Development of selective ssDNA micro-probe for PD1 detection as a novel strategy for cancer imaging

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Supplementary methods

Nomenclature of the aptamers

The nomenclature for individual aptamers followed the outlined scheme:

"P" - Selection for the PD1 protein (to distinguish from other projects),

"1c" or "2c" - Cluster number,

"1s" - "8s" - Sequence number (within the cluster).

The numbers for individual clusters and sequences are automatically generated during the bioinformatic analysis.

For the construction of mutants of the investigated aptamers, each aptamer under study was divided into fragments (with each fragment assigned a number). The names of individual aptamer mutants were assigned based on the excised fragment of the aptamer.

In vitro toxicity assay

Non-toxic doses of aptamers for *in vitro* binding tests were determined with LDH and MTT. Jurkat T-cells (non-transfected and stably expressing hPD1) were seeded on a 96-well plate (0.25×10^6 per well) and incubated in standard culture conditions. Cells were then incubated for 1h, 3h, 12h or 24h in the presence of 1nM, 25nM, or 50nM of aptamers (P2c2s, P34, negative control) at 37°C with 5% CO_2. After incubation, cytosolic lactate dehydrogenase

release was measured using Pierce LDH Cytotoxicity Assay Kit, according to the manufacturer protocol (Thermo Fisher Scientific, Waltham, MA, USA). For MTT analysis 150µl of growth media supplemented with MTT (0,45 mg/ml) was incubated with cells for 2h 30 minutes at 37°C. The medium was removed and 100 µl of DMSO with Methanol (1: 1) was added to each well to dissolve purple crystals of formazan. The absorbance was determined at $\lambda = 550$nm with the use of the Infinite 200 PRO multimode reader.

Analysis of antibody and aptamer binding to PD1 using ELISA

100 µl of PD1 (hPD1 extracellular domain (21-168) C-His-tag) was coated on a plate (10 µg/ml in PBS) overnight at +4°C.

Antibodies: The plate was washed twice with a washing buffer (0.05% Tween 20 / PBS). Serial dilutions (2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0 mg/L) of anti-PD1 antibody (rabbit anti-PD-1/CD279 Polyclonal Antibody (Bioss Antibodies: bs-1867R)) in reagent diluent (0.1% BSA, 0.05% Tween 20 / PBS) were added to the wells and incubated for 1.5 hours at room temperature. Unbound antibody was removed by washing the plate three times, then 100 µl of secondary antibodies (Goat anti-Rabbit IgG (H+L), Secondary Antibody, HRP 31460 Invitrogen) in reagent diluent was added and incubated for 1 hour at room temperature. Unbound secondary antibodies were removed away by washing the wells three times with washing buffer. Following this, 100 µl of the HRP reagent substrate (R&D Systems, Minneapolis, USA) was added and incubated for 30 minutes at room temperature.

Aptamers: The plate was washed twice with the SELEX buffer. Serial dilutions (62.5, 31.25, 15.62, 7.8, 3.9, and 0 nM) of biotinylated aptamers P34 and P2c2s (100 µl) in SELEX buffer were added to the wells and incubated for 30 minutes. Unbound aptamers were removed by extensive washing with the selection buffer. 100 µl of horse-radish-peroxidase (HRP)-conjugated streptavidin (1:200 dilution; R&D Systems, Inc., Minneapolis, USA) was added to the wells. After 20 minutes of incubation, the unbound streptavidin was removed by washing with the selection buffer. Following this, 100 µl of the HRP reagent substrate (R&D Systems, Minneapolis, USA) was added and incubated for 5 minutes at room temperature.

