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Supplemental information

SeqStain is an efficient method for multiplexed, spatialomic profiling of human and murine tissues

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SUPPLEMENTARY FIGURES

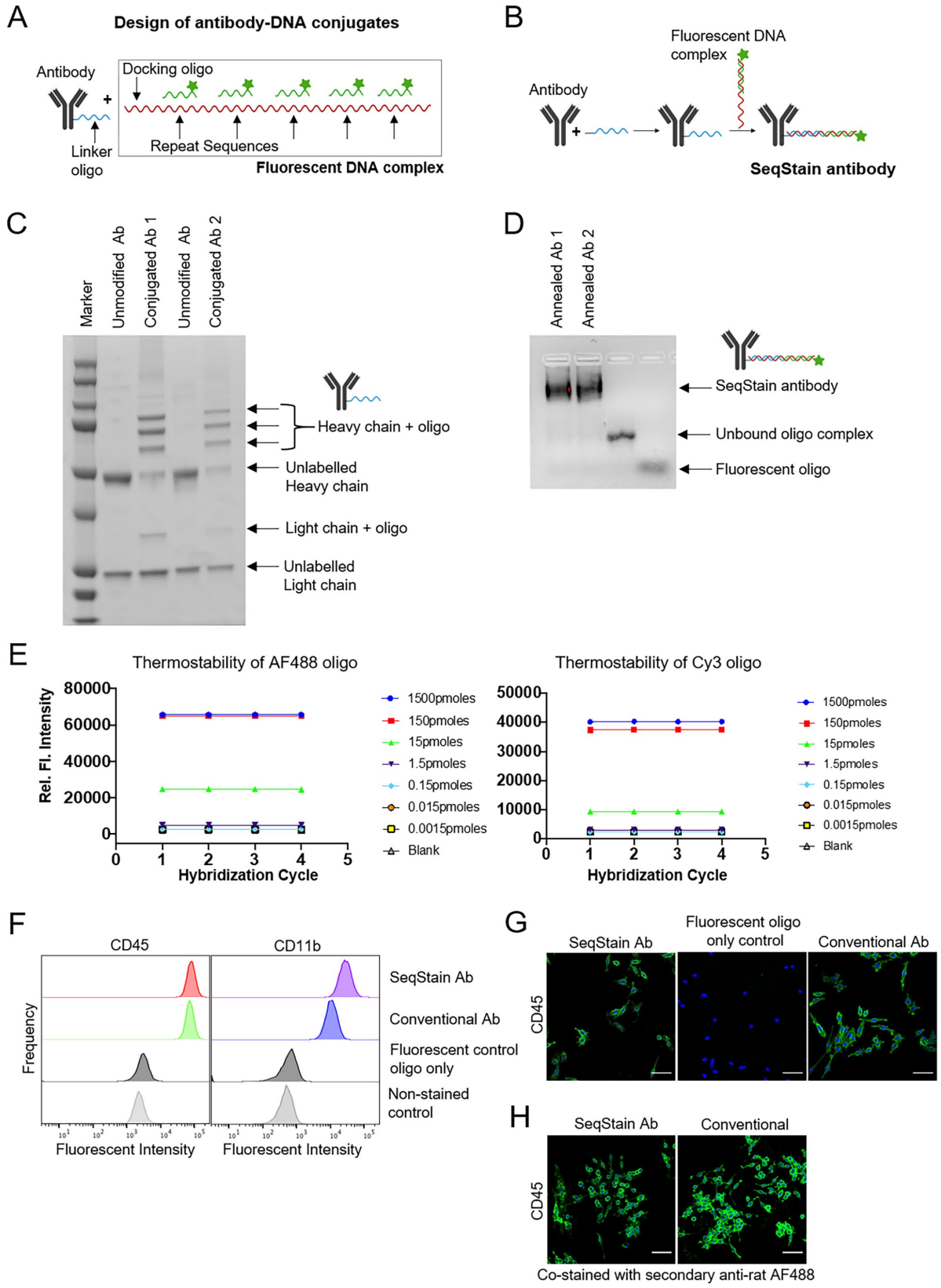

Figure S1. **Generation and staining with SeqStain antibodies, related to Figure 1. A**. Schematic showing the layout of the different oligos used for SeqStain modification. **B**. Schematic detailing the steps of antibody modification for SeqStain. In Step1, the antibodies are conjugated to the linker oligo using various conjugation chemistries (methods). In Step 2, the rest of the oligo complex containing the docking oligo and the fluorescent

oligo is annealed to the linker oligo that is conjugated to the antibody. **C**. Image of SDS-PAGE gel showing analysis of antibody conjugation to linker oligos (Step 1). Arrows show the bands representing the conjugated antibody heavy and light chains compared and the unmodified antibodies. The different bands correspond to the differences in the number of conjugated linker oligo per heavy chain. **D.** Image of agarose gel showing analysis of conjugated and annealed SeqStain antibodies. The annealed SeqStain-ready antibodies can be seen as a shifted band compared to the unbound oligo complex. Arrows show the bands representing the fluorescent DNA-hybridized antibodies and the unbound DNA complex. **D.** Graphs showing measurement of raw fluorescence intensities of fluorescent oligos with AF488 fluorochrome or Cy3 fluorochrome after repeated rounds of heating and cooling (Hybridization cycle) evaluated in a serial dilution series. **F.** Flow cytometric analysis of RAW264.7 cells stained with anti-CD45 and anti-CD11b SeqStain and conventional antibodies. Cells stained with fluorescent oligo alone and unstained cells were used as control. **G.** Representative immunofluorescence images of immobilized RAW 264.7 cells stained with CD45 SeqStain antibody or by the conventional immunostaining method. Cells stained with fluorescent oligo alone was used as a control. The antibodies were labelled using AF488 fluorophore (shown in green). Scale bar is 100µm. **H.** Representative immunofluorescent images showing RAW 264.7 cells stained antibody conjugated with linker oligo alone (without hybridization with fluorescent-DNA complex) (Left panel) or stained with the corresponding unmodified antibody (Right panel). The antibodies were co-stained with AF488 containing anti-rat secondary antibody. Scale bar is 100µm.

