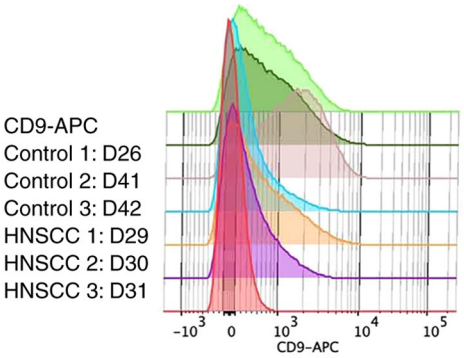
Figure S1. Bead-based flow cytometry analysis of EV surface markers using FlowJo™. (A) EV surface markers of plasma EV-EXÖBead® complexes (patients: n=3 and healthy controls: n=3) are shown as reduced geometric mean fluorescence intensity of CD9, CD63, CD81 and PD-L1 in the negative control. (B) $CD9^{+}$ $CD81^{+}$ $CD63^{+}$ $PD\text{-}L1^{+}$ of plasma EV-EXÖBead® complex (patients: n=3 and healthy controls: n=3). (C) $CD9^{+}$ $CD81^{+}$ $CD63^{Neg}$ $PD\text{-}L1^{+}$ of plasma EV-EXÖBead® complex (patients: n=3 and healthy controls: n=3). (D) $CD9^{+}$ $CD81^{Neg}$ $CD63^{Neg}$ $PD\text{-}L1^{+}$ of plasma EV-EXÖBead® complex (patients: n=3 and healthy controls: n=3). (E) $CD9^{+}$ $CD81^{Neg}$ $CD63^{+}$ $PD\text{-}L1^{+}$ of plasma EV-EXÖBead® complex (patients: n=3 and healthy controls: n=3). EV, extracellular vesicle.

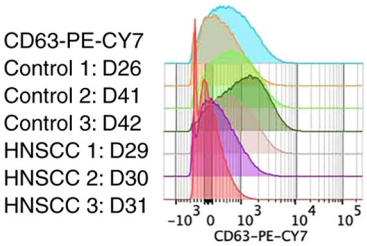
A

a



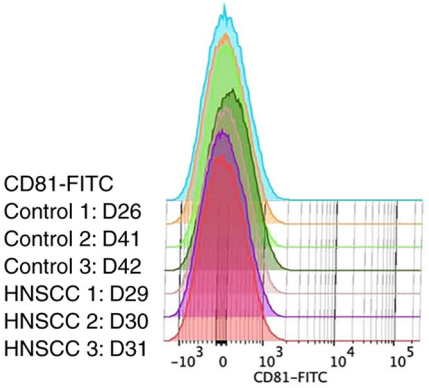
Sample Name	Geometric Mean : APC-A
Specimen_001_EF (USB)_005.fcs	55.6
Specimen_001_26 (USB plasma 250 ul)+ 1 ml EXOBUFFER_006.fcs	308
Specimen_001_41 (USB plasma 250 ul)+ 1 ml EXOBUFFER_010.fcs	506
Specimen_001_42 (USB plasma 250 ul)+ 1 ml EXOBUFFER_011.fcs	262
Specimen_001_29 (USB plasma 250 ul)+ 1 ml EXOBUFFER_007.fcs	1331
Specimen_001_30 (USB plasma 250 ul)+ 1 ml EXOBUFFER_008.fcs	682
Specimen_001_31 (USB plasma 250 ul)+ 1 ml EXOBUFFER_009.fcs	615

b



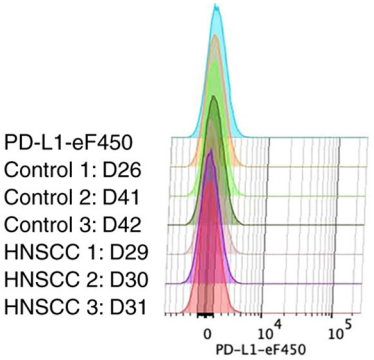
Sample Name	Geometric Mean : PE-Cy7-A
Specimen_001_EF (USB)_005.fcs	38.0
Specimen_001_26 (USB plasma 250 ul)+ 1 ml EXOBUFFER_006.fcs	292
Specimen_001_29 (USB plasma 250 ul)+ 1 ml EXOBUFFER_007.fcs	638
Specimen_001_30 (USB plasma 250 ul)+ 1 ml EXOBUFFER_008.fcs	1065
Specimen_001_31 (USB plasma 250 ul)+ 1 ml EXOBUFFER_009.fcs	720
Specimen_001_41 (USB plasma 250 ul)+ 1 ml EXOBUFFER_010.fcs	360
Specimen_001_42 (USB plasma 250 ul)+ 1 ml EXOBUFFER_011.fcs	578

c



Sample Name	Geometric Mean : FITC-A
Specimen_001_EF (USB)_005.fcs	63.3
Specimen_001_26 (USB plasma 250 ul)+ 1 ml EXOBUFFER_006.fcs	57.0
Specimen_001_29 (USB plasma 250 ul)+ 1 ml EXOBUFFER_007.fcs	97.0
Specimen_001_30 (USB plasma 250 ul)+ 1 ml EXOBUFFER_008.fcs	163
Specimen_001_31 (USB plasma 250 ul)+ 1 ml EXOBUFFER_009.fcs	88.7
Specimen_001_41 (USB plasma 250 ul)+ 1 ml EXOBUFFER_010.fcs	50.2
Specimen_001_42 (USB plasma 250 ul)+ 1 ml EXOBUFFER_011.fcs	59.0

d



Sample Name	Geometric Mean : BV421-A
Specimen_001_EF (USB)_005.fcs	575
Specimen_001_26 (USB plasma 250 ul)+ 1 ml EXOBUFFER_006.fcs	519
Specimen_001_29 (USB plasma 250 ul)+ 1 ml EXOBUFFER_007.fcs	583
Specimen_001_30 (USB plasma 250 ul)+ 1 ml EXOBUFFER_008.fcs	646
Specimen_001_31 (USB plasma 250 ul)+ 1 ml EXOBUFFER_009.fcs	594
Specimen_001_41 (USB plasma 250 ul)+ 1 ml EXOBUFFER_010.fcs	493
Specimen_001_42 (USB plasma 250 ul)+ 1 ml EXOBUFFER_011.fcs	545

Figure S1. Continued.

B

CD9+ CD81+CD63+ PD-L1+

Control 1: D26

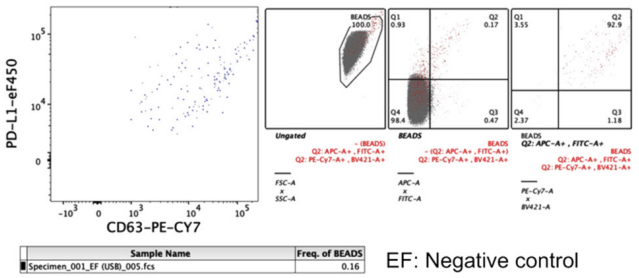
Control 2: D41

Control 3: D42

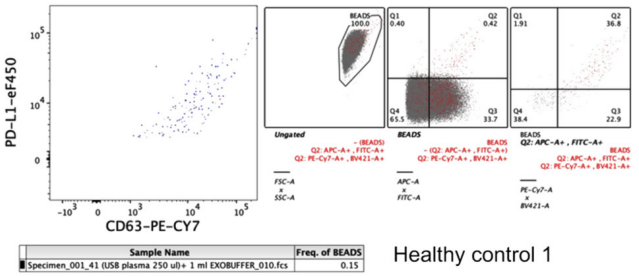
HNSCC 1: D29

HNSCC 2: D30

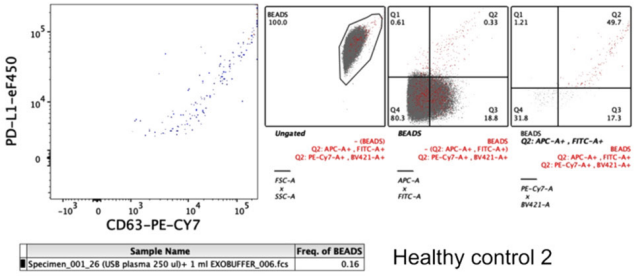
HNSCC 3: D31



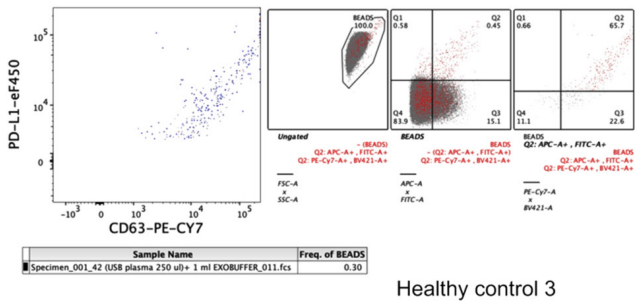
EF: Negative control



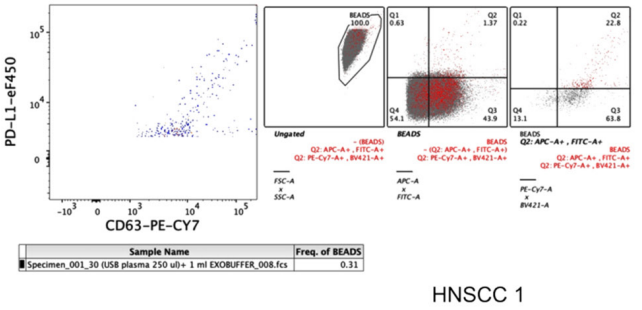
Healthy control 1



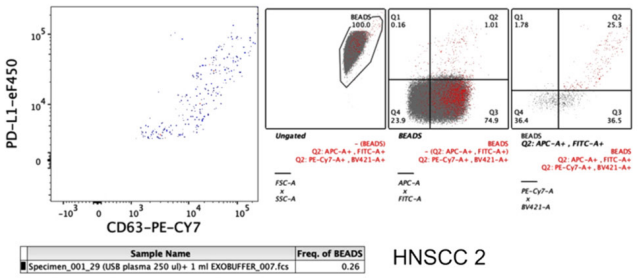
Healthy control 2



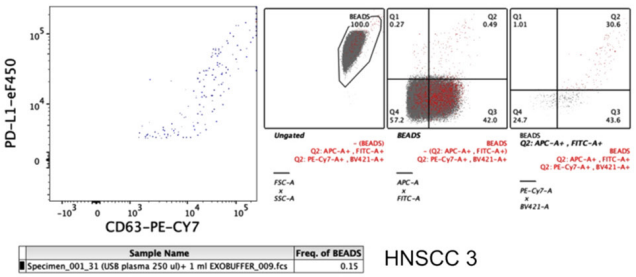
Healthy control 3



HNSCC 1



HNSCC 2

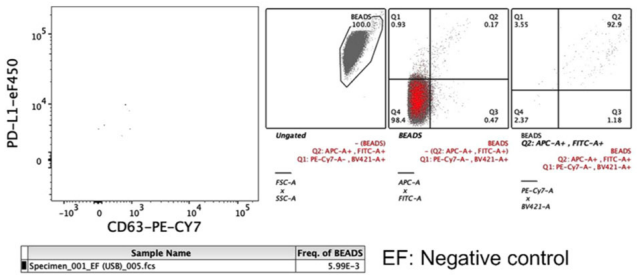


HNSCC 3

Figure S1. Continued.

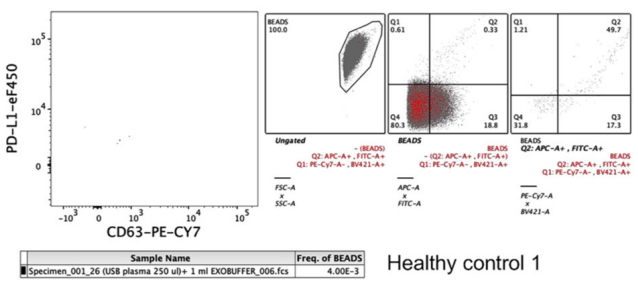
C

CD9+ CD81+CD63Neg PD-L1+
Control 1: D26
Control 2: D41
Control 3: D42
HNSCC 1: D29
HNSCC 2: D30
HNSCC 3: D31



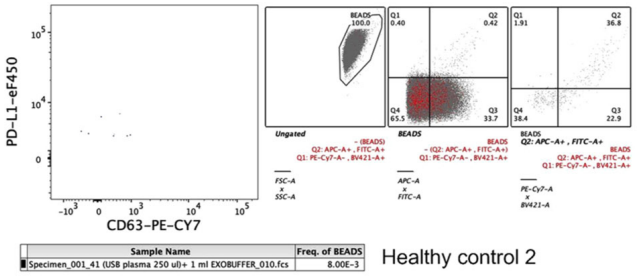
Sample Name	Freq. of BEADS
Specimen_001_EF (USB)_005.fcs	5.99E-3

EF: Negative control



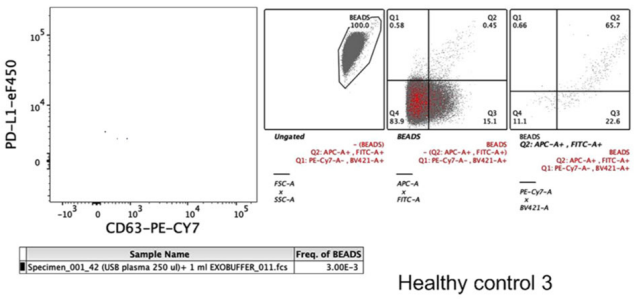
Sample Name	Freq. of BEADS
Specimen_001_26 (USB plasma 250 ul)+ 1 ml EXOBUFFER_006.fcs	4.00E-3

Healthy control 1



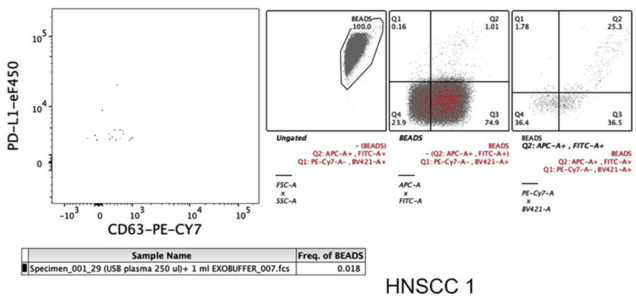
Sample Name	Freq. of BEADS
Specimen_001_41 (USB plasma 250 ul)+ 1 ml EXOBUFFER_010.fcs	8.00E-3

Healthy control 2



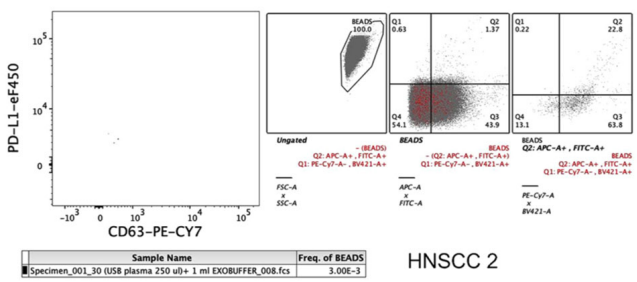
Sample Name	Freq. of BEADS
Specimen_001_42 (USB plasma 250 ul)+ 1 ml EXOBUFFER_011.fcs	3.00E-3

Healthy control 3



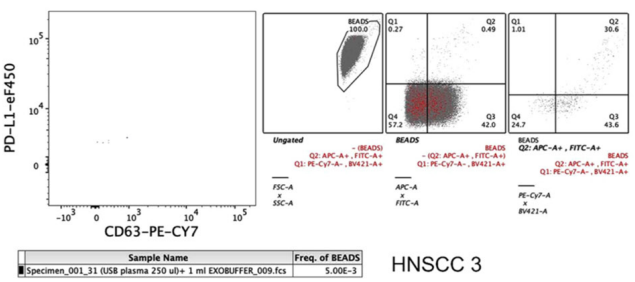
Sample Name	Freq. of BEADS
Specimen_001_29 (USB plasma 250 ul)+ 1 ml EXOBUFFER_007.fcs	0.018

HNSCC 1



Sample Name	Freq. of BEADS
Specimen_001_30 (USB plasma 250 ul)+ 1 ml EXOBUFFER_008.fcs	3.00E-3

HNSCC 2



Sample Name	Freq. of BEADS
Specimen_001_31 (USB plasma 250 ul)+ 1 ml EXOBUFFER_009.fcs	5.00E-3

HNSCC 3

Figure S1. Continued.

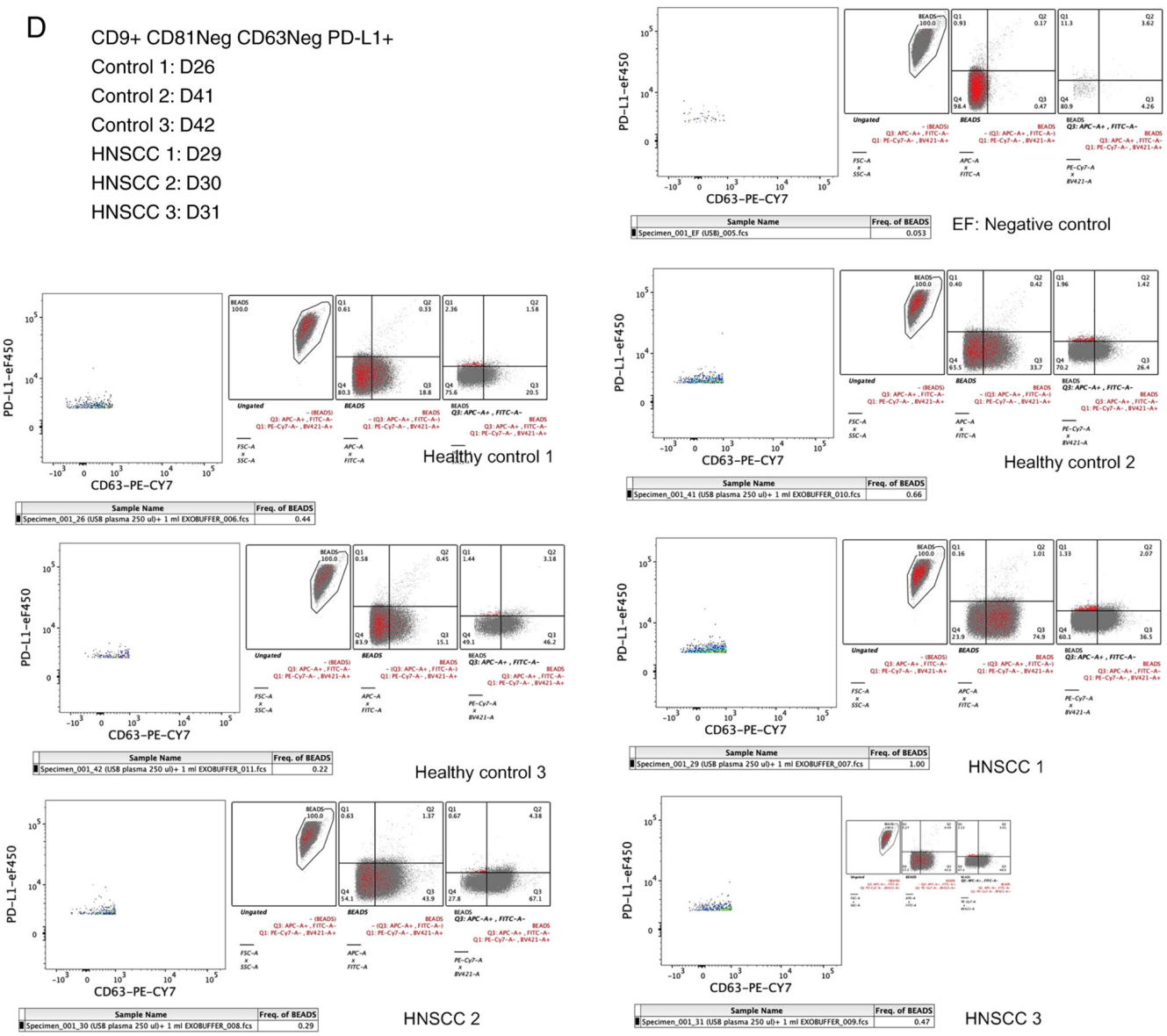


Figure S1. Continued.

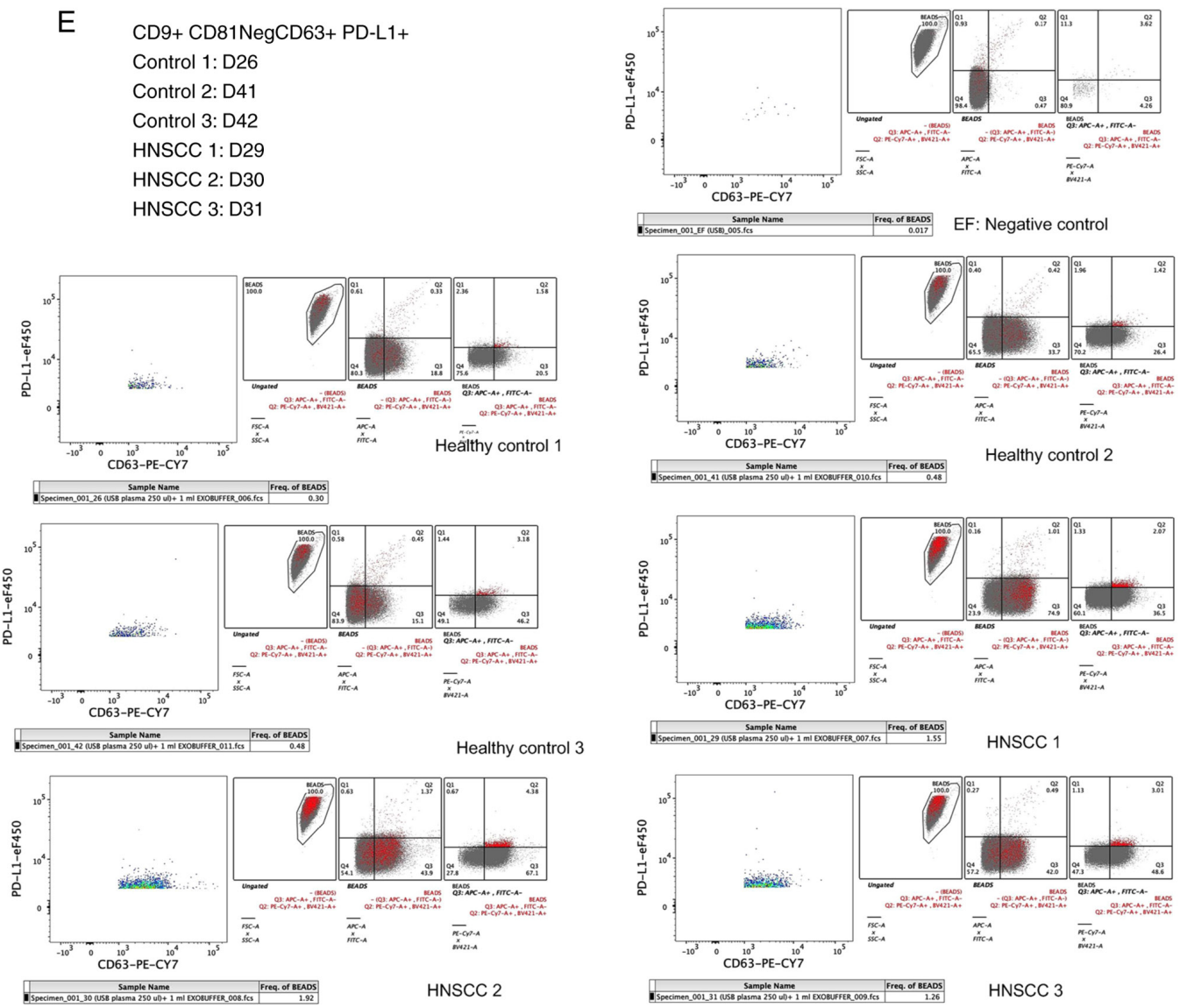
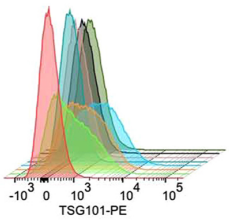


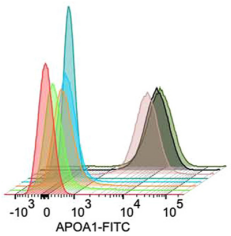
Figure S2. Bead-based flow cytometric analysis of EV intracellular marker and non-EV marker. (A) Intracellular EVs markers (TSG101) and non-EV markers (ApoA1) of plasma EVs-EXÖBead® complexes and unbound plasma fraction magnetic bead complexes (n=3) are shown as reduced geometric mean fluorescence intensity (MFI) in the negative control. (B) PanEV$^{+/Neg}$ and ApoA1$^{+/Neg}$ populations of the plasma EVs-EXÖBead® complex and SEC (Izon qEVoriginal 70) plasma EVs-magnetic beads complex are expressed as percentages by gating with FlowJo™. EV, extracellular vesicle.

A

Intracellular staining
Negative control of EF-Exobead complex: EF intra
EV-Exobead complex sample 1: C intra
EV-Exobead complex sample 2: 23_2 intra
EV-Exobead complex sample 3: 35_3 intra
Negative control of EF-beads complex: EF after super control intra
Unbound plasma-beads complex sample 1: C after super intra
Unbound plasma-beads complex sample 2: 23_2 after super intra
Unbound plasma-beads complex sample 3: 35_3 after super intra



	Sample Name	Geometric Mean : PE-A
	Specimen_001_EF intra_001.fcs	166
	Specimen_001_C intra_002.fcs	694
	Specimen_001_23_2 intra_003.fcs	945
	Specimen_001_35_3 intra_004.fcs	1346
	Specimen_001_EF after supe control intra_009.fcs	99.5
	Specimen_001_C after supe intra_010.fcs	133
	Specimen_001_23_2 after supe intra_011.fcs	153
	Specimen_001_35_3 after supe intra_012.fcs	185



	Sample Name	Geometric Mean : FITC-A
	Specimen_001_EF intra_001.fcs	40.0
	Specimen_001_C intra_002.fcs	183
	Specimen_001_23_2 intra_003.fcs	276
	Specimen_001_35_3 intra_004.fcs	179
	Specimen_001_EF after supe control intra_009.fcs	32.8
	Specimen_001_C after supe intra_010.fcs	6451
	Specimen_001_23_2 after supe intra_011.fcs	8291
	Specimen_001_35_3 after supe intra_012.fcs	6963

Figure S2. Continued.

B

Intracellular staining
Negative control of EF-Exobead complex: EF+EXOBEAD (030321)
EV-Exobead complex 1: C10 500UL+EXOBEAD (030321)
EV-Exobead complex 1: C11 500UL+EXOBEAD (030321)
EV-Exobead complex 1: C13 500UL+EXOBEAD (030321)
Negative control of EF-bead complex: EF+magnetic bead SEC (030321)
SEC-bead complex (qEV 70 mm column) 1: C10 SEC 7-10+ magnetic bead SEC (030321)
SEC-bead complex (qEV 70 mm column) 2: C11 SEC 7-10+ magnetic bead SEC (030321)
SEC-bead complex (qEV 70 mm column) 3: C13 SEC 7-10+ magnetic bead SEC (030321)

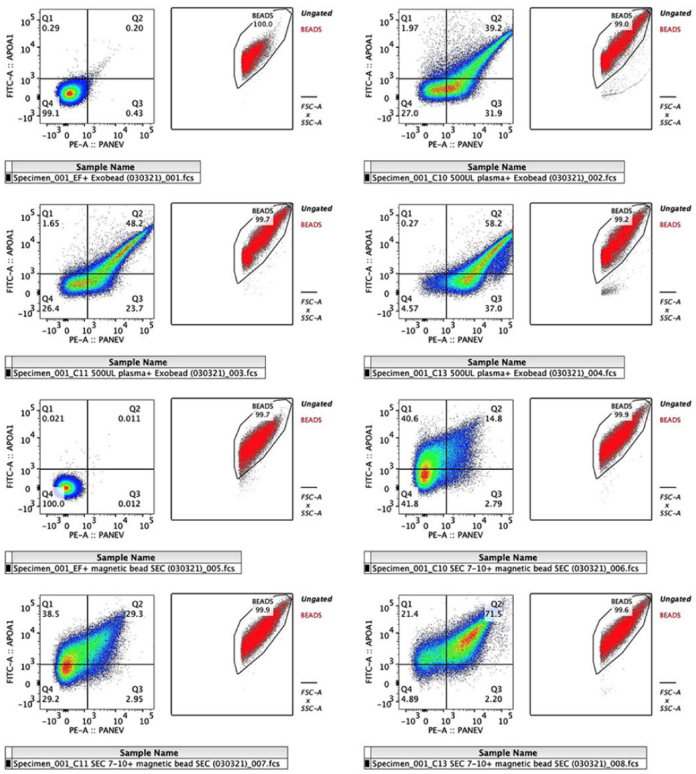


Figure S3. Bead-based flow cytometric analysis of HNSCC biomarkers, evaluated using FlowJo™. (A) EVs surface marker (PanEV), leukocyte common marker (CD45) and HNSCC markers (EpCAM, PanCK and PD-L1) of plasma EVs-EXÖBead® complexes (patients: n=9 and healthy controls: n=9 are shown as reduced geometric mean fluorescence intensity (MFI) in the negative control. (B) $PanEV^{+/Neg}$ $CD45^{+/Neg}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). (C) $PanEV^{+/Neg}$ $EpCAM^{+/Neg}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). (D) $PanEV^{+/Neg}$ $PD\text{-}L1^{+/Neg}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). (E) $PanEV^{+/Neg}$ $PanCK^{+/Neg}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). (F) $EpCAM^{Neg}$ $PD\text{-}L1^{+}$ $CD45^{Neg}$ $PanEV^{+}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). (G) $EpCAM^{+}$ $PD\text{-}L1^{+}$ $CD45^{Neg}$ $PanEV^{+}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). (H) $EpCAM^{+}$ $PD\text{-}L1^{Neg}$ $CD45^{Neg}$ $PanEV^{+}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). (I) $EpCAM^{Neg}$ $PD\text{-}L1^{Neg}$ $CD45^{Neg}$ $PanEV^{+}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). (J) $EpCAM^{Neg}$ $PanCK^{+}$ $PD\text{-}L1^{+}$ $CD45^{Neg}$ $PanEV^{+}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). (K) $EpCAM^{+}$ $PanCK^{+}$ $PD\text{-}L1^{+}$ $CD45^{Neg}$ PanEV+ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). (L) $EpCAM^{+}$ $PanCK^{Neg}$ $PD\text{-}L1^{+}$ $CD45^{Neg}$ $PanEV^{+}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). (M) $EpCAM^{Neg}$ $PanCK^{Neg}$ $PD\text{-}L1^{+}$ $CD45^{Neg}$ $PanEV^{+}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). (N) $EpCAM^{Neg}$ $PanCK^{+}$ $PD\text{-}L1^{+}$ $CD45^{+}$ $PanEV^{+}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). (O) $EpCAM^{+}$ $PanCK^{+}$ $PD\text{-}L1^{+}$ $CD45^{+}$ $PanEV^{+}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). (P) $EpCAM^{+}$ $PanCK^{Neg}$ $PD\text{-}L1^{+}$ $CD45^{+}$ $PanEV^{+}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9) were evaluated using FlowJo™. (Q) $EpCAM^{Neg}$ $PanCK^{Neg}$ $PD\text{-}L1^{+}$ $CD45^{+}$ $PanEV^{+}$ of single plasma EVs-EXÖBead® complex (patients: n=9 and healthy controls: n=9). EV, extracellular vesicle; HNSCC, head and neck squamous cell carcinoma.

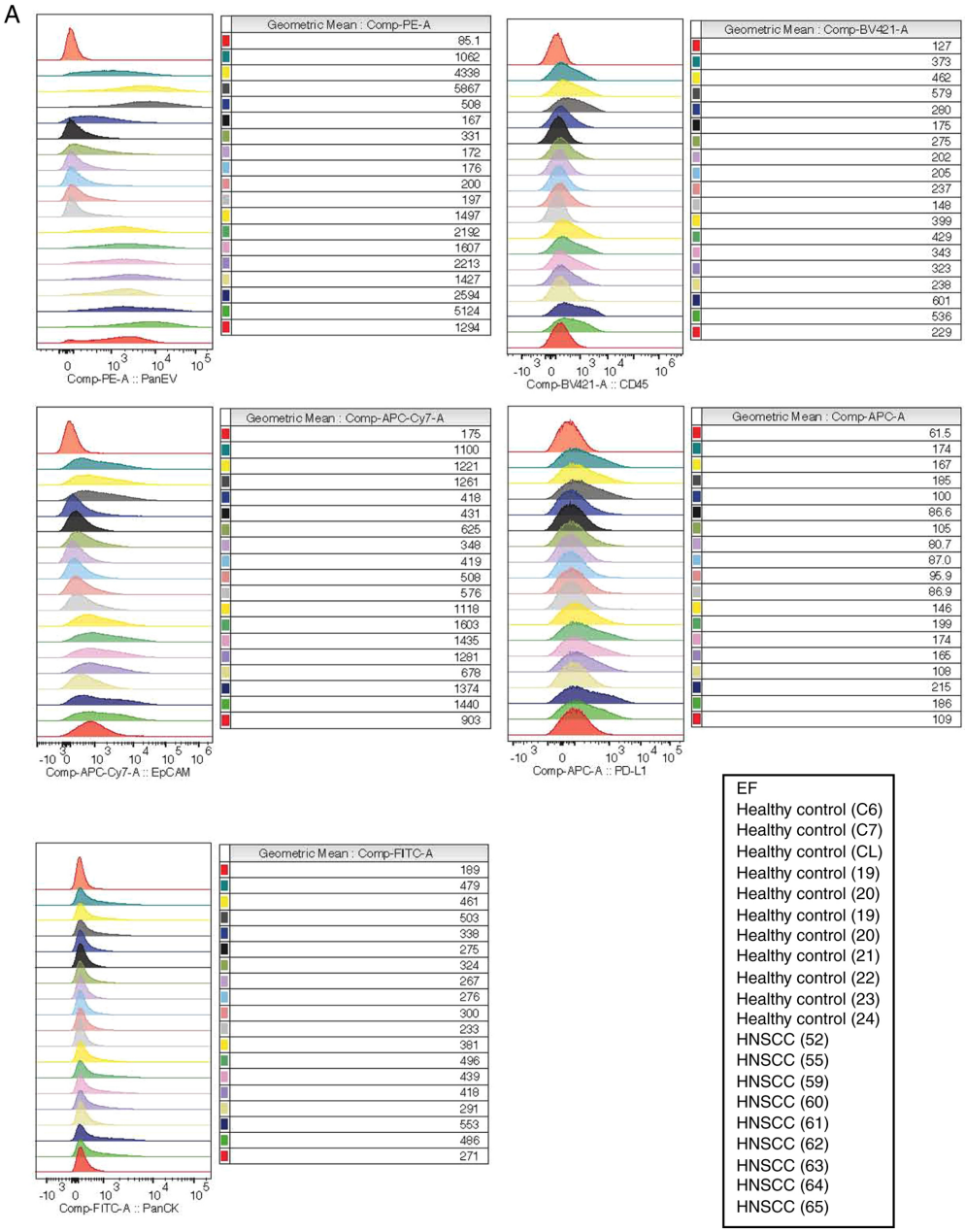


Figure S3. Continued.

