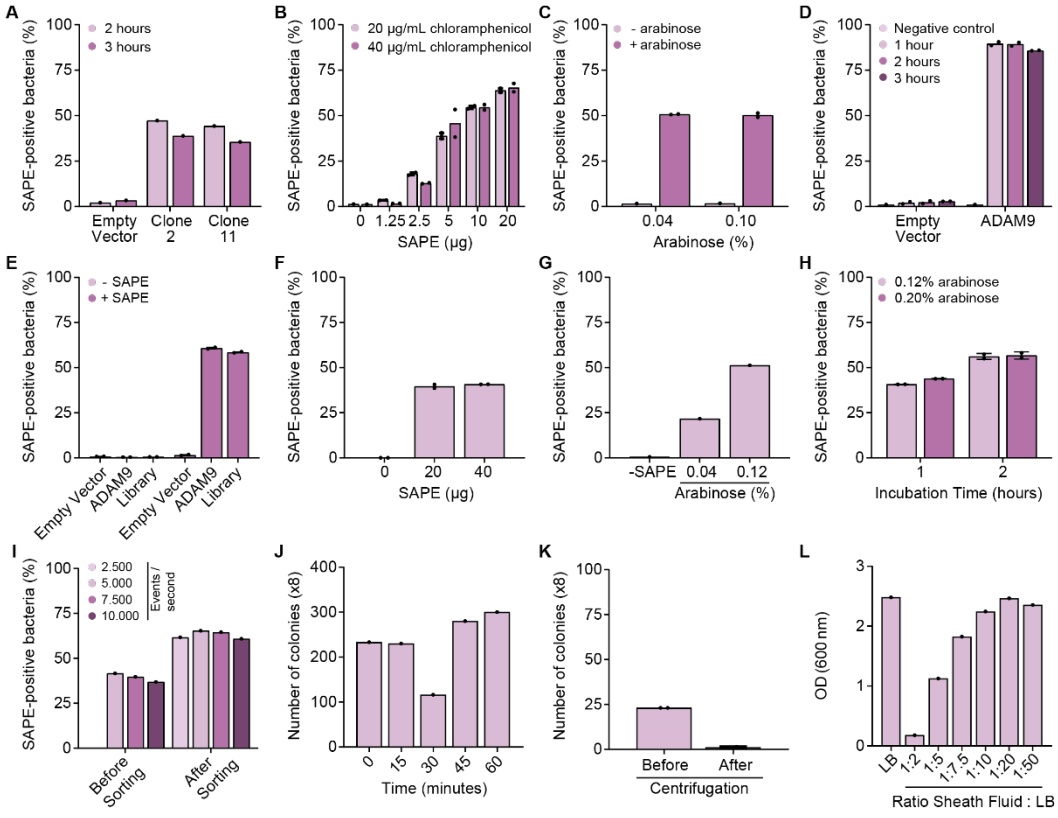
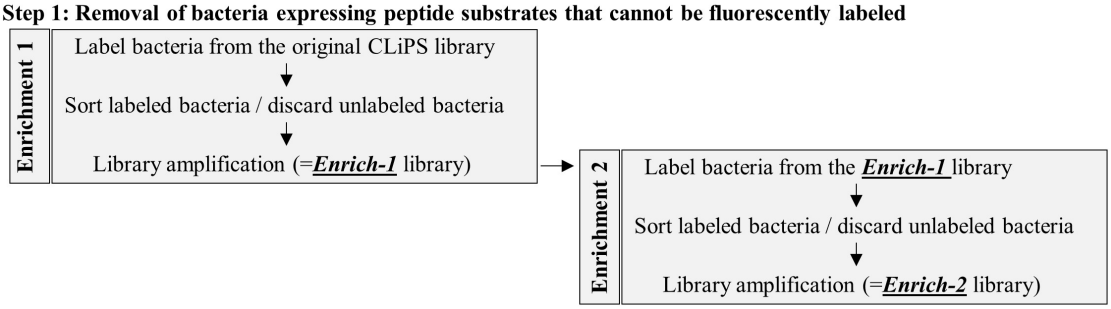
