

Supplementary information for:

***Leveraging dual-ligase recruitment to enhance protein degradation via a heterotrivalent proteolysis targeting chimera***

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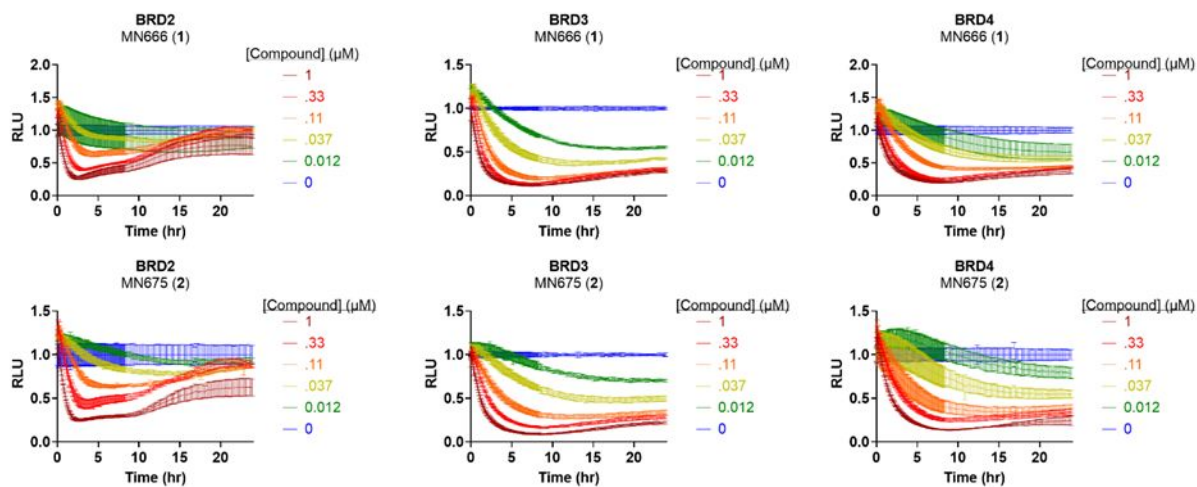
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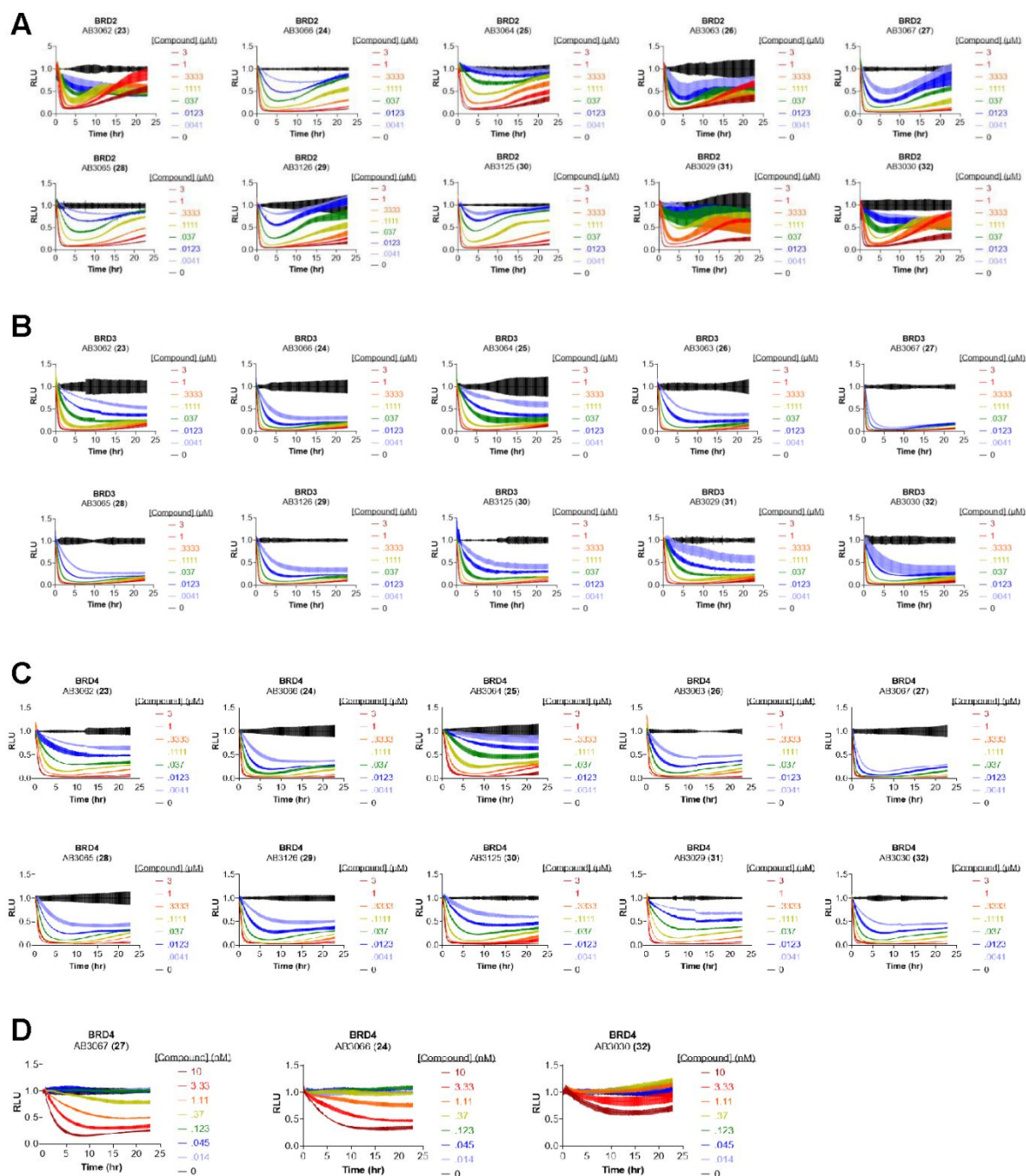
**Supplementary Figure 1** (related to Figure 3): Representative kinetic traces of first generation heterotrivalent PROTACs in HiBiT-BRD2, BRD3, and BRD4 HEK293 cells. Cells were treated with a dilution series ranging from 1  $\mu$ M - 12 nM compound. For all panels, each plot shows a single representative experiment with n=3 technical replicates; error bars represent S.D.

**Supplementary Table 1** (related to Figure 3): **Quantification of EC<sub>50</sub> values from cellular proliferation assays with dBET6, MZ1 and heterotrivalent PROTACs 1 and 2 in WT, CRBN KO, VHL KO and CRBN/VHL dKO RKO cell lines.**

RKO line	EC <sub>50</sub> (nM) <sup>a</sup>							
	dBET6	95% CI	MZ1	95% CI	MN666 (1)	95% CI	MN675 (2)	95% CI
WT	281	237 to 327	212	183 to 246	720	550 to 1001	489	435 to 552
CRBN KO	>10000	-	181	144 to 228	703	589 to 844	601	467 to 773
VHL KO	233	203 to 267	>10000	-	336	287 to 397	870	732 to 1079
CRBN/VHL dKO	>10000	-	>10000	-	1579	1184 to 2464	2063	1585 to 3112

<sup>a</sup> Data is colour scaled for lowest (green), median (yellow) and highest (red) EC<sub>50</sub> values in nM.





**Supplementary Figure 2** (related to Figure 4): (A-C) Kinetic traces of second generation heterotrivalent PROTACs in HiBiT-BRD2 (A), BRD3 (B), and BRD4 (C) HEK293 cells. Cells were treated with a dilution series ranging from 3  $\mu\text{M}$  - 4 nM compound. (D) Raw kinetic traces of HiBiT-BRD4 HEK293 cells treated with an extended nanomolar 3-fold dilution series ranging from 10 nM to 0.014 nM of compounds 27, 24, and 32. For all panels, each plot shows a single representative experiment with n=3 technical replicates; error bars represent S.D.

**Supplementary Table 2** (related to Figure 4B & Table 3): **Quantification of live-cell degradation parameter  $D_{\max 50}$  with heterotrivalent PROTACs 1, 2 and 23 – 32 against BET proteins in HiBiT-BRD knock-in HEK293 cells.**

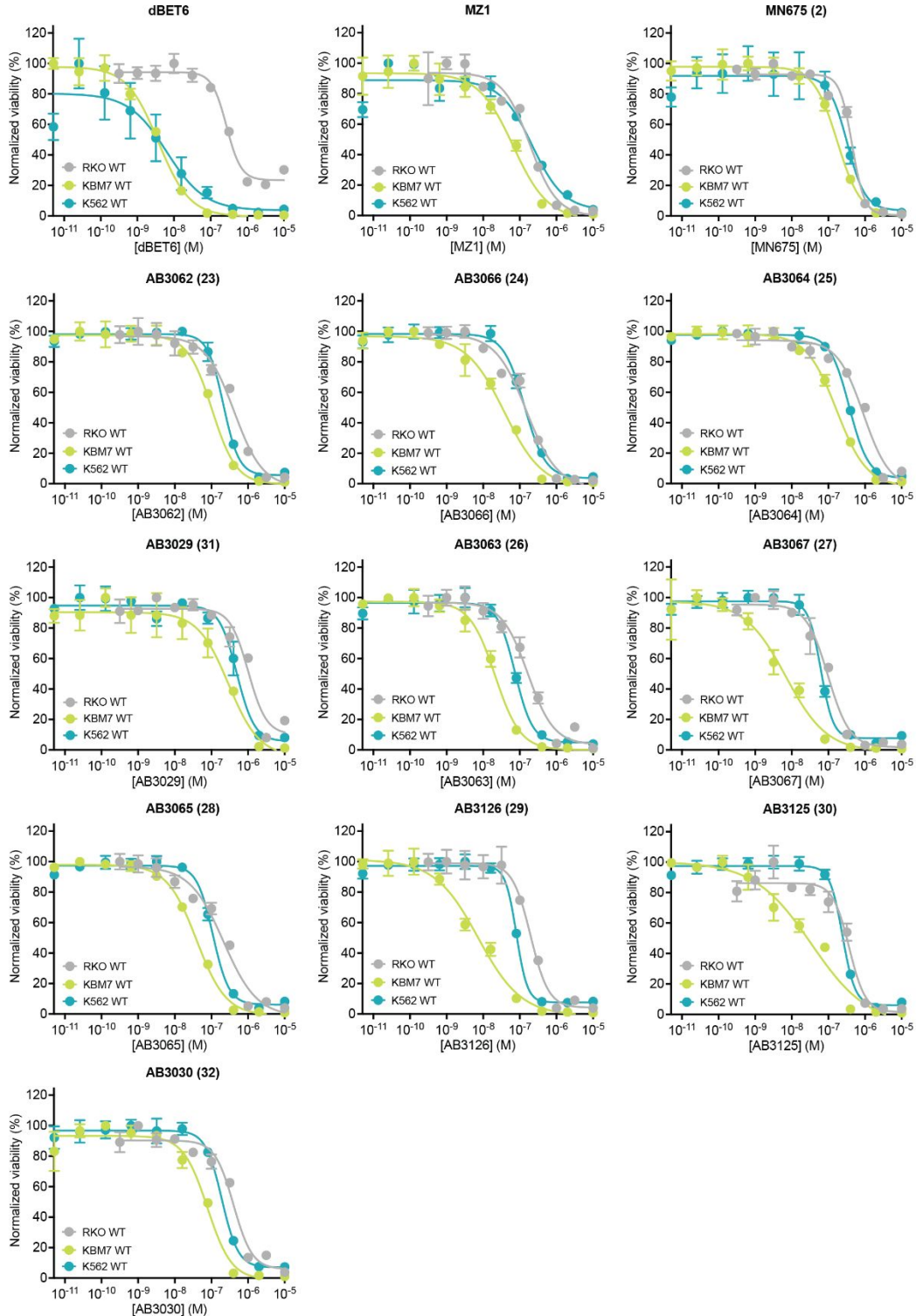
Compound	$D_{\max 50}$ , nM <sup>a</sup>					
	BRD4	95% CI	BRD3	95% CI	BRD2	95% CI
MN666 ( <b>1</b> )	37	29 to 48	-	-	-	-
MN675 ( <b>2</b> )	55	37 to 97	-	-	-	-
AB3062 ( <b>23</b> )	11	9.5 to 12	5.7	3.8 to 8.0	16	3.8 to und
AB3066 ( <b>24</b> )	3.0	2.6 to 3.5	0.12	und <sup>b</sup> to 1.4	7.3	5.3 to 9.2
AB3064 ( <b>25</b> )	31	24 to 40	8.1	5.8 to 12	74	59 to 93
AB3029 ( <b>31</b> )	15	13 to 16	6.2	4.8 to 7.9	160	61 to und <sup>b</sup>
AB3063 ( <b>26</b> )	3.0	2.6 to 3.3	1.5	0.70 to 2.2	4.7	2.7 to 7.5
AB3067 ( <b>27</b> )	0.64	0.50 to 0.80	0.085	und <sup>b</sup> to 0.43	2.0	0.37 to 3.5
AB3065 ( <b>28</b> )	2.9	2.4 to 3.4	1.2	0.92 to 1.5	23	20 to 27
AB3126 ( <b>29</b> )	4.0	3.6 to 4.5	1.9	1.2 to 2.4	18	15 to 22
AB3125 ( <b>30</b> )	8.2	7.2 to 9.3	3.0	2.2 to 3.9	31	22 to 29
AB3030 ( <b>32</b> )	7.4	5.6 to 10	1.7	0.47 to 2.8	25	12 to 130

<sup>a</sup> Data is colour scaled for lowest (green), median (yellow), and highest (red)  $D_{\max 50}$  values. <sup>b</sup> “und.” indicates the fit of the 95% CI is undefined.

**Supplementary Table 3** (related to Figure 4C & Table 3): **Quantification of live-cell degradation parameter degradation rate ( $\lambda_{\max}$ ) with heterotrivalent PROTACs 1, 2 and 23 – 32 against BET proteins in HiBiT-BRD knock-in HEK293 cells.**

Compound	$\lambda_{\max}$ , h <sup>-1</sup> <sup>a</sup>					
	BRD4	95% CI	BRD3	95% CI	BRD2	95% CI
AB3062 ( <b>23</b> )	1.40	1.36 to 1.44	2.06	1.99 to 2.13	1.12	0.93 to 1.33
AB3066 ( <b>24</b> )	2.16	2.09 to 2.24	2.66	2.55 to 2.78	2.04	1.88 to 2.21
AB3064 ( <b>25</b> )	1.09	1.06 to 1.11	1.69	1.62 to 1.75	1.34	1.25 to 1.44
AB3029 ( <b>31</b> )	1.99	1.94 to 2.04	2.36	2.23 to 2.51	1.90	1.75 to 2.06
AB3063 ( <b>26</b> )	1.98	1.89 to 2.07	2.54	2.45 to 2.64	1.72	1.57 to 1.88
AB3067 ( <b>27</b> )	2.68	2.56 to 2.81	3.31	3.15 to 3.47	2.37	2.21 to 2.53
AB3065 ( <b>28</b> )	1.51	1.46 to 1.55	2.14	2.06 to 2.23	1.46	1.34 to 1.58
AB3126 ( <b>29</b> )	1.69	1.68 to 1.79	2.62	2.54 to 2.71	2.35	2.19 to 2.52
AB3125 ( <b>30</b> )	1.76	1.70 to 1.84	2.60	2.48 to 2.72	2.63	2.54 to 2.72
AB3030 ( <b>32</b> )	2.19	2.10 to 2.29	2.97	2.86 to 3.09	2.04	1.90 to 2.20

<sup>a</sup> Data is colour scaled for highest (green), median (yellow), and lowest (red)  $D_{\max 50}$  values.



**Supplementary Figure 3: Cell viability evaluation of heterotrivalent PROTACs 23 - 32 in RKO, KBM7 & K562 cell lines.** Effect on cellular proliferation of dBET6, MZ1 and heterotrivalent PROTACs 2 and 23 - 32 after 316 pM to 10  $\mu$ M treatment in WT RKO or 5.12 pM to 10  $\mu$ M treatment in WT KBM7 and K562 cell lines. Mean  $\pm$  S.D.;  $n = 3$  biological replicates. EC<sub>50</sub> values are tabulated in Supplementary Table 2.

**Supplementary Table 4** (related to Supplementary Figure 2): **Quantification of EC<sub>50</sub> values from cellular proliferation assays with dBET6, MZ1 and heterotrivalent PROTACs 2 and 23 – 32 in WT RKO, KBM7 and K562 cell lines.**

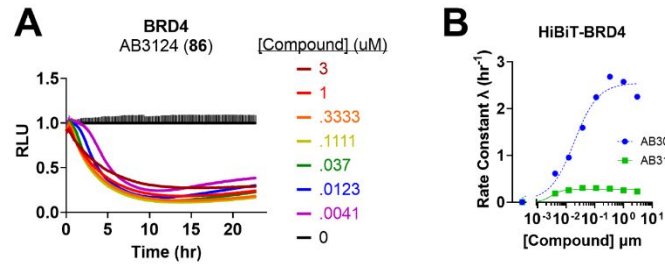
Compound	EC <sub>50</sub> (nM) <sup>a</sup>					
	RKO	95% CI	KBM7	95% CI	K562	95% CI
dBET6	271.6	223.0 to 322.6	3.7	3.0 to 4.6	6.4	2.3 to 20.1
MZ1	187.9	134.5 to 259.9	66.2	44.9 to 97.7	216.3	121.2 to 478.8
MN675 (2)	444.6	390.4 to 510.5	175.4	144.1 to 214.0	327.3	219.1 to 513.0
AB3062 (23)	419.8	319.8 to 563.7	104.2	87.7 to 124.4	212.3	187.9 to 239.3
AB3066 (24)	151.4	115.7 to 196.3	38.0	28.4 to 50.7	133.7	113.4 to 158.5
AB3064 (25)	903.6	668.5 to 1363.0	168.3	141.3 to 201.5	369.0	337.9 to 403.2
AB3029 (31)	1016.2	729.0 to 1332.0	294.4	185.4 to 507.9	494.3	394.1 to 648.5
AB3063 (26)	165.2	125.0 to 216.8	21.2	18.0 to 25.0	74.1	63.8 to 86.2
AB3067 (27)	97.5	78.6 to 119.4	6.9	4.3 to 10.8	62.1	53.4 to und <sup>b</sup>
AB3065 (28)	213.8	151.4 to 302.8	39.4	34.6 to 44.8	110.2	96.6 to 126.4
AB3126 (29)	195.4	161.5 to 236.1	7.3	5.2 to 10.2	80.5	74.9 to 87.1
AB3125 (30)	369.0	284.4 to 468.4	34.1	19.1 to 64.1	242.1	207.8 to 295.1
AB3030 (32)	401.8	311.7 to 509.8	78.0	58.3 to 102.7	192.3	158.0 to 231.7

<sup>a</sup> Data is colour scaled for lowest (green), median (yellow) and highest (red) EC<sub>50</sub> values in nM. <sup>b</sup> “und.” indicates the fit of the 95% CI is undefined.

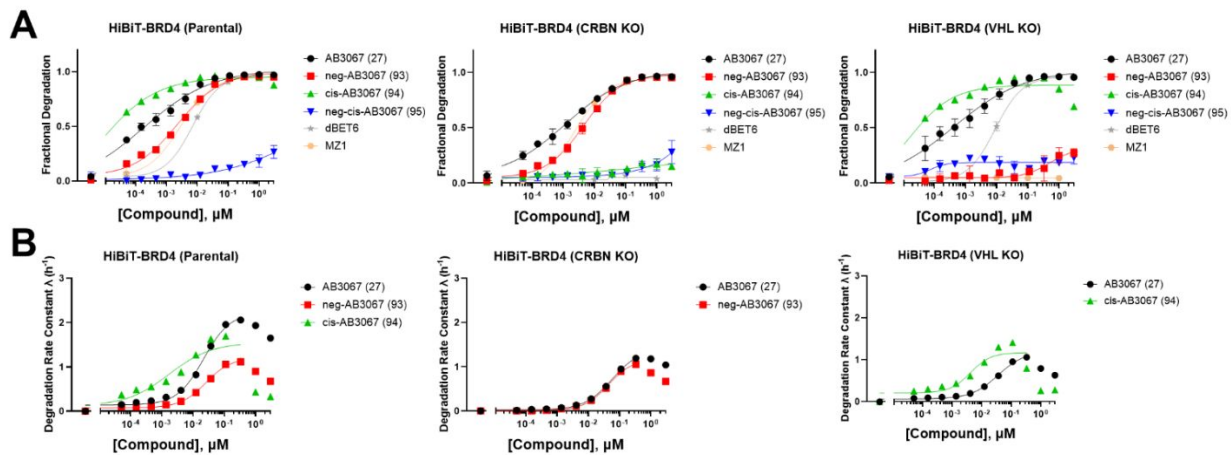
**Supplementary Table 5** (related to Figure 5 and Figure 8C): **Quantification of EC<sub>50</sub> values from cellular proliferation assays with heterotrivalent PROTACs 26, 27 and 86 in WT, CRBN KO, VHL KO and CRBN/VHL dKO RKO cell lines.**

RKO line	EC <sub>50</sub> (nM) <sup>a</sup>					
	AB3063 (26)	95% CI	AB3067 (27)	95% CI	AB3124 (86)	95% CI
WT	260	226 to 294	111	104 to 121	33	22 to 52
CRBN KO	408	335 to 501	180	149 to 215	34	26 to 44
VHL KO	819	651 to 1013	133	121 to 145	62	51 to 75
CRBN/VHL dKO	2235	1435 to 6027	988	695 to 1902	16	13 to 20

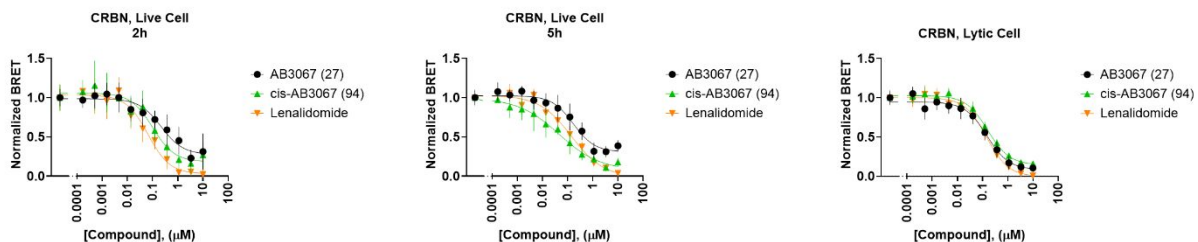
<sup>a</sup> Data is colour scaled for lowest (green), median (yellow) and highest (red) EC<sub>50</sub> values in nM.



**Supplementary Figure 4** (related to Figure 8): Kinetic traces associated with Figure 8. (A) 24-hour kinetic luminescence timecourse of HiBiT-BRD4 HEK293 cells supplied with an 8-point, 3-fold dilution series of **86** starting at 3  $\mu\text{M}$  ( $n=3$  technical replicates per concentration, error bars represent standard deviation). (B) BRD4 degradation rate constants mediated by **86** from the experiment in panel A of this figure) plotted alongside BRD4 degradation rate constants mediated by **27** (data replotted from Figure 4B) to allow for comparison of differences in degradation rate.



**Supplementary Figure 5: Degradation potency and rate of control compounds in HiBiT-BRD4 parental, VHL KO, and CRBN KO cells.** (A, B) Fractional degradation representing the  $D_{\text{max}}$  taken from individual kinetic profiles (A) and degradation rate constant determined from the kinetic profiles (B) of each cell line treated for 24h with an 8-point, 3-fold dilution series starting at 3  $\mu\text{M}$  of **27**, **93**, **94**, or **95**. A 3-point, 10-fold dilution series starting at 1  $\mu\text{M}$  of dBET6 and MZ1 were included in this experiment to show the specificity of KO of each E3 ligase.



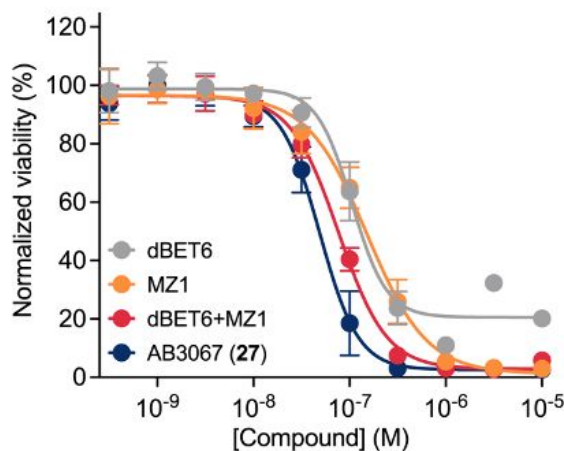


**Supplementary Figure 6. NanoBRET lytic and live cell target engagement assay of 27 and 94.** Competitive displacement profiles of HEK293 cells transiently transfected with NanoLuc-CRBN, which are incubated with a CRBN fluorescent tracer in the presence of serial dilutions of 27, 94 or lenalidomide in cells lysed with digitonin (Lytic cell) or in live cells for 2 h or 5 h. Data are represented as NanoBRET ratios normalized to 0  $\mu$ M compound. Error bars are expressed as S.D. of the mean of  $n = 2$  biological replicates (each consisting of 3 technical replicates) for 2 h Live Cell and Lytic and  $n=1$  biological replicate (consisting of 4 technical replicates) for 5 h Live Cell.

**Supplementary Table 6 (related to Figure 10): Quantification of EC<sub>50</sub> values from cellular proliferation assays with dBET6, MZ1 and heterotrivalent PROTACs 27 and 93 - 95 in WT, CRBN KO, VHL KO and CRBN/VHL dKO RKO cell lines.**

RKO line	EC <sub>50</sub> (nM) <sup>a</sup>			
	MZ1	95% CI	<i>neg</i> -AB3067 (93)	95% CI
WT	160	136 to 189	107	96 to 124
CRBN KO	136	118 to 158	105	94 to 117
VHL KO	>5000	-	391	324 to 504
CRBN/VHL dKO	>5000	-	420	328 to 550
RKO line	dBET6	95% CI	<i>cis</i> -AB3067 (94)	95% CI
WT	256	215 to 302	48	40 to 58
CRBN KO	>10000	-	832	652 to 1079
VHL KO	291	236 to 357	48	39 to 60
CRBN/VHL dKO	>10000	-	804	626 to 1051
RKO line	AB3067 (27)	95% CI	<i>neg-cis</i> -AB3067 (95)	95% CI
WT	73	66 to 81	594	460 to 786
CRBN KO	82	71 to 94	586	403 to 924
VHL KO	110	94 to 129	522	388 to 726
CRBN/VHL dKO	818	641 to 1078	657	449 to 1014

<sup>a</sup> Data is colour scaled for lowest (green), median (yellow) and highest (red) EC<sub>50</sub> values in nM



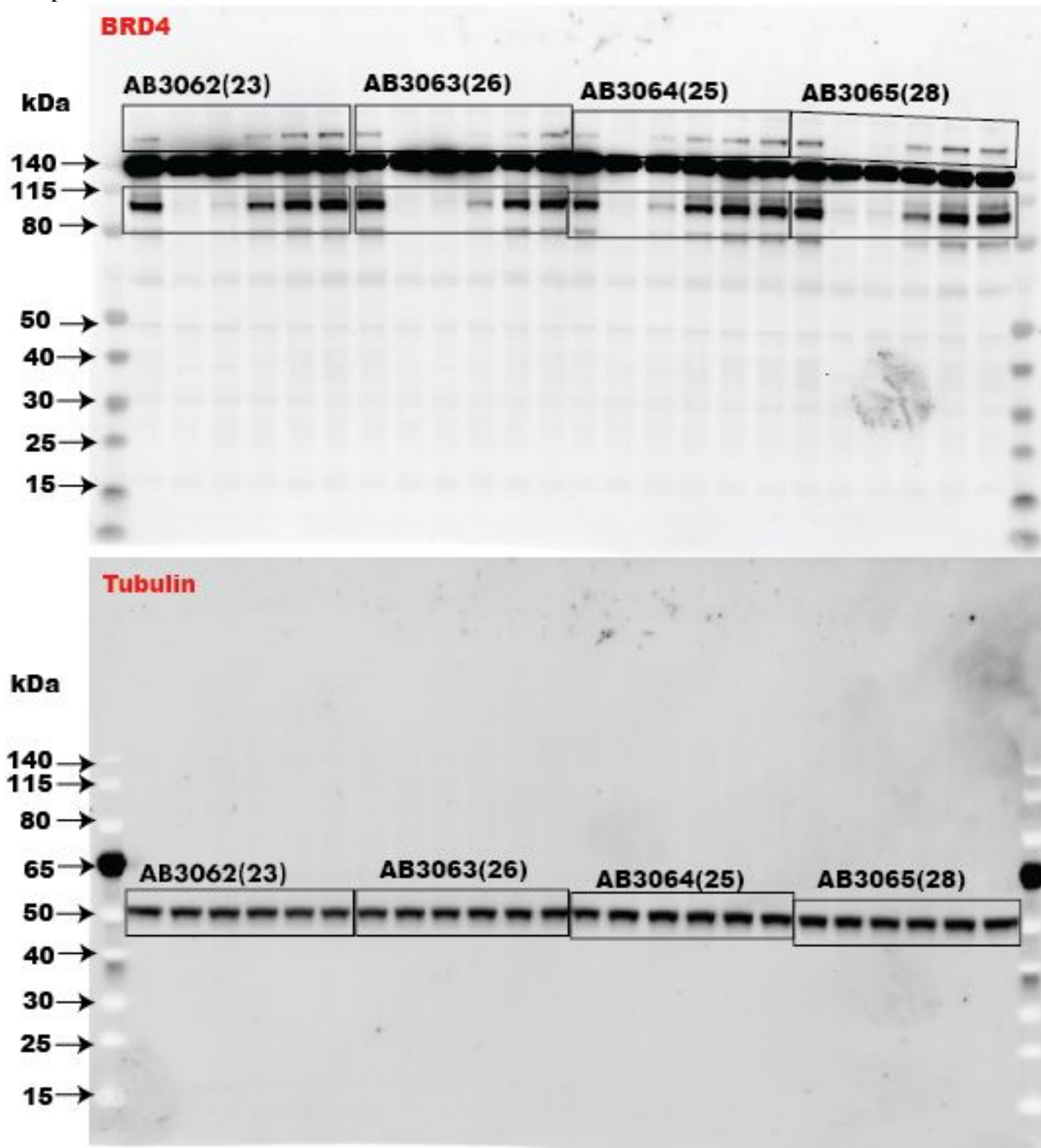
**Supplementary Figure 7** (related to Figure 10). **Cell viability assay with dBET6, MZ1, 27 and a 1:1 combination of dBET6 and MZ1 in BET sensitive RKO WT cells.** Effect on cellular proliferation of dBET6, MZ1, 27 and a 1:1 combination of dBET6 and MZ1 after 316 pM to 10  $\mu$ M treatment in WT RKO cells. dBET6 and MZ1 were added at 5  $\mu$ M for a total starting concentration of 10  $\mu$ M BET PROTAC and diluted therein. Mean  $\pm$  S.D.;  $n = 6$  biological replicates.

