SUPPLEMENTAL TABLE LEGENDS

Supplemental Table 1: Primary antibodies

Supplemental Table 2: Secondary antibodies

Supplemental Table 3: Staining variability

The mean channel value ± SD is shown for the number of replicas in the column “n”. Data are expressed also as % variation over 256 channels. The wavelength in which the variation is measured is also shown

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Fig. 1

A- Effect of incubation time on staining

The variation in intensity, measured as channel variation, after one hour (100%) and overnight (O/N) incubation is plotted.

B- Effect of double indirect vs single indirect staining of intensity.

The variation in intensity, measured as channel variation, after one (100%) or two rounds of indirect immunofluorescent staining are plotted.

C- Effect of mounting media on antigenicity.

Buffered 60% Glycerol as mounting medium has minimal or no effect on antigen preservation, compared to a polyvinyl alcohol or a Glycerol-Gelatin one. Antigen-retrieved sections were mounted with the media shown, immunostained and quantified. Values are % channel variations over the baseline (100%).

Supplemental Fig. 2

A- Tissue autofluorescence in four tissues.

Tissue autofluorescence was obtained by multiple exposure time in four tissues (placenta, kidney, skin and squamous epithelium) in each of four excitation/emission combinations. Mean fluorescence intensity (Y) is plotted against exposure time (milliseconds, X). Note the linear response.

B- A portion of a kidney biopsy stained for cytokeratin 8 was acquired with the AF (top) and with the FITC (middle, KER8+AF) specific filter sets. The AF image, corrected for the exposure factor, was subtracted from the cytokeratin image. The result is shown at the bottom, showing exclusively the specific staining (KER8). Note the absence of nuclear DAPI staining in the top image. Scale bar = 500 µm.

C and D- Pixel-by-pixel comparison of AF values in placenta and kidney.

Comparison of the intensity values pixel by pixel are shown for the AF channel (420exc/530em) versus the FITC and the TRITC channels in placenta (left) and kidney (right). FITC vs TRITC comparison is also shown. Note in kidney the spread of the values for AF vs TRITC and for FITC vs TRITC.

E- Changes in autofluorescence before AR, after AR and after stripping (5 cycles).

Autofluorescence images were obtained from placenta sections, illuminating with the 480 ± 17nm filter and collecting with the triband dichroic and the 520 ±28nm filter. WSI were acquired before AR, after AR and after five cycles-equivalent (2,5 hr) of 2ME/SDS stripping. The WSI were all registered and a 30,000 pixel selection placed in an excel file. Bivariate plot for PreAR-PostAR (red dots) and PostAR-PostStrip (black circles) are shown. Note the multiple pixel

populations with divergent AF before and after AR, while before and after stripping the vast majority of pixels line up along the middle of the bivariate graph.

F- Pixel-by-pixel comparison of AF values over 10 stripping cycles.
Comparison of AF values of kidney tubules (left) and an immune infiltrate (right) at time 0 (abscissa) and after 10 cycles (ordinate) shows an overall reduced AF values in the immune cells and a greater variation of the pre- and post-values in kidney tubules.

Supplementary Fig. 3
A- Channel intensity for six markers and six negative controls over ten staining and stripping 2ME/SDS.
One single section for every three markers was stained (t0), sequentially stripped with the 2ME/SDS method and re-stained alternatively with a negative control antibody or for the same markers 10 times. Staining intensity variation is expressed as a box plot. Note the separation of the negative controls from the positive stains. CD3 and Pax5 in FITC, bcl-2 and CD79a in TRITC, CD20 and CD44 in Cy5. Primary Ab incubation time: 1 hr, 2nd Ab: 30 min, single indirect IF.

B- Channel variations for individual antibody pools over stripping.
Percent variations from the t0 channel position over 256 channels for each three-antibody pool at each stripping cycle with the buffers listed on top of each column.

C- Variation of the positive pixel area for ten stripping cycles.
The positive pixel area, expressed as percentage of the area analyzed, over ten staining and stripping cycles is depicted for GnHCl (blue boxes), 2ME/SD (red boxes) and DAB-stained serial

sections (black boxes). Note the overlap of the results over the three methods for most, but not all antigens.

Supplemental Fig. 4

Variability for nine markers over ten staining and stripping GnHCl, 6Murea, no AR cycles.
One single section for every three markers was stained (t0) and sequentially stripped and re-stained for the same markers 10 times. Variation in staining intensity is expressed as fraction of the 256 8bit channels over the initial staining intensity. Primary Ab incubation time: 1 hr, 2nd Ab: 30 min, single indirect IF. Note the decrease below the baseline value for all the markers tested.

Supplemental Fig. 5

A, B- Effect of 26mM $NaBH_4$ in EtOH 95% and Tris Buffer 0.05M pH 9.
$NaBH_4$ causes changes in AF when applied at 1 mg/ml (26mM) in 95% EtOH (A) or in buffer pH9 (B). The reduction at 30 min in buffer is a $NaBH_4$-specific degradation of the section. Changes in AF in three filter combos are expressed as % over 256 channels.

C- Effect of 15mM $NaBH_4$ in EtOH 95% and Tris Buffer 0.05M pH 9 on AF.
A $NaBH_4$ specific effect is seen in EtOH at 10 min and disappears at 30 min. The reduction at 30 min in buffer is a $NaBH_4$-specific degradation of the section.
$NaBH_4$ is applied at 15mM in 95% EtOH or in buffer pH9. An equivalent amount of NaOH, the diluent, is applied as a comparison. The reduction at 30 min in buffer is a $NaBH_4$-specific degradation of the section. Changes in AF in three filter combos are expressed as % over 256 channels.

