Supporting Information

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SI Materials and Methods

Fluorescence Activated Cell Sorting of Hematopoietic Populations. All murine tissues were extracted from 6- to 8-wk-old mixed sex C57BL/6J mice into PBS solution with 2% FCS. All samples were lysed with NH_3CL (StemCell Technologies) on ice as described (1) and cells were stained with the antibody combinations listed in Table S1. After staining, the cells were kept on ice and a maximum of 7,000 cells were sorted into PBS with 2% FCS. All samples were sorted on the Influx sorter (BD Bioscience), the FACS Vantage DIVA SORP instrument (BD Biosciences), or the Vantage SE (BD Biosciences) at 4 °C. Immediately after sorting, the purified cells were centrifuged at 5,000 rpm (Beckman Microfuge 18, Rotor F241.5P) for 10 min at 4 °C, transferred to 0.2-mL PCR tubes and processed to cDNA as described below. The purity of all samples was confirmed by resorting a small fraction of the cells. All samples used for miRNA studies were at least 90% pure except erythroblasts (Eb), which were >70% pure. In case of extremely rare cell populations such as E-SLAM 150^+ and E-SLAM 150^-, the cell numbers limited us to only one technical replicate.

miRNA Reverse Transcription. Cells were spun down in PCR tubes, washed 3 times with PBS, supernatants were removed, and 25 μL of Lysis Master Mix (3.1 μL of 10× RT buffer, 0.2 μL of 20 U/μL RNase inhibitor, and 21.7 μL of H_2O, all reagents from High Capacity cDNA Reverse Transcription Kit; ABI) were added to the cell pellet and mixed. Cells were incubated at 95 °C for 7 min, and the resulting lysate then was divided among six tubes of 4.13 μL each. Then 0.22 μL of solution of 96 stem-loop primers (from TaqMan MicroRNA Assays miRBase v12 panel; ABI) for reverse transcription were added to each of the tubes and heated to 95 °C for 5 min. There were three different pools covering 288 miRNAs (see Dataset S2 for the list of primers) that were added in duplicates as technical replicates of each biological sample. Finally 0.65 μL of Enzyme Master Mix (0.25 μL of 100 mM dNTPs, 0.335 μL of 50 U/μL MultiScribe reverse transcriptase, 0.0325 μL of 20 U/μL RNase inhibitor, and 0.0325 μL of H_2O, all reagents from High Capacity Reverse Transcription Kit; ABI) were added to each reaction. The final concentration of RT primers in each 5 μL of reverse transcription reaction was 0.24×. The RT reaction was performed under the following pulsed temperature conditions: 2 min at 16 °C, followed by 60 cycles of 30 seconds at 20 °C, 30 seconds at 42 °C, and 1 second at 50 °C. RT enzyme was inactivated at 85 °C (5 min). After generating the cDNA, the reactions were stored at −20 °C and processed later.

cDNA Amplification. The RT reactions were diluted with 20 μL of PCR mix consisting of 12.5 μL of TaqMan Universal PCR Master No AmpErase UNG mix, 1 μL of 25 mM $MgCl_2$, 1 μL of 100 mM dNTPs, 1.25 mkL of 5 U/μL AmpliTaq Gold, and 2.5 μL of 96 pooled 20× TaqMan assays (miRBase v12 panel; Applied Biosystems). PCRs were thermocycled under the following conditions: 10 min at 95 °C followed by 18 cycles of 15 seconds at 95 °C and 4 min at 60 °C. The final concentration of TaqMan miRNA assays in the PCR was 0.02×.

cDNA Detection. Amplified cDNA was diluted five times by adding 100 μL of H_2O to the 25-μL PCR mix from the previous step and assayed in Fluidigm 48.48 Dynamic Array devices. For this 48 sample, the inlets of the device were loaded with 6.67 μL of diluted PCR sample and enzyme mix [3.7 μL of TaqMan Universal PCR Master No AmpErase UNG mix; ABI), 0.37 μL of DA Sample Loading reagent (Fluidigm), 0.93 μL of TE buffer], 1.67 and 5 μL, respectively. The 48 assay inlets were loaded with an equal mix (2.5 μL each) of DA Assay Loading reagent (Fluidigm) and 20× TaqMan miRNA single assays (ABI).

miRNA Detection in Single Cells. A protocol similar to the above was also used for miRNA analysis in single cells. For this analysis, single cells from FACS-purified suspension of hematopoietic populations were manually picked up by pulled glass capillary, released into 4.35 μL of RT reaction mix, and processed in similar fashion to multiple cells. Tween (1%) was added to RT reaction mix and the cDNA amplification mix to minimize miRNA adhesion on tube walls. The final concentration of RT primers in 5 μL of reverse transcription reaction was 12.5 nM (0.05×). In addition, all samples were spiked with a known quantity (1 pg) of synthetic *Caenorhabditis elegans* miRNA cel-2 as an internal exogenous control (2).