**Supplementary Table 7** (related to Supplementary Figure 7): **Quantification of EC<sub>50</sub> values from cellular proliferation assays with dBET6, MZ1, 27 and 1:1 combination of dBET6 and MZ1 in BET sensitive RKO WT cells.**

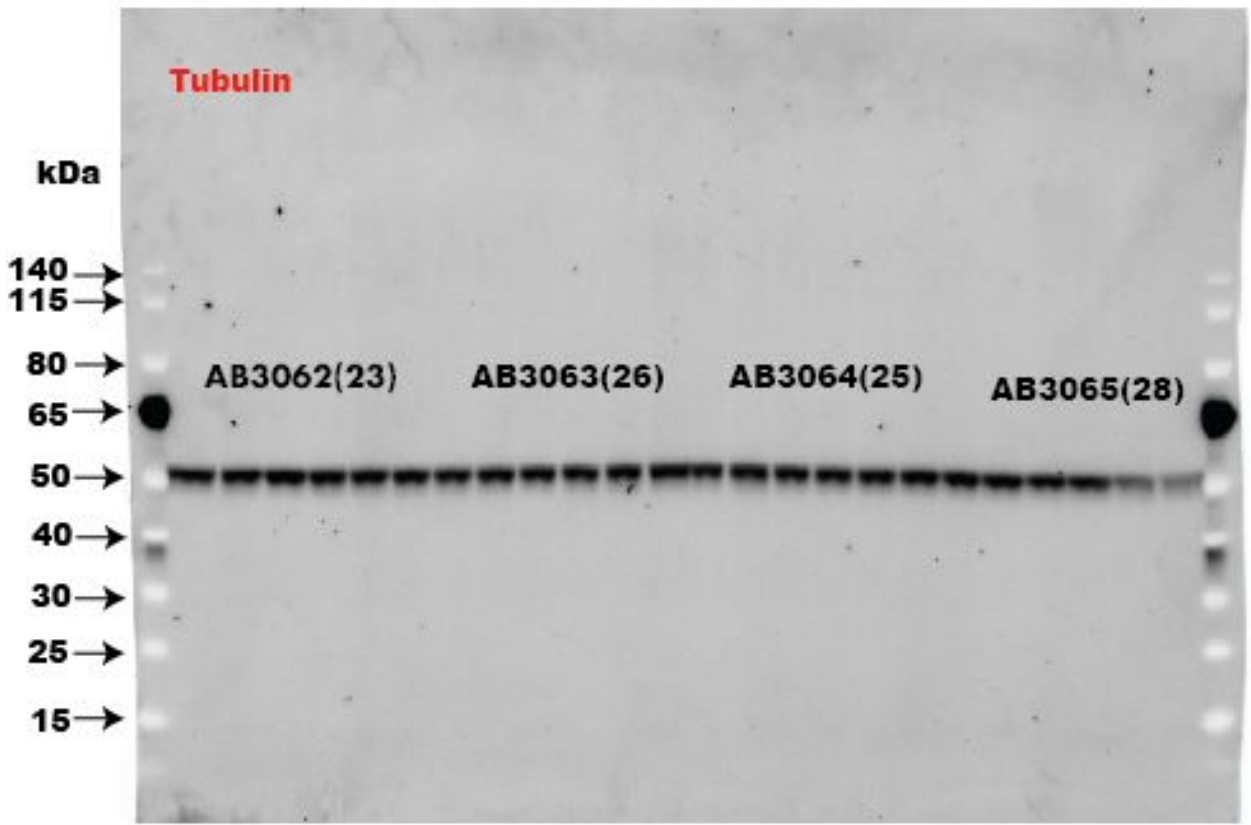
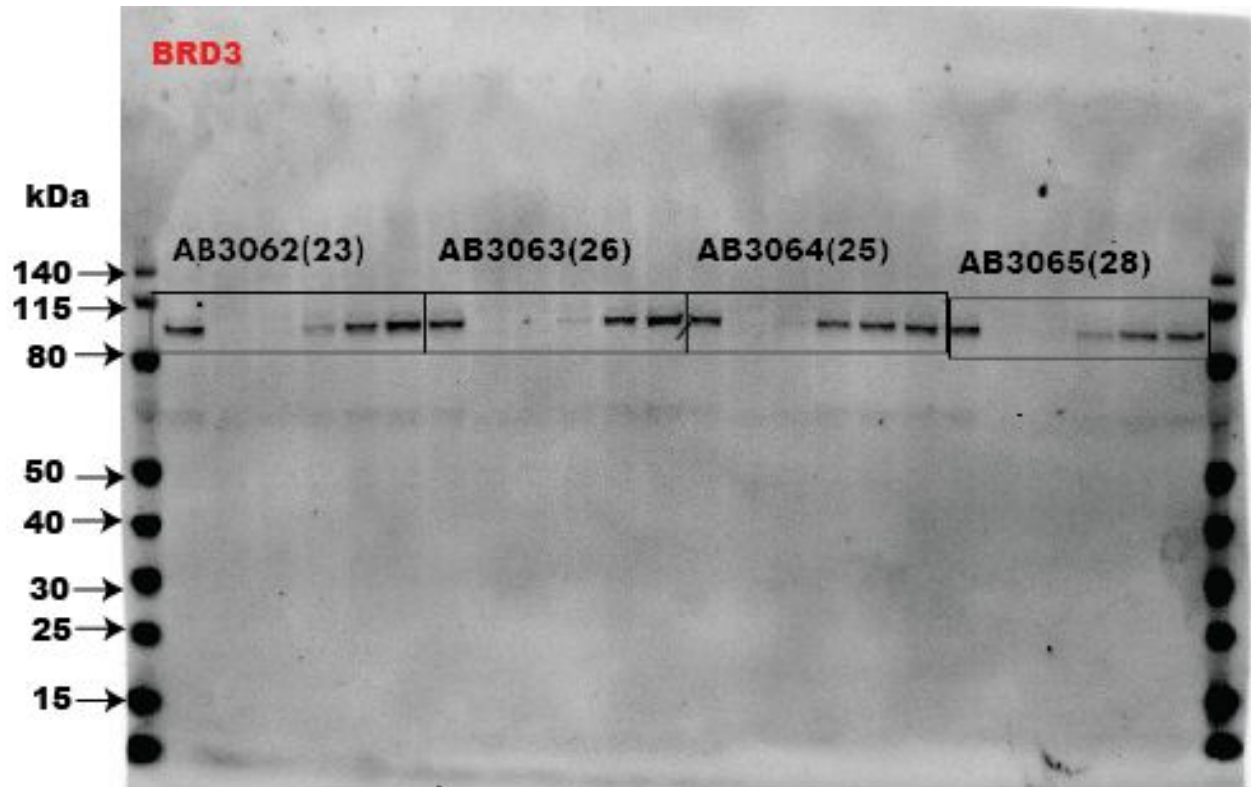
Compound	EC <sub>50</sub> (nM) <sup>a</sup>	
	RKO	95% CI
dBET6	106.0	89 to 123
MZ1	152.0	132 to 175
dBET6 + MZ1	77.0	71 to 83
AB3067 (27)	49.0	44 to 54

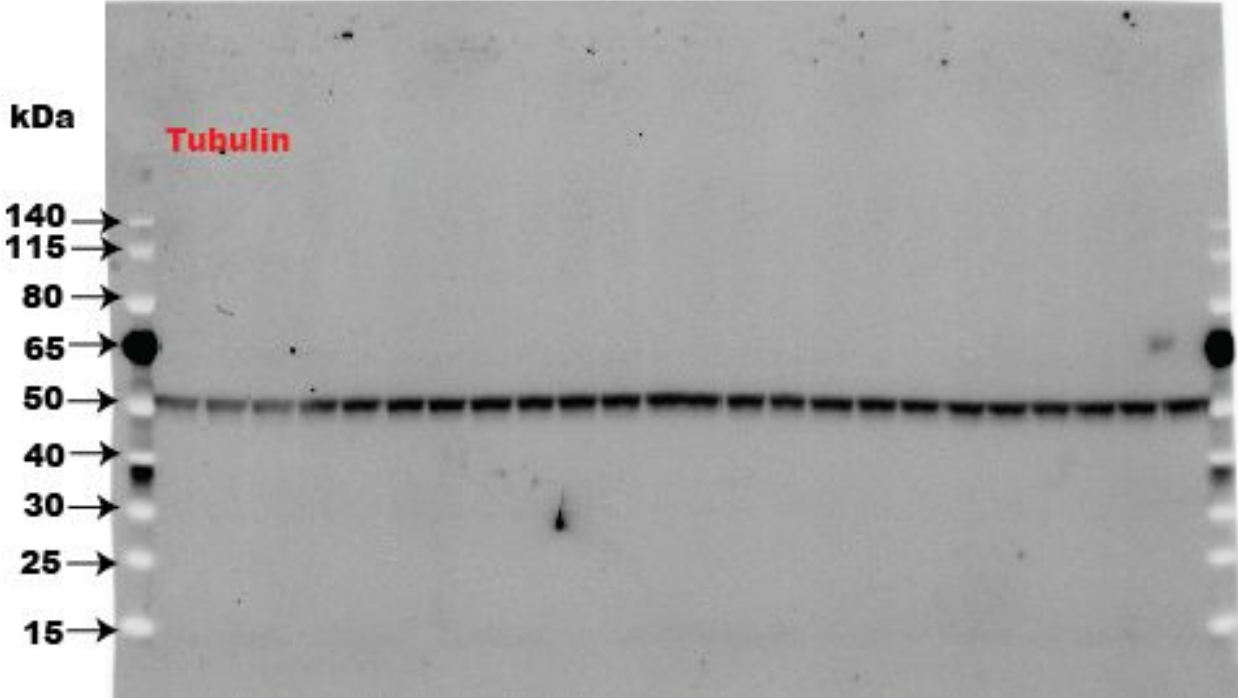
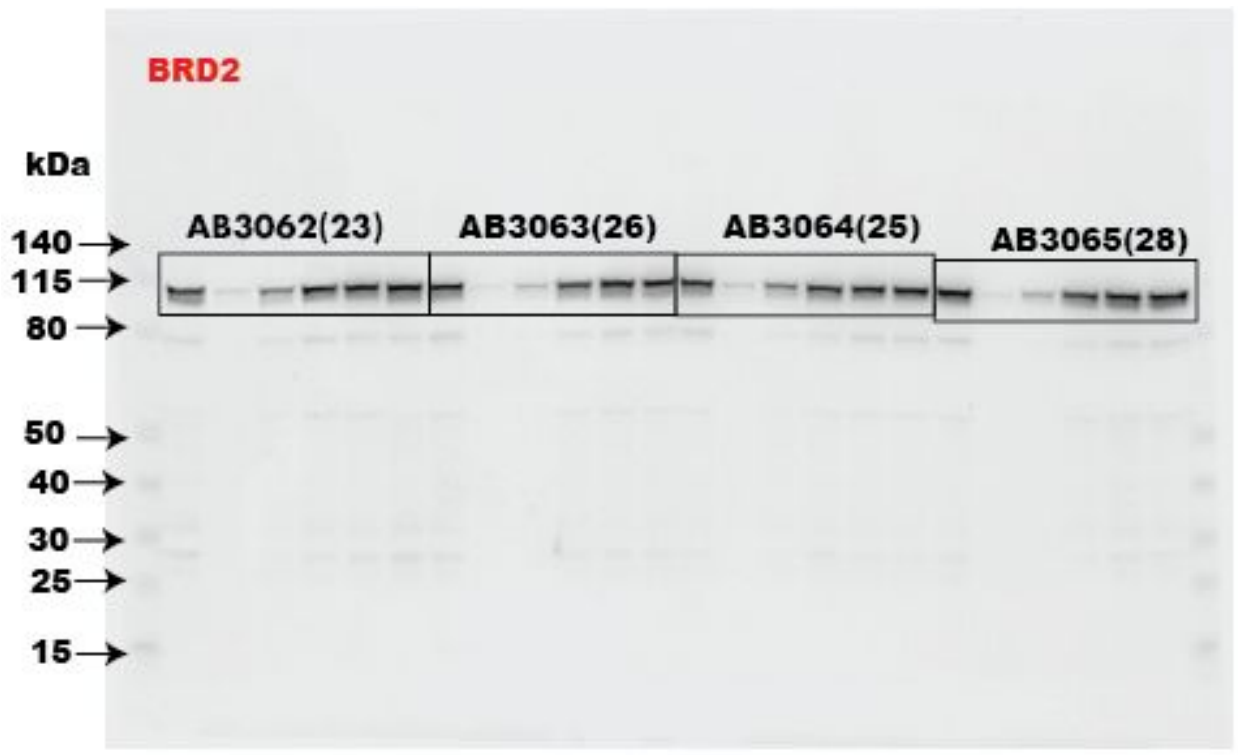
<sup>a</sup> Data is colour scaled for lowest (green), median (yellow) and highest (red) EC<sub>50</sub> values in nM.

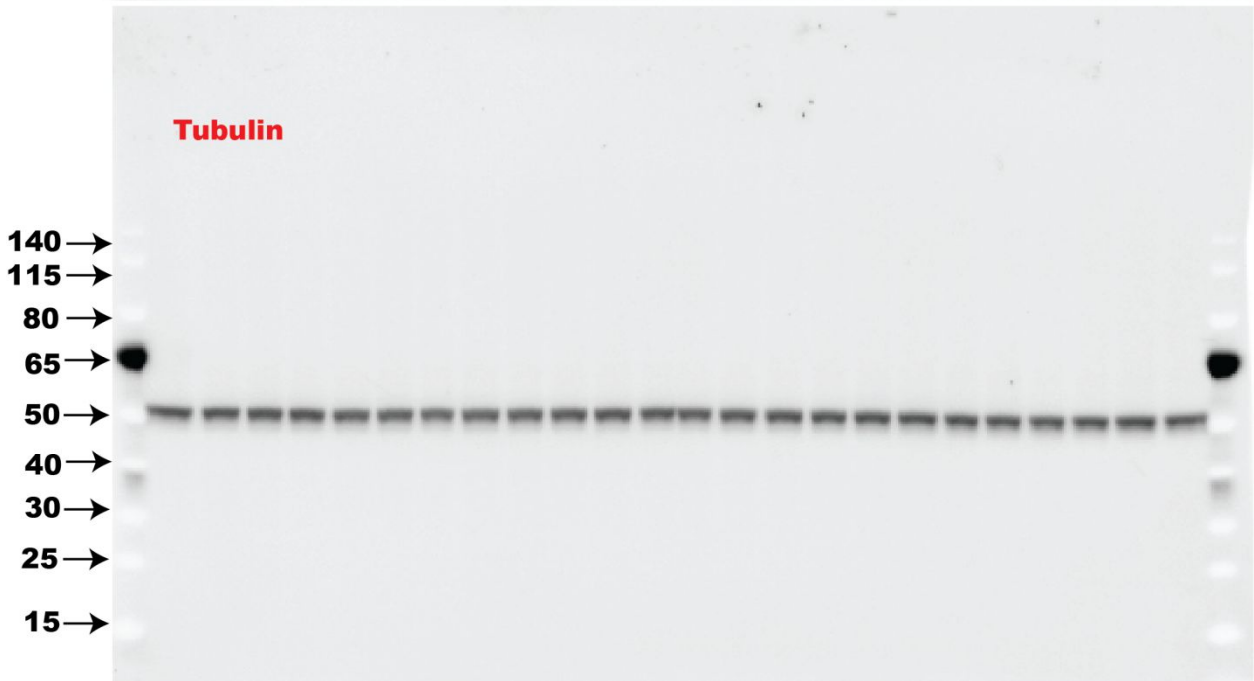
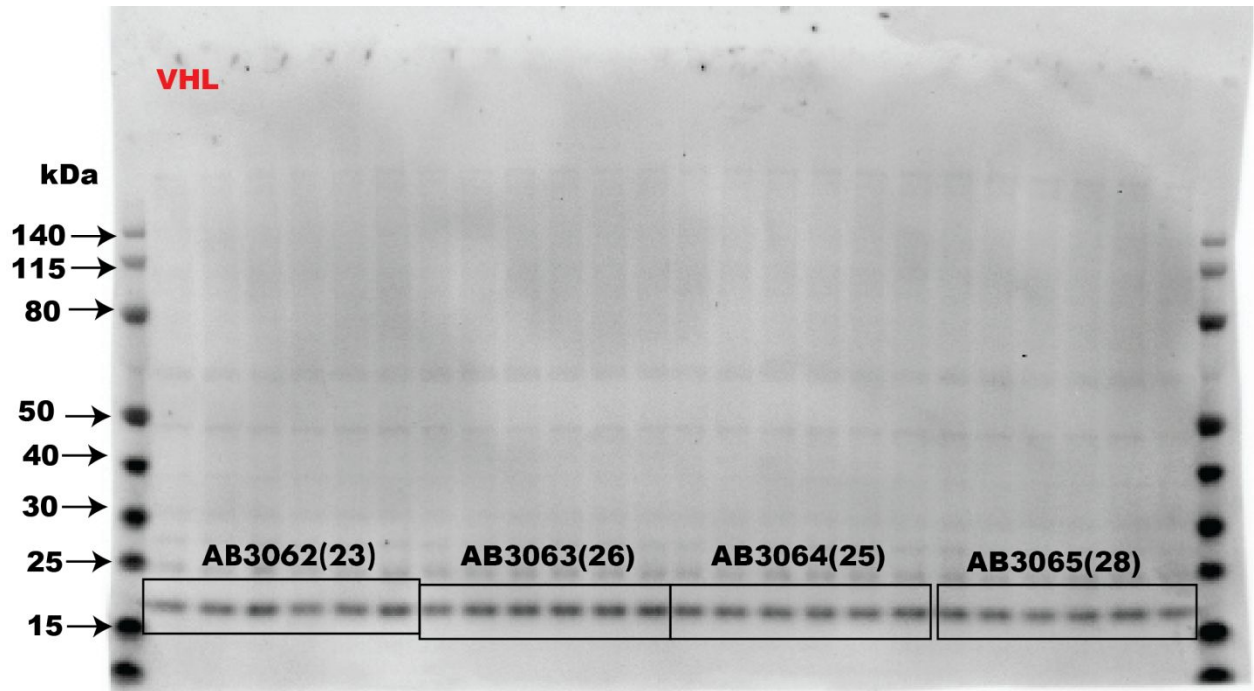
Supplementary Figure 8 (related to Figure 4). **Uncropped Western Blotting images of the evaluation of heterotrivalent PROTACs 23 – 32 & 86 in HEK293 cells.** The boxed region represents the cropped area containing the target protein expression upon treatment with compound.

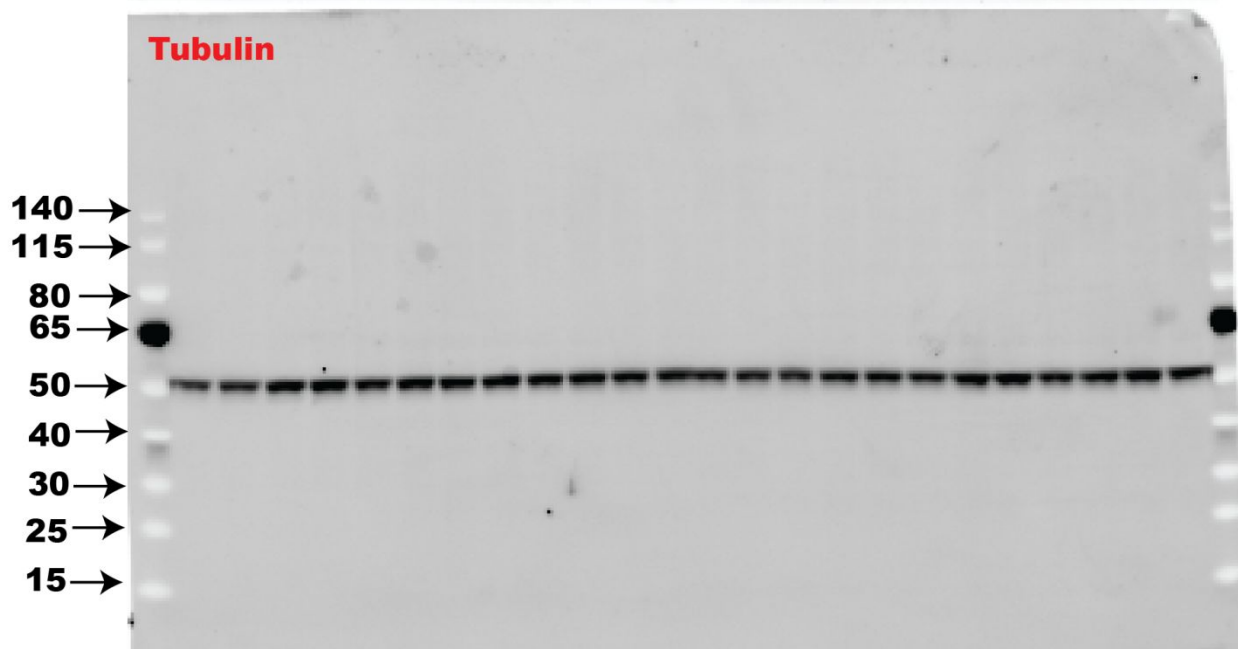
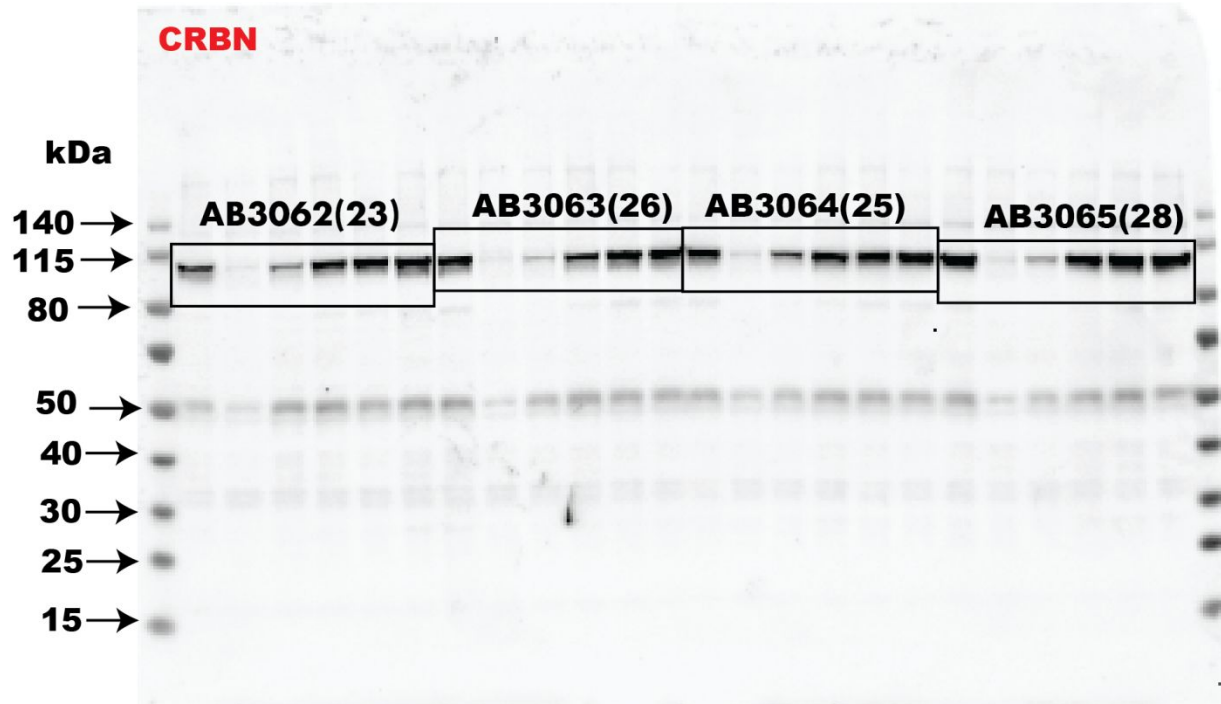


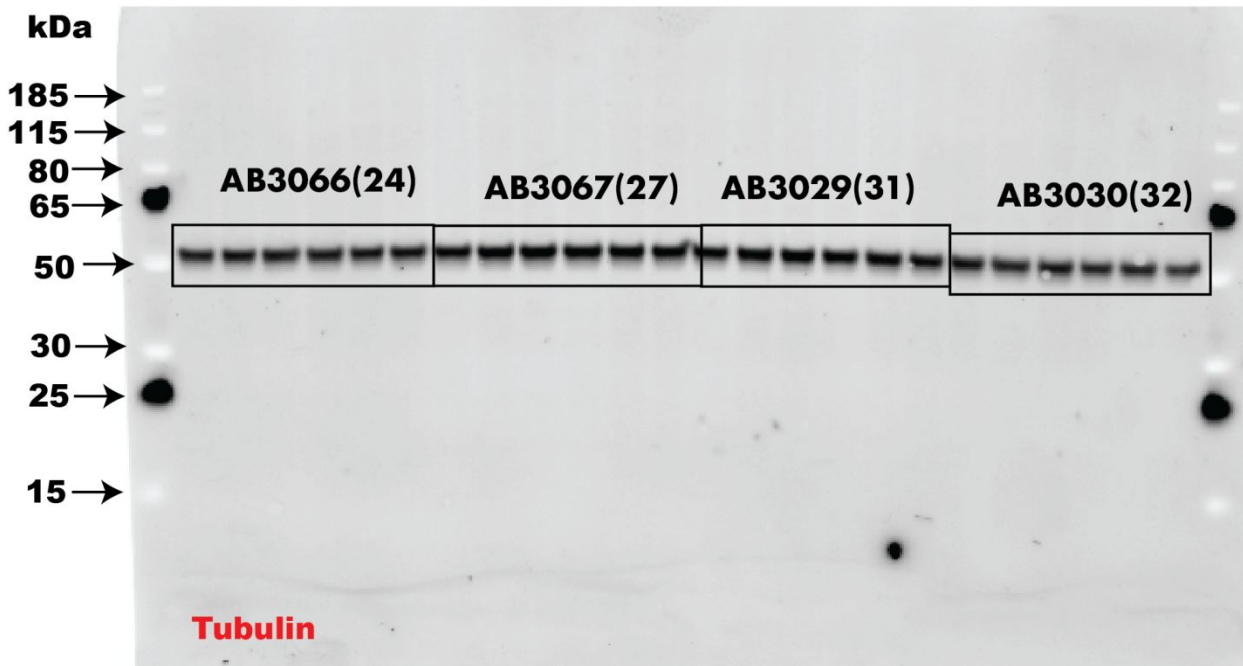
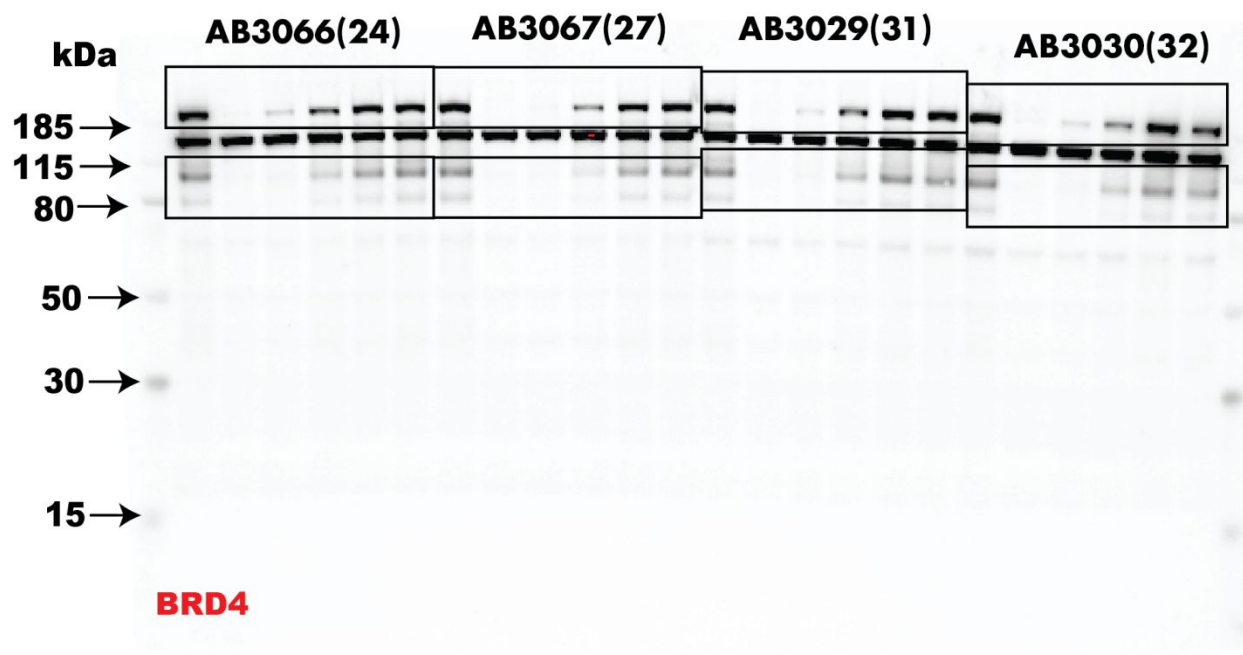