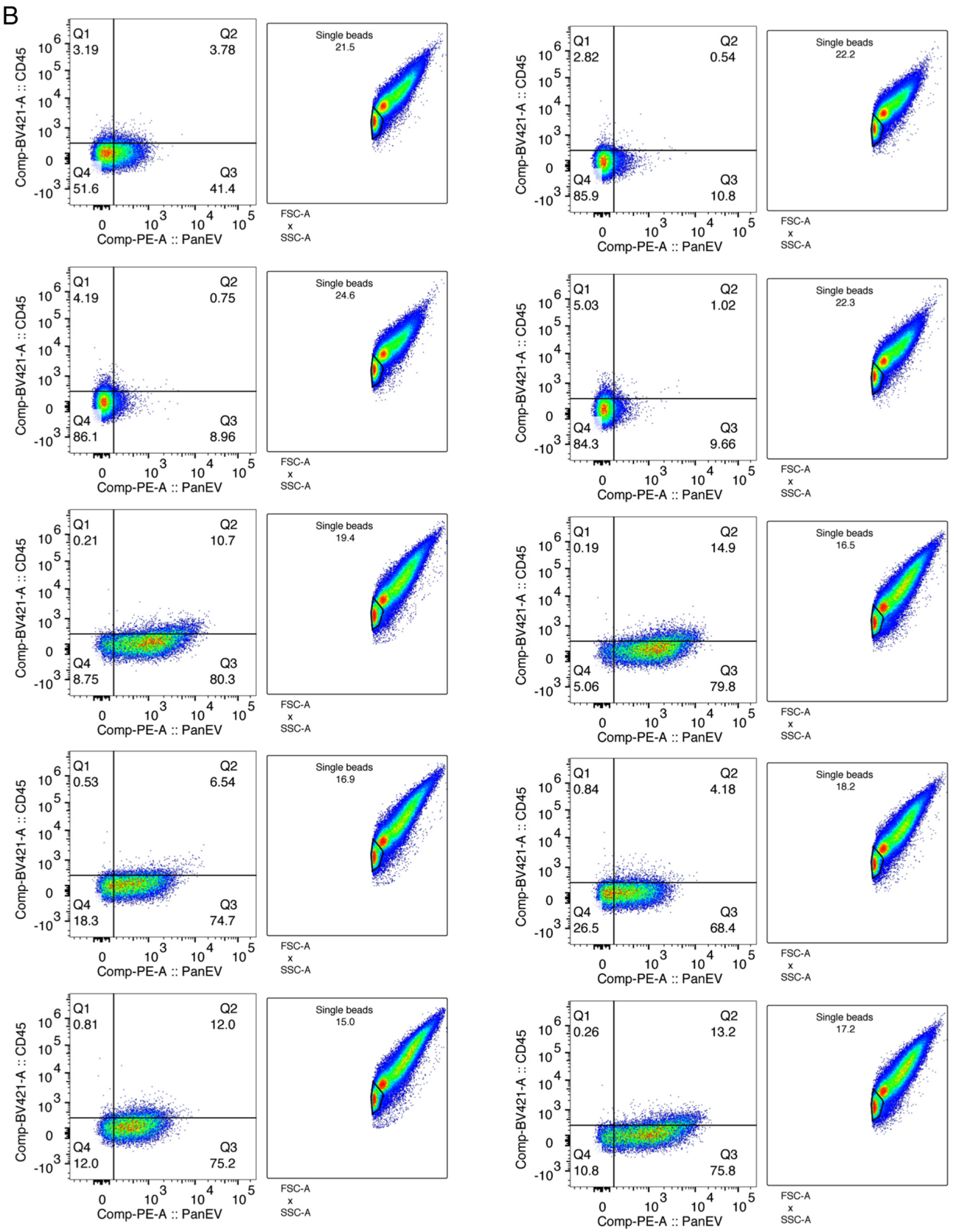
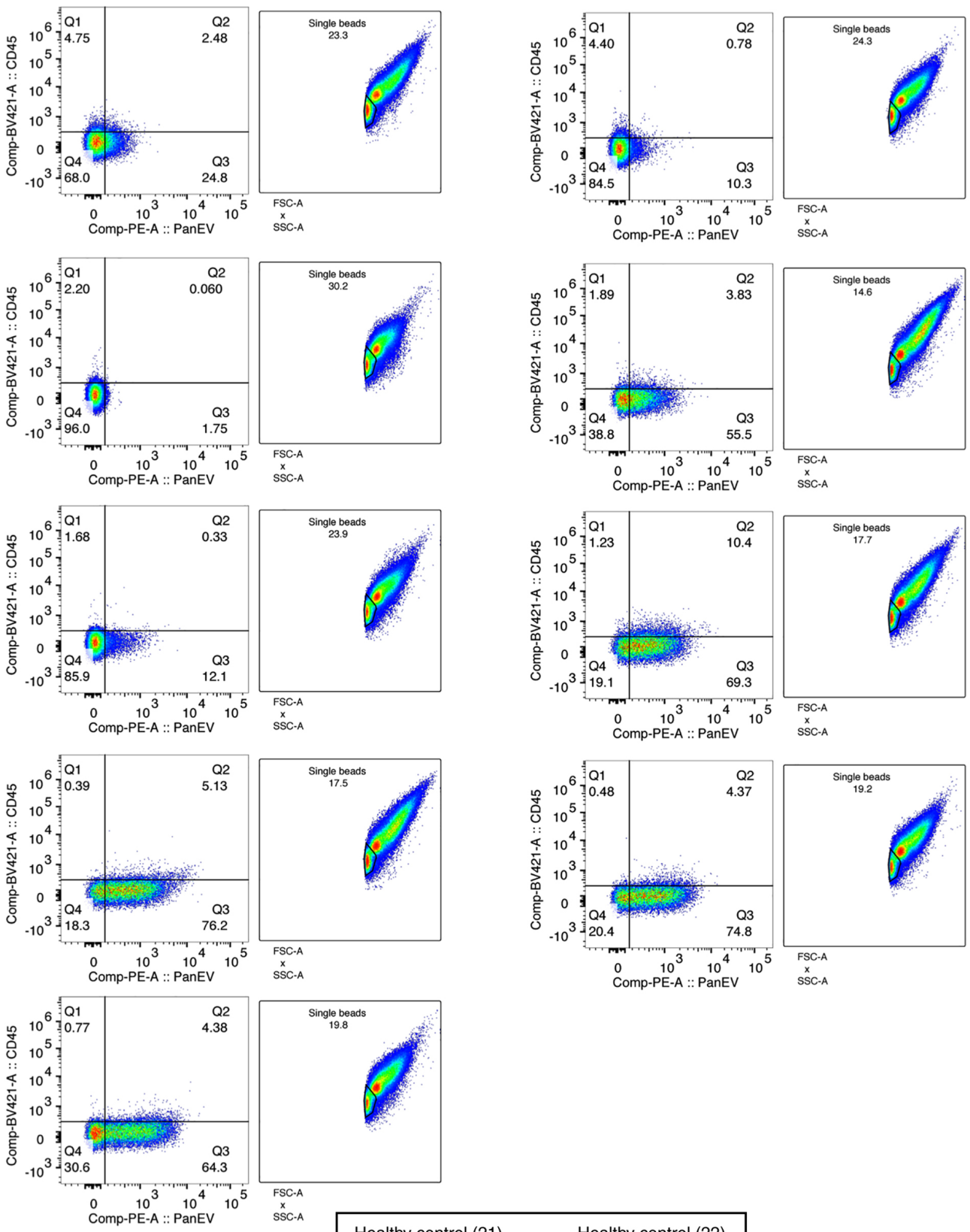


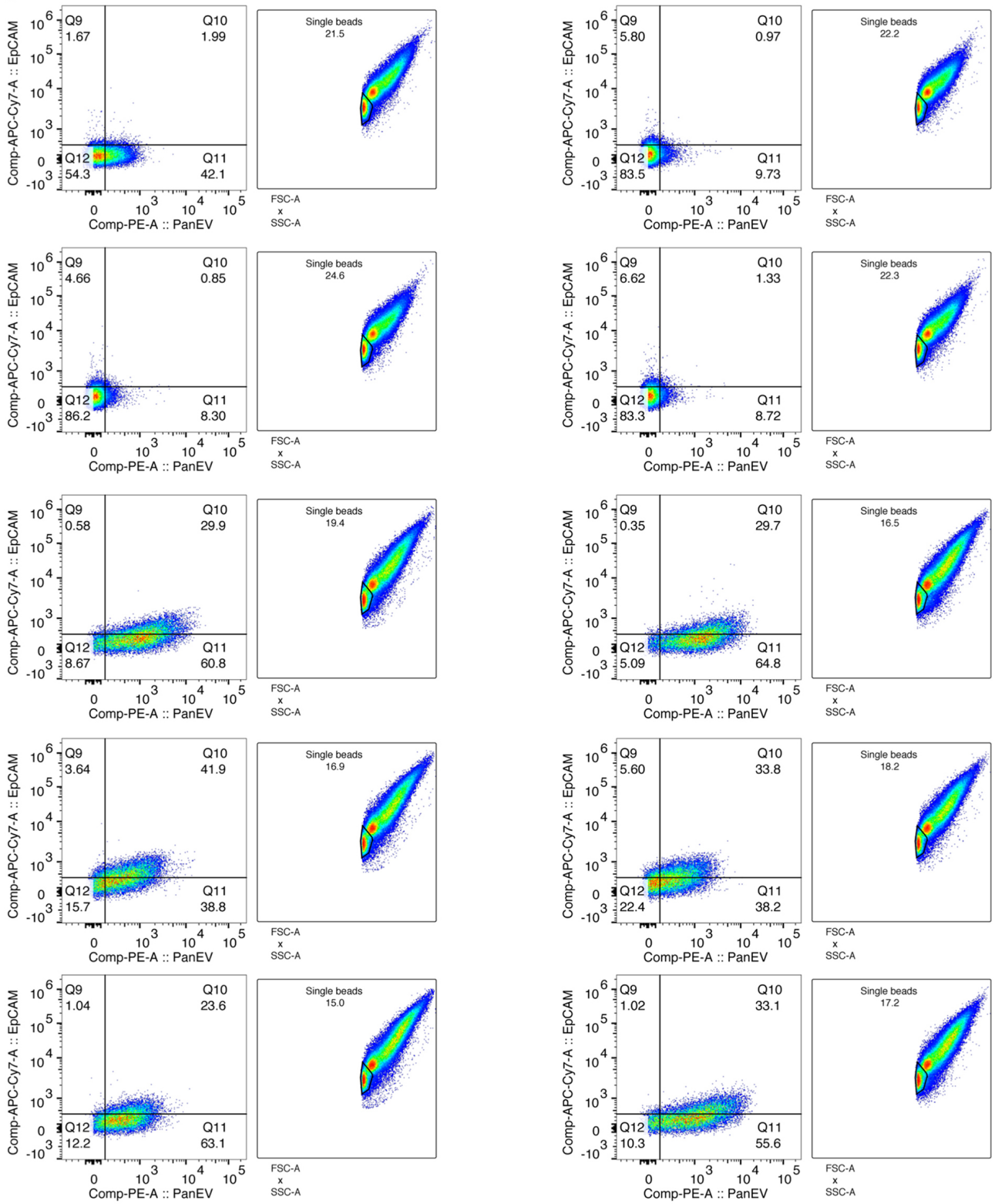
Figure S3. Continued.



Healthy control (21)	Healthy control (22)
EF (Negative control)	Healthy control (C6)
HNSCC (52)	HNSCC (55)
HNSCC (61)	HNSCC (62)
HNSCC (65)	

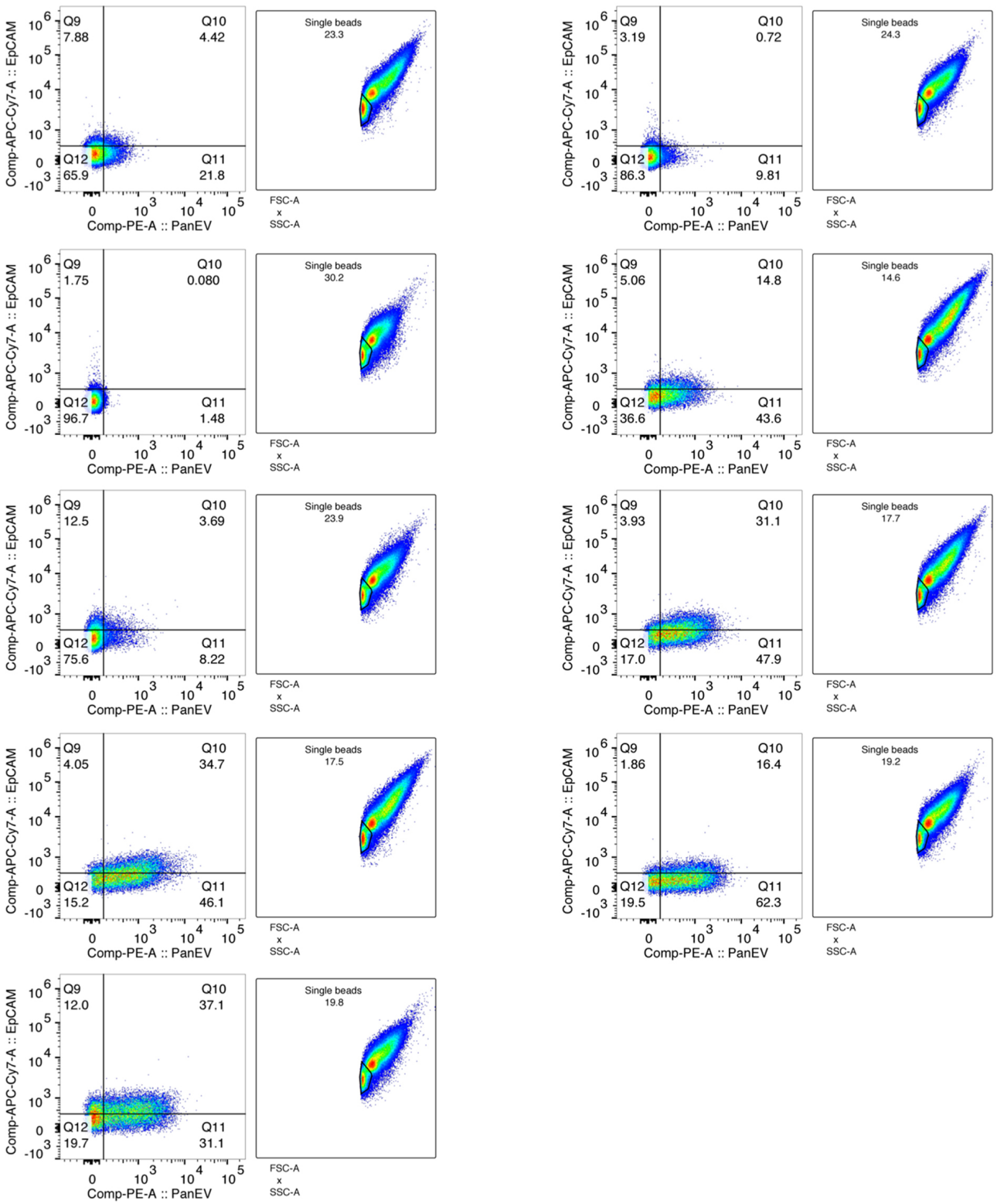
Figure S3. Continued.

C



Healthy control (19)	Healthy control (20)
Healthy control (23)	Healthy control (24)
Healthy control (C7)	Healthy control (CL)
HNSCC (59)	HNSCC (60)
HNSCC (63)	HNSCC (64)

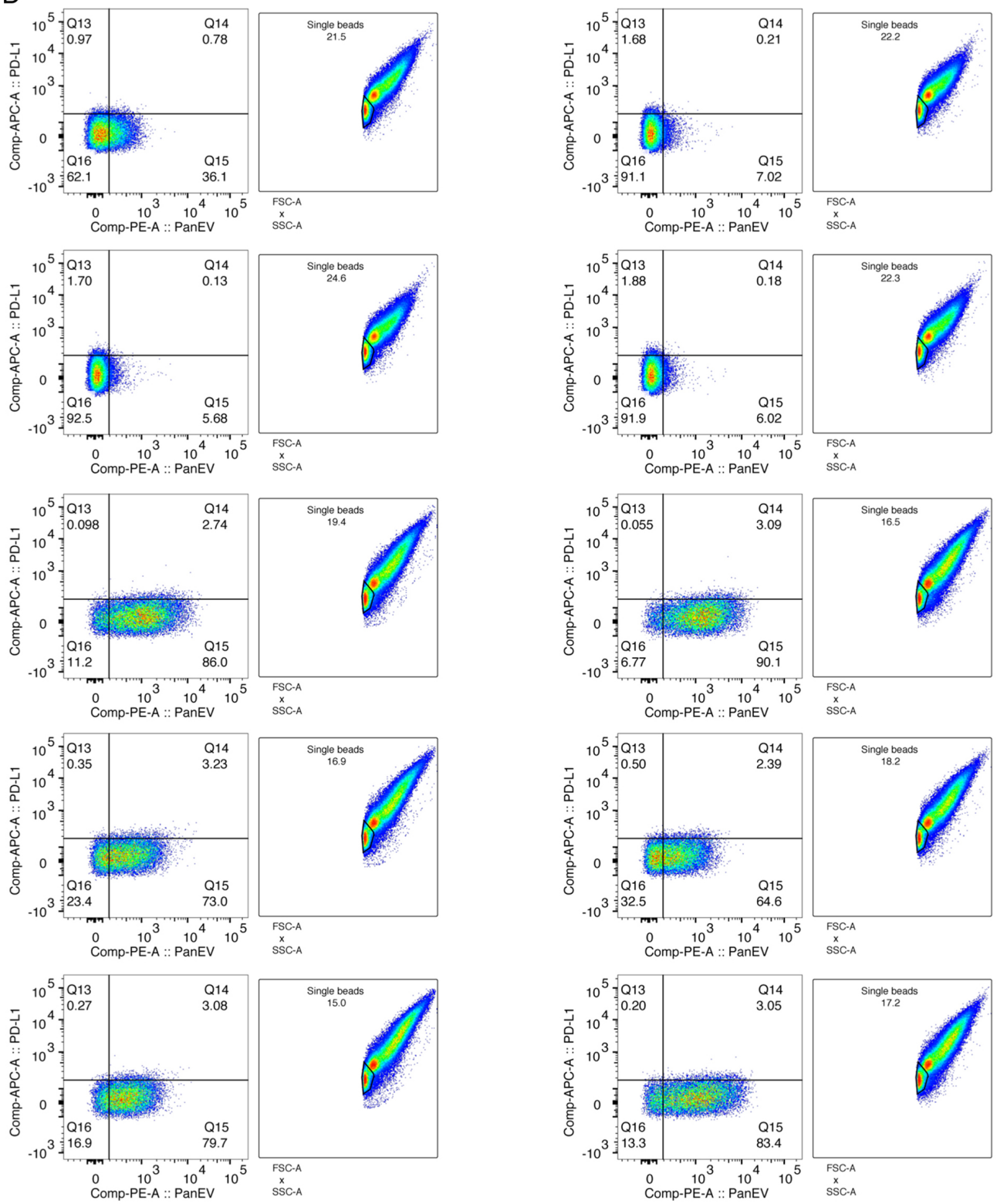
Figure S3. Continued.



Healthy control (21)	Healthy control (22)
EF (Negative control)	Healthy control (C6)
HNSCC (52)	HNSCC (55)
HNSCC (61)	HNSCC (62)
HNSCC (65)	

Figure S3. Continued.

D



Healthy control (19)	Healthy control (20)
Healthy control (23)	Healthy control (24)
Healthy control (C7)	Healthy control (CL)
HNSCC (59)	HNSCC (60)
HNSCC (63)	HNSCC (64)

Figure S3. Continued.

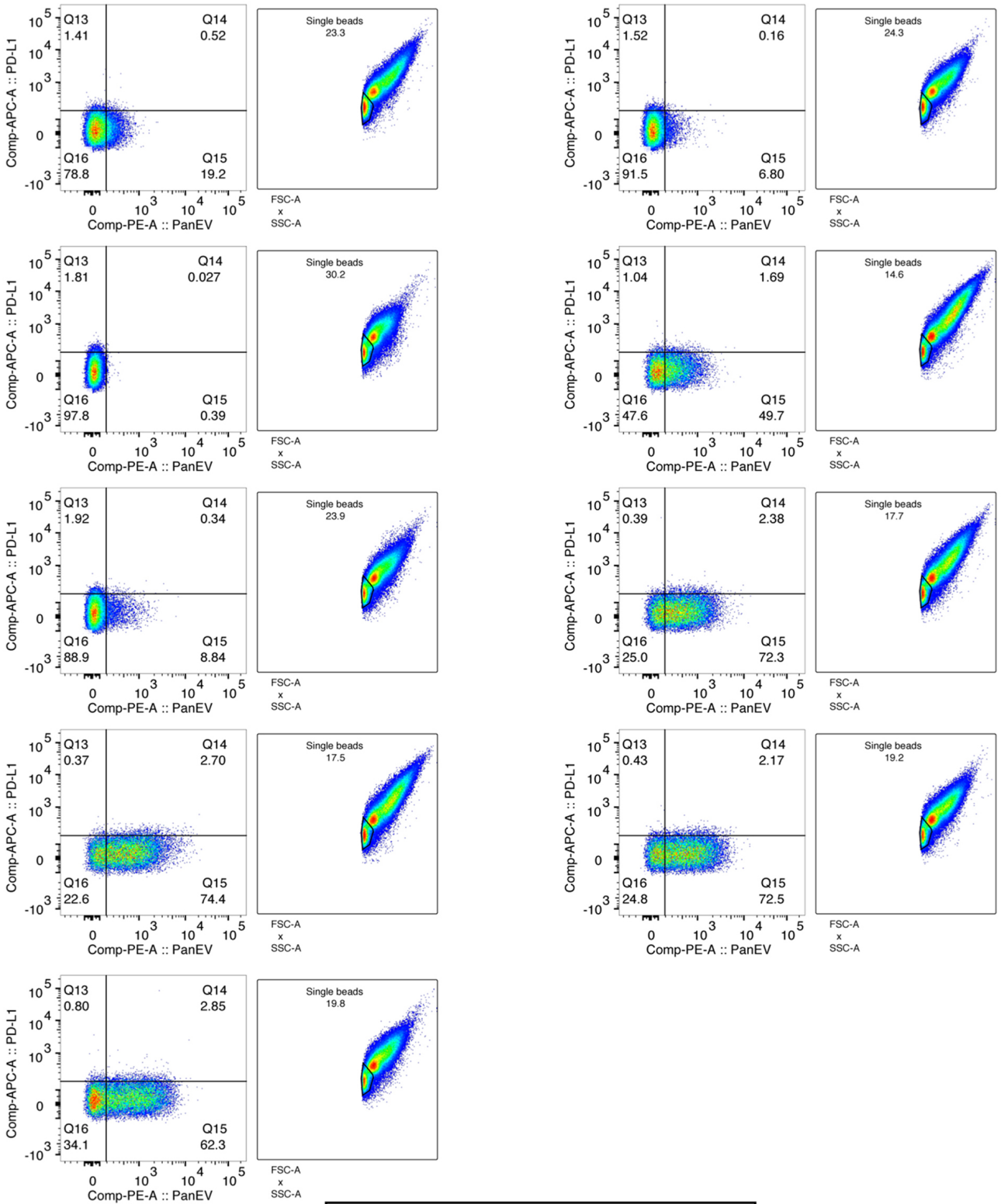
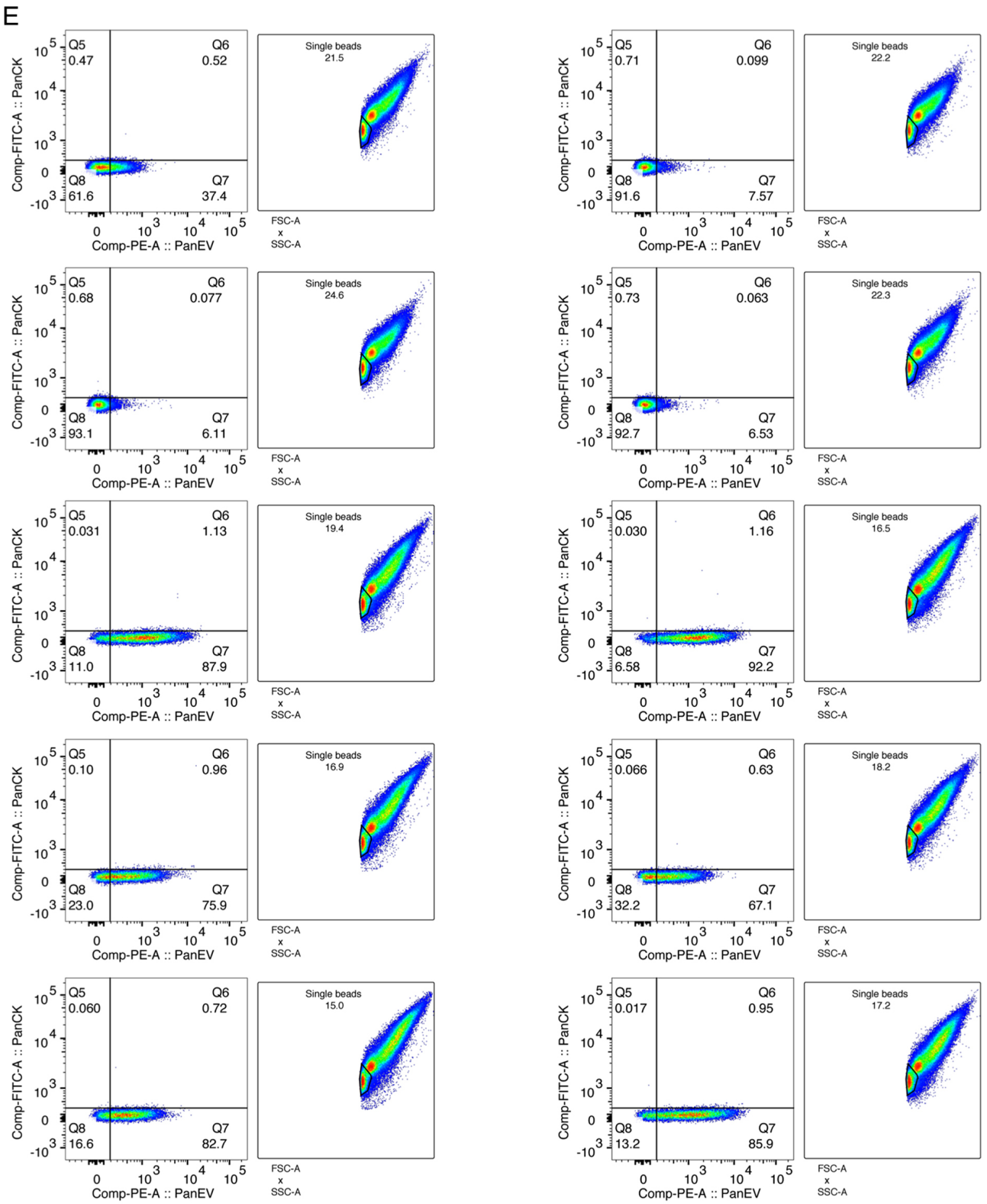
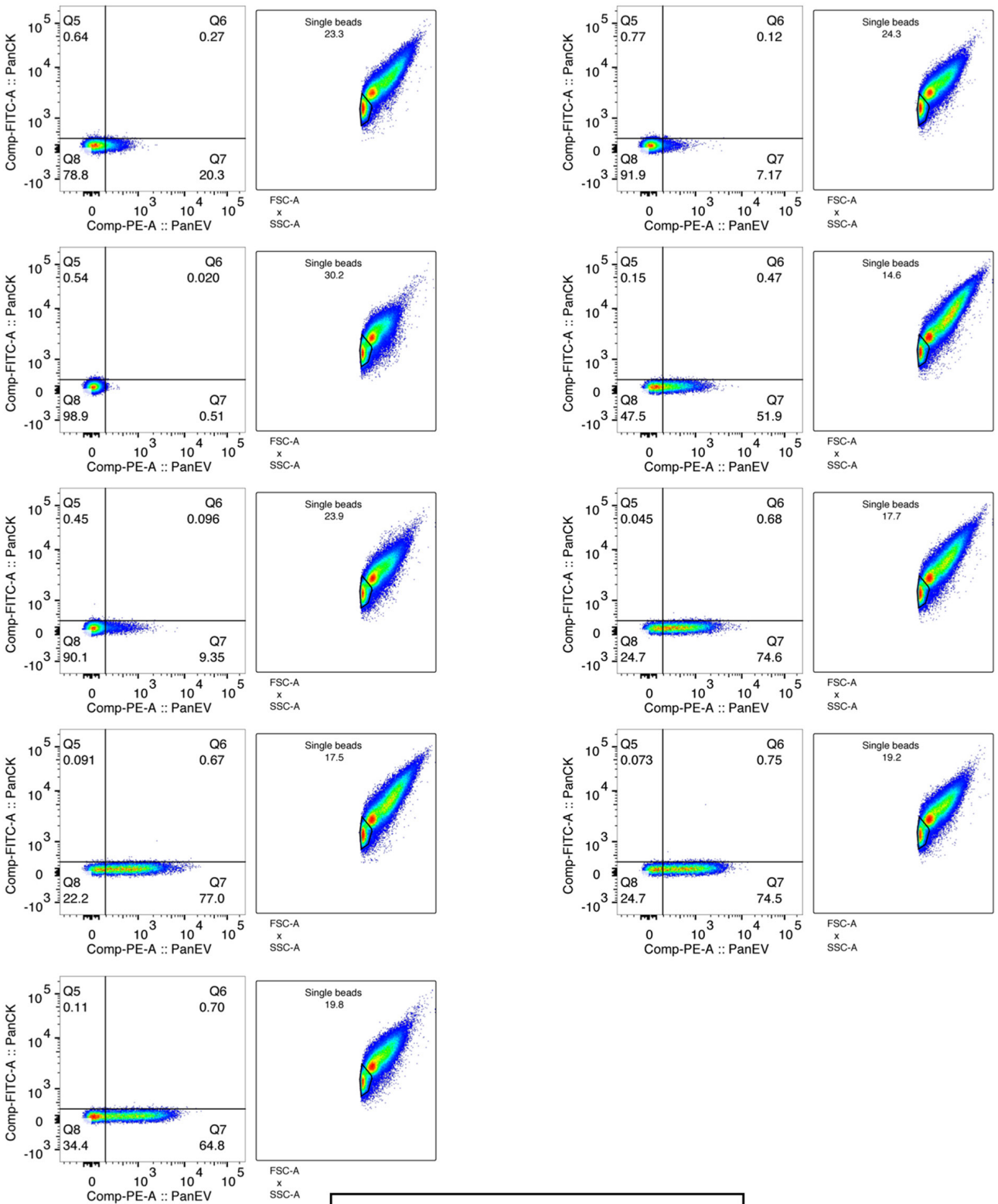


Figure S3. Continued.



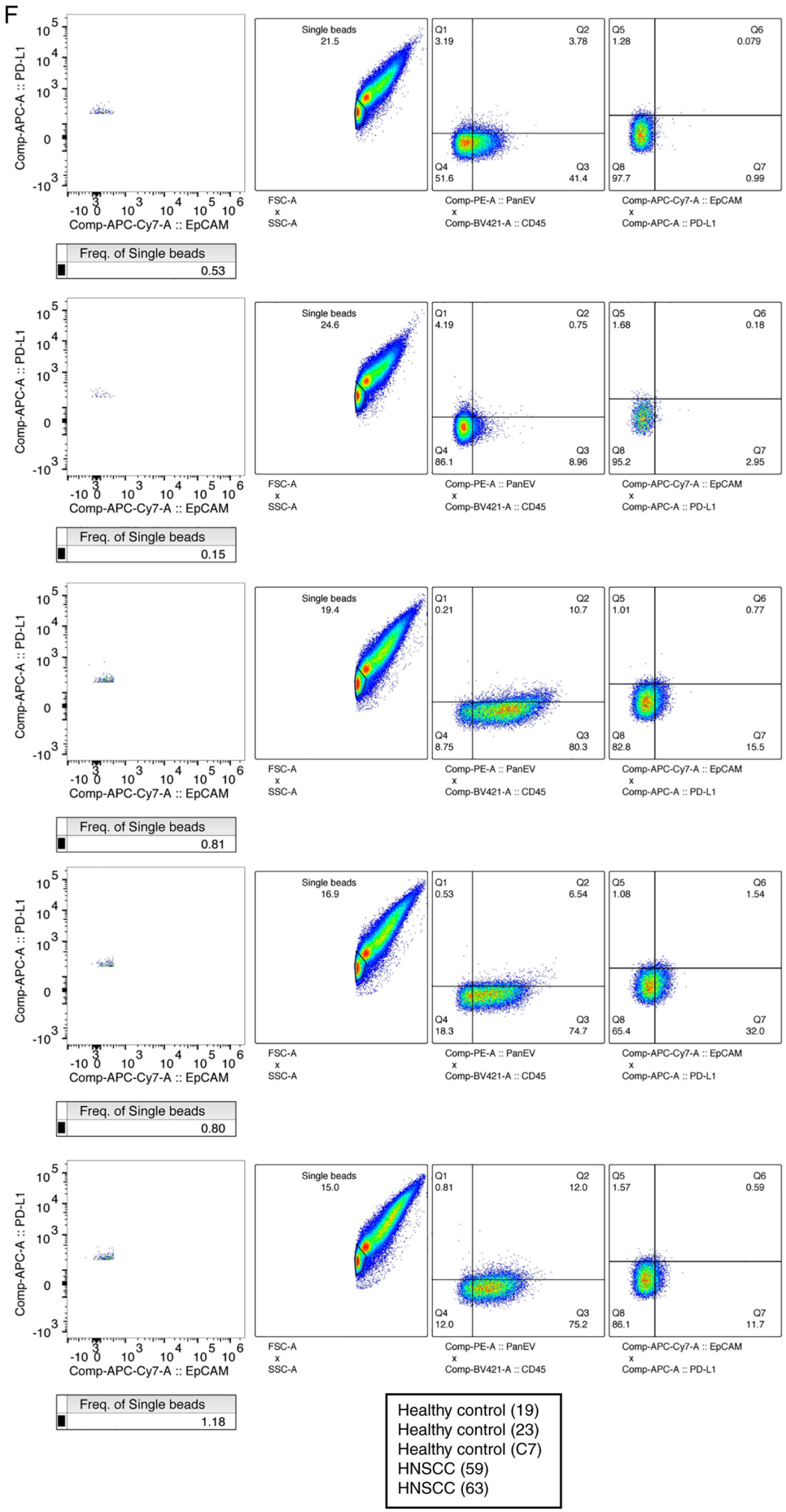
Healthy control (19)	Healthy control (20)
Healthy control (23)	Healthy control (24)
Healthy control (C7)	Healthy control (CL)
HNSCC (59)	HNSCC (60)
HNSCC (63)	HNSCC (64)

Figure S3. Continued.



Healthy control (21)	Healthy control (22)
EF (Negative control)	Healthy control (C6)
HNSCC (52)	HNSCC (55)
HNSCC (61)	HNSCC (62)
HNSCC (65)	

Figure S3. Continued.



