Supplementary Materials and Methods

All experimental steps were compliant with the Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) (1) (full details available on request).

Patient demographics

Clinico-pathological details are listed in Table 1. The initial discovery-phase study set represented 54 serum samples from 54 patients. The tumor group (n=34) included 18 (53%) male patients and 16 (47%) females with a median age ± standard deviation of 63 ± 59 months. There were no significant differences in age between the male and female patients within the tumor group (p=0.35; two-tailed unpaired t-test). The control group (n=20) included 11 (55%) male patients and 9 (45%) females with a median age of 49 ± 53 months. There were no significant differences in age between the male and female patients within the control group (p=0.92). There were also no significant differences by age between the tumor and control groups (p=0.74).

Processing of samples

All serum samples were collected in serum separator tubes as part of standard clinical care, processed and centrifuged within four hours of receipt. After routine clinical testing had been performed, residual serum was frozen and stored at -80^{o}C and only thawed for RNA isolation and extraction, as described (2). Only samples without macroscopic evidence of hemolysis on visual inspection were utilized for this study, to avoid substantial technical variation due to the release of intracellular microRNAs from red blood cells (3).

RNA extraction

All RNA extractions were performed using the miRNeasy Mini-Kit (Qiagen, Crawley, UK). Serum was thawed on ice and centrifuged for five minutes in a cooled 4^{o}C microcentrifuge before 200µl of sample serum was added to a 750µl Qiazol mix [containing 1.25µg/ml MS2 non-human

bacteriophage RNA (Roche, Welwyn Garden City, UK) to maximise yield, as described (4)]. In addition, a set amount of UniSp2 RNA was added as a spike-in kit (Exiqon, Vedbaek, Denmark) to each sample to allow subsequent assessment of the efficiency of RNA isolation (5). Next, 200µl of chloroform was added and the mixture vortexed briefly before the samples were left for three minutes at room temperature. The samples were then centrifuged at 13,200 rpm for 30 minutes at 4^{o}C and the aqueous phase retained, to which 1.5 volumes of 100% ethanol was added and mixed thoroughly. The sample was then loaded into a MinElute spin-column (Qiagen) and centrifuged for 30 seconds at 13,200 rpm at room temperature as per the manufacturer's instructions. After rinsing with 700µl of RWT-Mini-Kit buffer, the column was centrifuged at 13,200 rpm for one minute at room temperature, followed by another rinse with 500µl of RPE-Mini-Kit buffer. After allowing the column to dry, the RNA was extracted by the addition of 50µl of RNase-free water to the membrane before centrifugation at 13,200 rpm for one minute. The RNA eluate was then stored at -80^{o}C until used.

Reverse transcription (RT) and initial quality control (QC) qRT-PCR

Fifteen µl of RNA eluate was reverse transcribed using the miRCURY-LNA Universal-RT microRNA PCR, polyadenylation and cDNA synthesis Kit (Exiqon) in a 75µl reaction, as per the manufacturer's instructions. Set amounts of UniSp6 RNA (Exiqon) were added to each sample at this stage to allow subsequent assessment of RT and final qRT-PCR efficiency, as described (5). The RT assay was carried out in 10µl PCR reactions using 50x diluted complementary DNA (cDNA), according to the manufacturer's instructions. Prior to undertaking the full discovery-phase global microRNA profiling study, initial qRT-PCR quantification of four 'housekeeping' microRNAs was performed using an aliquot of diluted cDNA. These microRNAs are transcribed from independent chromosomal loci [miR-23a-3p (chromosomal locus 19p13.3), miR-30c-5p (6q13), miR-103a-3p (20p13) and miR-191-5p (3p21.31)] and have previously been shown to be abundant and stably expressed in serum from adults (6). Linear regression analysis was performed on housekeeping

microRNA levels in the samples to assess their suitability for this purpose ($p<0.05$ considered significant).

In addition to visual inspection for hemolysis, serum levels of a microRNA highly expressed in red blood cells [miR-451a; (7)] were compared with serum levels of a housekeeping microRNA unaffected by hemolysis, namely miR-23a-3p, as described (6). Delta Ct (miR-23a-3p - miR-451a) values of <8 indicated that hemolysis levels were acceptable for the sample to be utilized in further downstream analysis (6).

Full discovery-phase serum microRNA qRT-PCR profiling of diagnostic samples

For the full global discovery-phase serum qRT-PCR profiling, 741 human mature microRNAs were assayed in singlicate by qRT-PCR using the microRNA Ready-to-Use PCR Human Panels I&II (Exiqon). The panels were manufactured with UniSp3 DNA dried in the base of the PCR wells, to act as a marker of PCR efficiency. In addition, the efficiency of both the RT step and the PCR step was assessed by UniSp6 quantification. Negative template controls (NTC), which excluded cDNA template, were also profiled to confirm specificity of results, as described (1). Reactions were run using 384-well plates in a LightCycler-480 Real-Time PCR-System (Roche). Cycling conditions were 95^{o}C for 10 minute, 40 cycles of 95^{o}C for 10 seconds and 60^{o}C for one minute. The temperature was then increased in 1.6^{o}C increments from 60^{o}C to 95^{o}C for melting curve analysis. Amplification curves were analyzed using Roche-LC software for determination of Ct values and for melting curve analysis. MicroRNAs with Ct values≤37 were considered expressed; those with a Ct value of >37 were arbitrarily assigned a Ct value of 40. To evaluate the specificity of the PCR products, melting curve analysis was undertaken for each reaction, as described (1). Similar QC analyses were performed on the full discovery-phase qRT-PCR profiling study, as for the initial qRT-PCR assessment, prior to normalization.

Statistical analyses for discovery-phase serum microRNA quantification

In addition to the statistical analyses used (Materials and Methods), the Robust Rank Aggregate (RRA) method was also employed, as described (8), to add stringency to our findings of differentially expressed serum microRNAs in different tumor groups. For this analysis, samples were processed using the BioConductor package '*RobustRankAggreg*' in the statistical software environment *R*. This algorithm detected microRNAs that were ranked consistently higher than expected under the null hypothesis. This was performed by studying the position of each microRNA in the over-expressed list (ranked by fold-change) for each specific tumor type, compared with the 'other tumor' and control group. A significance (*p*-value) score was subsequently assigned for each gene, which calculated how much higher it was positioned in the ranked list than expected by chance, as described (8). This *p*-value was adjusted for multiple testing using a Bonferroni correction and was used both for re-ranking the genes and as an indicator of the significance of each gene. Adjusted RRA scores of $p<0.01$ were considered significant.

Serum microRNA qRT-PCR for technical confirmation

To maximise sensitivity in the technical confirmation study, cDNA was diluted 1:7.5, rather than the standard 1:50 dilution. To ensure robustness for this phase of the study, previously extracted RNA from each serum sample was assessed in technical triplicate. Each RT product was analyzed by PCR, using the cycling conditions adopted in the discovery-phase full profiling study. The means of the three triplicate values for each microRNA for each sample were calculated.

Supplementary Results

RNA extraction and initial QC analysis

UniSp2 quantification confirmed the efficiency of RNA isolation (Supplementary Figure S1A). Initial qRT-PCR quantification of the four housekeeping microRNAs demonstrated that 53 of the 54 serum samples (98.1%) were suitable for subsequent global profiling (Supplementary Figures S1B and S1C), with failure of RNA extraction and housekeeping microRNA expression in a single sample (WT-4), which was excluded from further analysis. Linear regression analysis on the 53 samples showed that these four housekeeping microRNAs individually displayed a strong positive correlation with each other across the whole cohort ($p<0.0001$), excluding disease-related contributions to their serum expression levels and thereby confirming their suitability for initial QC purposes (Supplementary Figures S2A to S2F).

Hemolysis assessment by both miR-451a (Supplementary Figure S1D) and delta Ct (miR-23a-3p - miR-451a) (Supplementary Figure S1E) values showed that all 53 samples were suitable for full discovery-phase qRT-PCR profiling, with 52 of 53 samples (98.1%) having a delta Ct of <8 and only a single sample (MYCN-NB-2) having a borderline value of 8.5 (Supplementary Figure S1E).