Suppl.Fig 6

Residual anti-intermediate filaments (keratins 8 and 19, Vimentin) primary antibodies after GnHCl and 2ME/SDS stripping

Selected high-power fields are shown from a kidney section stained for rabbit anti Keratin 8, mouse IgG2a anti Keratin 19 and mouse IgG1 anti Vimentin, before (top, control) and after stripping. Note that the control images are unmodified, acquired with the exposure time selected. The stripped images instead, taken with the same setting, have been modified with the automatic increase in brightness and contrast of the ImageJ software (Maximum value <20, Brightness ~ -10)

SUPPLEMENTAL TABLE 1

Name	**clone**	Isotype	**cat n.**	Source	**Concentr**	**Dilution**
BCL-2	Bcl-2-100	IgG1	B3170-.2ML	Sigma Aldrich	5,1 mg/ml	1 µg/ml
BCL6	BCL6 (N3)	Rb Ab	sc-858	SCBT	200 µg/ml	1 µg/ml
BCL6	LN22	IgG2b	MS-1936-S0	Thermo	supn	1:100
Blimp-1	6D3	rat IgG2a	sc-47732	SCBT	1 mg/ml	1 µg/ml
C-MAF	M-153	Rb Ab	sc-7866	SCBT	200 µg/ml	1 µg/ml
CD10	NCL-CD10-L-270	IgG1		Leica-Microystem/ Novocastra	30 µg/ml	1 µg/ml
CD123	NCL-L-CD123	IgG2b		Leica-Microystem/ Novocastra	90 µg/ml	1:100
CD138	MI15	IgG1	MA5-12400	Thermo	supn	1:100
CD14	HPA002127	Rb Ab	HPA002127	Sigma Aldrich	400 µg/ml	1 µg/ml
CD141 / Thrombomodulin	EPR4051	Rb Mab	2927-1 ab109189	Abcam/Epitomics	unknown	1:200
CD141 / Thrombomodulin	D-3	IgG2a	sc-13164	SCBT	200 µg/ml	1 µg/ml
CD16	2H7	IgG2a	NCL-CD16	Leica-Microystem/ Novocastra	unknown	1:100
CD163 (Macrophages, M2)	10D6	IgG1	MA5-11458	Thermo	100 µg/ml	1:100
CD1a	EP3622	Rb Mab	ab108309	Abcam/Epitomics	supn	1:200
CD1c	UMAB46	IgG1	UM500042	Origene	2,48mg/ml	1 µg/ml
CD20	L26	IgG2a	sc-58985	SCBT	200 µg/ml	1 µg/ml
CD21	1F8	IgG1	MA1-27120	Thermo	100 µg/ml	1 µg/ml
CD27/ TNFRSF7	EPR8569	Rb Mab	ab131254	Abcam/Epitomics	1450 µg/ml	1 µg/ml
CD271 (NGF-Rp75)	EP1039Y	Rb Mab	1812-1	Abcam/Epitomics	unknown	1:2000
CD271 (NGF-Rp75)	NGFR 5	IgG1	MS-394-Px	Neomarkers	200 µg/ml	1 µg/ml
CD30	CON6D/ C2	IgG2a	MA5-12632	Thermo	200 µg/ml	1 µg/ml
CD34	EP373Y	Rb Mab	2150-1	Abcam/Epitomics	unknown	1:200
CD3e		Rb Ab	C7930-.2ML	Sigma Aldrich	unknown	1:500
CD4	EPR6855	Rb Mab	ab133616	Abcam/Epitomics	140 µg/ml	1:200
CD43	DF-T1	IgG1	sc-6256	SCBT	200 µg/ml	1 µg/ml
CD44	156-3C11	IgG2a	MS-668-Px	Neomarkers	200	1:500
CD44	poly	Rb Ab	HPA005785	Sigma Aldrich	190 µg/ml	1 µg/ml
CD45	EP322Y	Rb Mab	1691-1	Abcam/Epitomics	unknown	1:50
CD45	Bra-55	IgG1	MS-240 - P0	Thermo	200 µg/ml	1 µg/ml
CD45RB	PD7/26 CD45RB Ab-3	IgG1	MS-1846-P0	Thermo	200 µg/ml	1 µg/ml
CD56 (N-CAM)	123C3.D5	IgG1	sc-7326	SCBT	200 µg/ml	1 µg/ml
CD68	KP1	IgG1	sc-20060	SCBT	200 µg/ml	1 µg/ml
CD69		Rb Ab	HPA050525	Sigma Aldrich	50 µg/ml	1:100
CD7	CBC.37	IgG2b	M7255	Dako	47 µg/ml	1 µg/ml
CD7	SP94	Rb Mab	SAB5500071-100UL	Sigma Aldrich	unknown	1:100