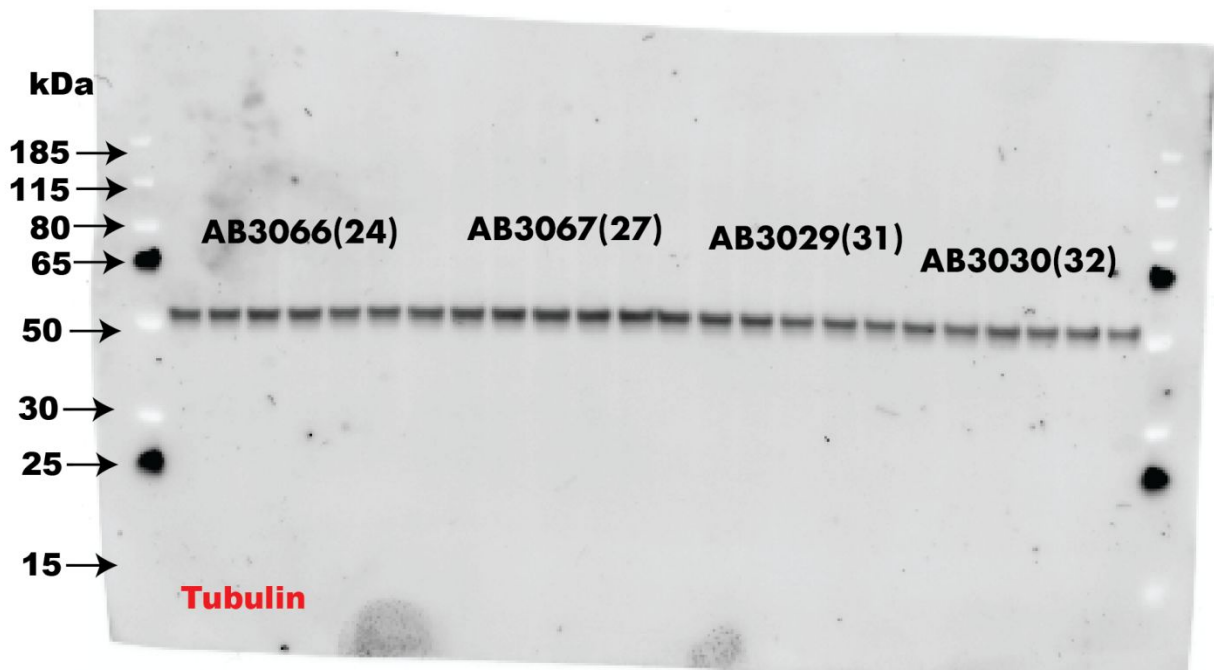
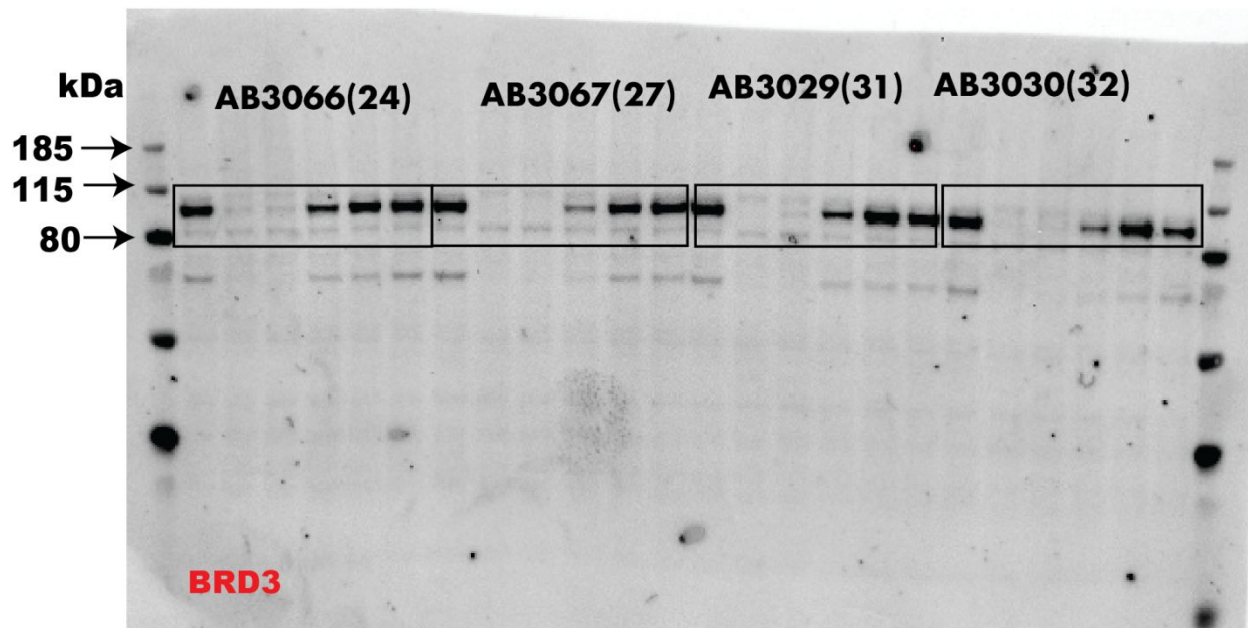




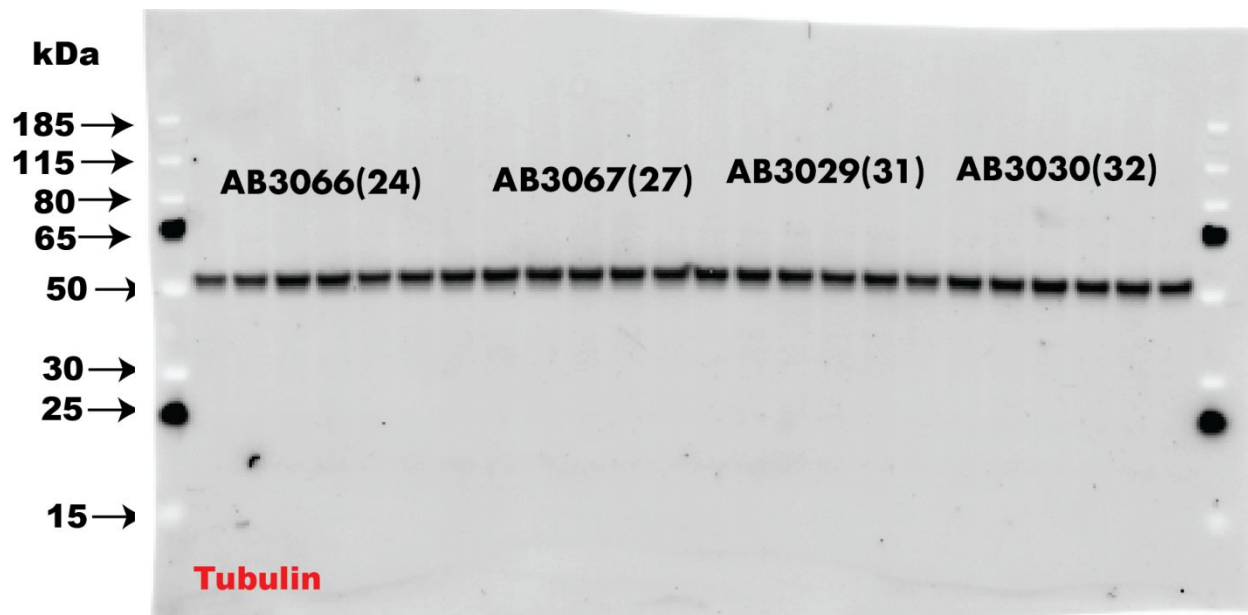
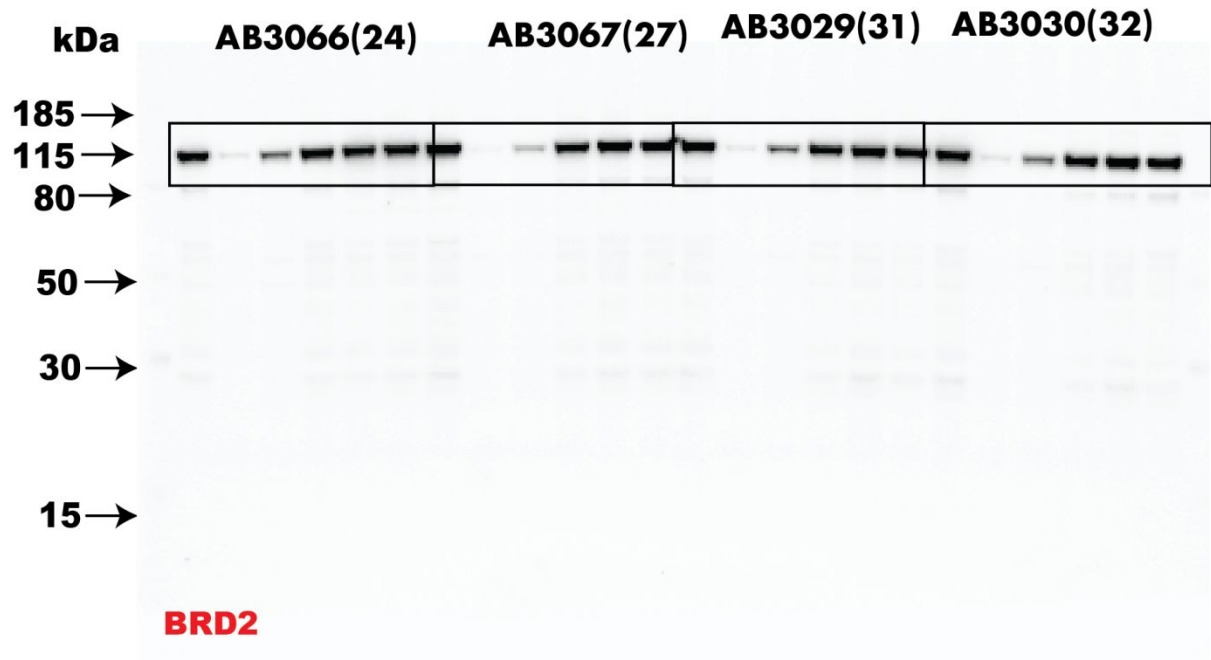


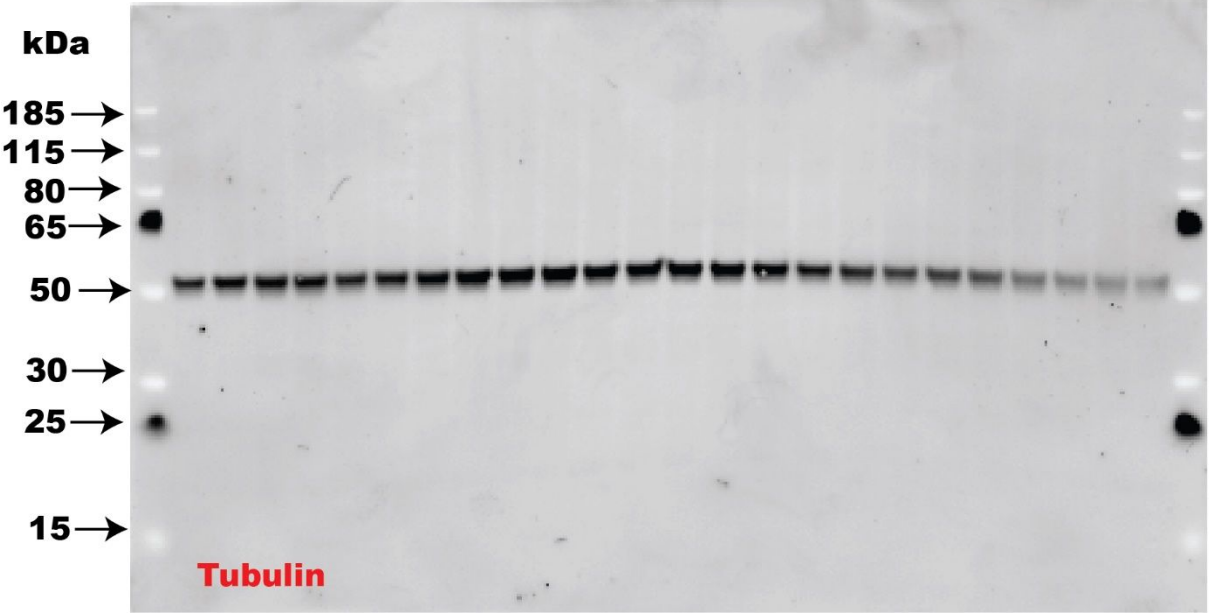
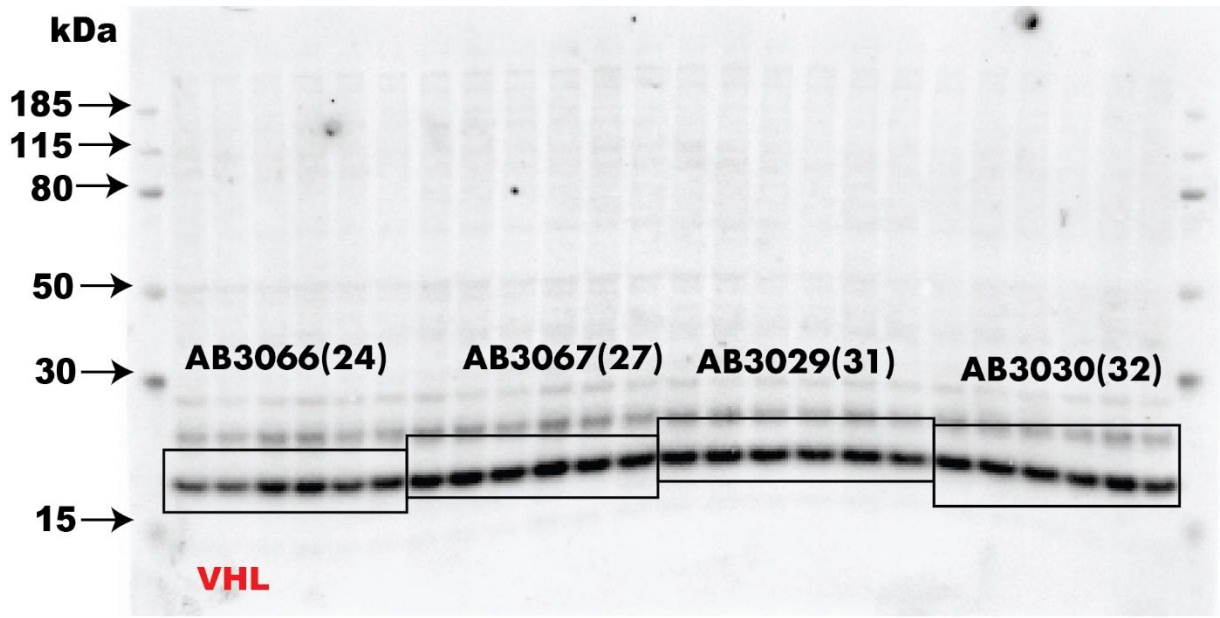




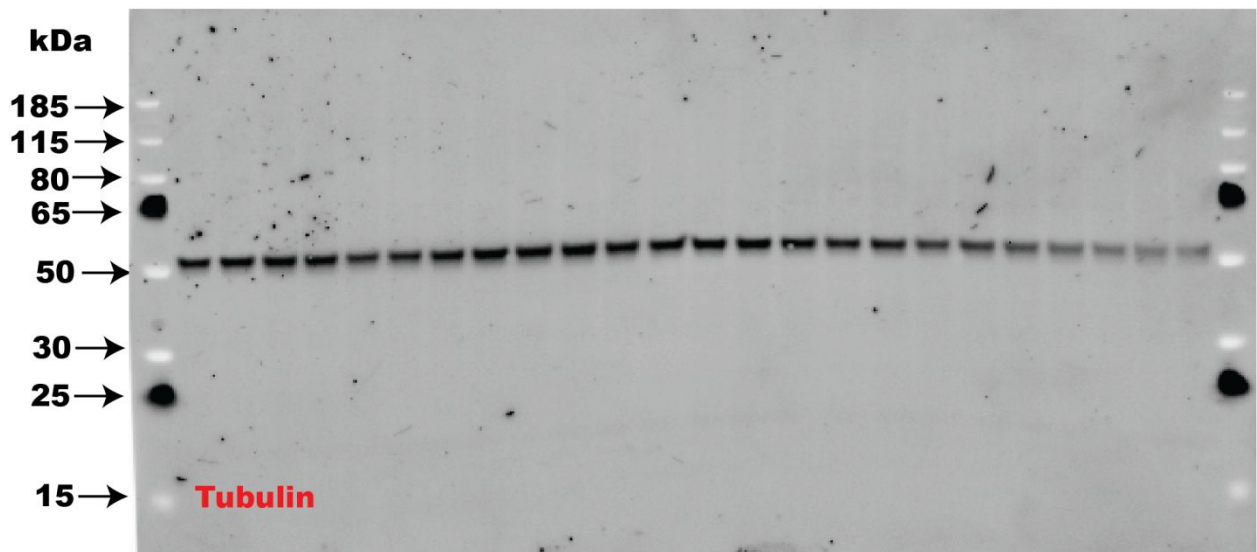
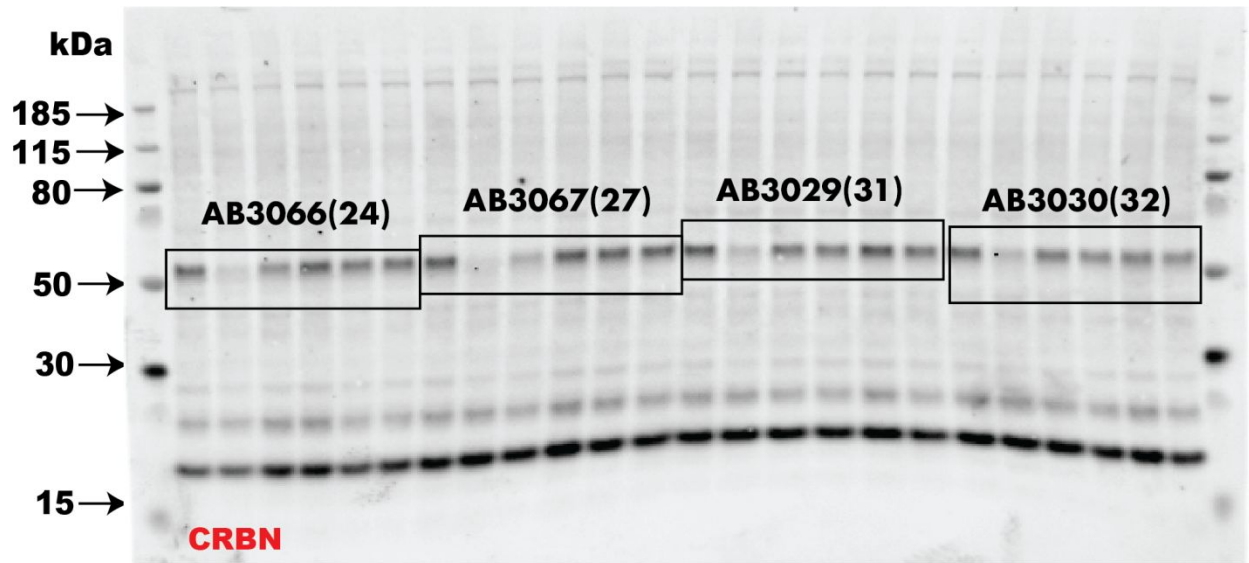


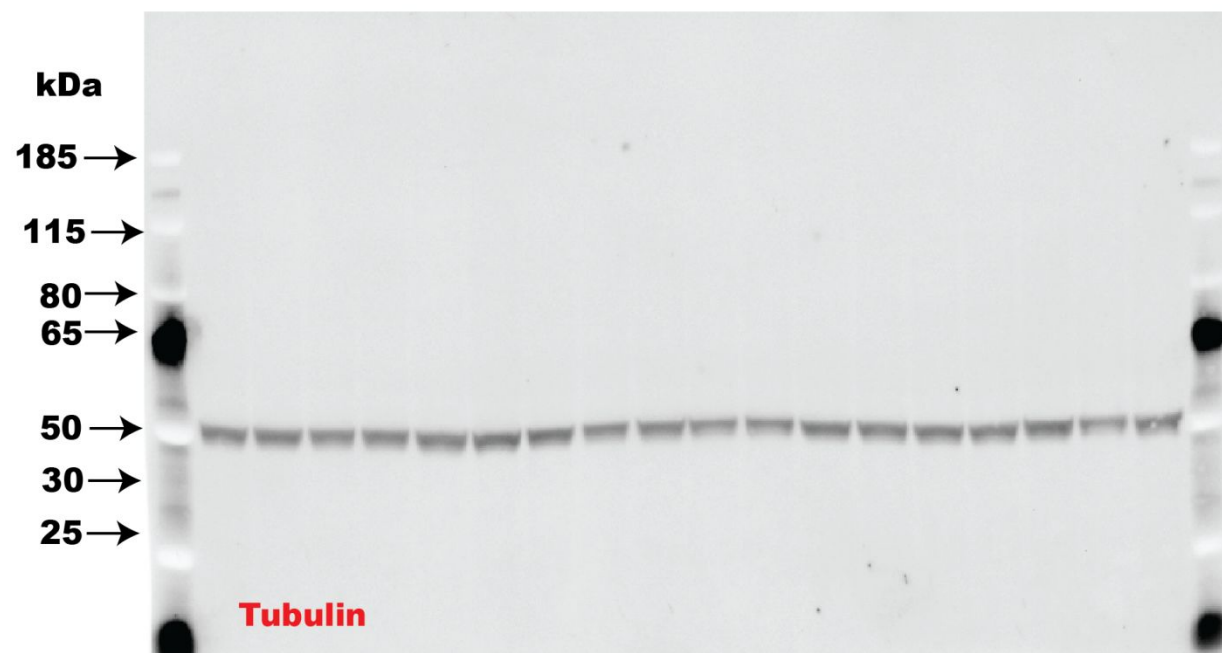
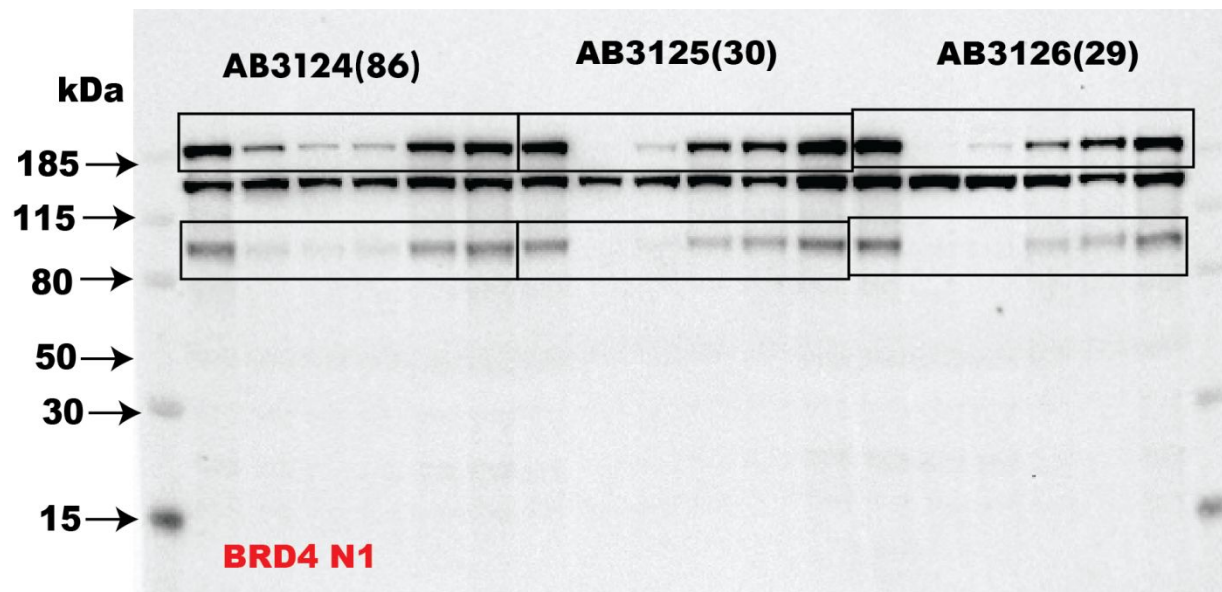