QC assessment of the full discovery-phase qRT-PCR profiling study

Following confirmation of suitably uniform UniSp2 recovery in all 53 samples as a marker of RNA extraction efficiency (Supplementary Figure S1A), assessment of the RT and qRT-PCR steps using UniSp3 and UniSp6 levels also showed similar efficiencies (Supplementary Figure S3A). Analysis of the expression of the four housekeeping microRNAs (6) (Supplementary Figure S3B) and their mean raw Ct values (Supplementary Figure S3C) for all samples in the full discovery-phase qRT-PCR profiling study were very similar to the levels obtained in the initial QC qRT-PCR (Figure 1A), and were confirmed by linear regression analysis ($p<0.0001$) (Supplementary Figure S3D). The same findings were also seen for hemolysis assessment for serum levels of miR-451a (Supplementary

Figure S4A) and delta Ct (miR-23a-3p – miR-451a) (Supplementary Figure S4B) in the full discovery-phase qRT-PCR study, again confirmed by linear regression analyses (p<0.0001) (Supplementary Figures S4C and S4D, respectively). The high levels of reproducibility observed between serum microRNA expression levels assessed by singlicate qRT-PCR quantification on different occasions gave confidence to any subsequent disease-related results obtained.

Following these approaches, a further quality assessment was performed by calculating the mean Ct value for all microRNAs detected in each sample, across the whole cohort of 53 samples, which was 33.6 (range 32.5 to 34.5) (Supplementary Figure S5A). This corresponded to the mean number of microRNAs detected in each sample of 228 (range 123 to 328) (Supplementary Figure S5B), i.e. 30.8% of all 741 microRNAs profiled and 40.1% of the 568 microRNAs that were expressed in at least one sample in the cohort. This confirmed that all 53 samples had adequate microRNA expression levels to progress to the normalization step (see below), although control samples C-8, C-11 and C-14 had slightly lower numbers of detectable microRNAs (123, 139 and 134, respectively) than the other 50 samples (Supplementary Figure S5B). Linear regression analysis confirmed the expected negative correlation between the mean raw Ct value and the number of microRNAs expressed in each sample (p<0.0001) (Supplementary Figure S5C).

Supplementary Discussion

Various tumor-specific serum microRNAs are of potential clinical value and biological significance. Of note, miR-205, one of the microRNAs in the hepatoblastoma (HB) panel that we identified, has been shown to contribute to hepatocyte differentiation (9). The serum panel for Wilms tumor (WT) included miR-141-5p, which is down-regulated in WT (10) but present at elevated levels in the serum of WT patients at diagnosis, as for miR-122-5p in HB. Passive leakage of microRNAs from tumor cells into the bloodstream, due to abnormal cellular function, may account for these findings. Alternatively, there may be active and selective microRNA release mechanisms from tumor cells, which may subsequently promote an environment suitable for tumor cell growth and metastasis (11). For rhabdomyosarcoma (RMS), serum levels of miR-183-5p were elevated at diagnosis. This is consistent with over-expression of miR-183-5p in RMS tissues, leading to down-regulation of the tumor suppressor transcription factor *EGR1* and increased cell migration (12). For osteosarcoma (OS), miR-512-5p and miR-519a-3p were over-expressed in the serum, potentially as a result of amplification of the 19q13 genomic locus (13).

Finally, our detection of a panel of differentially expressed serum microRNAs in central nervous system gliomas (GL) (Supplementary Table-S13) is likely to reflect disturbance of the blood brain barrier, allowing transfer of microRNAs into the bloodstream. Indeed, similar findings have been reported recently (14). The serum GL panel included miR-155-5p and miR-124-3p. In GL tissues, miR-155 levels were noted to be higher than in normal brain, associated with higher pathological grade and poor clinical outcomes, revealing miR-155 expression as a potential independent prognostic factor and therapeutic target for human glioma (15). Furthermore, in a study investigating altered microRNA expression during GL malignant transformation, both miR-155-5p (increased) and miR-124-3p (decreased) showed altered expression between GL stem cells and neural stem cells (16). Interestingly, apart from MYCN-NB samples, the GL group was the only other in which we could detect miR-124-3p in the serum (Figure 2, panel 1). MiR-124-3p is under-expressed in pediatric GL

(pilocytic astrocytoma) compared with non-neoplastic brain tissue (17), and again detection in the serum is likely due to active release or passive leak of miR-124-3p from the GL tumor cells across the disrupted blood brain barrier.

Cancer Type	Number of over-expressed serum microRNAs	
	≥2 fold-change *vs.* control group AND 'other tumor' group	**Robust Rank Aggregate (RRA) adjusted *p*-value <0.01**
MYCN-amplified HR neuroblastoma	35*	18
LR neuroblastoma	38	23
Hepatoblastoma	49	18
Wilms tumor	32	23
Rhabdomyosarcoma	25	23
Ewings sarcoma	35	20
Osteosarcoma	44	26
B-cell non-Hodgkin's lymphoma	22	21
Hodgkin's disease	26	17
Pleuropulmonary blastoma	41	25
Central nervous system glioma	24	16

Supplementary Table S1

Tumor Specificity	*microRNA*	*miRBase v20 accession number*	*5' to 3' Nucleotide Sequence*	*Chromosomal location*	*Mean tumor value*	*'Other Tumor' mean value*
MYCN-amplified HR neuroblastoma	miR-124-3p	MIMAT0000422	UAAGGCACGCGGUGAAUGCC	8p23.1	181.70	4.57
MYCN-amplified HR neuroblastoma	miR-9-3p	MIMAT0000442	AUAAAGCUAGAUAACCGAAAGU	1q22	164.39	1.79
MYCN-amplified HR neuroblastoma	miR-218-5p	MIMAT0000275	UUGUGCUUGAUCUAACCAUGU	4p15.31	182.78	2.88
MYCN-amplified HR neuroblastoma	miR-490-5p	MIMAT0004764	CCAUGGAUCUCCAGGUGGGU	7q33	25.18	2.35
MYCN-amplified HR neuroblastoma	miR-1538	MIMAT0007400	CGGCCCGGGCUGCUGCUGUUCCU	16q22.1	10.72	2.41
MYCN-amplified HR neuroblastoma and neuroblastoma	miR-129-5p	MIMAT0000242	CUUUUUGCGGUCUGGGCUUGC	7q32.1	121.23	3.03
Wilms tumor	miR-143-3p	MIMAT0000435	UGAGAUGAAGCACUGUAGCUC	5q32	5.30	1.17
Hepatoblastoma	miR-122-5p	MIMAT0000421	UGGAGUGUGACAAUGGUGUUUG	18q21.31	18.00	4.00
Hepatoblastoma	miR-483-3p	MIMAT0002173	UCACUCCUCUCCUCCCGUCUU	11p15.5	91.31	2.67
Hepatoblastoma	miR-205-5p	MIMAT0000266	UCCUUCAUUCCACCGGAGUCUG	1q32.2	11.26	0.71

Supplementary Table S2

Supplementary Table and Figure Legends.

Supplementary Table S1. Number of over-expressed serum microRNAs in solid tumor types of childhood. The Table lists the number of microRNAs identified with a) ≥2 fold -change versus both the control group and the 'other tumor' group (central column) and b) following the additional application of the Robust Rank Aggregate (RRA) method (right column). HR=high-risk; LR=low-risk. * number of microRNAs over-expressed in both MYCN-NB samples (see text).

Supplementary Table S2. Ten over-expressed serum microRNAs selected for the technical confirmation qRT-PCR study. The Table lists the over-expressed microRNAs, the tumor group for which they are potentially specific, their sequence information, chromosomal location and relevant fold-changes in the technical confirmation qRT-PCR study of 25 samples.

Supplementary Tables S3 to S13. Over-expressed serum microRNAs in individual types of solid tumor of childhood. The Tables list the over-expressed microRNAs in individual types of solid tumor of childhood compared with the 'other tumor' group and control group, their fold-changes, co-efficient of variation and robust rank aggregate (RRA) score. Key: FC = fold-change; CV = coefficient of variation. Table S3 = *MYCN*-amplified INRG high-risk neuroblastoma (MYCN-NB); Table S4 = non-*MYCN*-amplified INRG low-risk neuroblastoma (NB); Table S5 = hepatoblastoma (HB); Table S6 = Wilms tumor (WT); Table S7 = rhabdomyosarcoma (RMS); Table S8 = Ewings sarcoma (ES); Table S9 = osteosarcoma (OS); Table S10 = B-cell non-Hodgkin's lymphoma (B-NHL); Table S11 = Hodgkin's disease (HD); Table S12 = pleuropulmonary blastoma (PPB) and Table S13 = Central nervous system glioma (GL).