CD79a	JCB117	IgG1	sc-53209	SCBT	200 µg/ml	1 µg/ml
CD79a	HM47	IgG1	SAB4700229-100UG	Sigma Aldrich	1mg/ml	1 µg/ml
CD8	F5	IgG2a	sc-25277	SCBT	200 µg/ml	1 µg/ml
CD8	C8/144B	IgG1	sc-53212	SCBT	200 µg/ml	1 µg/ml
Cleaved Caspase 3	Cleaved Caspase-3 (Asp175)	Rb Ab	#9661	CST	unknown	1:200
Cleaved Caspase 8	Cleaved Caspase-8 (Asp391) (18C8)	Rb Mab	#9496	CST	unknown	1:200
Cleaved PARP	Cleaved PARP (Asp214) (D64E10) XP® Rabbit mAb	Rb Mab	#5625	CST	unknown	1:200
CLEC10A / CD301		Rb Ab	HPA021937	Sigma Aldrich	200 µg/ml	1 µg/ml
CLEC9A / CD370	14N8D7	IgG1	MA5-16262	Thermo	500 µg/ml	1 µg/ml
CTNNB1	14	IgG1	610154	BD Pharmingen	250 µg/ml	1:300
CXCL13	53610	IgG1	MAB801-100	R&D	500 µg/ml	1:100
Cytokeratin Abs	Cytokeratin 8 (1E8)	IgG2a	sc-58736	SCBT	200 µg/ml	1 µg/ml
Cytokeratin Abs	Cytokeratin 19 (A53-B/A2)	IgG2a	sc-6278	SCBT	200 µg/ml	1 µg/ml
Cytokeratin Abs	Cytokeratin 19	Rb Ab	HPA002465-100UL	Sigma Aldrich	200 µg/ml	1 µg/ml
Cytokeratin Abs	Cytokeratin 8	Rb Ab	HPA049866-100µl	Sigma Aldrich	50 µg/ml	1 µg/ml
E-cadherin	4A2C7	IgG1		BD Pharmingen	250 µg/ml	1 µg/ml
E2-2/TCF4	6H5-3	IgG2a	T5817	Sigma Aldrich	1mg/ml	1 µg/ml
E2A/E47/TCF3	N-649	Rb Ab	sc-763	SCBT	200 µg/ml	1 µg/ml
Estrogen Receptor Alpha	1D5	IgG1	sc-56833	Dako	166 µg/ml	1:100
Estrogen Receptor Alpha		Rb Ab	HPA000449	Sigma Aldrich	80 µg/ml	1 µg/ml
Foxp3	FOXP3 antibody [236A/E7]	IgG1	ab20034	Abcam/Epitomics	1000 µg/ml	1 µg/ml
gH2AX	rb Mab	Rb Mab	#9718	CST	ND	1:200
gH2AX	JBW301 IgG1	IgG1	05-636	Millipore	1000 µg/ml	1 µg/ml
Granzyme B	GRB7	IgG2a	sc-73620	SCBT	supn	1:500
HLA-A	EMR8-5	IgG1	ab70328	Abcam/Epitomics	1000 µg/ml	1 µg/ml
HLA-DR	SPM289	IgG2b		Abcam/Epitomics	200 µg/ml	1 µg/ml
ID1	BCH-1/195-14	Rb Mab	50 µg (BCH-1/195-14-50)	Biocheck	1000 µg/ml	1 µg/ml
ID2	BCH-3/9-2-8	Rb Mab	50 µg (BCH-3/9-2-8-50)	Biocheck	500 µg/ml	1 µg/ml
ID3	BCH-4/17-3	Rb Mab	50 µg (BCH-4/17-3-50)	Biocheck	1000 µg/ml	1 µg/ml
IRF4	EP5699	Rb Mab	5391-1 ab133590	Abcam/Epitomics	unknown	1:500
IRF4	IRF-4 (M-17)	goat	sc-6059	SCBT	100 µg/ml	1 µg/ml
IRF4		Rb Ab	HPA002038-100UL	Sigma Aldrich	100 µg/ml	1 µg/ml
IRF8	E-9	IgG2b	sc-365042	SCBT	200 µg/ml	1 µg/ml
IRF8		Rb Ab	HPA002531	Sigma Aldrich	100 µg/ml	1:200
ITF	H-425	IgG1	sc-81954	SCBT	100 µg/ml	1:500

Ki-67	SP6	Rb Mab	RM-9106 - S0/S1/S	Neomarkers	unknown	1:100
Ki-67	UMAB107	IgG2a	UMAB107	OriGene	1mg/ml	2 µg/ml
Ki-67	8D5	IgG1	SAB5300425	Sigma Aldrich	unknown	1:500
Lamin B1	CL3929	IgG1	AMAB91251 Sigma	Sigma Aldrich	1000 µg/ml	1 µg/ml
Langerin / CD207		Rb Ab	HPA011216	Sigma Aldrich	100 µg/ml	1 µg/ml
MCM5	E-10	IgG2b	sc-165994	SCBT	200 µg/ml	1 µg/ml
MYC	Y69	Rb Mab	1472-1	Abcam/Epitomics	unknown	1:200
MYC	c-Myc (N-262)	Rb Ab	sc-764	SCBT	200 µg/ml	1 µg/ml
p16 CDKN2A	F-12	IgG2a	sc-1661	SCBT	200 µg/ml	1 µg/ml
p16 CDKN2A	JC8	IgG2a	sc-56330	SCBT	200 µg/ml	1 µg/ml
p21	EA10	IgG1	OP64-20UG	Merck	100 µg/ml	1 µg/ml
p27 CDKN1B	Ab-1(Clone DCS-72.F6)	IgG1	MS-256-P0, 1, -	Neomarkers	200 µg/ml	1 µg/ml
p27 CDKN1B	Rb poly C-term	Rb Ab	RB-9019-Px	Neomarkers	200 µg/ml	1 µg/ml
p57, Kip2	C-20	Rb Ab	sc-1040	SCBT	200 µg/ml	1:200
Pax5	DAK-Pax5	IgG1	M7307	DAKO	157 µg/ml	1 µg/ml
Pax5	1EW	IgG1	NCL-L-Pax5	Leica-Microystem/ Novocastra	supn	1:20
Pax5	A-11	IgG2a	sc-13146	SCBT	100 µg/ml	1 µg/ml
Pax5	1H9	rat IgG2a	05-1573 EMD Millipore	Sigma Aldrich	100 µg/ml	1 µg/ml
Pax5	SP34	Rb Mab	MA5-16389	Thermo	unknown	1:50
PD-1 / CD279	UMAB197	IgG2a	UMAB197	Origene	1000 µg/ml	1 µg/ml
PD-L1(B7-H1, CD274)	Anti-PD-L1 antibody [28-8]	Rb Mab	ab205921	Abcam/Epitomics	1192 µg/ml	1 µg/ml
Progesterone Receptor	636	IgG1		Dako	54 µg/ml	1 µg/ml
S-100 alpha chain	4C4.9 - biotin conjugate	IgG2a	MA5-12966	Thermo	200 µg/ml	1 µg/ml
S-100 β chain	N-15	goat	sc-7852	SCBT	200 µg/ml	1 µg/ml
Serpin B9 / PI9	PI-9-17	IgG1	sc-57531	SCBT	supn	1:200
TCR beta	G-11	IgG1	sc-5277	SCBT	200 µg/ml	1 µg/ml
TCR delta	H-41	IgG1	sc-100289	SCBT	100 µg/ml	1 µg/ml
TP53	DO-7	IgG2b		Dako	137 µg/ml	1 µg/ml
Vimentin	V9	IgG1		Dako	156 µg/ml	1 µg/ml
ZEB1		Rb Ab	HPA027524	Sigma Aldrich	200 µg/ml	1 µg/ml