The reactions were stopped by adding 50 µl of 2N H_2SO_4. Absorbance at 450 nm and 570 nm (correction for optical plate imperfections) was determined using the Infinite 200 PRO multimode reader (Tecan Group Ltd., Switzerland). Affinity analysis was performed using Prism - GraphPad software

Supplementary results

Prediction of the 2D and 3D structure of aptamers P2c2s and its derivative P34

The selected aptamer, P2c2s, consists of 88 nucleotides. Further modification of this aptamer led to the creation of its shortened mutant, aptamer P34, comprising 68 nucleotides (shortened compared to the original aptamer due to the flanking sequence at the 3' end). The software available on the website http://www.unafold.org allowed the generation of three 2D structures for aptamer P2c2s with similar folding properties (ΔG) (Figure S3 A, B and C). Simultaneously, the analysis of the 2D structure for aptamer P34 generated one structure (Figure S3 D). Using the software accessible at https://rna.urmc.rochester.edu (enabling the analysis of common structures for two molecules), it was possible to determine the putative structures of P2c2s (Figure S3 C) and P34 (Figure S3 D) that shared a core for nucleotides 1-68 of the aptamer.

Further analysis of the structure of aptamer P34 was conducted using software available at http://biophy.hust.edu.cn. Five possible conformations of the aptamer were identified (Figure S4).