Supplementary Figure S1. Quality control (QC) assessments in the initial qRT-PCR run. The graphs show raw [A)-D)] or delta [E)] Ct values for the 53 samples that were subsequently used for the full discovery-phase qRT-PCR profiling study. A) Expression of the spike-in UniSp2; B) Expression of four housekeeping microRNAs (miR-30c-5p, miR-23a-3p, miR-191-5p and miR-103a-3p); C) Mean expression of the four housekeeping microRNAs listed in B); D) Expression of miR-451a and E) Delta Ct (miR-23a-3p–miR-451a) for hemolysis assessment. Key: QC = quality control; HK = housekeeper. For tumor abbreviations see Table 1.

Supplementary Figure S2. Confirmation of suitability of the four serum QC housekeeping microRNAs for screening samples for the full discovery-phase qRT-PCR run. Linear regression analysis was performed, comparing each of the four housekeeping microRNAs with each other across the 53 serum samples in the initial QC qRT-PCR run. A) miR-23a-3p *vs.* miR-30c-5p; B) miR-23a-3p *vs.* miR103a-3p; C) miR-23a-3p *vs.* miR-191-5p; D) miR-30c-5p *vs.* miR-103a-5p; E) miR-30c-5p *vs.* miR-191-5p and F) miR-103a-3p *vs.* miR-191-5p. Key: QC = quality control.

Supplementary Figure S3. QC assessments in the full discovery-phase qRT-PCR run. Graphs A) to C) show raw Ct values for all 53 samples in the full discovery-phase profiling study. A) Expression of the spike-ins UniSp3 and UniSp6; B) Individual expression of the four QC housekeeping microRNAs (miR-30c-5p, miR-23a-3p, miR-191-5p and miR-103a-3p); C) Mean expression of the four housekeeping microRNAs listed in B); D) Linear regression analysis of the mean raw Ct values for the four QC housekeeping microRNAs for all 53 samples in the initial QC qRT-PCR run (*x*-axis) versus the full discovery-phase run (*y*-axis). Key: QC = quality control; HK = housekeeper. For tumor abbreviations see Table 1.

Supplementary Figure S4. Hemolysis assessment of the 53 serum samples in the initial QC and full discovery-phase qRT-PCR run. Comparison of A) miR-451a raw Ct values and B) Delta Ct

(miR-23a-3p-miR-451a) values; and C) and D) Linear regression analysis for the values shown in A) and B) above, respectively. Key: QC = quality control. For tumor abbreviations see Table 1.

Supplementary Figure S5. Further QC assessment of the 53 serum samples in the full discovery-phase qRT-PCR run. A) Average raw Ct value of all expressed microRNAs (C_t 37); B) The number of microRNAs expressed per sample; C) Linear regression analysis of average raw Ct value (*y*-axis) versus the number of microRNAs expressed per sample (*x*-axis). For tumor abbreviations see Table 1.

Supplementary Figure S6. Top-ranking overlapping housekeeping microRNAs identified by NormFinder and geNorm algorithms, suitable for targeted serum qRT-PCR studies. The Venn diagrams show the overlap between the top-ten most stable, and therefore highest ranking, housekeeping microRNAs in the serum, as identified by the NormFinder and geNorm algorithms. The combined ranking score was obtained by adding the individual ranking score for each microRNA under consideration, as assigned by the different methods. Accordingly, the lower the combined ranking score, the higher-ranking that microRNA as a stable housekeeping gene. For the technical confirmation study, the six top-ranking overlapping microRNAs identified here (miR-140-3p, miR-30b-5p, miR-26a-5p, miR-15b-5p, miR-30c-5p and miR-191-5p) were used for normalization purposes.

Supplementary Figure S7. Comparison of expression of miR-122-3p and miR-122-5p in the 53 serum samples in the full discovery-phase qRT-PCR run. The graph shows raw Ct values for miR-122-3p and miR-122-5p, demonstrating the higher serum abundance of miR-122-5p, which was therefore chosen for the technical confirmation study, rather than its -3p counterpart. For tumor abbreviations see Table 1.

Supplementary Figure S8. Comparison of the average raw Ct values in the 21 serum samples common to both the full discovery-phase and technical confirmation qRT-PCR run. A) Comparison of the average raw Ct values for the 16 microRNAs analyzed at 1:50 cDNA dilution (full discovery-phase run) and 1:7.5 dilution (technical confirmation run). B) Linear regression analysis of the average raw Ct values for the two different cDNA dilutions. For tumor abbreviations see Table 1.

Supplementary Figure S9. QC assessment in the technical confirmation qRT-PCR run for all 25 serum samples used. A) Average raw Ct values for the spike-in UniSp6 for the 25 samples in the technical confirmation study (21 from the original set plus four additional MYCN-NB/NB samples). B) Average raw Ct values of the six top-ranking overlapping housekeeper microRNAs identified using *geNorm* and *NormFinder* (Supplementary Figure S6). All samples were run in technical triplicate. Error bars=standard deviation. For tumor abbreviations see Table 1.

Supplementary Figure S10. Serum microRNA expression in osteosarcoma. The boxplots show the raw Ct values (*y*-axis) of three selected microRNAs (miR-500a-5p, miR-512-5p and miR-519a-3p) in osteosarcoma (OS), compared with 'other tumors' and controls across the 53 samples in the full discovery-phase qRT-PCR study. Whiskers represent 1.5x inter-quartile range. Dots represent outlier samples. For tumor abbreviations see Table 1.

Supplementary References

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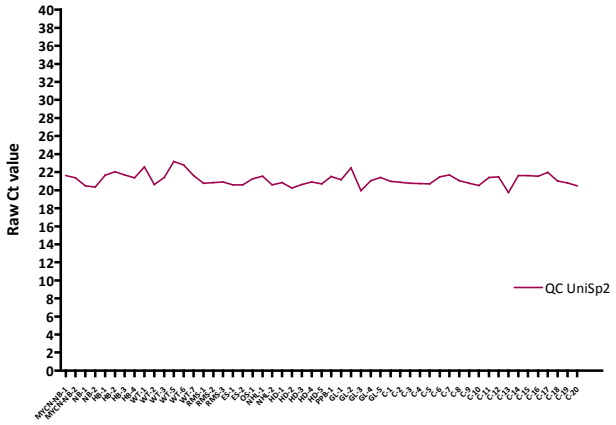
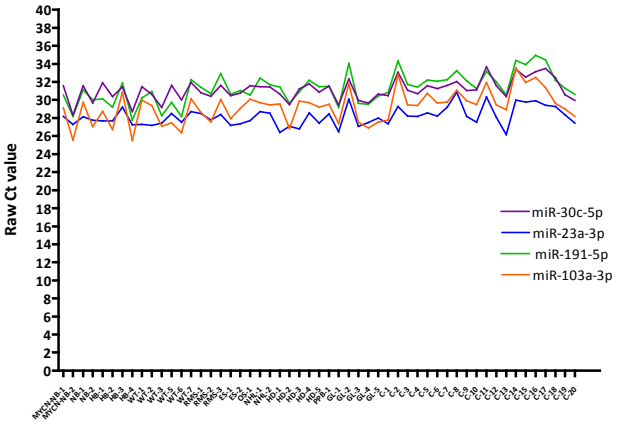
A


UniSp2 raw Ct value in QC qRT-PCR run
Raw Ct value
QC UniSp2

B


Four housekeeping microRNAs in QC qRT-PCR run
Raw Ct value
miR-30c-5p
miR-23a-3p
miR-191-5p
miR-103a-3p