SUPPLEMENTAL TABLE 2

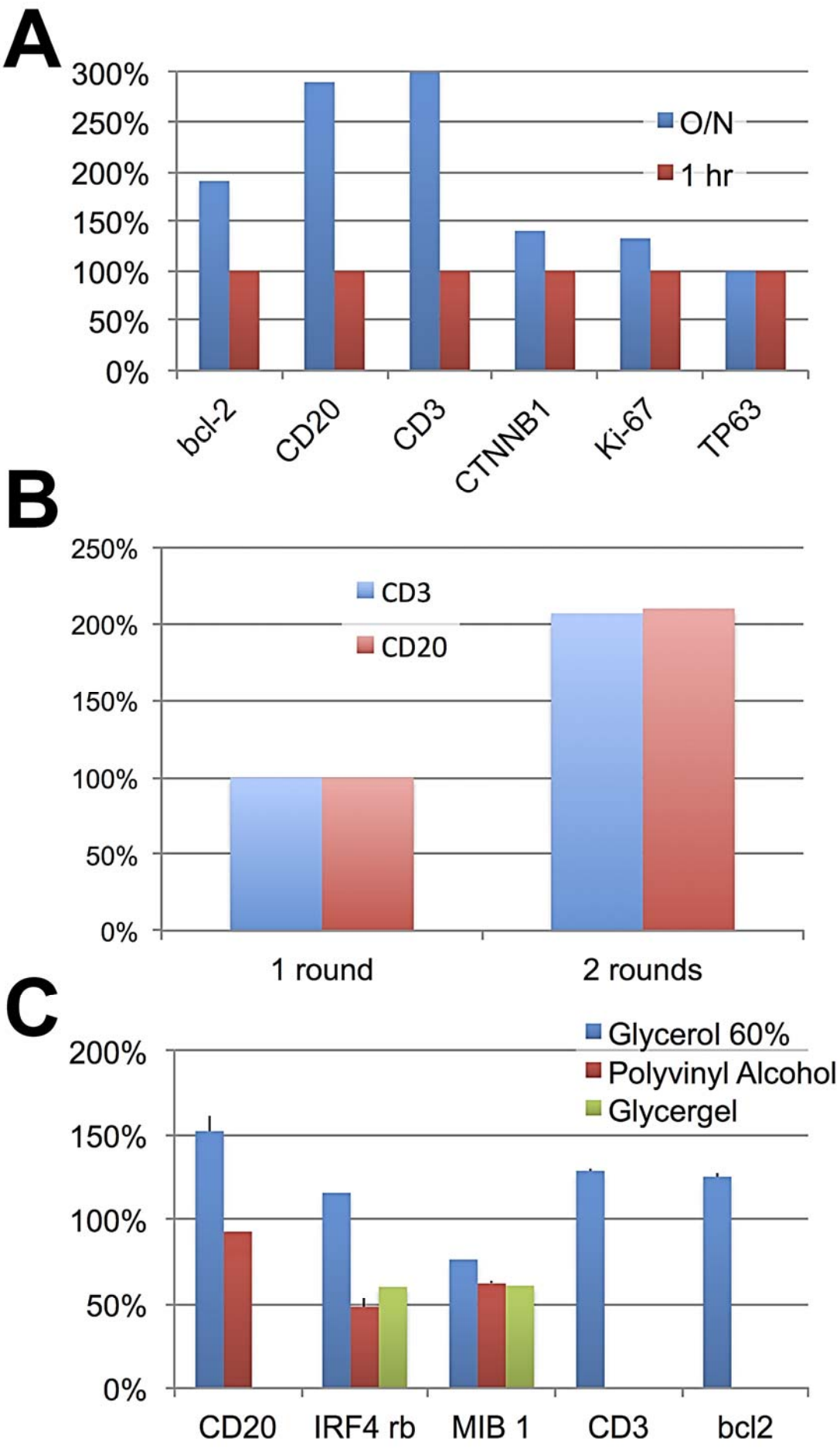
Host	Target	**Reagent**	**Absorbed**	**cat n.**	ditta
Dk	Rb	Donkey Anti-Rabbit IgG (H+L) DyLight 488	Multi	711-545-152	Jackson Immunoresearch
Gt	Rb	Goat Anti-Rabbit IgG (H+L) Alexa Fluor® 488	Hu, Mo, Rt	111-545-144	Jackson Immunoresearch
Dk	Mouse	Donkey anti Mouse Ig - DyLight 488	Multi (not rat)	715-485-150	Jackson Immunoresearch
Gt	Mouse	Goat Anti-Mouse IgG (H+L) Alexa Fluor® 488	Hu, Bov, Hrs, Rb, Rt	115-545-166	Jackson Immunoresearch
Gt	IgG1	Goat Anti-Mouse IgG1 Alexa Fluor 488	Hu, Bov, Rb	115-545-205	Jackson Immunoresearch
Gt	IgG2a	Goat Anti-Mouse IgG2a ATTO488	Hu, Bov, Rb	610-152-041	Rocklan d
Gt	IgG2a	Goat Anti-Mouse IgG2a Alexa Fluor® 488	Hu, Bov, Rb	115-545-206	Jackson Immunoresearch
Dk	Gt	Donkey Anti-Goat IgG (H+L) Alexa Fluor® 488	Multi	705-545-147	Jackson Immunoresearch
Dk	Rb	Donkey anti Rabbit Ig - DyLight 549	Multi	711-505-152	Jackson Immunoresearch
Dk	Rb	Donkey Anti-Rabbit IgG (H+L) Rhodamine Red™-X (RRX)	Multi	711-295-152	Jackson Immunoresearch
Gt	Rb	Goat Anti-Rabbit IgG (H+L) Rhodamine Red™-X (RRX)	Hu, Mo, Rt	111-295-144	Jackson Immunoresearch
Dk	Mouse	Donkey Anti-Mouse IgG (H+L) Rhodamine Red™-X (RRX)	Multi	715-295-150	Jackson Immunoresearch
Gt	Mouse	Goat Anti-Mouse IgG (H+L) Rhodamine Red™-X (RRX)	Hu, Bov, Hrs, Rb, Rt	115-295-166	Jackson Immunoresearch
Gt	IgG1	Goat Anti-Mouse IgG1 Rhodamine Red™-X (RRX)	Hu, Bov, Rb	115-295-205	Jackson Immunoresearch
Gt	IgG2a	Goat Anti-Mouse IgG2a Rhodamine Red™-X (RRX)	Hu, Bov, Rb	115-295-206	Jackson Immunoresearch
Gt	IgG2b	Goat Anti-Mouse IgG2b Rhodamine Red™-X (RRX)	Hu, Bov, Rb	115-295-207	Jackson Immunoresearch
Gt	IgG3	Goat Anti-Mouse IgG3 Rhodamine Red™-X (RRX)	Hu, Bov, Rb	115-295-209	Jackson Immunoresearch
Gt	Rat	Goat Anti-Rat IgG (H+L) Rhodamine Red™-X (RRX)	Hu, Bov, Hrs, Ms, Rb	112-295-167	Jackson Immunoresearch
Dk	Gt	Donkey Anti-Goat IgG (H+L) Alexa Fluor® 660	Multi	A21083	Invitroge n
Dk	Rb	Donkey Anti-Rb IgG (H+L) Alexa Fluor® 680	Multi	A10043	Invitroge n
Gt	Rb	Goat anti Rabbit IgG (H&L) ATTO 647N	Multi	611-156-122	Rocklan d
Gt	Mouse	Goat Anti-Mouse IgG (H+L) Alexa Fluor® 660	Multi	A21055	Invitroge n
Dk	Mouse	Donkey Anti-Mouse IgG (H+L) Alexa Fluor® 647	Multi	715-605-151	Jackson Immunoresearch
Gt	IgG1	Goat Anti-Mouse IgG1 Alexa Fluor® 680	Hu, Bov, Rb	A31562	Invitroge n
Gt	IgG1	Goat Anti-Mouse IgG1 Alexa Fluor® 647	Hu, Bov, Rb	115-605-205	Jackson Immunoresearch
Gt	IgG2a	Gt a Mouse IgG2a Alexa Fluor® 647	Hu, Bov, Rb	115-605-206	Jackson Immunoresearch

