
rno-miR-543	24	27	23	25	20	20	45	19	24	27	33	26

Positive NF	1.0	0.96	1.08	0.9	0.7	1.0	1.08	1.1	0.99	0.88	1.01	1.03
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	4			3	8	3		9				
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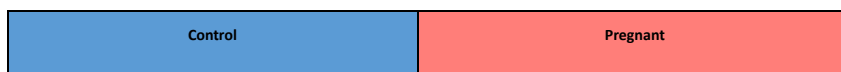
rno-let-7b	394	235	222	128	126	112	99	25	87	91	364	634
rno-miR-122	656	2745	1186	203	164	112	241 1	56	578 4	200 7	105 6	523 8
rno-miR-130a	162	205	123	126	85	50	30	27	109	203	159	491
rno-miR-150	145	186	324	610	753	282	104	40	270	647	179 0	182 3
rno-miR-20a+ rno-miR-20b-5p	196	176	287	392	228	186	97	32	240	369	743	807
rno-miR-22	474	597	406	378	423	128	286	64	1042	798	171 2	212 3
rno-miR-23a	189	151	102	163	168	86	33	27	95	210	455	975
rno-miR-25	129	102	124	218	139	168	64	18	114	213	674	572
rno-miR-27a	115	145	130	179	178	56	40	29	93	238	438	636
rno-miR-27b	189	176	154	192	186	96	60	23	203	226	717	114 2
rno-miR-3571	43	37	40	34	40	29	44	37	44	40	37	36
rno-miR-3578	51	39	32	46	40	42	47	43	32	57	51	36
rno-miR-3579	32	32	28	29	42	26	29	24	29	31	28	29
rno-miR-3581	35	37	32	27	40	34	32	45	30	30	39	34
rno-miR-3585-3p	34	33	39	37	37	27	40	35	36	38	41	46
rno-miR-3589	56	51	39	55	55	59	54	46	50	49	43	56
rno-miR-3590-3p	47	34	33	27	28	26	34	31	35	24	42	36
rno-miR-3590-5p	88	87	89	78	82	117	76	66	83	75	133	81
rno-miR-3592	42	39	40	35	29	32	39	35	37	36	40	32
rno-miR-3593-5p	110	102	88	92	112	105	73	107	93	84	105	85
rno-miR-3594-5p	132	102	111	111	126	103	112	91	106	97	119	107
rno-miR-3595	50	48	42	52	59	47	45	39	49	48	40	51

rno-miR-3596c	81	74	75	73	93	84	77	72	65	83	86	95
rno-miR-376b-5p	35	25	27	22	19	31	21	29	23	19.	20	31
rno-miR-421	109	82	95	92	69	73	83	75	71	106	94	84
rno-miR-448	31	20	20	19	19	20	21	29	26	24	19	30
rno-miR-449c-3p	155	152	136	158	153	142	166	124	149	138	159	157
rno-miR-450a	72	56	60	66	61	58	57	40	59	101	205	255
rno-miR-451	392	143	427	774	277	573	156	40	303	593	249	126
rno-miR-455	70	67	73	71	59	62	69	50	72	57	85	74
rno-miR-465	37	43	40	43	37	54	44	40	42	31	43	40
rno-miR-466b	54	56	39	62	66	45	49	55	50	60	74	54
rno-miR-466d	77	65	87	79	74	74	72	65	62	81	62	74
rno-miR-501	76	69	57	63	60	84	61	48	77	52	73	79
rno-miR-539	60	39	34	49	55	67	40	35	49	39	70	42
rno-miR-543	23	28	21	27	26	19	42	16	24	31	33	25

GeoMean	87	81	78	83	78	67	63	41	81	91	126	138
Arithmetic mean of GeoMeans	85											
Total miRNA Normalisation Factor	1.0			0.9	0.9	0.8		0.4				
	3	0.96	0.92	8	3	0	0.75	9	0.96	1.07	1.49	1.63

The raw count values were first divided by the positive control normalisation factor [Supplementary Table 3], the geometric mean (GeoMean) was calculated, and the arithmetic mean of these GeoMeans was taken. The Total RNA normalisation factor was then calculated by dividing the corresponding GeoMean by the arithmetic mean.