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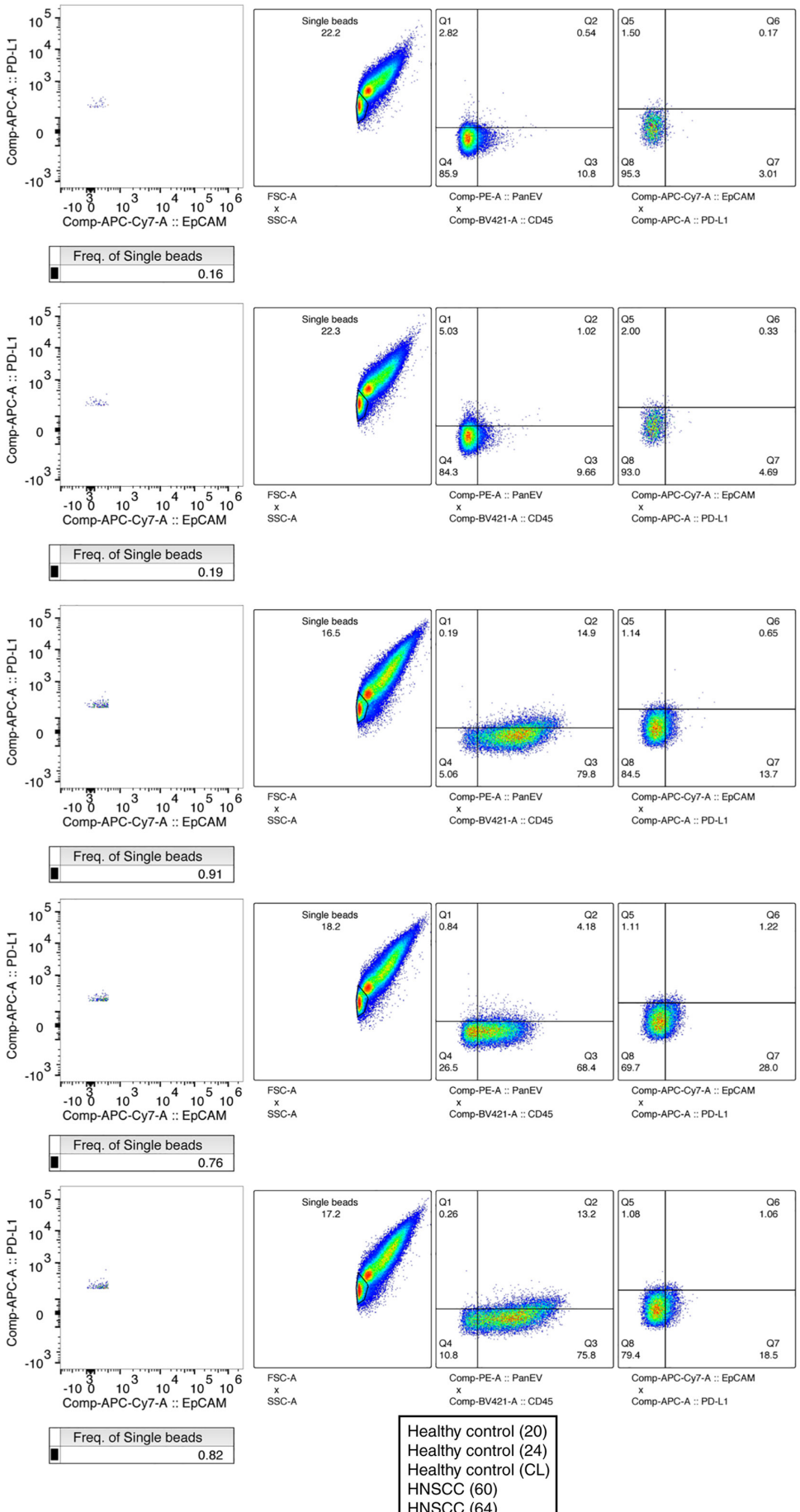

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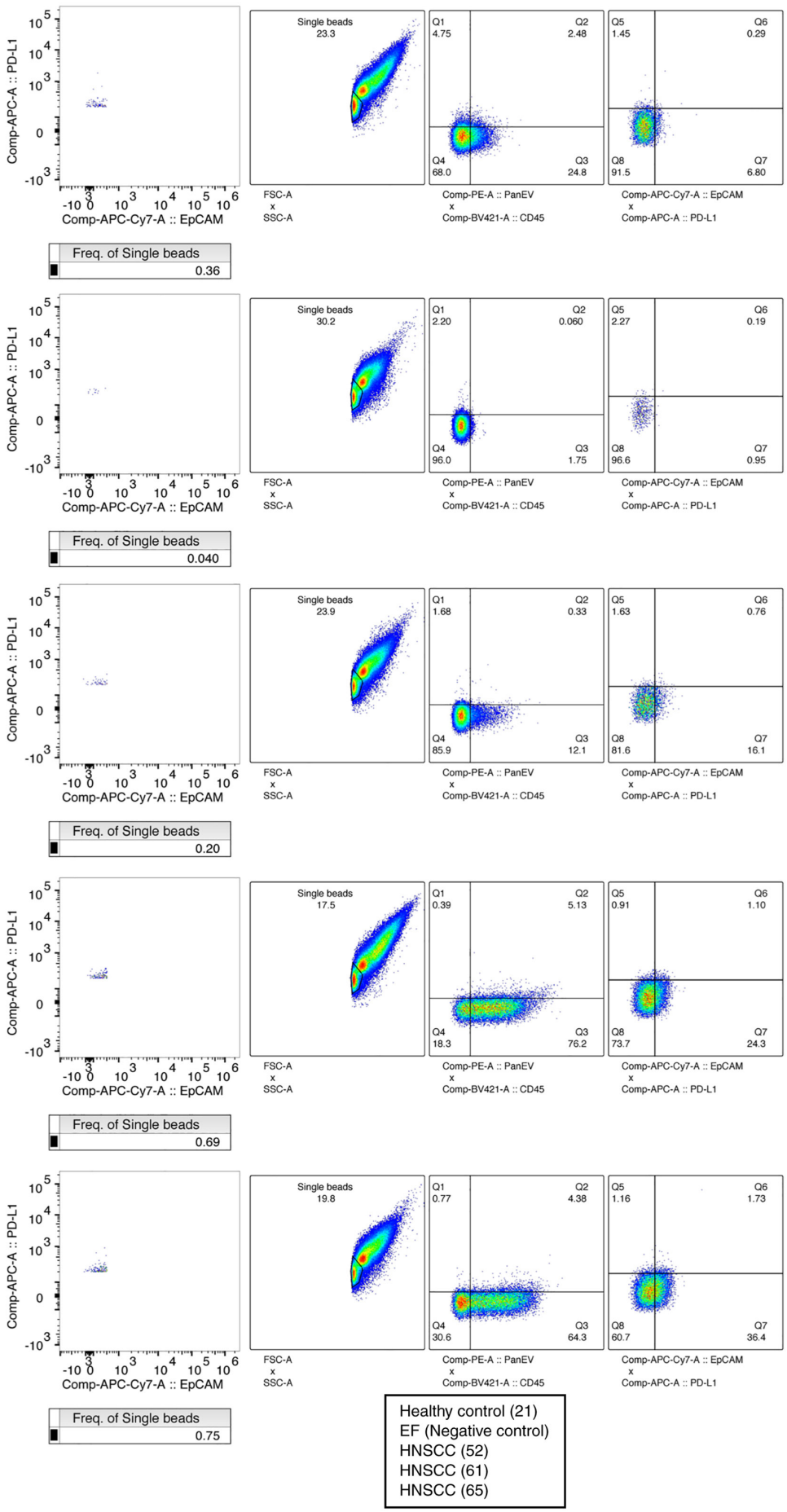

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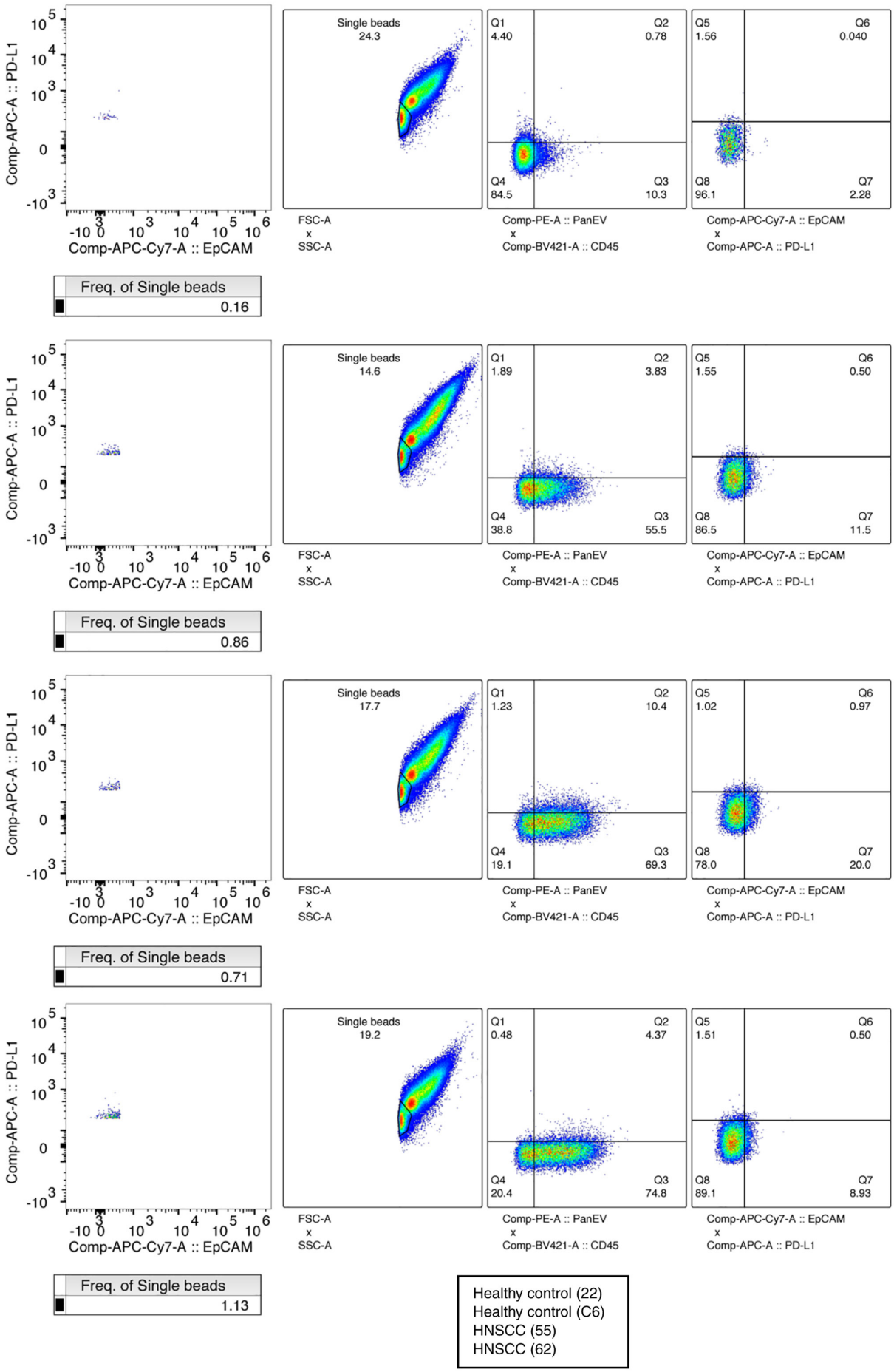

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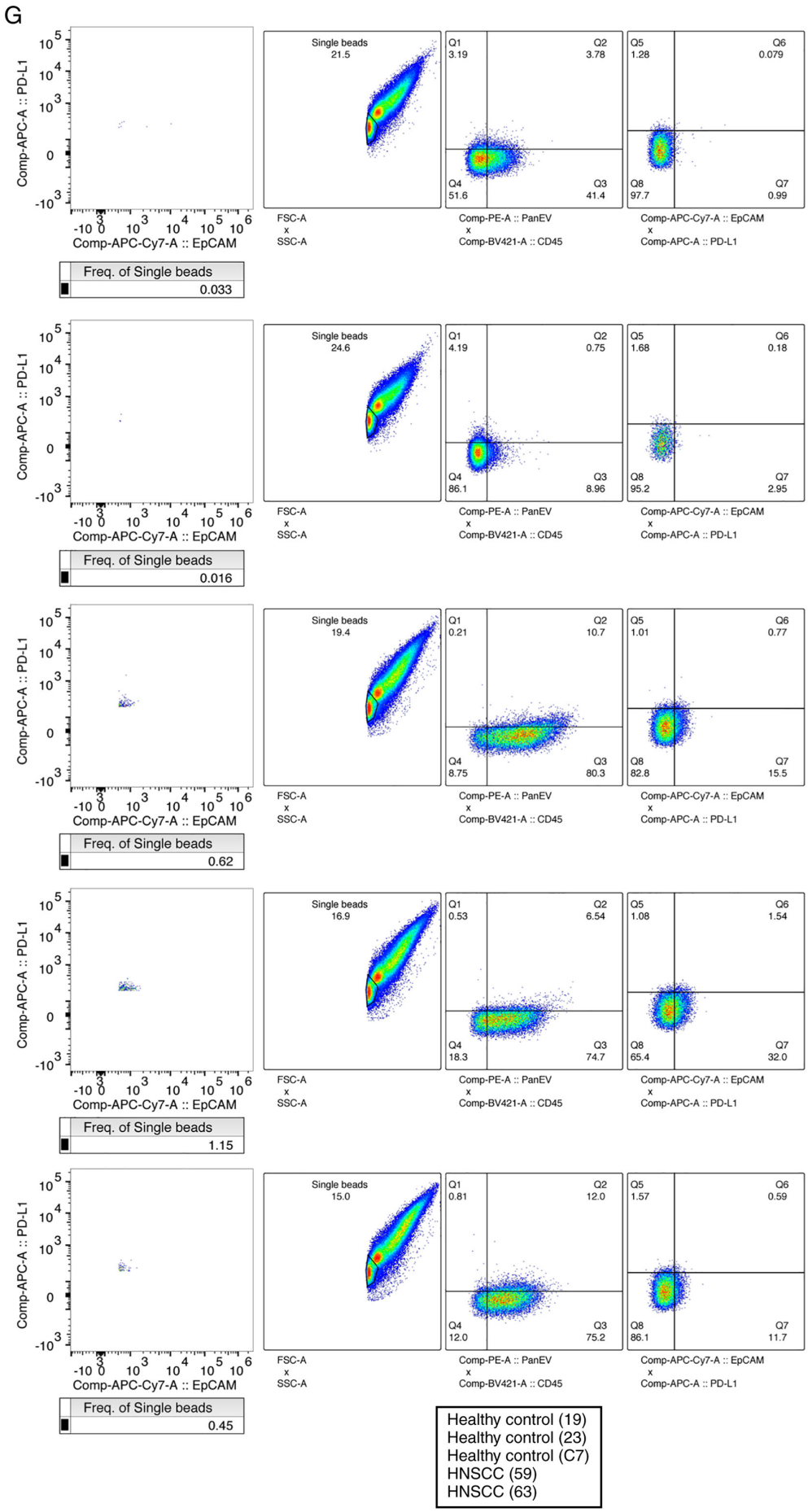

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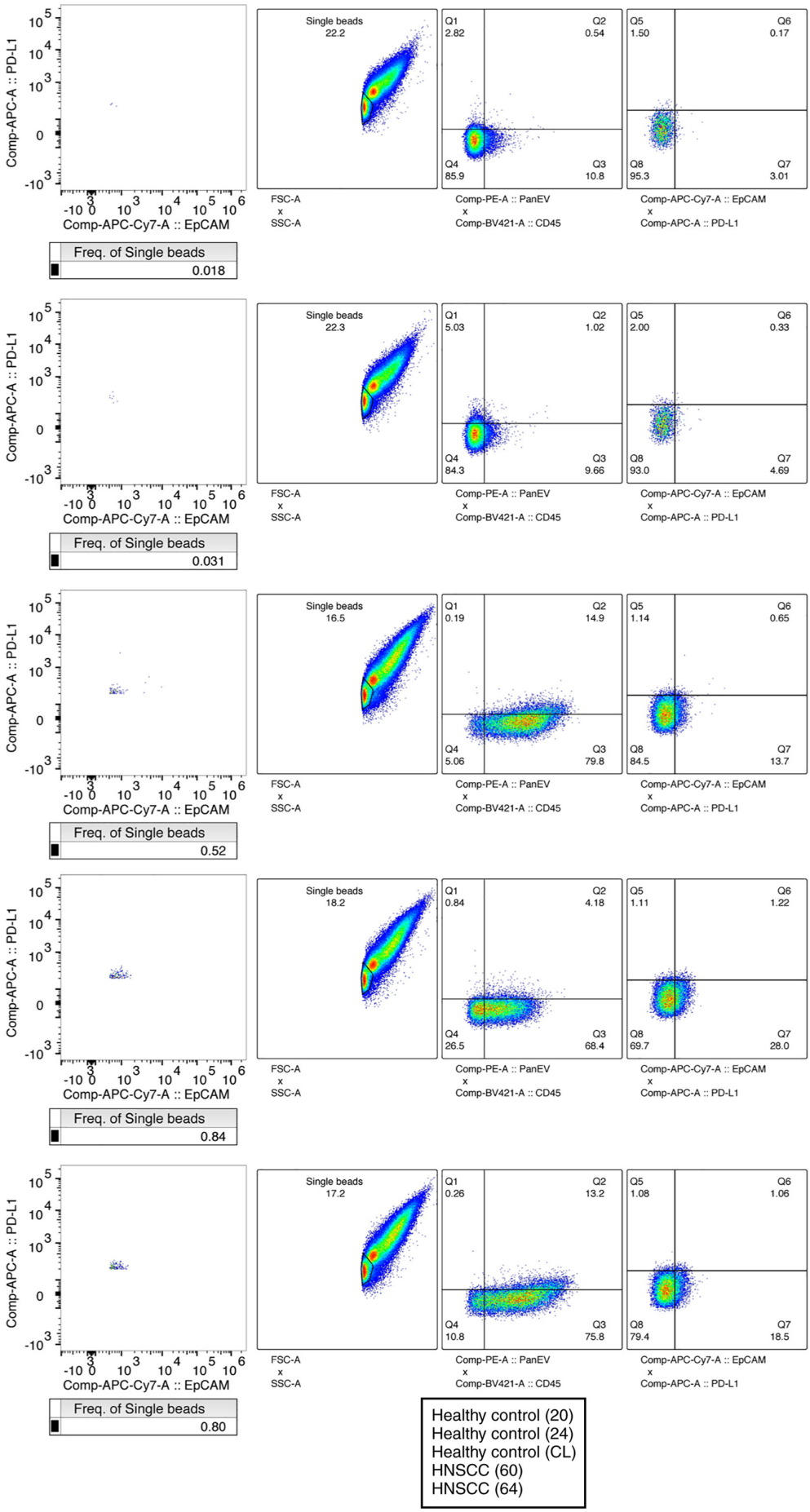

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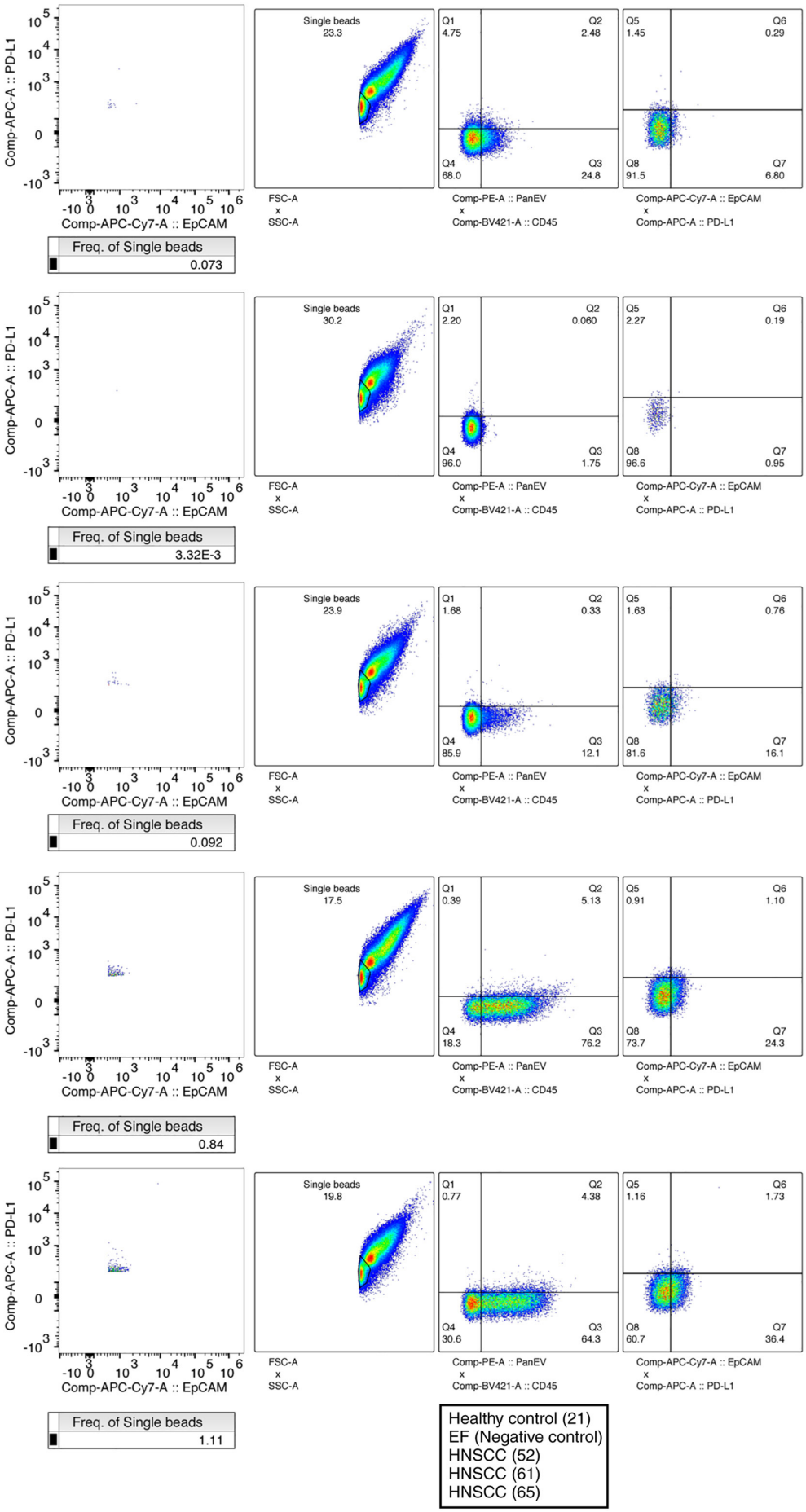

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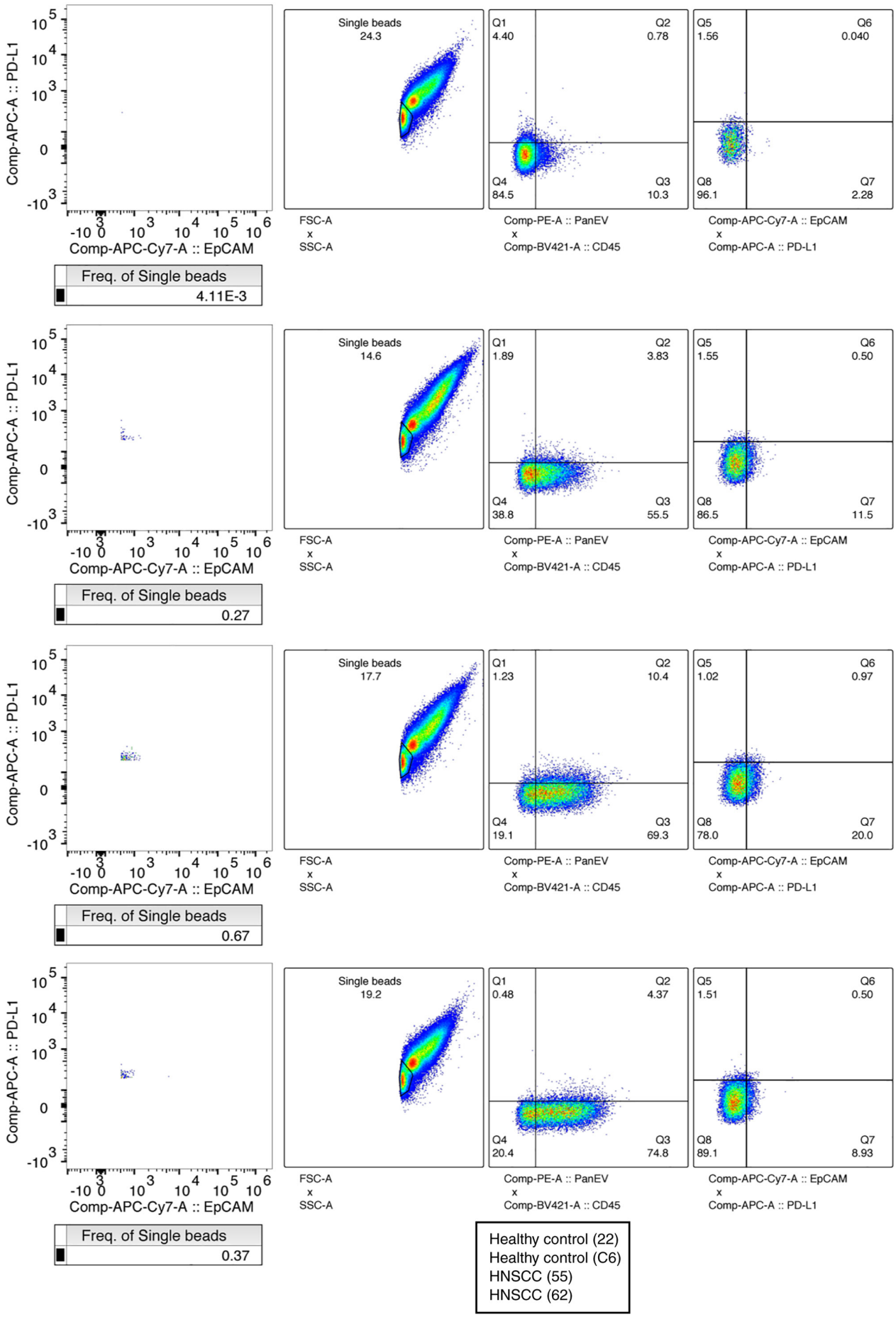

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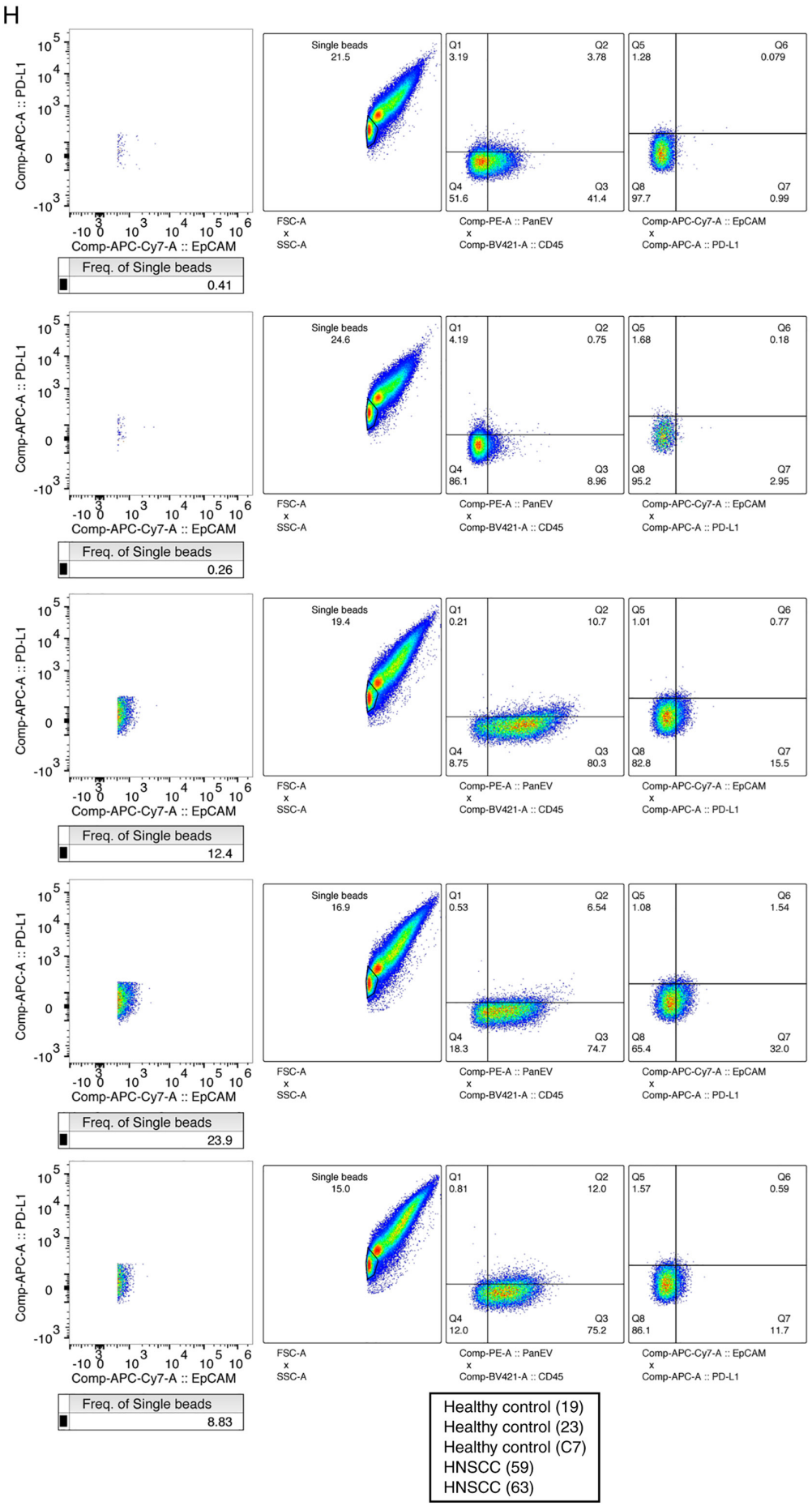

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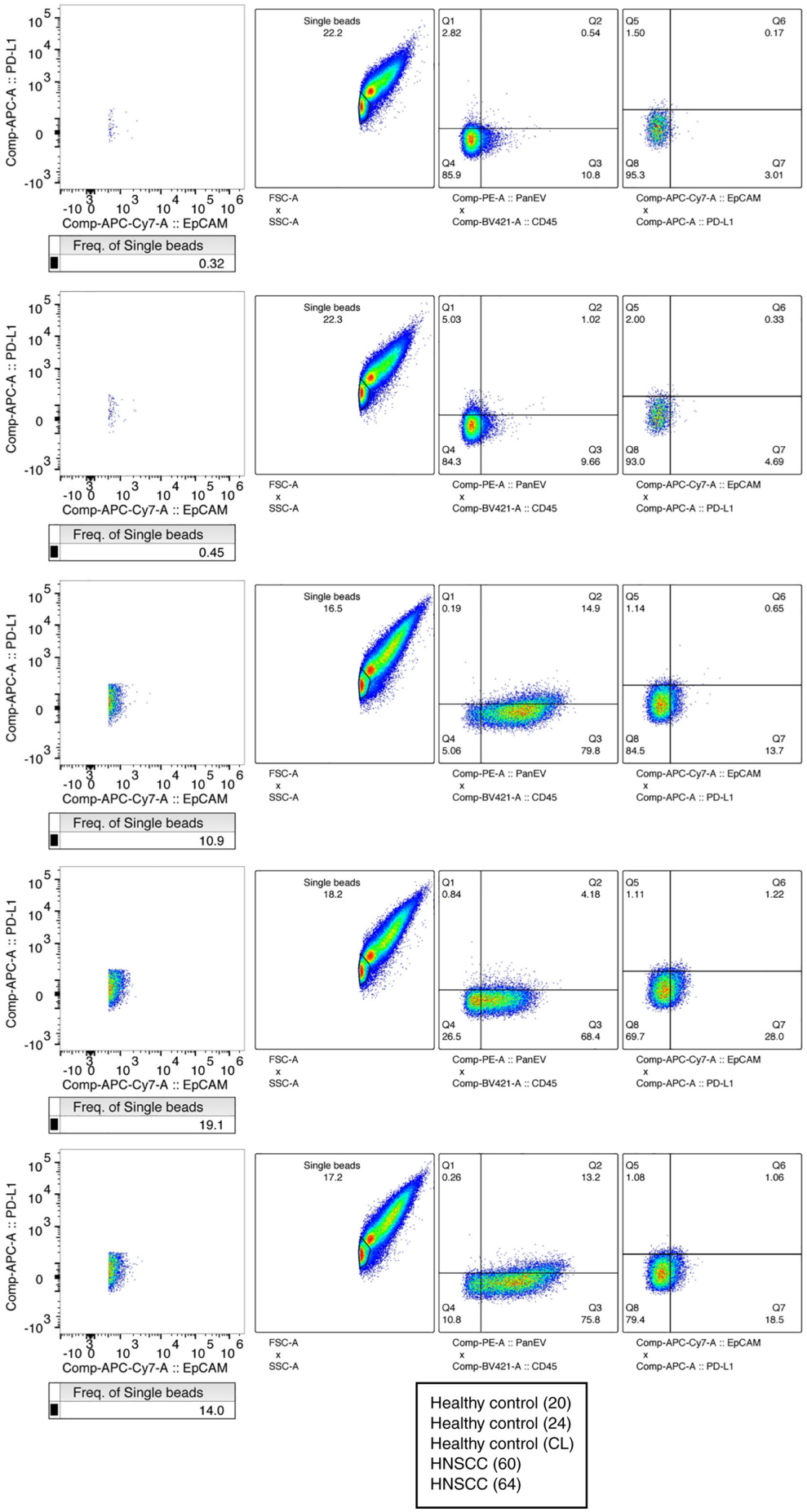

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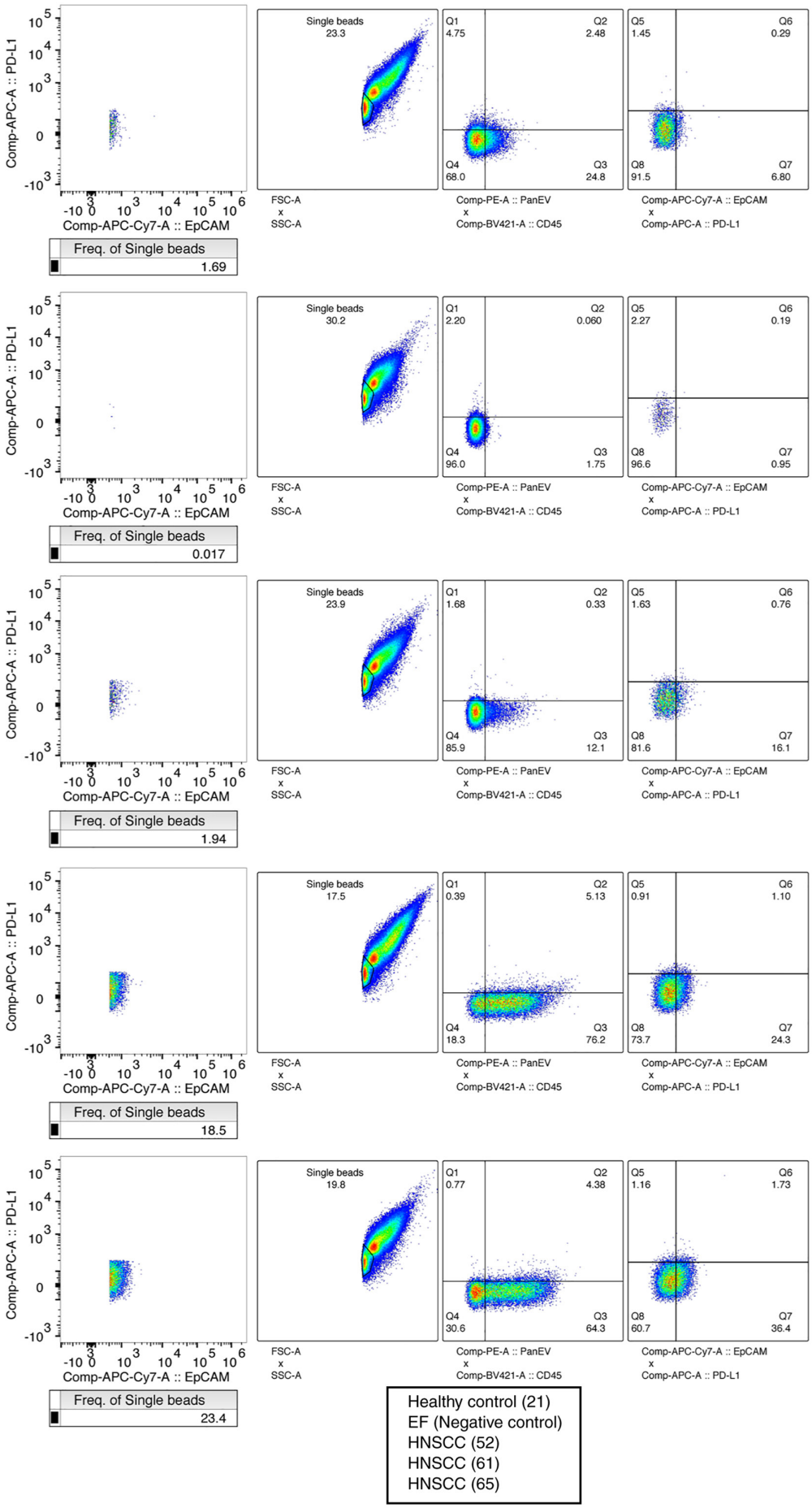

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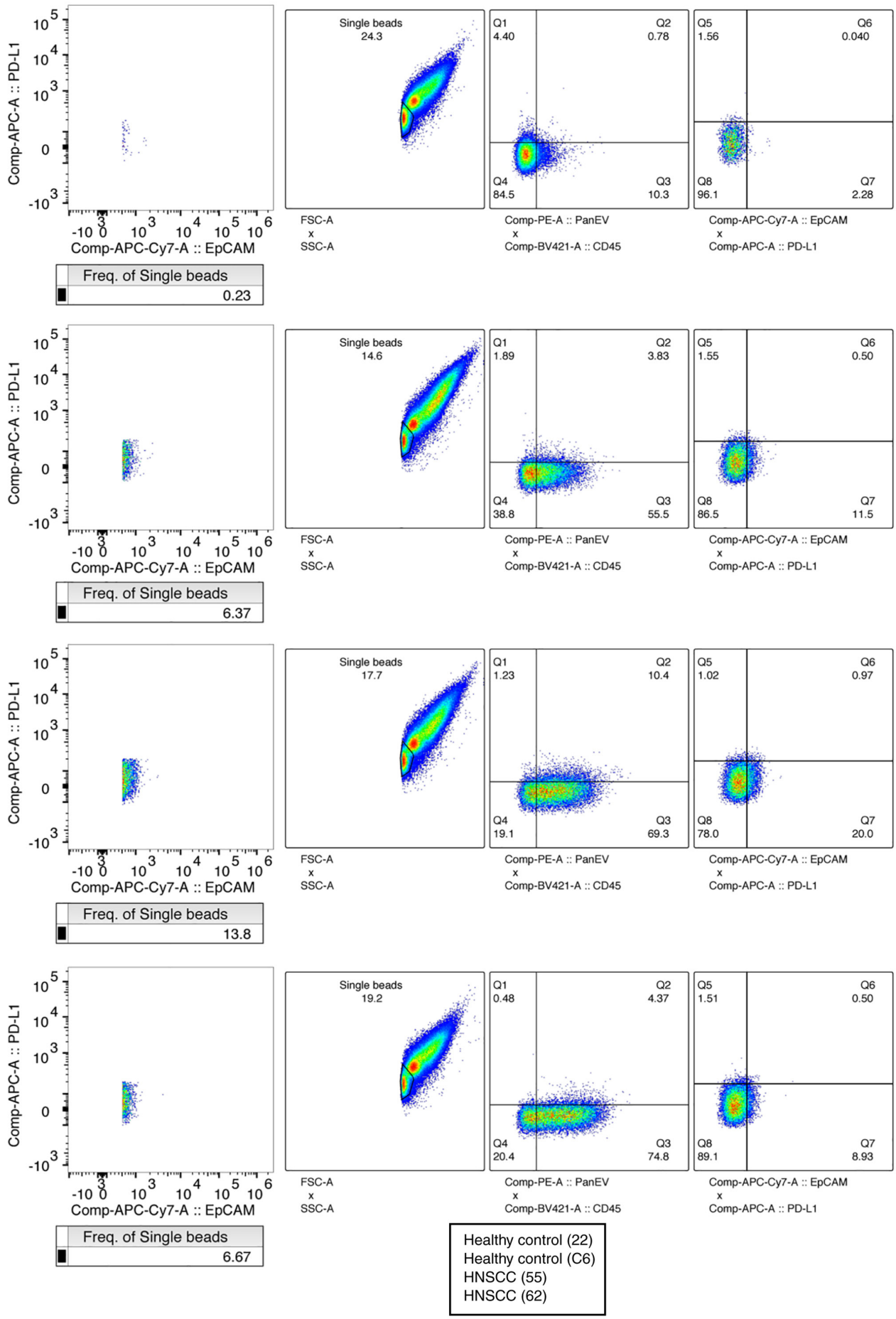