Gt	IgG2b	Goat Anti-Mouse IgG2b Alexa Fluor® 647	Hu, Bov, Rb	115-605-207	Jackson Immunoresearch
Dk	Rat	Donkey Anti-Rat IgG (H+L) Alexa Fluor® 647	Multi	712-605-153	Jackson Immunoresearch
Mouse		Mouse Gamma Globulin 10mg		015-000-002	Jackson Immunoresearch
Rb		Rabbit Gamma Globulin 10 mg		011-000-002	Jackson Immunoresearch

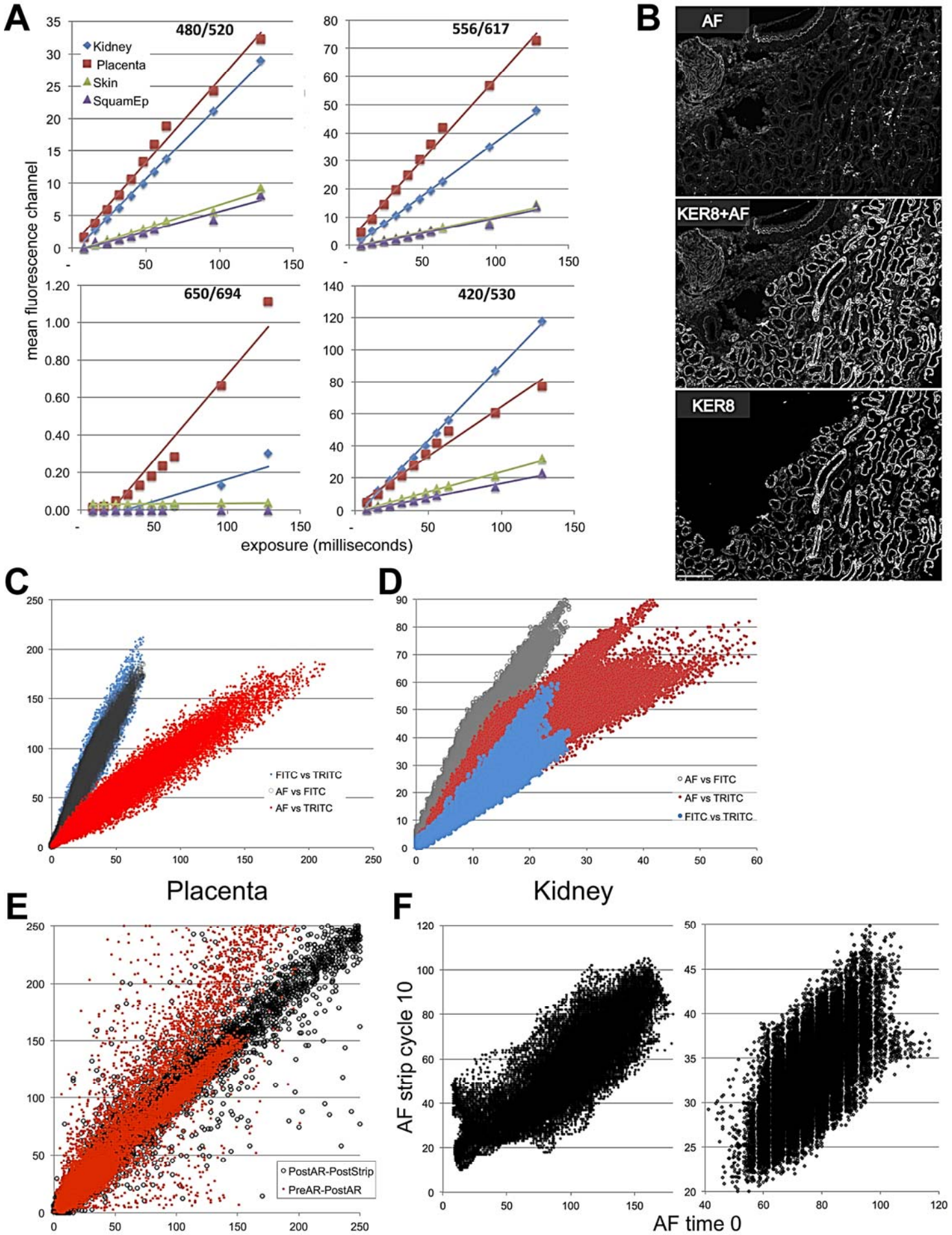
Absorbed: Multi

Supplemental Table 3