Supplementary Table 8. *In silico* recalculation: Background Thresholding plus NormFinder Normalisation recalculations for the 4 miRNA targets



	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-450a Raw values	75	54	64	62	48	60	61	48	58	89	208	263	
Positive Normalisation	72.11	56.32	59.50	66.34	61.19	58.26	56.58	40.36	58.71	101.48	205.12	255.27	
NormFinder	104.68	81.23	87.23	81.58	83.78	153.10	170.64	283.68	74.98	91.21	84.03	79.75	T-test
Mean	104.68	81.23	87.23	81.58	83.78	153.10	170.64	283.68	74.98	91.21	84.03	79.75	0.403
Mean+1SD	104.68	81.23	87.23	81.58	83.78	153.10	170.64	283.68	74.98	91.21	84.03	79.75	0.403
Mean+2SD	104.68	81.23	87.23	81.58	83.78	153.10	170.64	283.68	74.98	91.21	84.03	79.75	0.403

	Control						Pregnant						T-test
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-183 Raw values	4	2	3	4	2	2	6	4	4	5	24	18	
Positive Normalisation	3.85	2.09	2.79	4.28	2.55	1.94	5.57	3.36	4.05	5.70	23.67	17.47	
NormFinder	5.58	3.01	4.09	5.26	3.49	5.10	16.78	23.64	5.17	5.12	9.70	5.46	T-test
Mean	13.13	11.25	13.38	10.13	8.50	11.75	16.78 (13.25)	23.64 (12.62)	12.38	9.38	14.38	14.25	0.12
Mean+1SD	18.43	16.64	16.88	15.09	13.07	15.44	21.73	23.64 (17.52)	17.27	14.39	18.41	19.69	0.07
Mean+2SD	23.73	22.03	20.38	20.06	17.63	19.14	30.21	23.64 (22.42)	22.17	19.40	22.44	25.14	0.09

	Control						Pregnant						T-test
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-431 Raw values	19	17	25	16	17	16	16	10	13	9	22	26	
Positive Normalisation	18.27	17.73	23.24	17.12	21.67	15.53	14.84	8.41	13.16	10.26	21.70	25.24	
NormFinder	26.52	25.57	34.07	21.05	29.67	40.83	44.76	59.10	16.81	9.22	8.89	7.88	T-test
Mean	26.52	25.57	34.07	21.05	29.67	40.83	44.76	59.10 (12.62)	16.81	9.38	14.38	14.25	0.73

Mean+1SD	26.52	25.57	34.07	21.05	29.67	40.83	44.76 (21.73)	59.10 (17.52)	17.27	14.39	18.41	19.69	0.94
Mean+2SD	26.52 (23.73)	25.57 (22.03)	34.07	21.05 (20.06)	29.67 (17.63)	40.83 (19.14)	44.76 (30.21)	59.10 (22.42)	22.17	19.40	22.44	25.14	0.73

	Control						Pregnant						T-test
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-196c Raw values	19	13	26	15	19	31	12	10	17	13	25	22	
Positive Normalisation	18.27	13.56	24.17	16.05	24.22	30.10	11.13	8.41	17.21	14.82	24.65	21.35	
NormFinder	26.52	19.56	35.44	19.74	33.16	79.10	33.57	59.10	21.98	13.32	10.10	6.67	
Mean	26.52	19.56	35.44	19.74	33.16	79.10	33.57 (13.25)	59.10 (12.62)	21.98	13.32	14.38	14.25	0.44
Mean+1SD	26.52	19.56 (16.64)	35.44	19.74 (15.09)	33.16	79.10	33.57 (21.73)	59.10 (17.52)	21.98 (17.27)	14.39	18.41	19.69	0.51
Mean+2SD	26.52 (23.73)	22.03	35.44	20.06	33.16	79.10	33.57 (30.21)	59.10 (22.42)	22.17	19.40	22.44	25.14	0.61