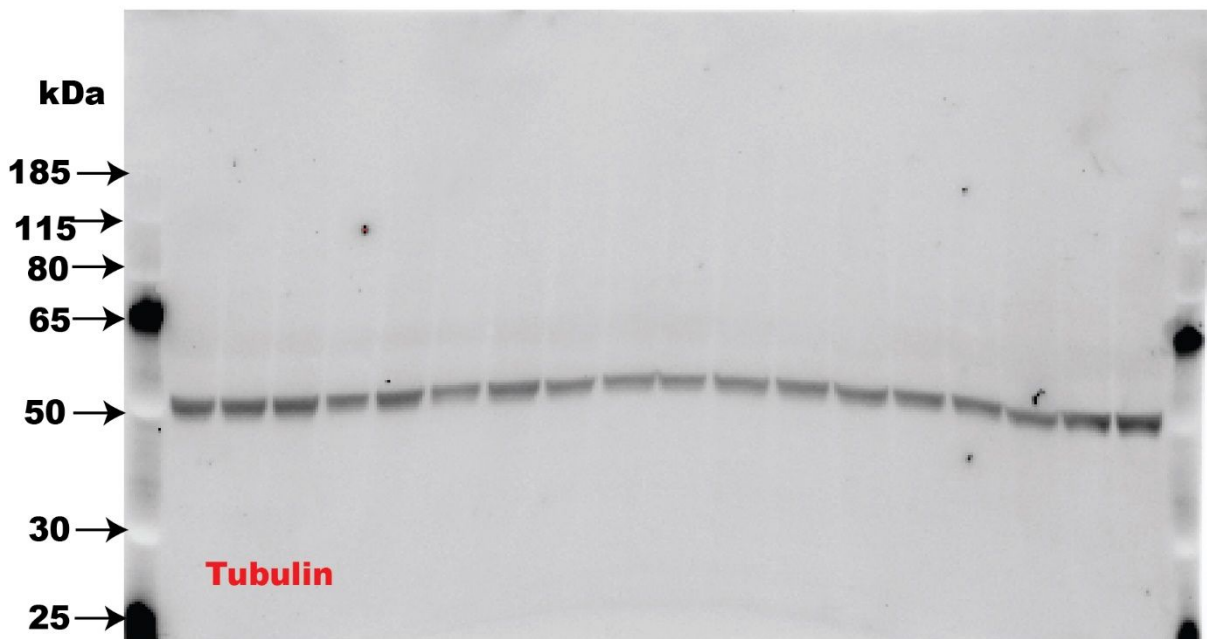
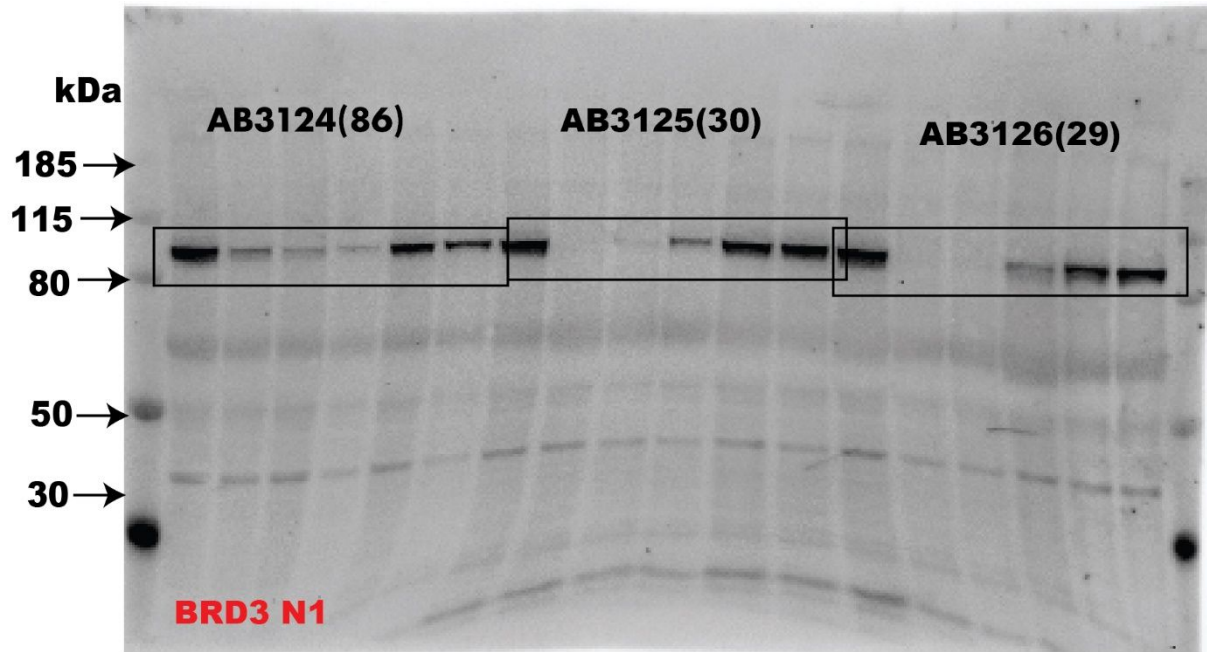


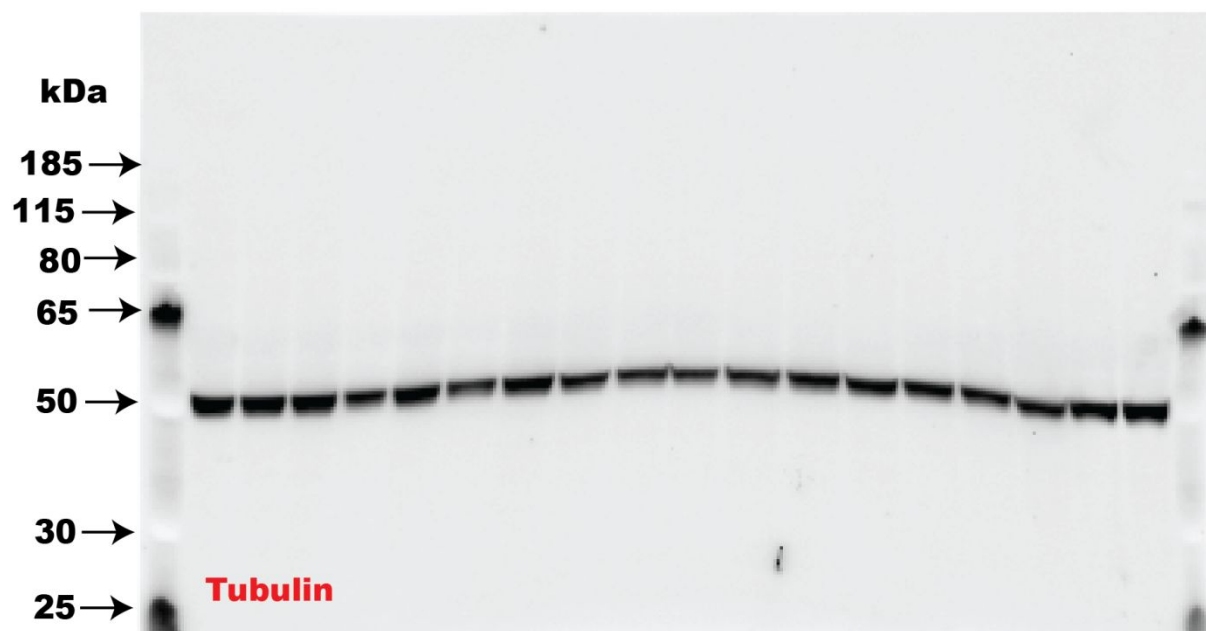
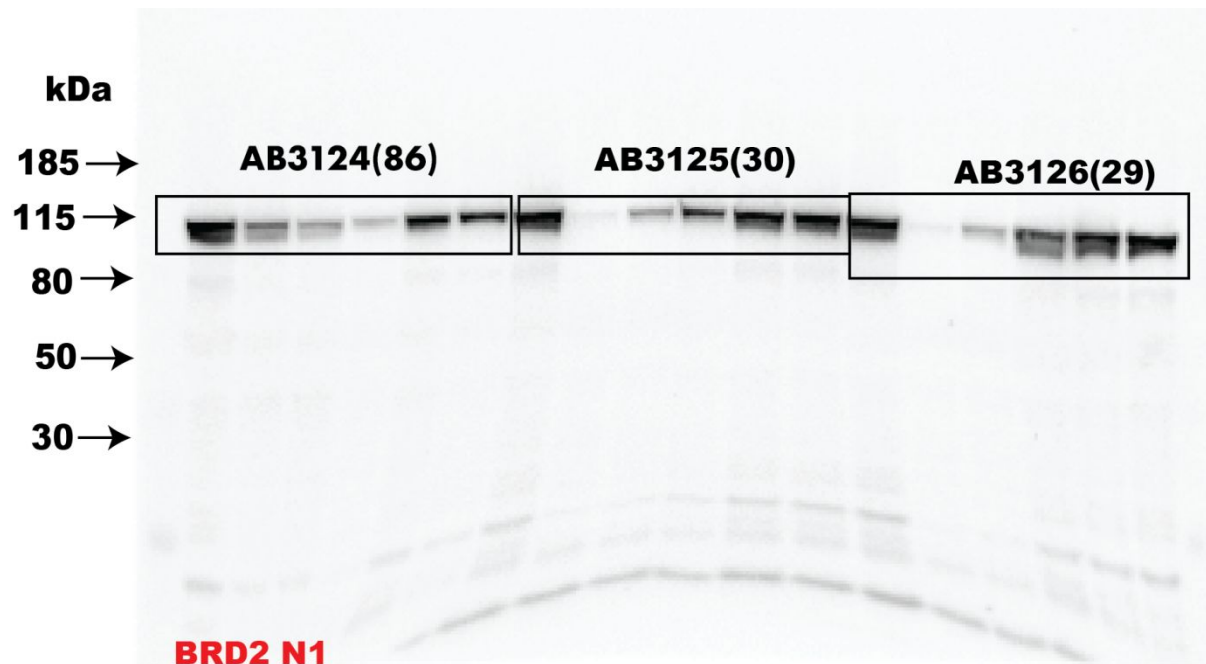


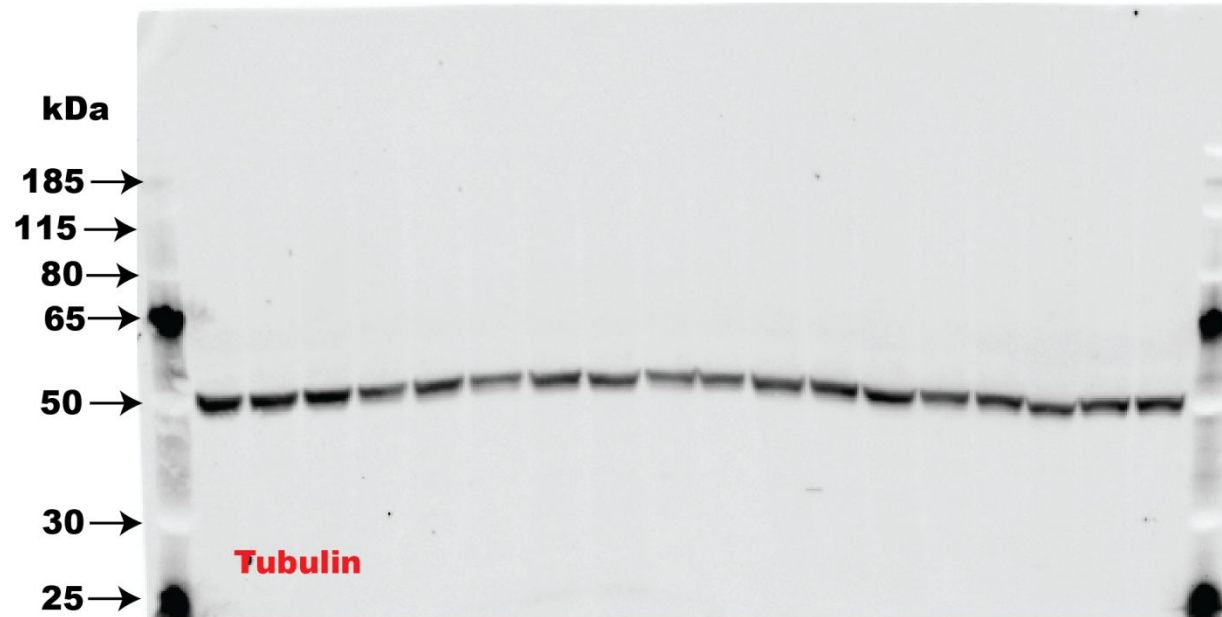
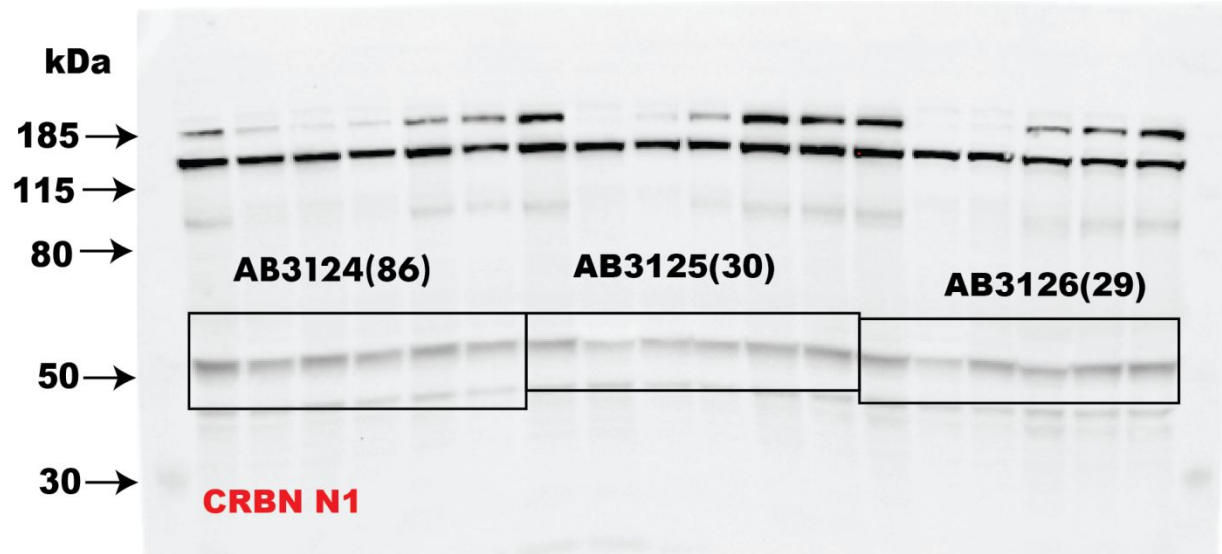




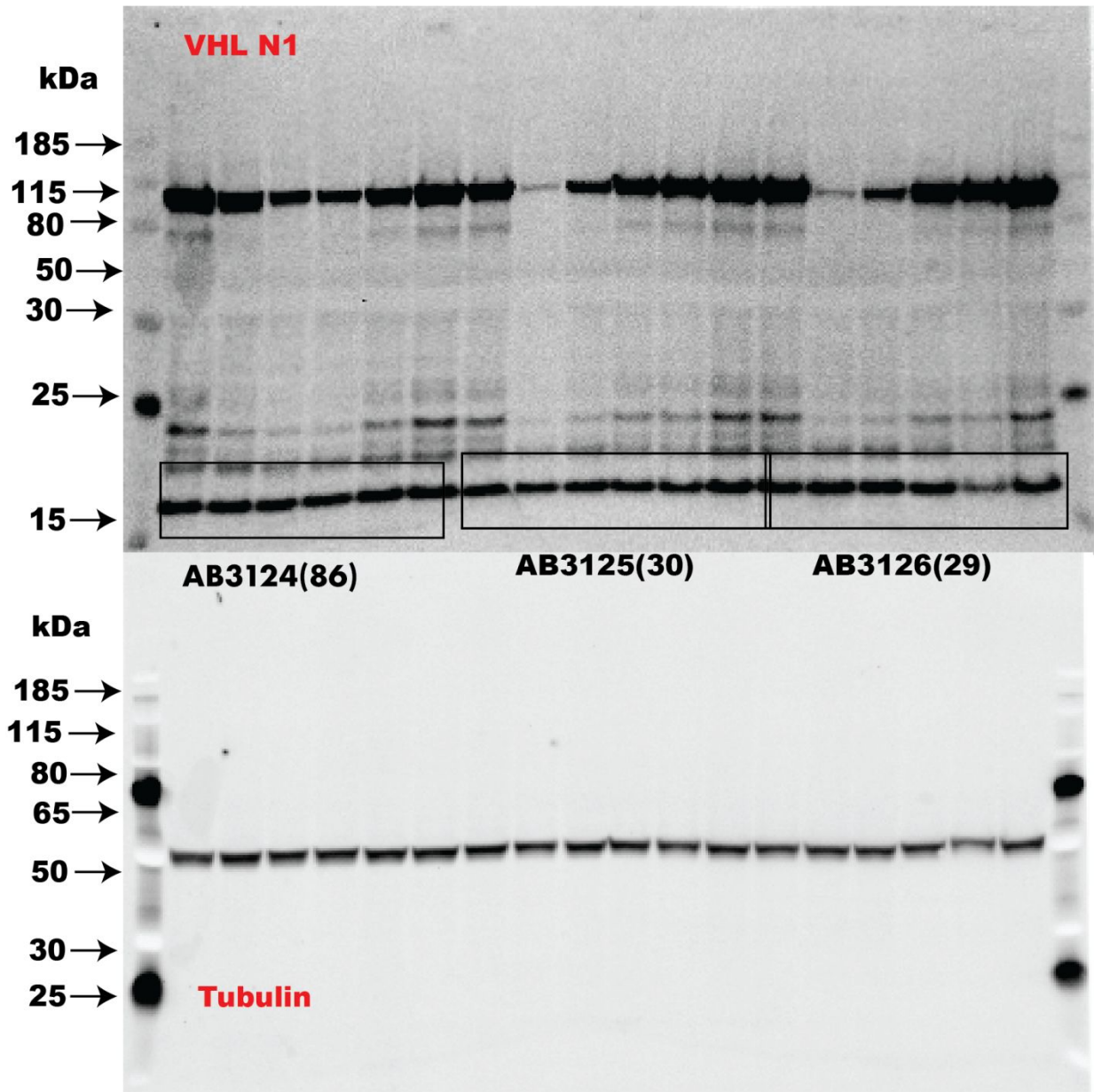




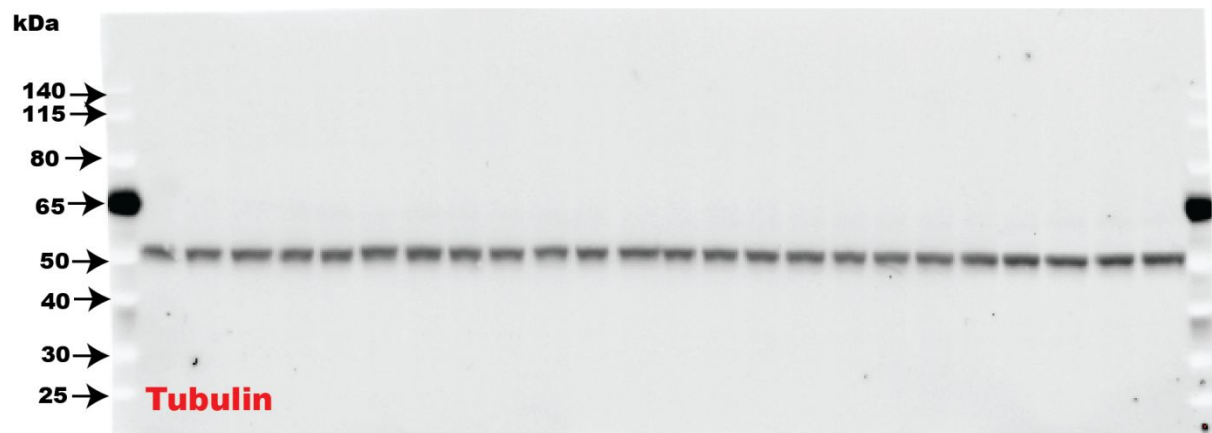
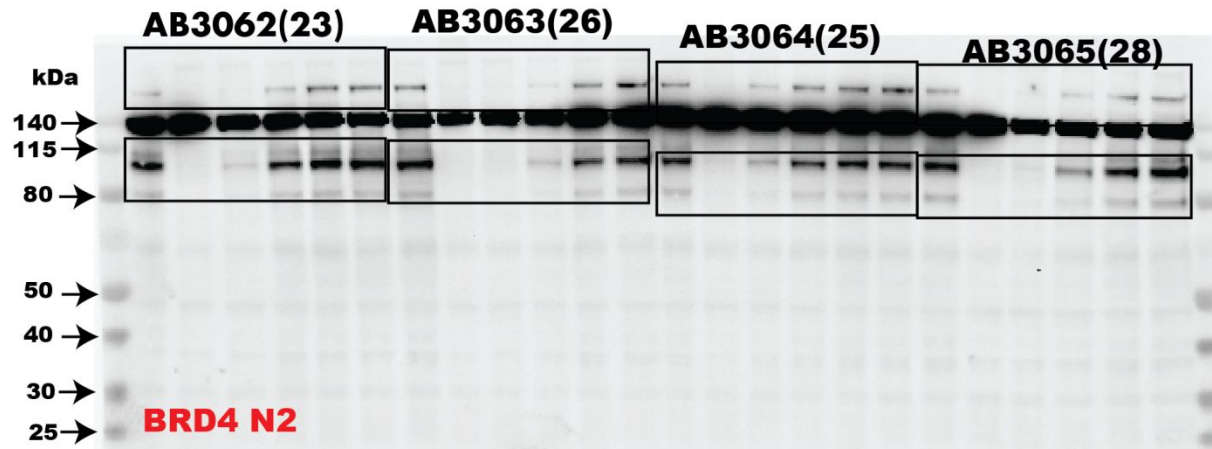


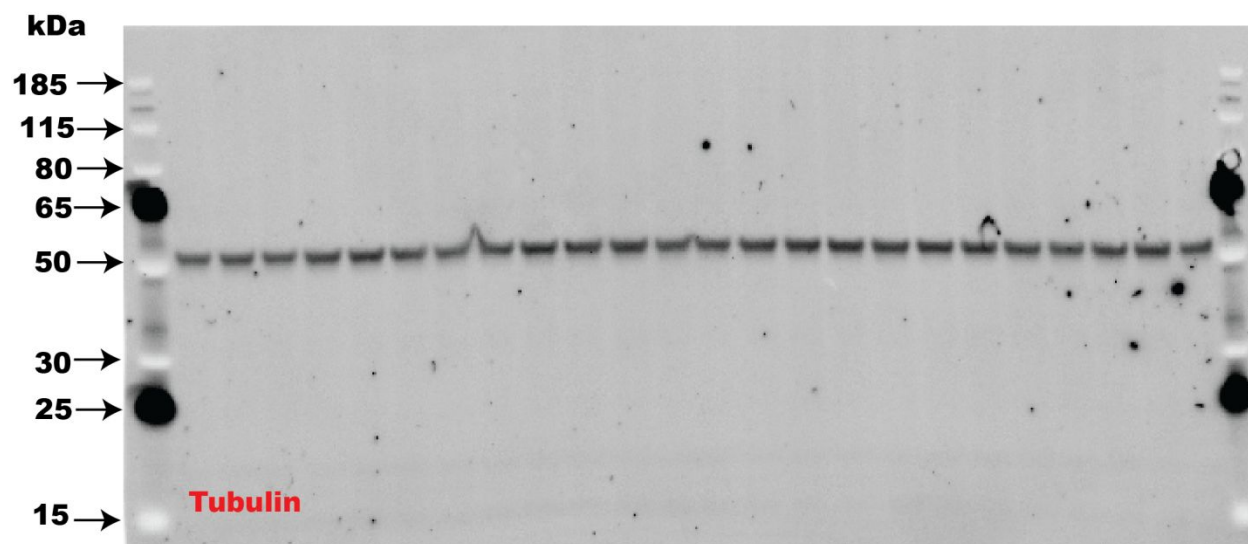
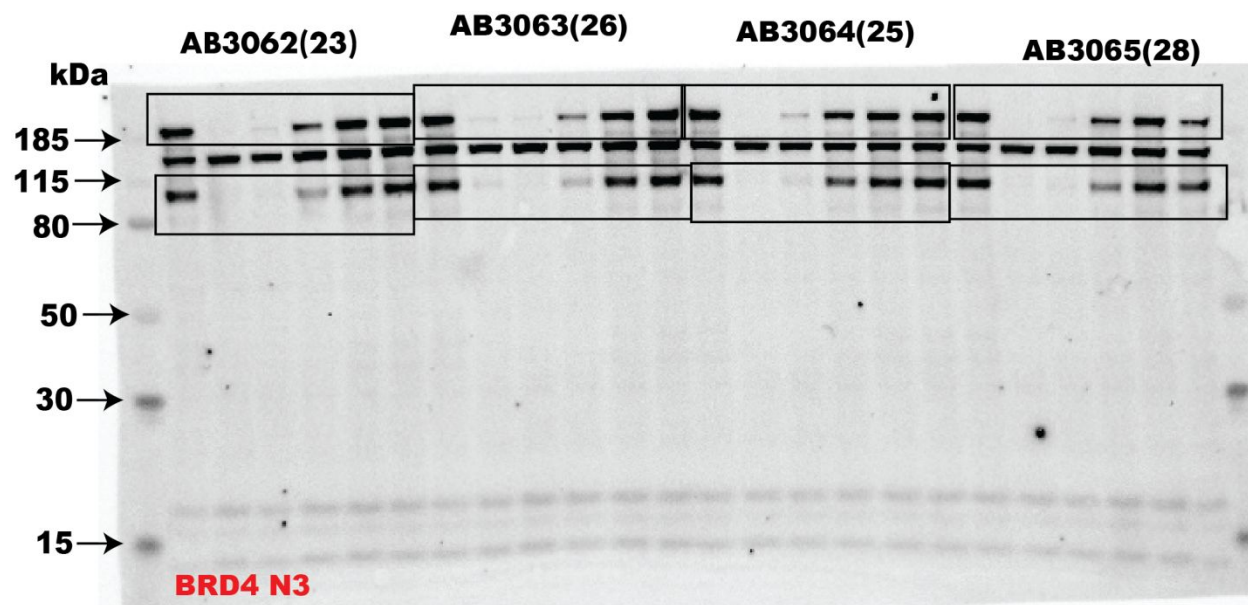




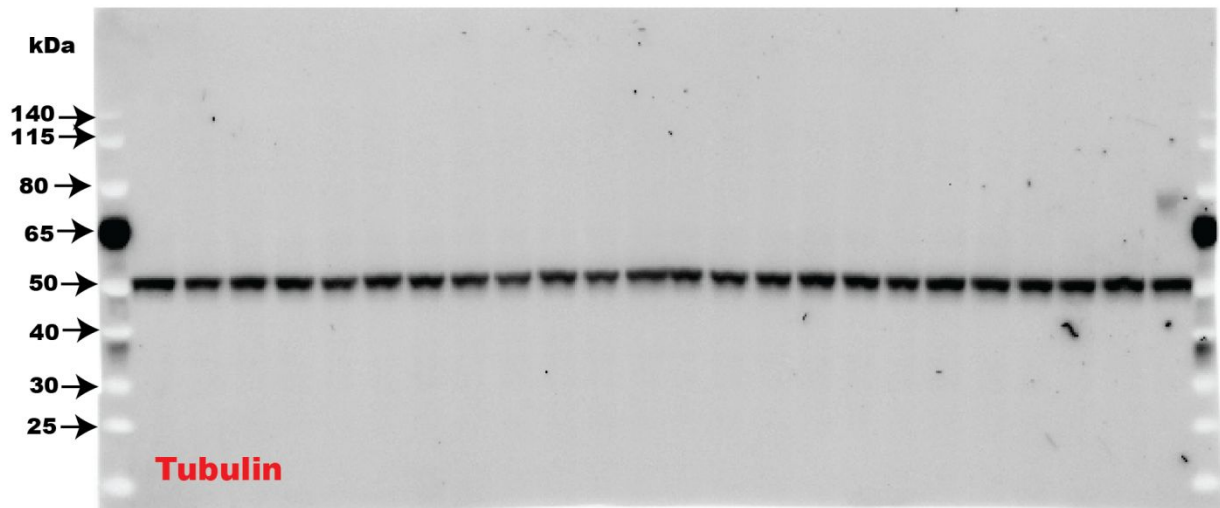
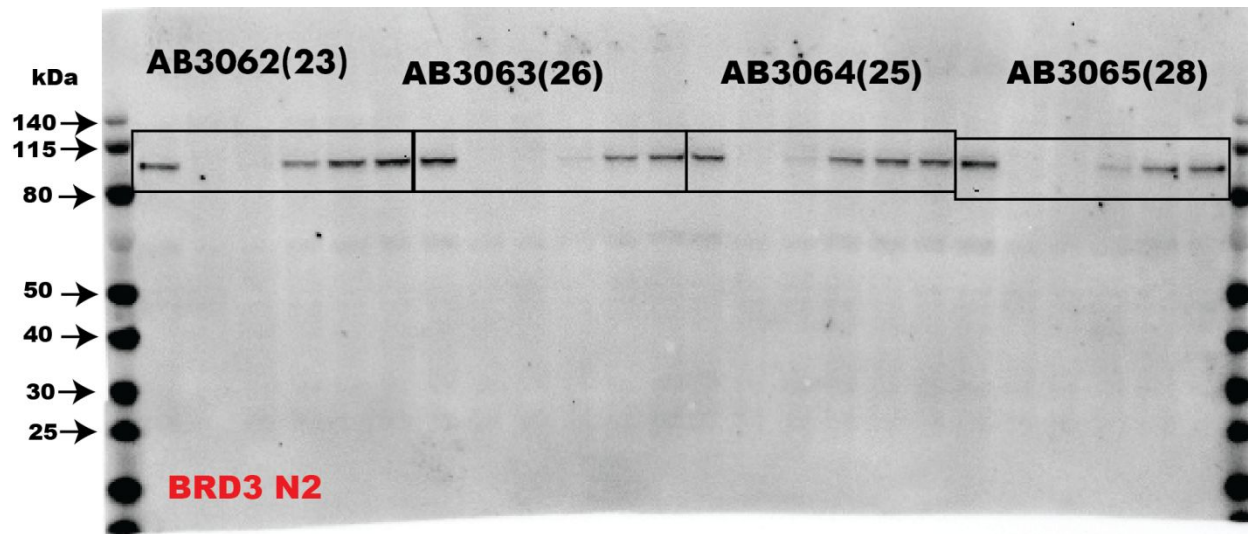


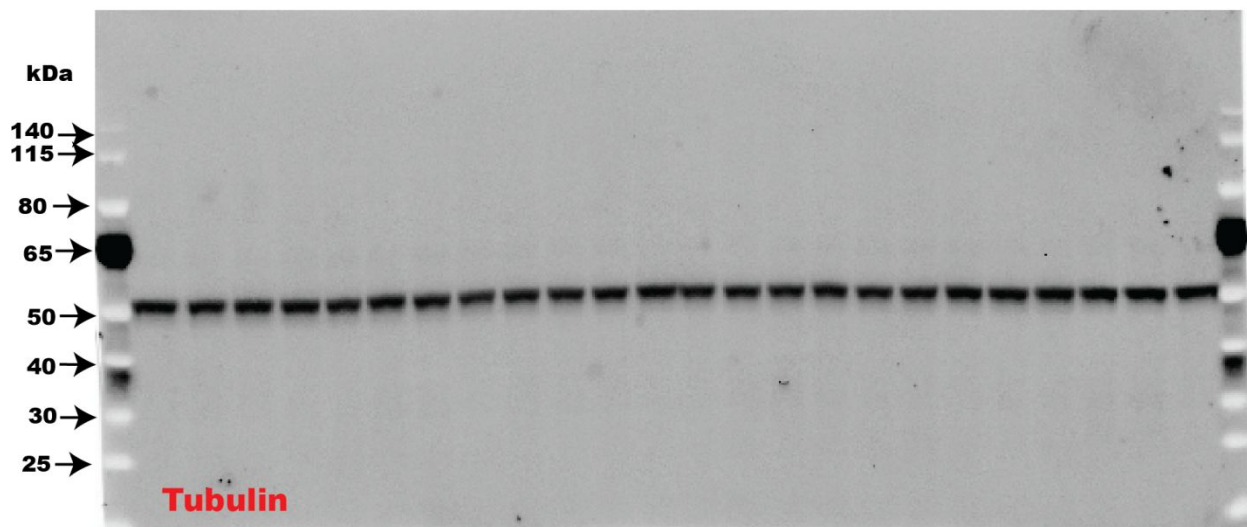
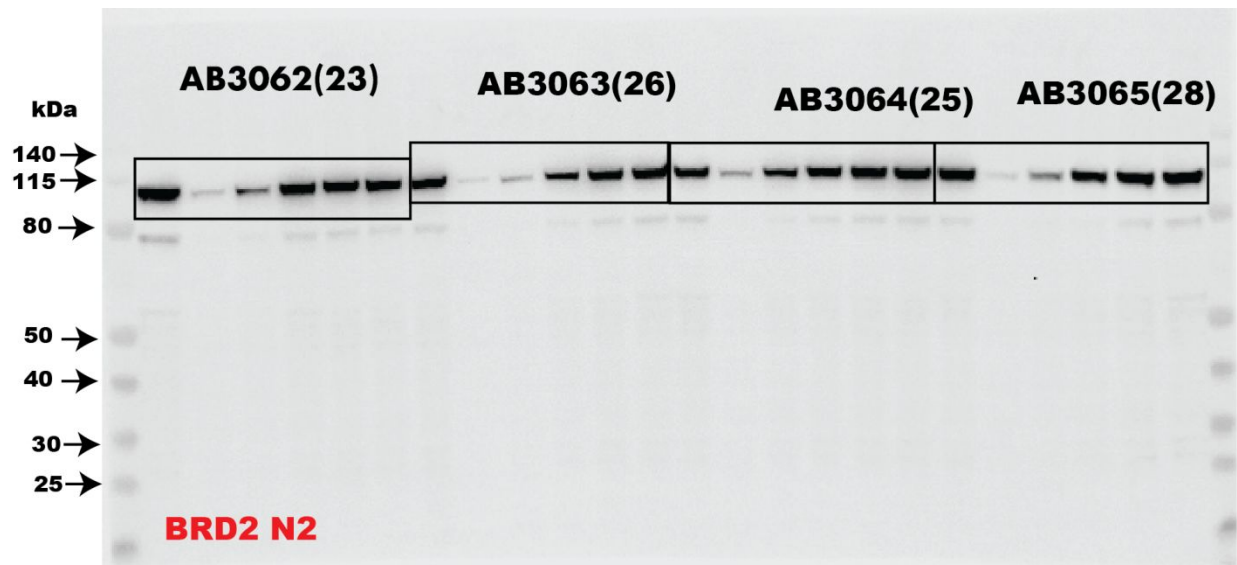
Supplementary Figure 9 (related to Figure 4). Uncropped independent replicate western blotting images of the evaluation of heterotrivalent PROTACs 23 – 32 & 86 in HEK293 cells.