Supplementary figures

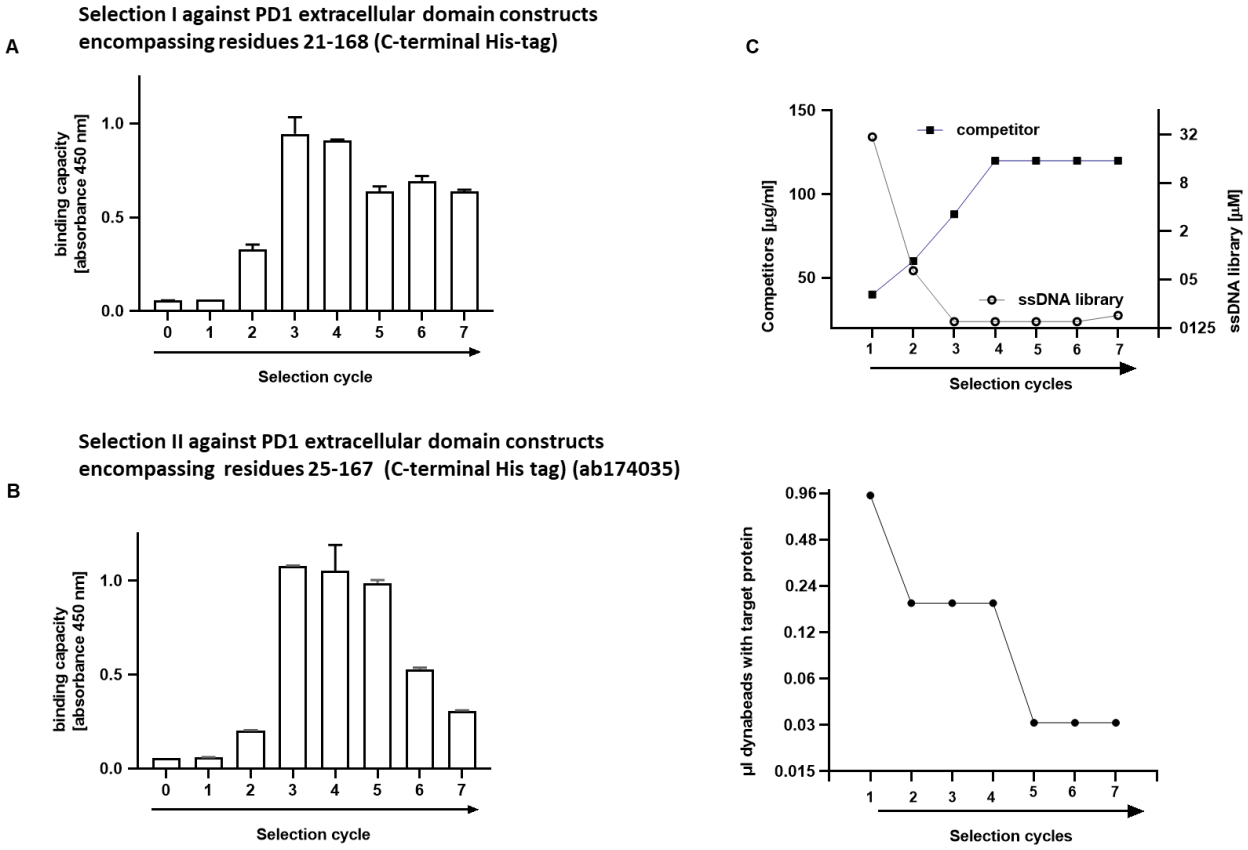

Figure S1 Selection of PD1 targeting aptamers. (A, B) Enrichment of ssDNA pool with aptamers recognizing the extracellular domain of PD1 (constructs indicated) determined using ELISA. (C) Gradual increase in selection pressure at subsequent selection cycles was obtained by increasing the concentration of the competitor (yeast tRNA) while simultaneously decreasing the concentration of ssDNA library and target protein.