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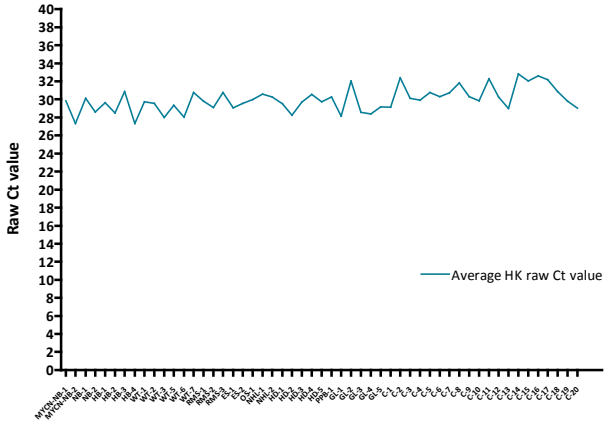
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Raw Ct value
Average HK raw Ct value

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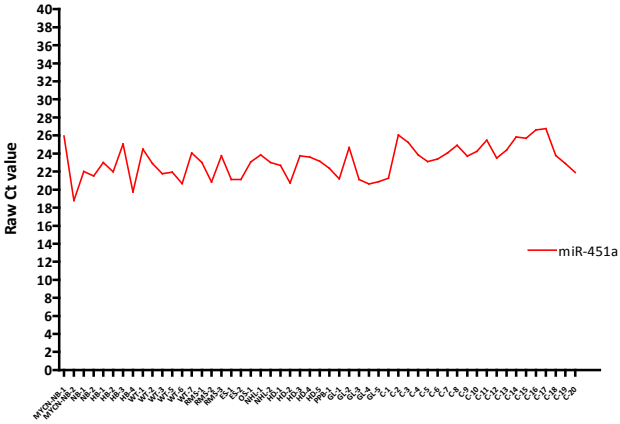
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Raw Ct value
miR-451a

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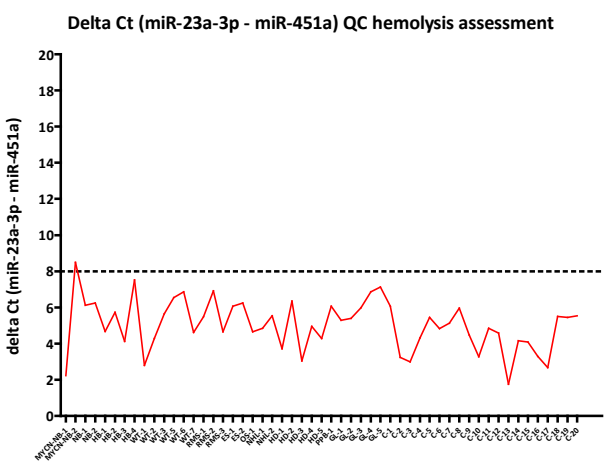
Delta Ct (miR-23a-3p - miR-451a) QC hemolysis assessment
delta Ct (miR-23a-3p - miR-451a)

A

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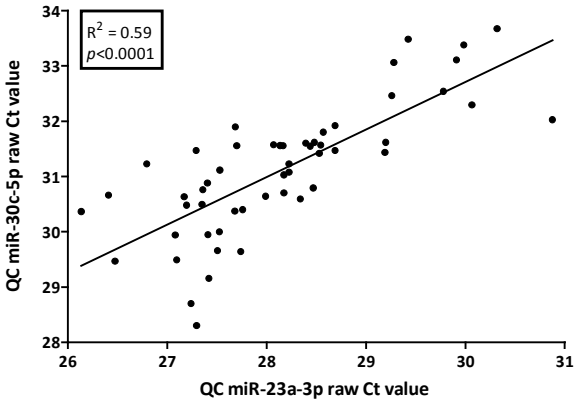

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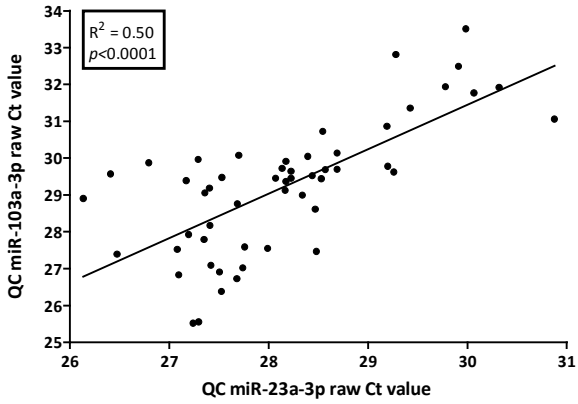
Linear regression of QC housekeeping miR-23a-3p *vs.* miR-103a-3p



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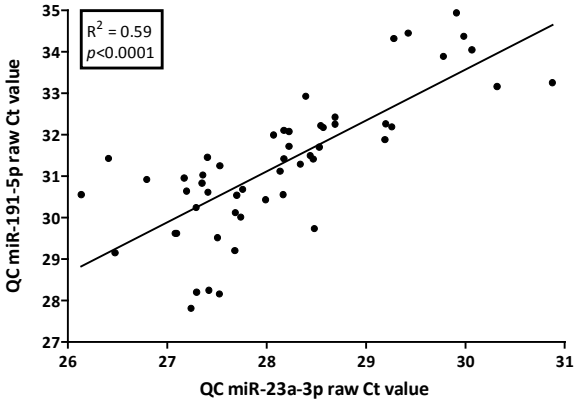
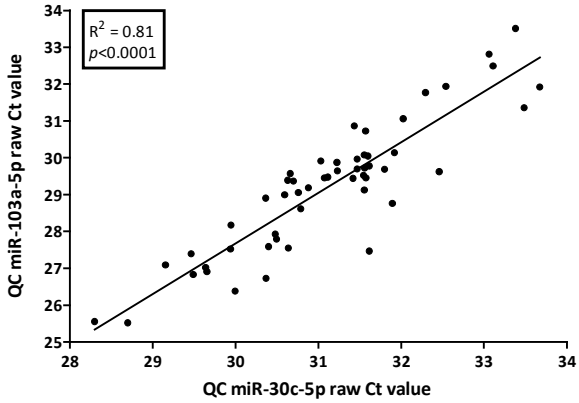