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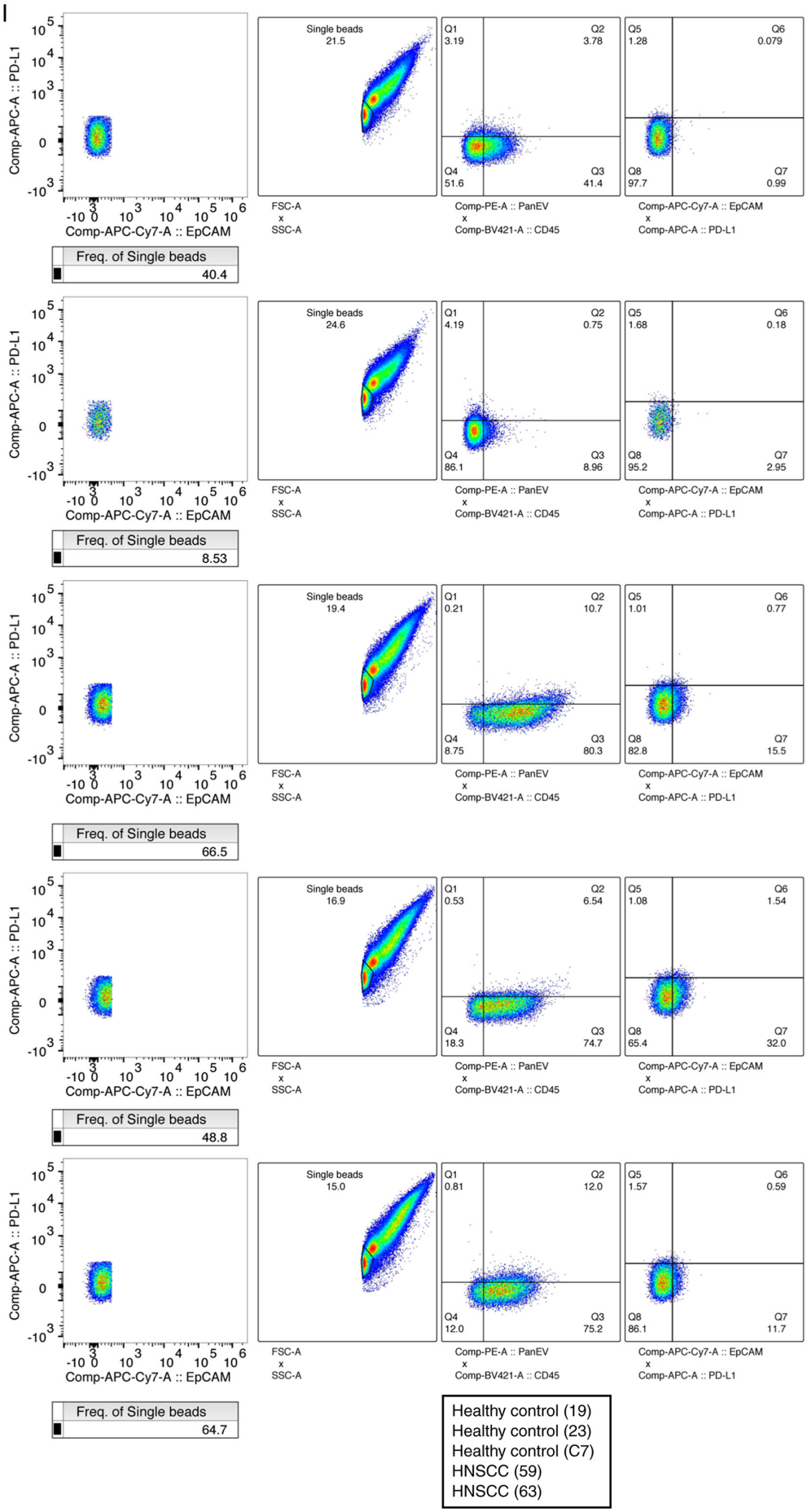

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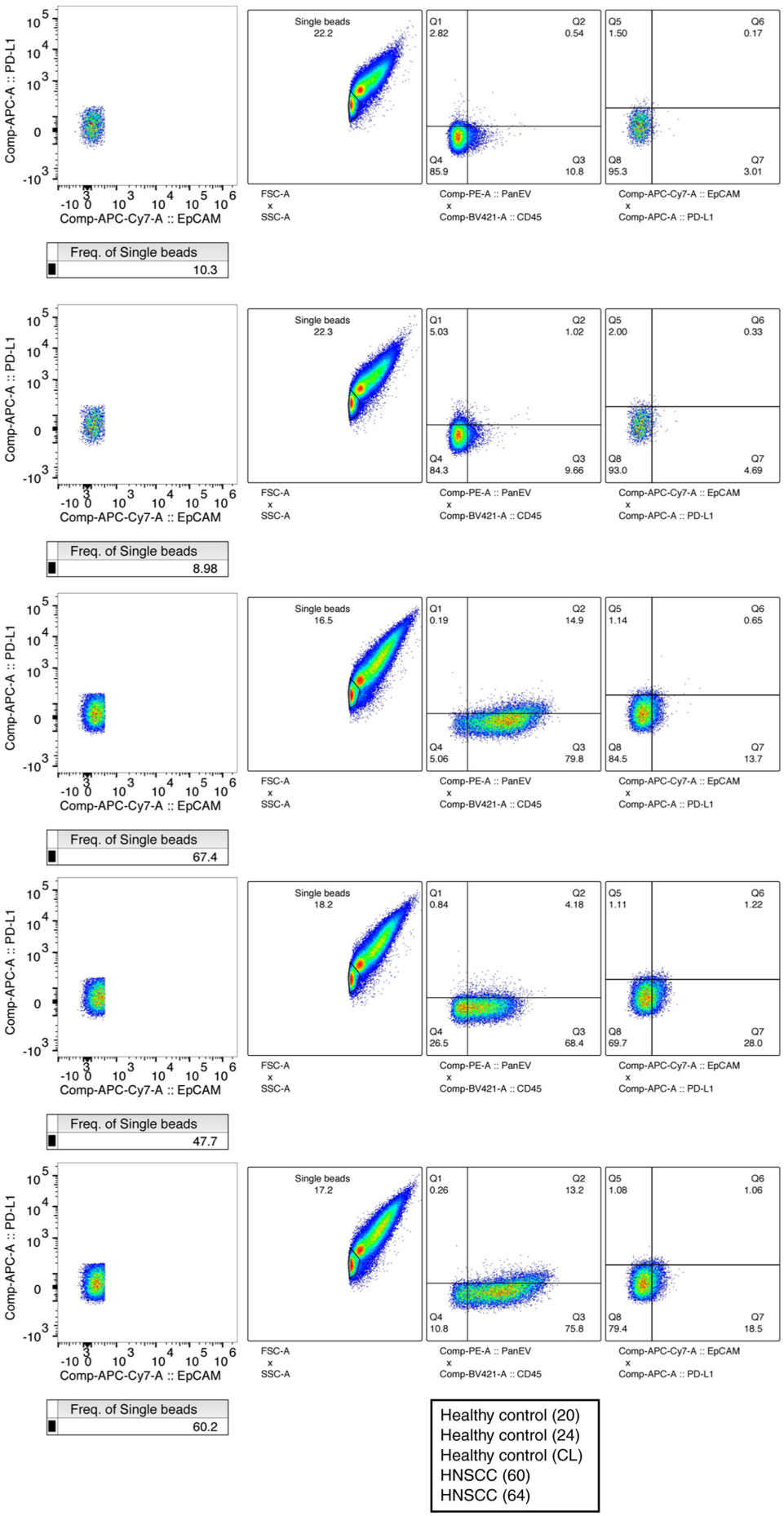

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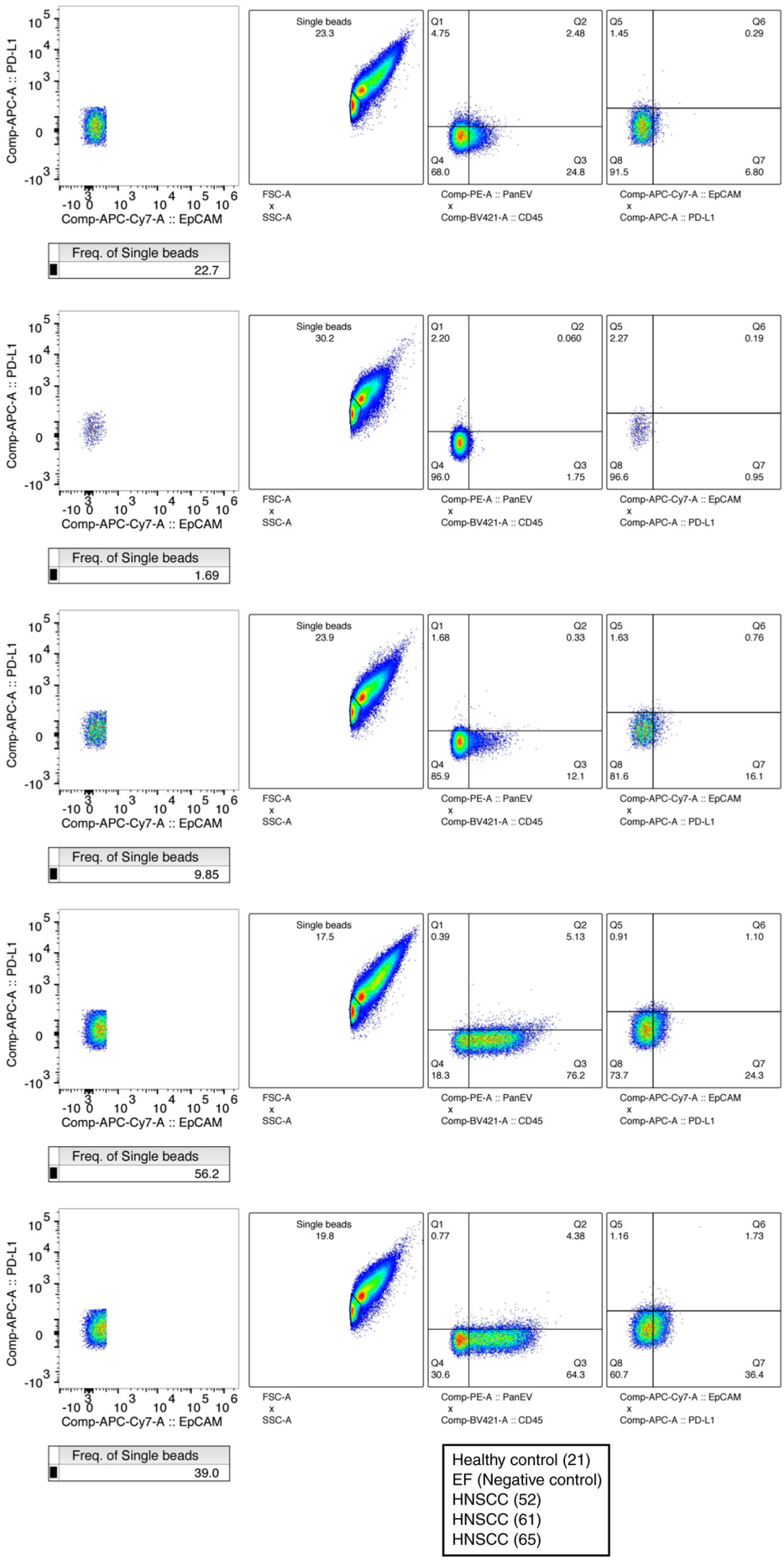

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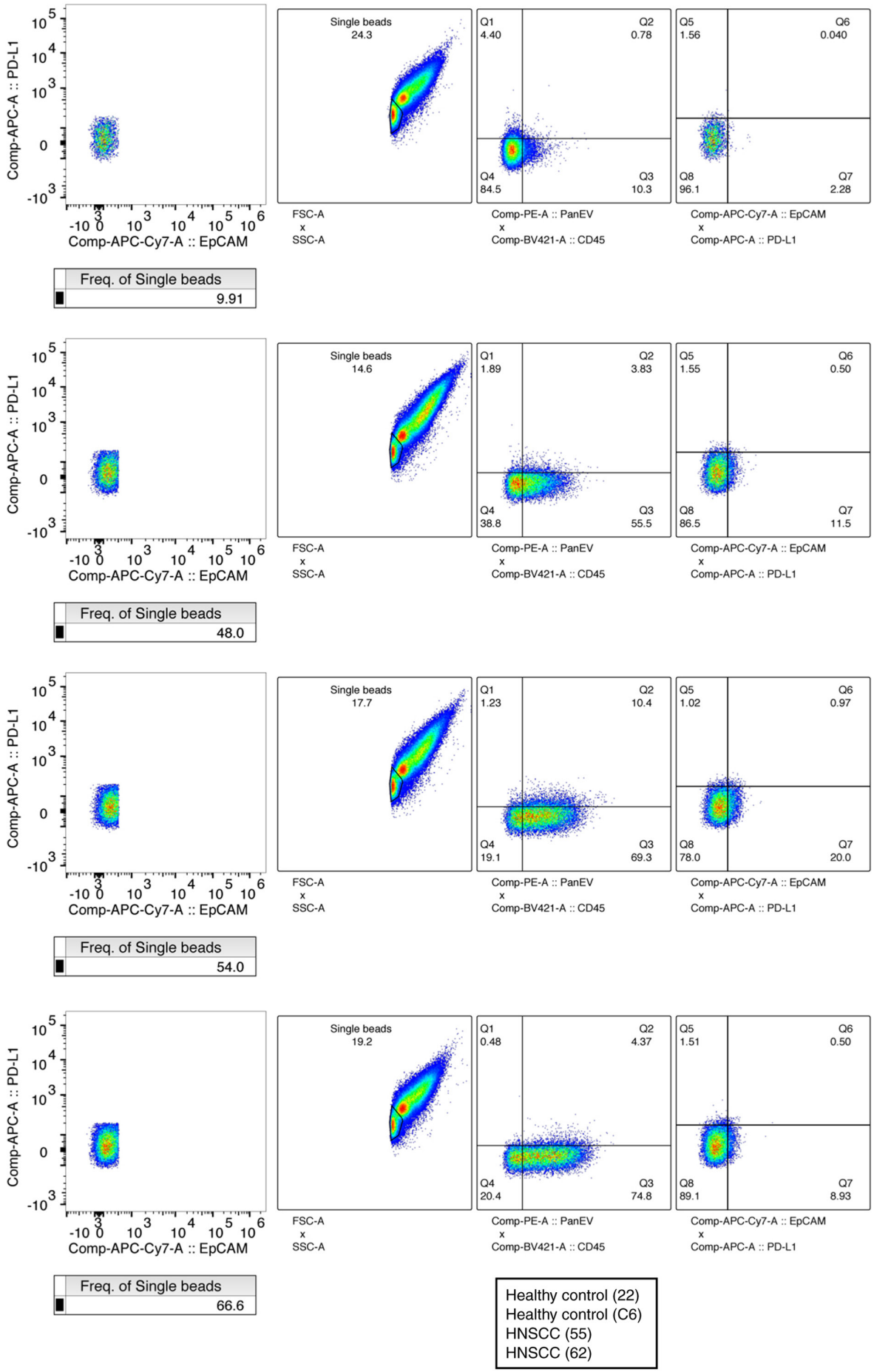

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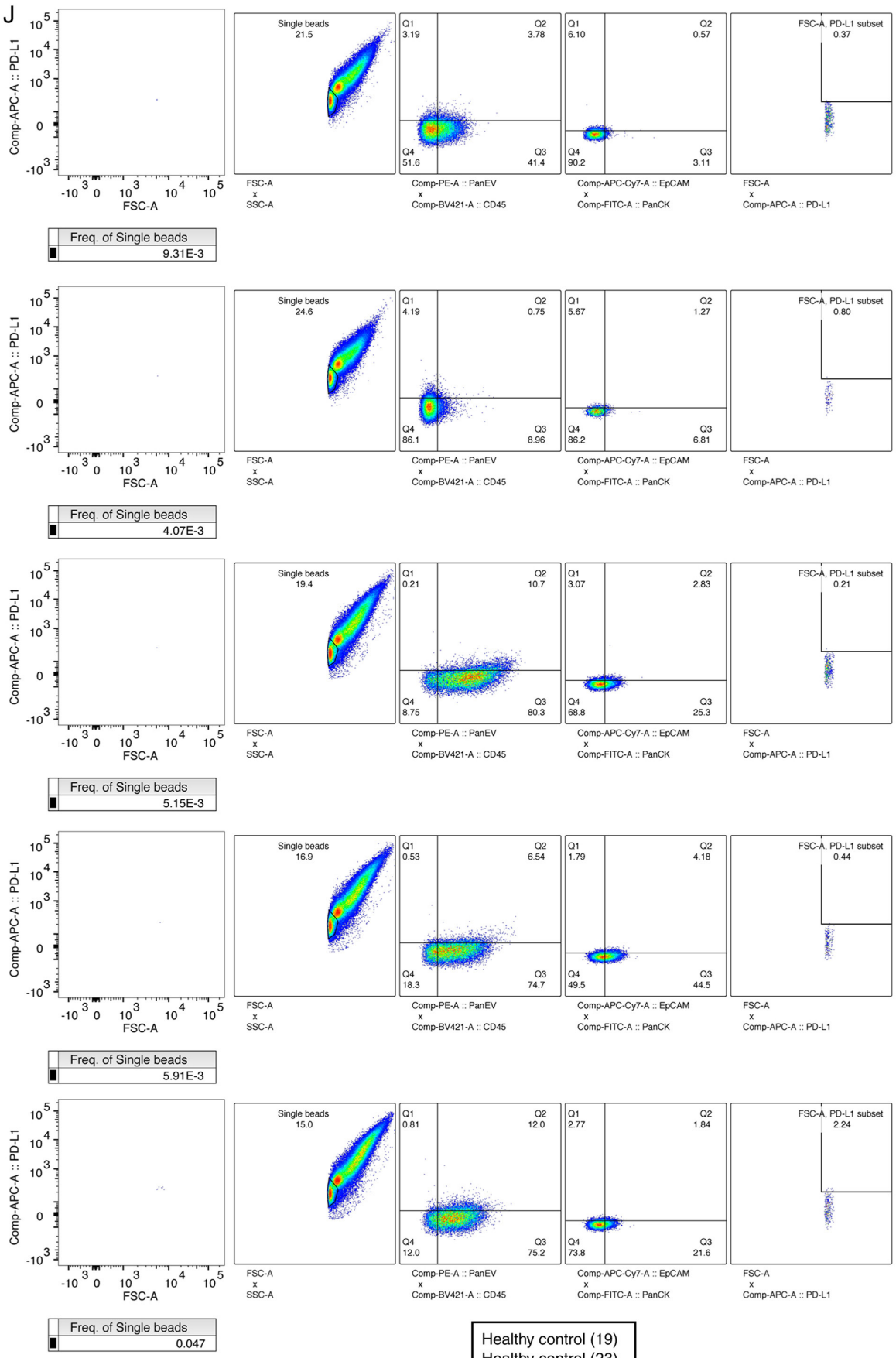

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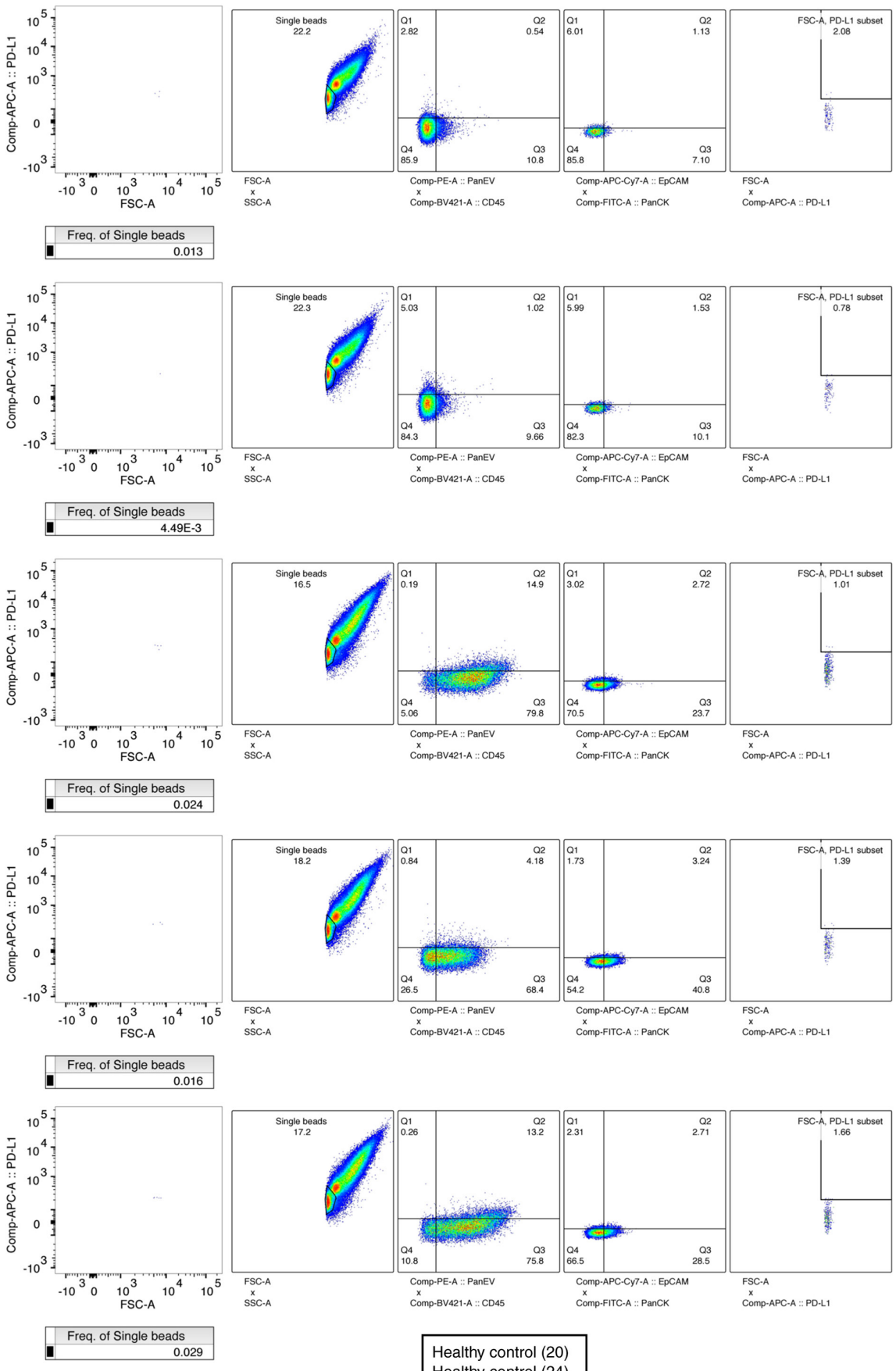
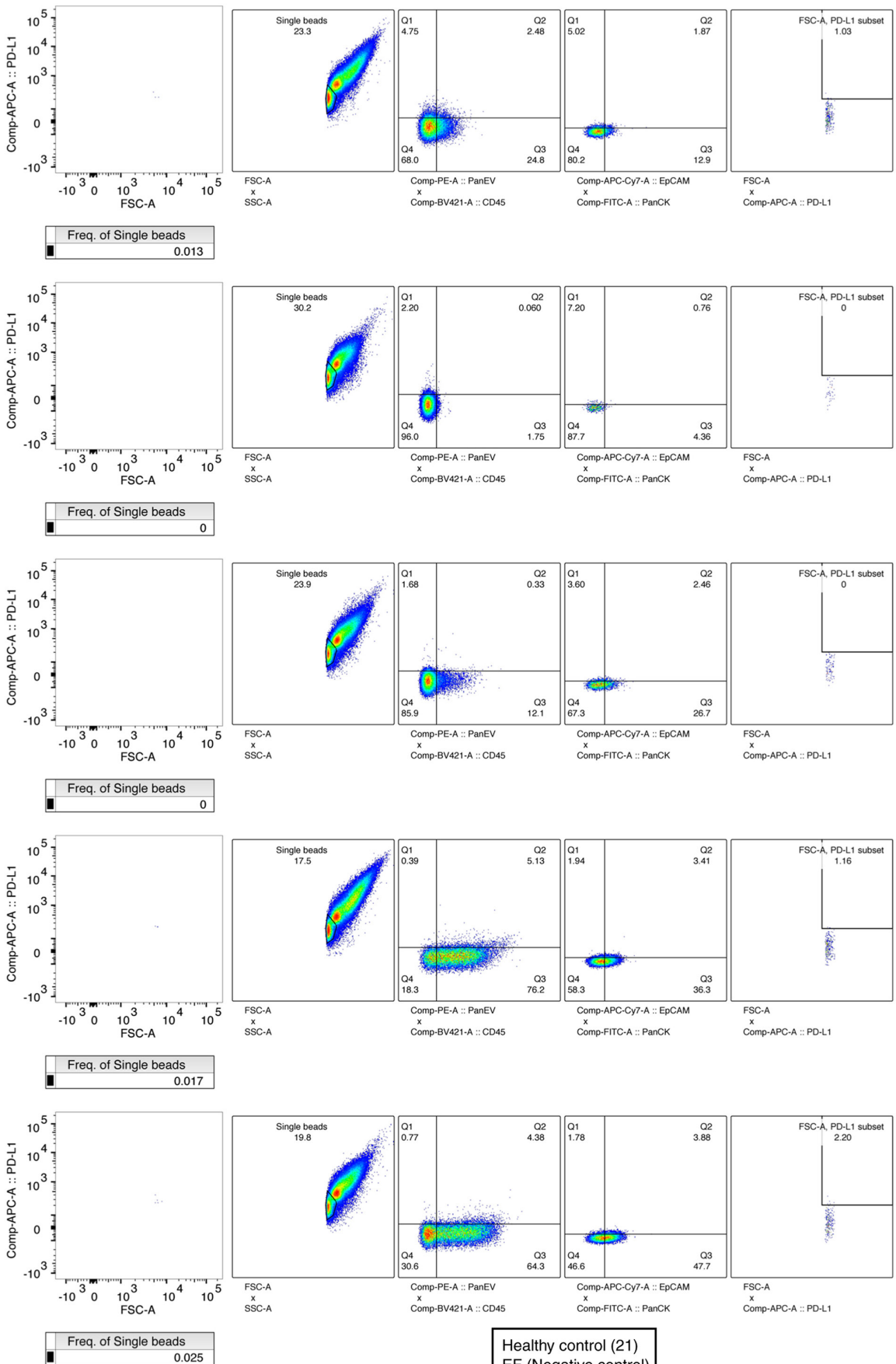


Healthy control (20)
Healthy control (24)
Healthy control (CL)
HNSCC (60)
HNSCC (64)

Figure S3. Continued.



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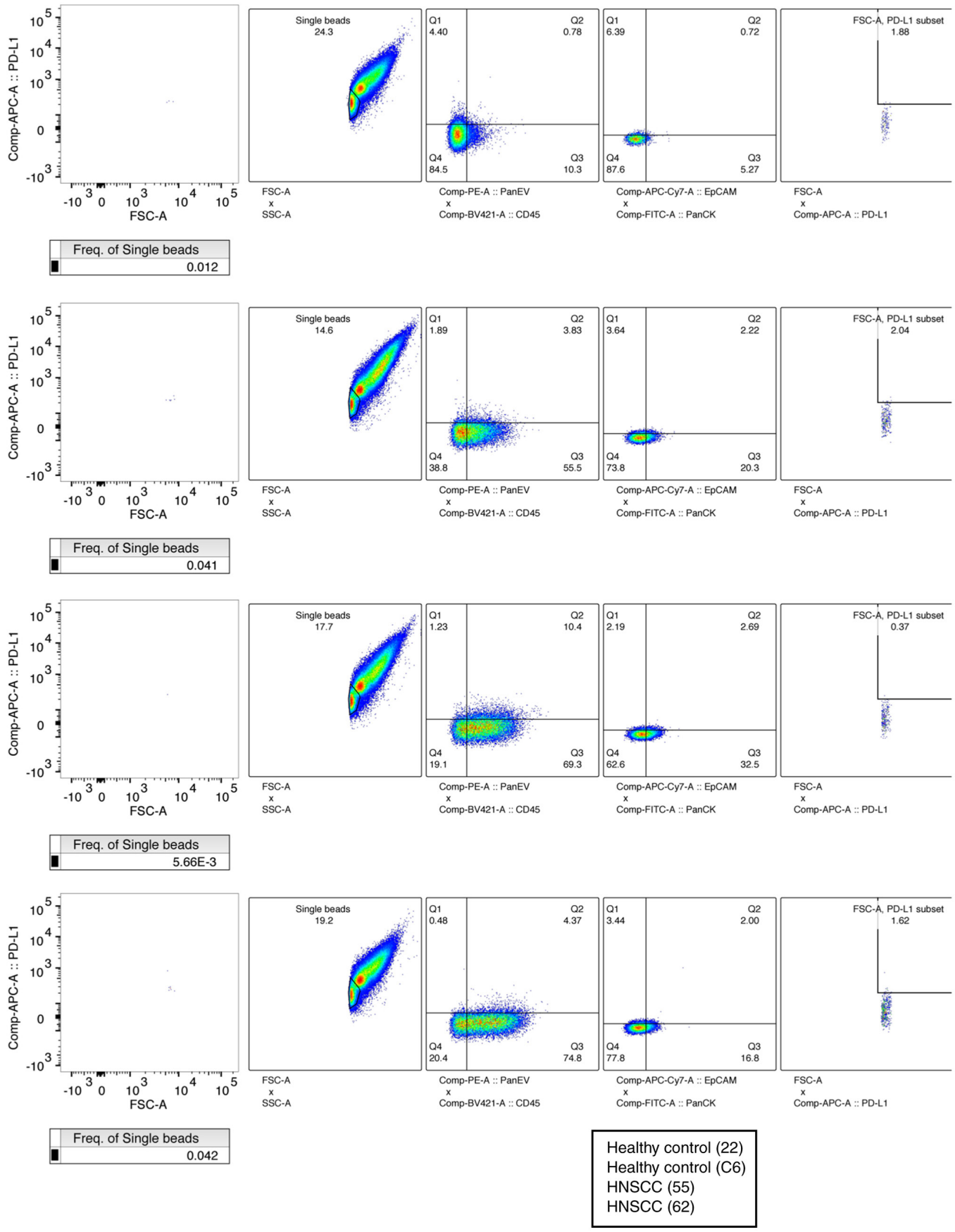

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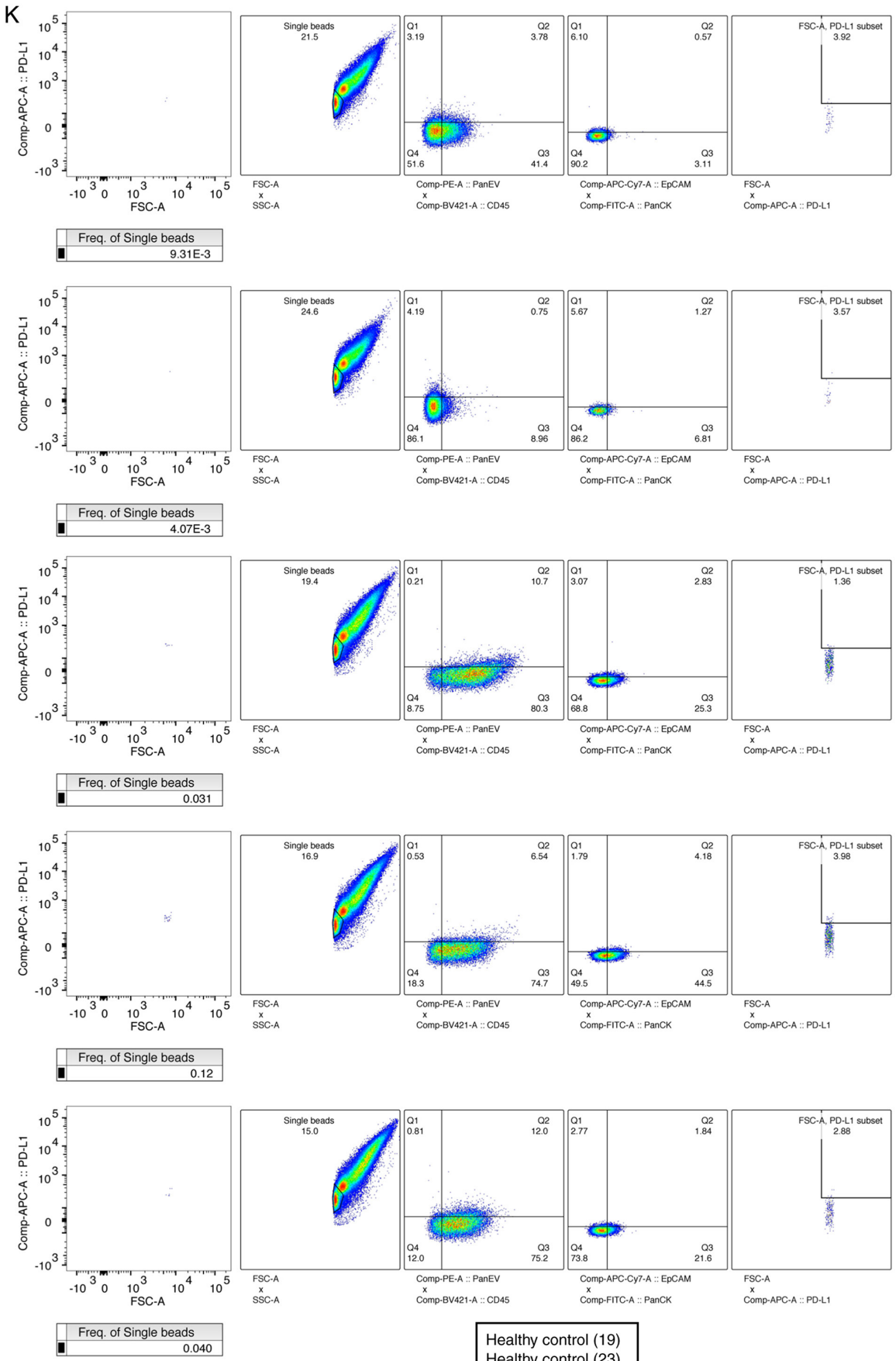

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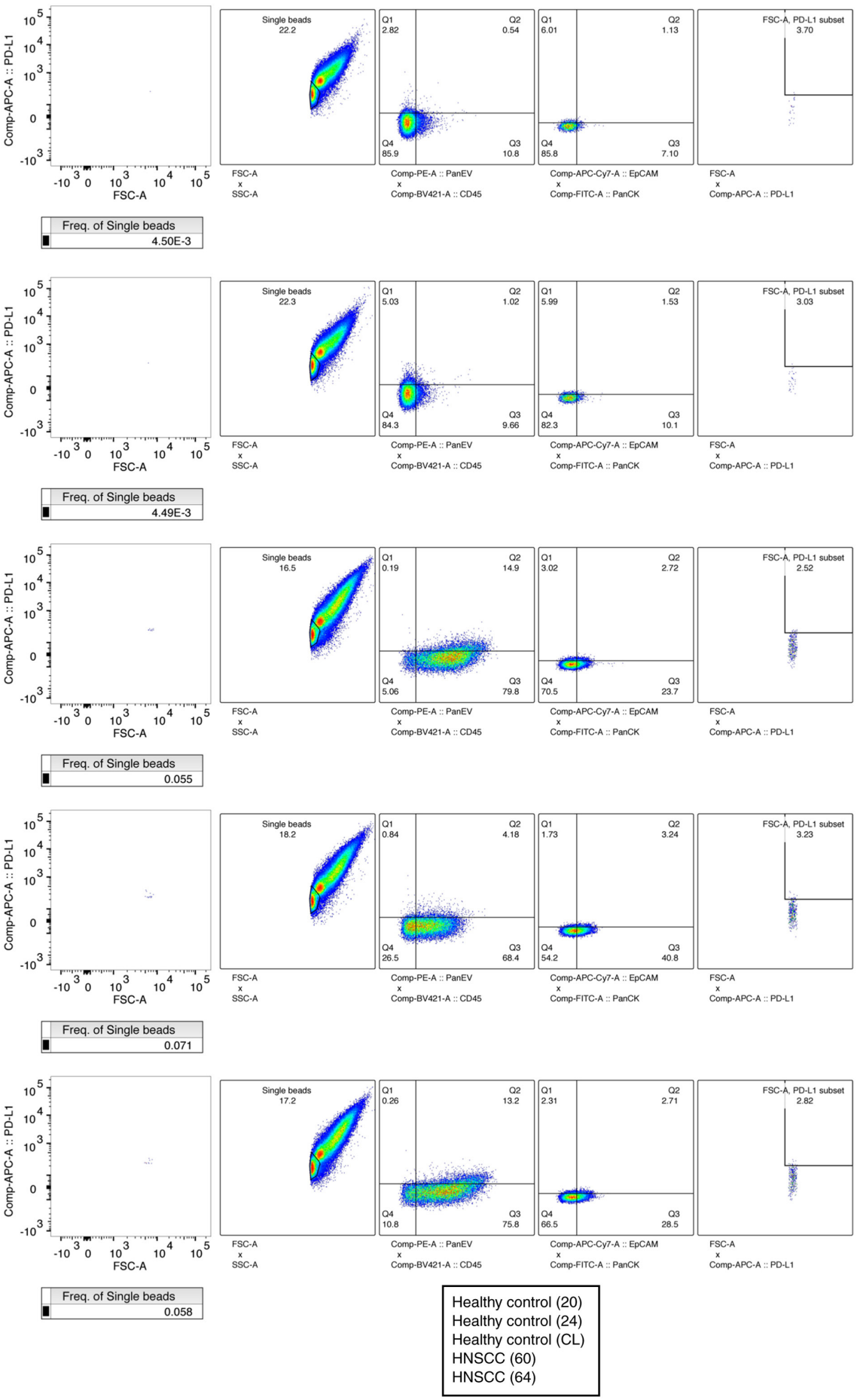

