Supplementary Information

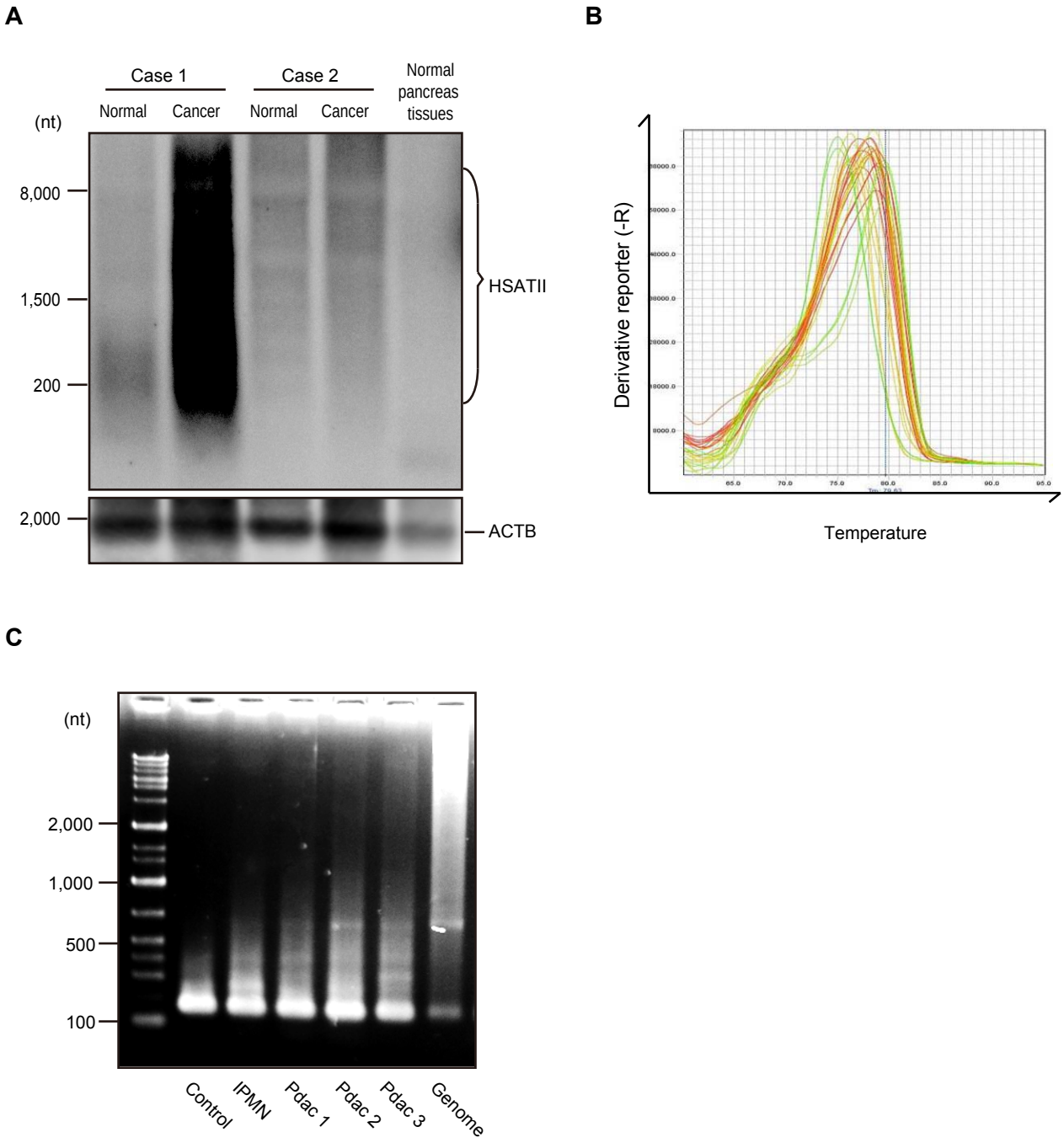
Quantitation of circulating satellite RNAs in pancreatic cancer patients

Takahiro Kishikawa, Motoyuki Otsuka, Takeshi Yoshikawa, Motoko Ohno, Keisuke Yamamoto, Natsuyo Yamamoto, Ai Kotani, and Kazuhiko Koike

Inventory of Supplementary Information:

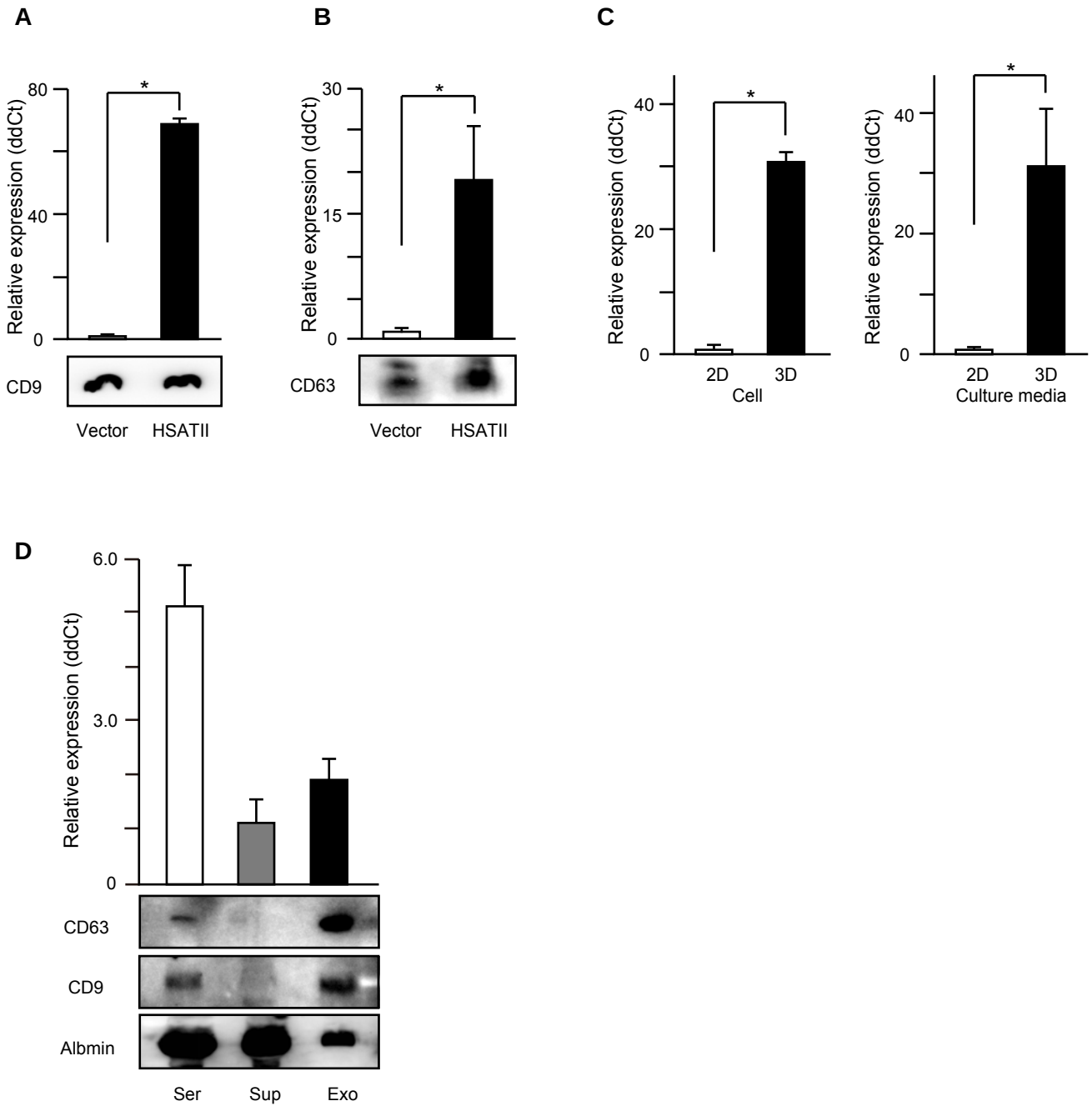
Supplementary Figures 1-5 with legends

Supplementary Figure 1



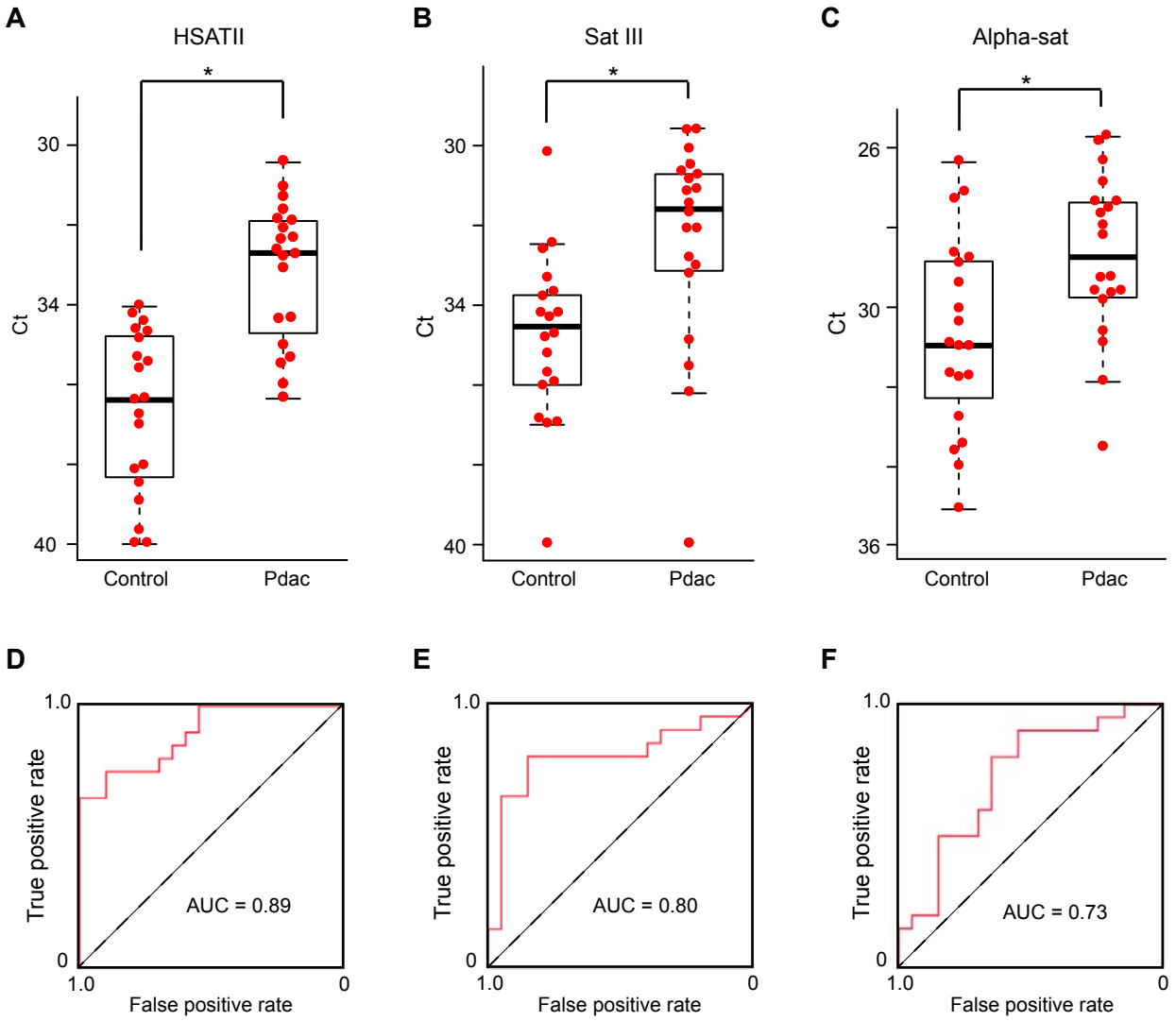
Supplementary Figure 1. Human satellite II (HSATII) RNA was detected in pancreatic ductal adenocarcinoma (Pdac) tissues but not in normal pancreas tissue. (A) Northern blotting using total RNA from two cases of human Pdac and patient-matched adjacent non-cancerous tissues and in normal pancreatic tissues. HSATII RNA could be detected as smear bands in cancer tissues. ACTB mRNA was detected by re-probing the same membrane to confirm approximately equal loading. nt, nucleotides. **(B)** Melting curves when detecting HSATII RNA by real-time PCR. Serum RNAs from control and patients with intraductal papillary mucinous neoplasm (IPMN) or Pdac were subjected to real-time PCR in triplicate. Melting peaks were highly variable in different samples and even in the repeated experiments using the same sample. Each sample was analyzed in triplicate. yellow, control (n = 5); green, IPMN (n = 2); red, Pdac (n = 3). **(C)** Difficulties in accurately quantifying HSATII RNA levels by conventional reverse transcription (RT)-PCR methods. Serum RNAs from controls (n = 1) and patients with IPMN (n = 1) or Pdac (n = 3) were amplified by RT-PCR. Genomic DNA was also tested as a comparison. The PCR products were separated by electrophoresis in an agarose gel. Each sample shows multiple ladder bands from approximately 150 to 800 nt. nt, nucleotides. IPMN: intraductal papillary mucinous neoplsm.