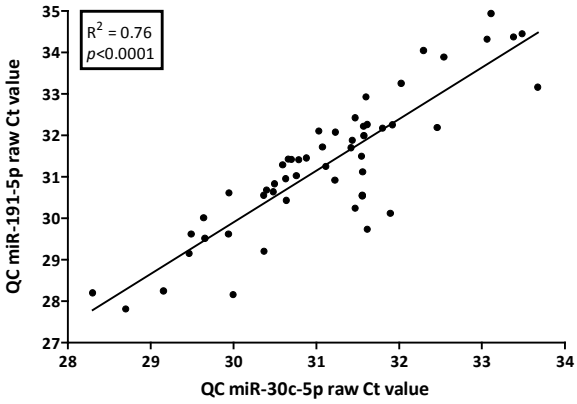
D

Linear regression of QC housekeeping miR-30c-5p *vs.* miR-103a-5p



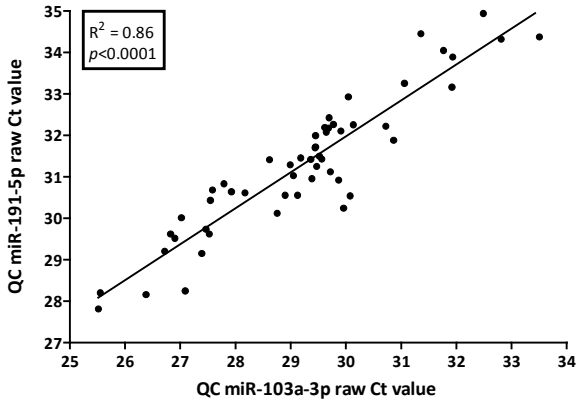
E

Linear regression of QC housekeeping miR-30c-5p *vs.* miR-191-5p

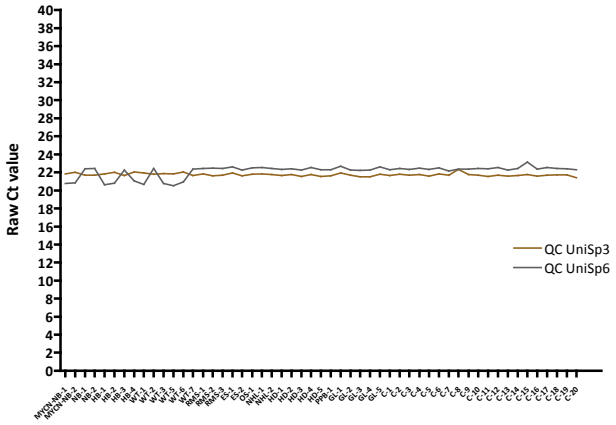


F

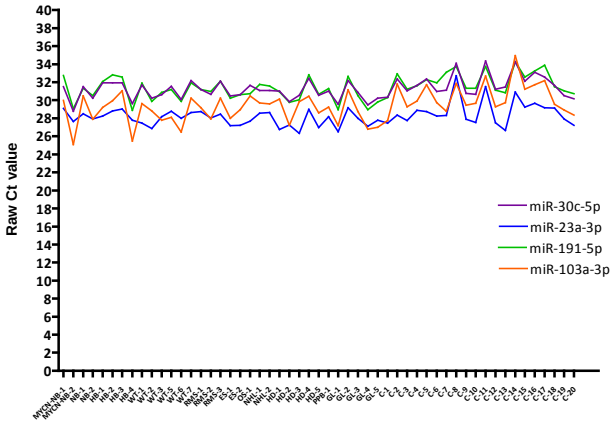
Linear regression of QC housekeeping miR-103a-3p *vs.* miR-191-5p



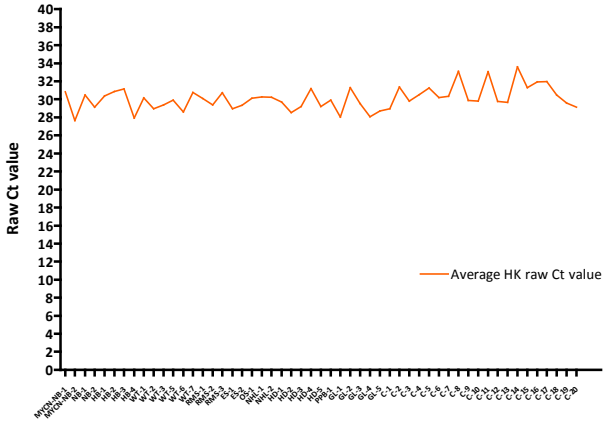
A


UniSp RNA and DNA in full qRT-PCR run
Raw Ct value
QC UniSp3
QC UniSp6

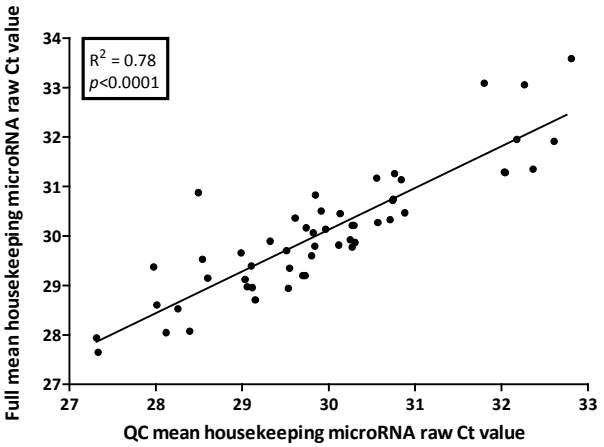
B


Four housekeeping microRNAs in full qRT-PCR run
Raw Ct value
miR-30c-5p
miR-23a-3p
miR-191-5p
miR-103a-3p

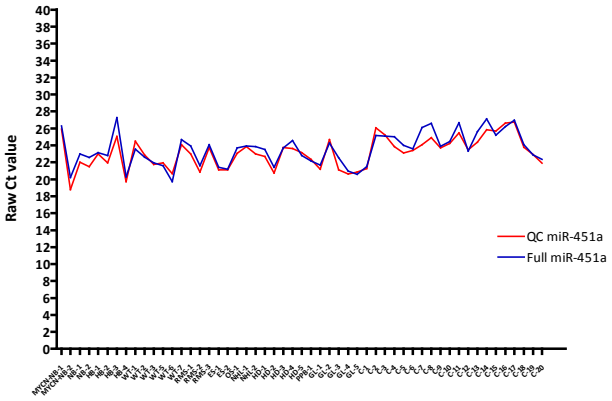
C


Mean of four housekeeping microRNAs in full qRT-PCR run
Raw Ct value
Average HK raw Ct value

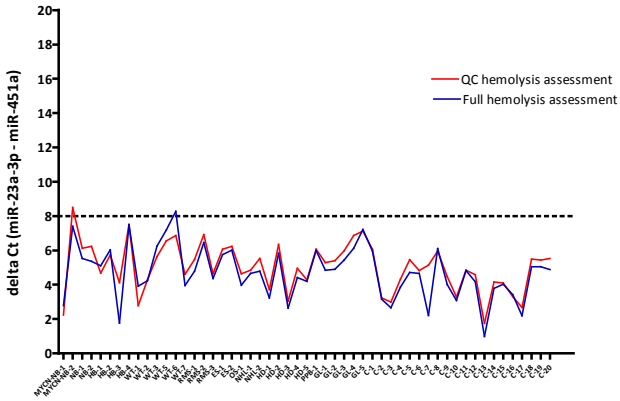
D


Full vs. QC mean housekeeping microRNA raw Ct values
miR-23a-3p, miR-30c-5p, miR-103a-3p, miR-191-5p
R2 = 0.78
p<0.0001
Full mean housekeeping microRNA raw Ct value
QC mean housekeeping microRNA raw Ct value

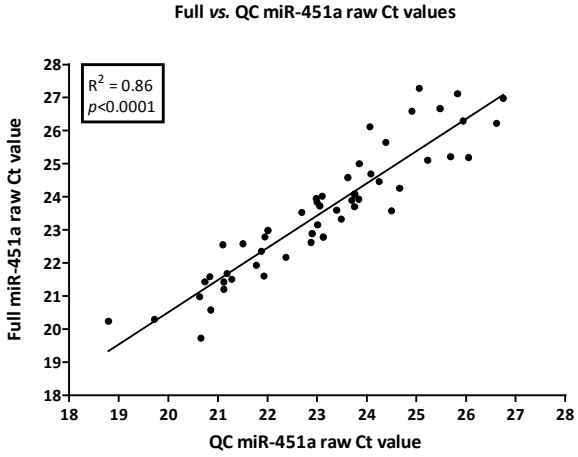
A


miR-451a level for hemolysis assessment in QC and full qRT-PCR run
Raw Ct value
QC miR-451a
Full miR-451a

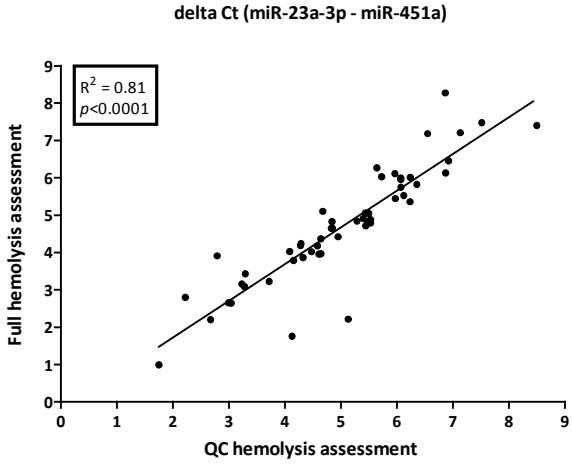
B


QC and main qRT-PCR run hemolysis assessment
delta Ct (miR-23a-3p - miR-451a)
QC hemolysis assessment
Full hemolysis assessment