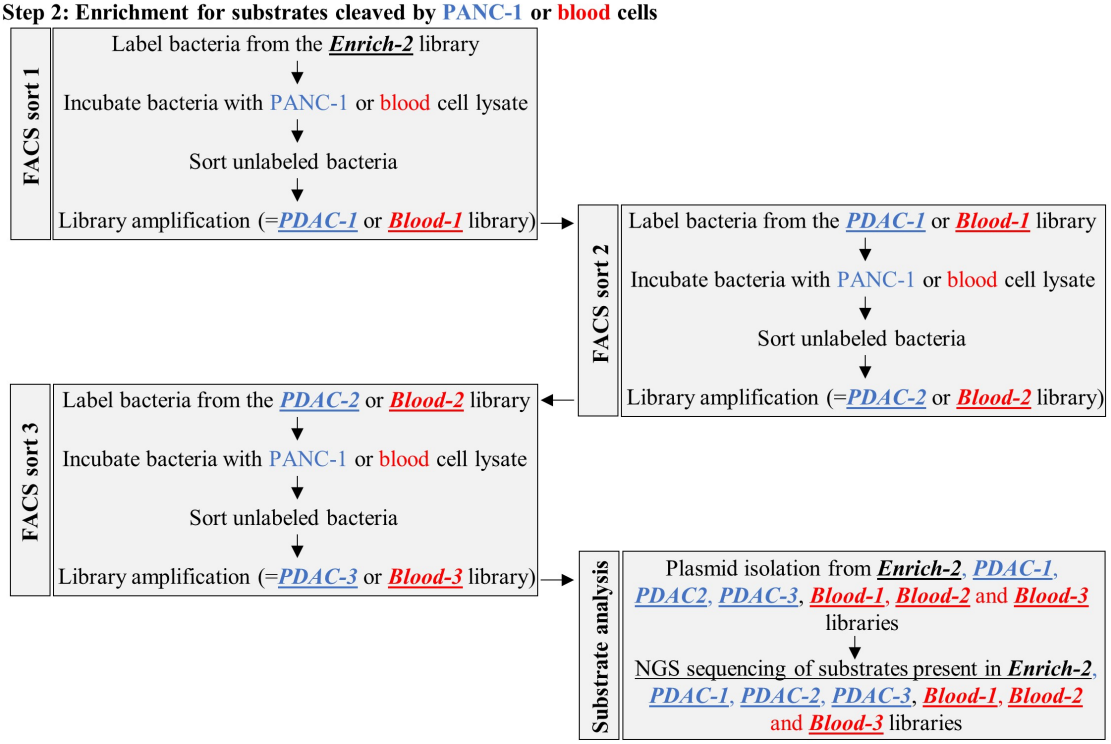