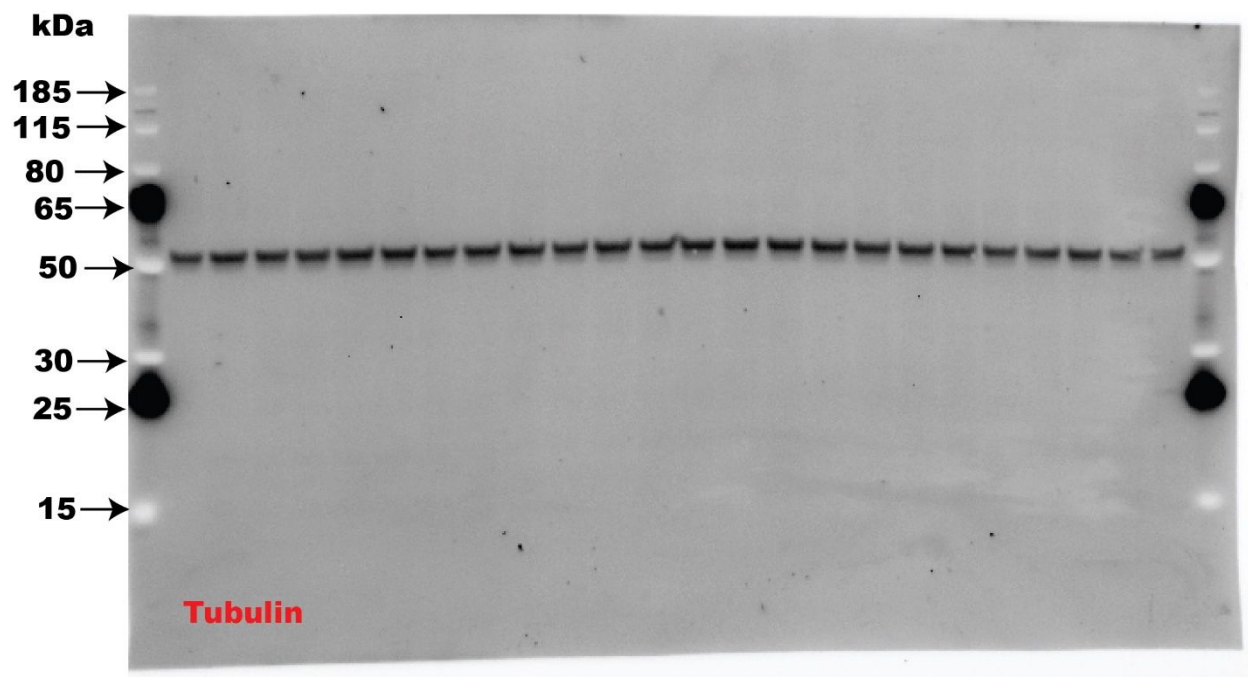
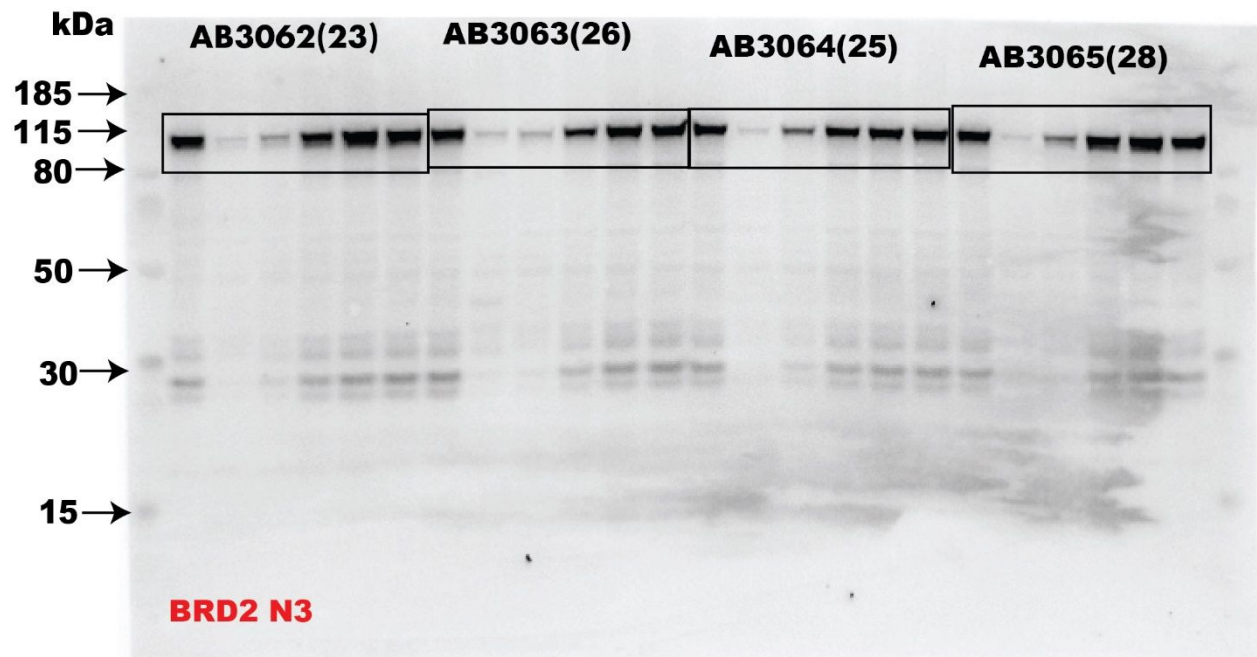


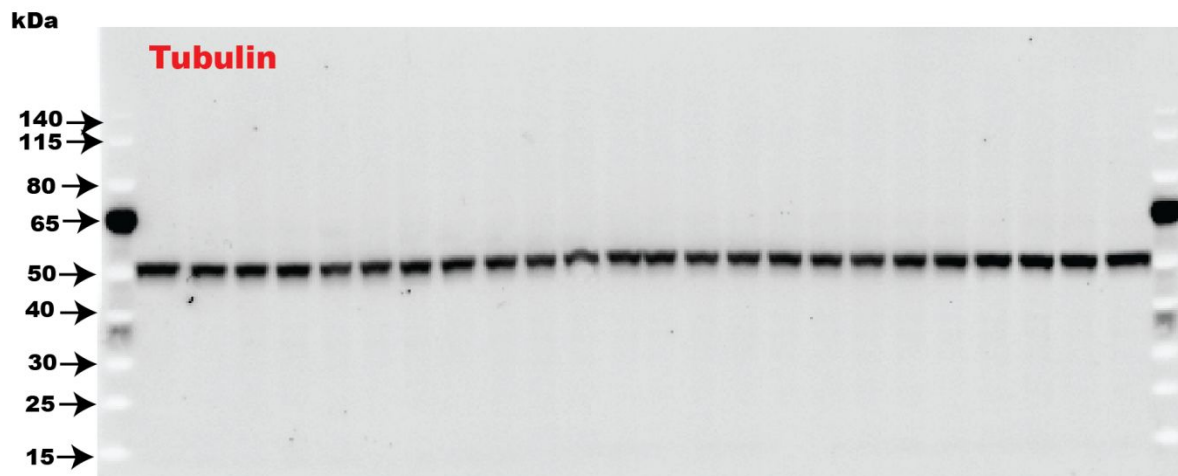
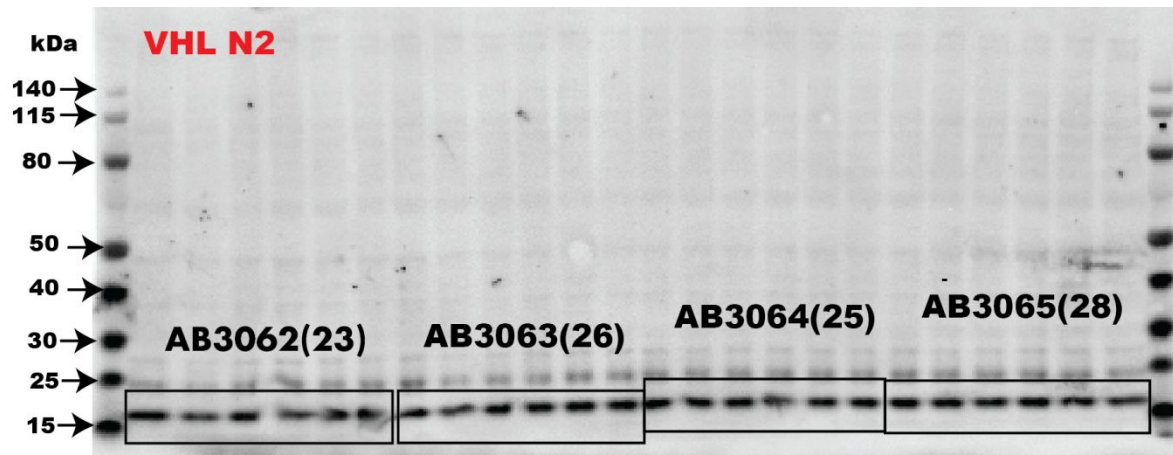


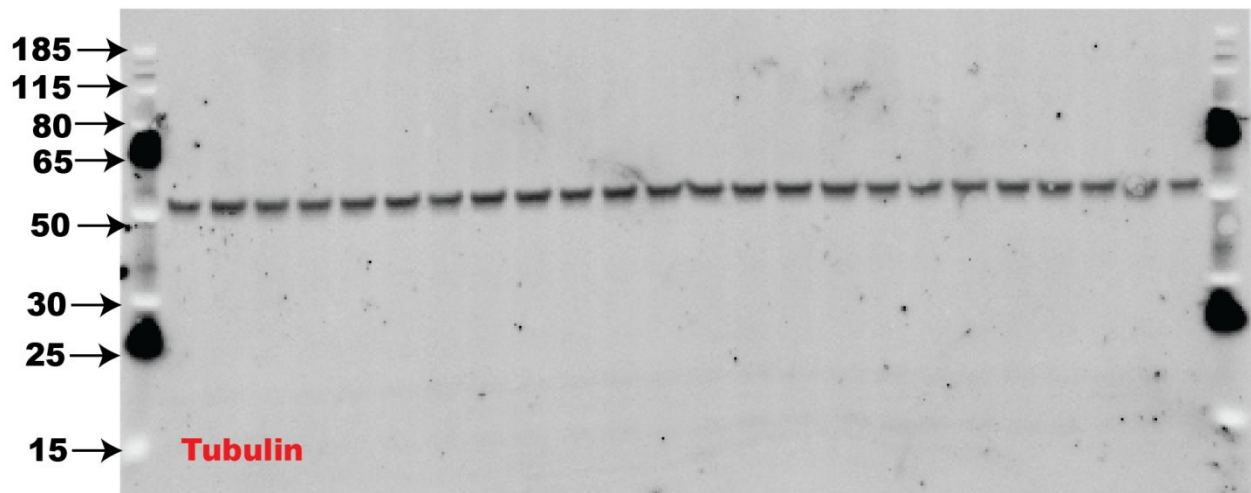
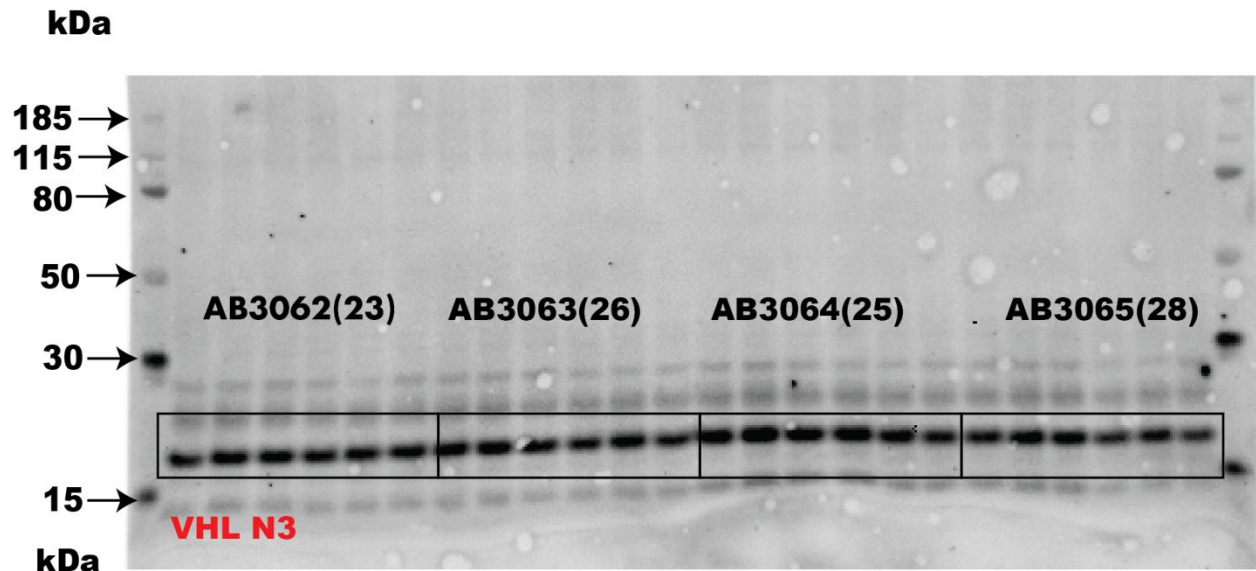




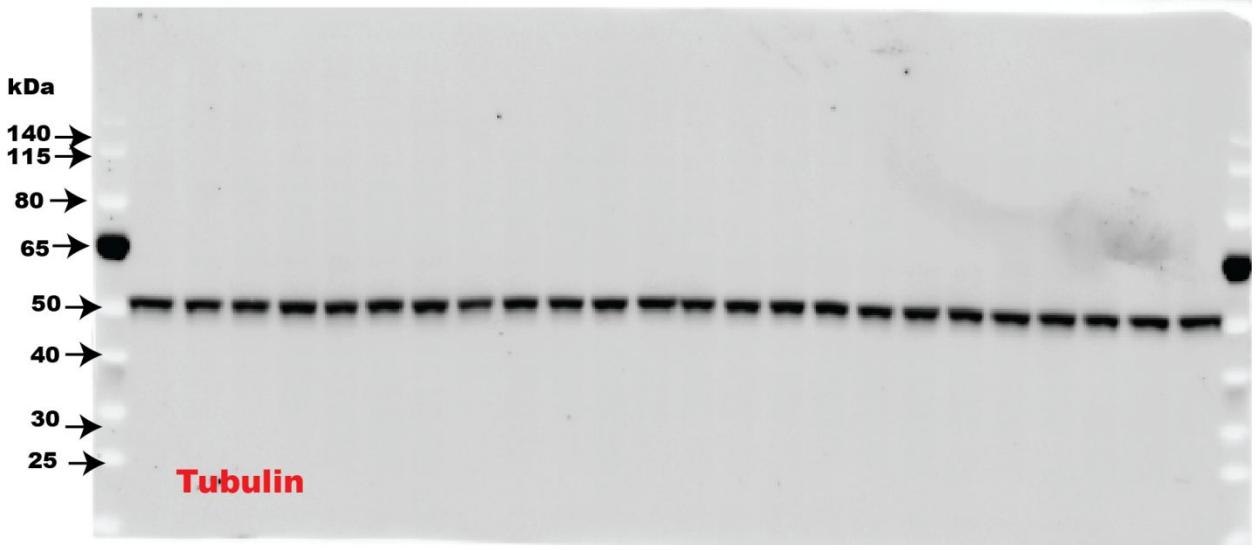
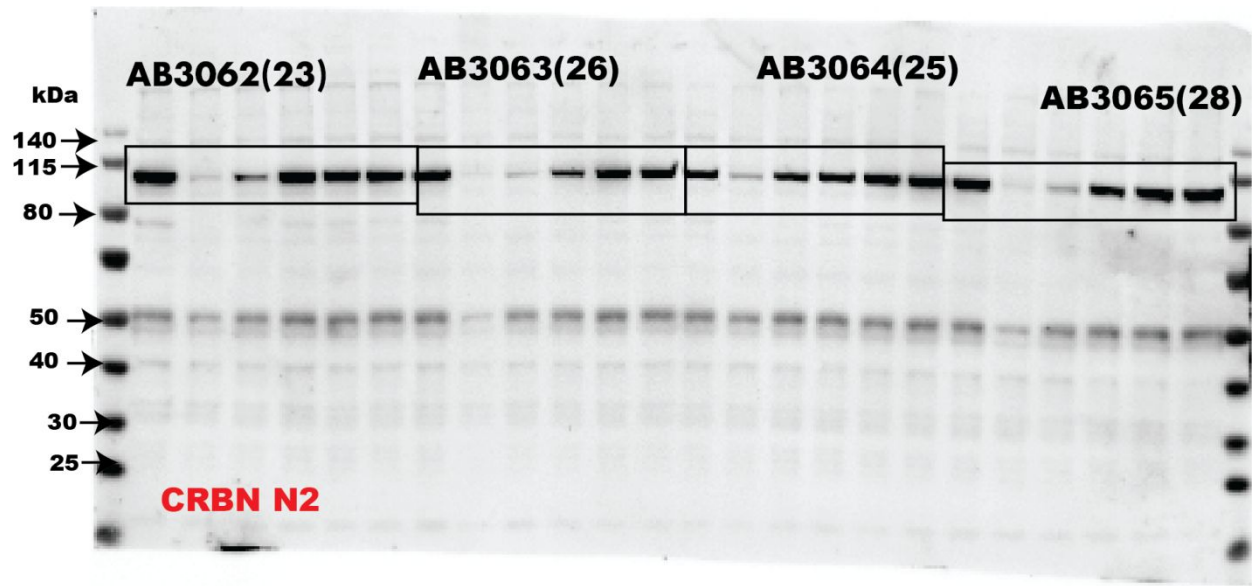


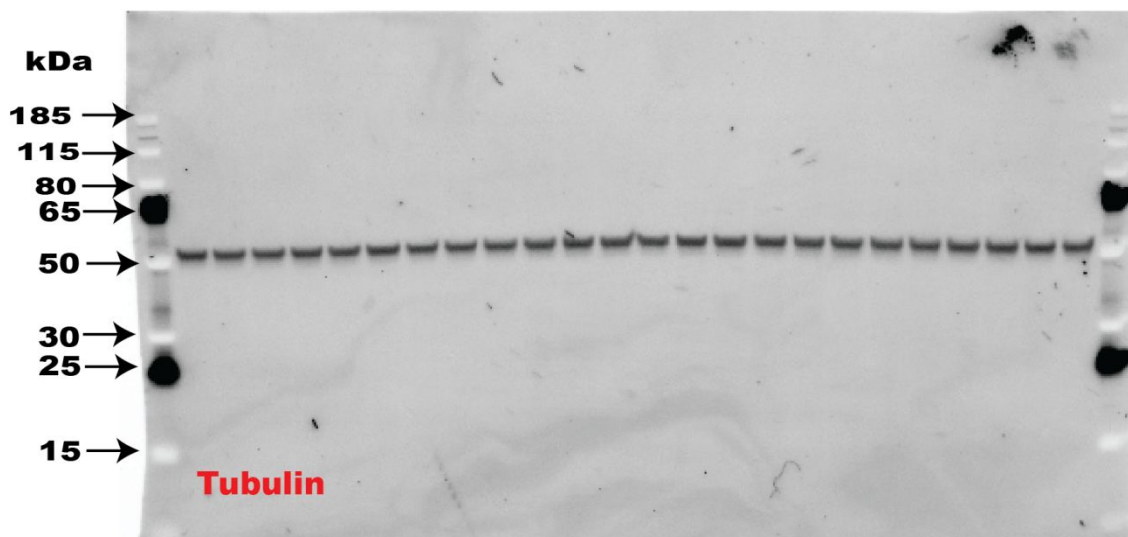
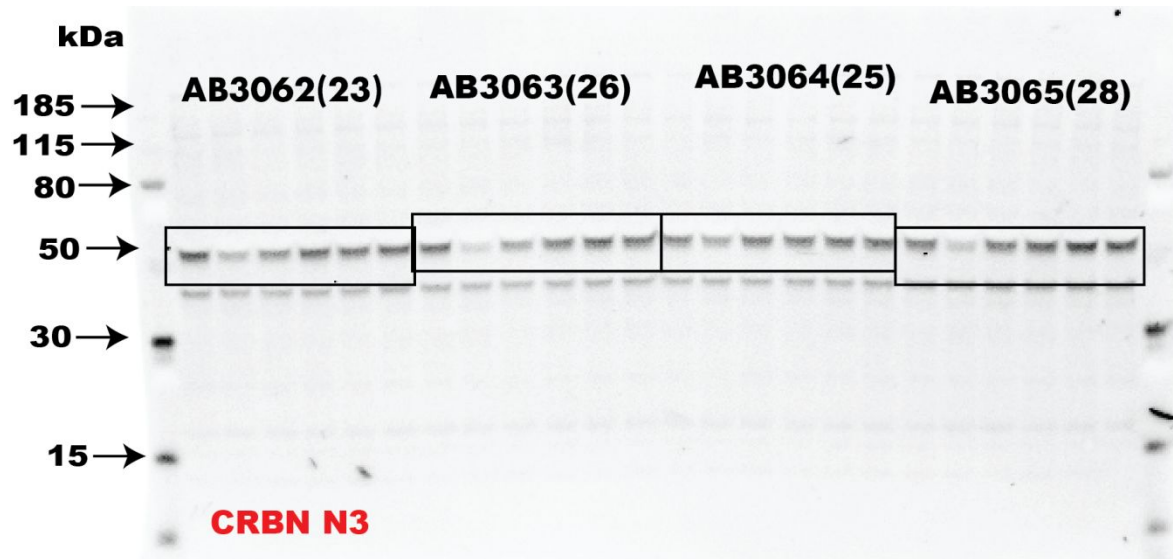


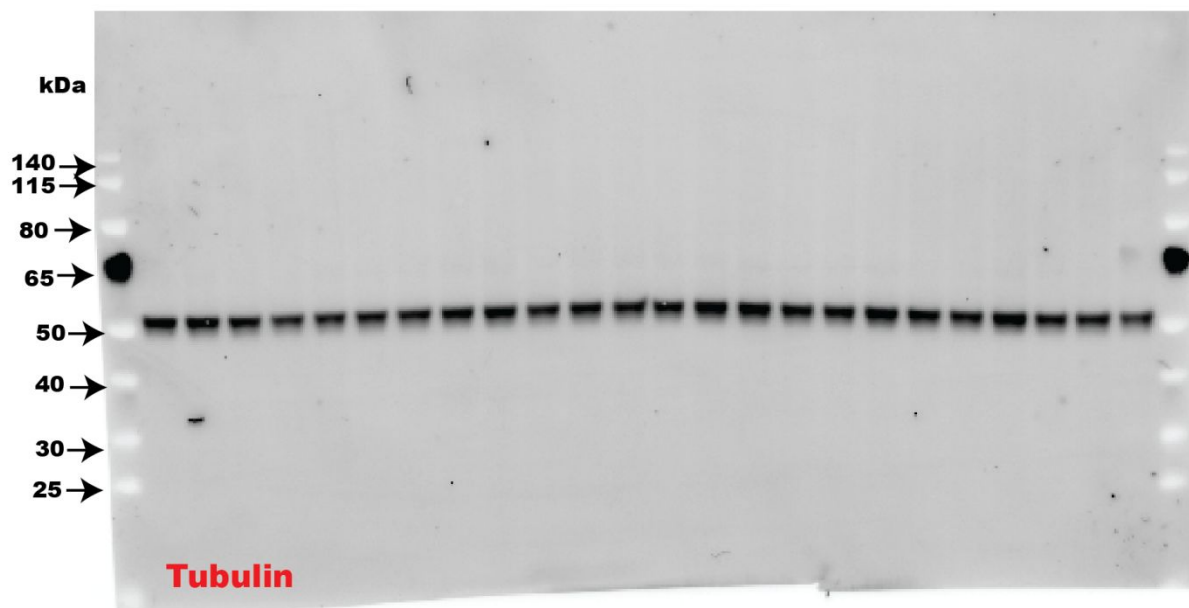
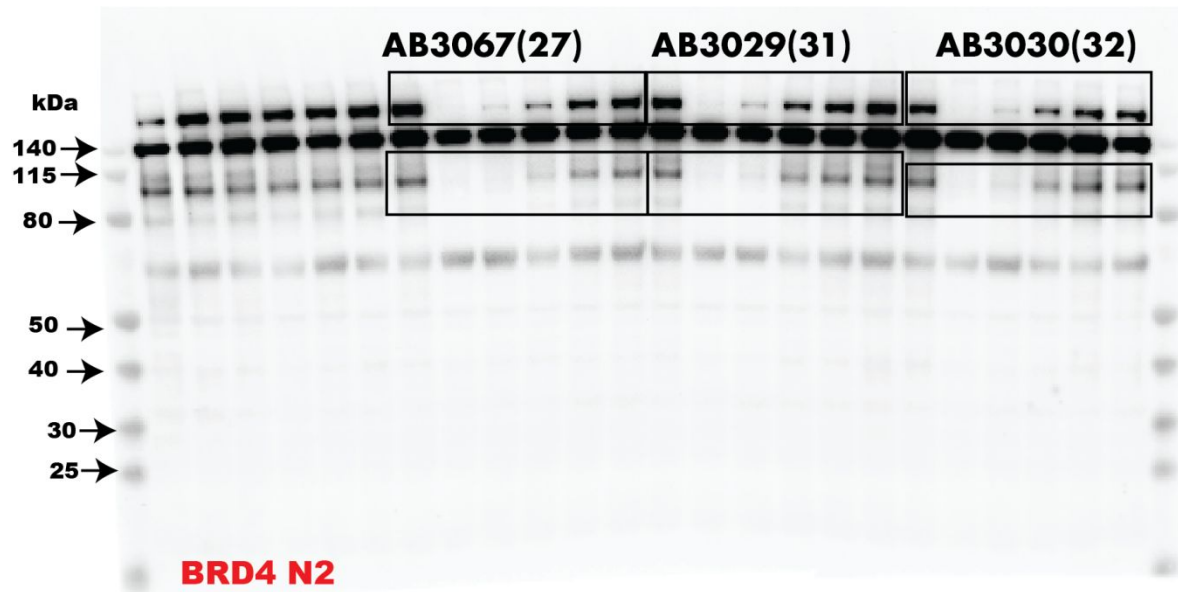