Supplementary Figure 2



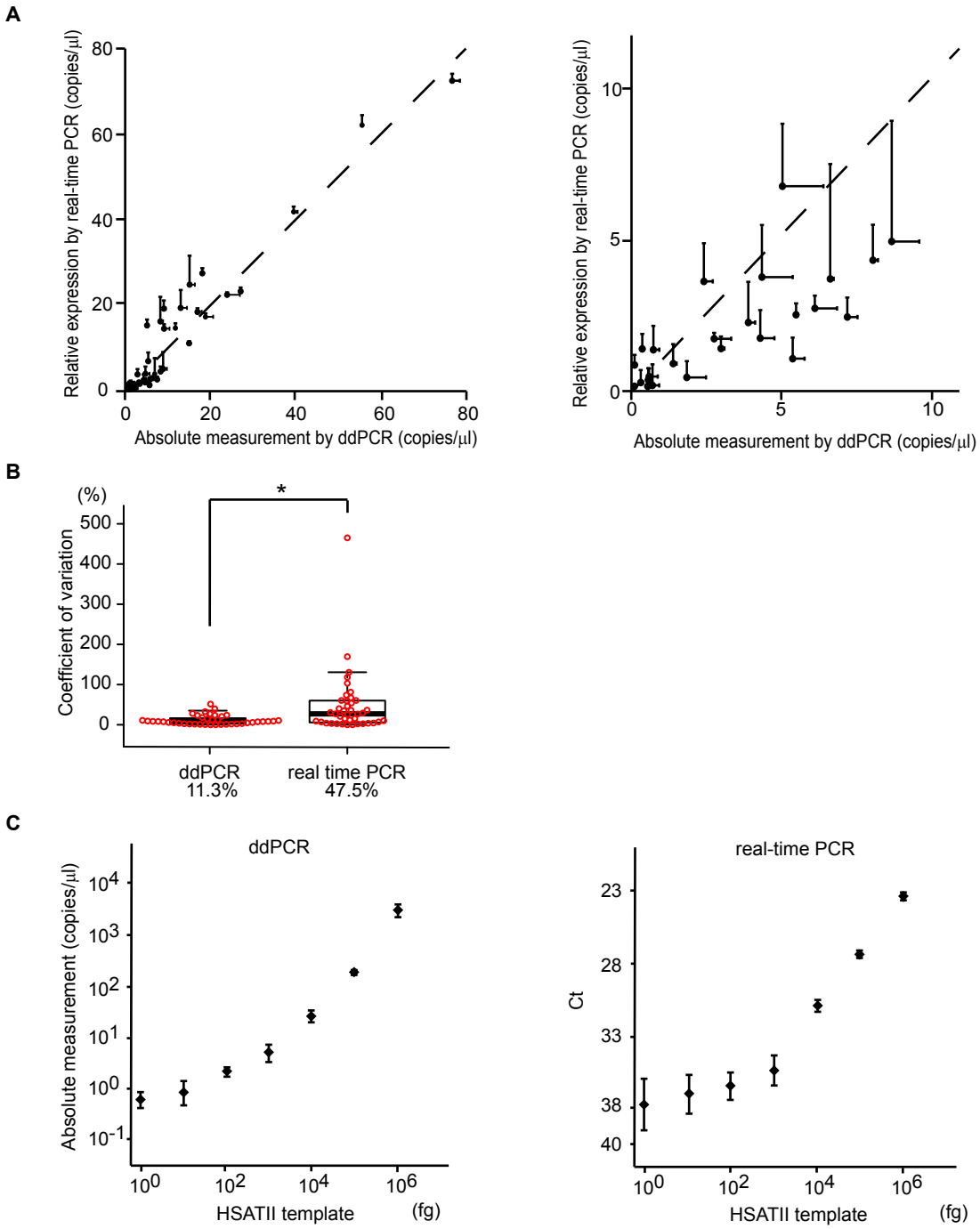
Supplementary Figure 2. Human satellite II (HSATII) RNA was mainly detected in the exosome fraction of the cell culture media and the serum. (A)(B) HSATII RNAs in the culture media of BxPC3 cells (A) and hTERT-HPNE cells **(B)**, which were transfected with control (vector) or HSATII-expressing plasmids (HSATII). The upper panels show the relative levels of HSATII RNA as determined by the Tandem Repeat Amplification with nuclease Protection (TRAP)-real-time PCR method. The data represent the mean ± S.E. of three independent experiments. *, $p < 0.01$, by two-tailed Student's t-test. Western blotting of CD9 (A) and CD63 (B) are shown as exosome markers. **(C)** BxPC3 cells were cultured on a standard 2D culture plate and in 3D on the Nunclon Sphera plate for 4 days. HSATII RNA expression in cell lysates (left panel) and cultured media (right panel) was determined by TRAP real-time PCR. BxPC3 cells cultured in 3D showed expression of HSATII RNA, but those cultured in 2D did not. Data represent the mean ± S.E. of three independent PCR assays. *, $p < 0.01$, by two-tailed Student's t-test. **(D)** HSATII RNA in the serum of a patient with pancreatic ductal adenocarcinoma (Pdac). The upper panel shows HSATII RNA levels in the total serum, in the extracted exosome fraction, and in the supernatant from 500 µl of serum. Data represent the mean ± S.E. of three independent experiments. Lower panels show the results of western blotting against CD63 and CD9 as exosome markers and against albumin as a serum marker.

