Supporting information for

Development and characterization of Protein Kinase B/AKT isoform-specific Nanobodies

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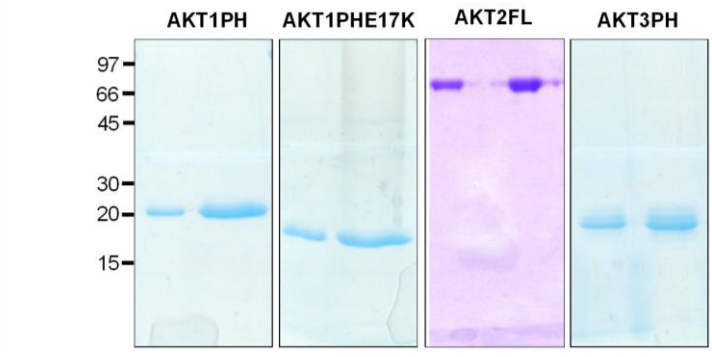

Figure S1: Antigens used for immunization. From left to right 1µg and 2µg of the AKT1 Pleckstrin homology domain (**AKT1PH**), the oncogenic mutant ATK1 PH-domain (**AKT1PHE17K**), full-length AKT2 (**AKT2FL**) and the AKT3 PH-domain (**AKT3PH**) respectively.