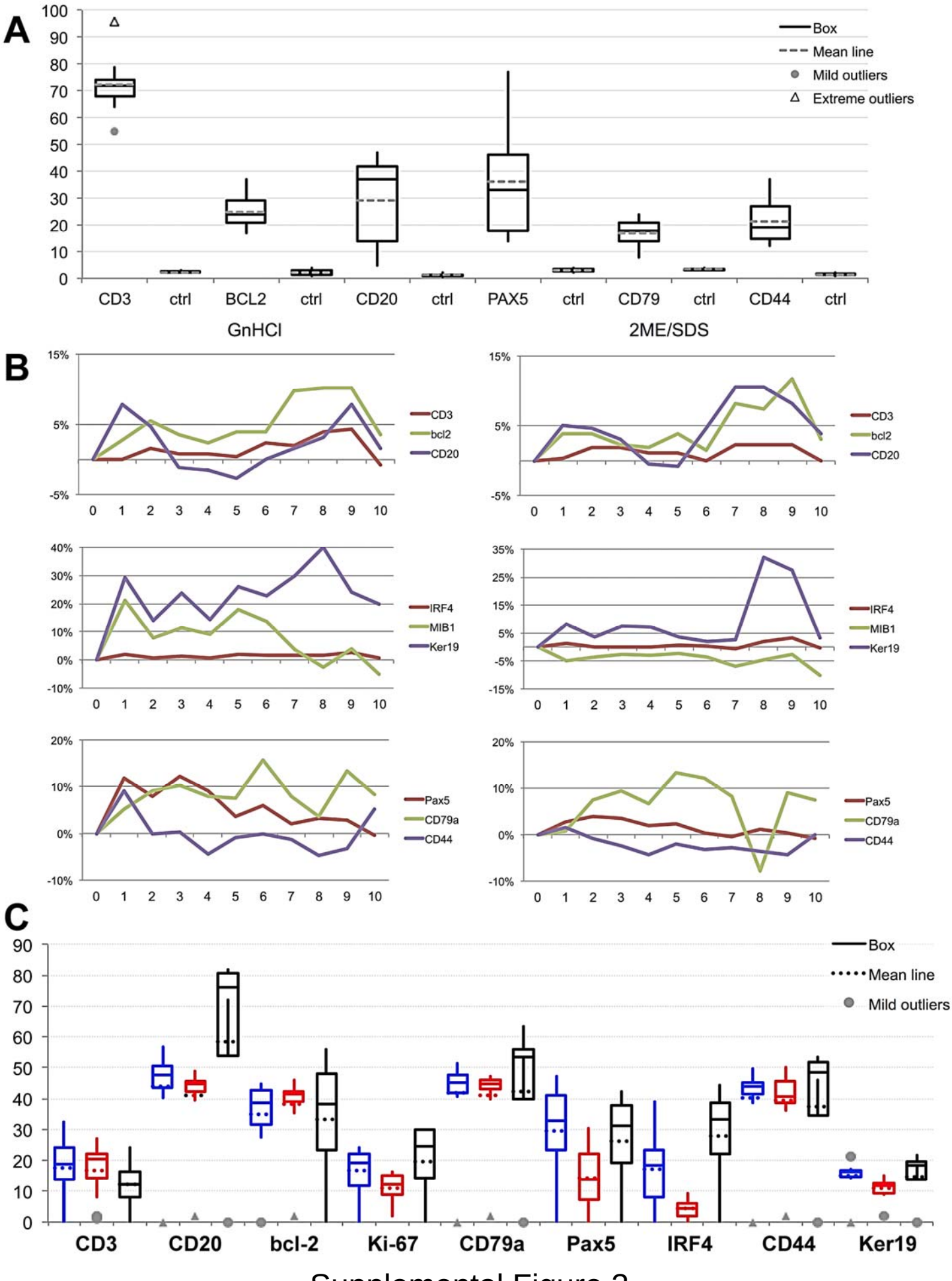
	Mean channel value	±SD	n	fraction of 256 ch	wavelenght measured
bcl-2	18.3	7.8	3	3.0%	TRITC
CD20	28.0	2.6	3	1.0%	Cy5
CD3	6.3	2.3	3	0.9%	FITC
CD44	26.0	1.4	2	0.5%	Cy5
CD79a	30.0	0.0	2	0.0%	TRITC
IRF4	6.3	2.1	3	0.8%	FITC
Ker19	80.3	31.4	6	12.3%	Cy5
Ker8	93.5	21.4	6	8.4%	FITC
MIB1	16.0	1.0	3	0.4%	TRITC
Pax5	7.5	0.7	2	0.3%	FITC
Vim	152.5	16.3	6	6.4%	TRITC
average				3.1%	