Supplemental Fig 1. Protocol optimization results in efficient E. coli labeling (**A-H**) and viable FACS sorting procedure (**I-L**). (**A**) Percentage SAPE-positive ADAM9-responsive bacteria of two randomly picked clones after transformation. Influence on labeling efficacy of chloramphenicol and SAPE concentration (**B**), arabinose (**C**) and SAPE-incubation time (**D**). (**E**) Comparison of labeling efficacy of ADAM9-responsive bacteria with random peptide Library bacteria. Influence of SAPE concentration (**F**), arabinose (**G**) and arabinose-incubation time (**H**) on percentage SAPE-positive Library bacteria. (**I**) To assess the influence of sorting speed on sorting efficiency, the percentage of SAPE-labeled bacteria was determined before and after sorting at various speeds using a SH800 Cell Sorter (Sony Biotechnology, San Jose, CA, USA). (**J**) Bacteria were suspended in FACS Flow buffer for various periods to test the influence on viability. (**K**) Influence of centrifugation (11.000 x g for 2 minutes) on bacteria recovery after sorting. (**L**) Influence of FACS Flow buffer on bacterial outgrowth.

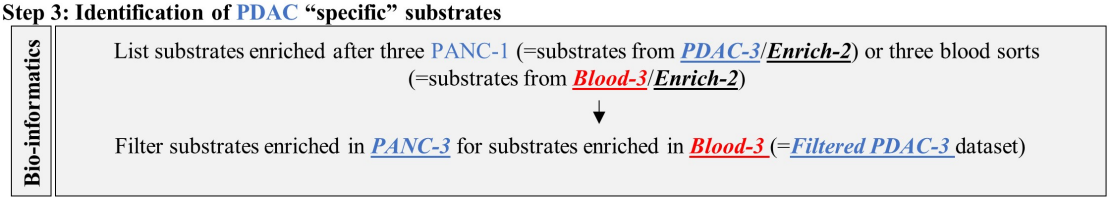
Step 1: Removal of bacteria expressing peptide substrates that cannot be fluorescently labeled



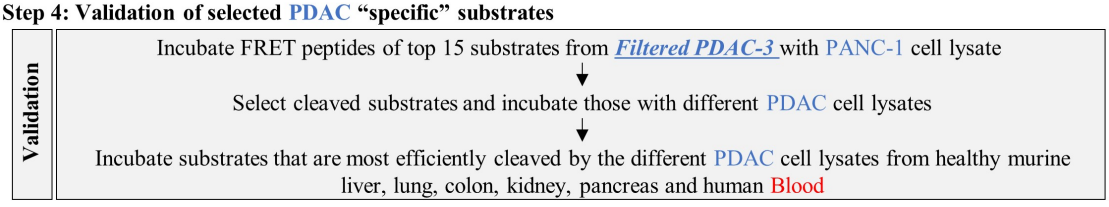
Step 2: Enrichment for substrates cleaved by PANC-1 or blood cells



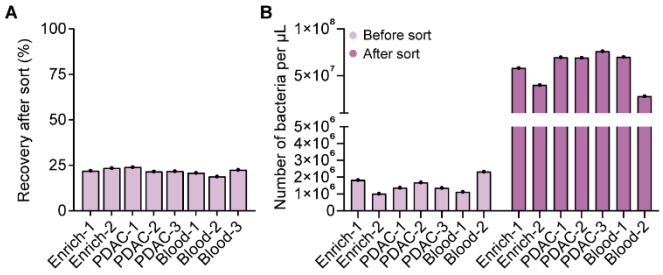
Step 3: Identification of PDAC "specific" substrates



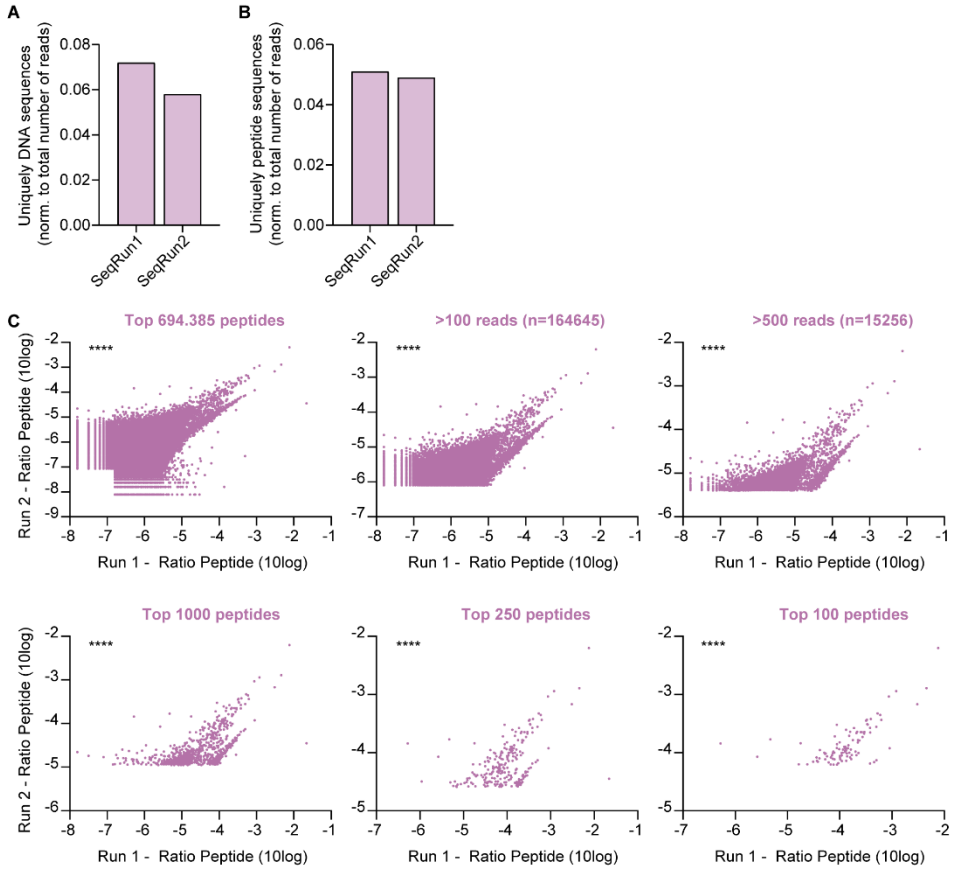
Step 4: Validation of selected PDAC "specific" substrates