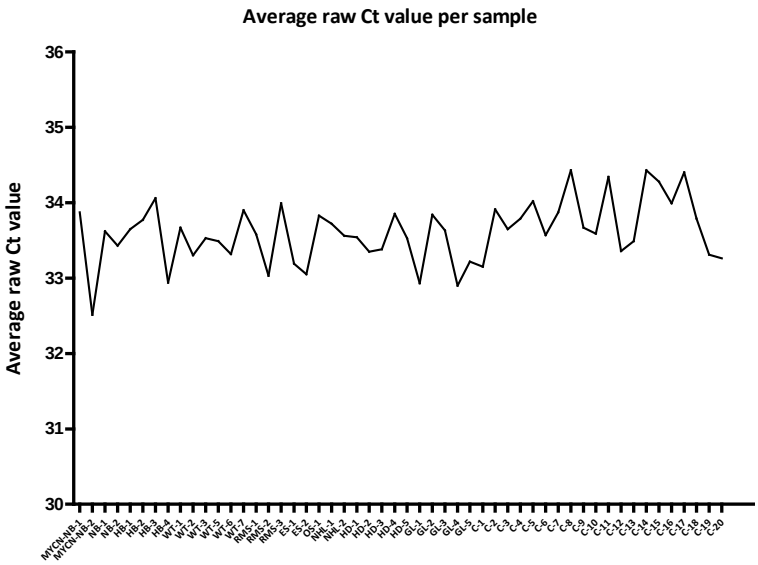
C


Full vs. QC miR-451a raw Ct values
R2 = 0.86
p<0.0001
Full miR-451a raw Ct value
QC miR-451a raw Ct value

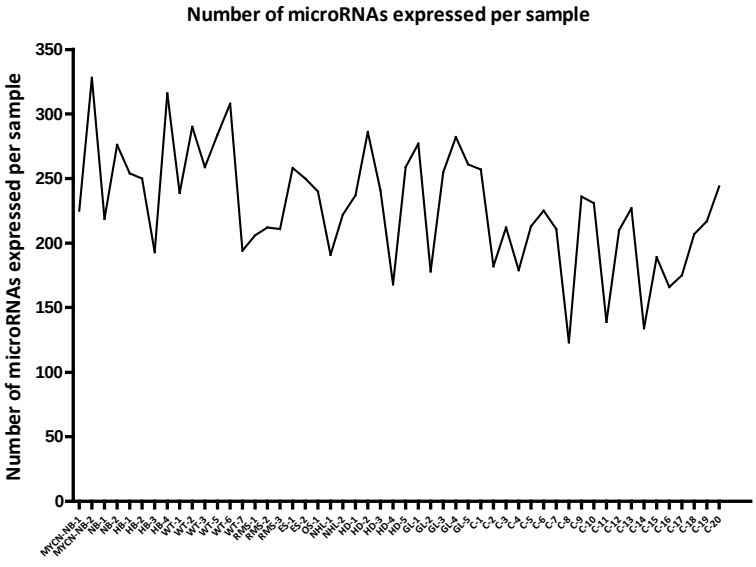
D


Full vs. QC hemolysis assessment
delta Ct (miR-23a-3p - miR-451a)
R2 = 0.81
p<0.0001
Full hemolysis assessment
QC hemolysis assessment

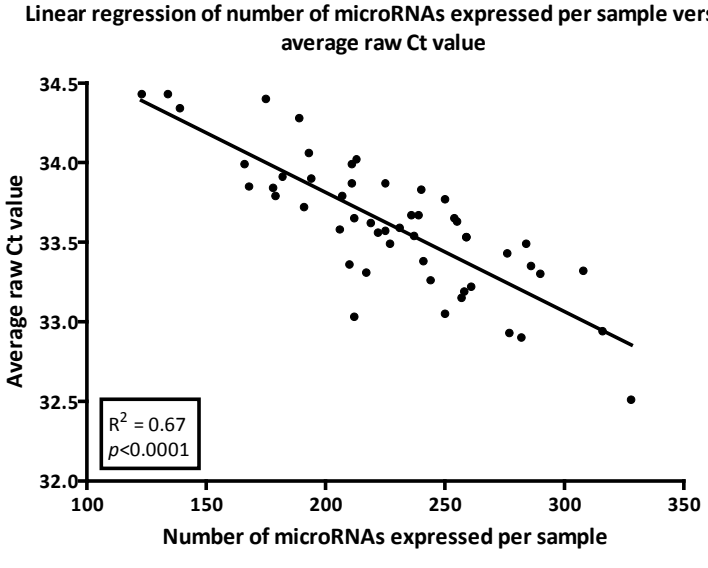
A


Average raw Ct value per sample
Average raw Ct value
36
35
34
33
32
31
30

B


Number of microRNAs expressed per sample
Number of microRNAs expressed per sample
350
300
250
200
150
100
50
0

C


Linear regression of number of microRNAs expressed per sample versus average raw Ct value
Average raw Ct value
34.5
34.0
33.5
33.0
32.5
32.0
R2 = 0.67
p<0.0001
100
150
200
250
300
350
Number of microRNAs expressed per sample