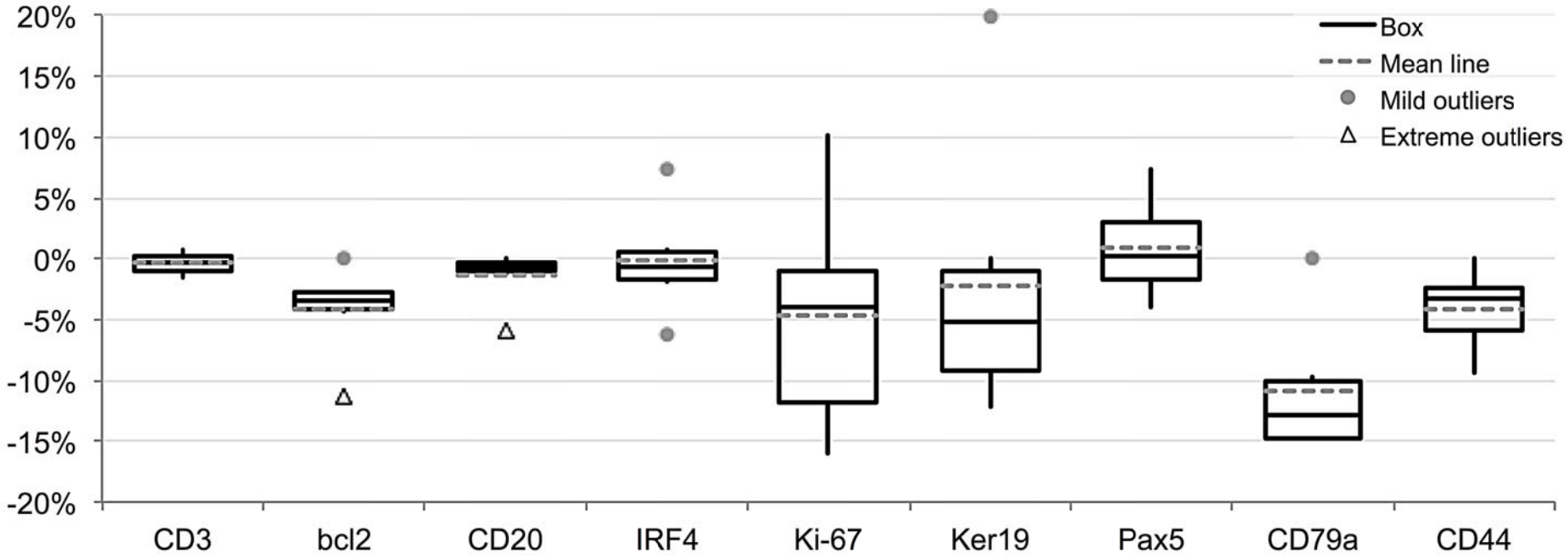
Supplemental Figure 1



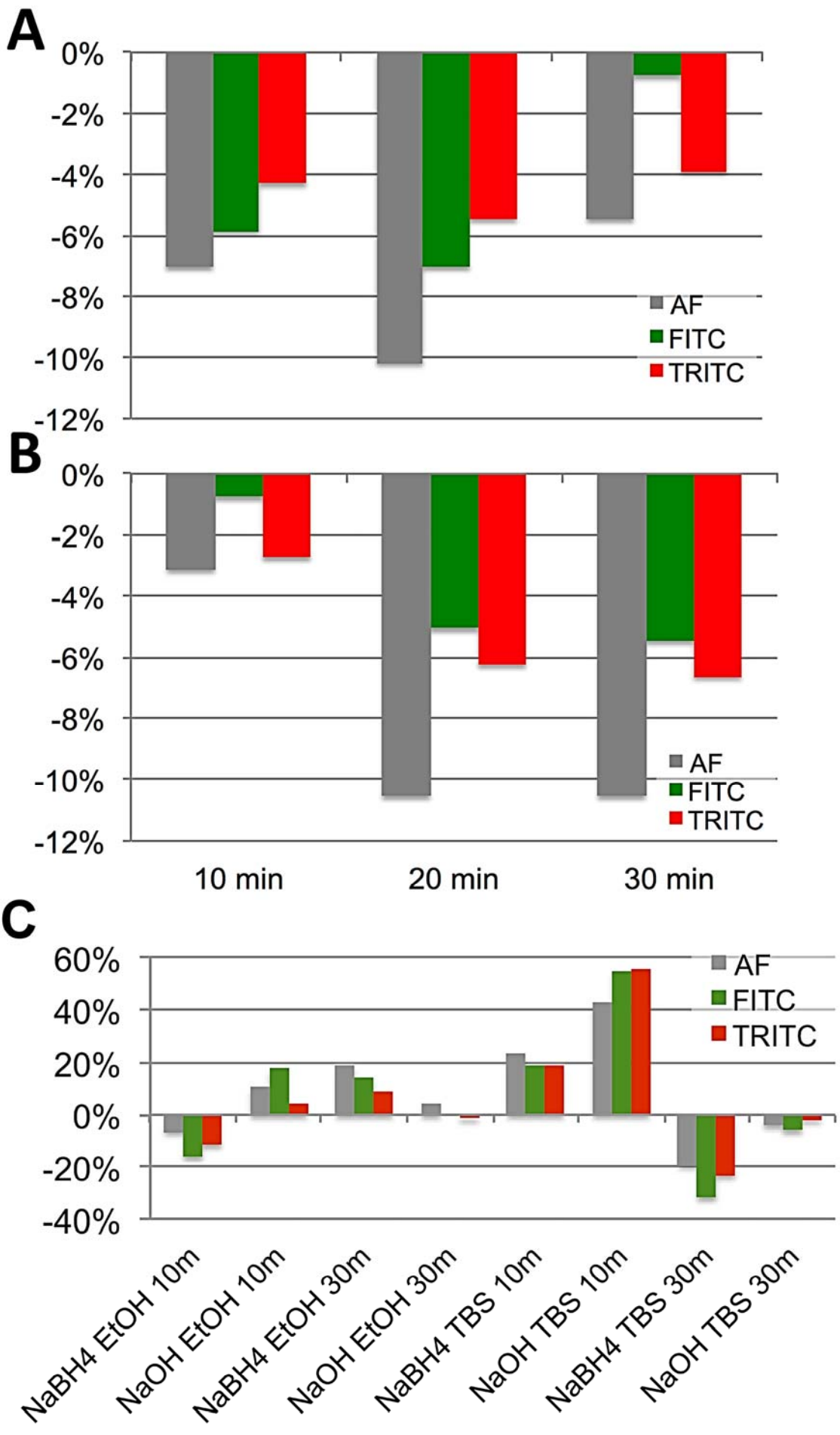