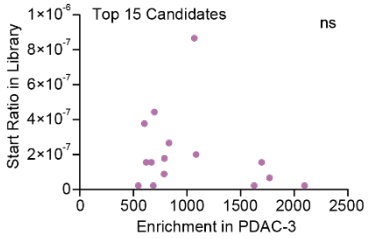
Supplemental Fig 2. Schematic overview of the procedure to identify and validate PDAC specific substrates.



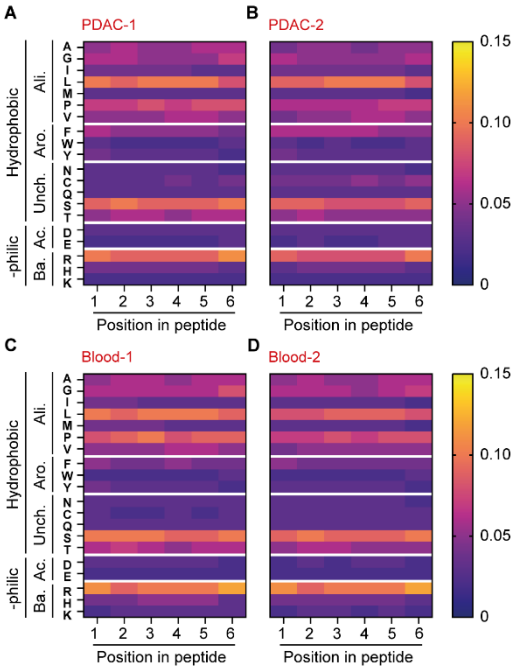
Supplemental Fig 3. Sorting procedure is reproducible as evident from similar bacteria recovery and bacteria abundance after outgrowth of sorted bacteria. (**A**) Percentage of bacteria recovered after sorting determined by dividing the number of colonies counted by the total amount of sorted cells as determined by the SH800 Cell Sorter. (**B**) Calculation of the number of bacteria in stock before and after sorting and overnight growth. Enrich; untreated bacteria, PDAC; PANC-1 cell lysate-treated bacteria, Blood; whole blood cell lysate-treated bacteria. The number corresponds to the treatment round. An overview of the sorting procedure can be seen in Fig 3A.