The raw values were first subjected to positive normalisation [Supplementary Table 3], followed by NormFinder normalisation [Supplementary Table 6]. The final values were then compared to the background levels [Supplementary Table 2], and if the value fell below the background value for that lane, the value was replaced by the background value. Red values highlight values that show discrepancies between the recalculation and nSolver, with nSolver values in brackets. Significance was tested using Welch's t-test. *P*-values highlighted in orange are those that were shown to be statistically significant (*P*-value < 0.05) by nSolver but were found to be non-significant upon recalculation.

Supplementary Table 9. *In silico* recalculation: Background Thresholding plus Total RNA Normalisation recalculations for the 4 miRNA targets

Control						Pregnant					
R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19

miR-450a Raw Values	75	54	64	62	48	60	61	48	58	89	208	263	
Positive Normalisation	72.11	56.32	59.37	52.13	48.59	68.42	60.16	46.59	53.93	95.22	265.18	255.35	
Total miRNA Normalisation	69.78	58.94	64.49	67.96	66.04	73.26	75.52	82.87	61.15	94.61	137.63	156.52	T-test
Mean	69.78	58.94	64.49	67.96	66.04	73.26	75.52	82.87	61.15	94.61	137.63	156.52	0.073
Mean+1SD	69.78	58.94	64.49	67.96	66.04	73.26	75.52	82.87	61.15	94.61	137.63	156.52	0.073
Mean+2SD	69.78	58.94	64.49	67.96	66.04	73.26	75.52	82.87	61.15	94.61	137.63	156.52	0.073

	Control						Pregnant						
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-183 Raw Values	4	2	3	4	2	2	6	4	4	5	24	18	
Positive Normalisation	3.85	2.09	2.78	3.36	2.02	2.28	5.92	3.88	3.72	5.35	30.60	17.48	
Total miRNA Normalisation	3.72	2.18	3.02	4.38	2.75	2.44	7.43	6.91	4.22	5.32	15.88	10.71	T-test
Mean	13.13	11.25	13.38	10.13	8.50	11.75	13.25	12.63	12.38	9.38	15.88	14.25	0.199
Mean+1SD	18.43	16.64	16.88	15.09	13.07	15.44	21.73	17.52	17.27	14.39	18.41	19.69	0.107
Mean+2SD	23.73	22.03	20.38	20.06	17.63	19.14	30.21	22.42	22.17	19.40	22.44	25.14	0.111

	Control						Pregnant						
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-431 Raw Values	19	17	25	16	17	16	16	10	13	9	22	26	
Positive Normalisation	18.27	17.73	23.19	13.45	17.21	18.24	15.78	9.71	12.09	9.63	28.05	25.24	
Total miRNA Normalisation	17.68	18.55	25.19	17.54	23.39	19.54	19.81	17.27	13.71	9.57	14.56	15.47	T-test
Mean	17.68	18.55	25.19	17.54	23.39	19.54	19.81	17.27 <i>(12.62)</i>	13.71	9.57 <i>(9.38)</i>	14.56	15.47	0.021
Mean+1SD	18.43	18.55	25.19	17.54	23.39	19.54	21.73	17.52	17.27	14.39	18.41	19.69	0.193
Mean+2SD	23.73	22.03	25.19	20.06	23.39 <i>(17.63)</i>	19.54 <i>(19.14)</i>	30.21	22.42	22.17	19.40	22.44	25.14	0.479