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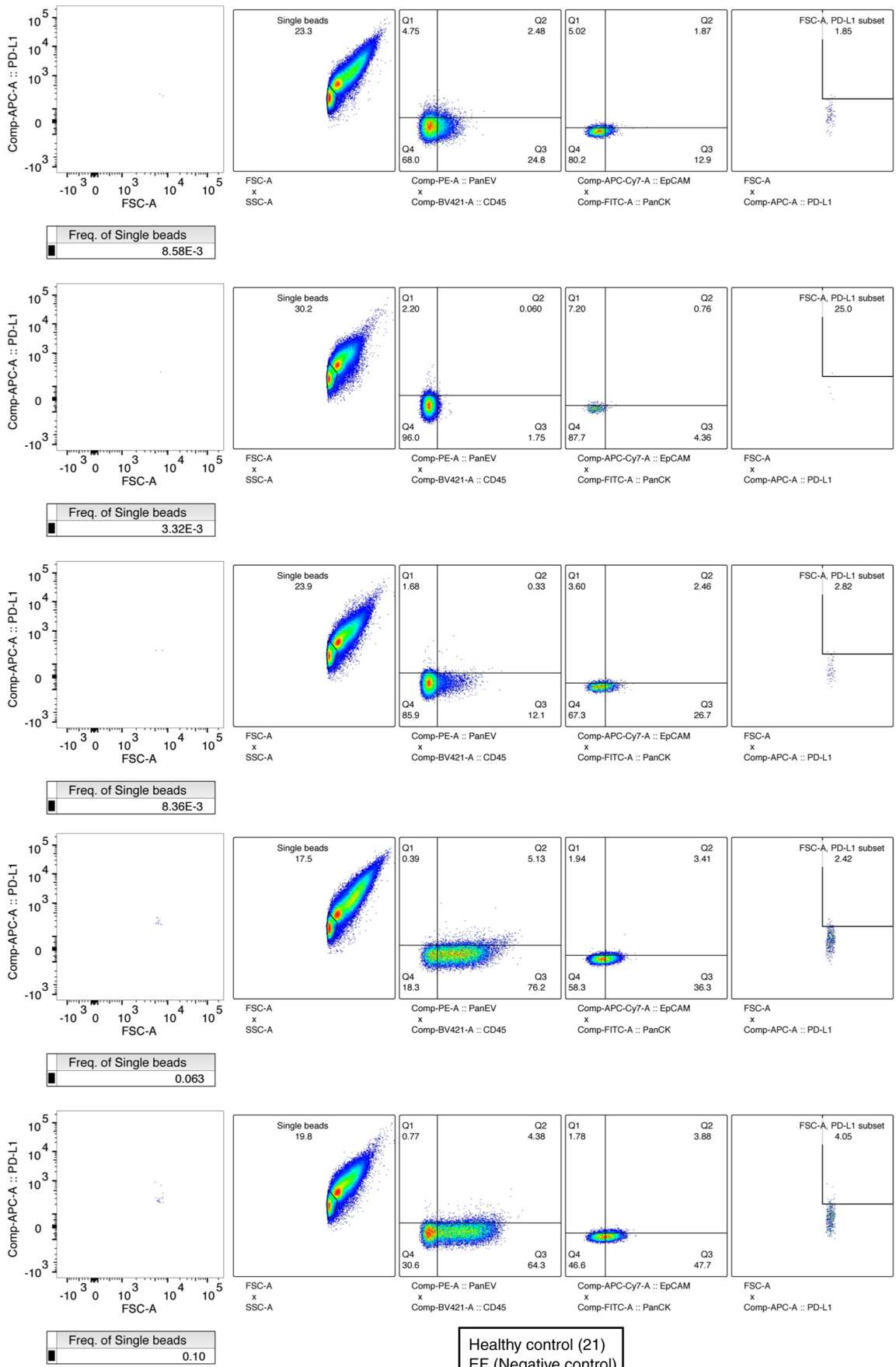

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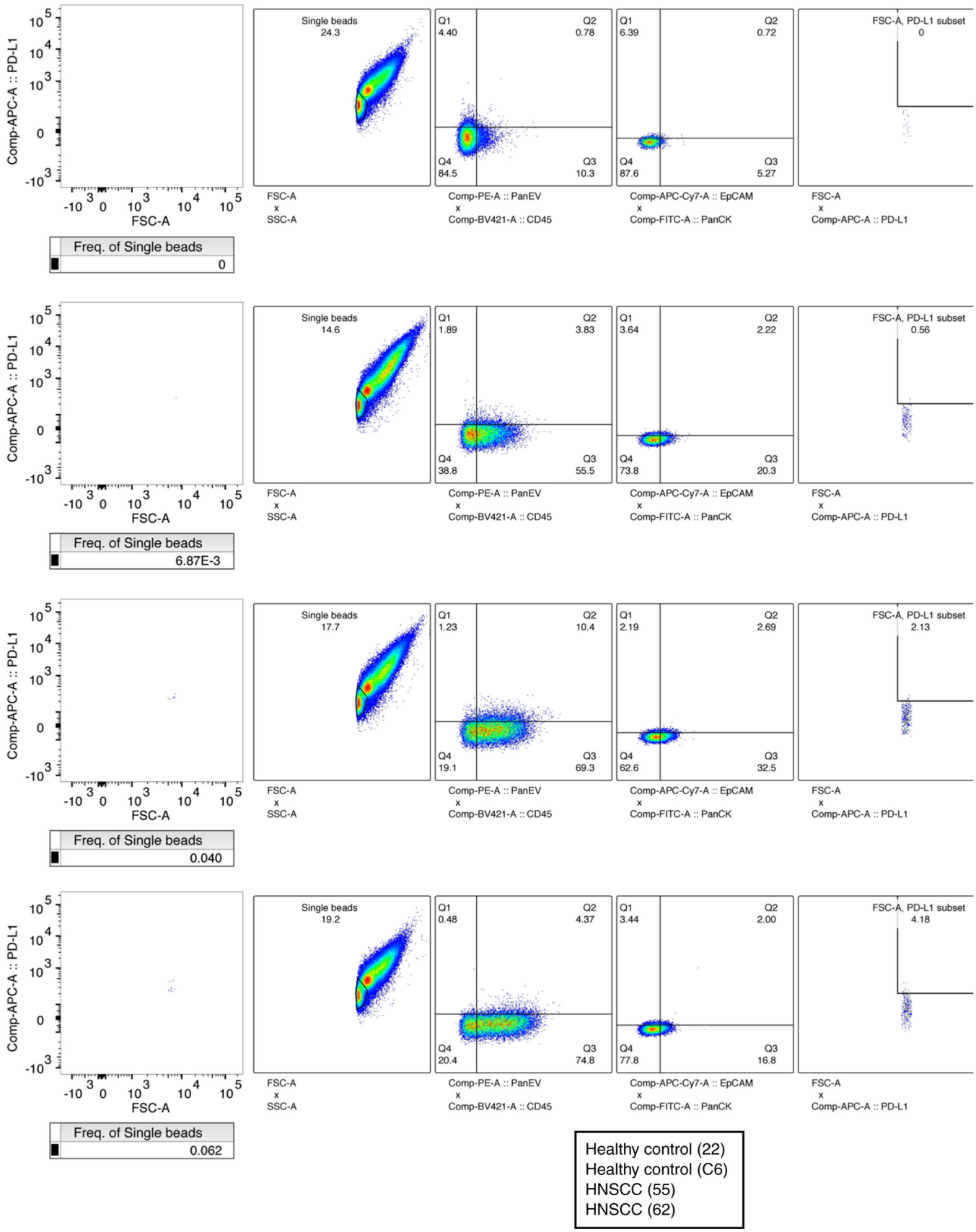

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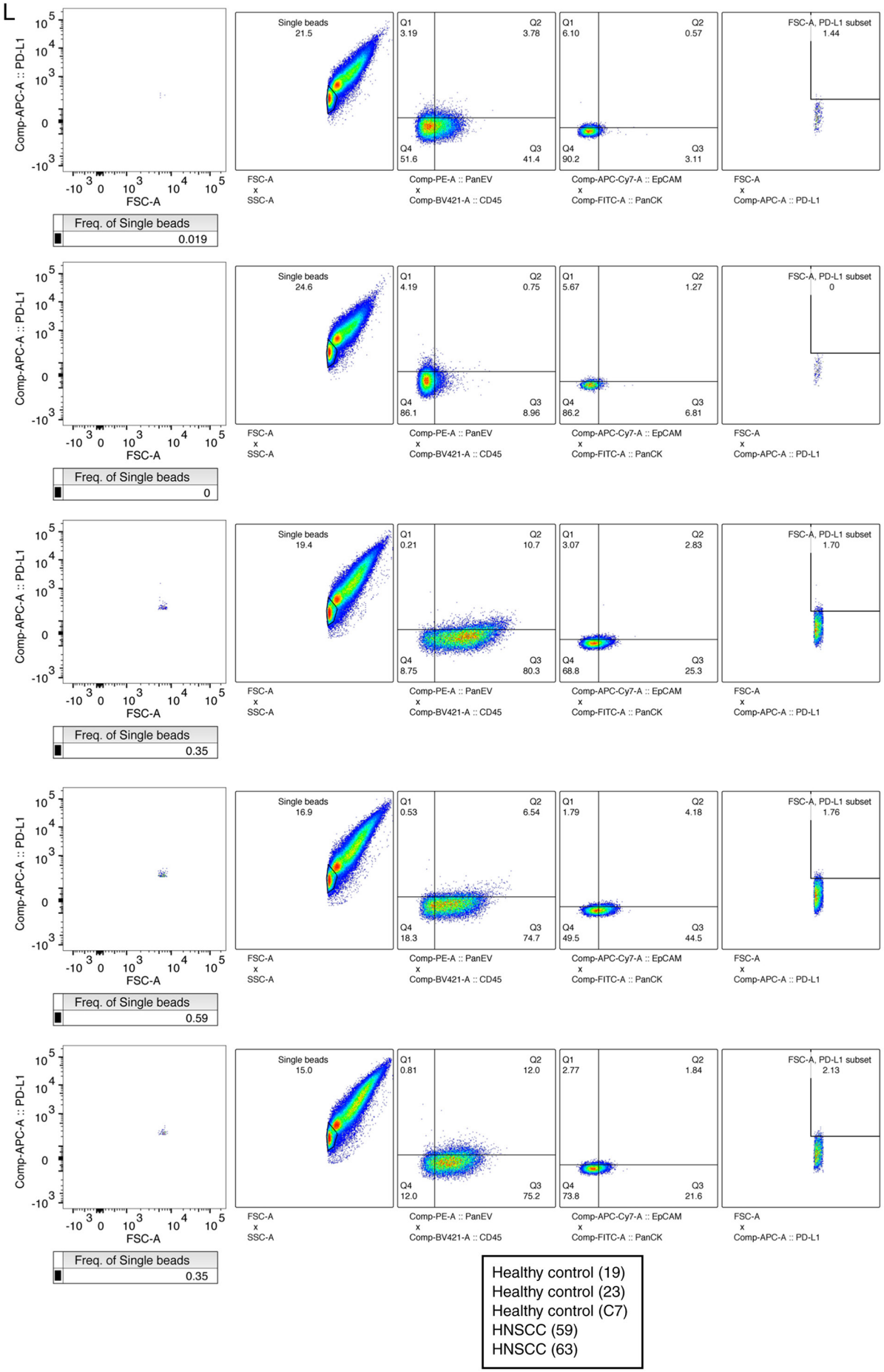

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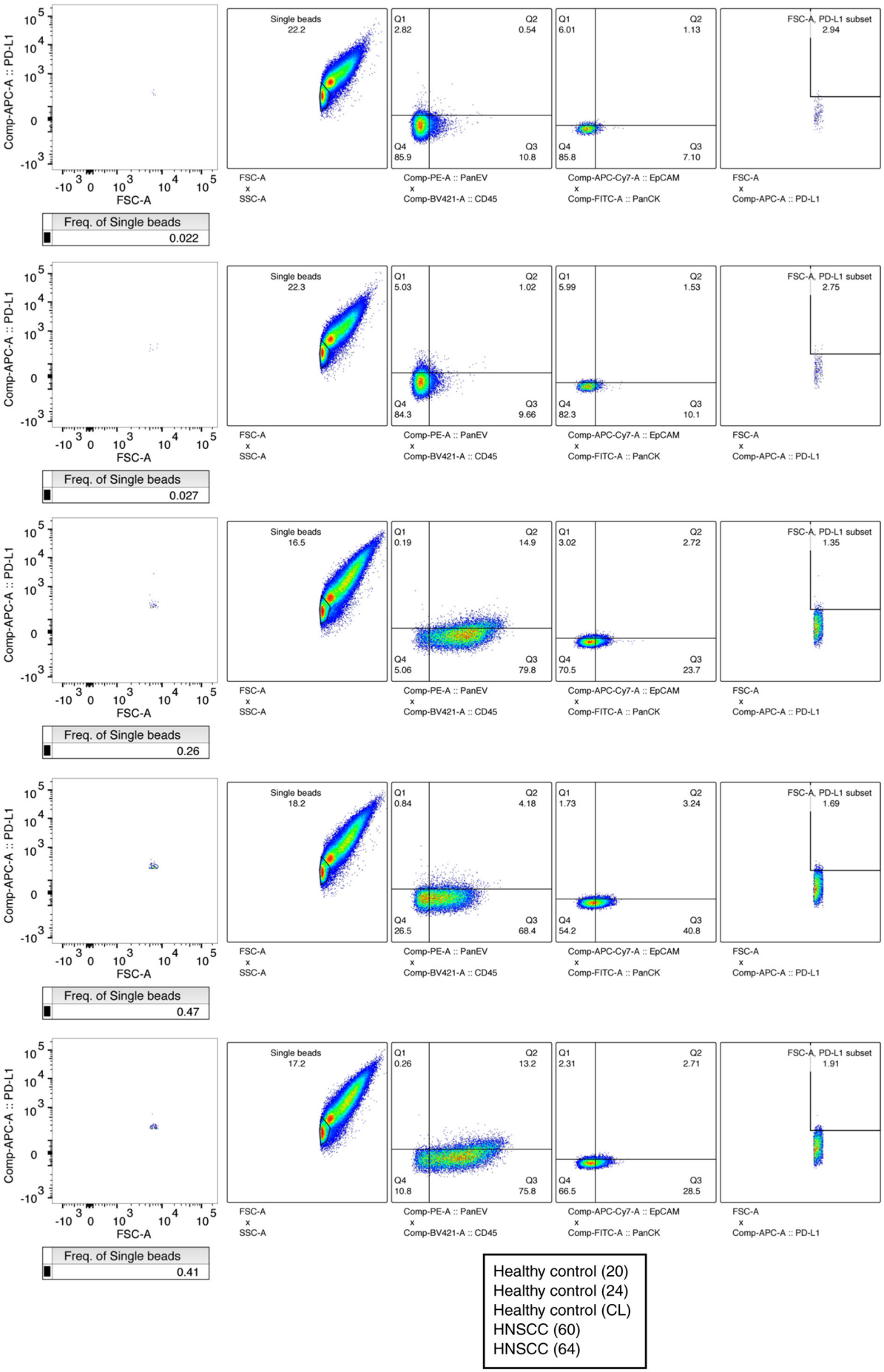

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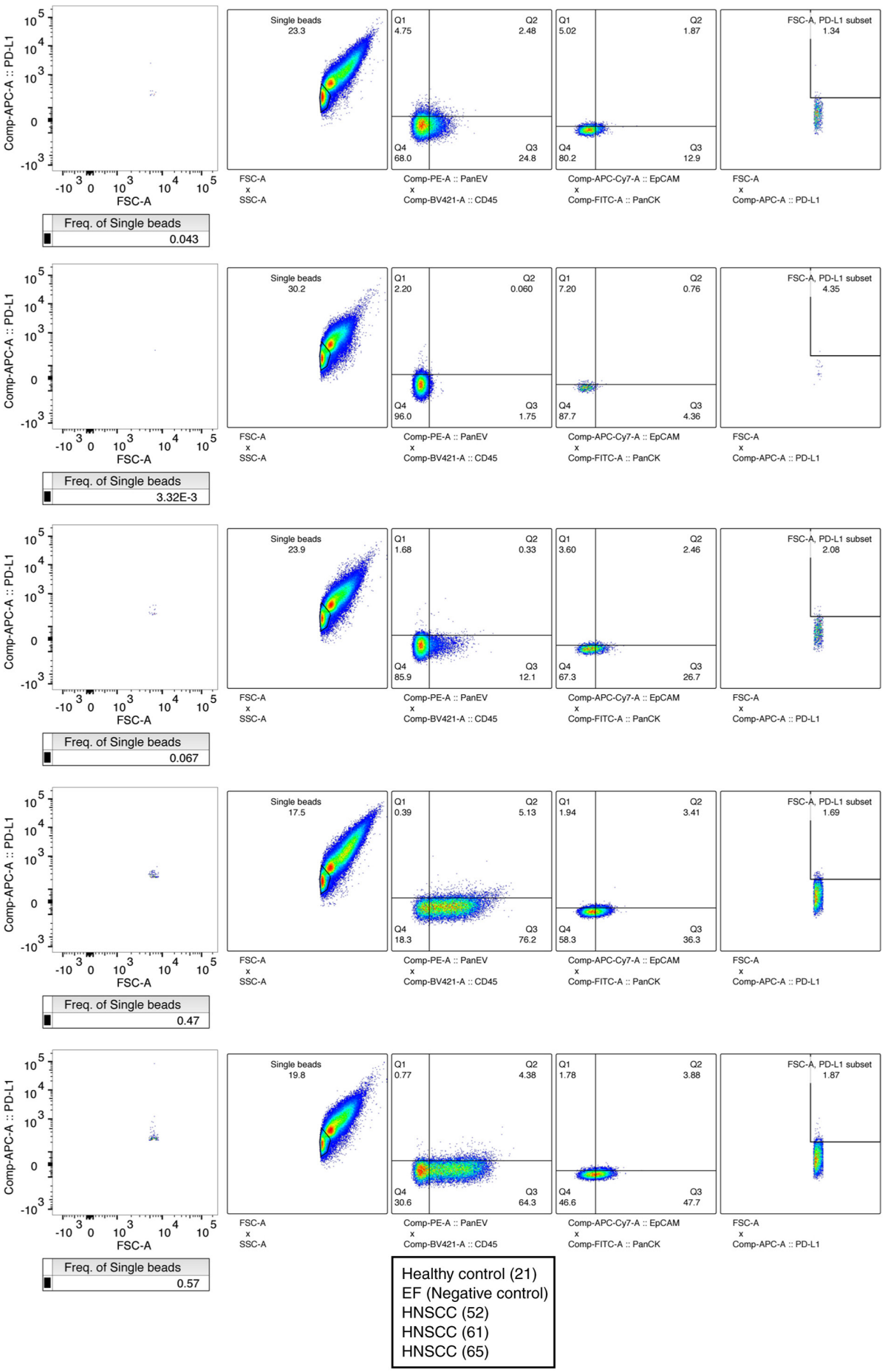

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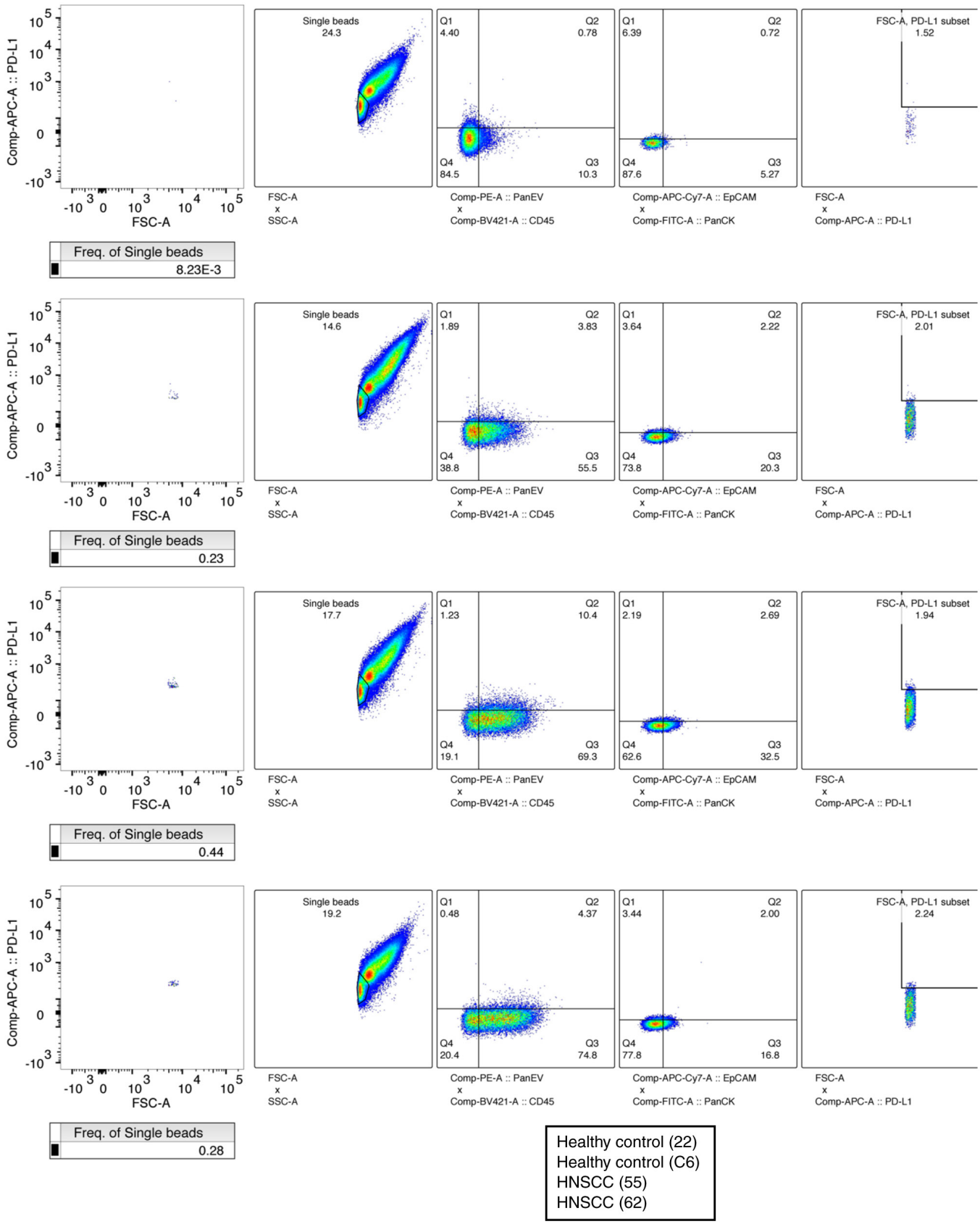

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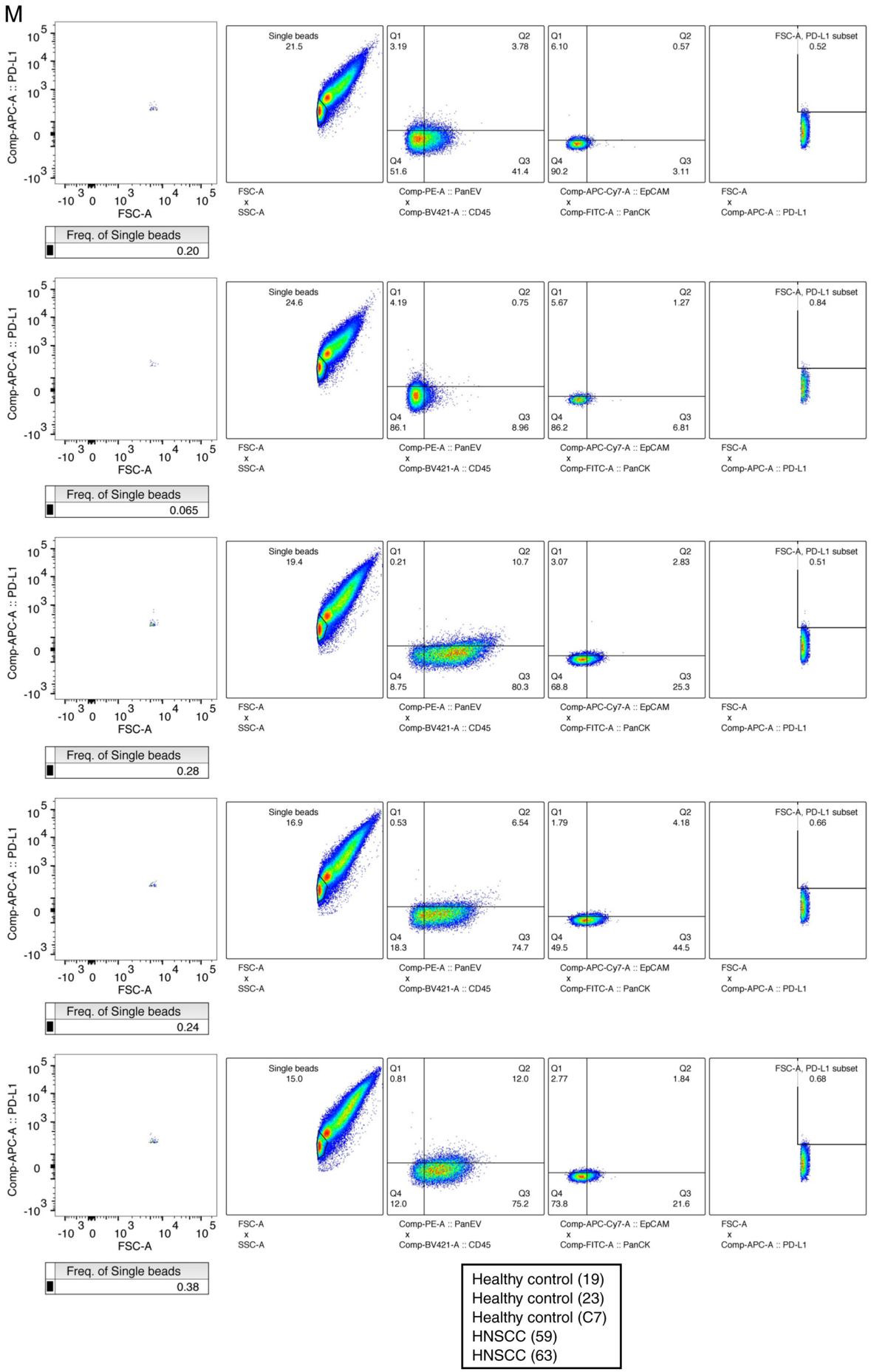

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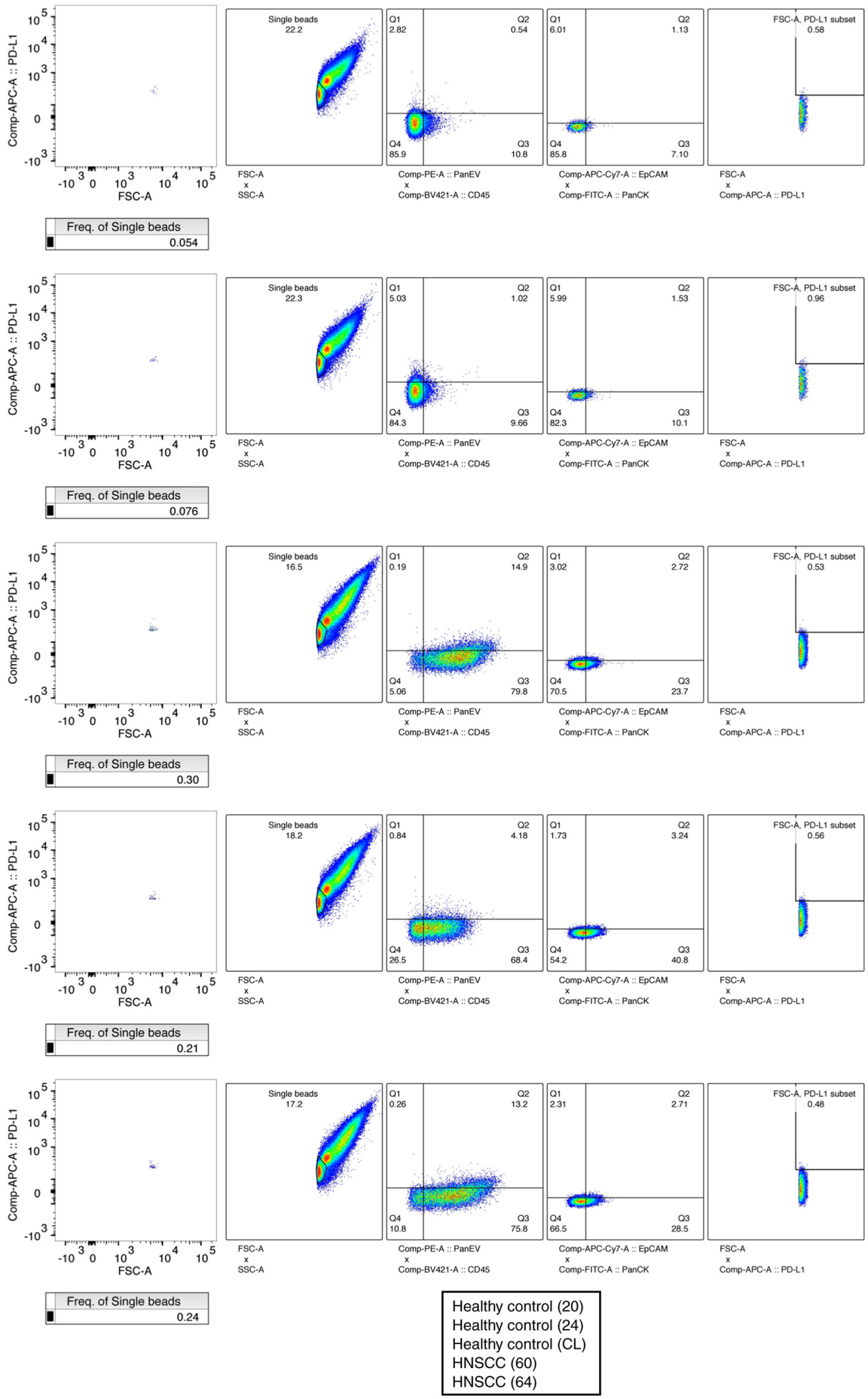

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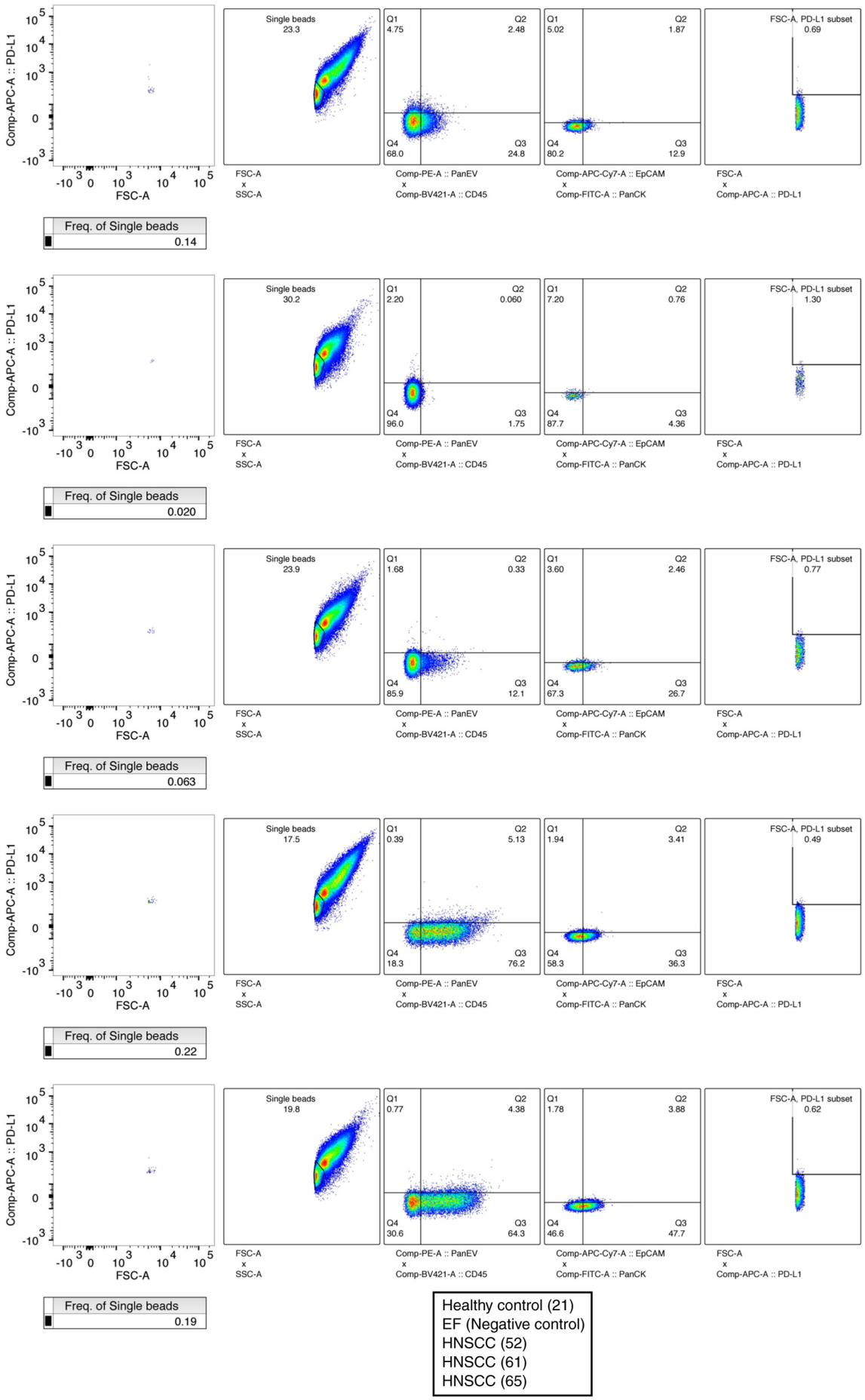

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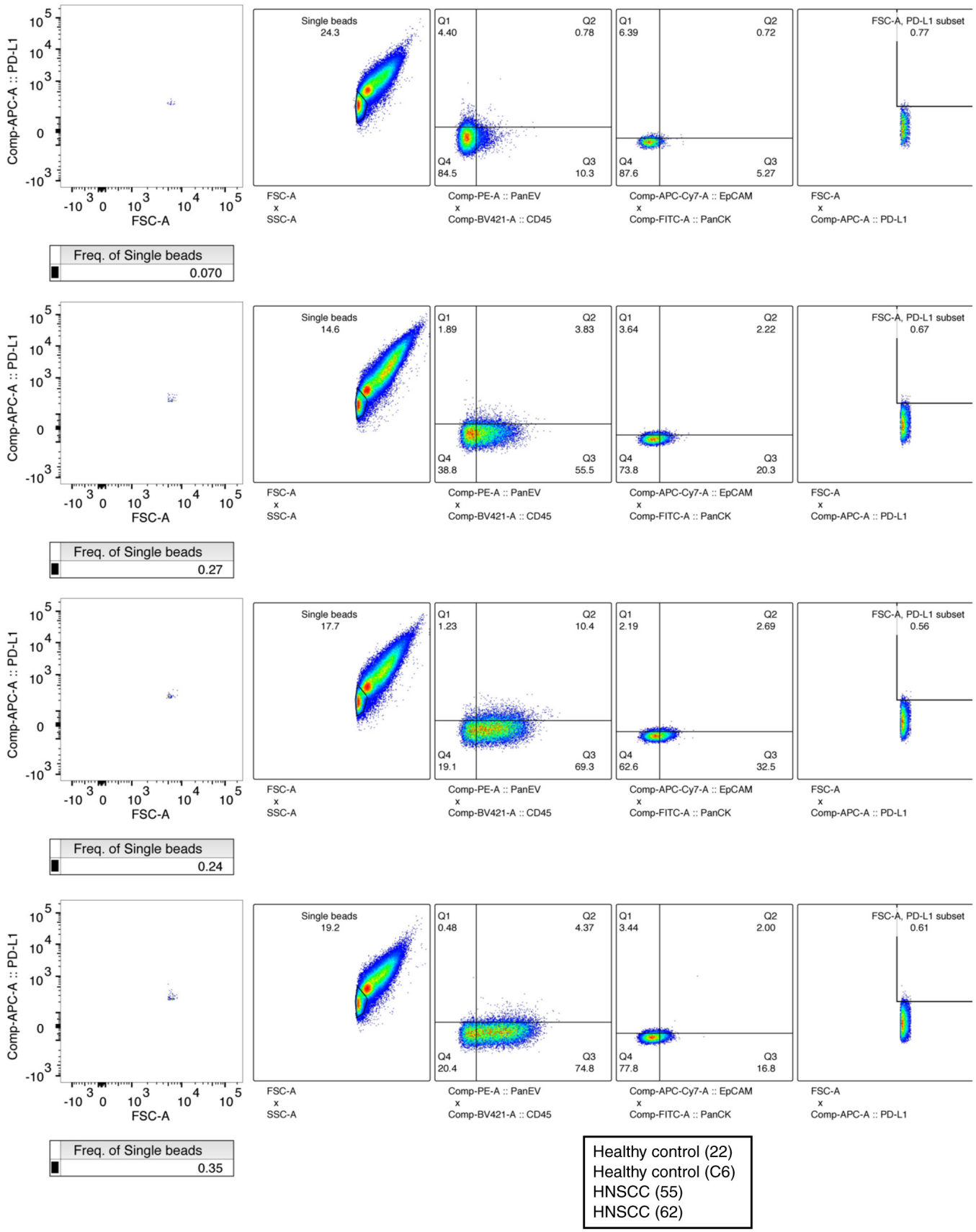

