Balancing Affinity and Chelator Density: Random Versus Site-Specific Single-Domain Antibody Radiolabelling for PET imaging of B7-H3 expressing tumors.

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Supplementary sheet.

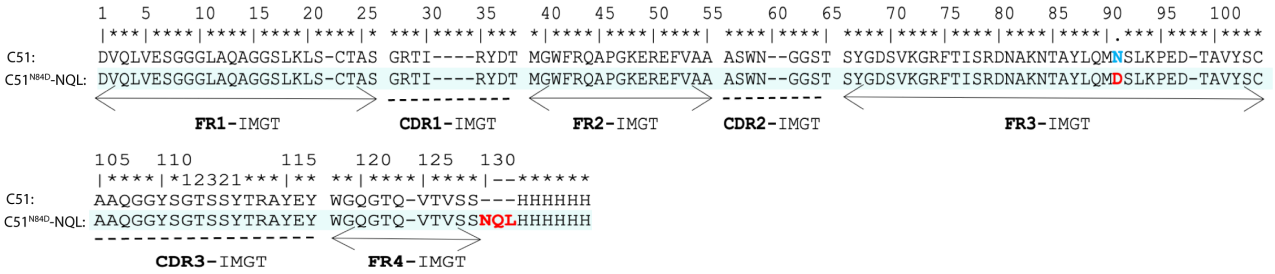

Fig. S1 Pairwise amino acid sequence alignment of native C51 and mutated C51^{N84D}-NQL sdAbs. Framework and CDR regions are highlighted (arrows and dotted lines, respectively), as well as the mutated amino acid in position 84 (91-IMGT numbering) and the inserted -NQL tag. C51, native C51 sdAb; C51^{N84D}-NQL, modified C51 sdAb containing VyPAL2-recognition-tag and a mutation.