	Control						Pregnant						T-tes t
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-196c Raw Values	19	13	26	15	19	31	12	10	17	13	25	22	
Positive Normalisation	18.27	13.56	24.17	16.05	24.22	30.10	11.13	8.41	17.21	14.82	24.65	21.35	
Total miRNA Normalisation	17.68	14.19	26.26	20.92	32.92	32.23	13.97	14.96	19.51	14.73	12.80	13.09	
Mean	17.68	14.19	26.26	20.92 (16.44)	32.92 (26.14)	32.23 (37.85)	13.97 (13.25)	14.96 (12.62)	19.51 (17.92)	14.73 (13.82)	14.38 (16.54)	14.25	0.039
Mean+1SD	18.43	16.64	26.26	20.92 (15.09)	32.92 (26.14)	32.23 (37.85)	21.73	17.52	19.51 (17.27)	14.73 (14.39)	18.41	19.69	0.094
Mean+2SD	23.73	22.03	26.26	20.92 (20.06)	32.92 (26.14)	32.23 (37.85)	30.21	22.42	22.17	19.40	22.44	25.14	0.321

The raw values were first subjected to positive normalisation [Supplementary Table 3], followed by total miRNA normalisation [Supplementary Table 7]. The final values were then compared to the background levels [Supplementary Table 2], and of the value fell below the background value for that lane, the value was replaced by the background value. Red values highlight values that show discrepancies between the recalculation and nSolver, with nSolver values in brackets. Significance was tested using Welch's t-test. *P*-values highlighted in green are those that were found to be statistically significant (P -value < 0.05) by nCounter and agreed by the recalculation.

	Control						Pregnant						T-tes t
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-450a Raw Values	75	54	64	62	48	60	61	48	58	89	208	263	
Subtraction (Mean)	61.88	42.75	50.63	51.88	39.50	48.25	47.75	35.38	45.63	79.63	193.6 3	248.7 5	
Positive Normalisation	59.49	44.59	47.07	55.50	50.36	46.85	44.29	29.74	46.18	90.79	190.9 5	241.4 3	

		64.31		68.26		123.12		209.06	58.98	81.60	78.22	75.42	
	86.36	(66.49	69.00	(68.91	68.94	(133.86	133.57	(291.37	(61.45	(80.63	(75.81	(72.54	0.32
NormFinder	(89.2)	(71.6))	(70.1))	(154))))))	9
Subtraction											189.5	243.3	
(Mean+1SD)	56.57	37.36	47.12	46.91	34.93	44.56	39.27	30.48	40.73	74.61	9	1	
Positive Normalisation	54.39	38.96	43.81	50.19	44.54	43.26	36.43	25.63	41.22	85.08	186.9	236.1	T-tes
											7	5	t
	78.96	56.20	64.22	61.72	60.97	113.69	109.85	180.12	52.65	76.47	76.59	73.77	
	(82.57	(59.39	(66.59	(62.86	(63.07	(126.38	(143.35	(304.16	(55.77	(75.45	(72.71	(69.51	0.31
NormFinder))))))))))))	4
Subtraction											185.5	237.8	
(Mean+2SD)	51.27	31.97	43.62	41.94	30.37	40.86	30.79	25.58	35.83	69.60	6	6	
Positive Normalisation	49.29	33.34	40.56	44.87	38.71	39.67	28.56	21.51	36.27	79.36	182.9	230.8	T-tes
											9	7	t
	71.56	48.09	59.45	55.18	53.00	104.27	86.12	151.18	46.32	71.33	74.96	72.12	
	(75.79	(52.10	(61.55	(56.73	(58.89	(118.66	(137.79	(340.06	(49.98	(70.22	(69.56	(66.43	0.30
NormFinder))))))))))))	5

	Control						Pregnant						T-test
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-183 Raw Values	4	2	3	4	2	2	6	4	4	5	24	18	
Subtraction (Mean)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	9.63	3.75	
Positive Normalisation	0.96	1.04	0.93	1.07	1.27	0.97	0.93	0.84	1.01	1.14	9.49	3.64	T-test
NormFinder	1.40	1.50	1.36	1.32	1.75	2.55	2.80	5.91	1.29	1.02	3.89	1.14	
	(1.44)	(1.56)	(1.41)	(1.33)	(1.77)	(2.77)	(3.23)	(8.24)	(1.35)	(1.01)	(3.77)	(1.09)	0.258
Subtraction (Mean+1SD)	1	1	1	1	1	1	1	1	1	1	5.59	1	