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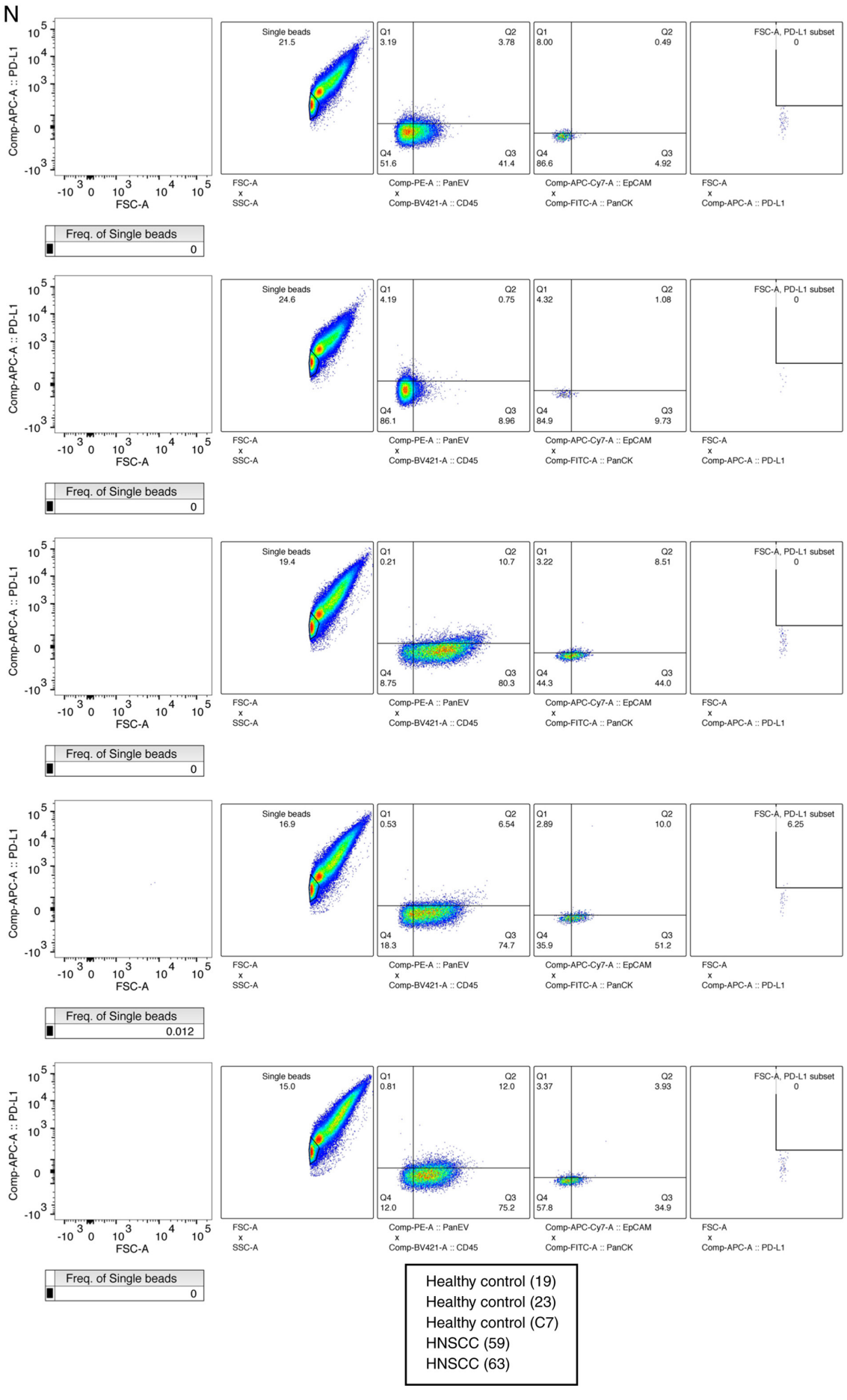

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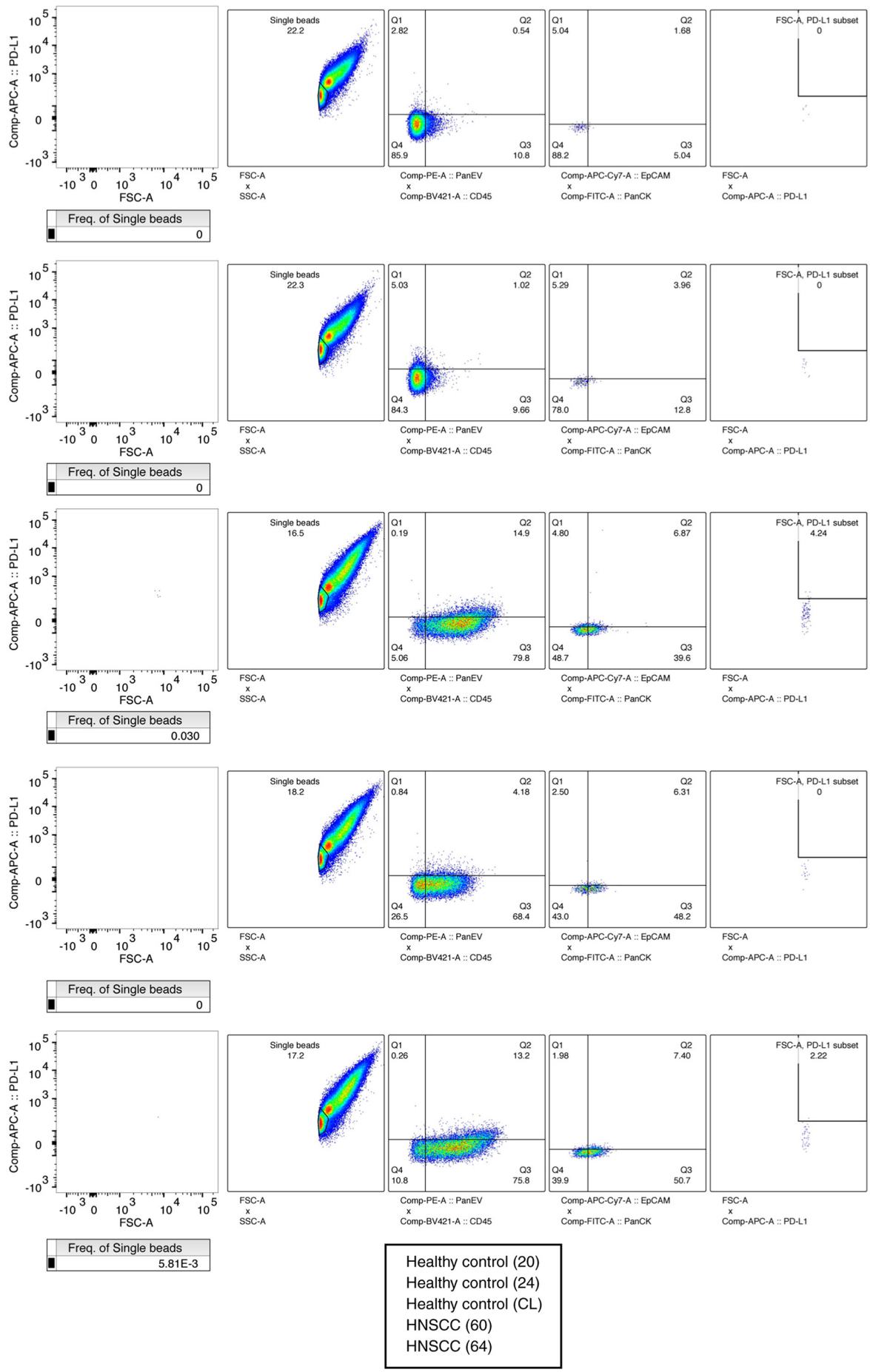

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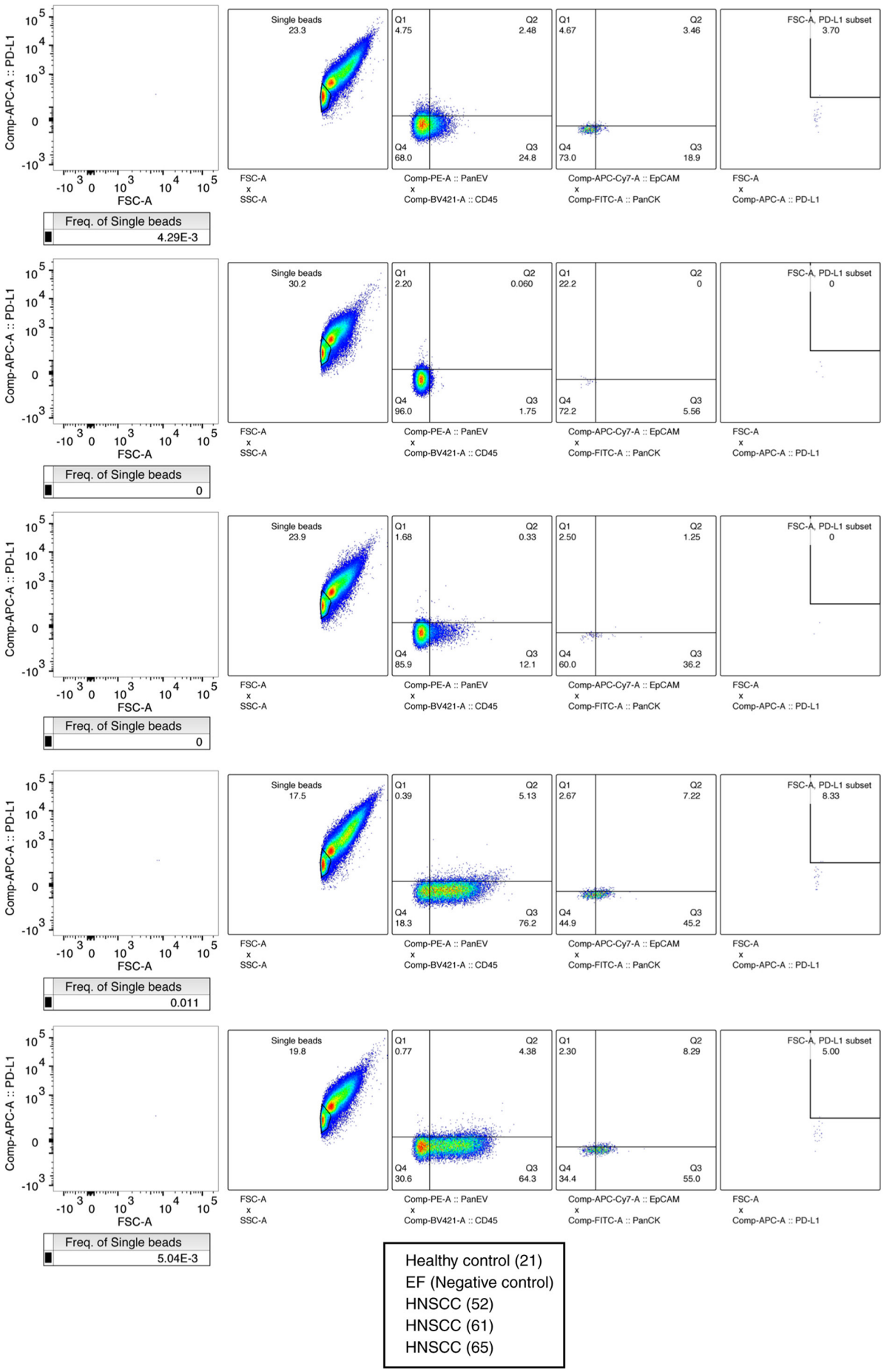

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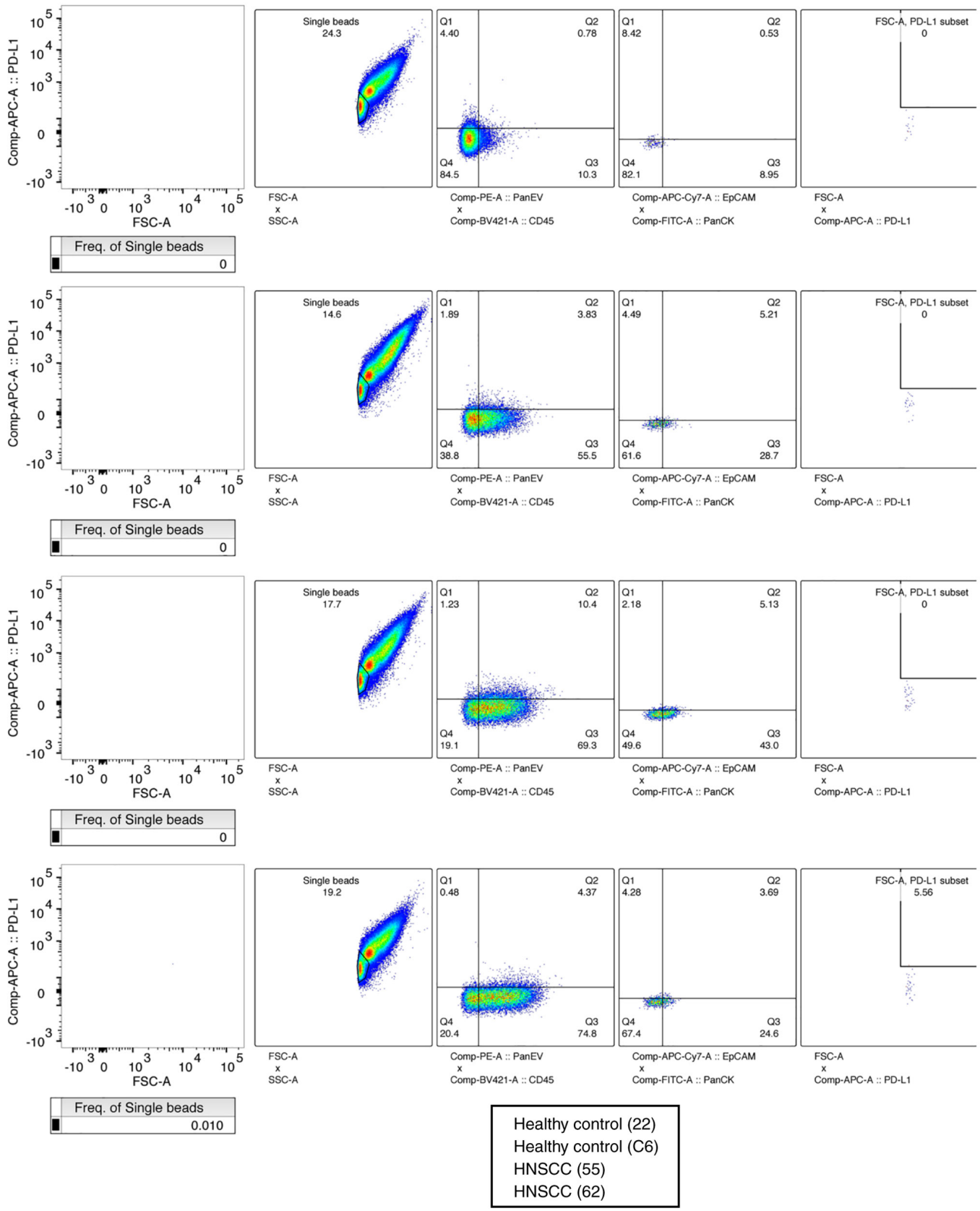

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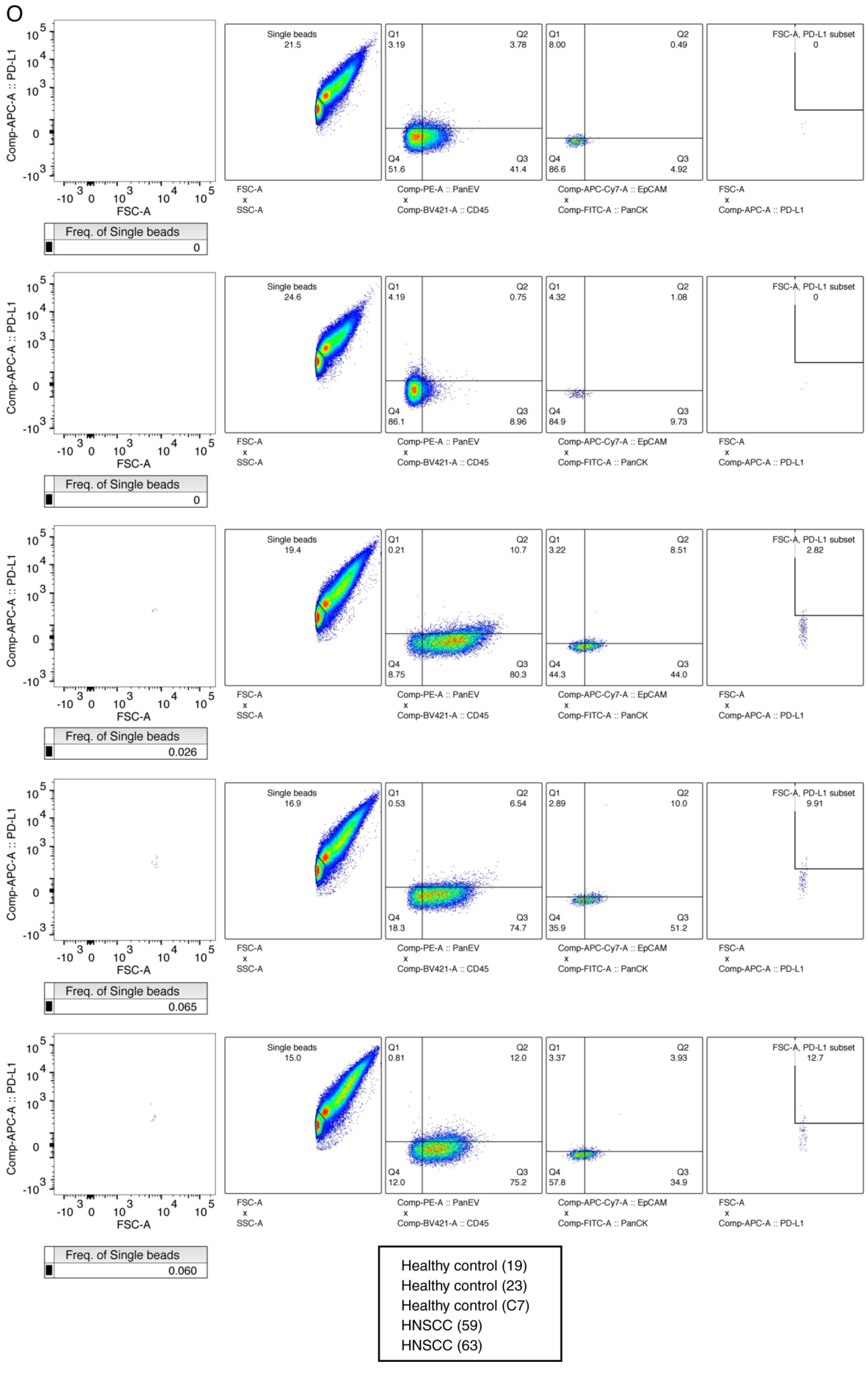

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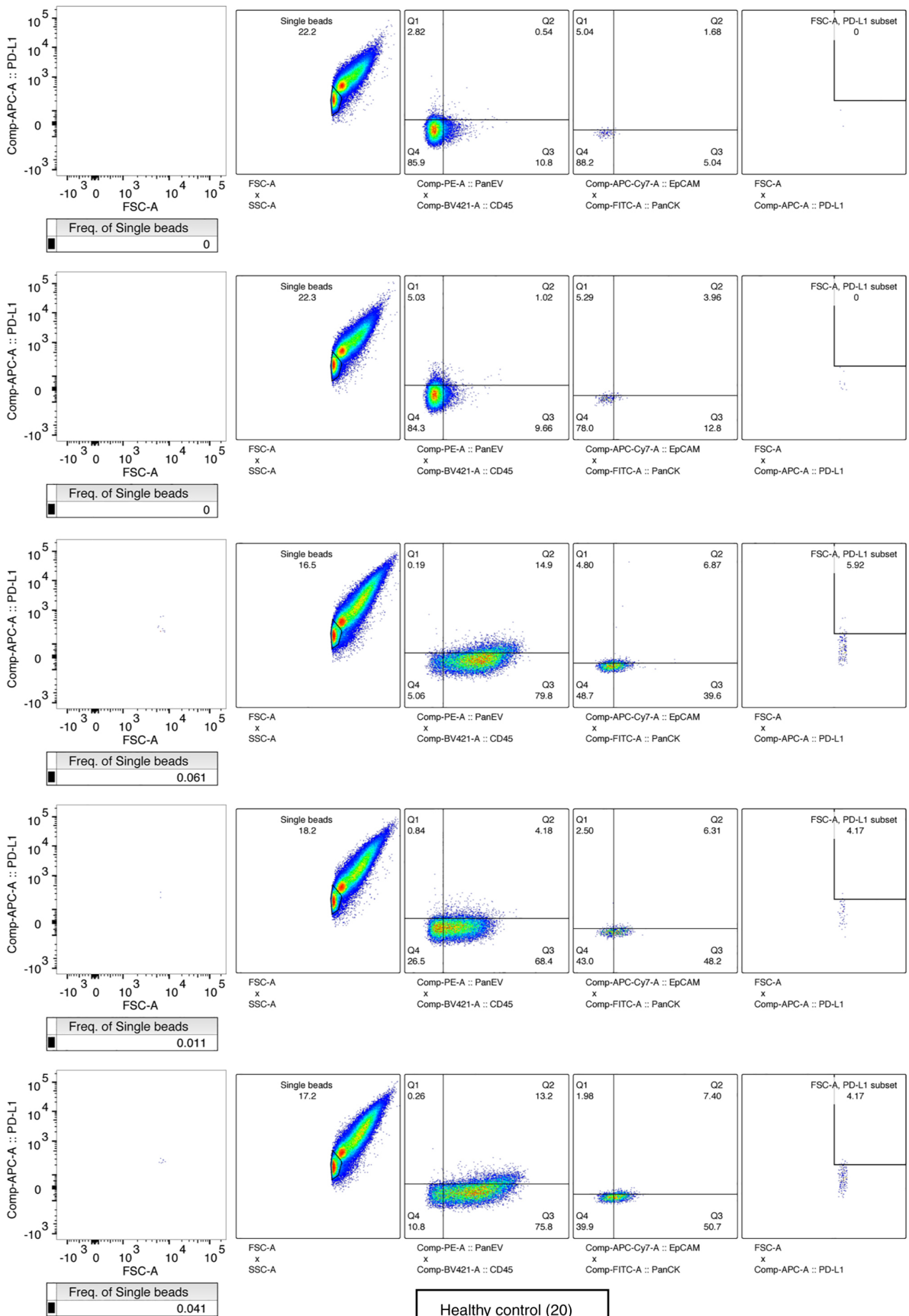

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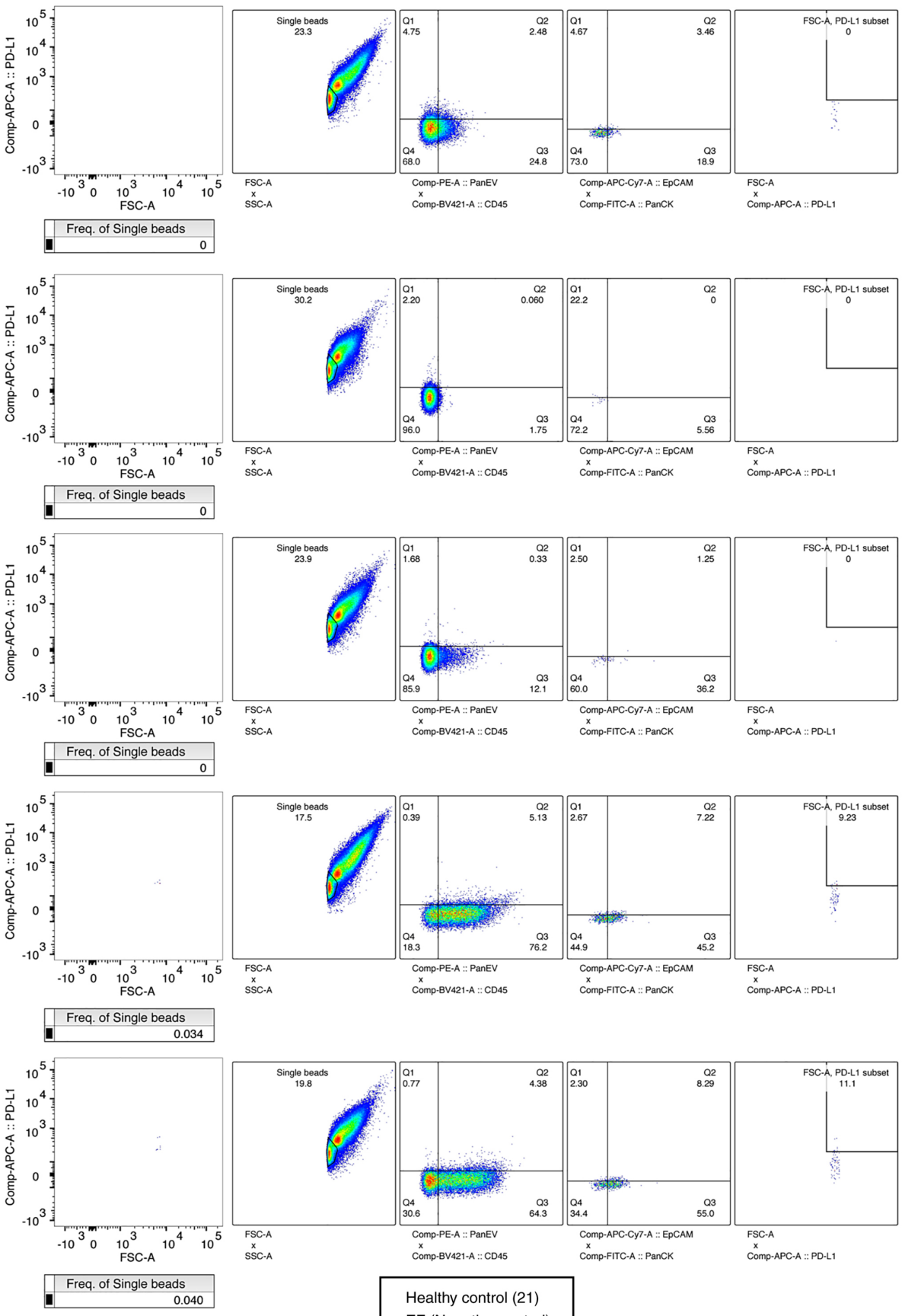

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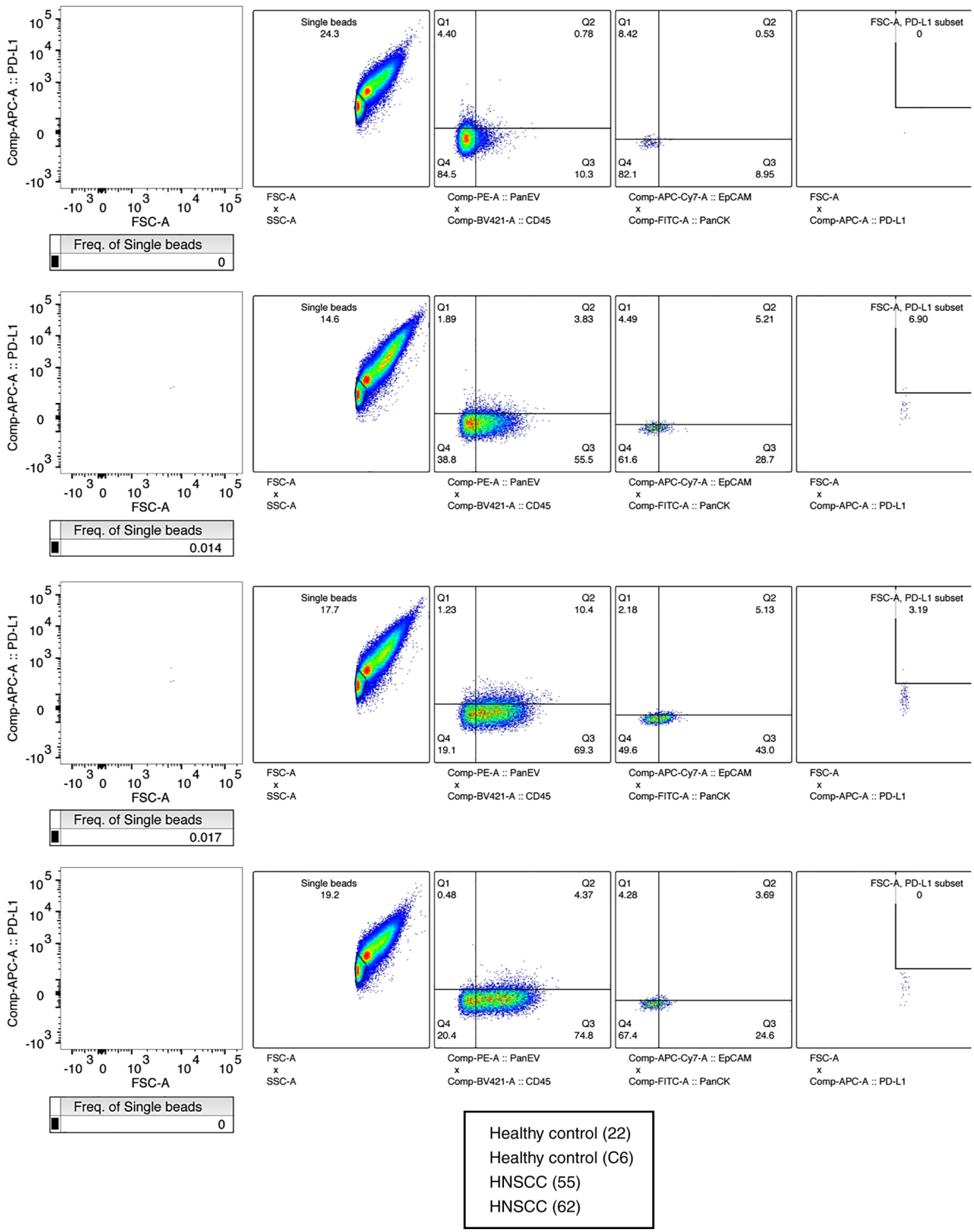

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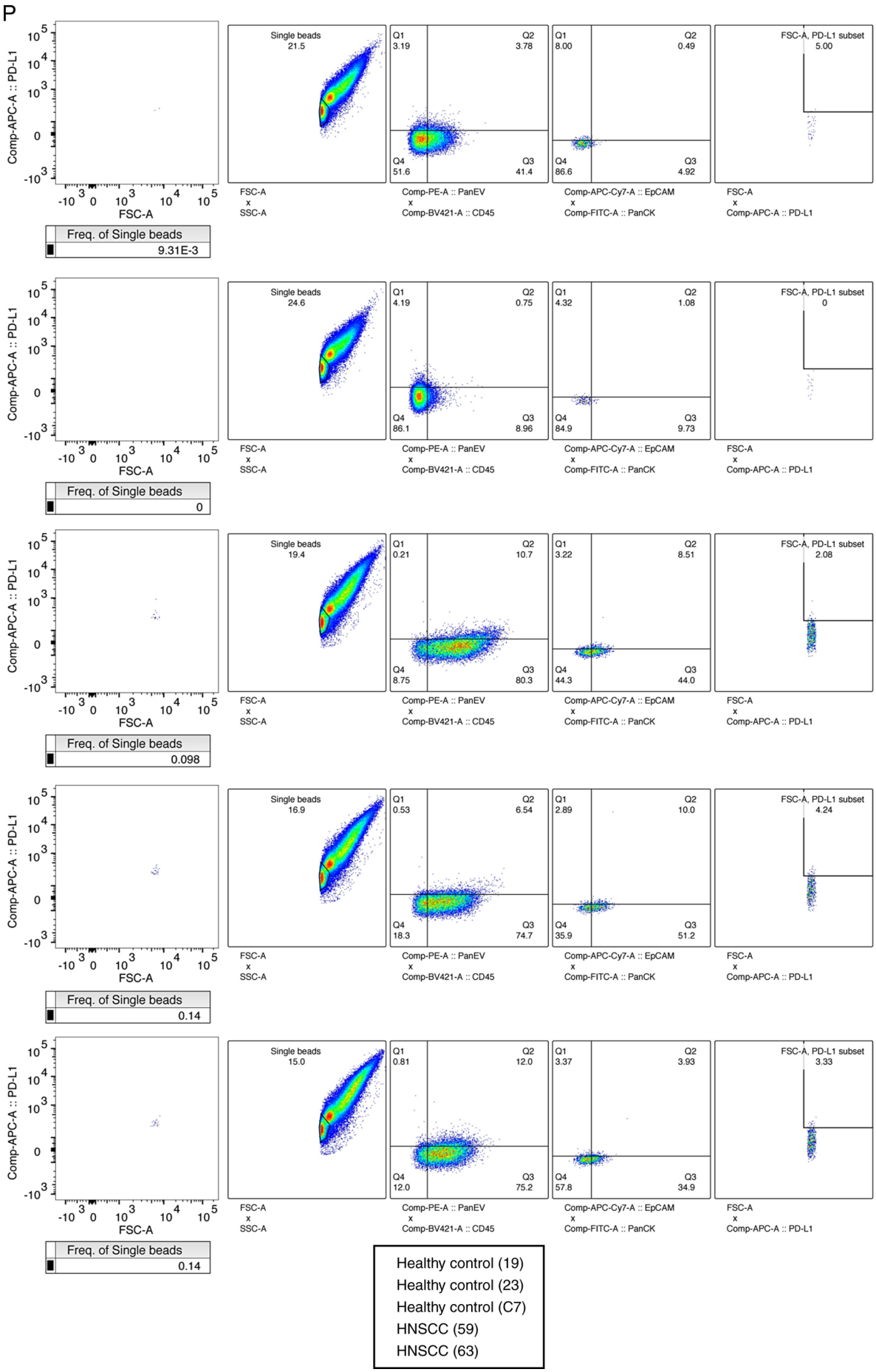

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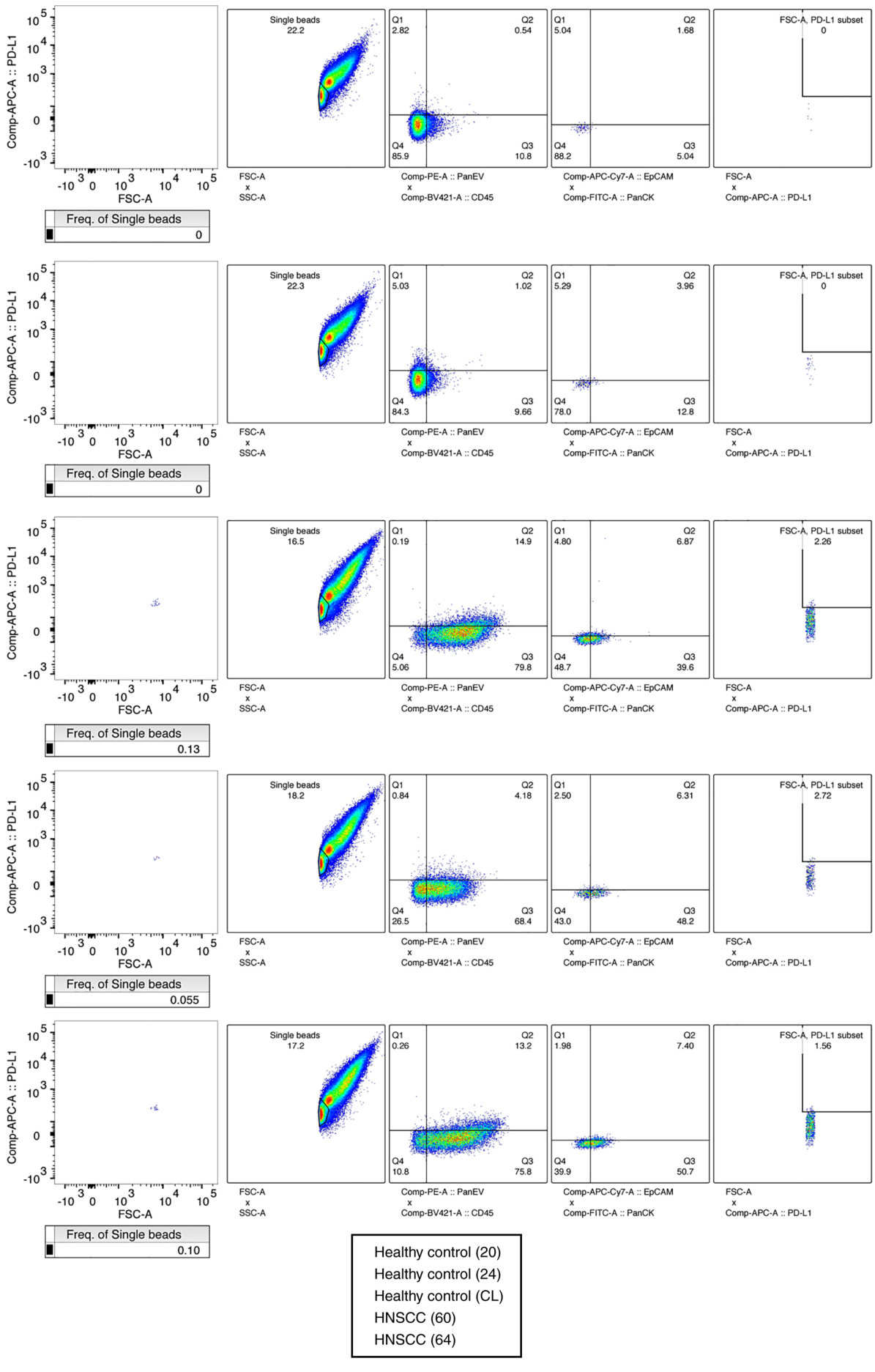