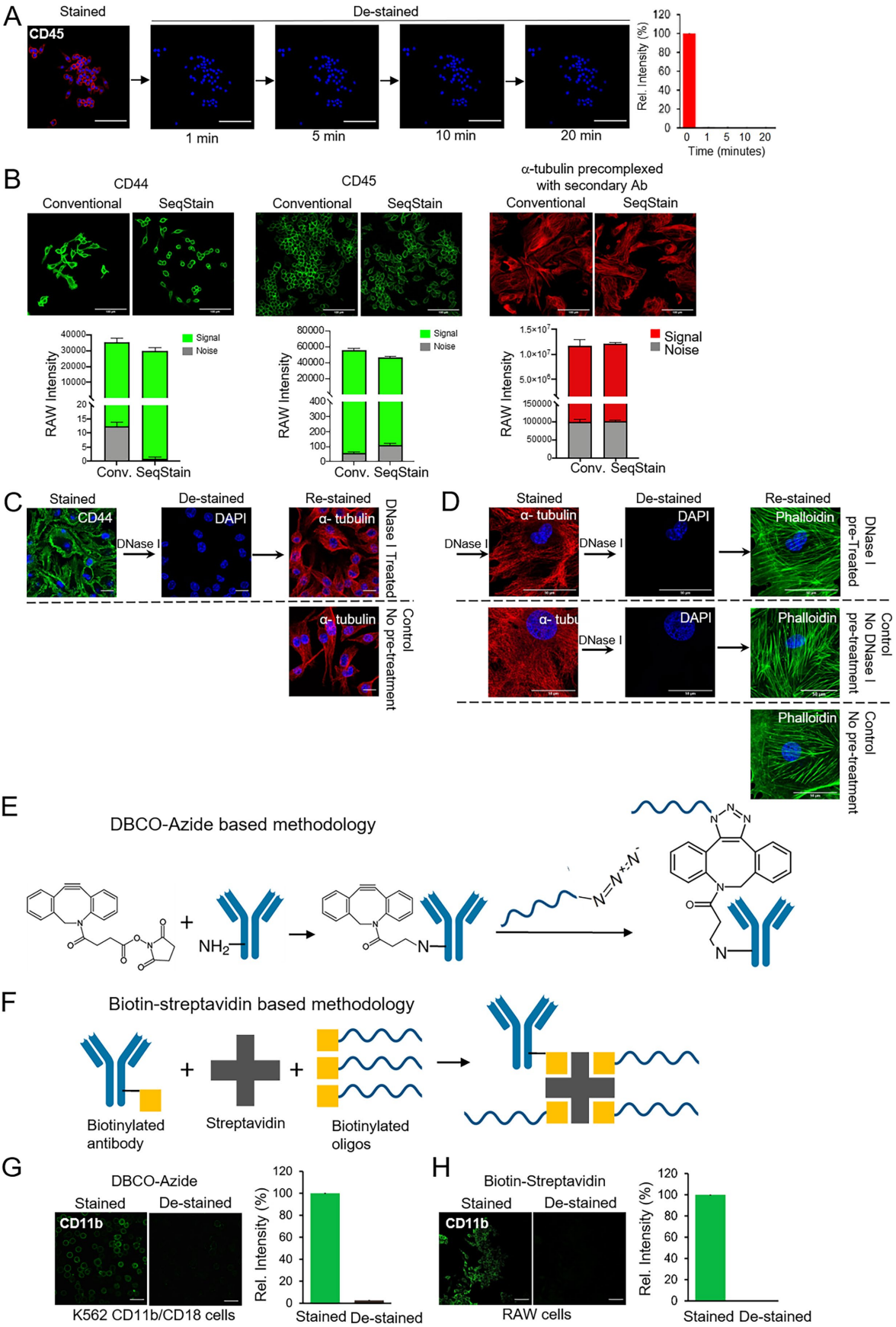

Figure S2. De-staining time course, signal to noise attributes and cell morphology post SeqStain, related to Figure 1. A. Immunofluorescence images showing RAW264.7 cells stained with anti-CD45 SeqStain

antibody, de-stained with DNase I and imaged at various time points. Images were acquired at different time points post DNase I addition as indicated in each panel. A bar graph showing quantification of fluorescence intensity after staining (red bar) and de-staining (brown bars) in each panel is also presented (bottom). Graphs show the mean ± standard deviation. Scale bar is 100μm. B. Immunofluorescence images of RAW cells stained with anti-CD44 (left panels), anti-CD45 SeqStain antibodies (middle panels) along with the conventional control. Immunofluorescence images of murine podocyte cell line stained with α-tubulin primary antibody pre-complexed with either conventional or SeqStain secondary antibodies (right panels). A bar graph showing quantification of raw signal intensities (Integrated Density) in regions with positive staining (signal) and regions in the absence of cell staining (noise) for each panel is presented below. Graphs show the mean ± standard deviation. **C.** Immunofluorescent images of RAW cells acquired at 60x magnification showing individual cells stained with anti-CD44 SeqStain antibody (left panel) and de-stained with DNase I (middle panel). The cells were subsequently stained with anti-α-tubulin primary antibody pre-complexed with SeqStain secondary antibody to visualize the microtubules of the cytoskeleton right panel. **D.** Immunofluorescent images of murine podocytes acquired at 60x magnification stained for α-tubulin with (Top left panel) and without (bottom left panel) DNase I pre-treatment using anti-α-tubulin primary antibody pre-complexed with SeqStain secondary antibody and de-stained using DNase I (middle panels). The cells were subsequently stained with phalloidin to visualize the actin filaments of the cytoskeleton (right panels). **E.-F.** Schematics showing preparation of SeqStain antibodies using (**E**) DBCO-Azide click chemistry or the (**F**) biotin-streptavidin chemistry to conjugate linker oligos to antibodies. **G.** Representative immunofluorescence images of K562 human myelogenous leukemia cells stably expressing CD11b and CD18 proteins and stained with anti-CD11b SeqStain antibody and de-stained with DNase I. The anti-CD11b antibody was conjugated to the linker oligo using DBCO-Azide click chemistry and subsequently hybridized to complementary fluorescent-DNA complex. The antibodies were labelled with AF488 fluorophore (shown in green). Scale bar is 100μm. A graph showing quantification of fluorescence intensity after staining (green bars) and de-staining (brown bars) in each panel is also presented. Graphs show the mean ± standard deviation. **H.** Representative immunofluorescence image of RAW264.7 cells stained with anti-CD11b SeqStain antibody and de-stained with DNase I. Scale bar is 100μm. The CD11b antibody was conjugated to the linker oligo using the biotin-streptavidin chemistry and subsequently hybridized to complementary fluorescent-DNA complex. The antibodies were labelled with AF488 fluorophore (shown in green). A graph showing quantification of fluorescence intensity after staining (green bars) and de-staining (brown bars) in each panel is also presented. Graphs show the mean ± standard deviation.