The primer and TaqMan assay pools contained 12 miRNA species including *C. elegans* miRNA cel-2. cDNA amplification protocol was adjusted to 24 cycles to achieve sufficient template concentration for the Fluidigm 48.48 Dynamic Array. Amplified cDNA was diluted four times by adding 75 μL of H_2O to the 25-μL PCR mix. In each experiment, standard curves were generated for each assay by making dilutions of hematopoietic cells (0.1 cell/mL to 10,000 cells/mL) and differential expression was calculated by taking into account the measured efficiency for each assay. Assays for which single-cell measurements fell outside of the linear sensitivity range were excluded from the analysis. To improve precision and reliability of profiling, we performed four technical replicates of each measurement. Equal volumes of supernatant to that taken with cells were included as no template controls.

Bioinformatics Analysis. Data retrieved in .csv format from the prototype Biomark machine by Fluidigm was converted to a tabular format including sample, miRNA, and replicate information along with the raw fluorescence value. PCR reactions that failed were marked as missing.

For each miRNA a set of 7 calibration samples was used for a linear regression fit to convert measured CT values to $\log_2$ molecule counts. 10% of assays (28/288) had a measured sensitivity of $<10^4$ copies per reaction and were excluded from further analysis. Multiple experiments were combined after normalizing to total expression of miRNA species. All measured miRNA expression were filtered based on the requirement of detection in at least 2 of all of the biological replicates tested with the exception of E-SLAM (stem) cells and mature thymocytes which were excluded from this filtering due to the low number of replicates. We assessed technical, biological and set deviation mean in entire dataset (Fig. S4).

A tree summarizing miRNA expression relationships between samples was constructed by using the Fitch Margoliash method, as implemented in the PHYLIP package (3–5). To improve the robustness of the sample distance computations, miRNAs that were detectable in fewer than three samples were not included.

The Consensus program, from the PHYLIP package (5, 6), was used to assess tree branch stability. With the Pearson correlation distance matrix as input, a minimum of 100 trees were generated with random order of input samples, and a consensus tree was generated with branches labeled with the proportion of occurrence.

Differential expression of miRNAs between samples was examined using a two-tailed Student *t* test with correction for unequal variance.

Clustering was performed by using Cluster 3.0 software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm#ctv) with hierarchical Spearman Rank correlation and average linkage.

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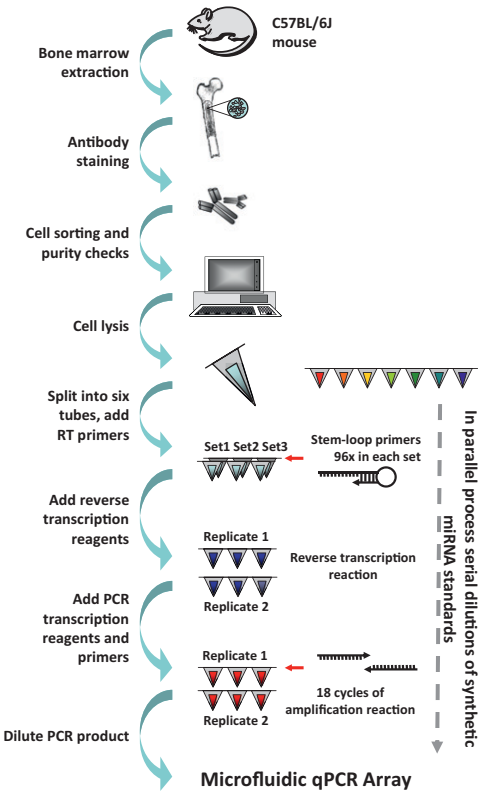
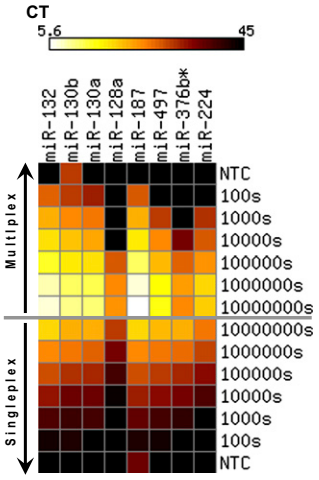