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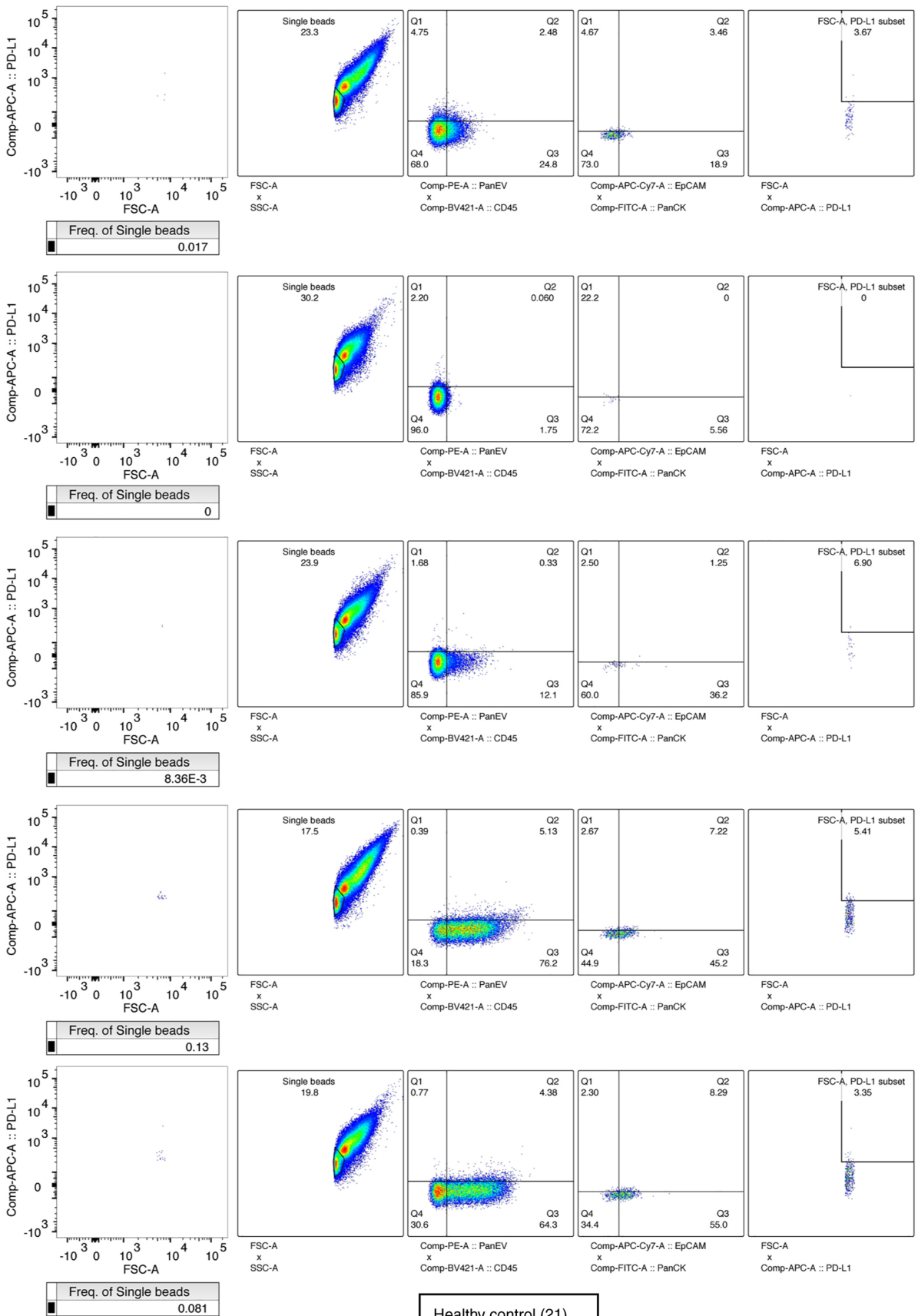

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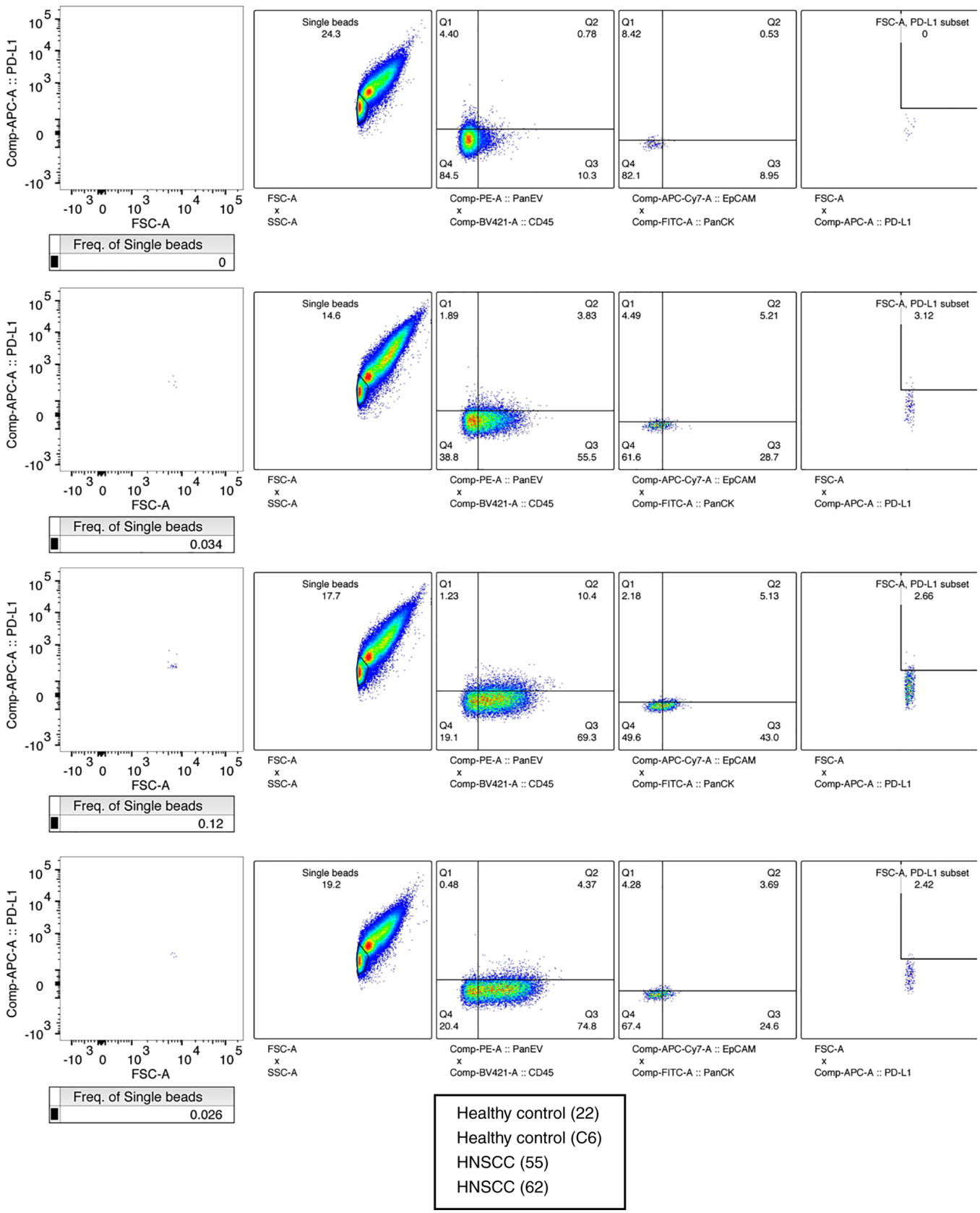

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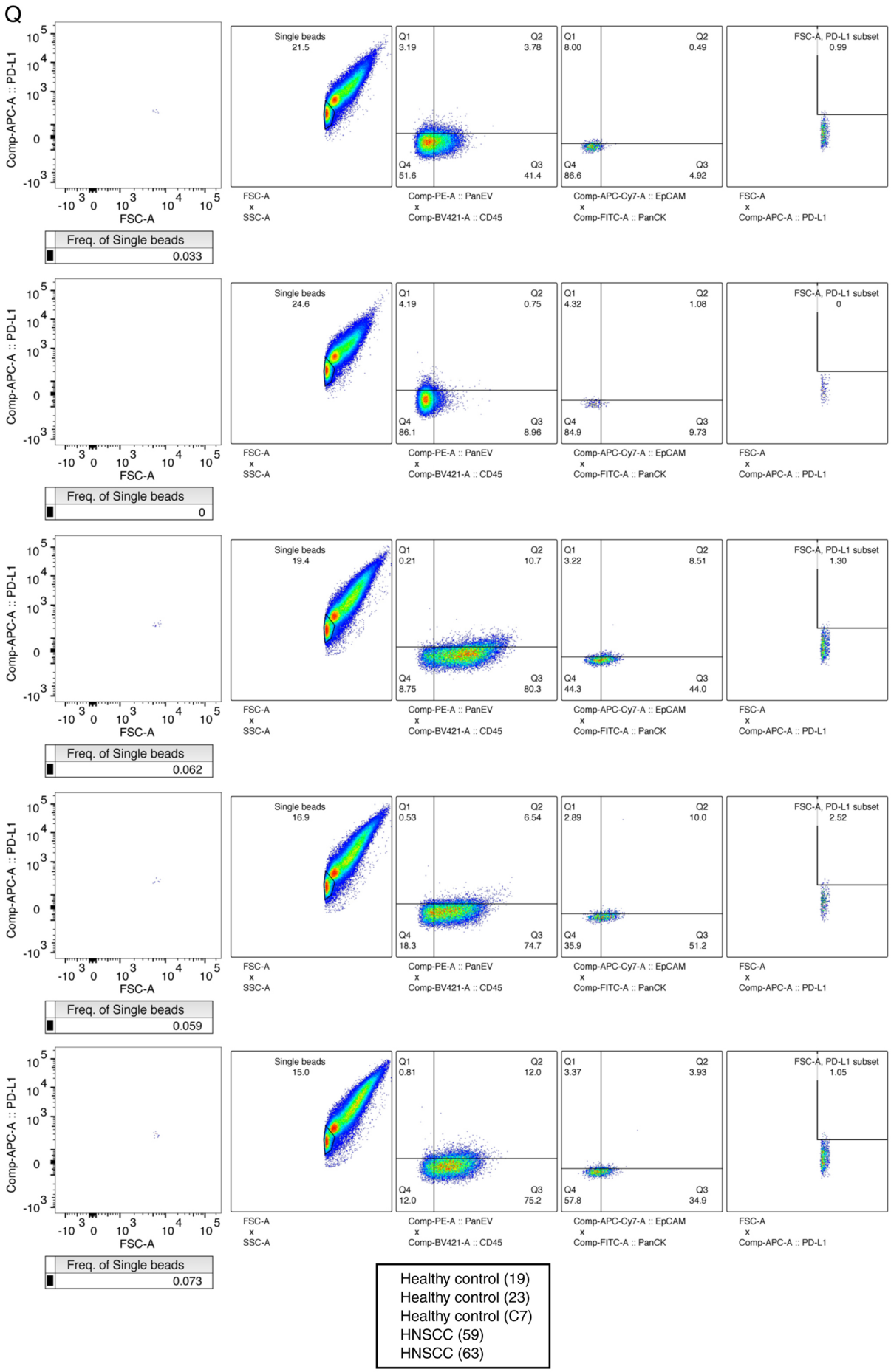

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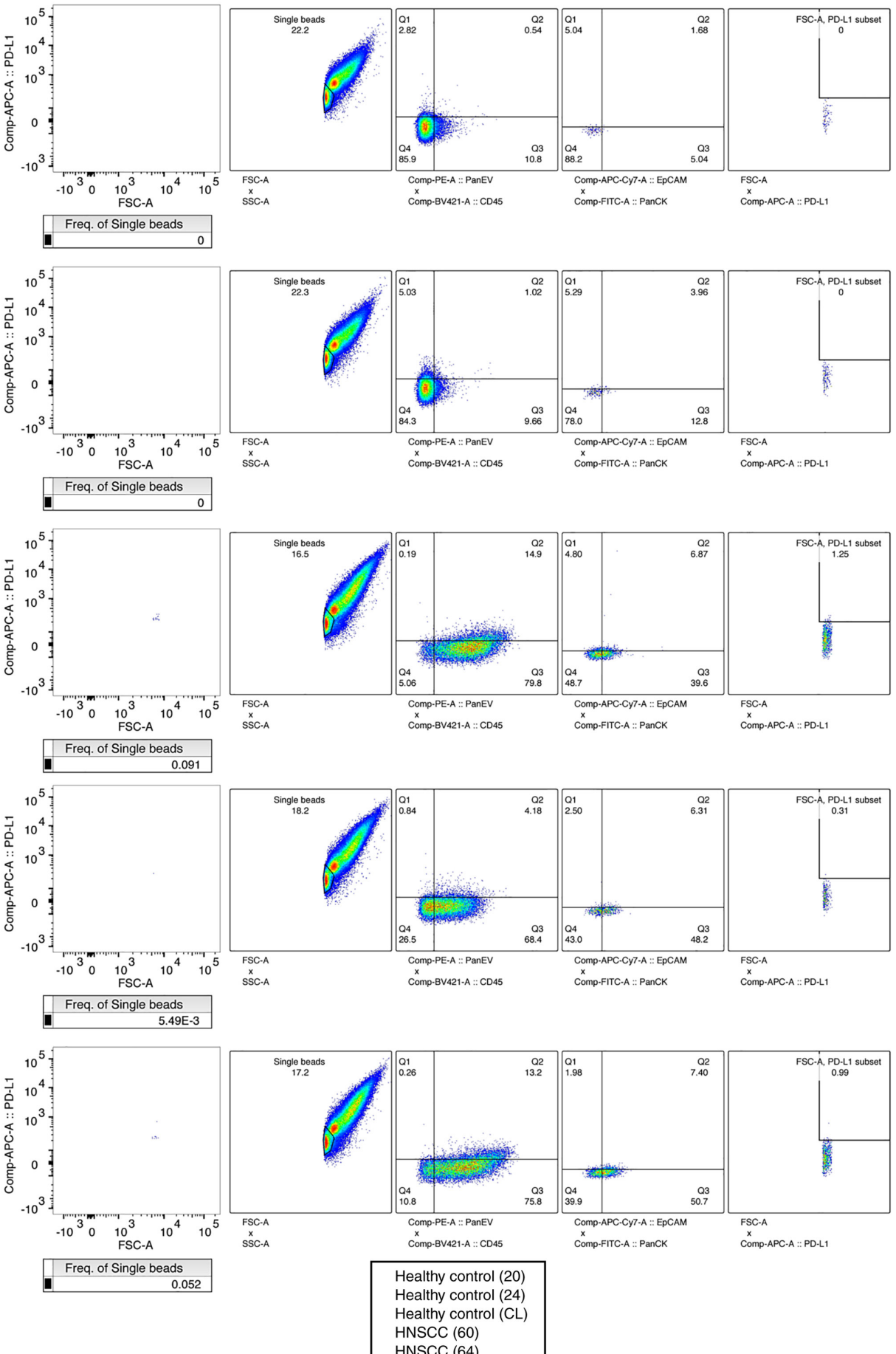

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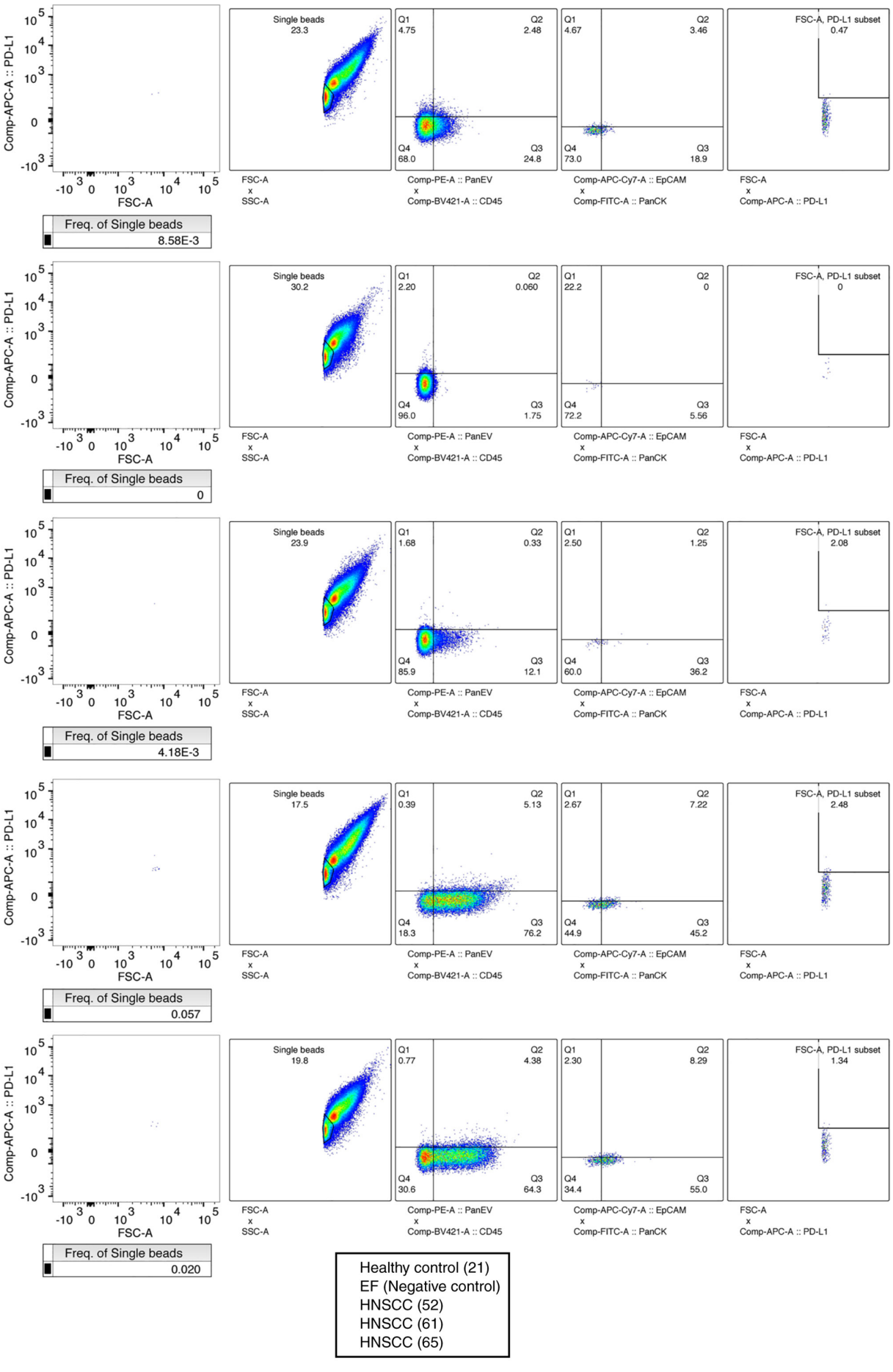

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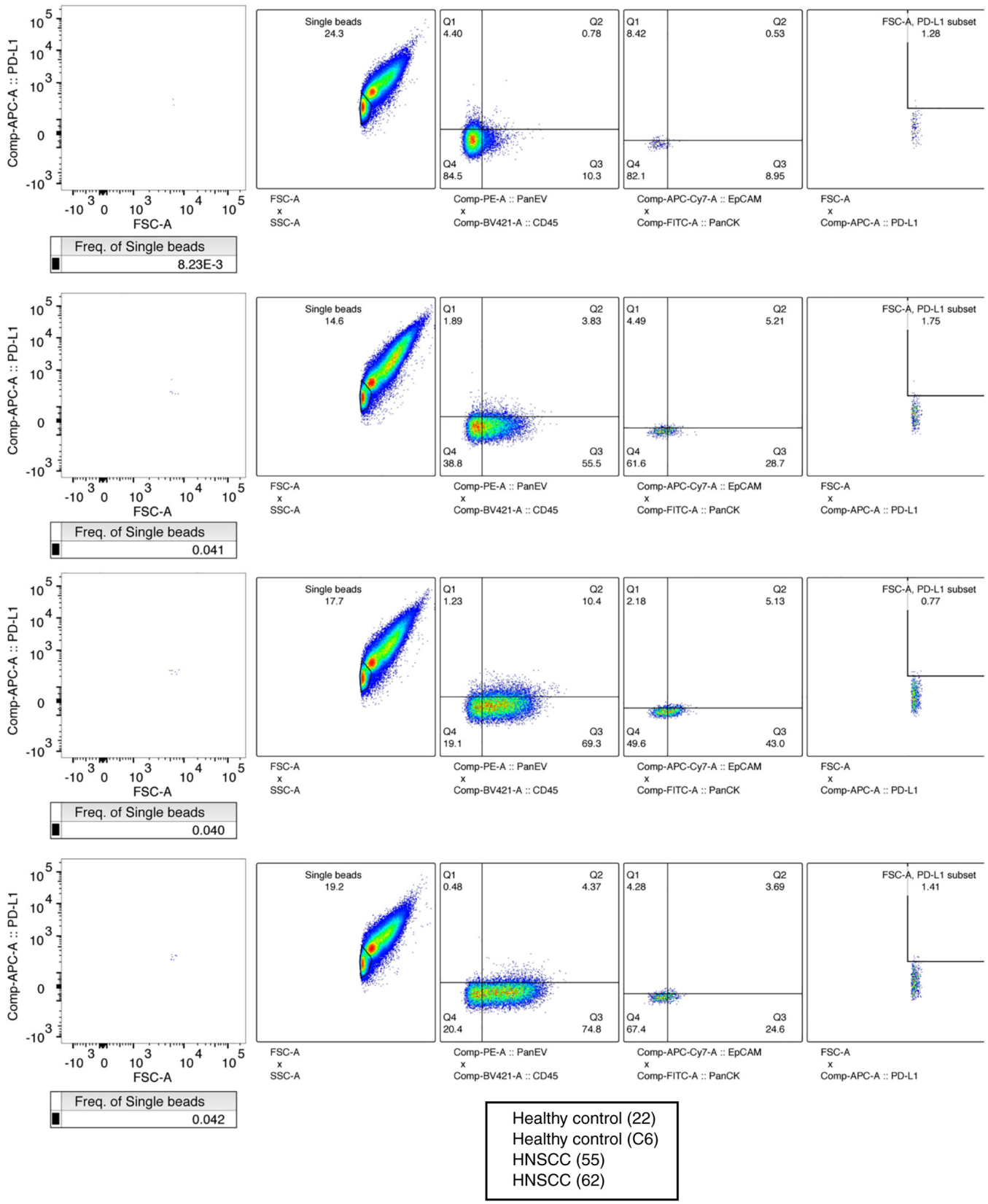
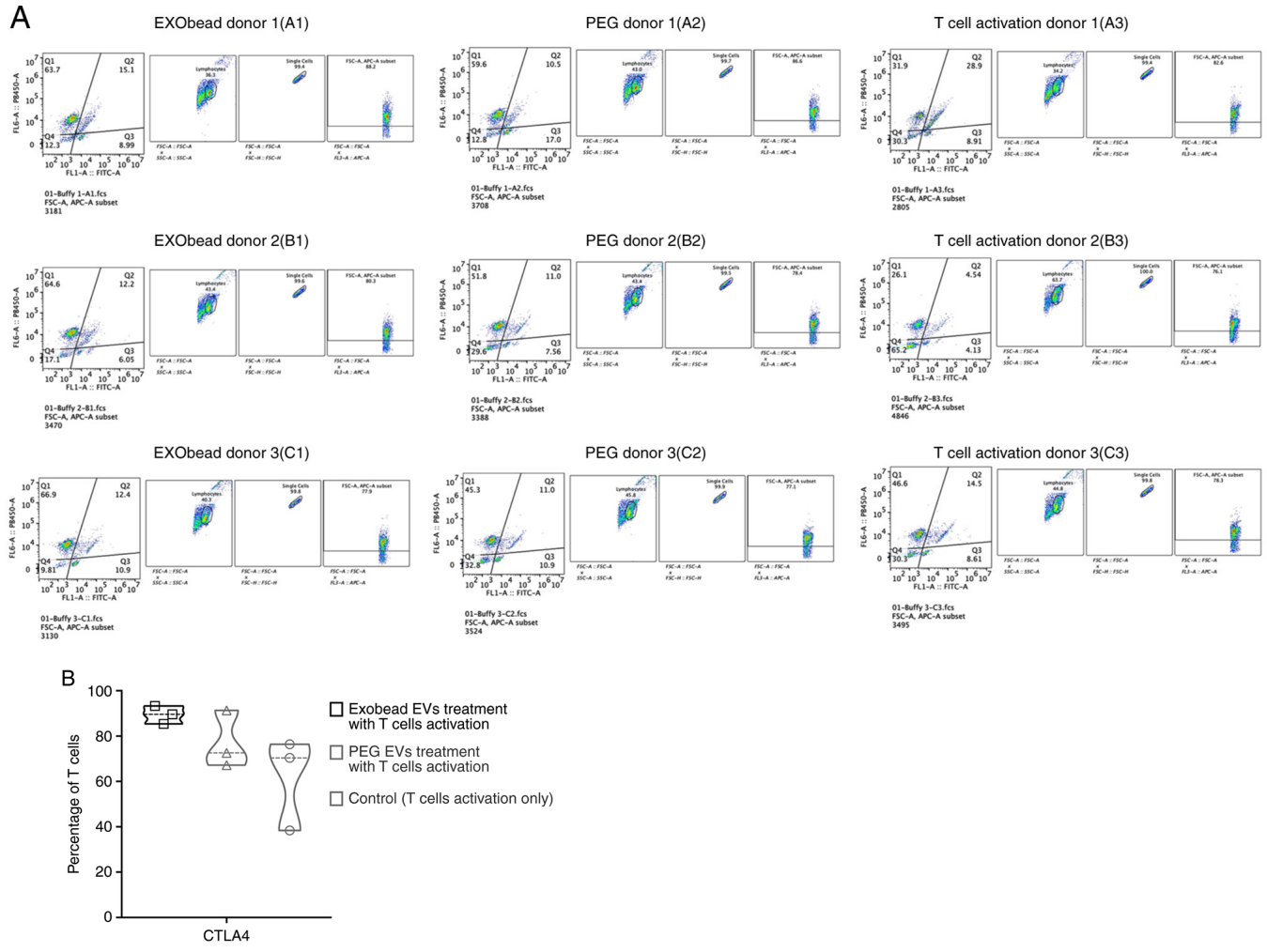


Figure S4. EVs functional assay of T cells and PBMCs activation. (A) 30 μl out of 200 μl eluted patient plasma EVs from EXÖBead® isolation and PEG EVs were treated with $CD4^+$ T cells in anti-CD2/3/28 antibodies activation condition. The Violin plot shows that $CTLA4^+$ $CD69^{Neg}$ T cells emerged only when treated with eluted patient plasma EVs from EXÖBead®, PEG EVs and T cells activation. (B) The Violin plot shows that $CTLA4^+$ T cells only appeared when treated with eluted patient plasma EVs from EXÖBead®, PEG-EVs and activated T cells (A). Significance was calculated using the nonparametric Kruskal-Wallis test with Dunn's multiple comparison test. (C) $5x10^7$ particles of eluted patient or control plasma EVs from EXÖBead® isolation were treated with $1x10^6$ PBMCs (ratio: 50:1) under anti-CD2/3/28 antibody activation conditions. Violin plot showed that $CD69^+$ $PD\text{-}L1^+$ live $CD4^+$ T cells were derived from treatment with elution buffer alone, from plasma EVs from HNSCC patients (n=13, with technological triplicate) and from EVs from healthy controls (n=3, with technological triplicate). (D) The violin plot shows that $CD69^+$ $PD1^+$ $CD4^+$ T cells showed no significant difference between treatment with elution buffer alone, treatment with plasma EVs from HNSCC patients (n=13, with technological triplicates), and treatment with EVs from healthy controls (n=3, with technological triplicates). (E) Significance was calculated using Brown-Forsythe and Welch's ANOVA test with Dunnett's T3 multiple comparison test. The violin plot shows that single positive $CD69^+$, $PD1^+$, or $PD\text{-}L1^+$ live $CD4^+$ T cells showed no significant difference between treatment with elution buffer alone, from plasma EVs of HNSCC patients (n=13, with technological triplicates), and from EVs of healthy controls (n=3, with technological triplicates). Significance was calculated using Brown-Forsythe and Welch's ANOVA test with Dunnett's T3 multiple comparison test. EV, extracellular vesicle; PBMC, peripheral blood mononuclear cells.



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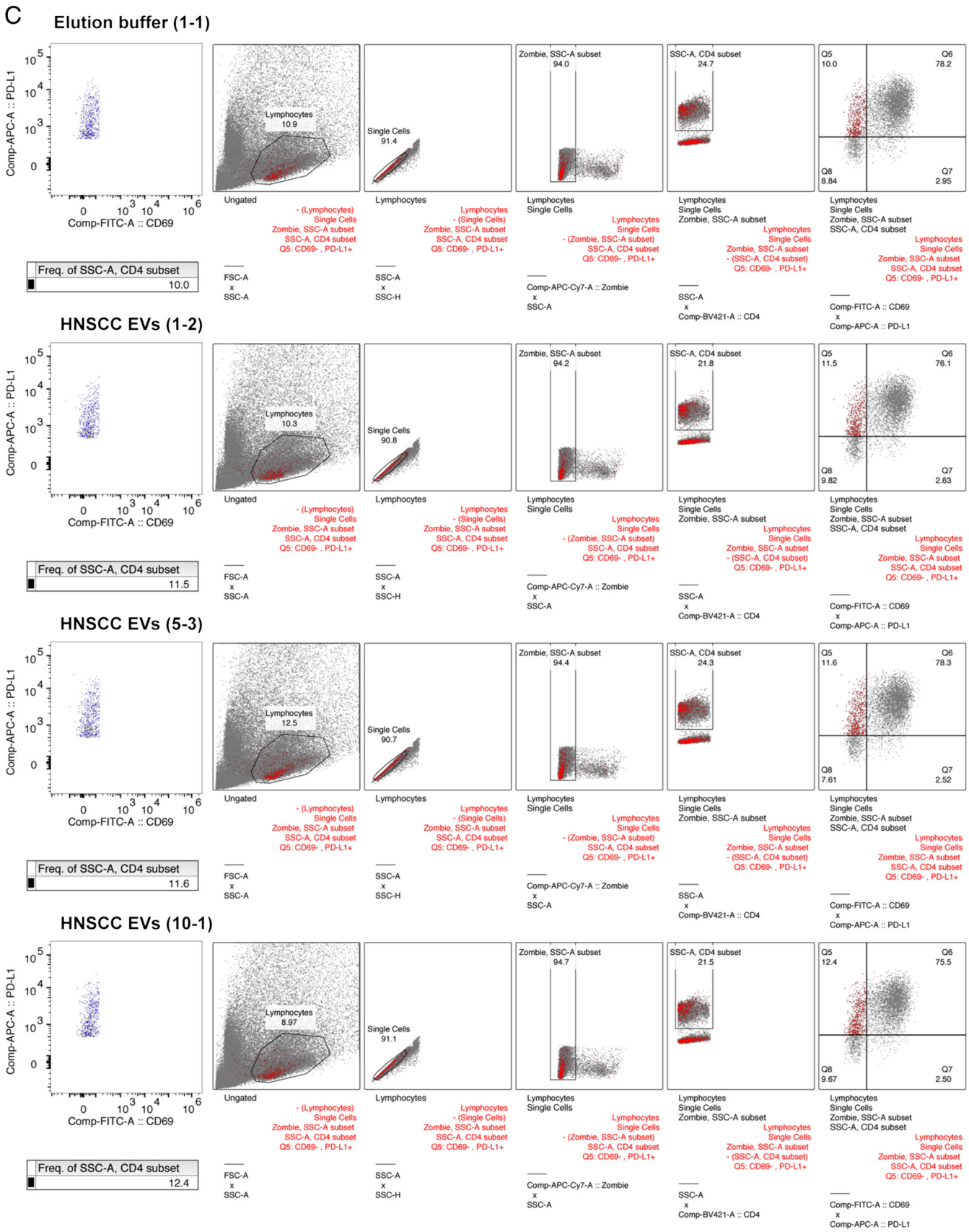

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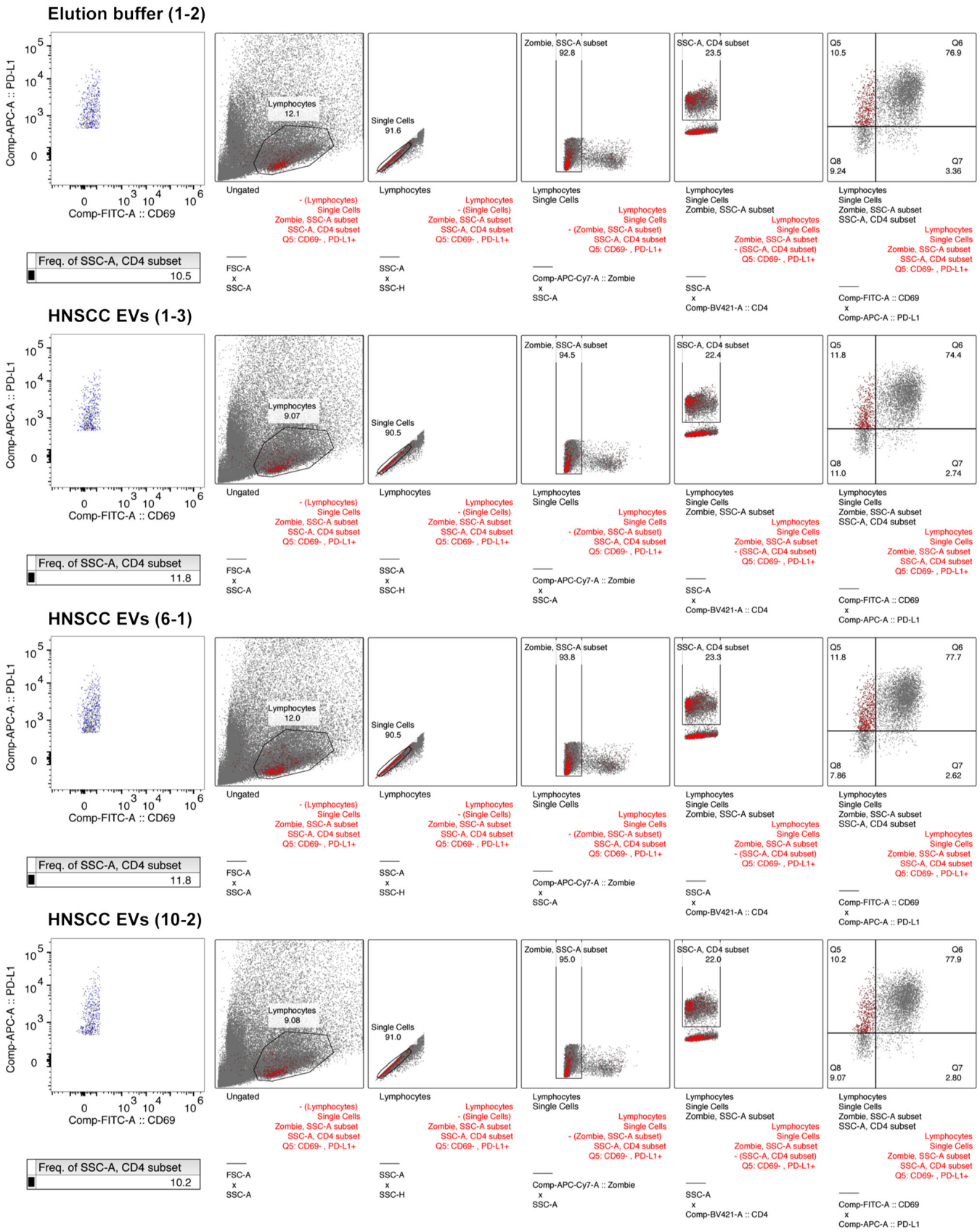

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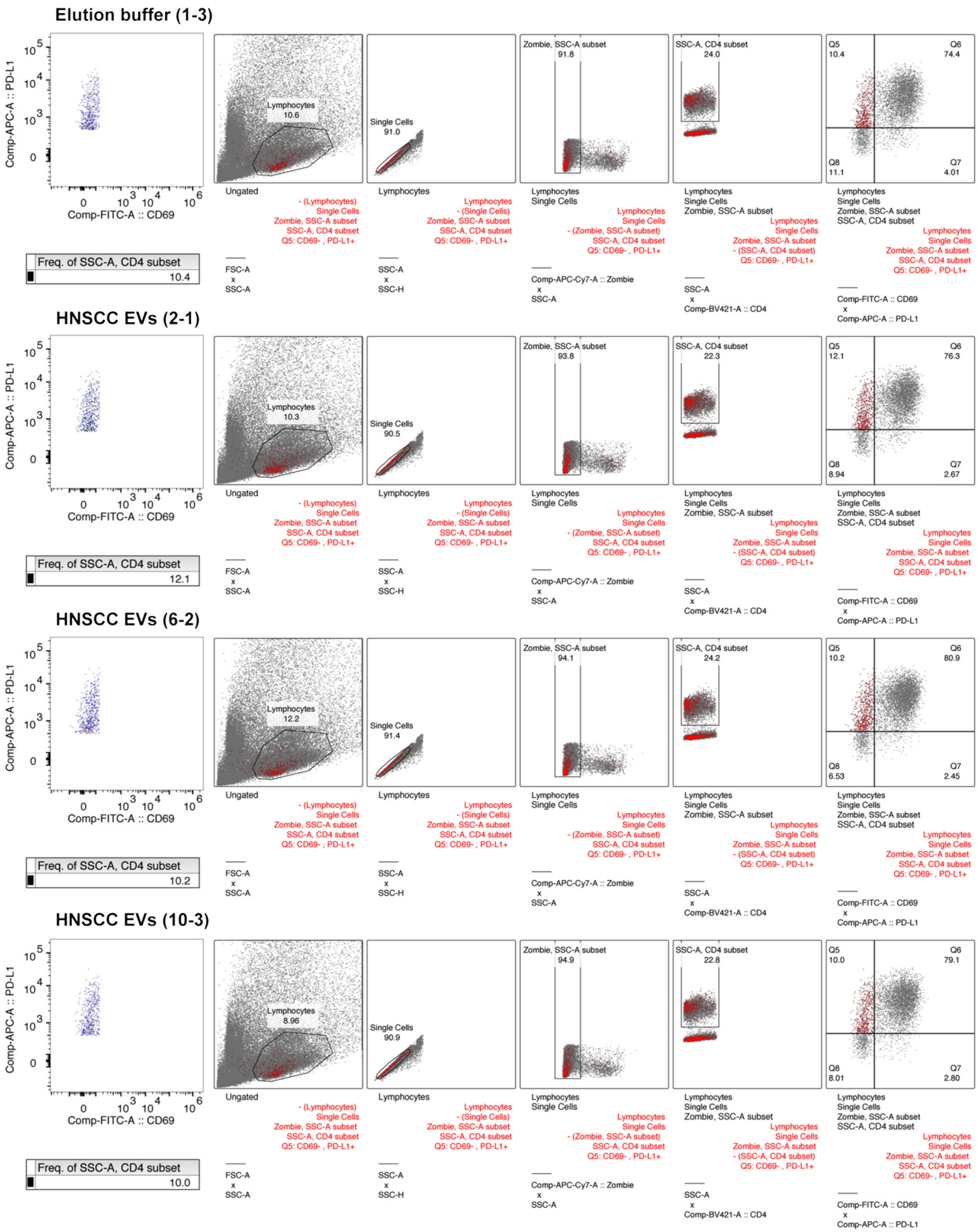