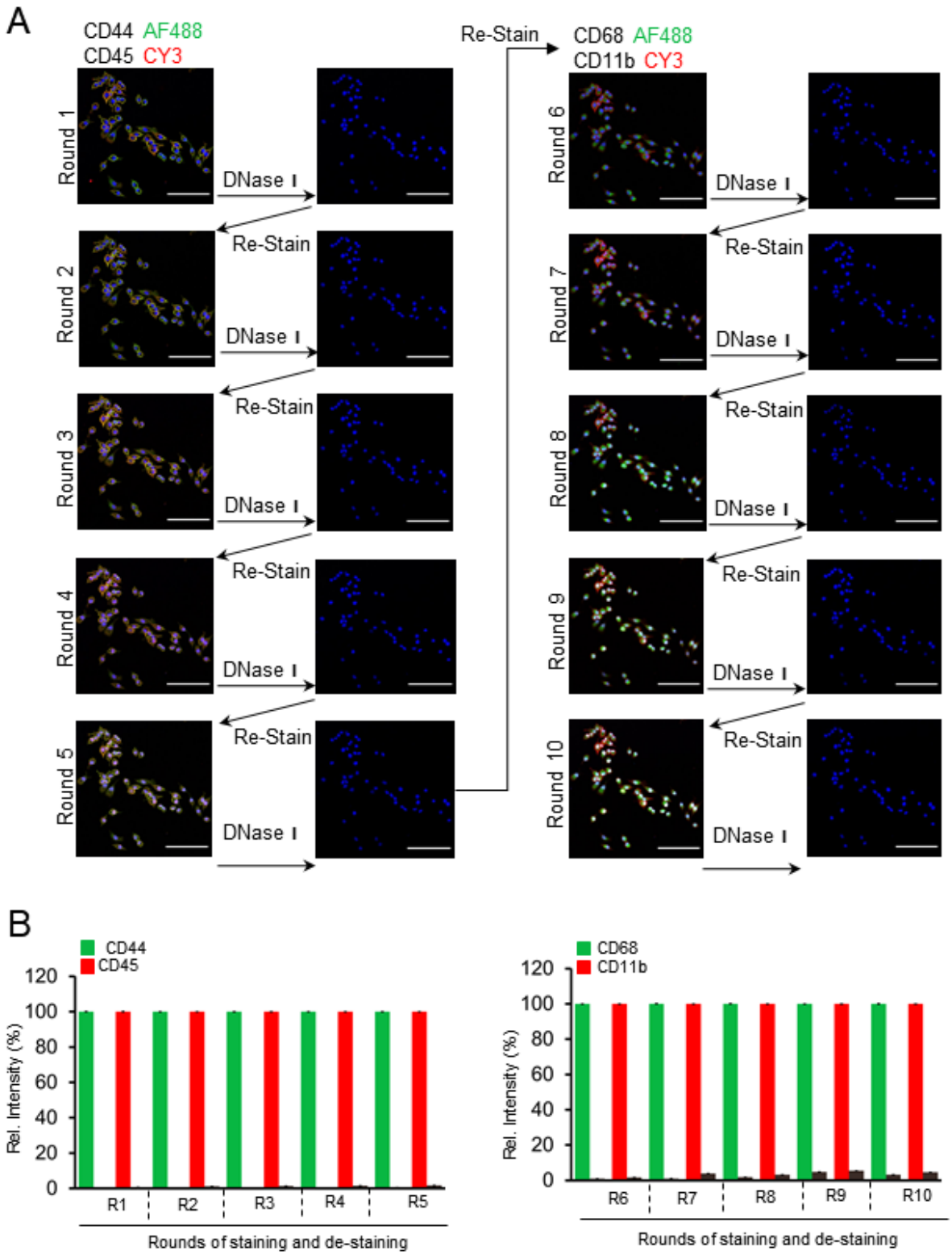

Figure S3. Repeat staining and de-staining of RAW264.7 cells with the same set of SeqStain antibodies, related to Figure 1. Immunofluorescence images of RAW264.7 cells repeatedly co-stained in the first five cycles with anti-CD44 SeqStain antibody bearing AF488 fluorophore and anti-CD45 SeqStain antibody bearing Cy3 fluorophore (left panel) and de-stained using DNase I (right panel). Subsequently, the cells were repeatedly stained with anti-CD68 SeqStain antibody bearing AF488 fluorophore and anti-CD11b SeqStain antibody bearing Cy3 fluorophore in the next five rounds. A graph showing quantification of fluorescence intensity after staining (green and red bars) and de-staining (brown bars) in each panel is also presented. Graphs show the mean ± standard deviation. Scale bar is 100μm.