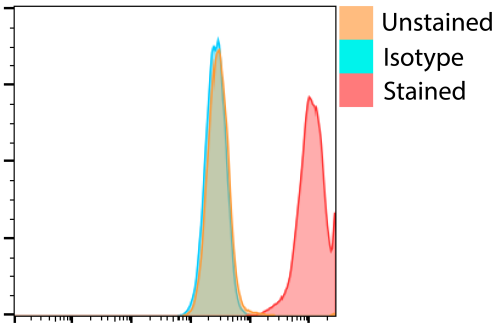

Fig. S2 Flow cytometry confirming native B7-H3 expression on U87-MG cells

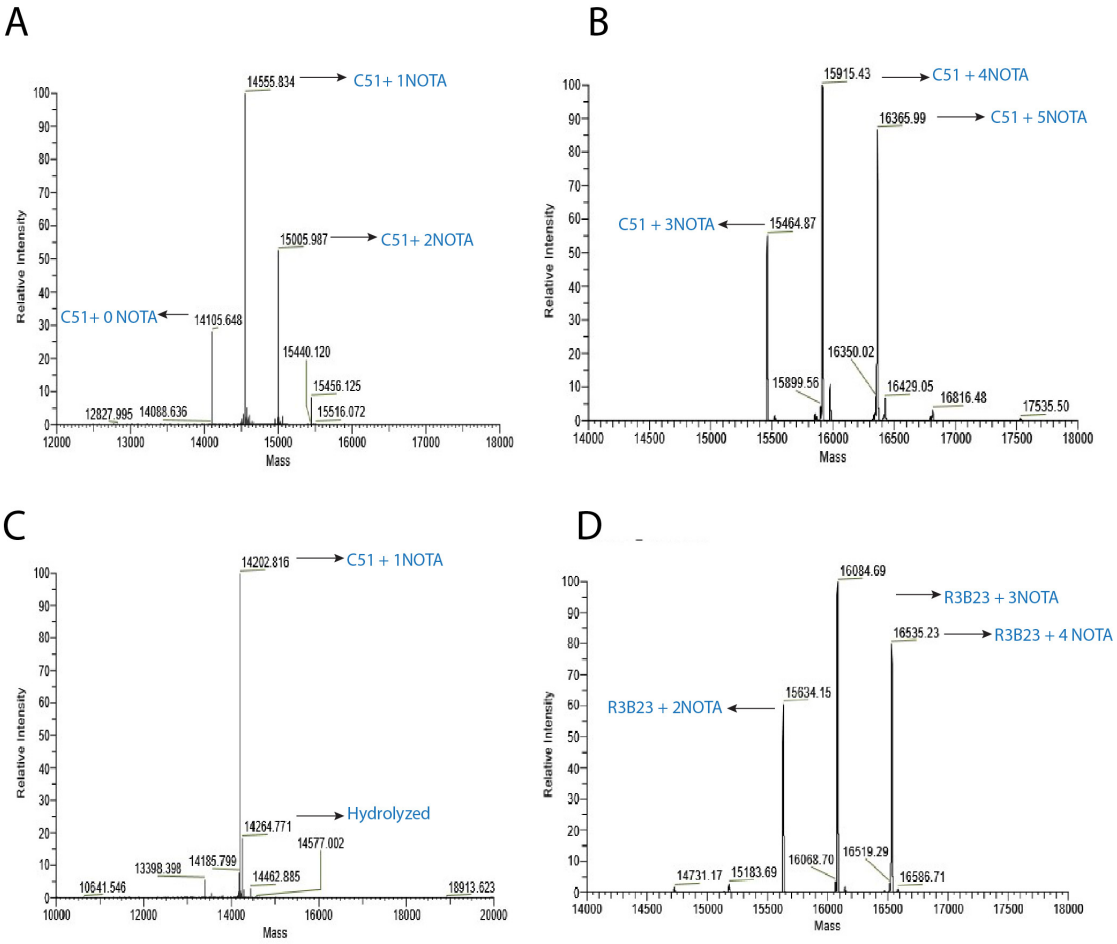

Fig. S3 Mass spectrometry profiles detailing different Chelator to sdAb ratios (CAR). (A) Randomly conjugated C51 construct with a ratio of 1.08 (CAR_L), (B) Randomly conjugated C51 with a ratio of 3.8 (CAR_H), (C) VyPAL2 mediated site-specific conjugated C51(C51-NGIGK-NOTA) with CAR of 0.96, and (D) R3B23-NOTA (CAR: 3.0)