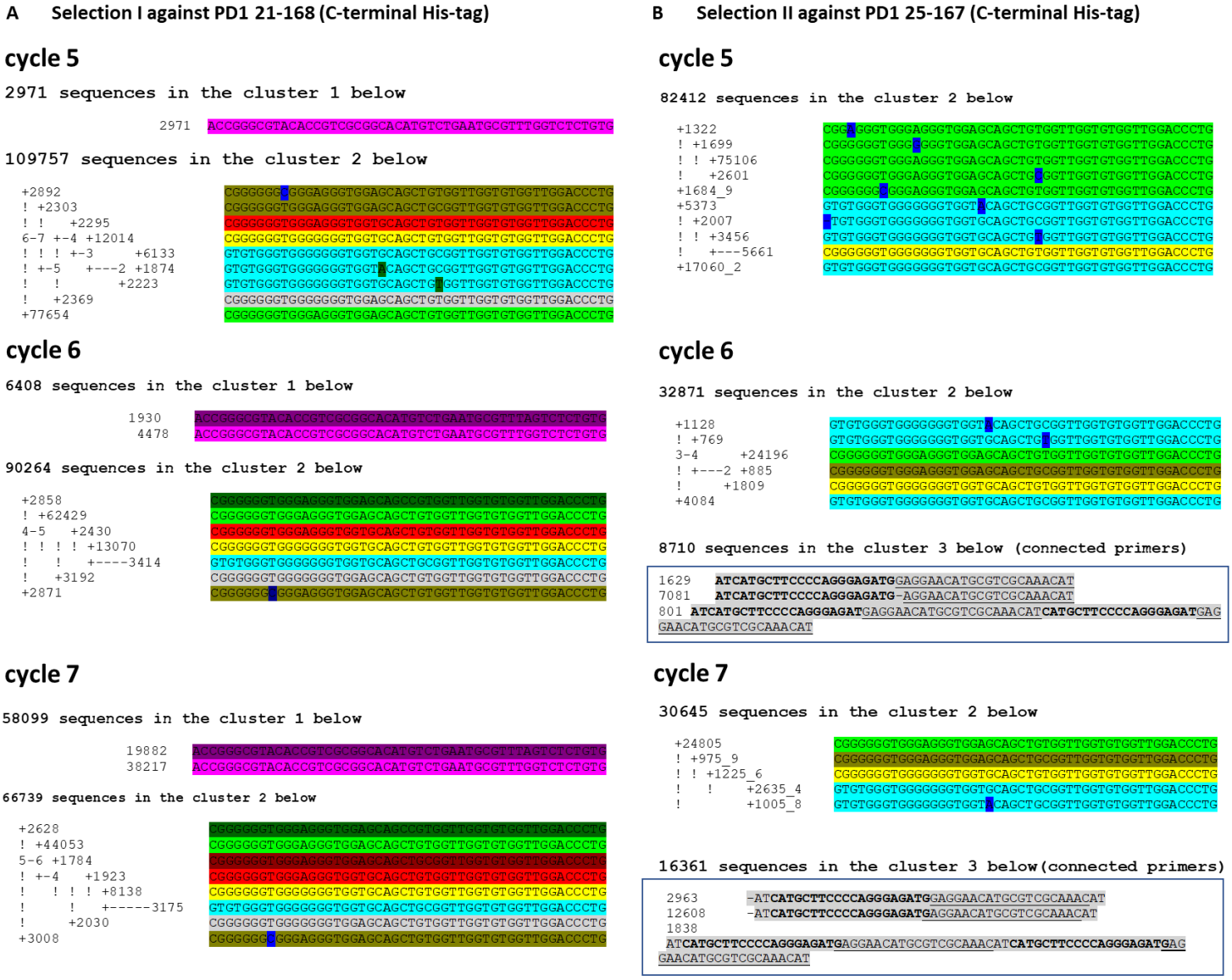

Figure S2 Qualitative analysis of sequences in the last 3 selection cycles. The decrease in affinity (Shown in Figure 1) of the aptamer pool in the following cycles is explained by: (A) preferential amplification of lower affinity sequences, (B) accumulation of unspecific PCR products. Color coding:

Purple: 1c1s
pink: 1c2s
dark green: 2c1s
light green: 2c2s (and single-nucleotide mutated variants)
brown: 2c3s
red: 2c4s
yellow: 2c5s (and single-nucleotide mutated variants)
blue: 2c6s
gray: 2c7s (and single-nucleotide mutated variants)
brown 2c8s
gray - boxed: composite sequences from linked primer sequences

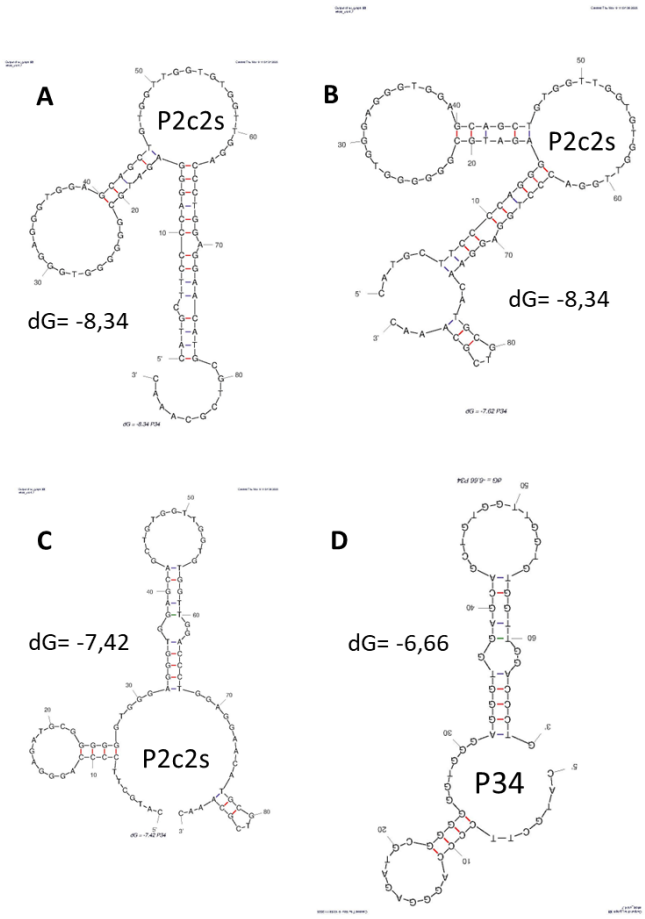

Figure S3 Prediction of the 2D structure of aptamers P2c2s and its derivative P34