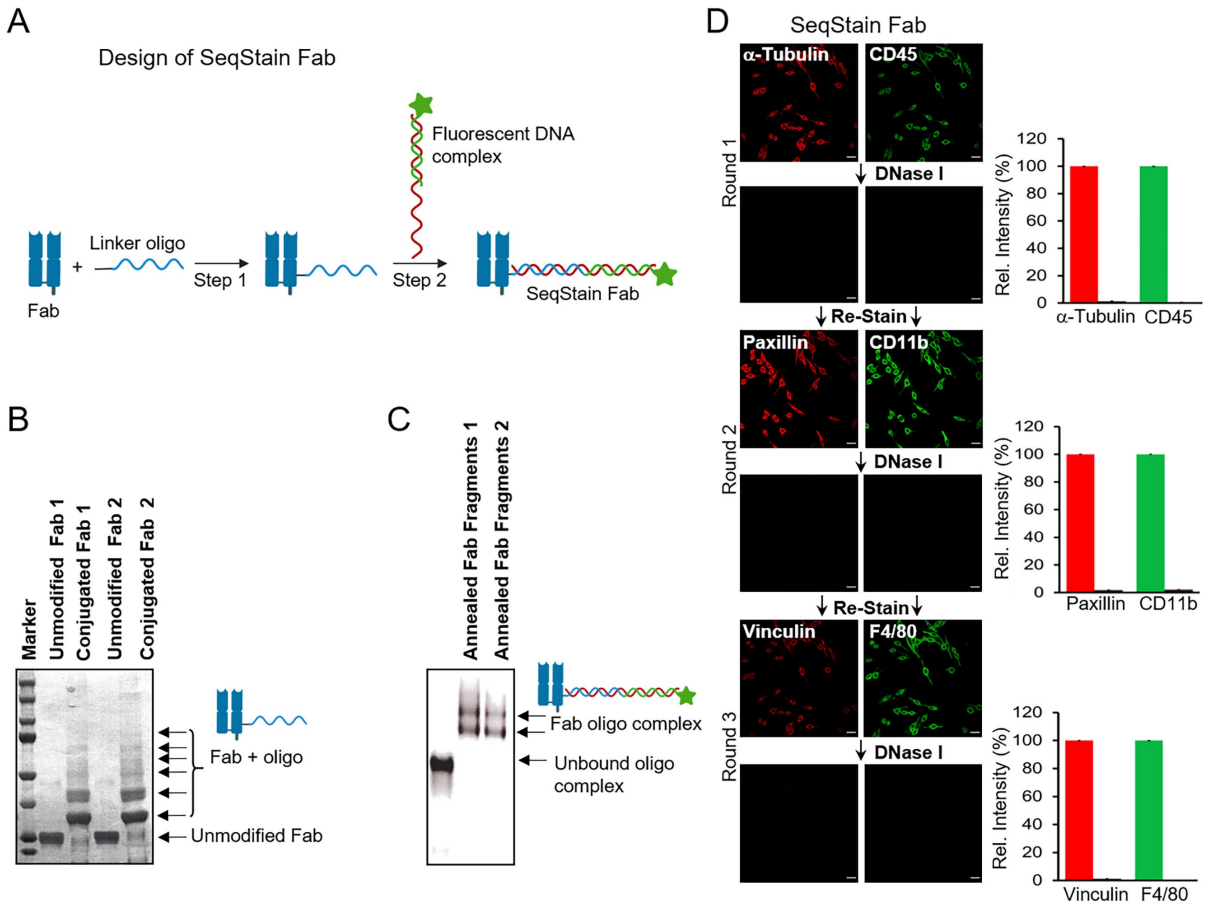

Figure S4. Generation of SeqStain Fabs, related to Figure 2. **A.** Schematic showing preparation of SeqStain Fabs. In step1, the Fab fragments are conjugated to the linker oligos. In step 2, the modified Fab is hybridized to fluorescent-DNA complex. **B**. Image of SDS-PAGE gel showing analysis of Fab conjugation to linker oligos (Step 1). The different bands correspond to the differences in the number of conjugated linker oligo per Fab. **C.** Image of agarose gel showing analysis of Fabs after the annealing step (Step 2). Arrows show the bands representing the fluorescent DNA-hybridized Fabs and the unbound DNA complex. **D.** Immunofluorescence images of RAW264.7 cells after each of the three rounds of staining with two unique antibodies pre-complexed with two different SeqStain Fabs (with Fabs labelled using the AF488 fluorophore shown in green and the Fabs labelled using the AF546 fluorophore shown in red) and after de-staining with DNase I. The primary antibodies used in each round are indicated in the panel. All images are representative of at least three replicates and different fields from each round are presented here to show representation. Scale bar is 100μm. A graph showing quantification of the fluorescence intensity after staining (green and red bars) and de-staining (brown bars) in each panel is also presented on the right. Graphs show mean ± standard deviation.

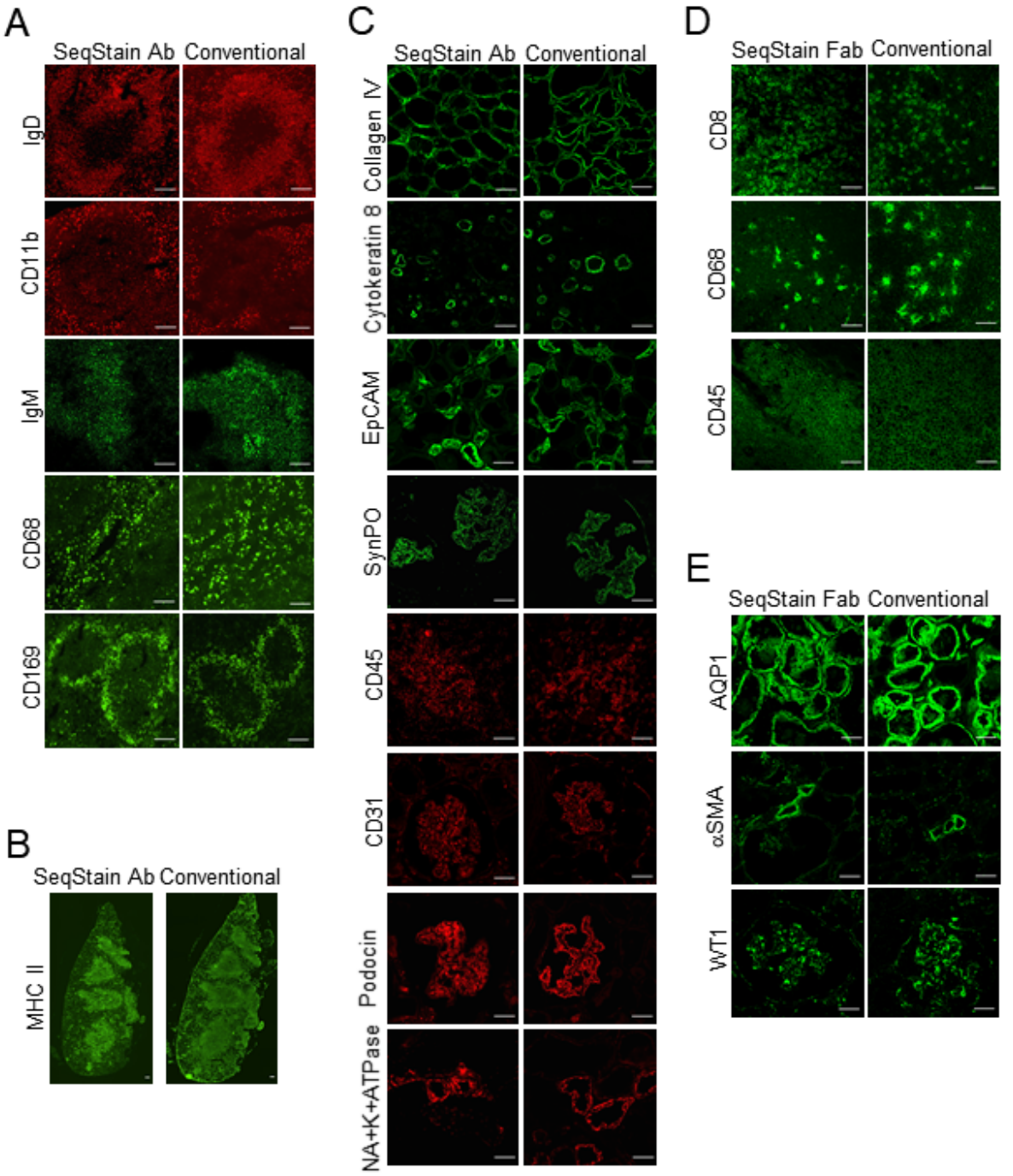

Figure S5. Comparison of SeqStain based staining with conventional immunofluorescence staining of various tissues, related to Figure 3. A. Immunofluorescence images showing mouse spleen tissue stained using SeqStain antibody based method (SeqStain Ab) or using conventional immunostaining method (conventional). The antibodies used are indicated in the panel, with antibodies labelled using the AF488 fluorophore shown in green and the antibodies labelled using the AF546 fluorophore shown in red. All images are representative of at least three replicates. Scale bar is 100µm. **B.** Immunofluorescence images showing whole slide scan of serial sections of mouse spleen tissue stained for MHC II using either the SeqStain antibody method (left panel) or the conventional method (right panel). Scale bar is 100µm. **C**. Immunofluorescence images showing human kidney tissue stained using SeqStain antibody based method (SeqStain Ab) or using conventional immunostaining method (conventional). The antibodies used are indicated in the panel, with antibodies labelled using the AF488 fluorophore shown in green and the antibodies labelled using the AF546 fluorophore and Cy3 shown in red. All images are representative of at least three replicates. Scale bar is 100µm. **D.** Immunofluorescence images showing mouse spleen tissue stained using SeqStain Fab based method