Positive Normalisation	0.96	1.04	0.93	1.07	1.27	0.97	0.93	0.84	1.01	1.14	5.51	0.97	T-test
NormFinder	1.40 (1.46)	1.50 (1.59)	1.36 (1.41)	1.32 (1.34)	1.75 (1.81)	2.55 (2.84)	2.80 (3.7)	5.91 (9.98)	1.29 (1.37)	1.02 (1.01)	2.26 (2.14)	0.30 (1)	0.490
Subtraction (Mean+2SD)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.56	1.00	
Positive Normalisation	0.96	1.04	0.93	1.07	1.27	0.97	0.93	0.84	1.01	1.14	1.54	0.97	T-test
NormFinder	1.40 (1.48)	1.50 (1.63)	1.36 (1.41)	1.32 (1.35)	1.75 (1.84)	2.55 (2.9)	2.80 (4.48)	5.91 (13.29)	1.29 (1.39)	1.02 (1.01)	0.63 (1)	0.30 (1)	0.708

Supplementary Table 10. *In silico* recalculation: Background Subtraction plus NormFinder Normalisation recalculations for the 4 miRNA targets

	Control						Pregnant						T-test
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-431 Raw Values	19	17	25	16	17	16	16	10	13	9	22	26	
Subtraction (Mean)	5.88	5.75	11.63	5.88	8.50	4.25	2.75	1	1	1	7.63	11.75	
Positive Normalisation	5.65	6.00	10.81	6.29	10.84	4.13	2.55	0.84	1.01	1.14	7.52	11.40	T-test
NormFinder	8.20 (8.47)	8.65 (8.94)	15.84 (16.44)	7.73 (7.8)	14.84 (15.09)	10.84 (11.79)	7.69 (8.87)	5.91 (8.24)	1.29 (1.35)	1.02 (1.01)	3.08 (2.99)	3.56 (3.43)	0.003

Subtraction (Mean+1SD)	1	1	8.12	1	3.93	1	1	1	1	1	3.59	6.31	
Positive Normalisation	0.96	1.04	7.55	1.07	5.01	0.97	0.93	0.84	1.01	1.14	3.54	6.12	T-test
NormFinder	1.40 (1.46)	1.50 (1.59)	11.07 (11.49)	1.32 (1.34)	6.86 (7.1)	2.55 (2.84)	2.80 (3.7)	5.91 (9.98)	1.29 (1.37)	1.02 (1.01)	1.45 (1.38)	1.91 (1.8)	0.371

Subtraction (Mean+2SD)	1	1	4.62	1	1	1	1	1	1	1	1	1	
Positive Normalisation	0.96	1.04	4.30	1.07	1.27	0.97	0.93	0.84	1.01	1.14	0.99	0.97	T-test
NormFinder	1.40 (1.48)	1.50 (1.63)	6.30 (6.52)	1.32 (1.35)	1.75 (1.84)	2.55 (2.9)	2.80 (4.48)	5.91 (13.29)	1.29 (1.39)	1.02 (1.01)	0.40 (1)	0.30 (1)	0.672

	Control						Pregnant						T-test
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-196c Raw Values	19	13	26	15	19	31	12	10	17	13	25	22	
Subtraction (Mean)	5.88	1.75	12.63	4.88	10.50	19.25	1.00	1	5	4	10.63	7.75	
Positive Normalisation	5.65	1.83	11.74	5.22	13.39	18.69	0.93	0.84	4.68	4.13	10.48	7.52	
NormFinder	8.20 (8.47)	2.63 (2.72)	17.21 (17.86)	6.41 (6.48)	18.33 (18.63)	49.12 (53.41)	2.80 (3.23)	5.91 (8.24)	5.98 (6.23)	3.72 (3.67)	4.29 (4.16)	2.35 (2.26)	0.123