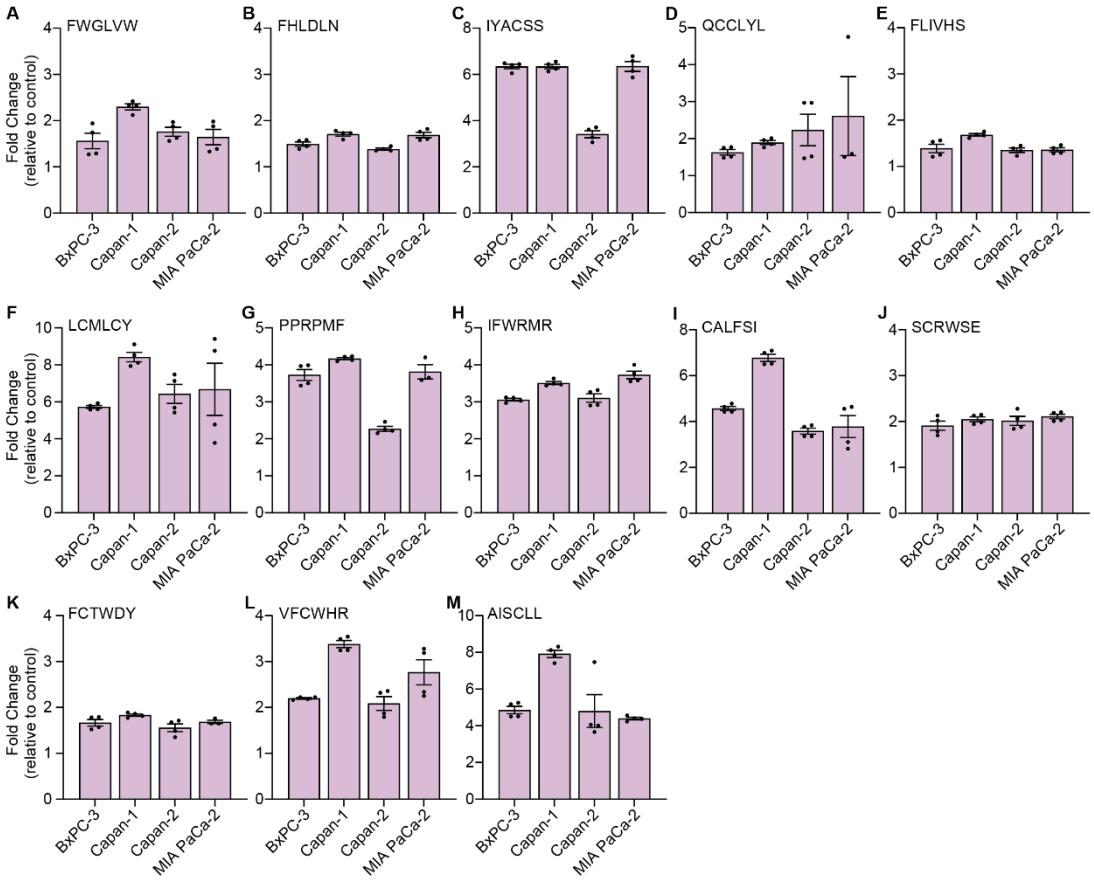
Supplemental Fig 4. Comparison of two separate rounds of library preparation and Next Generation Sequencing Runs shows excellent correlation. (**A**) Comparison of the number of unique DNA sequences in the library relative to the total amount of reads between two sequencing runs of the same sample. (**B**) Comparison of the number of unique peptide sequences in the library relative to total amount of reads between two sequencing runs of the same sample. (**C**) Correlation of matched peptides in sequencing run 1 and 2 for various parameters. Two-way ANOVA was used to compare the percentage of events in Q1. Levels of significance: **** $p < 0.0001$.



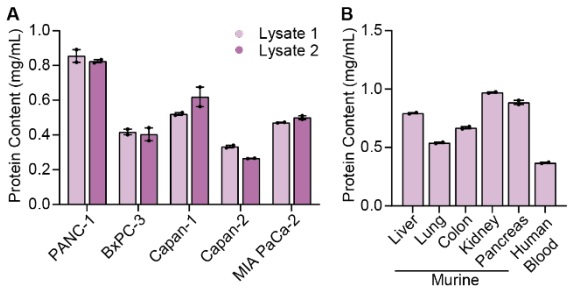
Supplemental Fig 5. Identification of top 15 substrates is independent from initial abundance in library. No significant correlation is observed between the fold enrichment of the top 15 hits (x-axis) and their corresponding abundance in the starting library (y-axis). This indicates that the substrates were enriched based on their specific properties rather than their starting concentrations. Significance was determined by Pearson correlation coefficient. Levels of significance: ns = not significant.