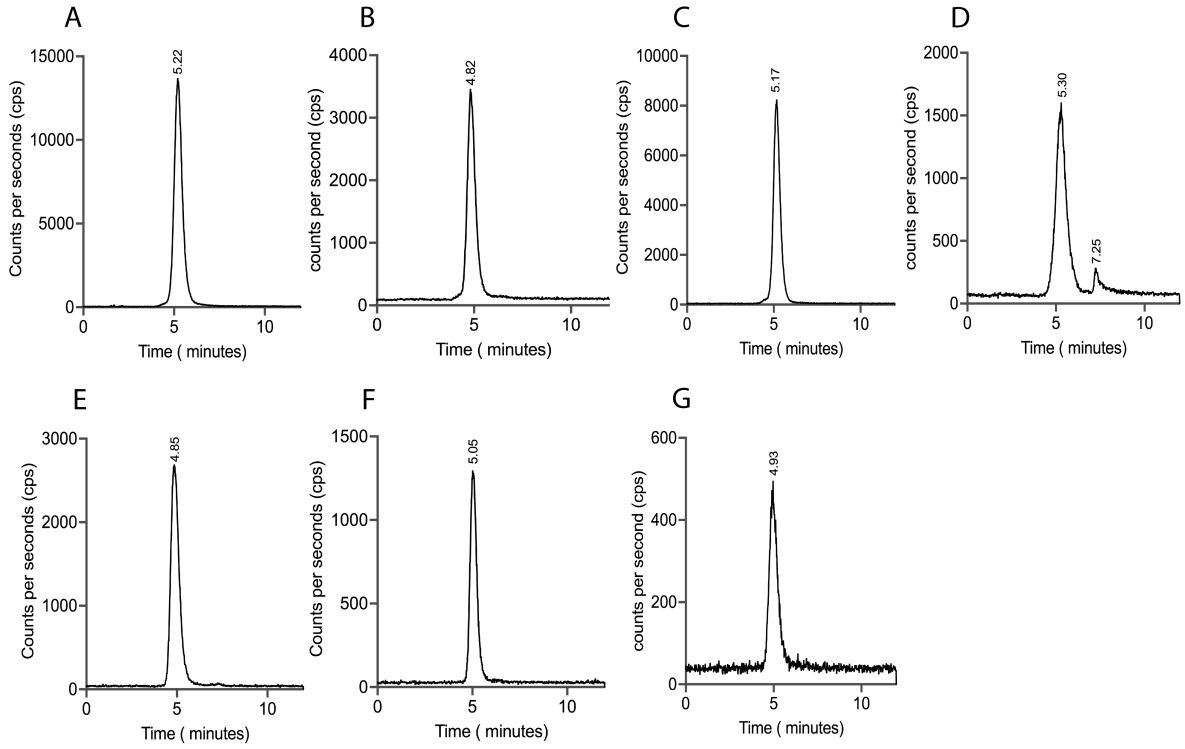

Fig S4 Radio size exclusion chromatograms of ^{68}Ga and ^{64}Cu radiolabeled tracers. (A) [^{68}Ga]Ga-NOTA-C51_CAR$_{L,}$ (B) [^{68}Ga]Ga-NOTA-C51_CAR$_{H,}$ (C) [^{68}Ga]Ga-NOTA-NGIGK-C51, (D) [^{68}Ga]Ga-NOTA-R3B23, free ^{68}Ga at 7.25', (E) [^{64}Cu]Cu-NOTA-C51, (F) [^{64}Cu]Cu-NOTA-NGIGK-C51, (G) [^{64}Cu]Cu-NOTA-R3B23.