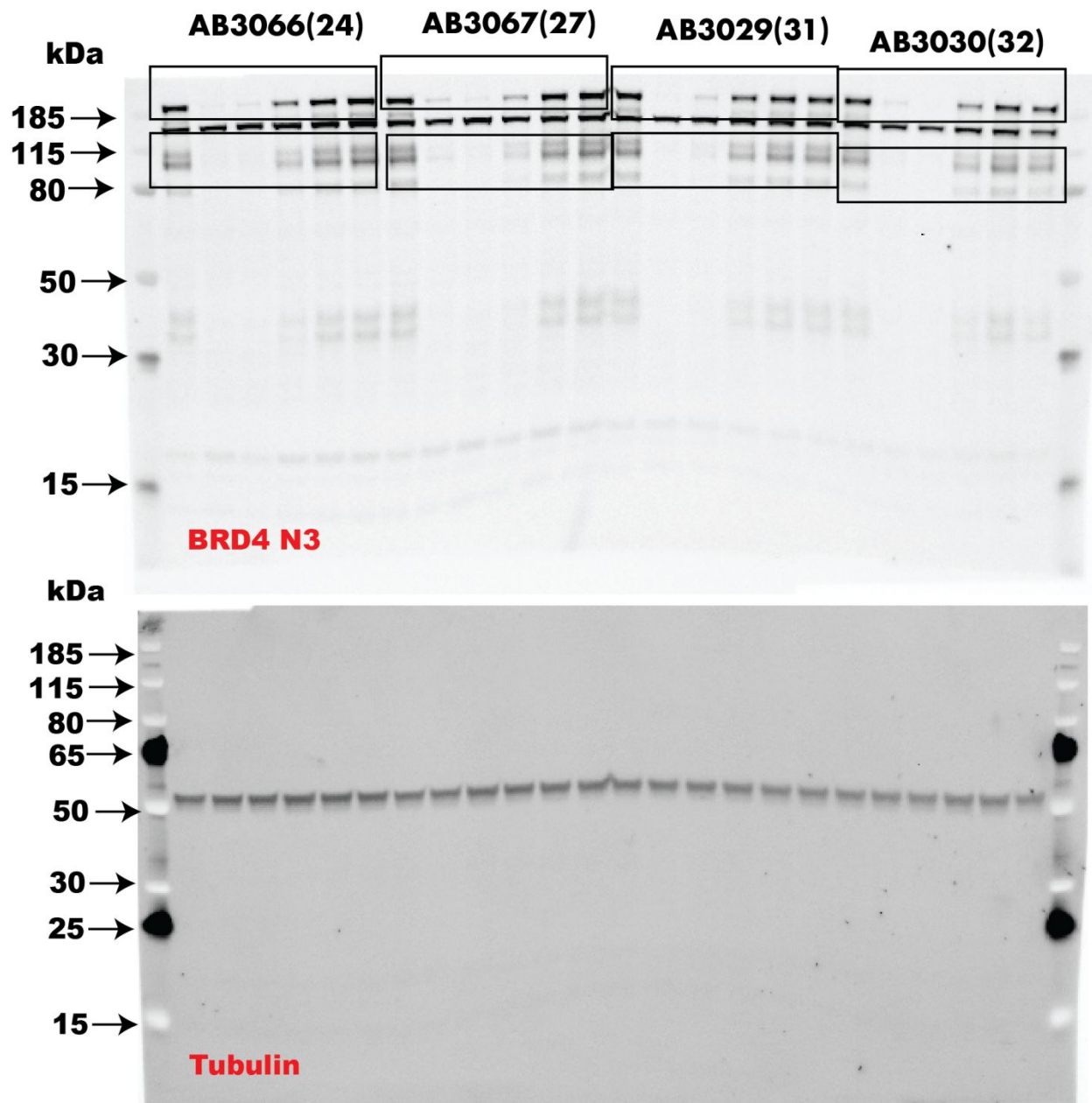


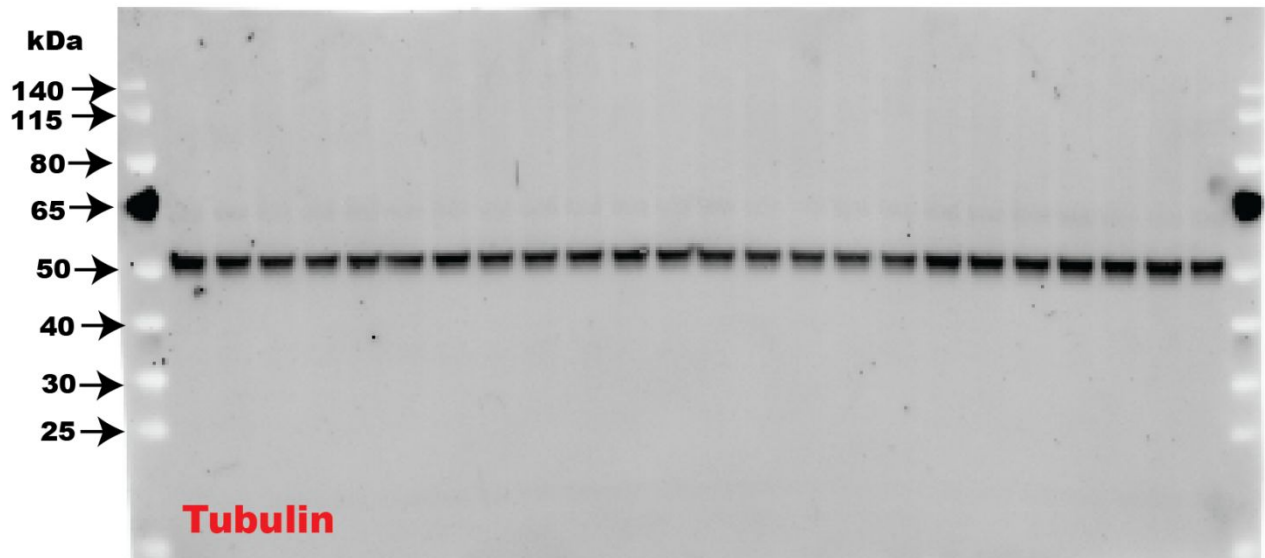
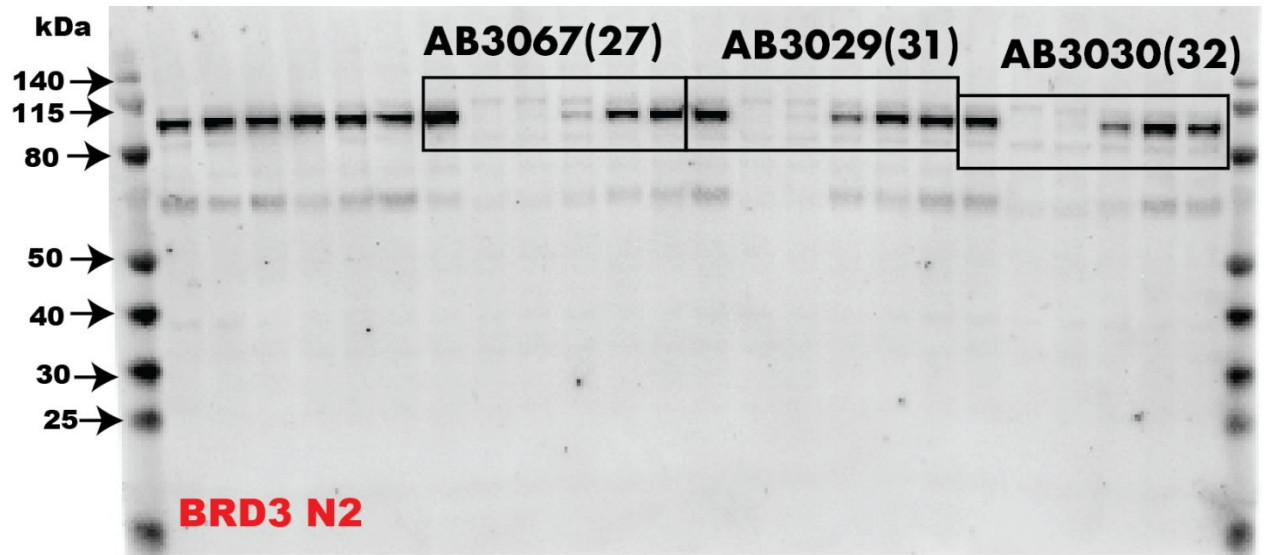


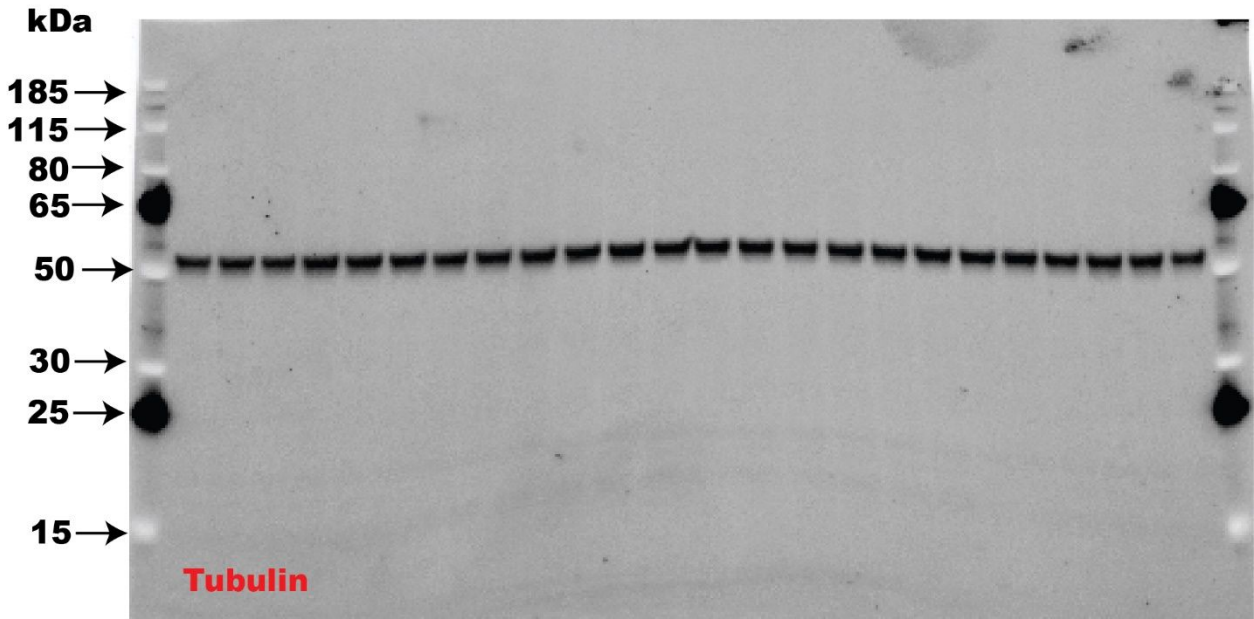
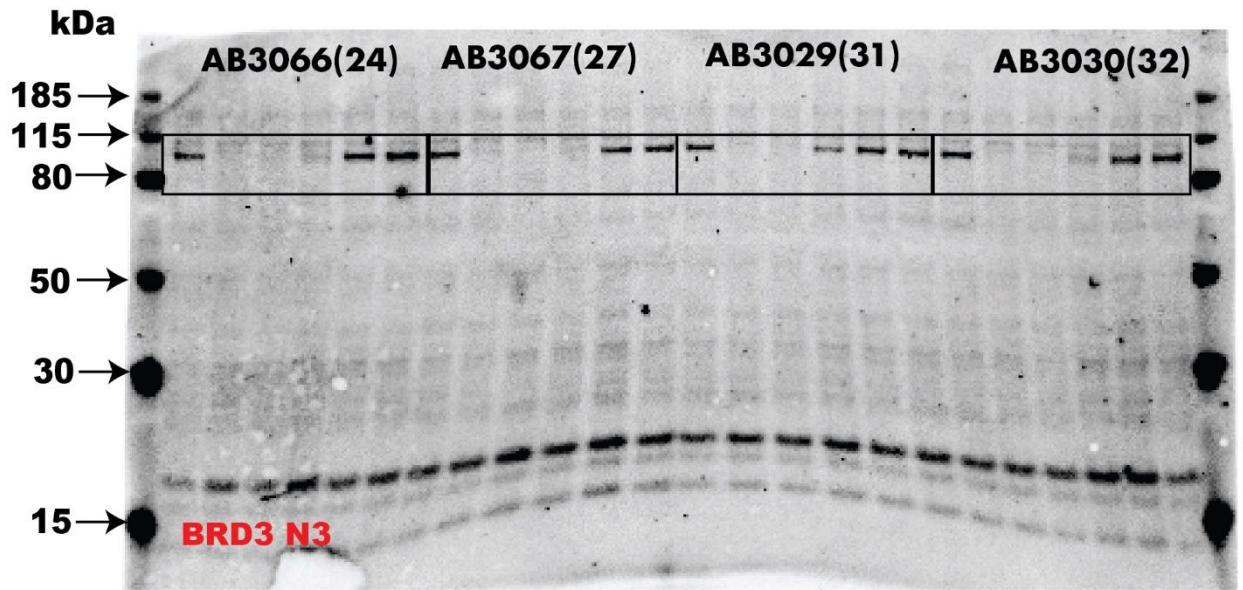


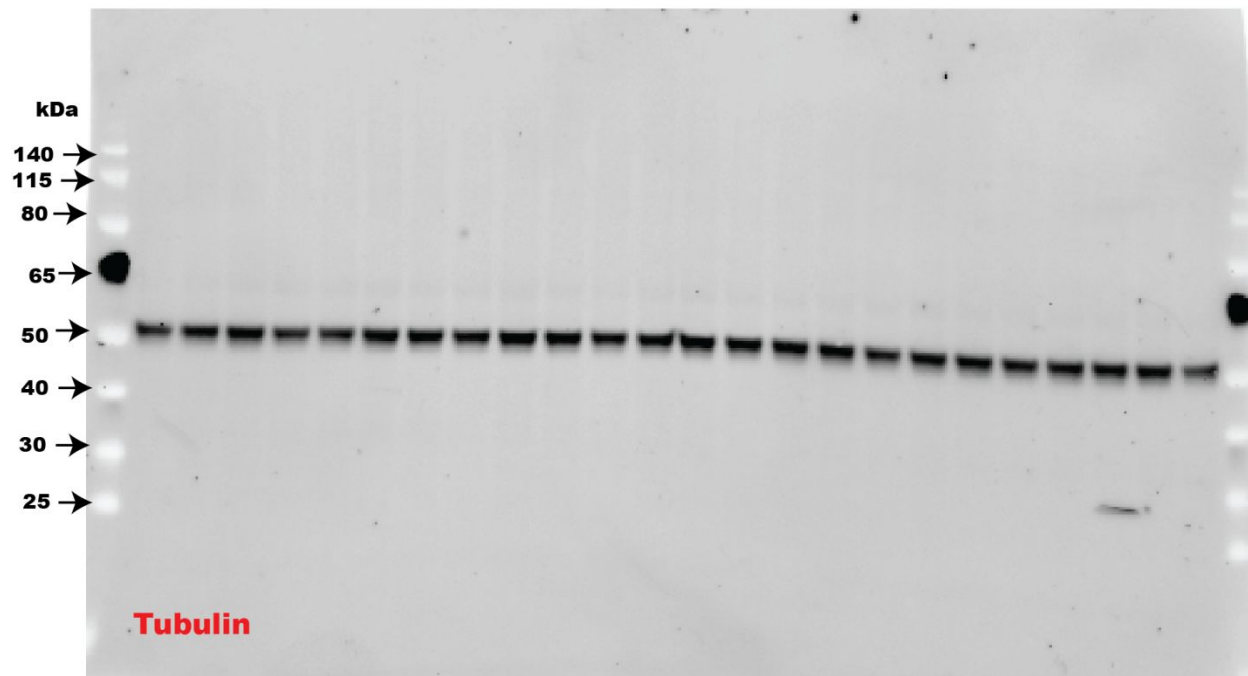
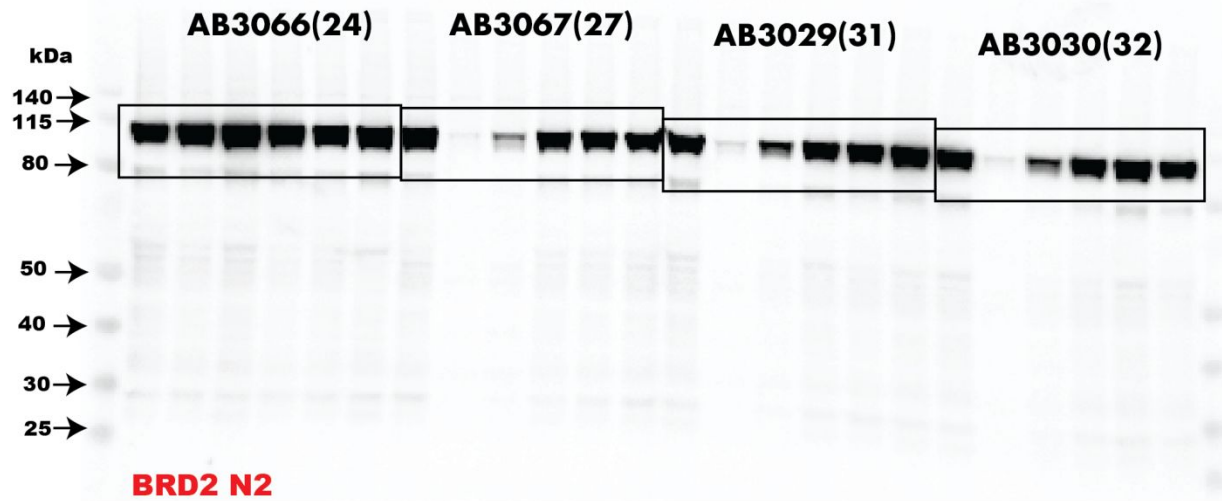




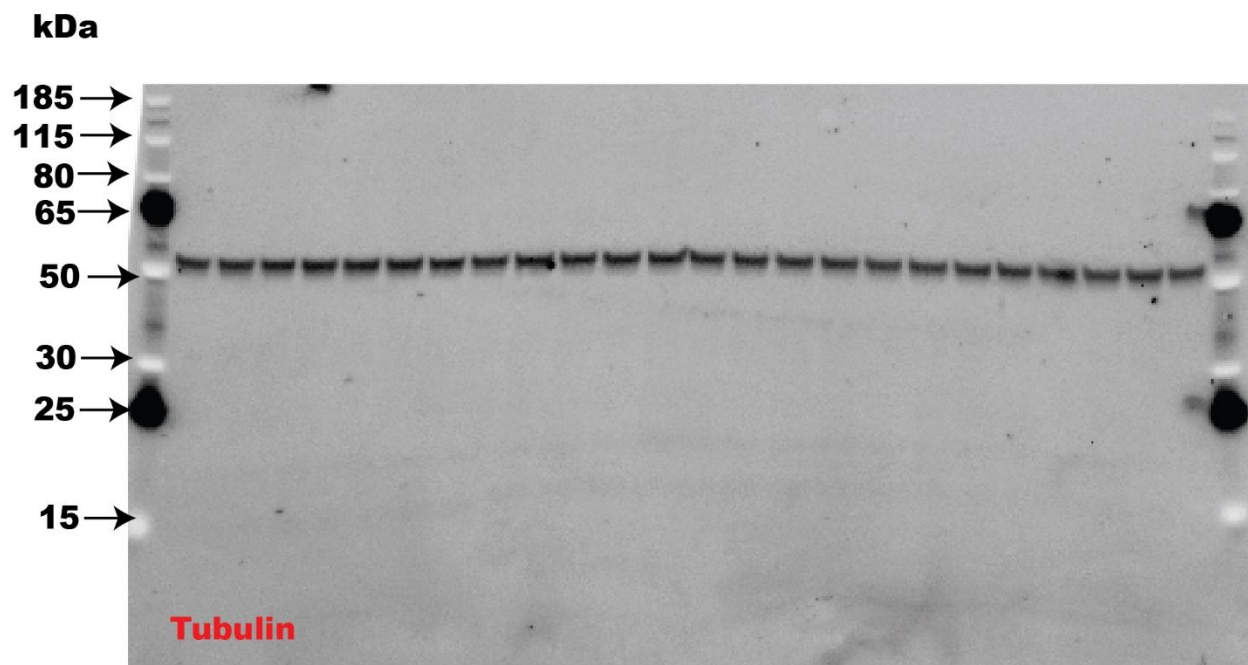
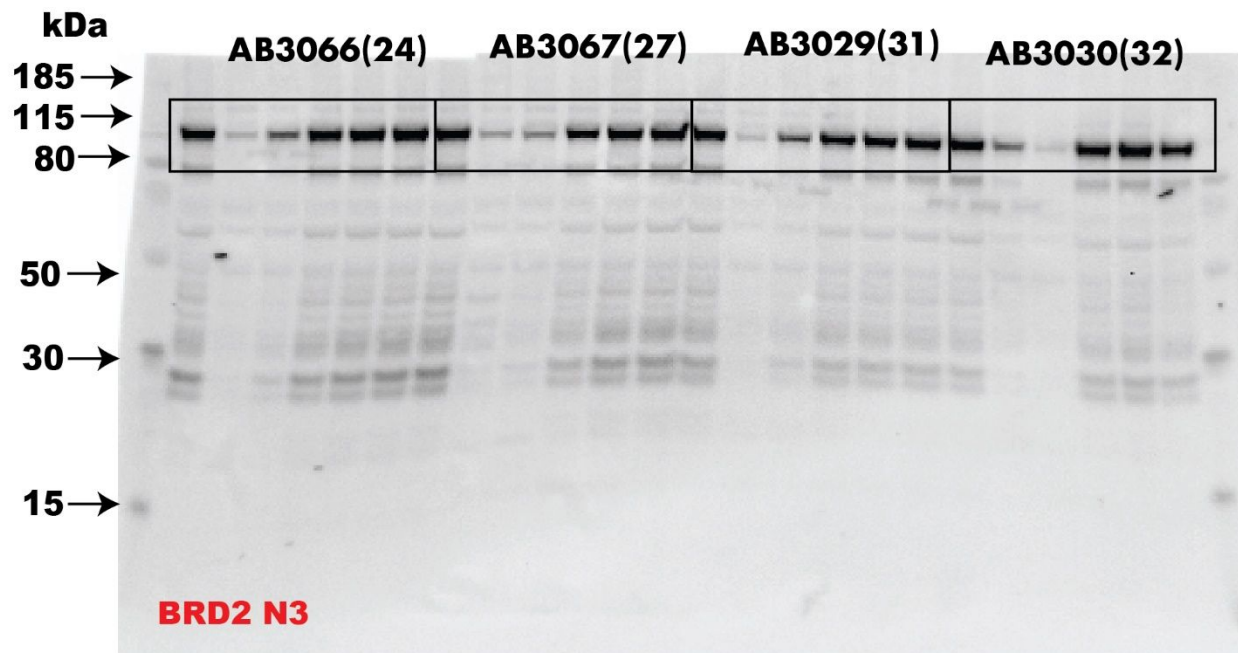


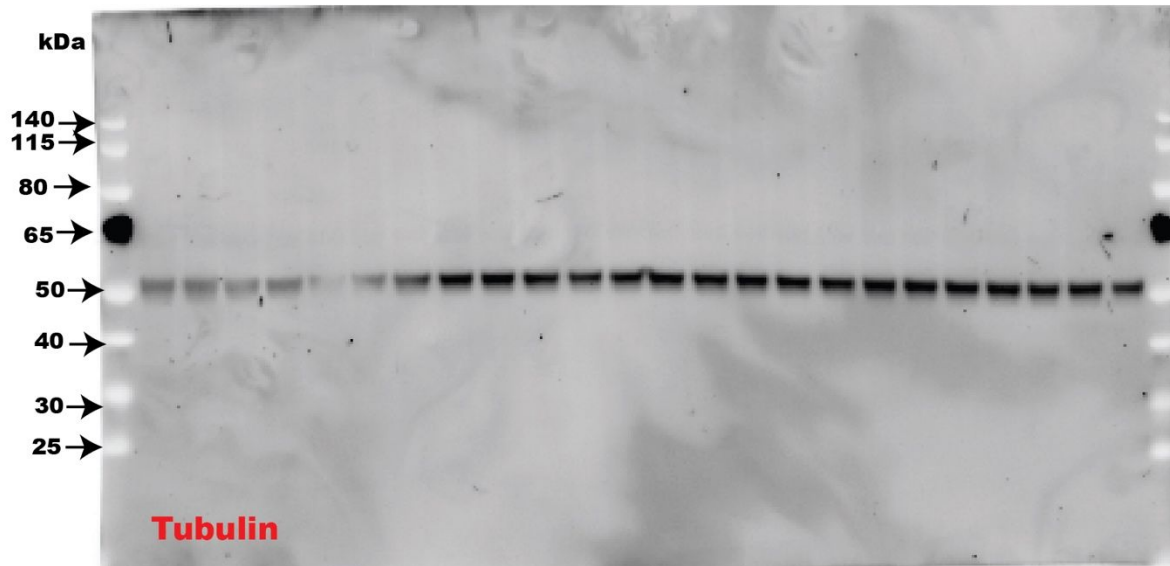
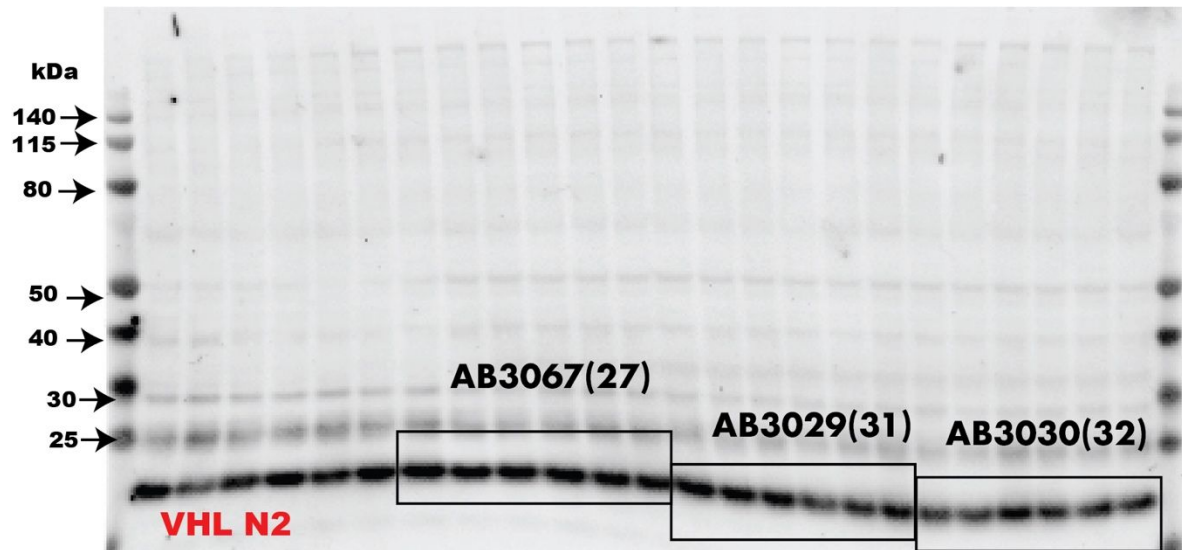