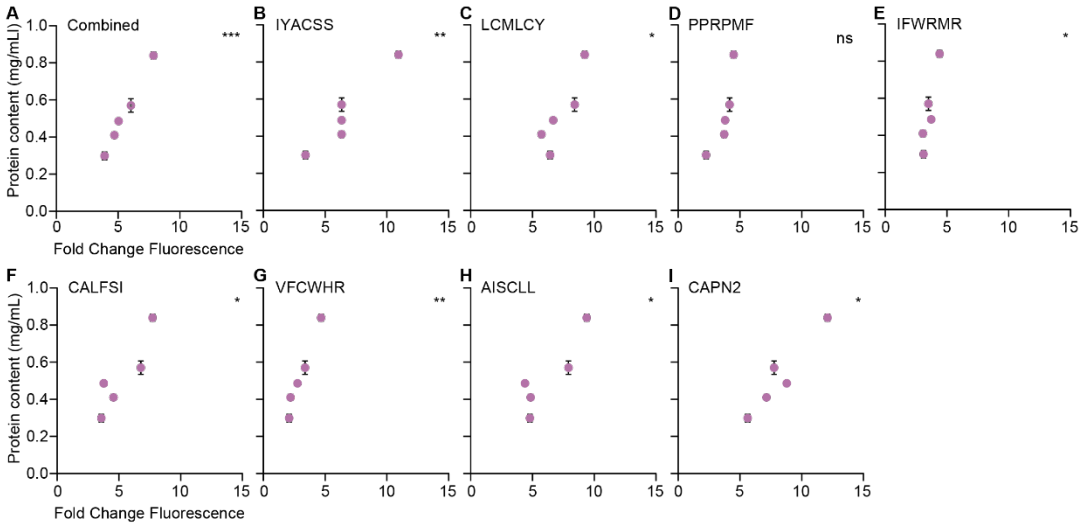
Supplemental Fig 6. Distribution of amino acids candidates for intermediate steps of PANC-1 and whole blood cell lysate-treated Library of obtained peptide candidates. (**A**) Amino acid distribution after one (PDAC-1) and two (PDAC-2) treatment rounds with PANC-1 cell lysate. (**B**) Amino acid distribution after one (Blood-1) and two (Blood-2) treatment rounds with whole blood cell lysate.



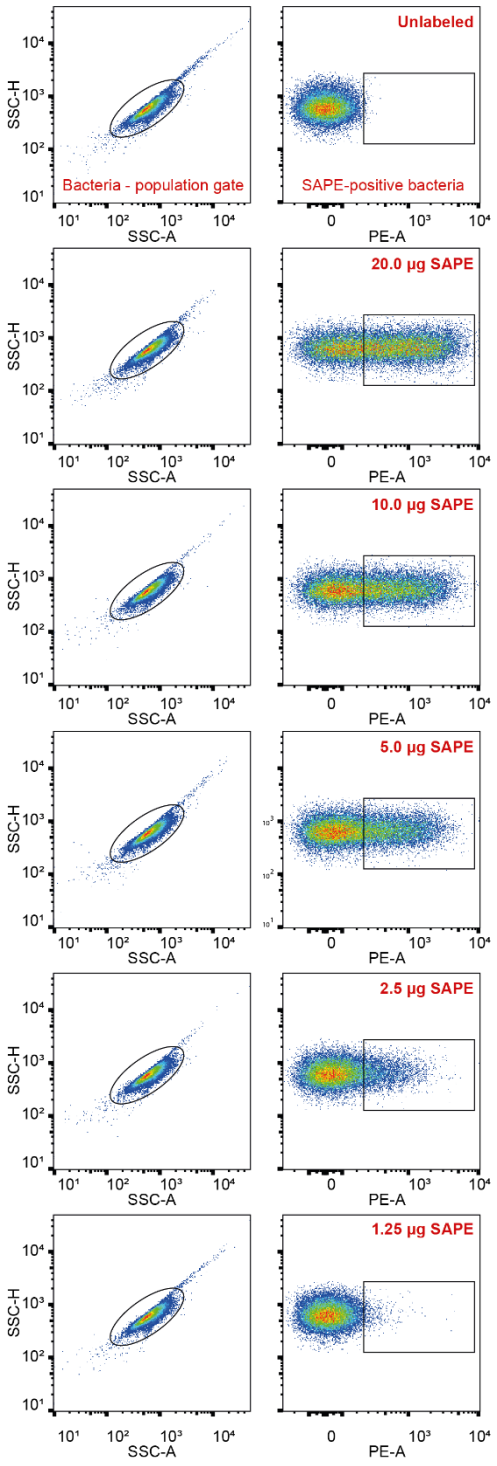
Supplemental Fig 7. Identified substrates are efficiently cleaved by cell lysates from multiple PDAC cell lines. One hour incubation of FRET-peptides with lysate from BxPC-3, Capan-1, Capan-2 or MIA PaCa-2 ($1x10^6$) cells as represented by fold change in measured fluorescence levels relative to PBS-treated FRET-peptide. Data of two representative experiments with *n* = 2 are shown.