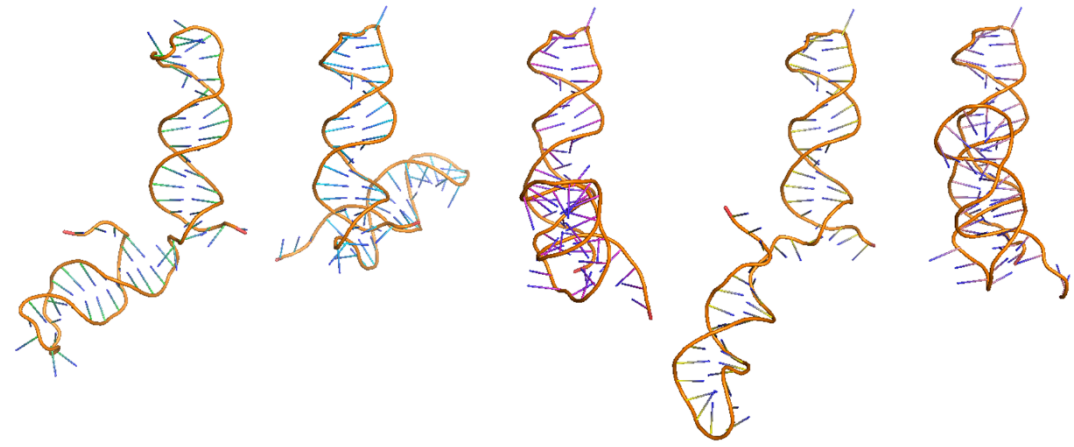

Figure S4 Prediction of the 3D structure of P34 aptamer (5 variants using software available at http://biophy.hust.edu.cn)

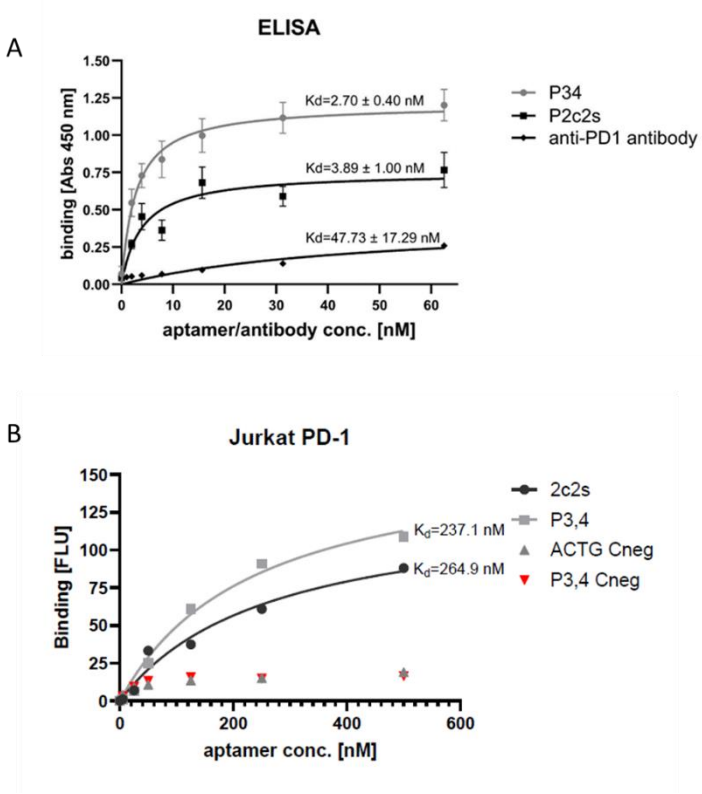

Figure S5 Affinity of P2c2s and P34 aptamers to PD1 receptor determined using: ELISA (rabbit anti-PD-1/CD279 Polyclonal Antibody was used as a positive control) (A) and flow cytometry (B).