(SeqStain Fab) pre-complexed with a primary antibody or using conventional immunostaining method (conventional). The antibodies used are indicated in the panel and were stained using the AF488 fluorophore. All images are representative of at least three replicates. Scale bar is 100μm. **E.** Immunofluorescence images showing human kidney tissue stained using SeqStain Fab based method (SeqStain Fab) pre-complexed with a primary antibody or using conventional immunostaining method (conventional). The antibodies used are indicated in the panel and were stained using the AF488 fluorophore. All images are representative of at least two replicates. Scale bar is 100μm.

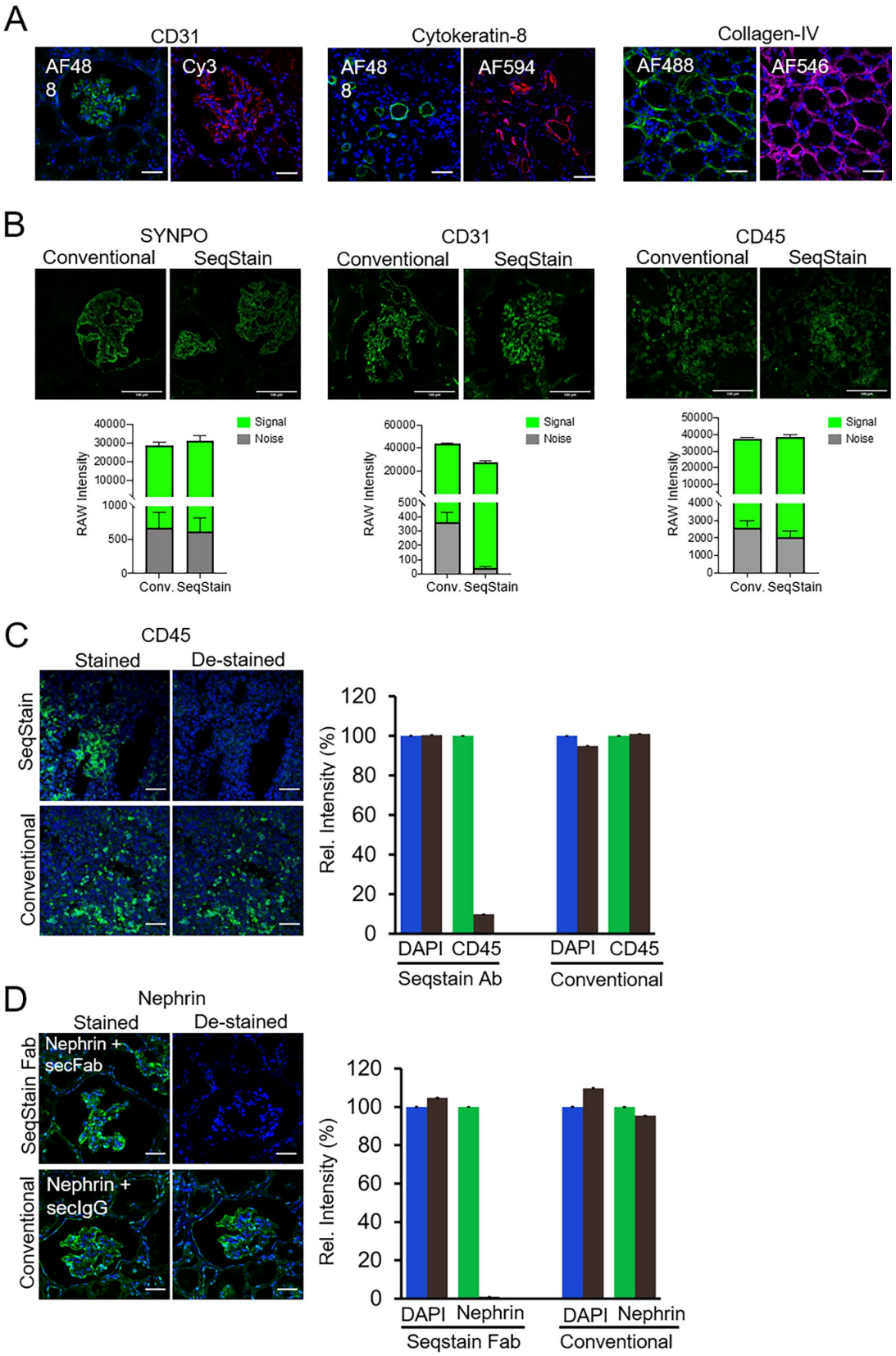

Figure S6. A. Staining tissues with SeqStain antibodies labelled with different fluorophores and signal to noise attribute of SeqStain, related to Figure 3. A. Representative immunofluorescence images of human kidney tissue sections stained with anti-CD31, anti-Cytokeratin-8 or anti-Collagen-IV SeqStain antibodies labelled with either AF488 fluorophore (Left panels) or a spectrally different fluorophore (as labelled, Right panels). All images are representative of at least three replicates. Scale bar is 100µm. **B**. Immunofluorescence

images of human kidney tissues stained with anti-synaptopdin (left panels), anti-CD31 (middle panels) or anti-CD45 (right panels) SeqStain antibodies along with the conventional control. A bar graph showing quantification of signal intensities (Integrated Density) in regions with positive staining (signal) and regions in the absence of tissue staining (noise) for each panel is presented below. Graphs show the mean ± standard deviation. **C**. Representative immunofluorescence images of murine tumor tissues (LLC tumors) after staining with DAPI (blue) and the anti-CD45 antibody (green) using either the SeqStain technique (top panel) or the conventional immunostaining methodology (bottom panel). Representative images post-destaining with DNase I are also presented (Right panels). The antibodies were labelled using the AF488 fluorophore. Scale bar is 100μm. A bar graph showing quantification of fluorescence intensity after staining (green and blue bars) and de-staining (brown bars) in each panel is also presented (right). Graph shows mean ± standard deviation. **D**. Representative immunofluorescence images of human kidney tissues after staining with DAPI (blue) and the anti-Nephrin antibody either pre-complexed with SeqStain Fab (top panel) or the conventional immunostaining method (bottom panel). Representative images acquired after DNase I treatment are also shown (right panels). Scale bar is 100μm. A graph showing quantification of fluorescence intensity after staining (green and blue bars) and de-staining (brown bars) in each panel is also presented. Graph shows the mean ± standard deviation.