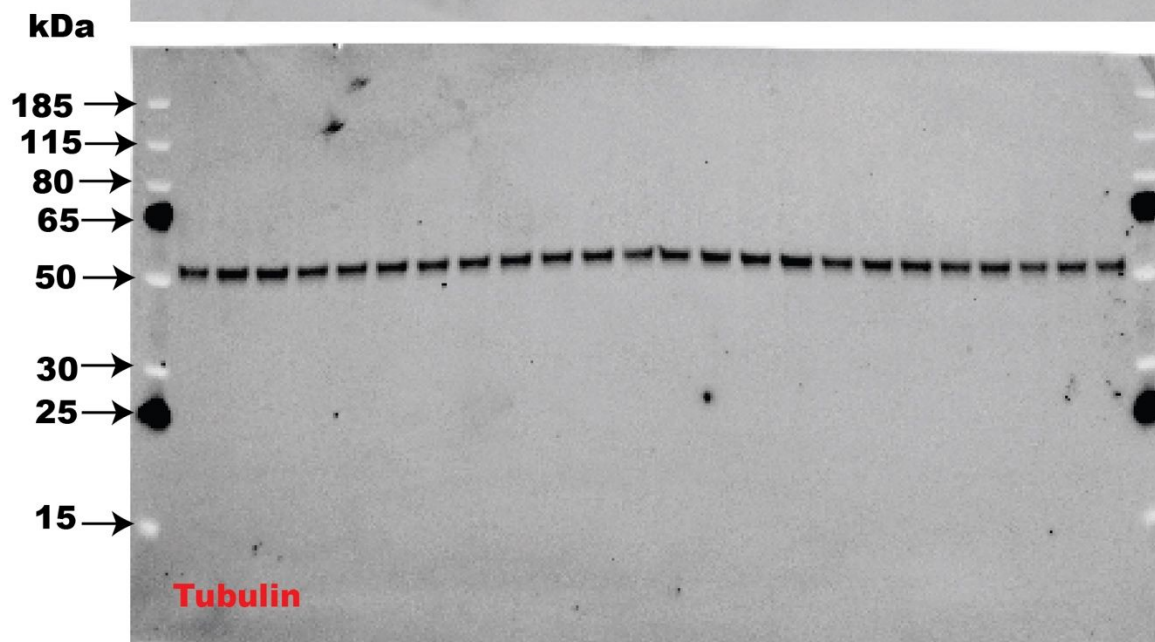
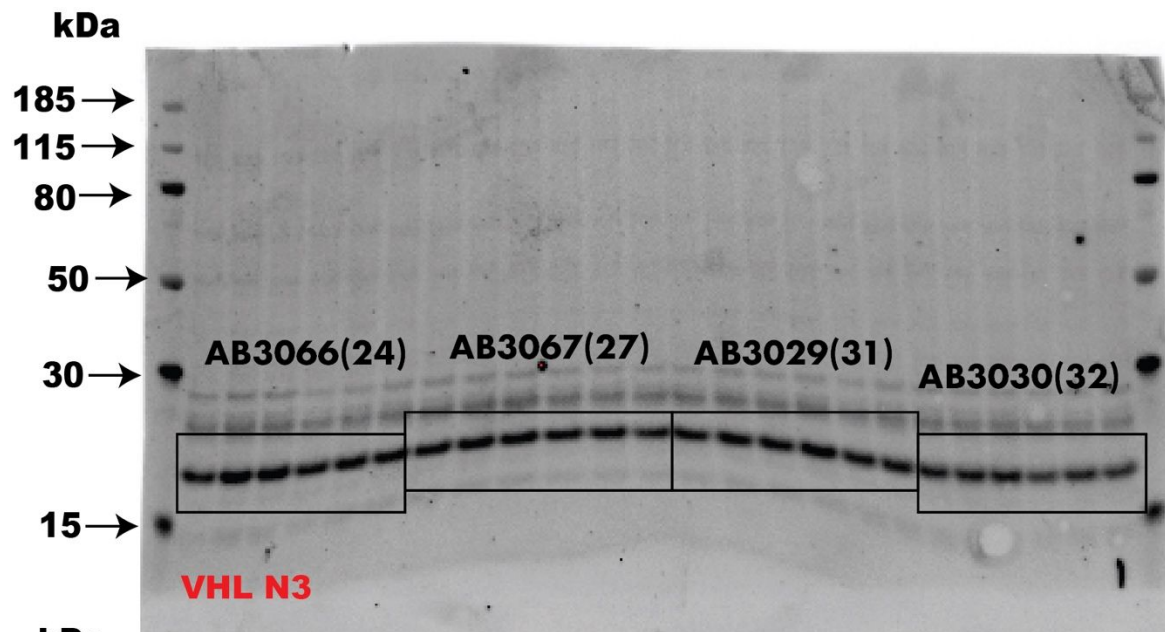


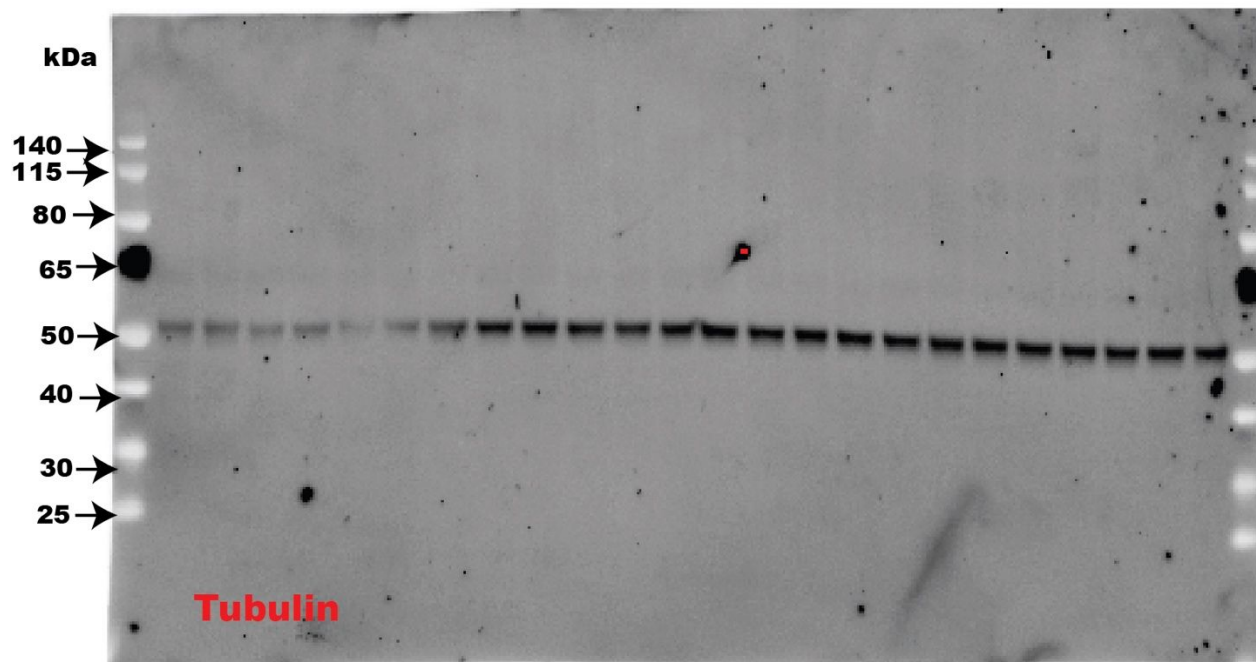
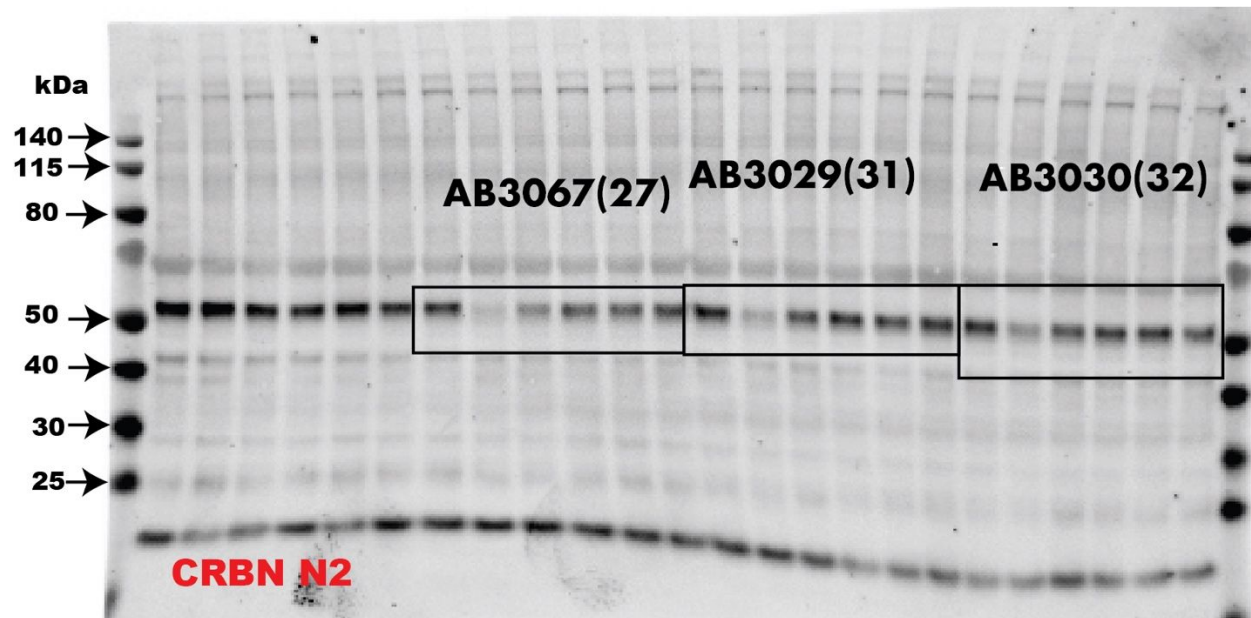


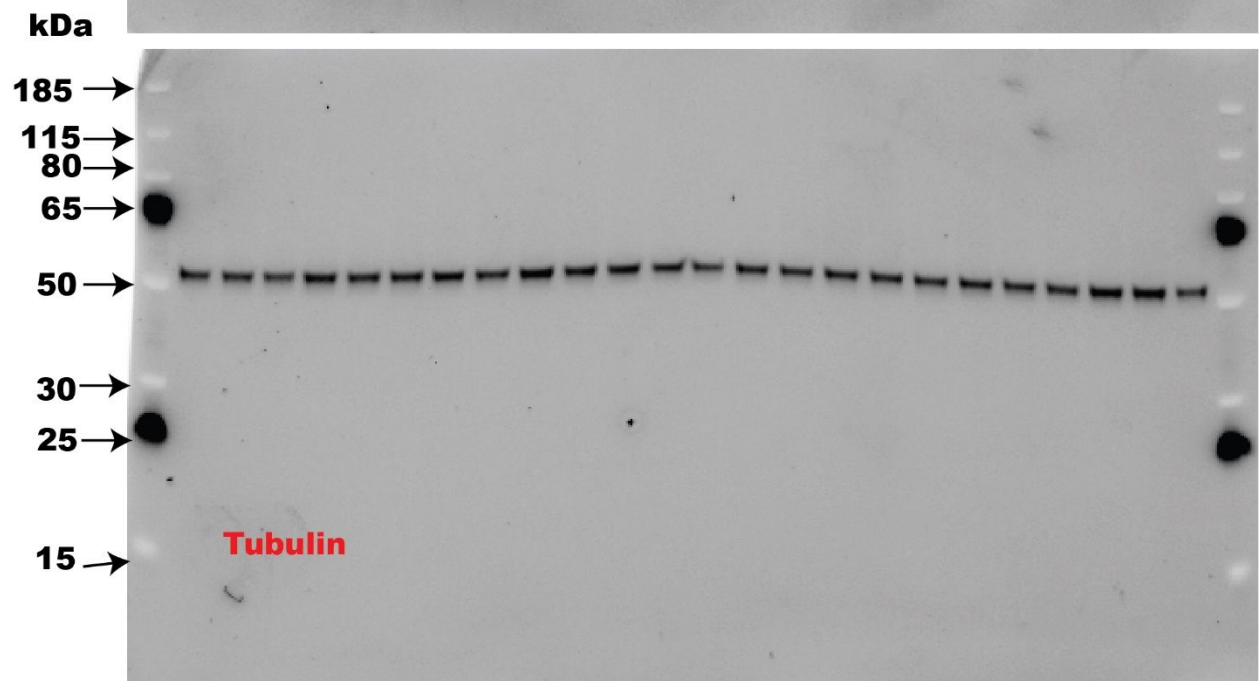
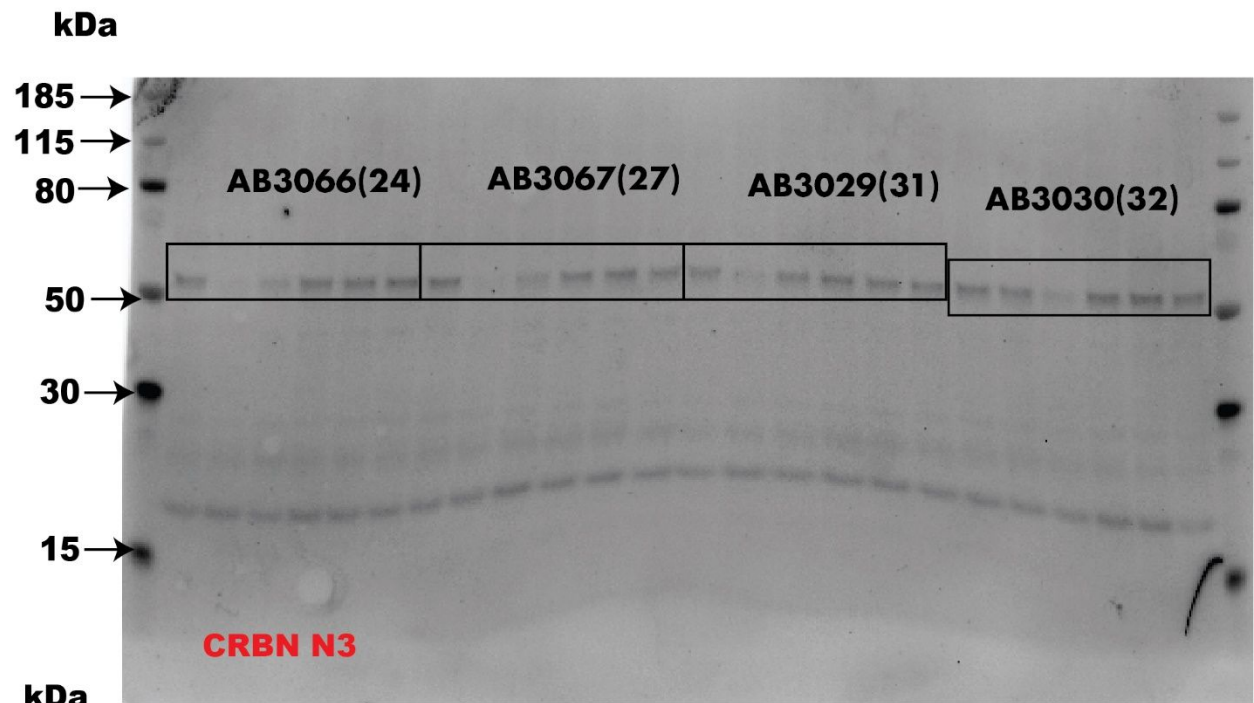


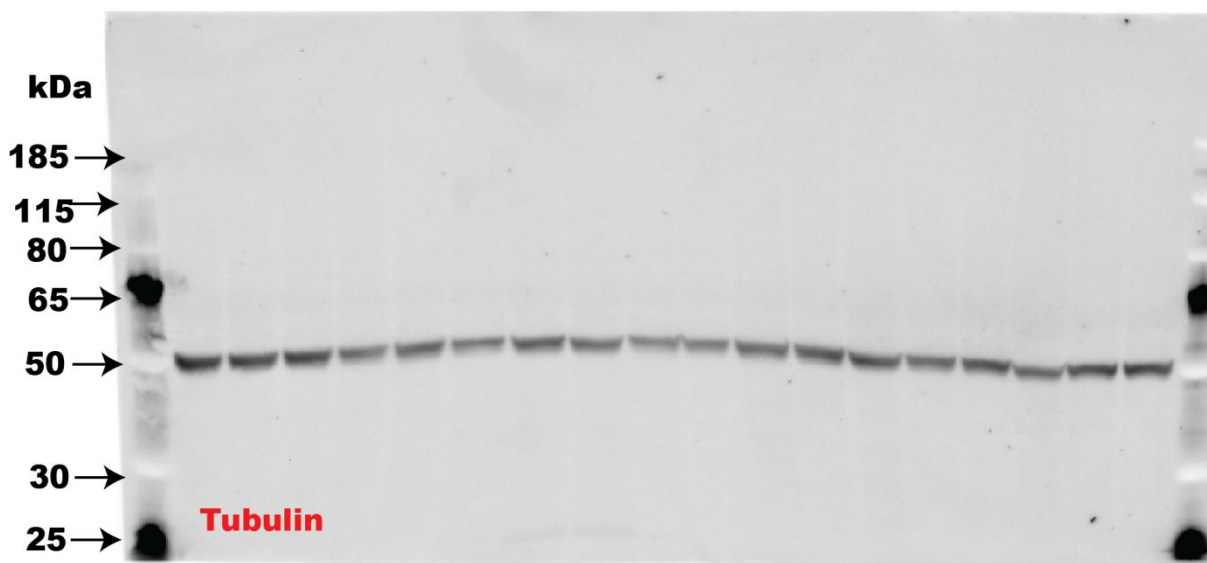
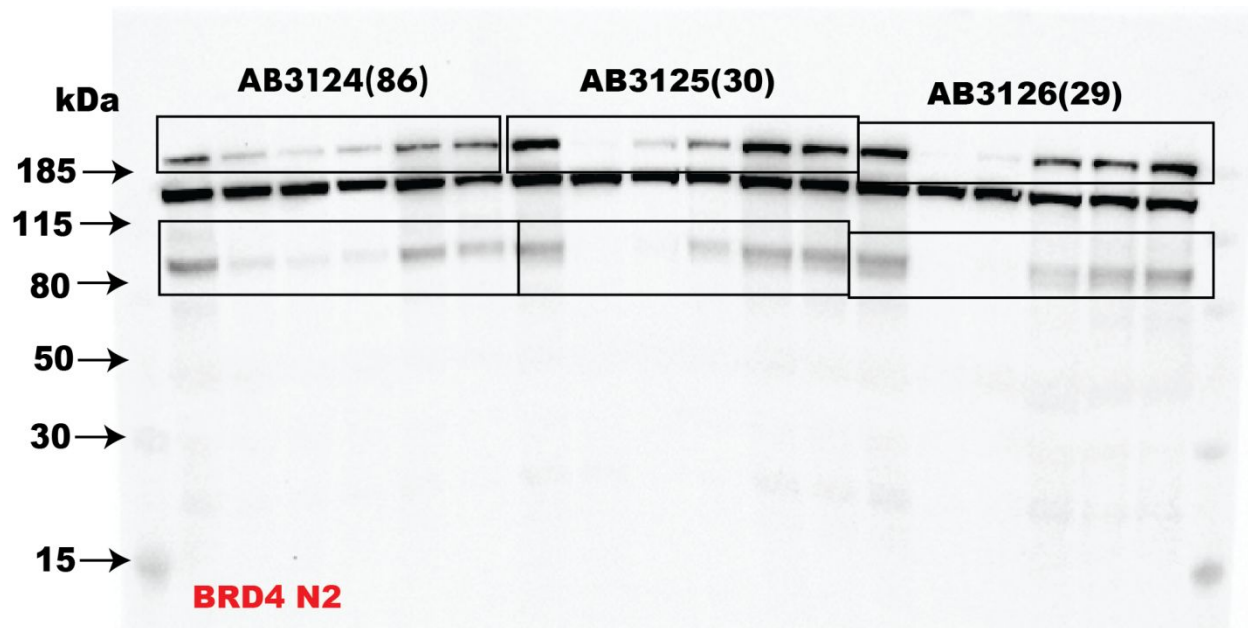




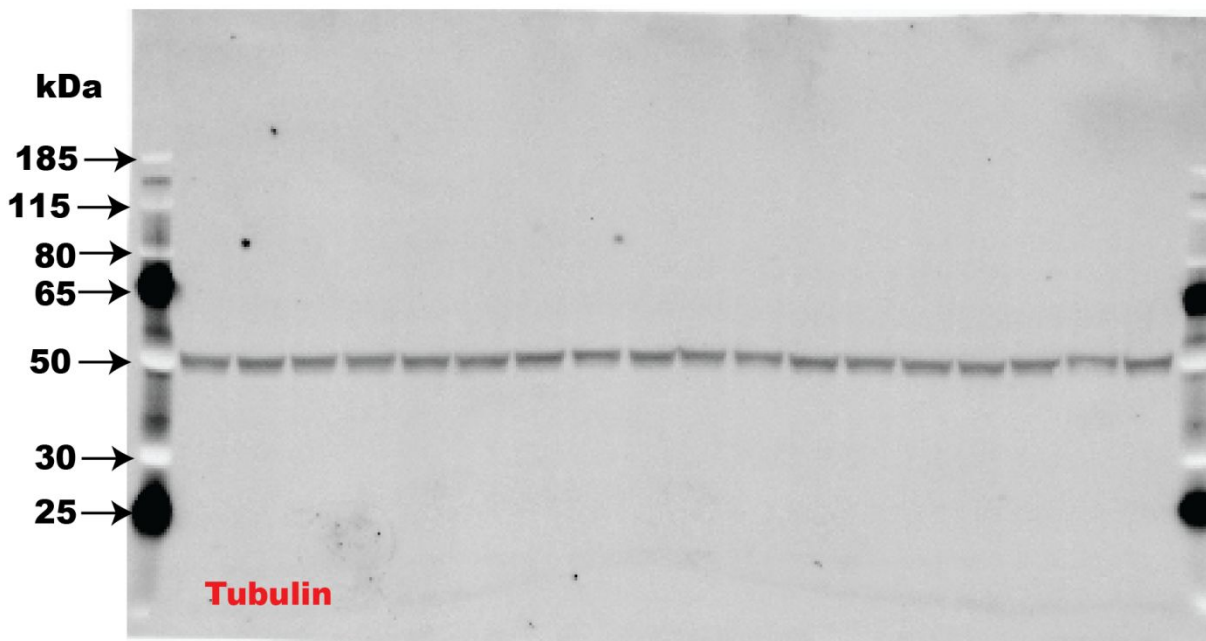
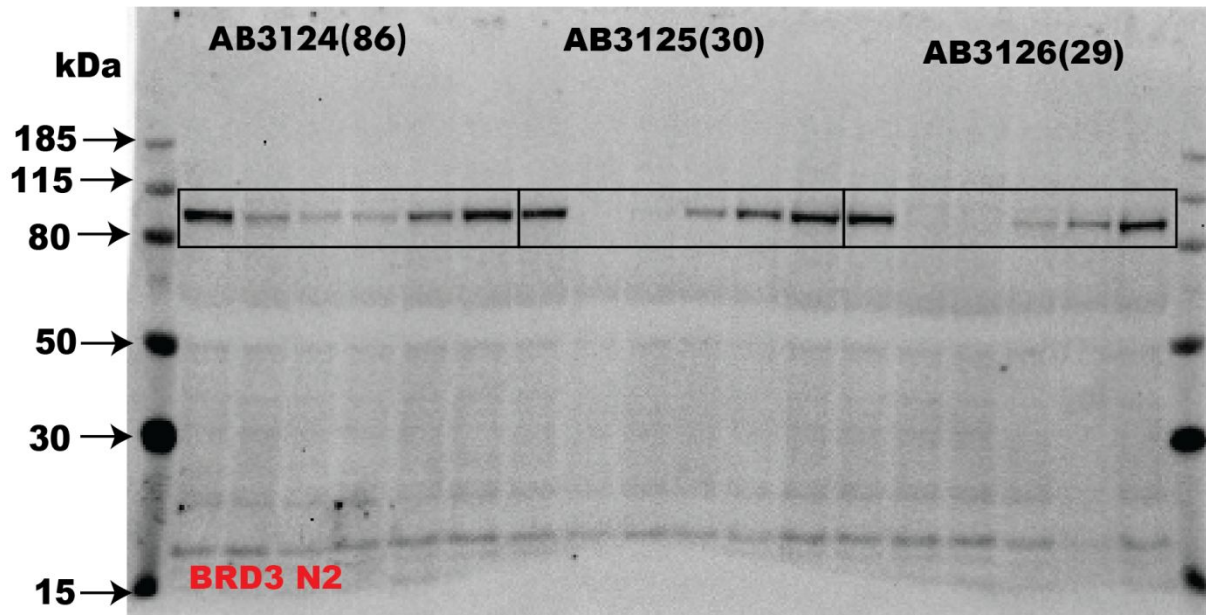


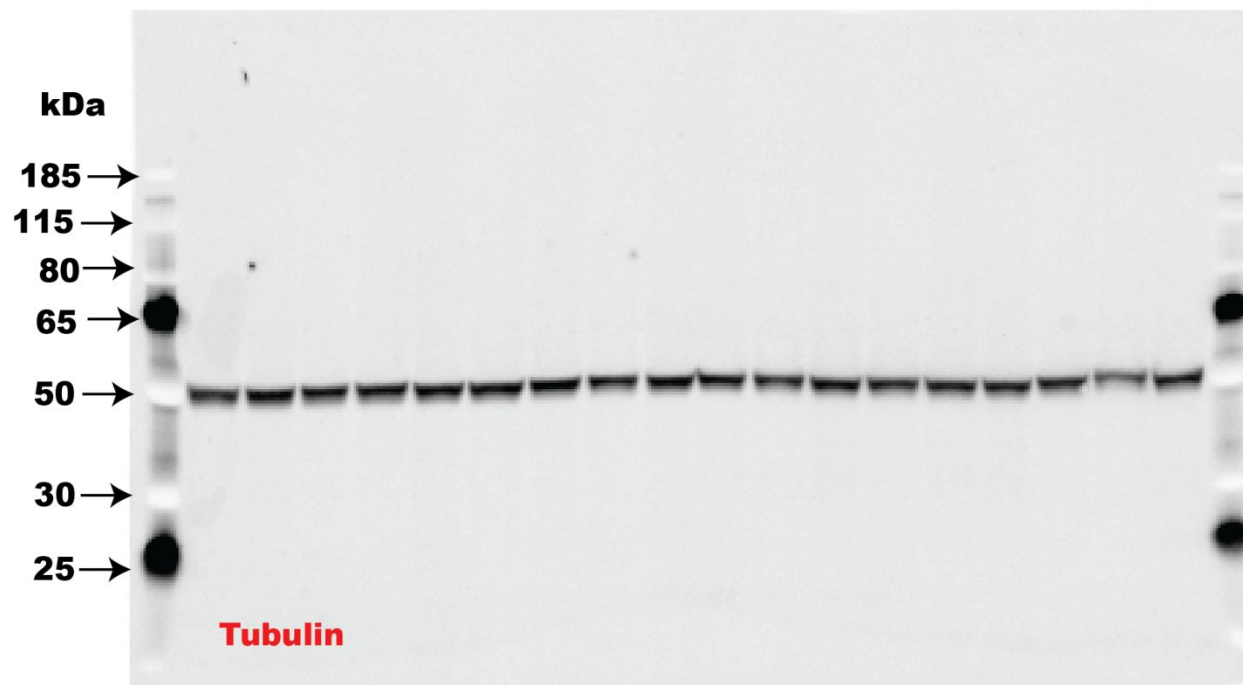
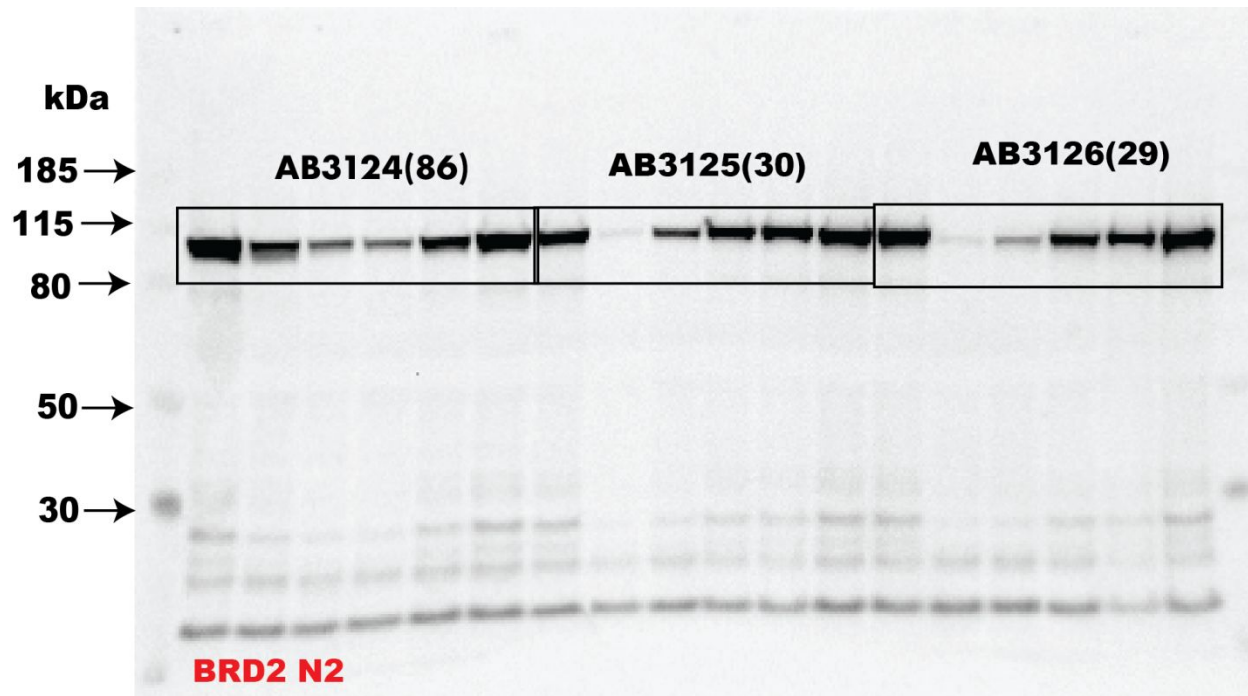