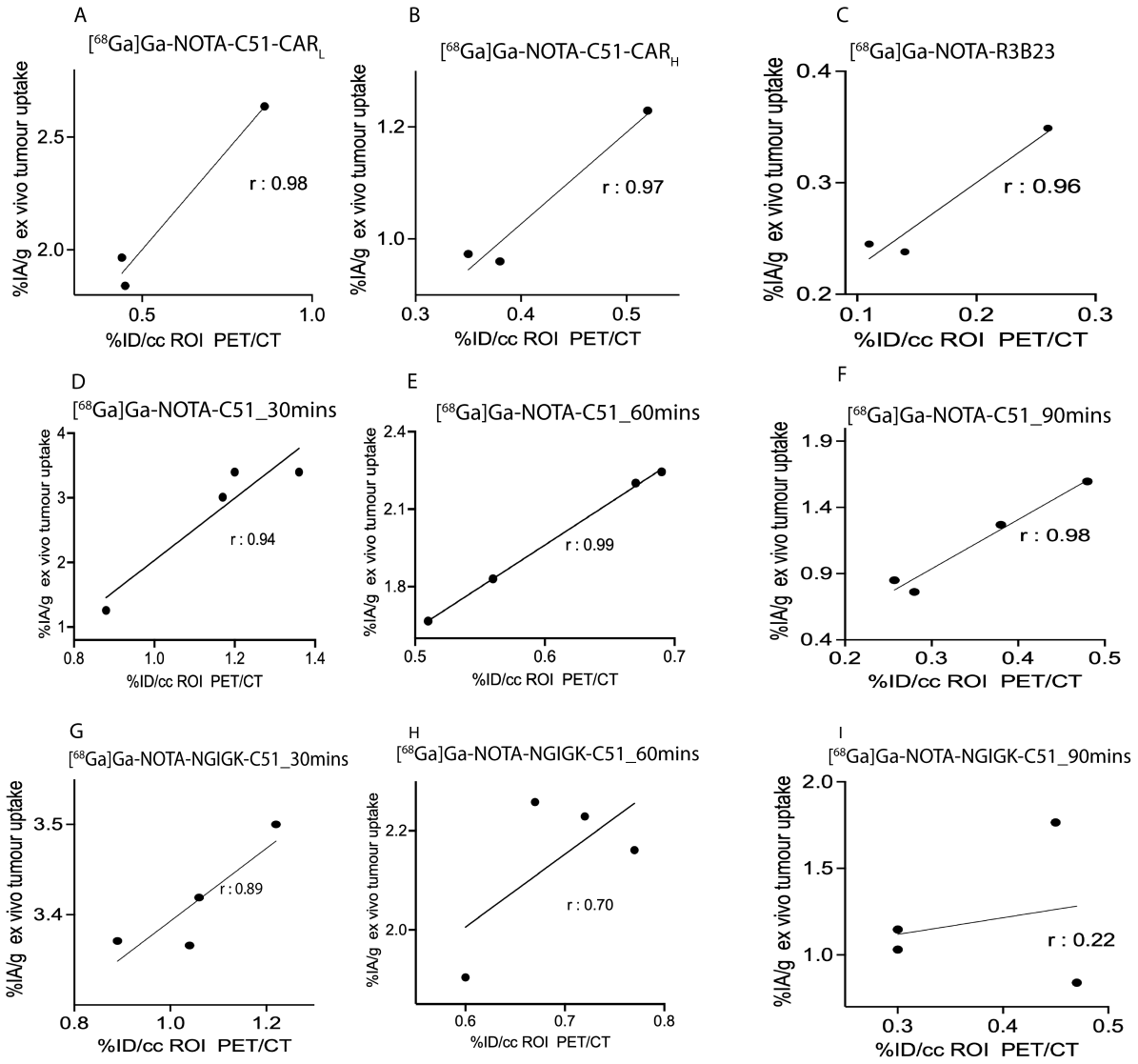

Fig. S5 Correlation plots of tumor radioactivity uptake measured via PET image quantification by drawing a region of interest (ROI) versus ex vivo measured radioactive uptake. (A-C) [^{68}Ga]Ga-NOTA-C51-CAR$_L$, [^{68}Ga]Ga-NOTA-C51-CAR$_H$, and [^{68}Ga]Ga-NOTA-R3B23, n = 3, (D-F) [^{68}Ga]Ga-NOTA-C51 at 30mins, 60 mins, and 90 mins p.i., n = 4, (G-I) [^{68}Ga]Ga-NOTA-NGIGK-C51 at 30 mins, 60 mins, and 90 mins p.i., n = 4.

Table S1 Radiochemical characteristics of ^{68}Ga- and ^{64}Cu radiotracers. Radiochemical purities (RCPs) were determined by radio size exclusion chromatography (Radio SE HPLC). Radiochemical yield (RCY) was calculated by measuring the activity of the end product and expressing it as a percentage of the starting activity, decay corrected. The specific activity was calculated by dividing the activity of the product by the recovered mass of sdAb in the product after purification.

Radiotracer	Decay corrected radiochemical yield (%)	Specific activity (MBq/ug)	RCP before purification (%)	RCP After purification (%)
[^{68}Ga]Ga-NOTA-C51 (CAR$_L$)	74.4 ± 6.1 (n=5)	4.9 ± 1.8 (n=5)	96.4 ± 0.6 (n =5)	98.7 ± 1.0 (n=5)
[^{68}Ga]Ga-NOTA-C51 (CAR$_H$)	70.0 ± 5.1 (n=2)	4.3 ± 2.8 (n=2)	96.5 ± 0 (n=1)	100 ± 00 (n=1)
[^{68}Ga]Ga-NOTA-KGIGN-C51	78.2 ± 5.1 (n=6)	5.0 ± 0.9 (n=6)	93.7 ± 0.9 (n=4)	97.9 ± 1.5 (n=4)

[^{68}Ga]Ga-NOTA-R3B23	70.0 ± 10.5 (n=3)	6.2 ± 0.7 (n=2)	94.7 ± 6.0 (n=2)	95.8 ± 5.8 (n=2)
[^{64}Cu]Cu-NOTA-C51 (CAR_L)	83.6 ± 5.5 (n=2)	2.4 ± 1.3 (n=2)	99.8 ± 0.4 (n=2)	99.6 ± 0.1 (n=2)
[^{64}Cu]Cu-NOTA-KGIGN-C51	87.2 ± 0.3 (n=2)	2.6 ± 1.3 (n=2)	98.7 ± 1.1 (n=2)	99.5 ± 0.1 (n=2)
[^{64}Cu]Cu-NOTA-R3B23	88.8 ± 0.0 (n=1)	1.6 ± 0.0 (n=1)	97.4 ± 0.0 (n=1)	98.9 ± 0.0 (n=1)