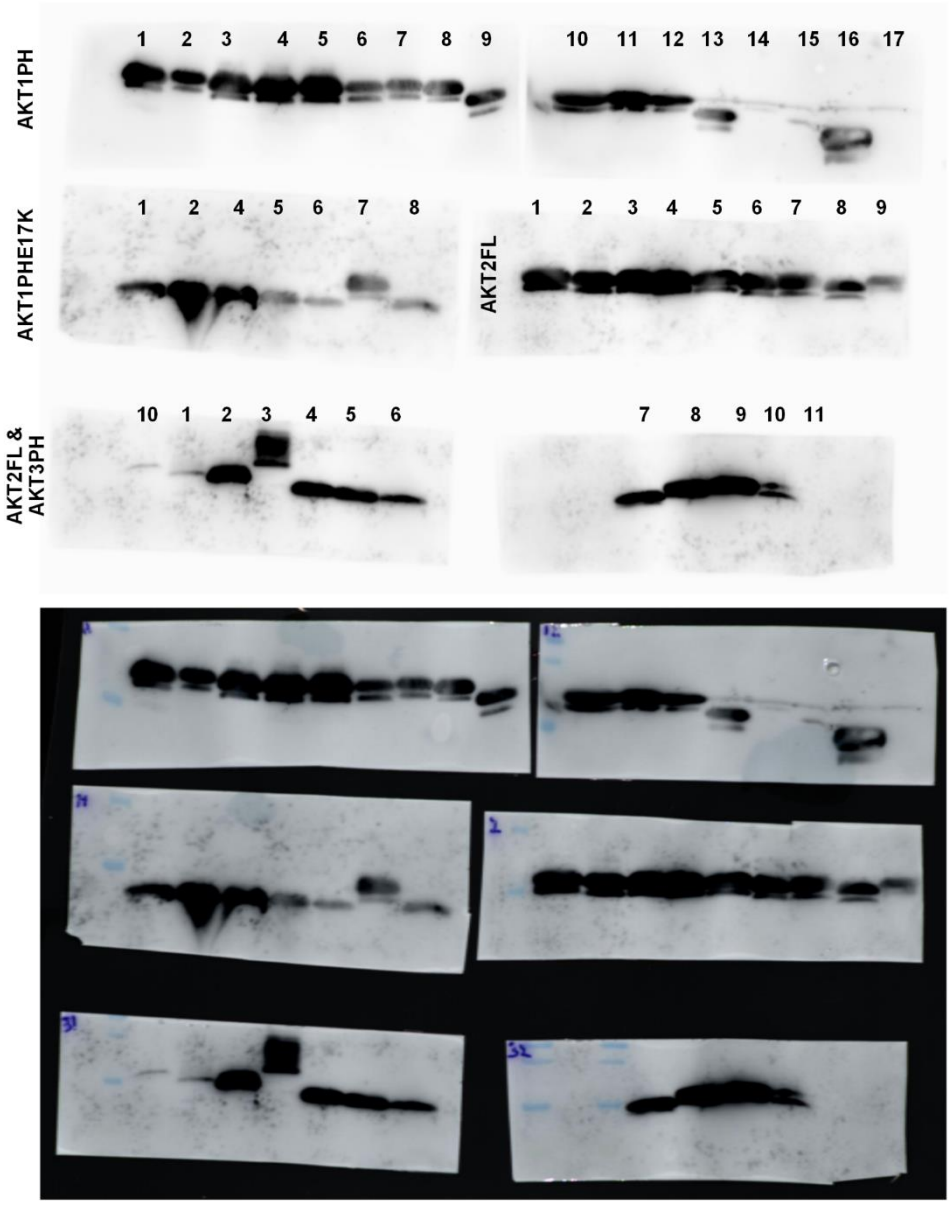

Figure S2: Expression of AKT nanobodies in WK6 *E. coli*. Uncropped Western blot images recorded using the Amersham Imager 680 blot and gel imager. AKT nanobodies present in the crude periplasmatic extract detected through enhanced-chemiluminescence using an anti-HA Ab and a secondary HRP-linked anti-mouse Ab. Nb signal is present at approximately 15kDa.