Supplemental Figure 2



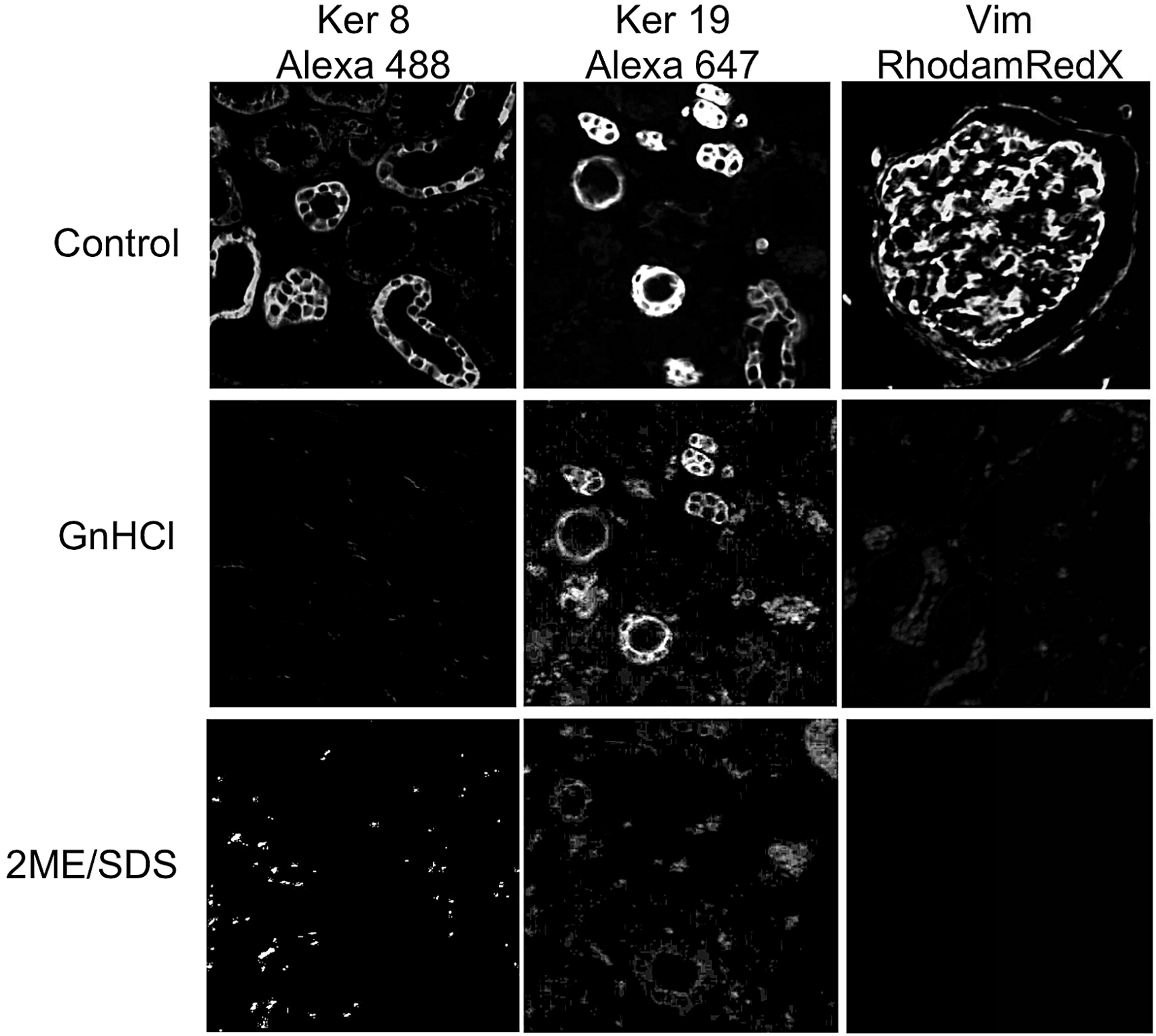
Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6