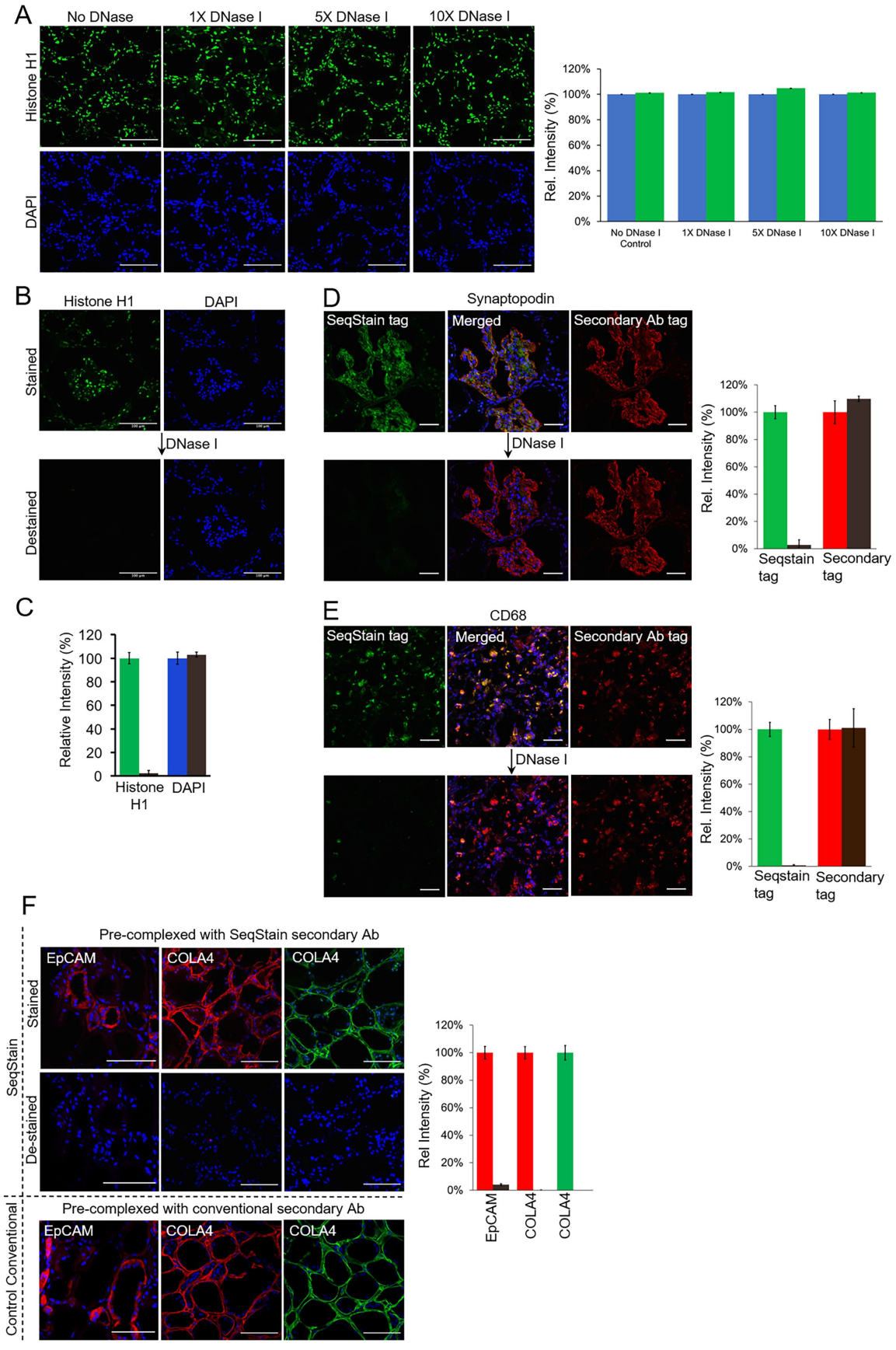

Figure S7. SeqStain de-staining step using DNase I treatment does not affect the nuclear DNA proteins or the DNA, related to Figure 3. A. Representative immunofluorescence images of human kidney sections treated

with DNase I either once (1X) or repeatedly (5X, 10X) and stained for a common DNA binding protein (Histone H1) by conventional immunofluorescence methodology. Top panels show Histone H1 immunofluorescence staining in each of the conditions and the bottom panels show DAPI staining. All images are representative of at least three replicates. Scale bar is 100μm. A bar graph showing quantification of the fluorescence intensity (green and blue bars) is also presented on the right. Graphs show mean ± standard deviation. **B.** Immunofluorescence images of human kidney tissue sections stained for the nuclear protein Histone H1 using anti-Histone H1 SeqStain antibody (Top left panel) and de-stained using DNase I (Bottom left panel). Panels on the right show DAPI staining before (Top right) and after de-staining (Bottom right). Scale bar is 100μm. **C.** A graph showing quantification of fluorescence intensity after staining (green and blue bars) and de-staining (brown bars) in each panel. Graph shows the mean ± standard deviation. **D.** Representative immunofluorescence images of human kidney tissue sections stained with anti-Synaptopodin SeqStain antibody (AF488, green) and counter-stained with corresponding secondary antibody (AF546, red). The individual channel images and the merged image are shown in individual panels (top). Representative images acquired after DNase I treatment are shown in the bottom panels. Scale bar is 100μm. A graph showing quantification of fluorescence intensity after staining (green and red bars) and de-staining (brown bars) in each panel is also presented. Graphs show the mean ± standard deviation. **E**. Representative immunofluorescence staining of human kidney tissue with anti-CD68 SeqStain antibody (AF488, green) and counter-stained with corresponding secondary antibody (AF546, red). The individual channel images and the merged image are shown in individual panels (top). Representative images acquired after DNase I treatment are shown in the bottom panels. Scale bar is 100μm. A graph showing quantification of fluorescence intensity after staining (green and red bars) and de-staining (brown bars) in each panel is also presented. Graphs show the mean ± standard deviation. **F.** Representative immunofluorescence images of human kidney tissue section stained with anti-EpCAM or anti-Collagen antibodies pre-complexed with SeqStain secondary antibodies (top panels) and de-stained using DNase I (bottom panels). Secondary antibodies bearing AF488 fluorophore are shown in green and with Cy3 fluorophore are shown in red. All images are representative of at least three replicates. Scale bar is 100μm. A graph showing quantification of the fluorescence intensity after staining (green and red bars) and de-staining (brown bars) in each panel is also presented on the right. Graphs show mean ± standard deviation.