Subtraction (Mean±1SD)	1	1	9.12	1	5.93	16	1	1	1	1	6.59	2.31	
Positive Normalisation	0.96	1.04	8.48	1.07	7.56	15.10	0.93	0.84	1.01	1.14	6.50	2.24	T-test
NormFinder	1.40 (1.46)	1.50 (1.59)	12.43 (12.89)	1.32 (1.34)	10.36 (10.71)	39.69 (44.12)	2.80 (3.70)	5.91 (9.98)	1.29 (1.37)	1.02 (1.01)	2.66 (2.53)	0.70 (1)	0.211

Subtraction (Mean±2SD)	1	1	5.62	1	1	12	1	1	1	1	3	1	
Positive Normalisation	0.96	1.04	5.23	1.07	1.74	11.52	0.93	0.84	1.01	1.14	2.52	0.97	T-test
NormFinder	1.40 (1.48)	1.50 (1.63)	7.66 (7.93)	1.32 (1.35)	2.38 (2.51)	30.27 (34.45)	2.80 (4.48)	5.91 (13.29)	1.29 (1.39)	1.02 (1.01)	1.03 (1)	0.30 (1)	0.307

The raw values were first subjected to background subtraction [Supplementary Table 2], followed by positive normalisation [Supplementary Table 3] and then NormFinder normalisation [Supplementary Table 6]. Red values highlight values that show discrepancies between the recalculation and nCounter, with final nSolver values in brackets. Significance was tested using Welch's t-test. *P*-values highlighted in green are those that were found to be statistically significant (*P*-value < 0.05) by nCounter and agreed by the recalculation.

	Control						Pregnant						T-test
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-450a Raw Values	75	54	64	62	48	60	61	48	58	89	208	263	
Subtraction (Mean)	61.88	42.75	50.63	51.88	39.50	48.25	47.75	35.38	45.63	79.63	193.63	248.75	
Positive Normalisation	59.49	44.59	47.07	55.50	50.36	46.85	44.29	29.74	46.18	90.79	190.95	241.43	t

Positive Normalisation	0.96	1.04	0.93	1.07	1.27	0.97	0.93	0.84	1.01	1.14	5.51	0.97	T-test
Total miRNA Normalisation	0.93 (1)	1.1	1.01 (1)	1.39 (1.06)	1.73 (1.38)	1.04 (1.18)	1.16 (1.53)	1.50 (2.03)	1.15 (1.05)	1.13 (1.04)	2.86 (3.71)	0.59 (1)	0.576

Subtraction (Mean + 2SD)	1	1	1	1	1	1	1	1	1	1	1.56	1	
Positive Normalisation	0.96	1.04	0.93	1.07	1.27	0.97	0.93	0.84	1.01	1.14	1.54	0.97	T-test
Total miRNA Normalisation	0.93 (1)	1.1	1.01 (1)	1.39 (1.1)	1.73 (1.38)	1.04 (1.14)	1.16 (1.85)	1.50 (2.12)	1.15 (1.09)	1.13 (1.08)	0.80 (1)	0.59 (1)	0.440

	Control						Pregnant						T-test
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-431 Raw Values	19	17	25	16	17	16	16	10	13	9	22	26	
Subtraction (Mean)	5.88	5.75	11.63	5.88	8.50	4.25	2.75	1	1	1	7.63	11.75	
Positive Normalisation	5.65	6.00	10.81	6.29	10.84	4.13	2.55	0.84	1.01	1.14	7.52	11.40	T-test
Total miRNA Normalisation	5.47 (5.32)	6.28 (6.19)	11.74 (12.01)	8.19 (6.25)	14.73 (11.37)	4.42 (5.24)	3.20 (3.54)	1.50 (1.91)	1.15 (1.07)	1.13 (1.03)	3.90 (5.14)	6.99 (6.98)	0.019