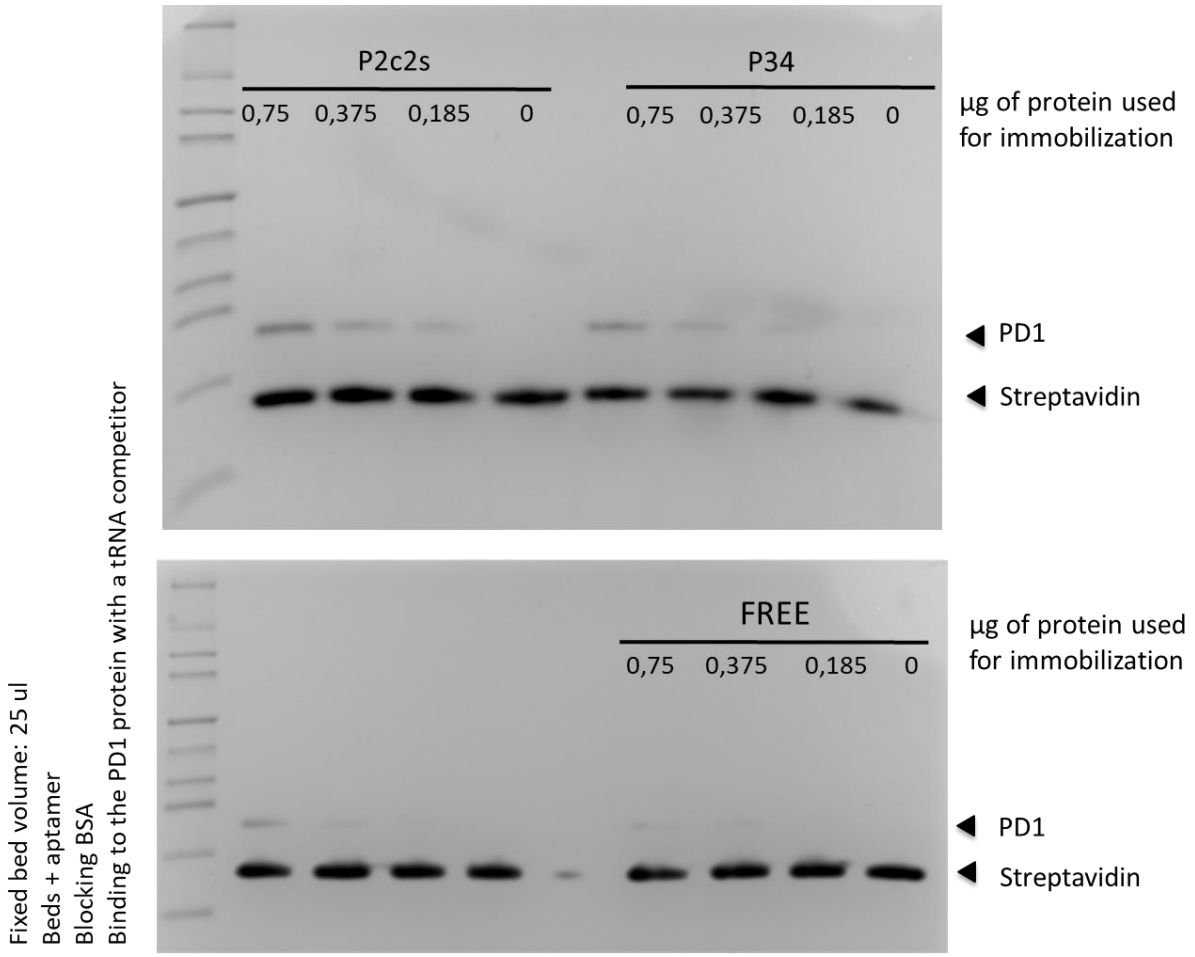

Figure S6 The original photographs of the gels in full size (presented in Figure 3C in the main manuscript).