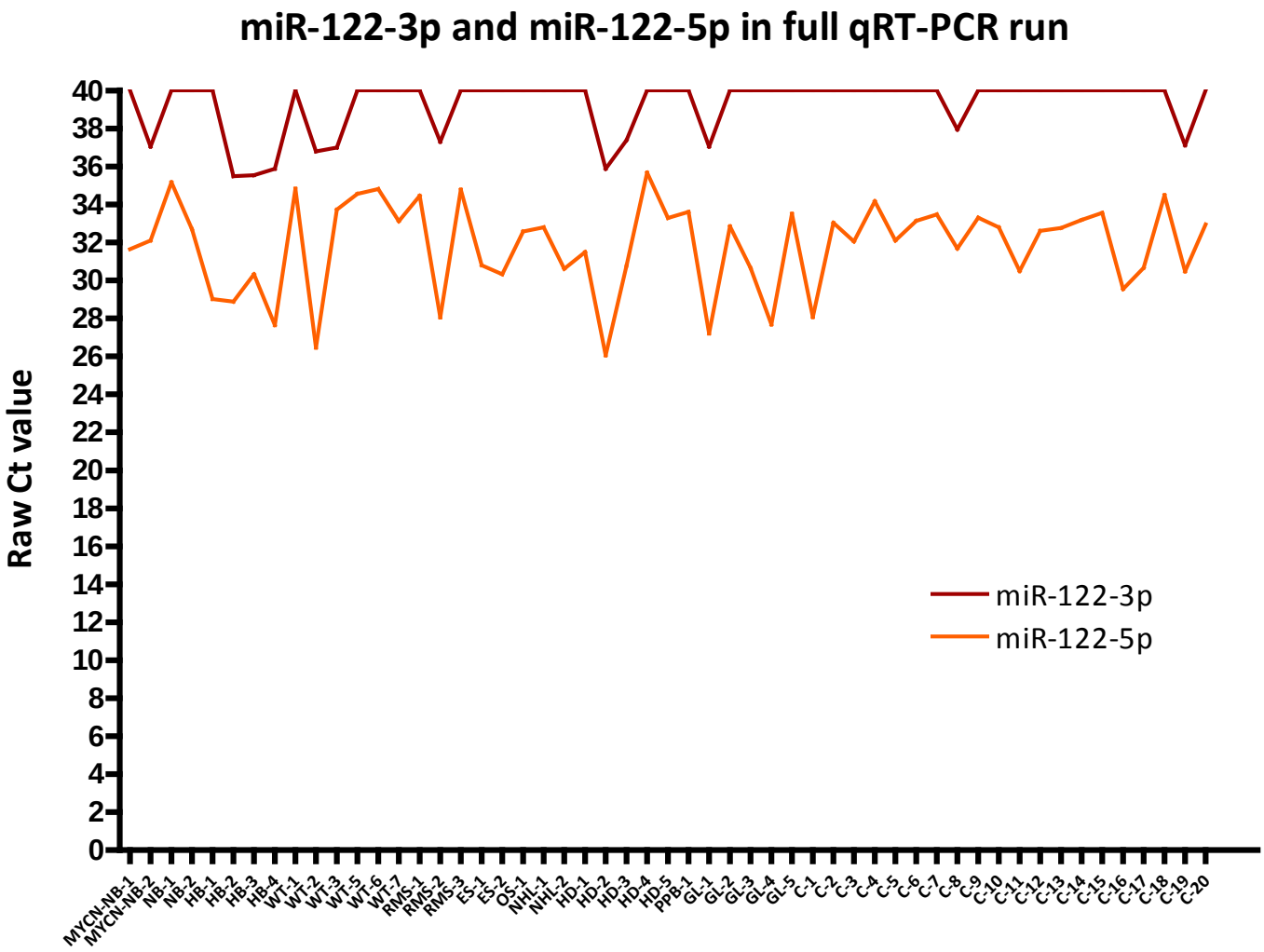
NormFinder

Ranking score	microRNA
6	hsa-miR-15a-5p
7	hsa-miR-23a-3p
8	hsa-miR-484

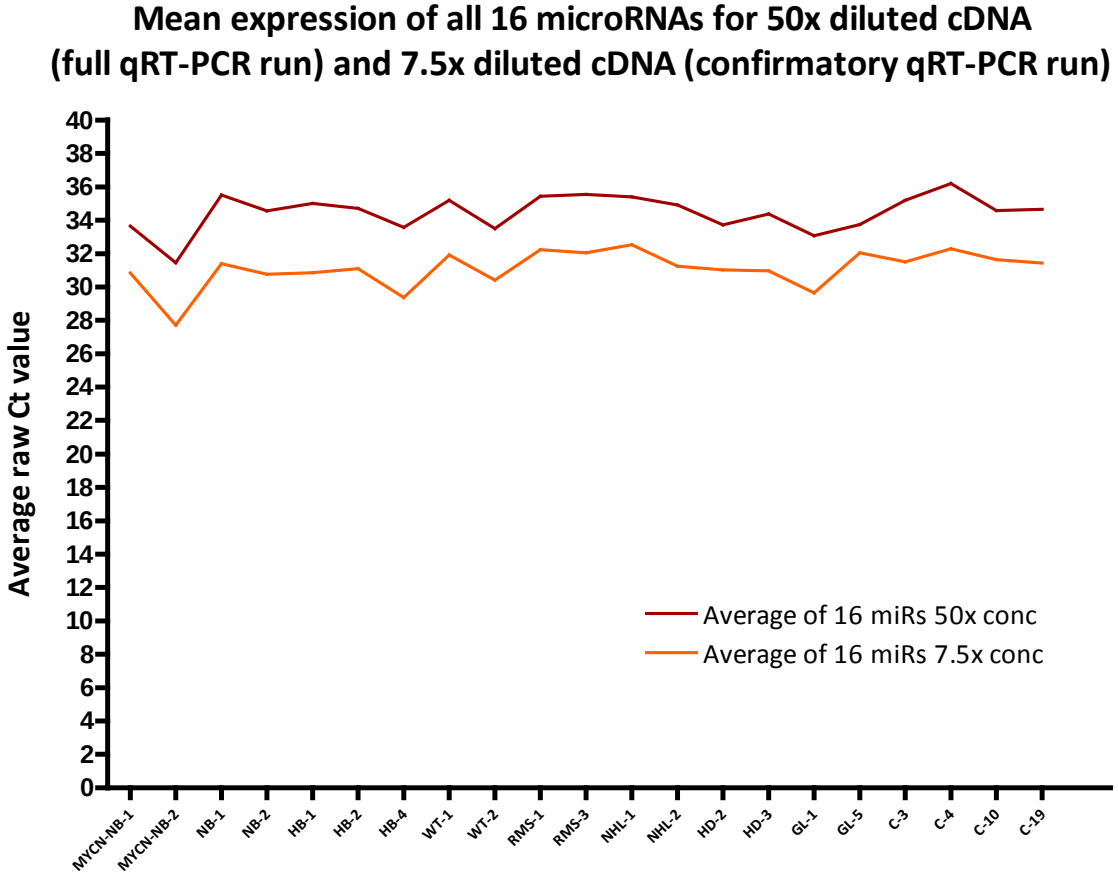
Combined ranking score	microRNA
6	hsa-miR-140-3p
8	hsa-miR-30b-5p
8	hsa-miR-26a-5p
8	hsa-miR-15b-5p
11	hsa-miR-30c-5p
13	hsa-miR-191-5p
19	hsa-miR-30e-5p

Ranking score	microRNA
3	hsa-miR-19a-3p
7	hsa-let-7i-5p
9	hsa-miR-425-5p

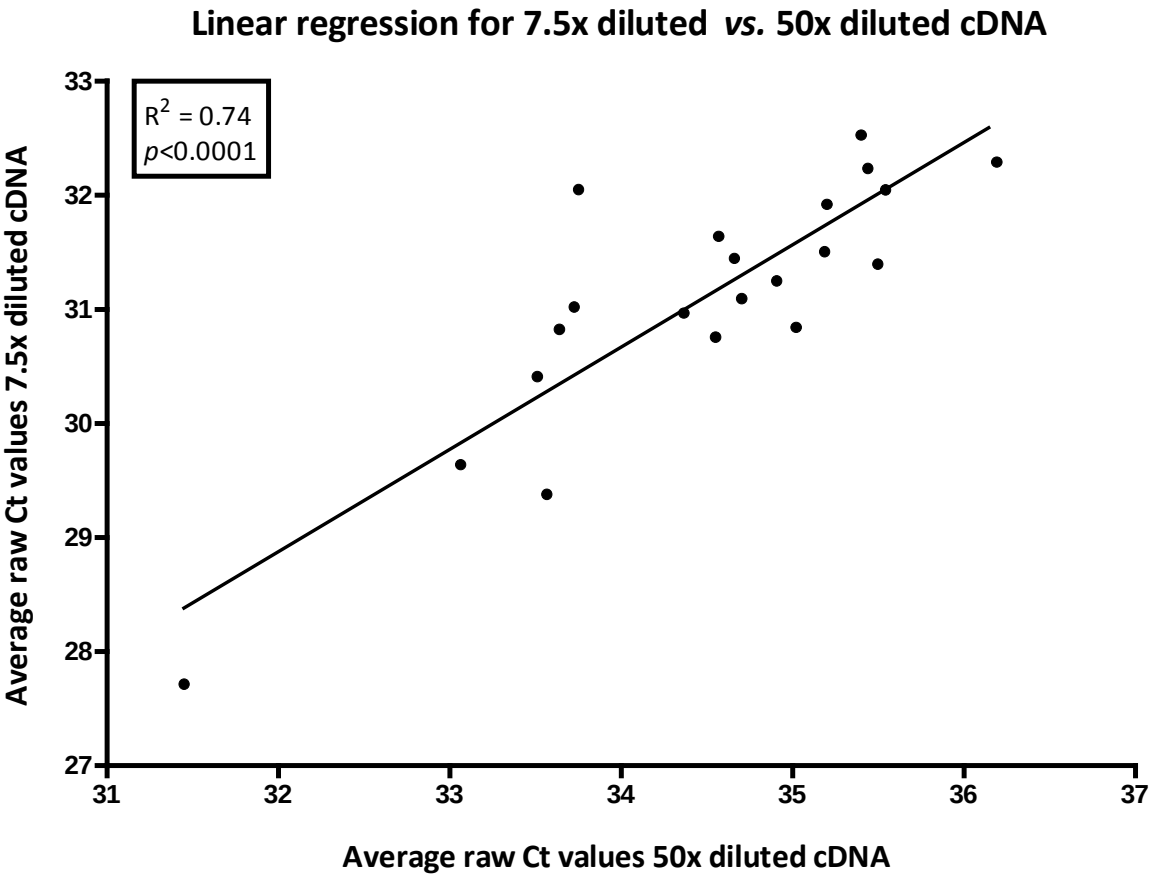
geNorm


miR-122-3p and miR-122-5p in full qRT-PCR run
Raw Ct value
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
miR-122-3p
miR-122-5p
MYCN-NB-1
MYCN-NB-2
NB-1
NB-2
HB-1
HB-2
HB-3
HB-4
WT-1
WT-2
WT-3
WT-5
WT-6
WT-7
RMS-1
RMS-2
RMS-3
ES-1
ES-2
OS-1
NHL-1
NHL-2
HD-1
HD-2
HD-3
HD-4
HD-5
PPB-1
GL-1
GL-2
GL-3
GL-4
GL-5
C-1
C-2
C-3
C-4
C-5
C-6
C-7
C-8
C-9
C-10
C-11
C-12
C-13
C-14
C-15
C-16
C-17
C-18
C-19
C-20