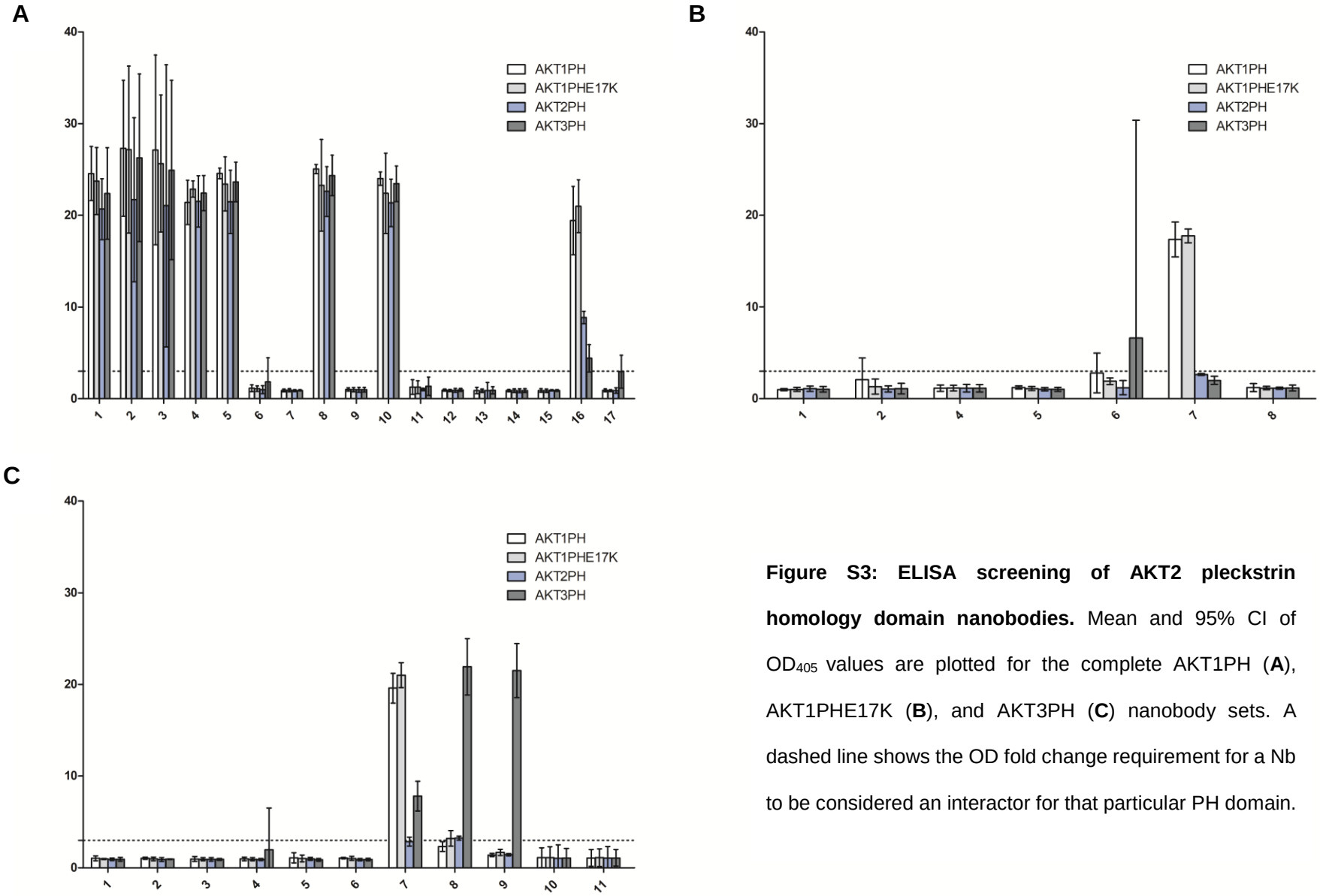

Figure S3: ELISA screening of AKT2 pleckstrin homology domain nanobodies. Mean and 95% CI of OD_{405} values are plotted for the complete AKT1PH (**A**), AKT1PHE17K (**B**), and AKT3PH (**C**) nanobody sets. A dashed line shows the OD fold change requirement for a Nb to be considered an interactor for that particular PH domain.