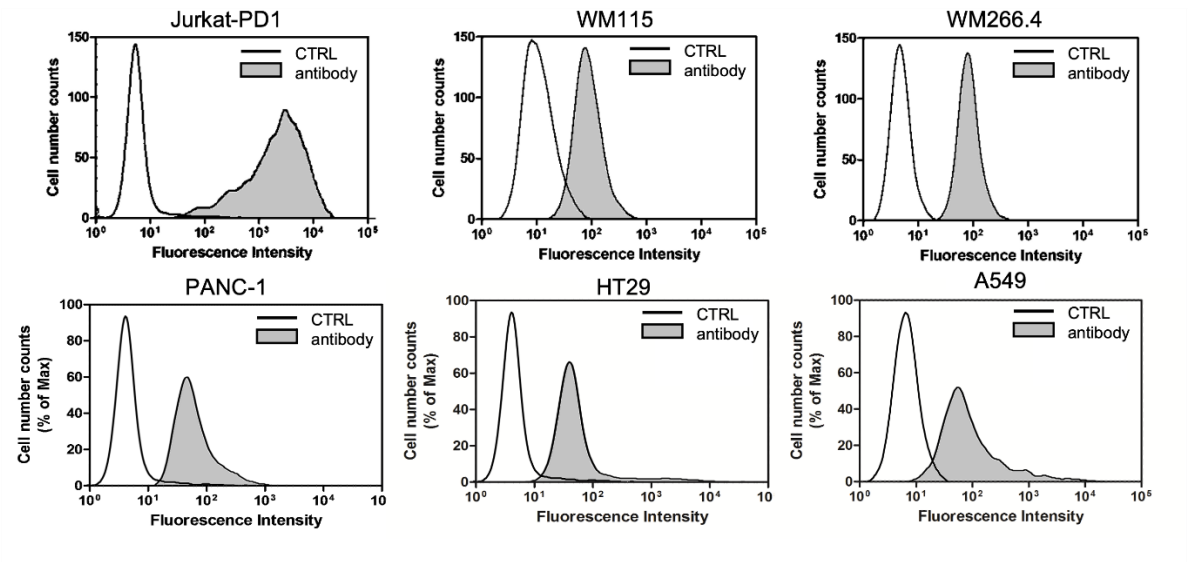

Figure S7 Detection of PD1 expression at the surface of representative cell lines by anti-PD1 antibody analyzed by flow cytometry. Jurkat T cells stably expressing human PD1, melanoma cells WM115 and WM266, PANC-1 (pancreatic cancer), HT29 (colon cancer) and A549 cells (lung cancer)