Table S2 *Ex vivo biodistribution results of [^{68}Ga]Ga-NOTA-C51 tracers in U87-MG tumor model (n=5) at 80 minutes post injection (p.i.). All results are represented as mean ± SD of injected activity per gram (%IA/g).*

Organ/Tissue	[^{68}Ga]Ga-NOTA-C51_CAR_L (%IA/g)	[^{68}Ga]Ga-NOTA-C51_CAR_H (%IA/g)	[^{68}Ga]Ga-NOTA-R3B23 (%IA/g)
Blood	0.22 ± 0.05	0.25 ± 0.03	0.24 ± 0.06
Lymph nodes	0.20 ± 0.09	0.19 ± 0.03	0.11 ± 0.08
Heart	0.12 ± 0.02	0.16 ± 0.03	0.16 ± 0.03
Lung	0.32 ± 0.03	0.47 ± 0.10	0.32 ± 0.09
Liver	0.31 ± 0.07	0.63 ± 0.09	0.53 ± 0.09
Pancreas	0.09 ± 0.01	0.16 ± 0.02	0.16 ± 0.03
Spleen	0.12 ± 0.01	0.18 ± 0.02	0.19 ± 0.05
R Kidneys	185.27 ± 20.17	117.07 ± 20.54	75.82 ± 10.48
Stomach	0.16 ± 0.02	0.21 ± 0.01	0.19 ± 0.04
Small intestine	0.13 ± 0.02	0.18 ± 0.03	0.16 ± 0.02
Large intestine	0.18 ± 0.02	0.21 ± 0.04	0.21 ± 0.07
Ovary	0.25 ± 0.03	0.23 ± 0.10	0.23 ± 0.08
Uterus	0.39 ± 0.10	0.38 ± 0.10	0.32 ± 0.05
Skin	0.28 ± 0.03	0.34 ± 0.04	0.36 ± 0.06
Muscle	0.10 ± 0.04	0.10 ± 0.04	0.10 ± 0.06
Bone	0.11 ± 0.06	0.12 ± 0.06	0.10 ± 0.02
Brain	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00
Tumor	2.20 ± 0.31	1.30 ± 0.50	0.28 ± 0.05

Table S3 *Ex vivo biodistribution over time for ^{68}Ga-labelled tracers (random Vs site-specific) in a U87-MG tumor model (n = 6) at 50, 80, and 110 minutes p.i. All results are represented as mean ± SD of injected activity per gram (%IA/g).*

	50 minutes		80 minutes		110 minutes	
ORGAN /TISSUE	[^{68}Ga]Ga-NOTA-C51 (%IA/g)	[^{68}Ga]Ga-NOTA-NGIGK-C51 (%IA/g)	[^{68}Ga]Ga-NOTA-C51 (%IA/g)	[^{68}Ga]Ga-NOTA-NGIGK-C51 (%IA/g)	[^{68}Ga]Ga-NOTA-C51 (%IA/g)	[^{68}Ga]Ga-NOTA-NGIGK-C51 (%IA/g)
Blood	1.01 ± 0.25	0.89 ± 0.24	0.30 ± 0.07	0.27 ± 0.16	0.14 ± 0.02	0.22 ± 0.03
L.nodes	0.48 ± 0.28	0.57 ± 0.21	0.17 ± 0.06	0.15 ± 0.09	0.10 ± 0.06	0.13 ± 0.04
Heart	0.42 ± 0.11	0.34 ± 0.11	0.14 ± 0.04	0.14 ± 0.03	0.08 ± 0.01	0.10 ± 0.02
Lung	0.87 ± 0.12	0.72 ± 0.17	0.41 ± 0.12	0.36 ± 0.14	0.22 ± 0.05	0.24 ± 0.04
Liver	0.51 ± 0.09	0.70 ± 0.08	0.34 ± 0.06	0.61 ± 0.14	0.33 ± 0.04	1.04 ± 0.23
Pancreas	0.29 ± 0.06	0.26 ± 0.07	0.12 ± 0.02	0.16 ± 0.07	0.07 ± 0.01	0.09 ± 0.02
Spleen	0.29 ± 0.03	0.32 ± 0.07	0.16 ± 0.03	0.33 ± 0.17	0.14 ± 0.03	0.42 ± 0.15