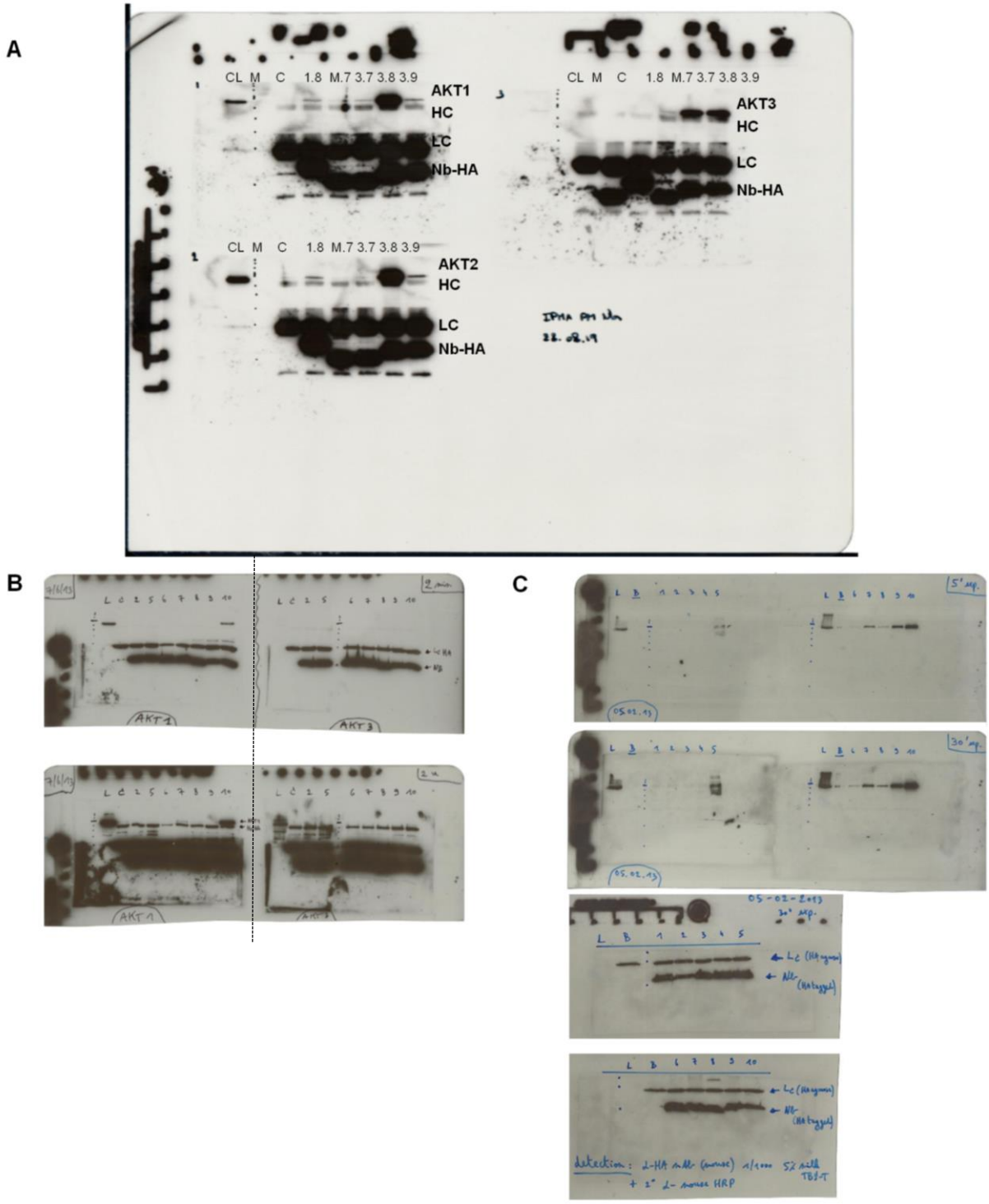

Figure S4: Co-IP of AKT isoforms using AKT1PH-, AKT1PHE17K, AKT2FL and AKT3PH-Nbs.

(**A**) Co-IP of AKT isoforms for PH-domain binding nanobodies selected through ELISA screening. **CL**: Crude lysate, **M**: protein ladder, **C**: control Co-IP using the EGFP Nb. (**B**) Co-IP screening of the AKT2FL nanobody set for AKT1 (**left**) and AKT3 (**right**) and (**C**) Co-IP screening of the AKT2FL nbs for AKT2.

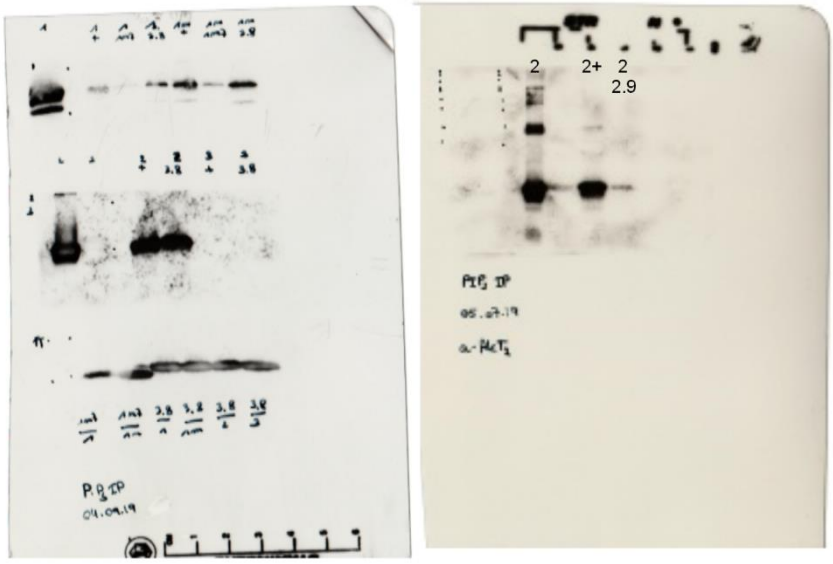

Figure S5: AKT nanobodies interfere with AKT-PH-PIP3 interaction. Uncropped western blots of pull-down experiments using PIP3-coated beads. For each PH-domain there is a lane containing only the PH-domain numbered **1**, **2** and **3**. Additionally a positive control where the PH domain is incubated with the beads without a Nb (**1+, 1m+, 2+, 3+**). For the test conditions the Nb that was added to the PH-domain prior to incubation with the beads is listed below the PH domain present in the mix: i.e. 1 / 1m7 indicated the AKT1 PH-domain was incubated with AKT1PHE17K Nb7.