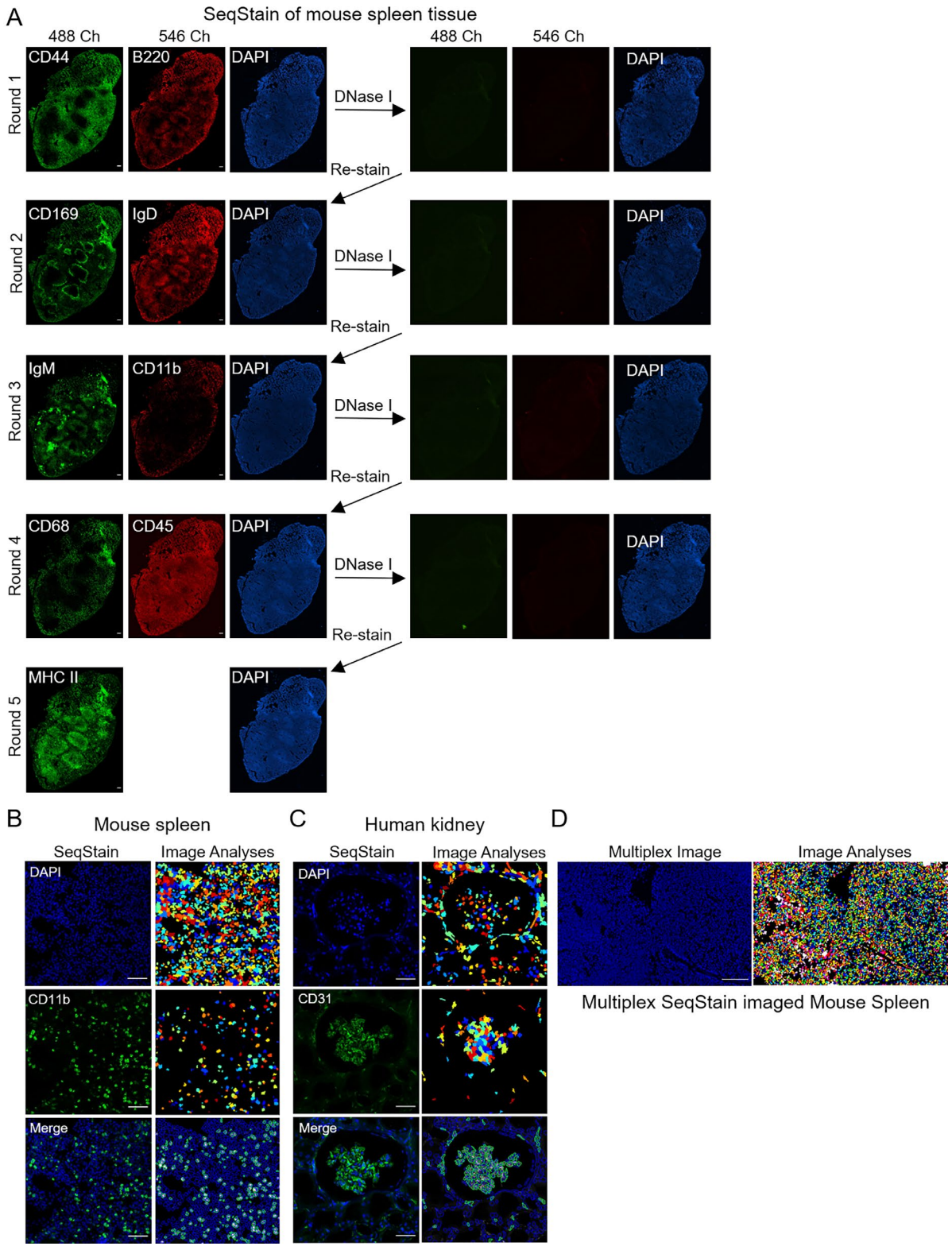

Figure S8. A. SeqStain based multiplexed staining and de-staining of murine spleen tissue, related to Figure 5. Whole slide images of murine spleen tissue acquired after each of the five cycles of staining with unique SeqStain antibodies and de-staining with DNase I. The antibodies used in each round are indicated in the panel, with SeqStain antibodies labelled using the AF488 fluorophore shown in green and the antibodies labelled using the AF546 fluorophore shown in red. Scale bar is 100µm. **B**. Immunofluorescent images (Left panels) and the corresponding Cell Profiler analysed images (Right panels) of mouse spleen tissue stained with CD11b SeqStain antibody. DAPI stained nuclei were demarcated and identified as primary objects (Top panels) while cells stained with CD11b was demarcated and identified as secondary objects (middle panel). The primary

and secondary objects were linked and quantified using Cell Profiler (Bottom panel). **C.** Immunofluorescent images (Left panels) and the corresponding Cell Profiler analysed images (Right panels) of human kidney tissue stained with CD31 SeqStain antibody. DAPI stained nuclei were demarcated and identified as primary objects (Top panels) while cells stained with CD31 was demarcated and identified as secondary objects (middle panel). The primary and secondary objects were linked and quantified using Cell Profiler (Bottom panel). **D**. Immunofluorescent image of DAPI stained nuclei from a representative region in the multiplex stained spleen tissue (Left panel) and the corresponding StarDist generated segmented nuclei (Right panel).