Fig. S1. Experimental protocol for profiling miRNA expression in small cell numbers. Cells were harvested from normal adult C57BL/6J mice and FACS purified to isolate 27 distinct populations. For each cell population ≈7,000 cells were sorted into three tubes, 1,024 cells each, in duplicates, each containing pools of 96 RT primers before heat lysis and RT. A series of 10× dilutions of equimolar synthetic miRNA was run in parallel for each dataset. Cells were subjected to heat lysis followed by the addition of RT enzyme and cDNA synthesis. RT product of each tube was diluted with PCR mix and subjected to 18 cycles of PCR pre-amplification, followed by dilution and qPCR detection in a microfluidic qPCR array.



	Singleplex	Multiplex
miR-132	10^2	10^2
miR-130b	10^2	10^2
miR-130a	10^2	10^2
miR-128a	10^4	10^5
miR-187	10^2	10^2
miR-497	10^2	10^3
miR-376b*	10^2	10^4
miR-224	10^3	10^3

Fig. S2. Comparison of TaqMan assay sensitivity under single-plex and multiplex conditions.

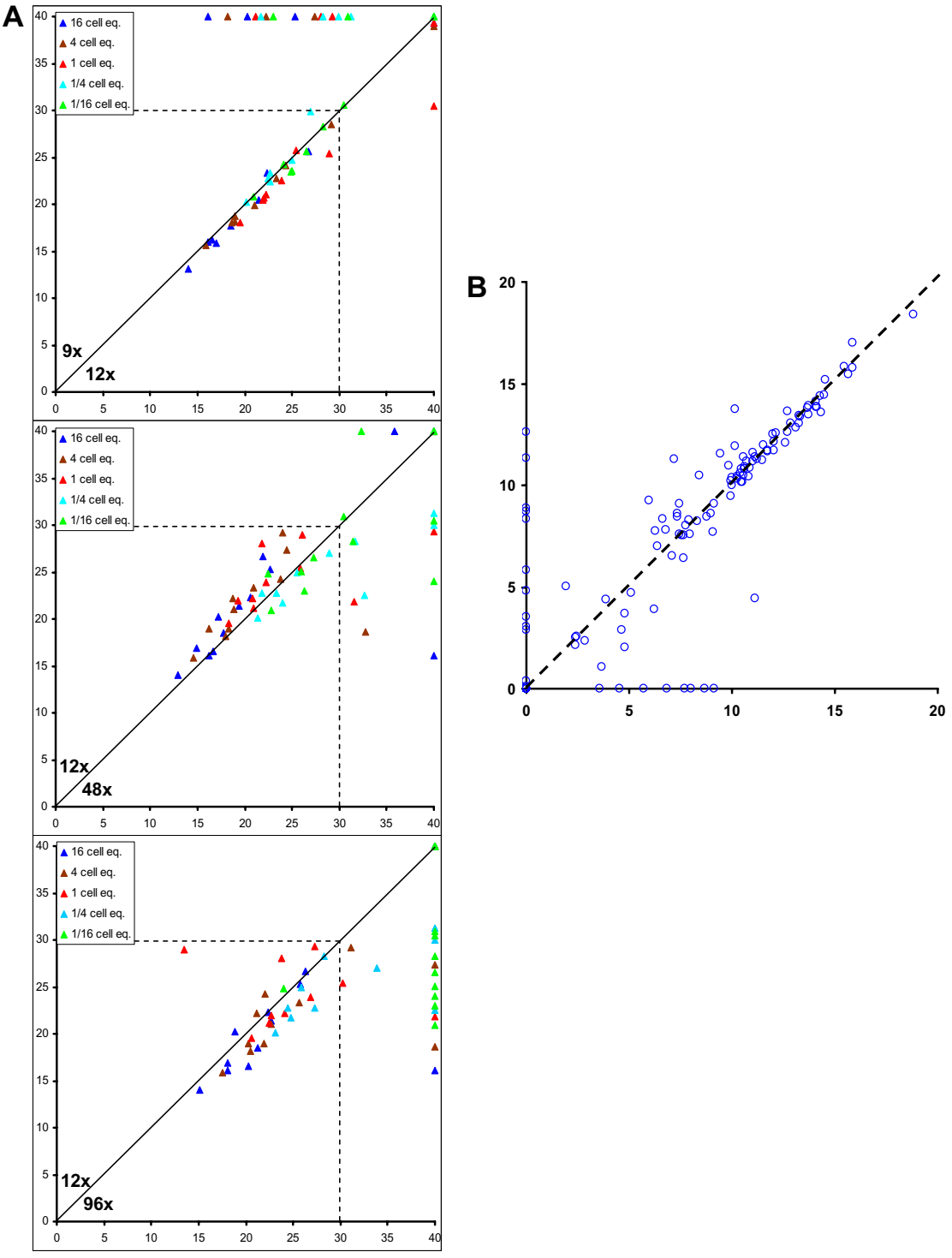


Fig. S3. Effect of multiplexing on TaqMan assay sensitivity. (*A*) Increasing the level of assay multiplexing results in a reduction in assay sensitivity as seen by the loss of detection for low template amounts. It should be noted that the deviation from the diagonal results from differing efficiency of assays at different multiplexing level and is not an indication of decreased assay precision. (*B*) Pairwise comparison shows the reproducibility of technical replicates of 124 TaqMan assays at 96× multiplexing. Both axes represent log2 molecule counts.

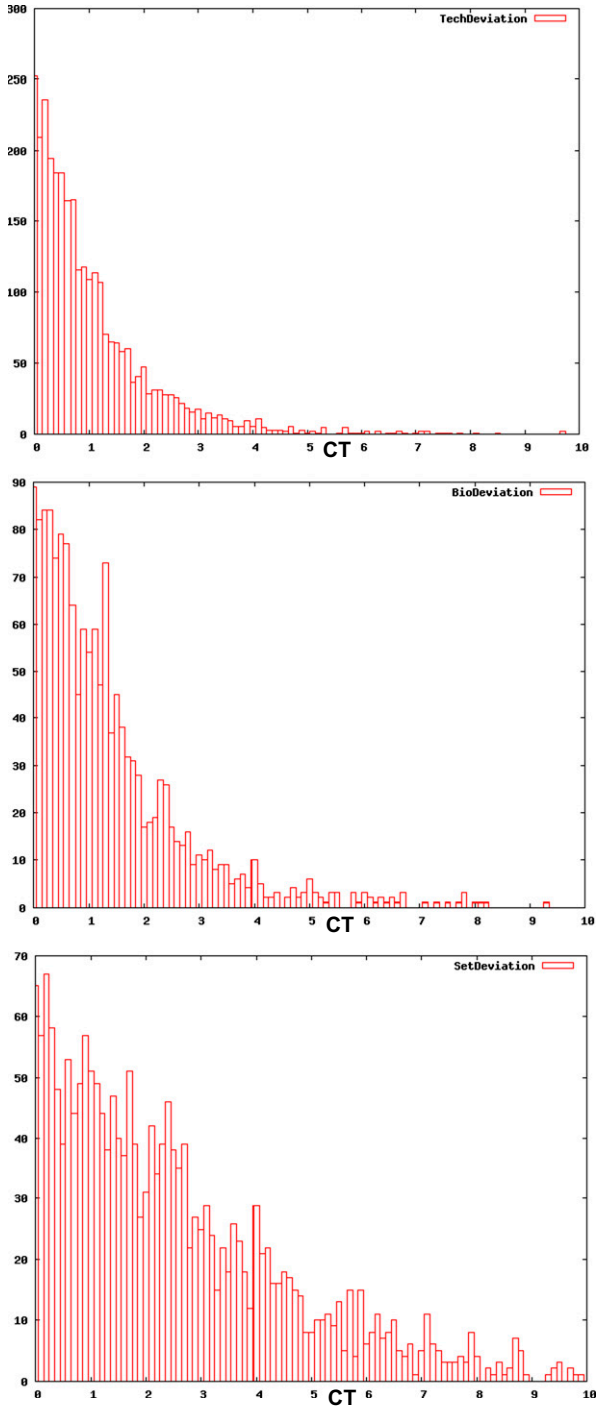


Fig. S4. Histograms of log-ratio of technical (*Top*), biological (*Middle*), and set (*Bottom*) replicates at 96× multiplexing level. Technical deviation mean is 1.08, biological deviation mean is 2.02, and set deviation mean is 2.6.