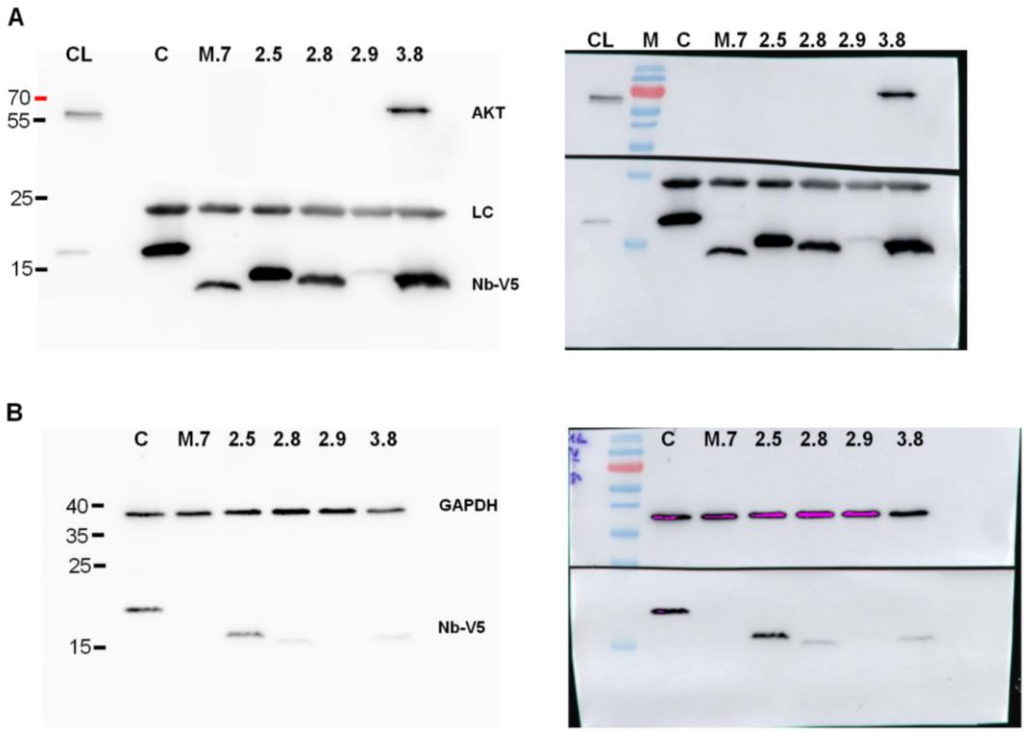

Figure S6: Co-IP of endogenous AKT from MDA-MB-231 cells with Nbs transiently expressed as intrabodies. A: Co-IP of AKT and Nbs. **CL**= crude lysate, **C**= negative control with transient expression of the EGFPNb. **LC**= Light Chain. **B:** Nb expression. 10µg crude lysate from transfected cells analysed through SDS-PAGE and western blotting. GAPDH signal was used as loading control. Blots were recorded images recorded using the Amersham Imager 680 blot and gel imager, the ECL signal is shown on the left. The right half of the figure shows the ECL signal superimposed on a photo of the blot membranes.

Table S1A

Characterization Of ATK1 PH-domain Nanobodies (AKT1PH set)								
	WK6 expression	ELISA specificity	Co-IP specificity	Epitope	PIP3 interfering	MDA-MB-231 expression	Intrabody Co-IP	Notes
1	✓	1, 1M, 2, 3		PH				Not specific, ELISA
2	✓	1, 1M, 2, 3		PH				
3	✓	1, 1M, 2, 3		PH				
4	✓	1, 1M, 2, 3		PH				
5	✓	1, 1M, 2, 3		PH				
6	✓							No interaction, ELISA
7	✓							
8	✓	1, 1M, 2, 3	1, 2	PH	N/A			Not specific, ELISA,IP
9	✓							No interaction, ELISA
10	✓	1, 1M, 2, 3		PH				Not specific, ELISA
11	✓							No interaction, ELISA
12	✓							
13	✓							
14	Low expression							
15	Low expression							
16	✓	1, 1M, 2, 3		PH				Not specific, ELISA
17	No expression							No interaction, ELISA