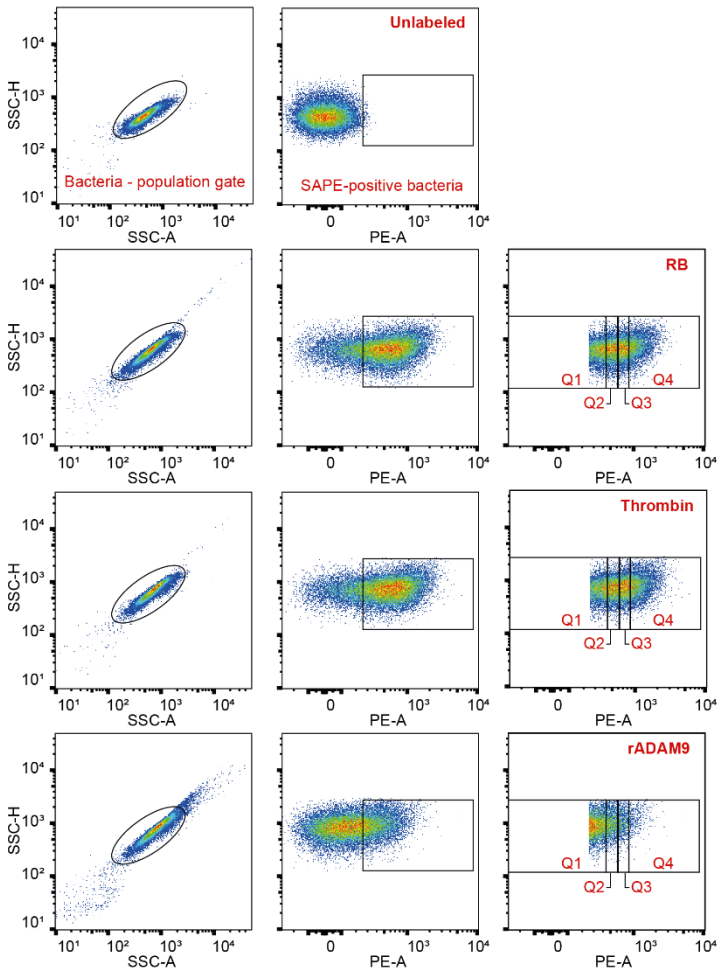
Supplemental Fig 8. Successful PDAC cell, murine organ and human blood cell lysis is confirmed by the presence of proteins in the lysis buffer. (**A**) Comparison of protein content of two separate lysates from PANC-1, BxPC-3, Capan-1, Capan-2 or MIA PaCa-2 after 5 rounds of 5 second pulses at 30% amplitude followed by 5 seconds of pause using a Vibra-Cell X-130 (Sonic & Materials Inc, CT, Newtown, USA). (**B**) Protein content in murine liver, lung, colon, kidney and pancreas, and human whole blood cell lysates obtained through similar sonication protocol. All samples were measured using a Nanodrop 2000 (Thermo Fisher, USA).



Supplemental Fig 9. Lysate protein content correlates to substrate cleavage efficiency. (**A**) Correlation between the protein content and fold change in fluorescence of 8 FRET-peptides combined. (**B-I**) Correlation of individual peptides. Fluorescence levels were measured using a Biotek Synergy HT plate reader (Biotek Instruments, Winooski, VT, USA) after 1 hour of incubation with either PANC-1, BxPC-3, Capan-1, Capan-2 or MIA PaCa-2 cell lysate. Data is normalized to untreated control. Data represents two representative experiments with $n = 2$. Significance was determined by Pearson correlation coefficient. Levels of significance: ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Supplemental Fig 10. Gating strategy to assess the percentage of fluorescent-labeled E. coli bacteria. Streptavidin-coupled phycoerythrin (SAPE)-positive bacteria are determined by setting the gate using unlabeled bacteria. An increase in SAPE-positive bacteria can be observed following increasing SAPE concentration compared to unlabeled cells (i.e. 0 ug SAPE condition).



Supplemental Fig 11. Gating strategy to assess cleavage of peptide substrate on the cell surface of E. coli bacteria. First, SAPE-positive bacteria are selected based on unlabeled bacteria. To determine the extent of substrate cleavage expressed on the cell surface of E. coli, gates are set to include 25% of the cells in every quartile based on RB-treated cells. Following this, the change in labeling can be observed after treatment with recombinant thrombin and ADAM9 compared to untreated cells (RB; reaction buffer). Gates are set to include 25% of the cells in every quartile based on RB-treated cells.