Supplementary Figure 3



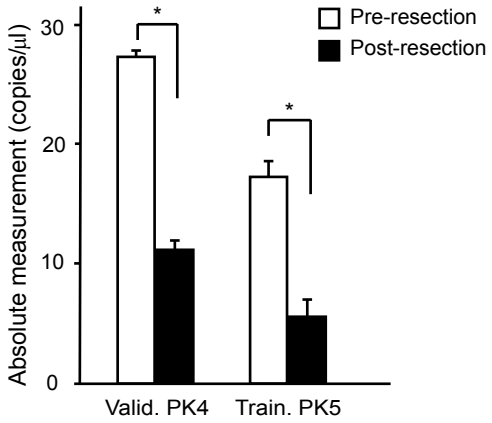
Supplementary Figure 3. Human satellite RNA levels in sera for the detection of pancreatic ductal adenocarcinoma (Pdac). (A)(B)(C) The Ct values of human satellite II (HSATII) (A), satellite III (satIII) (B), and alpha satellite (Alpha-sat) (C) determined with real-time PCR. Core sequences of each satellite RNA from the serum RNAs of the training set (n = 20 vs 20) were processed by the Tandem Repeat Amplification with nuclease Protection (TRAP) method, followed by the real-time PCR. Bee swarm dot plots and box plots are shown. The horizontal line in the middle of each box indicates the median. The top and bottom borders of the box mark the 75th and 25th percentiles, respectively. The whiskers above and below each box extend to the most extreme point that in no more than 1.5 times the interquartile range of the box. *, $p < 0.05$, by two-tailed Welch's t-test. **(D)(E)(F)** Receiver operating characteristic (ROC) curve analysis of each satellite RNA: HSATII (D), satIII (E), and Alpha-sat (F). AUC, areas under the curve.

Supplementary Figure 4



Supplementary Figure 4. Superior accuracy of measurements by droplet digital PCR (ddPCR) relative to real-time PCR. **(A)** Comparison of the standard deviations of the results acquired by real-time PCR and ddPCR after performing the Tandem Repeat Amplification with nuclease Protection (TRAP) method. Serum RNAs from the training set were amplified by the TRAP method and quantified by ddPCR and real-time PCR. Data represent the mean ± s.d. of two (ddPCR) or three (real-time PCR) independent experiments. n = 40. The right panel shows the results of the lower expression levels. **(B)** The differences of the distribution of the coefficient of variation (CV) of the results between ddPCR and real-time PCR in the training set. Bee swarm dot plots and box plots are shown as described in the legend of Figure 2. *, $p < 0.05$, by two-tailed Welch's t-test. **(C)** The lowest detection limits by ddPCR and real-time PCR were determined using 10-fold serial dilutions between 1 fg and 1 ng of the HSATII RNA template. Data represent the mean ± S.E. of two (ddPCR) or three (real-time PCR) independent PCR assays.

Supplementary Figure 5



Supplementary Figure 5. Decreased serum human satellite II (HSATII) RNA levels in Pdac patients after surgery. HSATII RNA levels in sera were determined by the Tandem Repeat Amplification with nuclease Protection (TRAP)-droplet digital PCR (ddPCR) method in two individuals who underwent surgery (PK4 in the validation set, "Valid. PK4"; and PK5 in the training set, "Train. PK5"). Sera samples acquired 5-7 months after operation were used as post-resection samples. In both cases, HSATII RNA levels were significantly decreased post-resection compared with pre-resection. Data represent the mean ± S.E. of two independent PCR assays. *, $p < 0.05$, by two-tailed Student's t-test.