Table S1B

Characterization Of ATK1 PHE17K-domain Nanobodies (AKT1PHE17K set)								
	WK6 expression	ELISA specificity	Co-IP specificity	Epitope	PIP3 interfering	MDA-MB-231 expression	Intrabody Co-IP	Notes
1	✓							No interaction, ELISA
2	✓							
4	✓							
5	Low expression							
6	Low expression							
7	Low expression	1, 1M	1	PH	YES	YES, low	No signal	Specific for AKT1, binds PH-domain and interferes with PIP3 interaction
8	Low expression							No interaction, ELISA

Table S1C

	Characterization Of ATK2FL Nanobodies (AKT2FL set)							
	WK6 expression	ELISA specificity	Co-IP specificity	Epitope	PIP3 interfering	MDA-MB-231 expression	Intrabody Co-IP	Notes
1	✓		No AKT signal					No AKT2 signal, Co-IP
2	✓		No AKT signal					
3	✓		No AKT signal					
4	✓		No AKT signal					
5	✓		2	LINK/ KINASE	N/A	Yes, high	No	AKT2-specific, high expression in MDA-MB-23
6	✓		2, Low Signal					Low AKT2 signal
7	✓		1, 2					Not specific
8	✓		2	REG	N/A	Yes, low	No	AKT2-specific, low expression in MDA-MB-231
9	✓		2	PH	YES	Yes, very low	No	AKT2-specific, PH-domain interactor, inter-
10	Low expression		1, 2, 3	REG				Not specific

Table S1D

	Characterization Of AKT3 PH-domain Nanobodies (AKT3PH set)							
	WK6 expression	ELISA specificity	Co-IP specificity	Epitope	PIP3 interfering	MDA-MB-231 expression	Intrabody Co-IP	Notes
1	Low expression							No interaction, ELISA
2	✓							
3	✓							
4	✓							
5	✓							
6	✓							
7	✓	1, 1M, 3	1, 3	PH	N/A			Not specific
8	✓	3	1, 2, 3	PH	NO	YES, low	Yes	Not isoform specific, signal for intrabody Co-IP, does not interfere with PIP3
9	✓	3	1, 2, 3	PH	N/A			Not specific
10	✓							Not specific
11	No expression							No interaction, ELISA

Table S1: Overview of the characterization for all AKT Nb sets. Summary of characterization for the AKT1PH- (**A**), AKT1PHE17K- (**B**), AKT2FL- (**C**) and AKT3PH-nanobody sets (**D**). Expression of the Nbs in WK6 *E. coli* was evaluated though SDS-PAGE & Western blot analysis of crude periplasmatic extracts. AKT isoform-specificity was assessed though both ELISA using recombinant AKT PH-domains (**1 = AKT1 PH-domain, 1M = AKT1 PHE17K-domain, 2 = AKT2 PH-domain and 3 = AKT3 PH-domain**) and a Co-IP using recombinant Nbs and the endogenous AKT isoforms from MDA-MB-231 crude lysates. A grey filled cell in the 'ELISA specificity' column for the AKT1PH-, AKT1PHE17K- and AKT3PH-Nb sets indicates this Nb did not meet the OD fold change requirement for any PH-domain (**Figure S3, mean OD fold change below the dashed line**), these Nbs are not included in further experiments. The AKT2FL Nbs were not included in the ELISA screening. The Co-IP was used as final criteria for specificity. AKT2FL Nbs were produced by immunization with full-length AKT2, an ELISA epitope mapping was performed to determine which domain(s) these Nbs bind (**PH= pleckstrin homology domain, LINK/KINASE = Linker and kinase domain, REG= regulatory domain**). Nbs that interact with an AKT PH-domain can interfere with the PIP3 interaction required for AKT activation. Using PIP3 coated beads, recombinant AKT PH-domains and Nbs we determined AKT1PHE17K Nb7 interferes with the interaction of the AKT1 PH-domain AND AKT1 PHE17K-domain with PIP3. AKT2FL Nb9 interferes with the interaction of the AKT2 PH-domain with PIP3. Using transient expression a selection of Nbs was expressed in mammalian cells (MDA-MB-231) and evaluated as intrabodies through a Co-IP of endogenous AKT.