Table S1. Selection markers used for FACS sorting

No.	Population	Surface markers for selection	Source	Branch	Designation on a tree	Ref.
1	B-lymphocyte	$CD19^+$	Spleen	Lymphoid	B-Cell	
2	Basophil	$FceR1\alpha^+$/c-kit^{neg}	Bone marrow	Myeloid	Basophil	1
3	Common Lymphoid Progenitor	lin^-/IL-7^+/$ckit^+$/sca-1^{low}	Bone marrow	Precursor	CLP	2
4	Common Myeloid Progenitor	lin^-/$ckit^+$/sca-1^- $CD34^+$/$Fc\gamma R^{low}$	Bone marrow	Myeloid	CMP	3
5	Erythroblast	$Ter119^{med}$/$CD71^{hi}$	Bone marrow	Erythroid	Eb	4
6	Erythroblast basophilic	$Ter119^+$/$CD71^{hi}$	Bone marrow	Erythroid	EbBas	4
7	Erythroblast orthochromatophilic and reticulocyte	$Ter119^+$/$CD71^{med-}$	Bone marrow	Erythroid	OrtER	4
8	Erythroblast polychromatic	$Ter119^+$/$CD71^{low}$	Bone marrow	Erythroid	EbPol	4
9	Granulocyte	$Mac1^+$/$Gr1^-$	Bone marrow	Myeloid	$Mac1^+$	5
10	Granulocyte precursor	$Mac1^+$/$Gr1^+$	Bone marrow	Myeloid	G Precursor	5
11	Granulocyte/Monocyte Progenitor	lin^-/$ckit^+$/sca-1^-/$CD34^+$/$Fc\gamma R^{high}$	Bone marrow	Precursor	GMP	3
12	HSCs with high self-renewal capacity	$CD45^+$/$EPCR^+$/$CD48^-$/$CD150^+$	Bone marrow	Stem cell	ES 150^+	6
13	HSCs with low self-renewal capacity	$CD45^+$/$EPCR^+$/$CD48^-$/$CD150^-$	Bone marrow	Stem cell	ES 150^-	6
14	Lin^- sca-$1^+$$ckit^+$ cells	EasySep/sca-1^+/$ckit^+$	Bone marrow	Stem cell/ precursor	LSK	7
15	Macrophage	$F4/80^+$/$Mac\text{-}1^+$	Peritoneum	Myeloid	Macrophage	8
16	Mast Cell	Spl CT(SCF derived) or Spl M (IL-3 derived)	Spleen, bone marrow	Myeloid	Mast Cell	9
17	Megakaryocyte	$CD41^+$	Bone marrow	Myeloid	Megakaryocyte	
18	Megakaryocyte/erythroid progenitor	lin^-/$ckit^+$/$Sca1^-$/$CD34^-$/$Fc\gamma R^-$	Bone marrow	Precursor	MEP	3
19	Monocyte	EasySep kit (Stem Cell Technologies)	Peripheral blood	Myeloid	Monocyte	
20	Natural killer cell	$NK1.1^+$/$CD3^-$	Bone marrow	Lymphoid	Natural Killer cell	10
21	Natural Killer cell progenitor	Lin^-/$NK1.1^-$/$CD122^+$	Bone marrow	Lymphoid	NK progenitor	11
22	Neutrophil	Magnetic Negative selection using EasySep containing CD3 CD49b (DX-5) CD45R/B220 Anti-Ia (Anti-MHC II) Ter119 followed by Histopaque gradient: 1119/1077-cells at interface were taken as neutrophils. Purity (>95%) was confirmed by Diff-Quik (Dade Behring Cat.B4132-1A) staining on cytospin preparations of freshly isolated cells.	Bone marrow	Myeloid	Neutrophil	Similar to 12
23	SLAM HSC ($CD150^+CD48^-$)	$CD150^+$/48^-, enrichment with CD45	Bone marrow	Stem cell	SLAM	13
24	T cell ($CD4^+$)	$CD4^+$	Spleen	Lymphoid	CD4 T cell	
25	T cell ($CD8^+$)	$CD8^+$	Spleen	Lymphoid	CD8 T cell	
26	Thymocytes double negative ($CD4^-CD8^-$)	Lin^- (CD3, CD8, Gr-1, Mac-1, Ter119, B220, CD11c, NK1.1)/$CD4^-$/$CD8^-$	Thymus	Lymphoid	Thymocyte DN	14
27	Thymocytes double positive ($CD4^+CD8^+$)	$CD4^+$/$CD8^+$	Thymus	Lymphoid	Thymocyte DP	14

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Other Supporting Information Files

Dataset S1 (XLS)
Dataset S2 (XLS)
Dataset S3 (XLS)
Dataset S4 (XLS)