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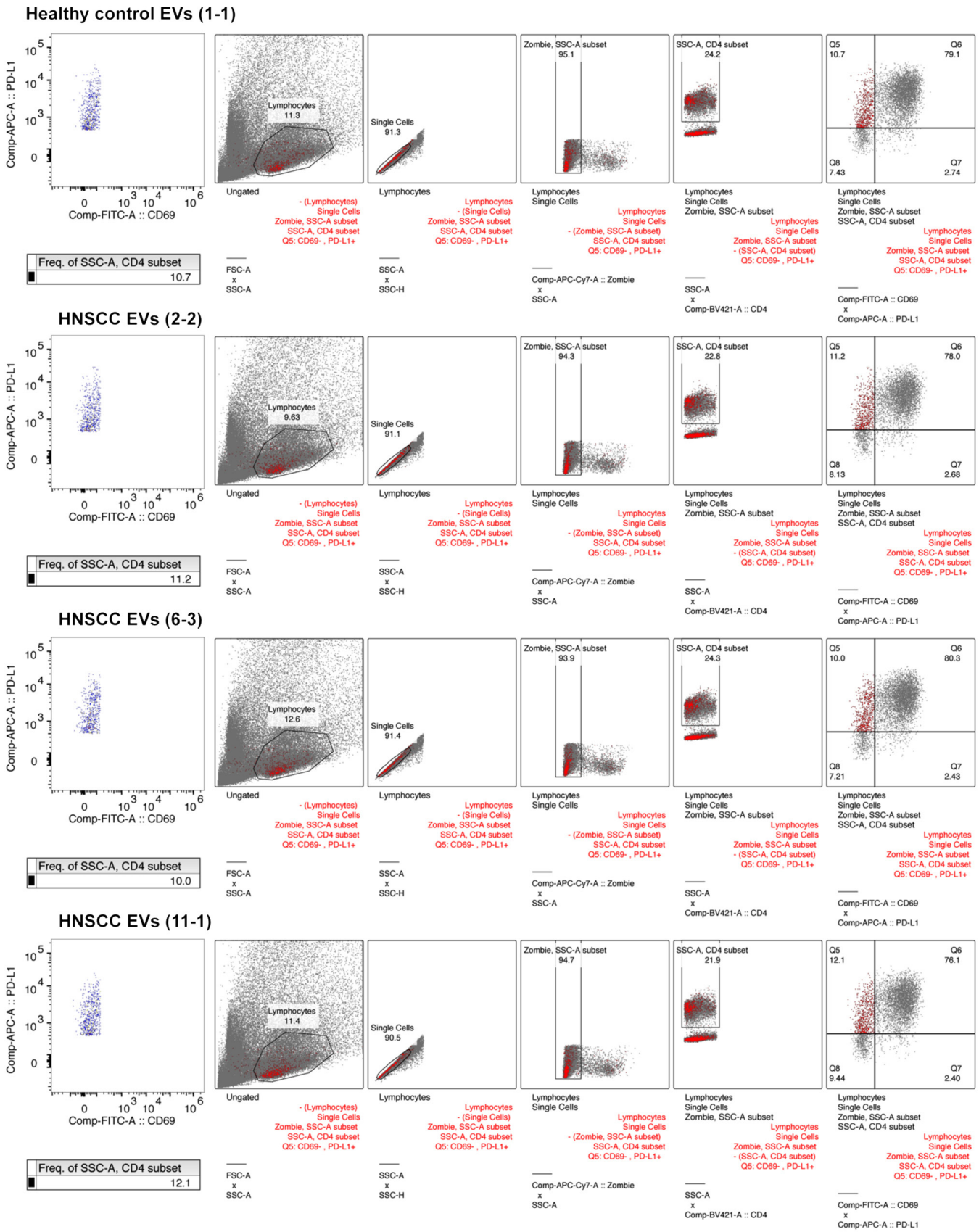

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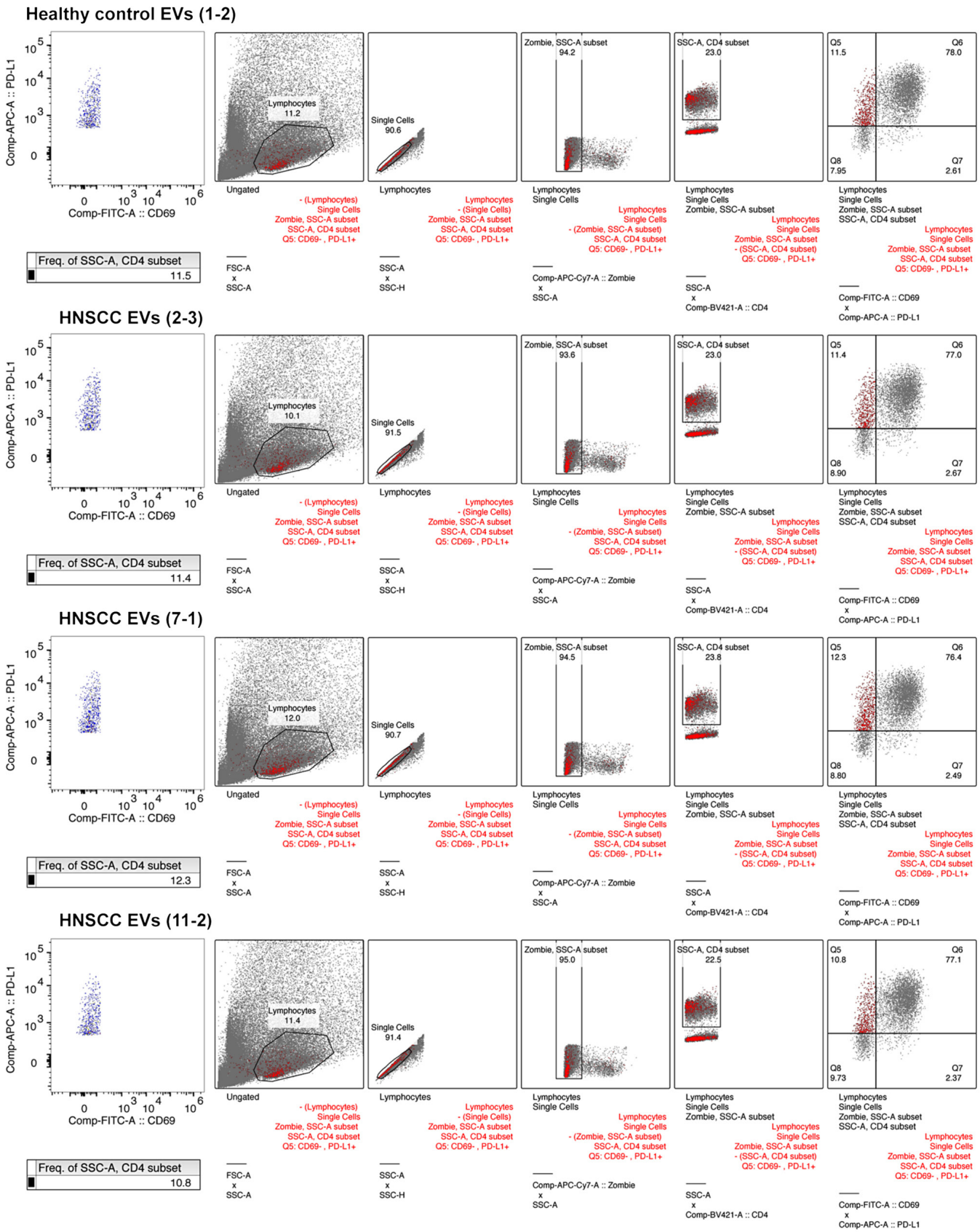

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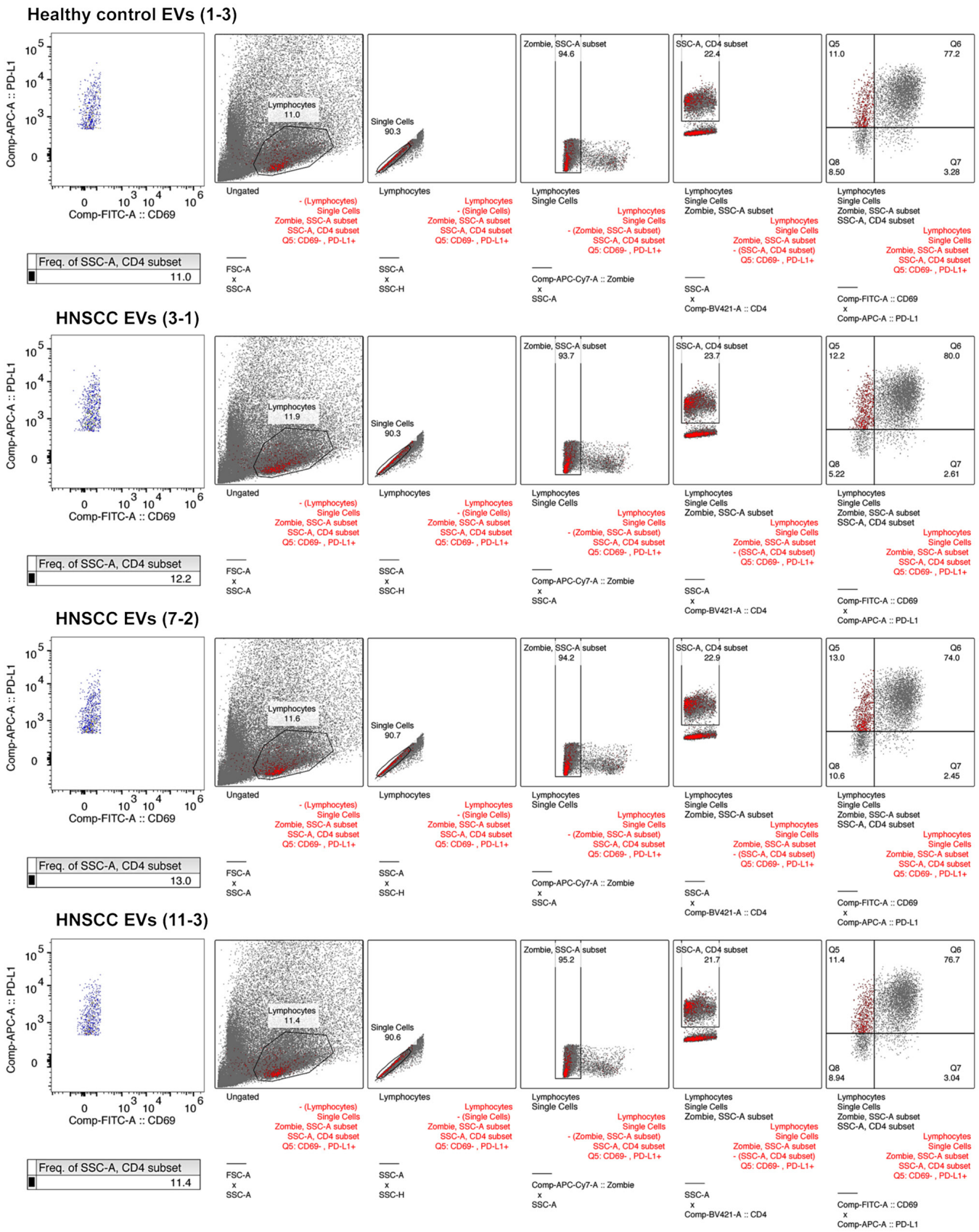

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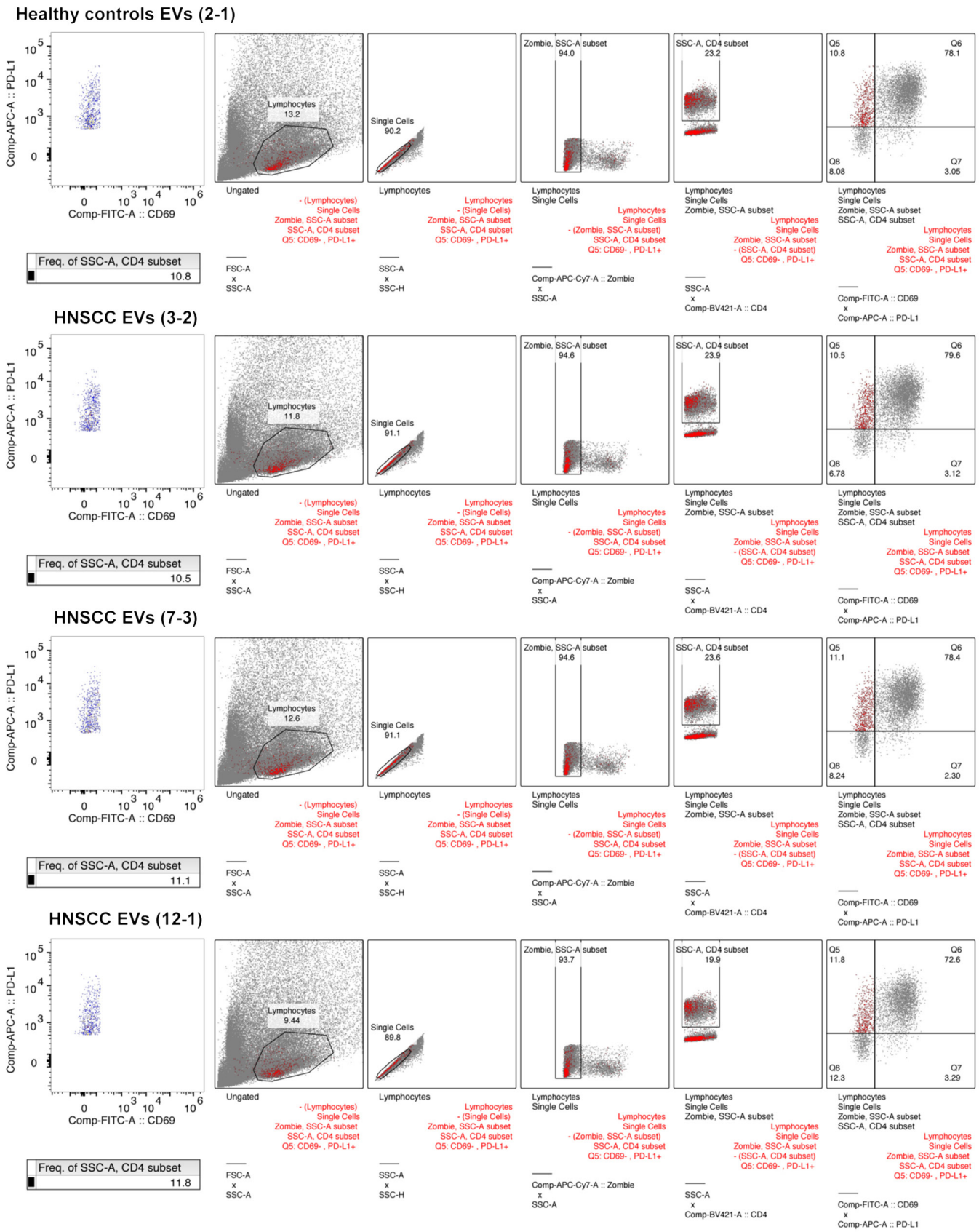

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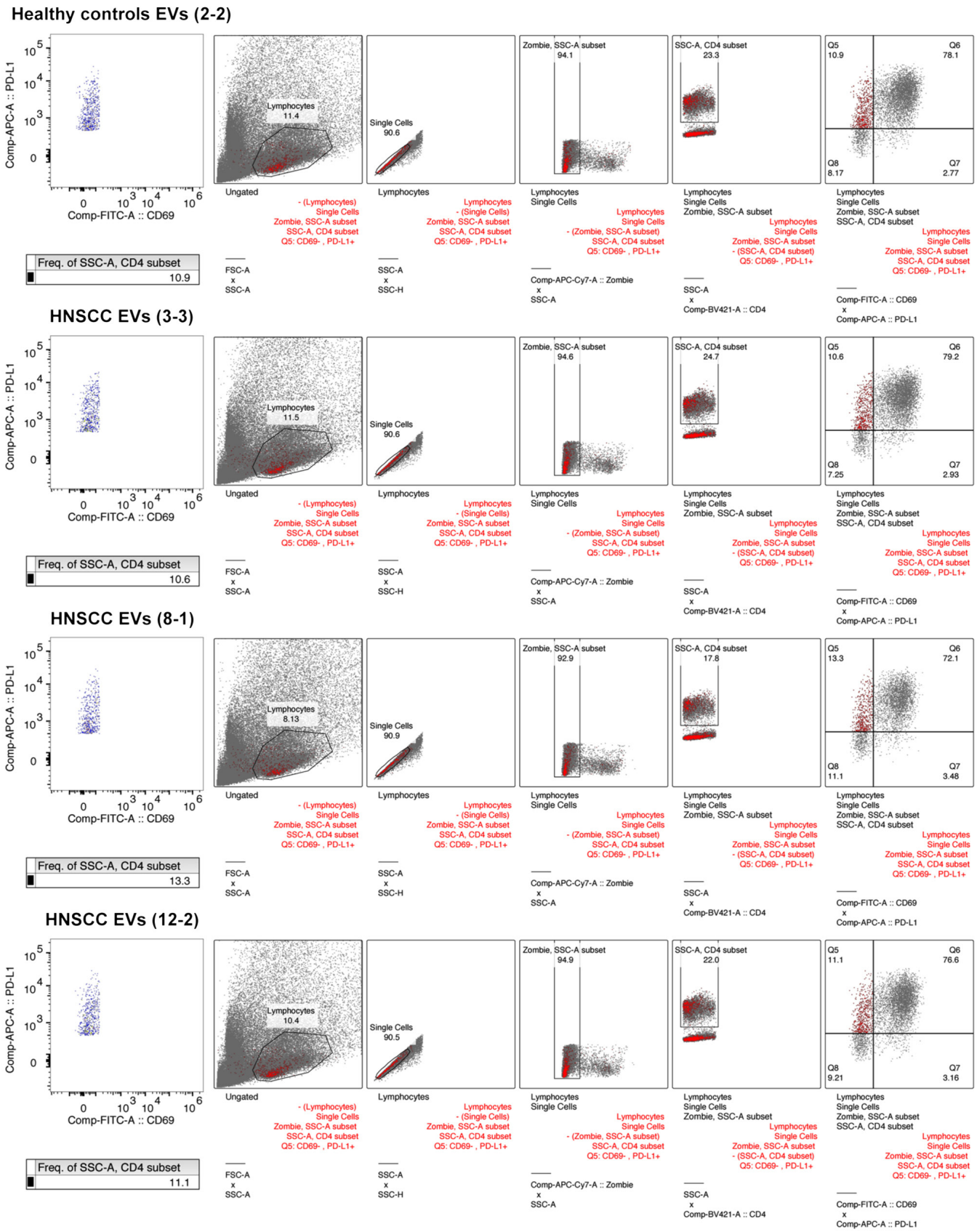

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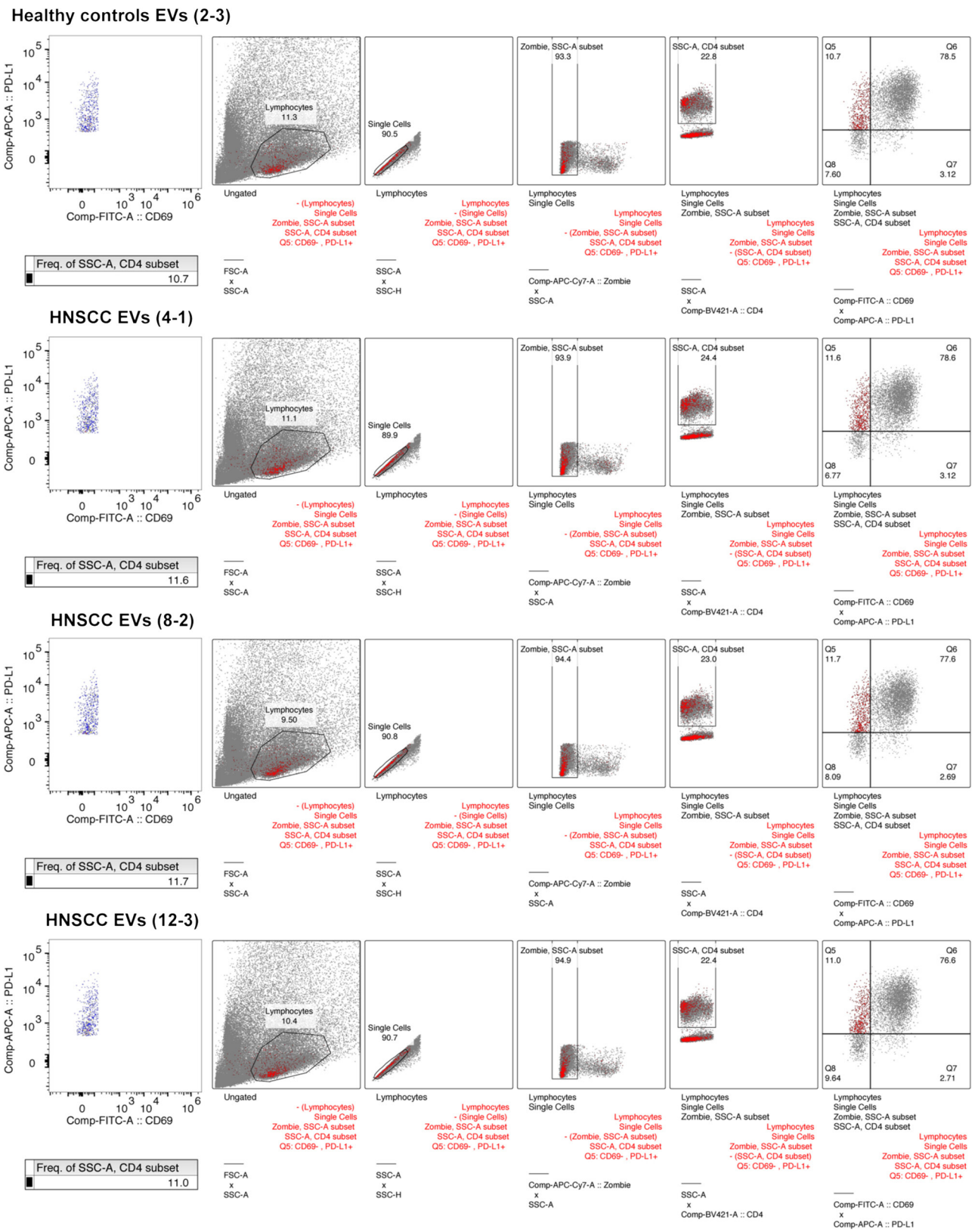

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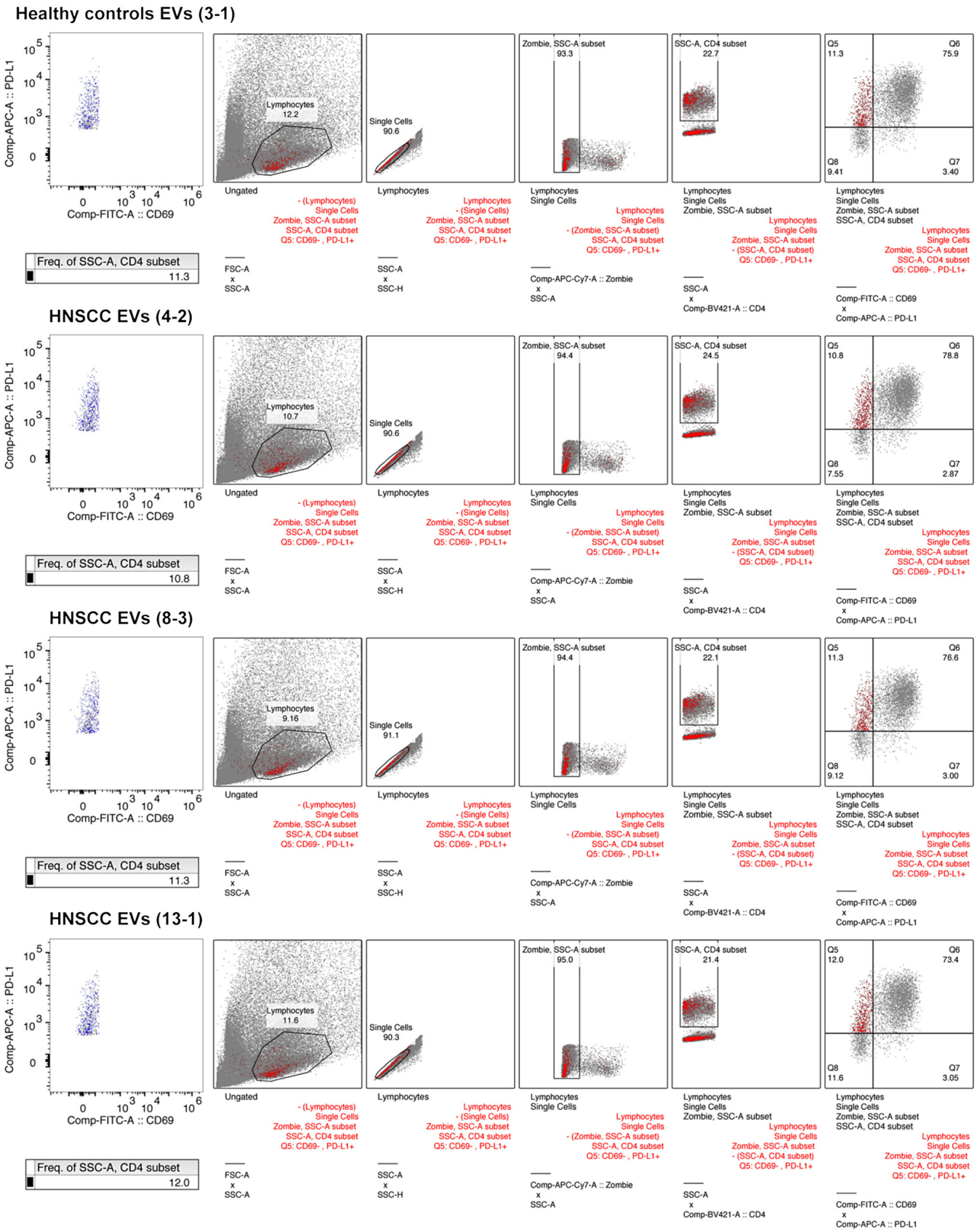

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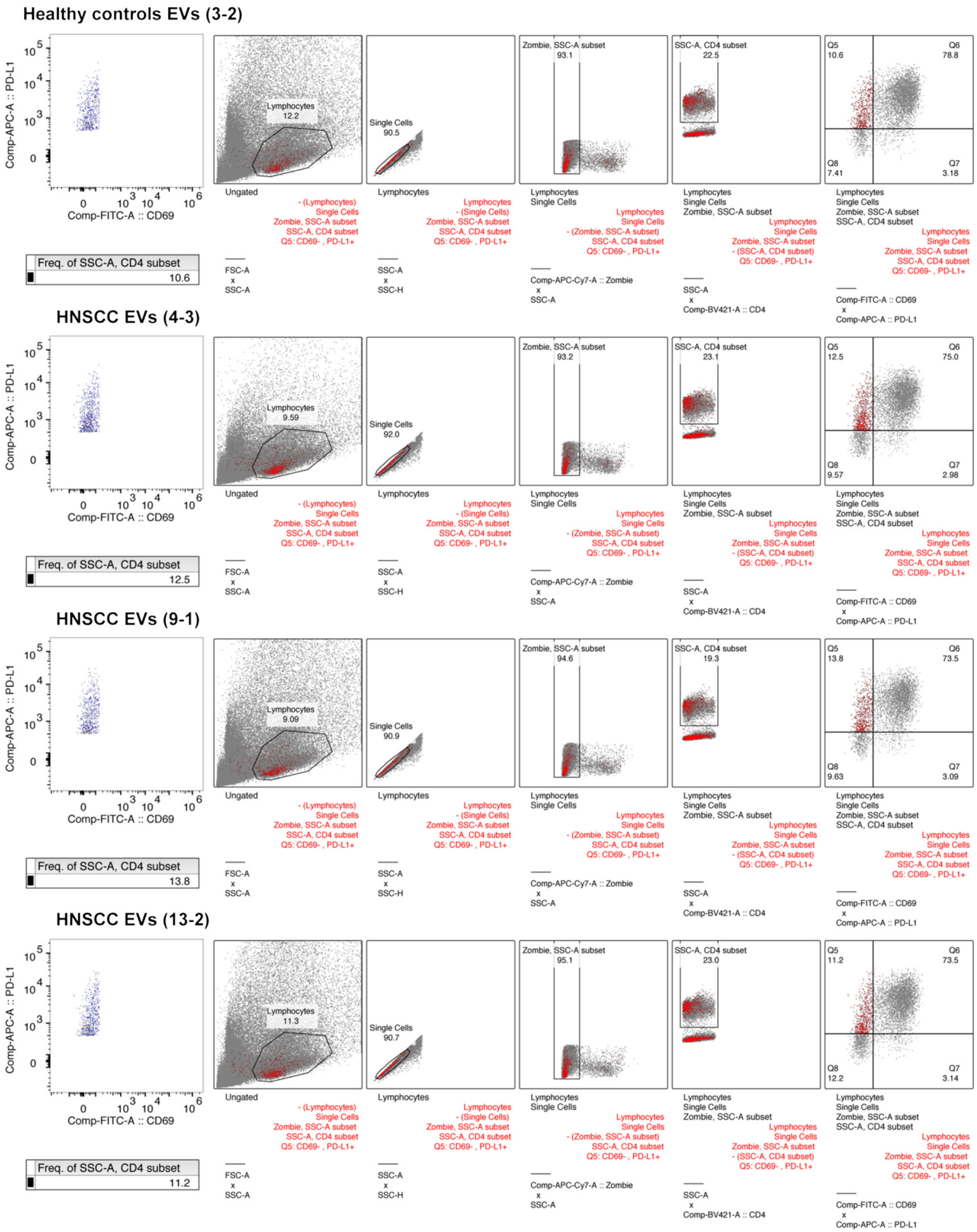

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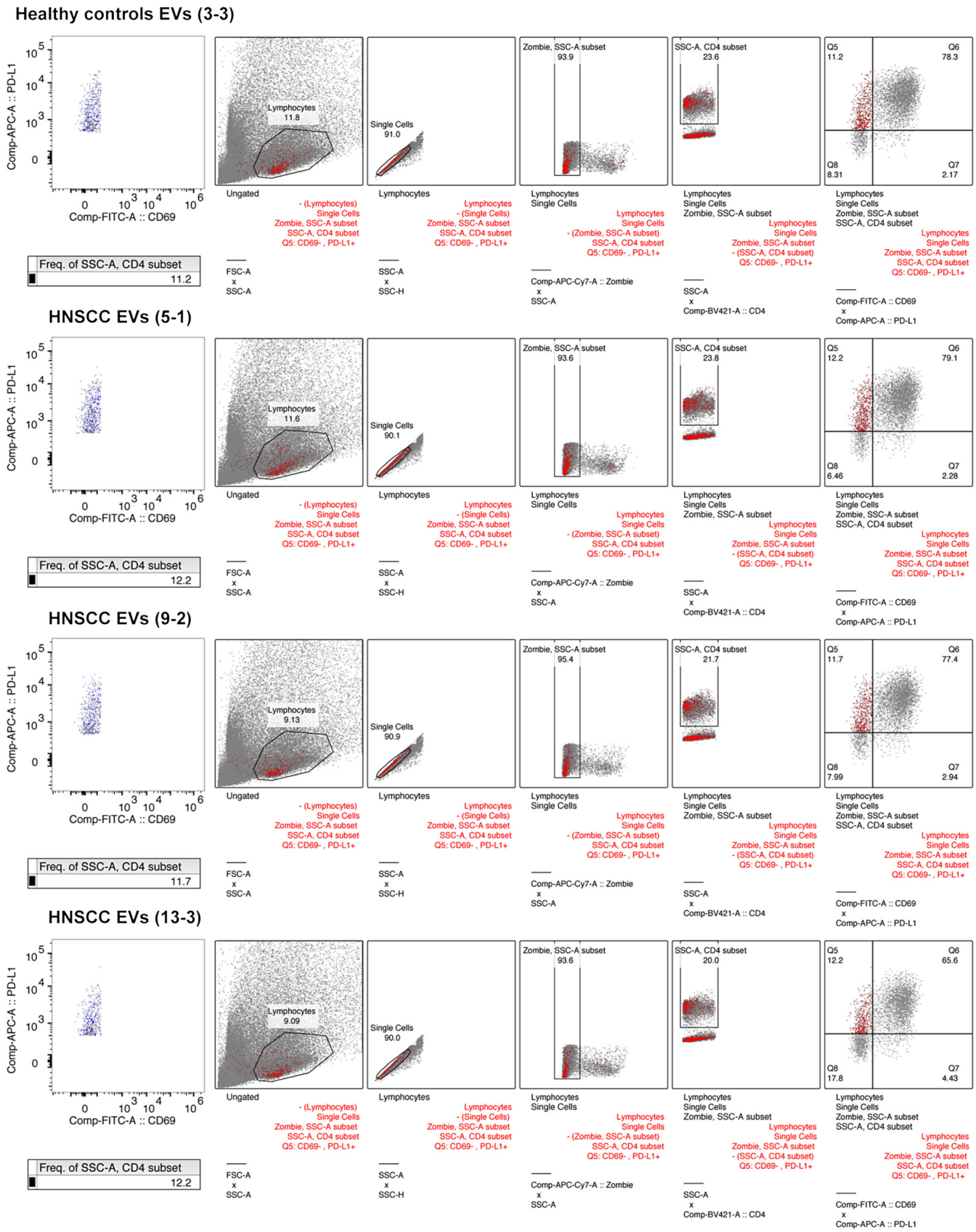

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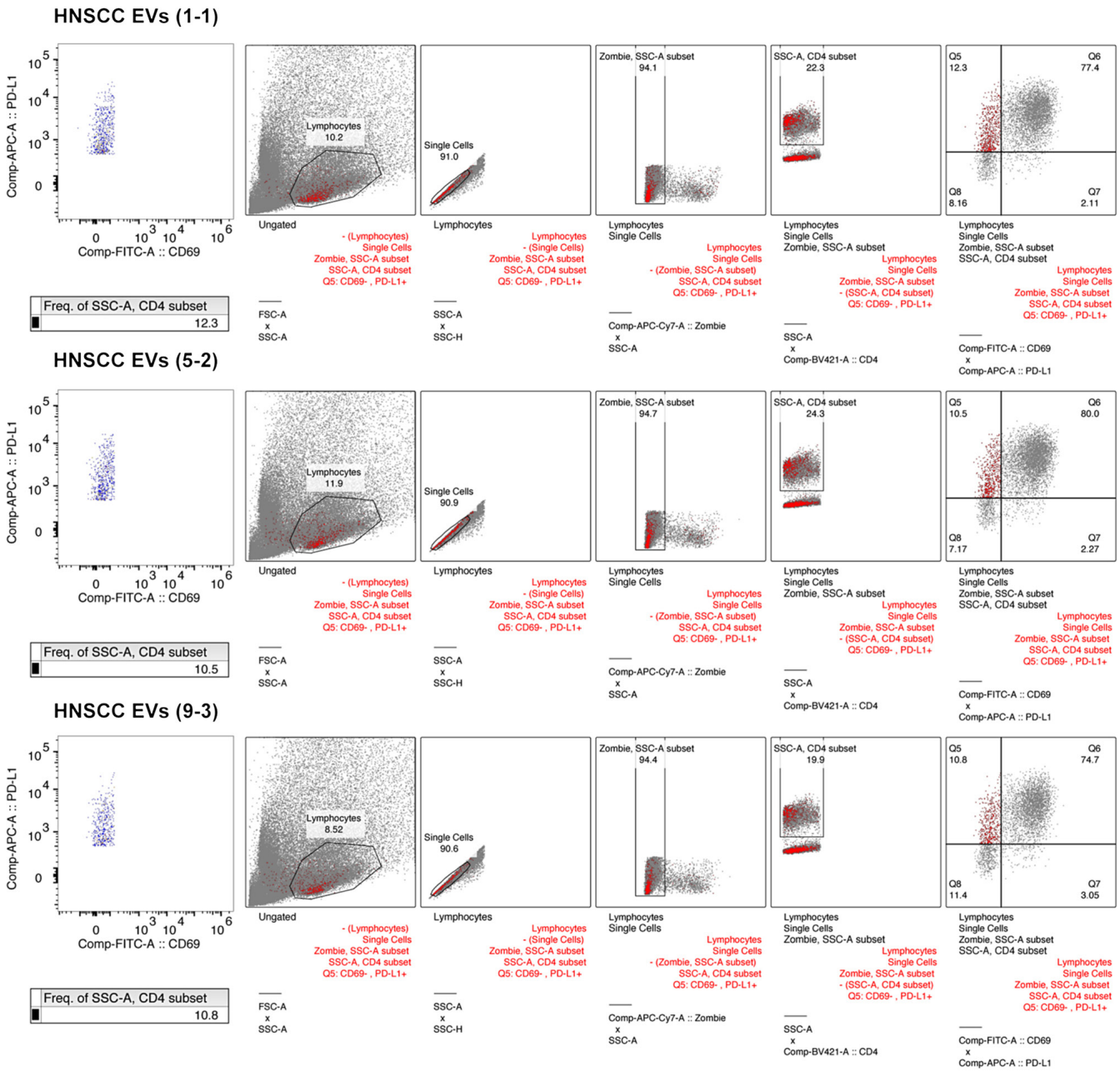

Figure S4. Continued.