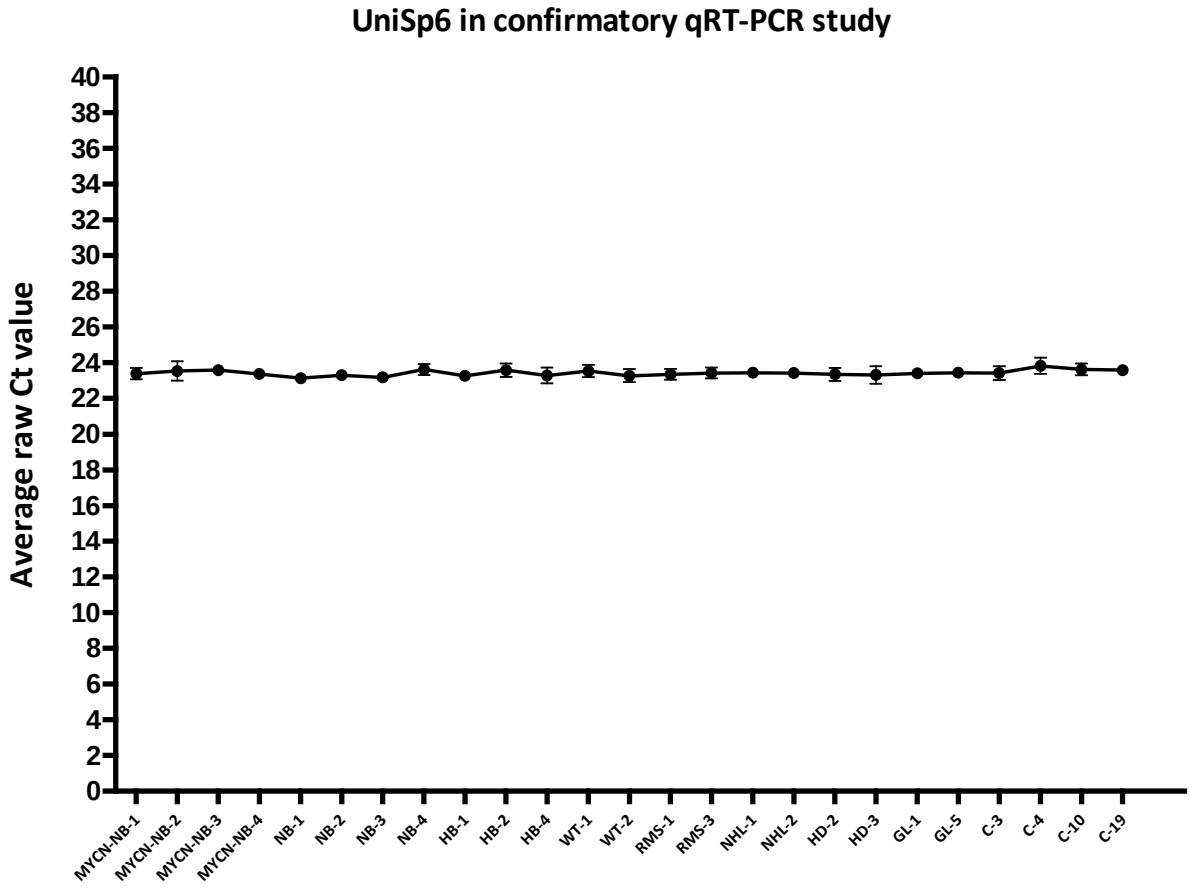
A


Mean expression of all 16 microRNAs for 50x diluted cDNA
(full qRT-PCR run) and 7.5x diluted cDNA (confirmatory qRT-PCR run)
Average raw Ct value
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
Average of 16 miRs 50x conc
Average of 16 miRs 7.5x conc
MYCN-NB-1
MYCN-NB-2
NB-1
NB-2
HB-1
HB-2
HB-4
WT-1
WT-2
RMS-1
RMS-3
NHL-1
NHL-2
HD-2
HD-3
GL-1
GL-5
C-3
C-4
C-10
C-19

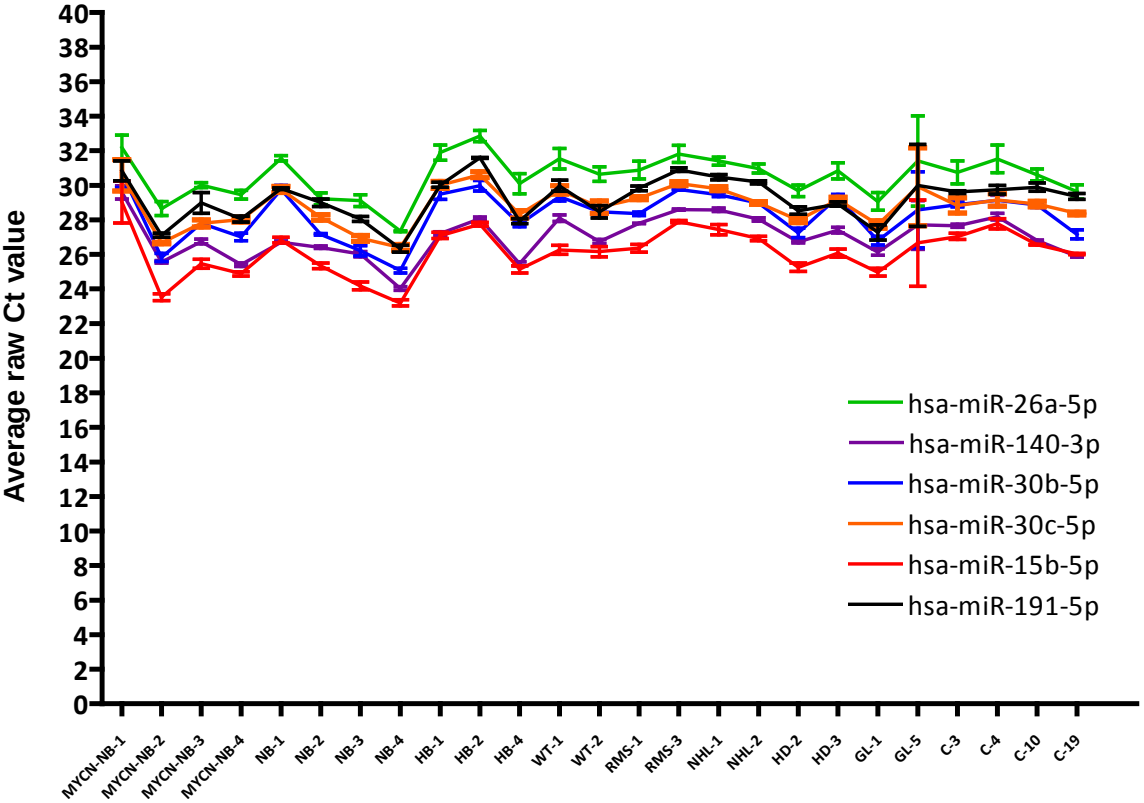
B


Linear regression for 7.5x diluted vs. 50x diluted cDNA
R2 = 0.74
p<0.0001
Average raw Ct values 7.5x diluted cDNA
33
32
31
30
29
28
27
31
32
33
34
35
36
37
Average raw Ct values 50x diluted cDNA

A


UniSp6 in confirmatory qRT-PCR study
Average raw Ct value
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
MYCN-NB-1
MYCN-NB-2
MYCN-NB-3
MYCN-NB-4
NB-1
NB-2
NB-3
NB-4
HB-1
HB-2
HB-4
WT-1
WT-2
RMS-1
RMS-3
NHL-1
NHL-2
HD-2
HD-3
GL-1
GL-5
C-3
C-4
C-10
C-19

B


Expression of 6 housekeeping microRNAs in the confirmatory qRT-PCR study
Average raw Ct value
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
MYCN-NB-1
MYCN-NB-2
MYCN-NB-3
MYCN-NB-4
NB-1
NB-2
NB-3
NB-4
HB-1
HB-2
HB-4
WT-1
WT-2
RMS-1
RMS-3
NHL-1
NHL-2
HD-2
HD-3
GL-1
GL-5
C-3
C-4
C-10
C-19
hsa-miR-26a-5p
hsa-miR-140-3p
hsa-miR-30b-5p
hsa-miR-30c-5p
hsa-miR-15b-5p
hsa-miR-191-5p

Supplementary Figure S10