Subtraction (Mean + 1SD)	1	1	8.12	1	3.93	1	1	1	1	1	3.59	6.31	
Positive Normalisation	0.96	1.04	7.55	1.07	5.01	0.97	0.93	0.84	1.01	1.14	3.54	6.12	T-test
Total miRNA Normalisation	0.93 (1)	1.1	8.20 (7.95)	1.39 (1.06)	6.82 (5.43)	1.04 (1.18)	1.16 (1.53)	1.50 (2.03)	1.15 (1.05)	1.13 (1.04)	1.84 (2.38)	3.75 (3.67)	0.336

Subtraction (Mean + 2SD)	1	1	4.62	1	1	1	1	1	1	1	1	1	
Positive Normalisation	0.96	1.04	4.30	1.07	1.27	0.97	0.93	0.84	1.01	1.14	0.99	0.97	T-test
Total miRNA Normalisation	0.93 (1)	1.1	4.67 (4.27)	1.39 (1.1)	1.73 (1.38)	1.04 (1.14)	1.16 (1.85)	1.50 (2.12)	1.15 (1.09)	1.13 (1.08)	0.51 (1)	0.59 (1)	0.235

	Control						Pregnant						T-test
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-196c Raw Values	19	13	26	15	19	31	12	10	17	13	25	22	
Subtraction (Mean)	5.88	1.75	12.63	4.88	10.50	19.25	1.00	1	5	4	10.63	7.75	
Positive Normalisation	5.65	1.83	11.74	5.22	13.39	18.69	0.93	0.84	4.68	4.13	10.48	7.52	
Total miRNA Normalisation	5.47 (5.32)	1.91 (1.89)	12.75 (13.05)	6.80 (5.18)	18.19 (14.04)	20.01 (23.72)	1.16 (1.29)	1.50 (1.91)	5.31 (4.93)	4.11 (3.72)	5.44 (7.17)	4.61	0.061

Subtraction (Mean±1SD)	1	1	9.12	1	5.93	16	1	1	1	1	6.59	2.31	
Positive Normalisation	0.96	1.04	8.48	1.07	7.56	15.10	0.93	0.84	1.01	1.14	6.50	2.24	T-test
Total miRNA Normalisation	0.93 (1)	1.09	9.21 (8.93)	1.39 (1.06)	10.28 (8.19)	16.17 (18.35)	1.16 (1.53)	1.50 (2.03)	1.15 (1.05)	1.13 (1.04)	3.37 (4.37)	1.37 (1.34)	0.118

Subtraction (Mean±2SD)	1	1	5.62	1	1	12	1	1	1	1	3	1	
Positive Normalisation	0.96	1.04	5.23	1.07	1.74	11.52	0.93	0.84	1.01	1.14	2.52	0.97	T-test
Total miRNA Normalisation	0.93 (1)	1.09 (1.10)	5.68 (5.20)	1.39 (1.10)	2.37 (1.89)	12.33 (13.54)	1.16 (1.85)	1.50 (2.12)	1.15 (1.09)	1.13 (1.08)	1.31 (1.54)	0.59 (1)	0.182

The raw values were first subjected to background subtraction [Supplementary Table 2], followed by positive normalisation [Supplementary Table 3] and then total miRNA normalisation [Supplementary Table 7]. Red values highlight values that show discrepancies between the recalculation and nCounter, with final nSolver values in brackets. Significance was tested using Welch's t-test. *P*-values highlighted in orange are those that were found to be statistically significant (P -value < 0.05) by nCounter but were not replicated in the recalculation. *P*-values highlighted in green are those that were found to be statistically significant (P -value < 0.05) by nCounter and agreed by the recalculation.

Supplementary Table 12. *In silico* recalculation: No Background Subtraction plus NormFinder Normalisation recalculations for the 4 miRNA targets

	Control						Pregnant					
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19
miR-450a Raw Values	75	54	64	62	48	60	61	48	58	89	208	263

Positive Normalisation	72.11	56.32	59.50	66.34	61.19	58.26	56.58	40.36	58.71	101.48	205.12	255.27	T-test
NormFinder	104.68	81.23	87.23	81.58	83.78	153.10	170.64	283.68	74.98	91.21	84.03	79.75	0.77