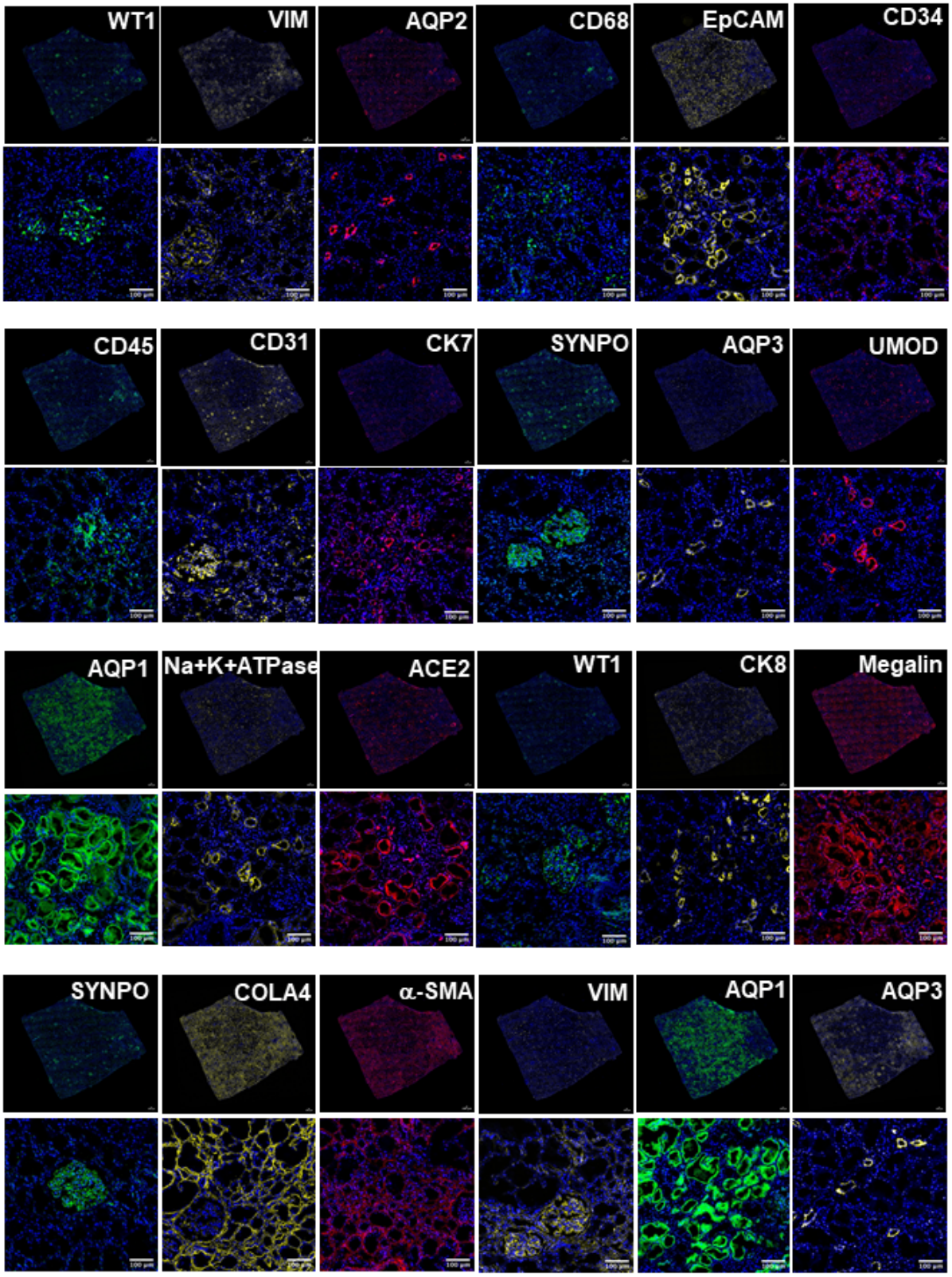

Figure S9. SeqStain-based 25-plex staining and imaging of whole human kidney tissue, related to Figure 6. Immunofluorescence images of whole kidney tissue sections stained with SeqStain antibodies and DAPI (as indicated in the panel) in 25-plex experiment. Antibody used in each staining step is labelled on the panel. Zoomed-in sections from each of the immunofluorescence images are presented below each panel. Images are representative of at least two replicates. Scale bar is 100μm.

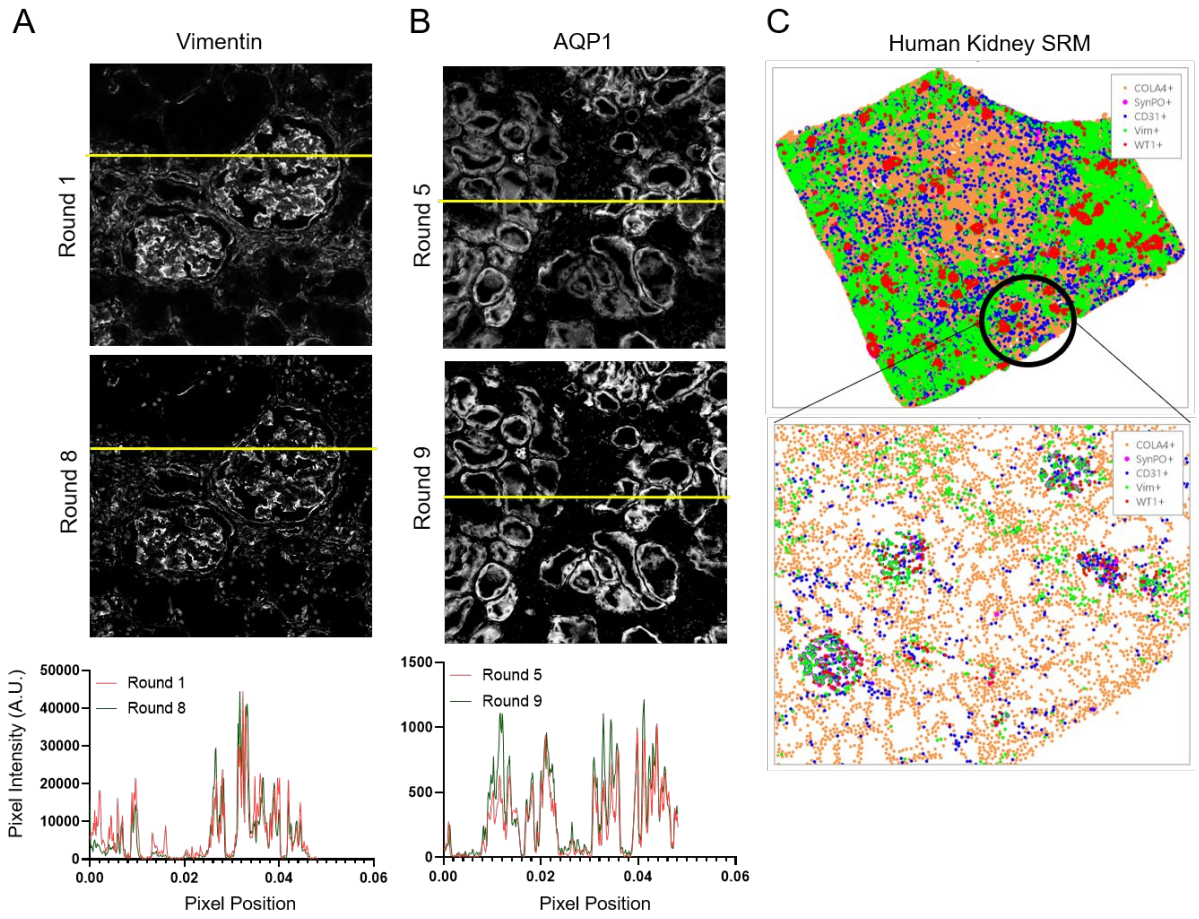

Figure S10. Signal integrity of the staining obtained with SeqStain antibodies, related to Figure 7. **A.-B**. The fluorescence intensity profile was compared for two of the markers (Vimentin and AQP1) that were repeated during the multiplex staining of human kidney. The intensity profile was measured by ImageJ plot profiler around the indicated yellow line. The cycle number is as indicated in the panel. **C.** Spatial Relationship Map generated by HALO spatial analysis of the indicated markers in the whole kidney tissue (Top panel). Zoomed-in region (bottom panel) shows individual cell types identified by HALO and their relative location in the kidney.