L kidney	159.27 ±13.28	130.23 ± 14.65	150.61 ± 17.59	147.05 ± 46.93	149.22 ± 6.25	136.36 ± 16.94
Stomach	0.58 ± 0.11	0.42 ± 0.06	0.21 ± 0.04	0.18 ± 0.05	0.10 ± 0.02	0.12 ± 0.02
S.intestine	0.35 ± 0.10	0.26 ± 0.06	0.22 ± 0.10	0.19 ± 0.14	0.10 ± 0.02	0.10 ± 0.02
L.intestine	0.37 ± 0.13	0.28 ± 0.08	0.22 ± 0.10	0.30 ± 0.22	0.11 ± 0.03	0.13 ± 0.04
Ovary	0.47 ± 0.17	0.31 ± 0.14	0.20 ± 0.11	0.19 ± 0.07	0.12 ± 0.07	0.12 ± 0.05
Uterus	1.17 ± 0.32	0.98 ± 0.32	0.42 ± 0.06	0.43 ± 0.23	0.18 ± 0.05	0.20 ± 0.04
Skin	1.28 ± 0.33	0.94 ± 0.16	0.40 ± 0.04	0.69 ± 0.51	0.20 ± 0.03	0.24 ± 0.06
Muscle	0.33 ± 0.08	0.27 ± 0.08	0.10 ± 0.01	0.10 ± 0.04	0.05 ± 0.01	0.07 ± 0.03
Bone	0.29 ± 0.08	0.42 ± 0.27	0.13 ± 0.04	0.11 ± 0.04	0.08 ± 0.01	0.11 ± 0.02
Brain	0.04 ± 0.01	0.04 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Tumor	3.25 ± 1.14	3.67 ± 0.58	2.05 ± 0.22	1.99 ± 0.48	1.18 ± 0.32	1.17 ± 0.32

Table S4 *Ex vivo biodistribution for ^{64}Cu-labelled tracers (random Vs site-specific) in a U87-MG tumor model (n=3) at 80 minutes p.i. All results are represented as mean ± SD of injected activity per gram (%IA/g).*

Organ/tissue	[^{64}Cu]Cu-NOTA-C51 (%IA/g)	[^{64}Cu]Cu-NOTA-NGIGK-C51 (%IA/g)	[^{64}Cu]Cu-NOTA-R3B23 (%IA/g)
Blood	0.24 ± 0.11	0.26 ± 0.06	0.11 ± 0.00
L. nodes	0.40 ± 0.15	0.47 ± 0.15	0.17 ± 0.09
Heart	0.22 ± 0.08	0.16 ± 0.02	0.12 ± 0.01
Lung	0.44 ± 0.19	0.44 ± 0.08	0.20 ± 0.05
Liver	0.95 ± 0.23	0.36 ± 0.02	0.49 ± 0.06
Pancreas	0.18 ± 0.04	0.13 ± 0.03	0.11 ± 0.00
Spleen	0.24 ± 0.05	0.13 ± 0.03	0.11 ± 0.01
L kidney	177.77 ± 30.76	195.97 ± 18.27	75.79 ± 8.26
Stomach	0.30 ± 0.06	0.21 ± 0.03	0.15 ± 0.02
S. intestine	0.32 ± 0.07	0.20 ± 0.01	0.17 ± 0.01
L. intestine	0.38 ± 0.13	0.23 ± 0.06	0.20 ± 0.04
Ovary	0.46 ± 0.09	0.33 ± 0.12	0.17 ± 0.03
Uterus	0.36 ± 0.07	0.59 ± 0.11	0.22 ± 0.06
Skin	0.37 ± 0.05	0.47 ± 0.06	0.29 ± 0.04
Muscle	0.10 ± 0.01	0.13 ± 0.02	0.05 ± 0.00
Bone	0.16 ± 0.01	0.16 ± 0.02	0.09 ± 0.02
Brain	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
Tumor	1.27 ± 0.40	1.89 ± 0.44	0.26 ± 0.09