	Control						Pregnant						
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-183 Raw Values	4	2	3	4	2	2	6	4	4	5	24	18	
Positive Normalisation	3.85	2.09	2.79	4.28	2.55	1.94	5.57	3.36	4.05	5.70	23.67	17.47	T-test
NormFinder	5.58	3.01	4.09	5.26	3.49	5.10	16.78	23.64	5.17	5.12	9.70	5.46	0.045

	Control						Pregnant						
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-431 Raw Values	19	17	25	16	17	16	16	10	13	9	22	26	
Positive Normalisation	18.27	17.73	23.24	17.12	21.67	15.53	14.84	8.41	13.16	10.26	21.70	25.24	T-test
NormFinder	26.52	25.57	34.07	21.05	29.67	40.83	44.76	59.10	16.81	9.22	8.89	7.88	0.30

	Control						Pregnant						
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-196c Raw Values	19	13	26	15	19	31	12	10	17	13	25	22	
Positive Normalisation	18.27	13.56	24.17	16.05	24.22	30.10	11.13	8.41	17.21	14.82	24.65	21.35	T-test
NormFinder	26.52	19.56	35.44	19.74	33.16	79.10	33.57	59.10	21.98	13.32	10.10	6.67	0.37

The raw values were first subjected to positive normalisation [Supplementary Table 3] followed by NormFinder normalisation[Supplementary Table 6]. Significance was tested using Welch's t-test. *P*-values highlighted in green are those that were found to be statistically significant (P -value < 0.05) by nCounter and agreed by the recalculation.

Supplementary Table 13. *In silico* recalculation: No Background Subtraction plus Total RNA Normalisation recalculations for the 4 miRNA targets.

Control	Pregnant
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	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-450a Raw Values	75	54	64	62	48	60	61	48	58	89	208	263	
Positive Normalisation	72.11	56.32	59.37	52.13	48.59	68.42	60.16	46.59	53.93	95.22	265.18	255.35	T-test
Total miRNA Normalisation	69.78	58.94	64.49	67.96	66.04	73.26	75.52	82.87	61.15	94.61	137.63	156.52	0.09

	Control						Pregnant						
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-183 Raw Values	4	2	3	4	2	2	6	4	4	5	24	18	
Positive Normalisation	3.85	2.09	2.78	3.36	2.02	2.28	5.92	3.88	3.72	5.35	30.60	17.48	T-test
Total miRNA Normalisation	3.72	2.18	3.02	4.38	2.75	2.44	7.43	6.91	4.22	5.32	15.88	10.71	0.03

	Control						Pregnant						
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-431 Raw Values	19	17	25	16	17	16	16	10	13	9	22	26	
Positive Normalisation	18.27	17.73	23.19	13.45	17.21	18.24	15.78	9.71	12.09	9.63	28.05	25.24	T-test
Total miRNA Normalisation	17.68	18.55	25.19	17.54	23.39	19.54	19.81	17.27	13.71	9.57	14.56	15.47	0.02

	Control						Pregnant						
	R5	R8	R20	R22	R23	R24	R9	R11	R12	R14	R18	R19	
miR-196ca Raw Values	19	13	26	15	19	31	12	10	17	13	25	22	
Positive Normalisation	18.27	13.56	24.17	16.05	24.22	30.10	11.13	8.41	17.21	14.82	24.65	21.35	T-test
Total miRNA Normalisation	17.68	14.19	26.26	20.92 (16.44)	32.92 (26.14)	32.23 (37.85)	13.97 (14.86)	14.96 (17.27)	19.51 (17.92)	14.73 (13.82)	12.80 (16.54)	13.09	0.03

The raw values were first subjected to positive normalisation [Supplementary Table 3] followed by total miRNA normalisation[Supplementary Table 7]. Significance was tested using Welch's t-test. *P*-values highlighted in green are those that were found to be statistically significant (*P*-value < 0.05) by nCounter and agreed by the recalculation. *P*-values highlighted in blue are those that were found to be significant

according to the recalculation but were not identified as a significant change by nSolver.