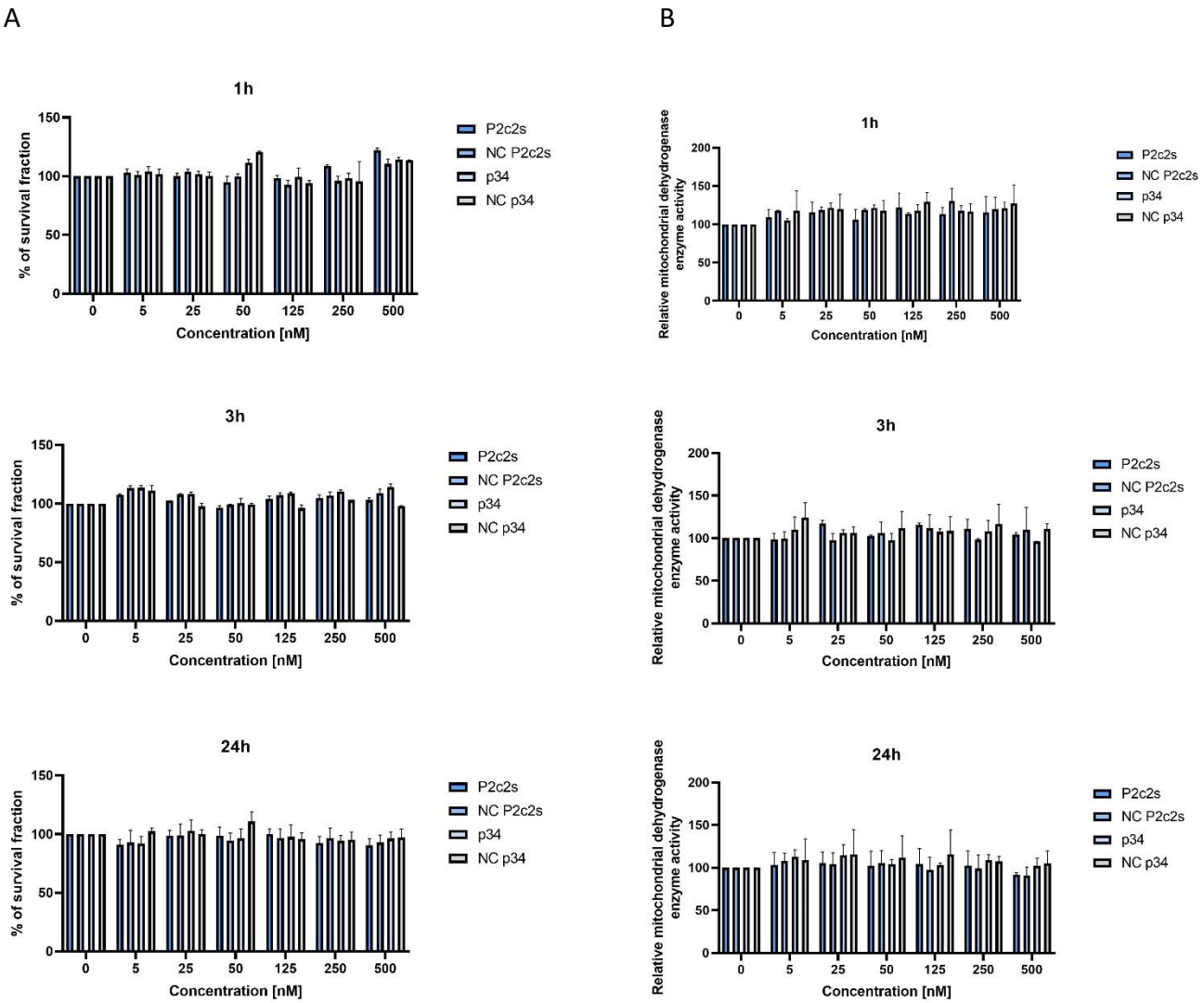

Figure S8 Analysis of aptamer toxicity (A) LDH (B) MTT tests.

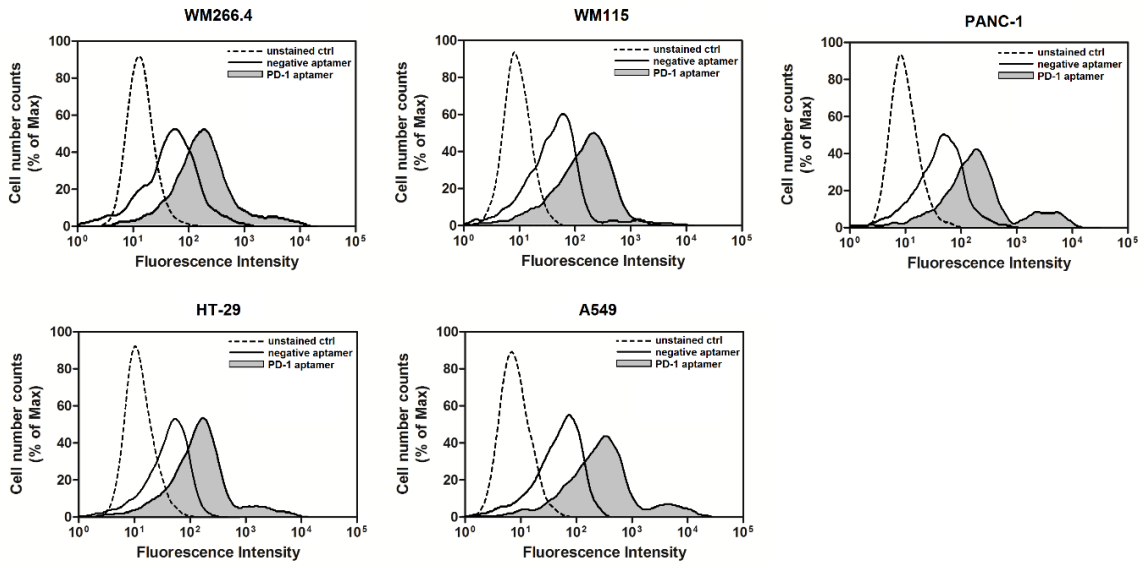

Figure S9. Detection of PD1 expression at the surface of representative cell lines characterized by high endogenous PD1 level as analyzed by flow cytometry. WM266 and WM115 cells (melanoma), PANC-1 (pancreatic cancer), HT29 (colon cancer) and A549 cells (lung cancer).