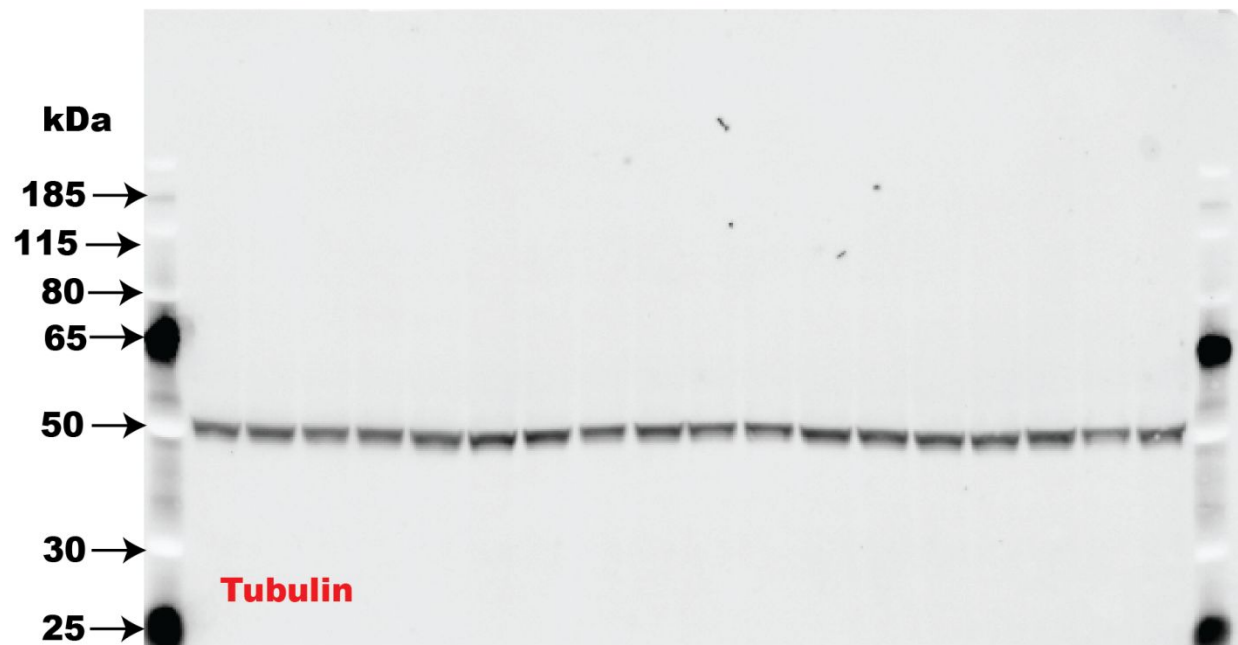
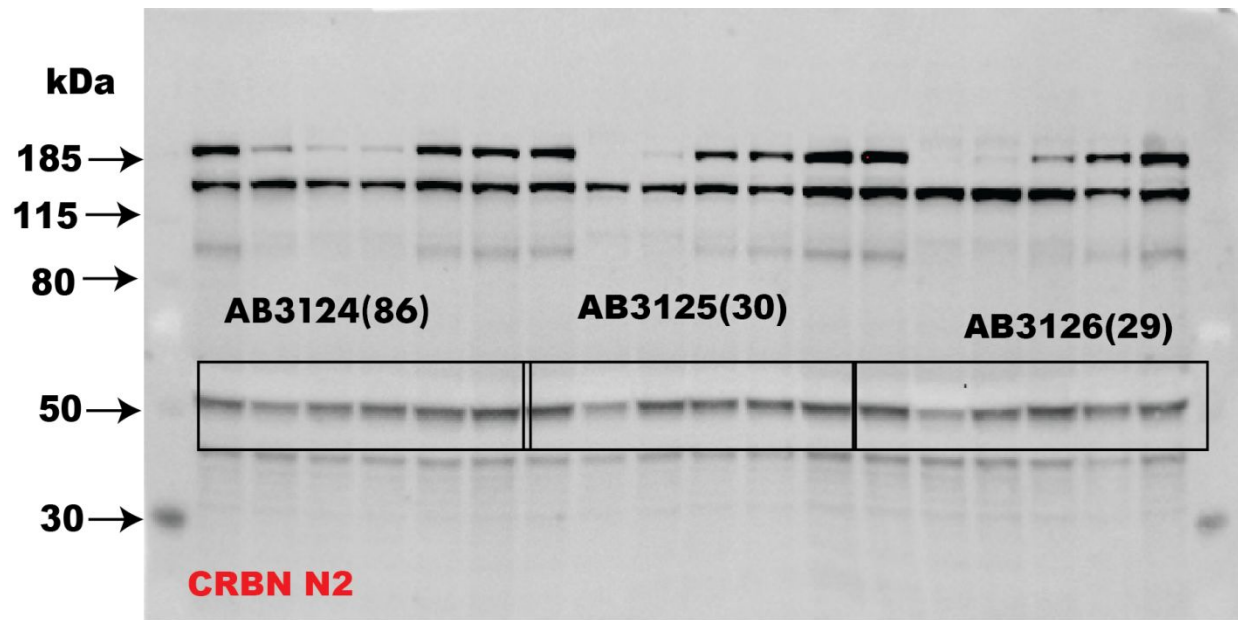




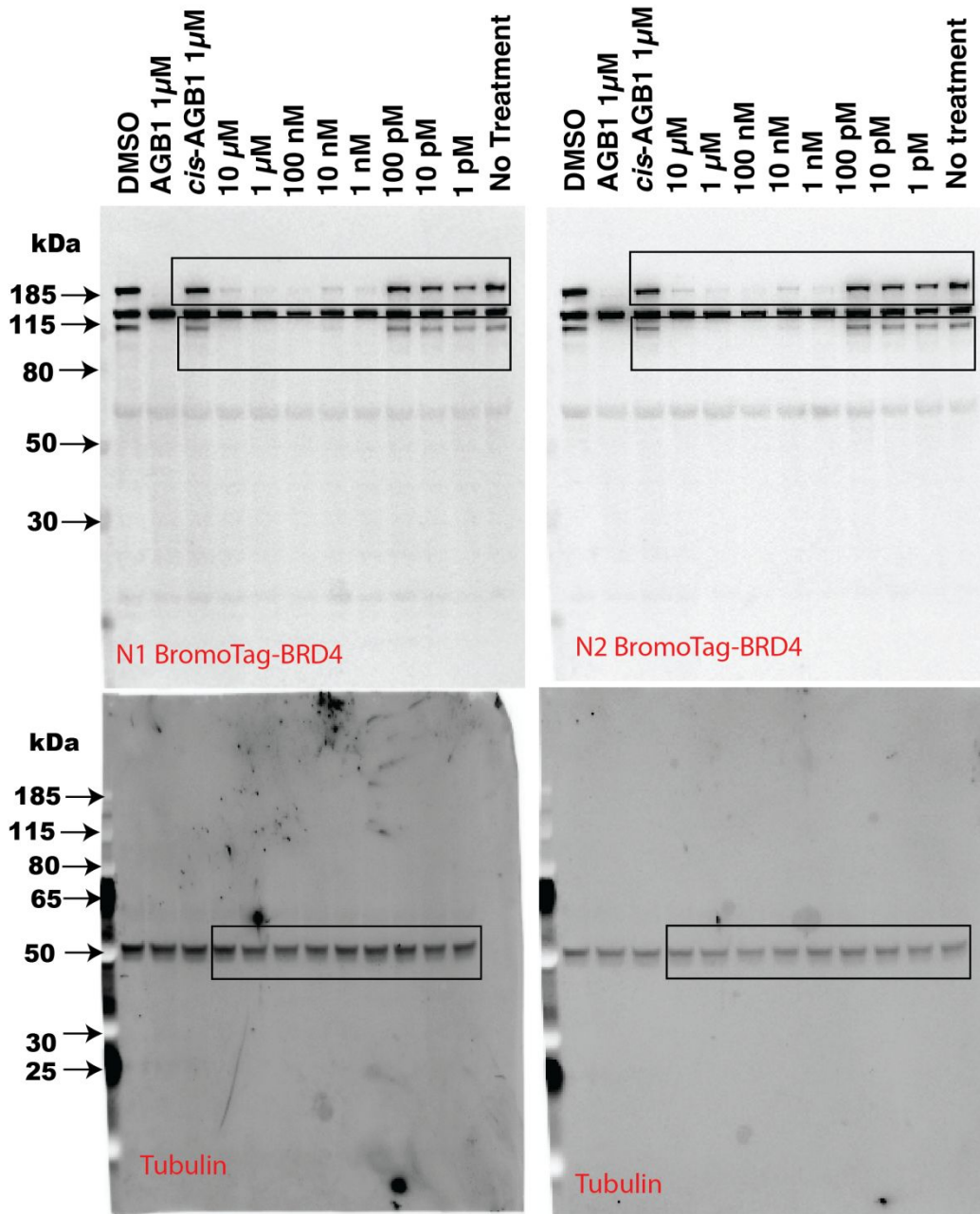


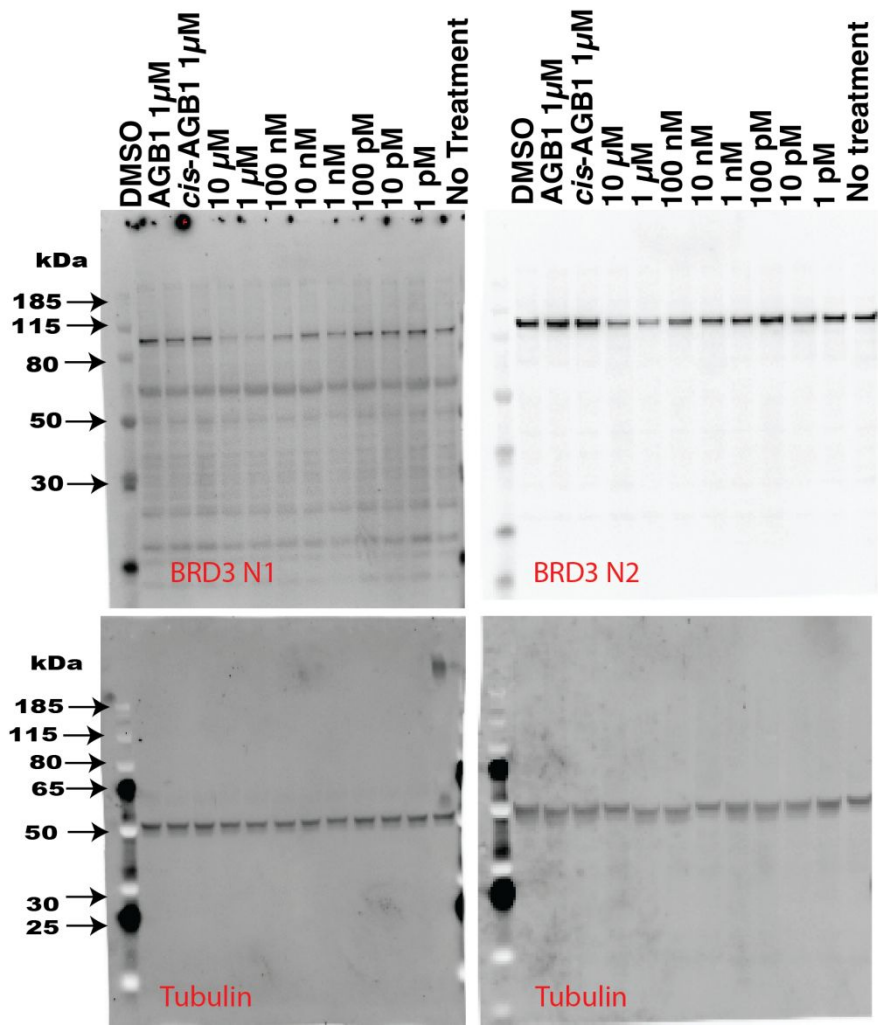


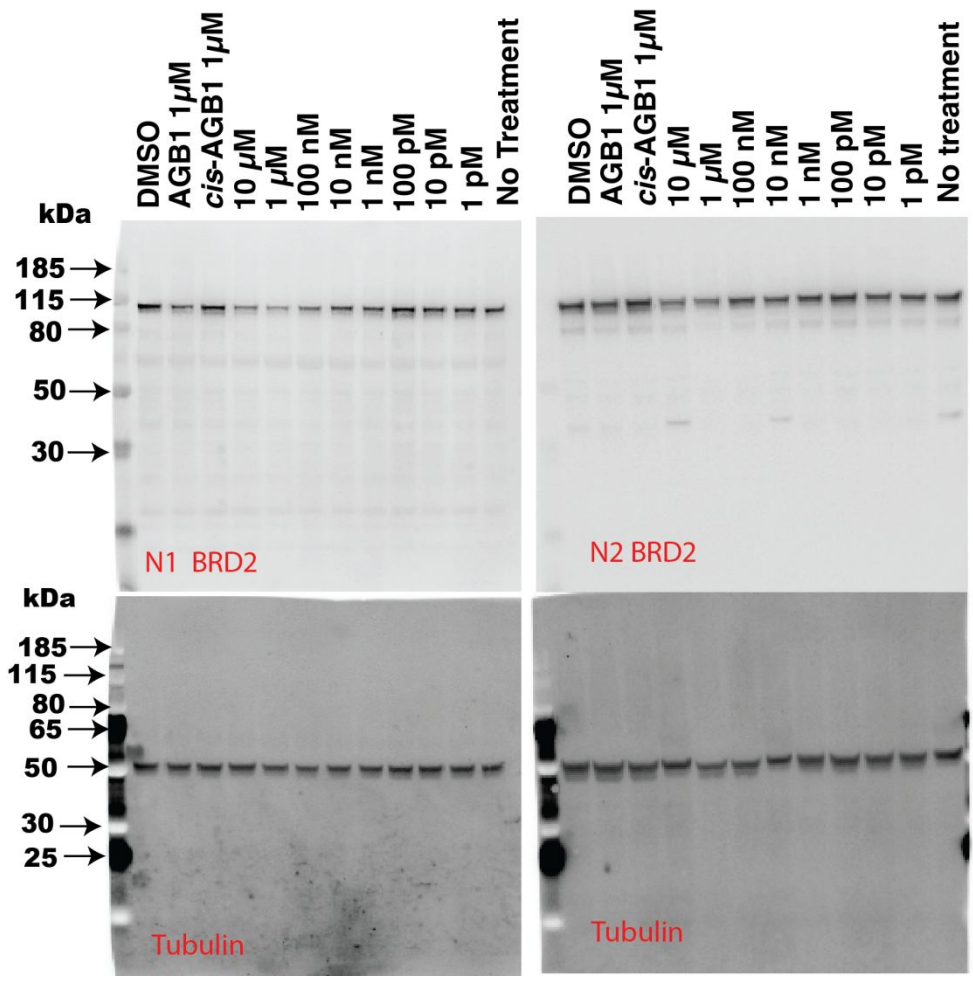


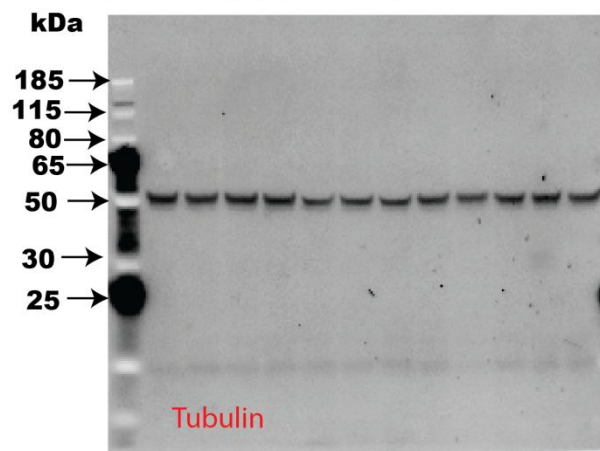
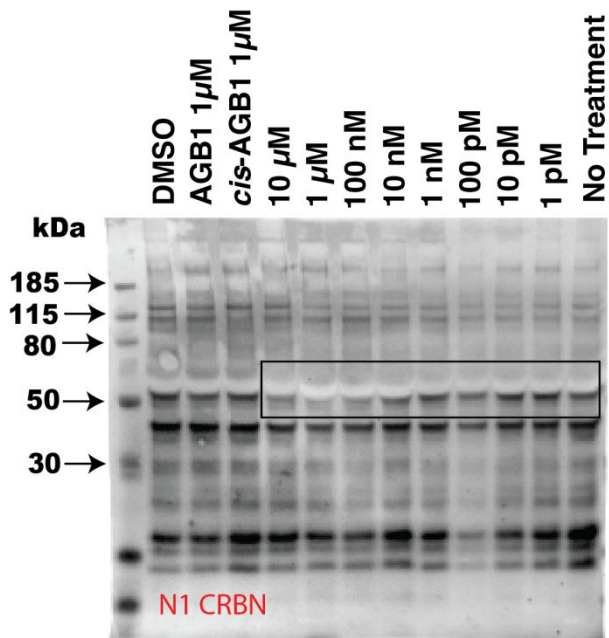


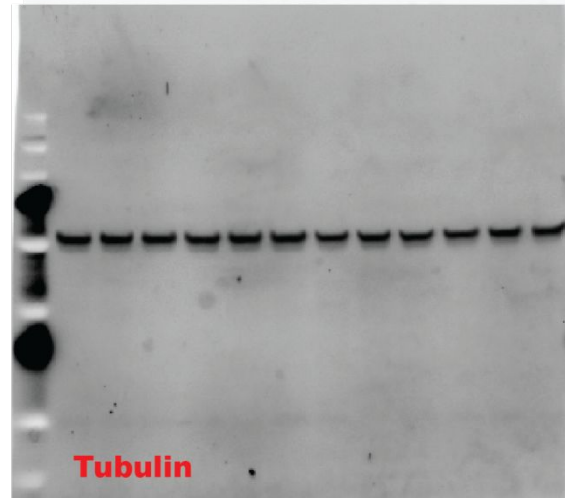
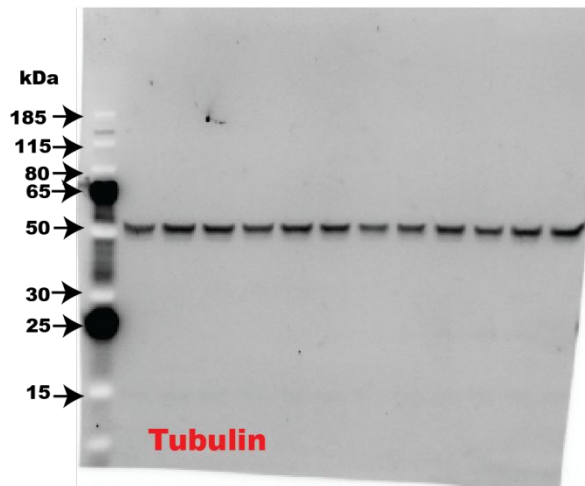
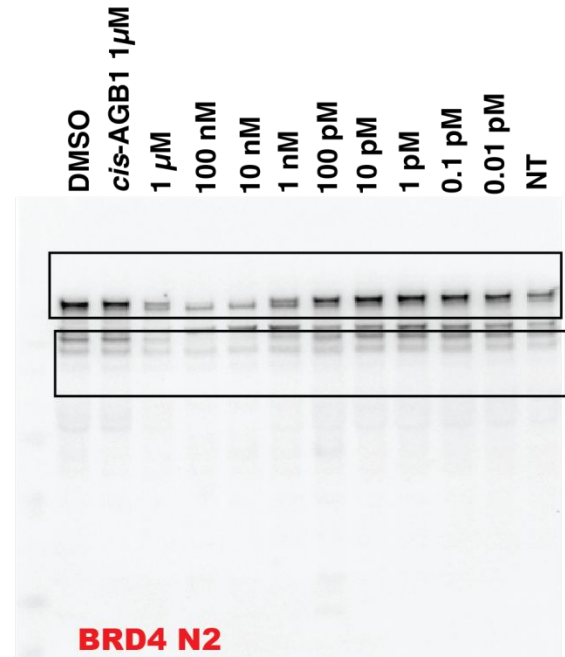
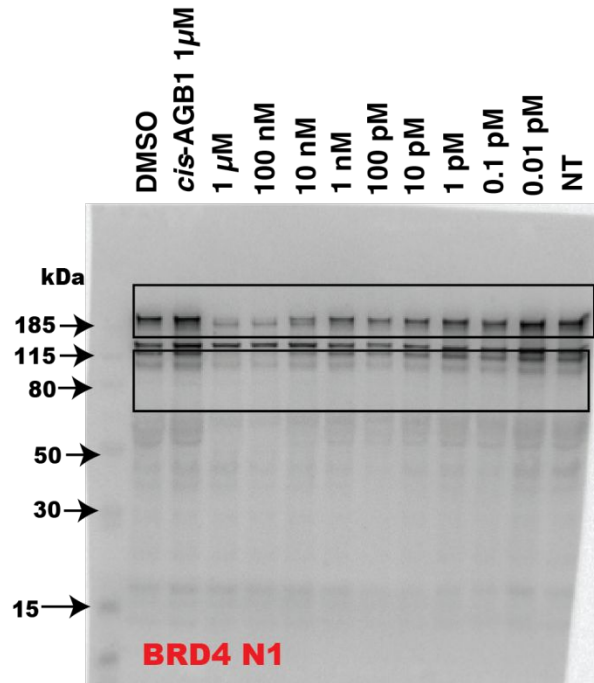
Supplementary Figure 10 (related to Figure 12). Uncropped independent replicate western blotting images of the evaluation of heterotrivalent AB3145 (97) & AGB1 in homozygous BromoTag-BRD4 HEK293 cells.



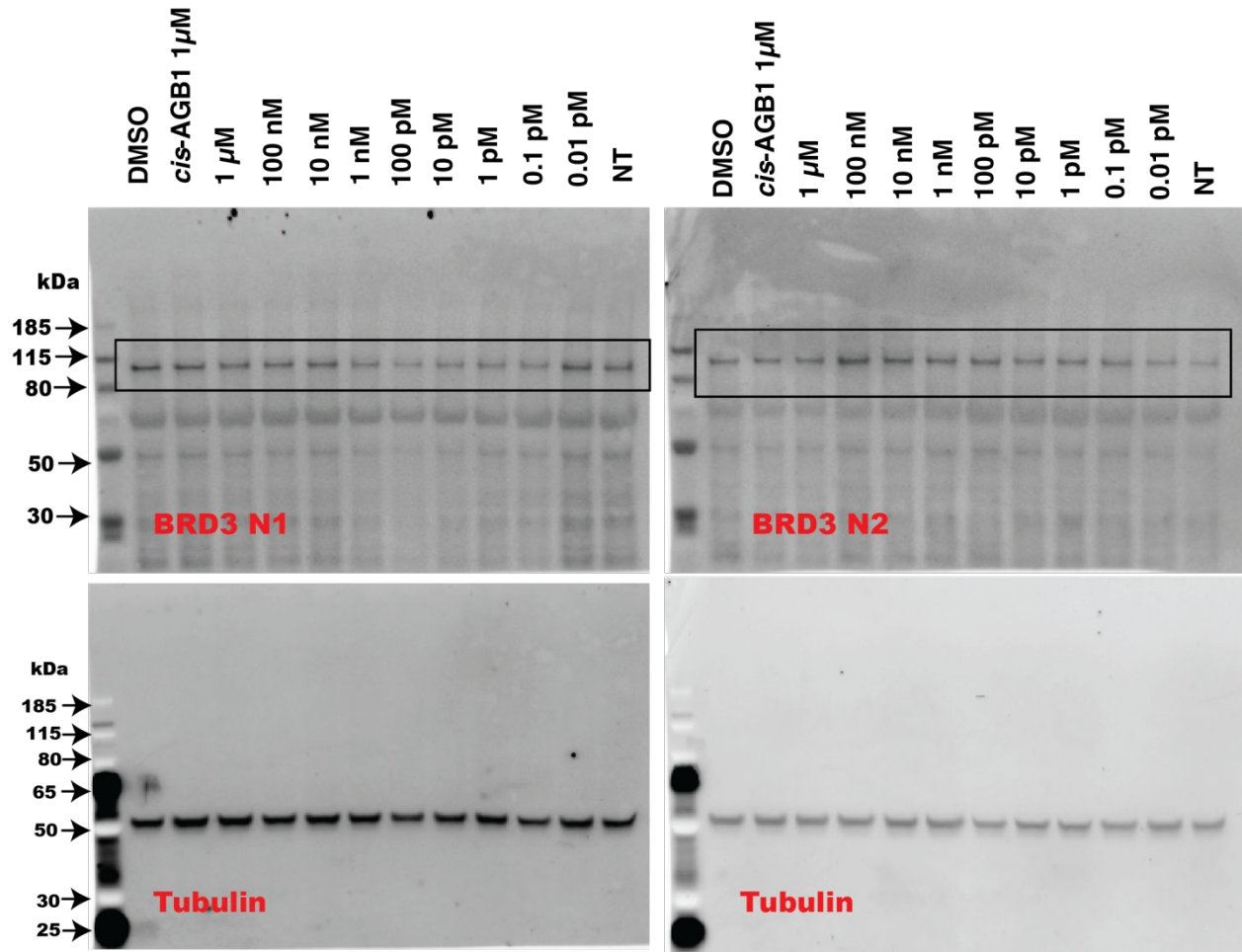


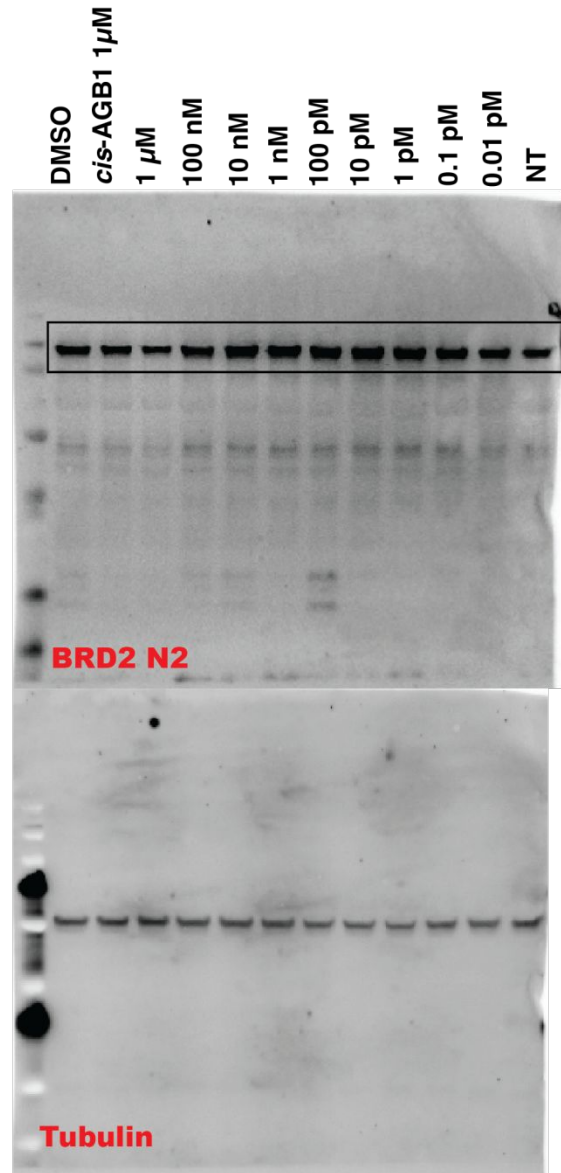
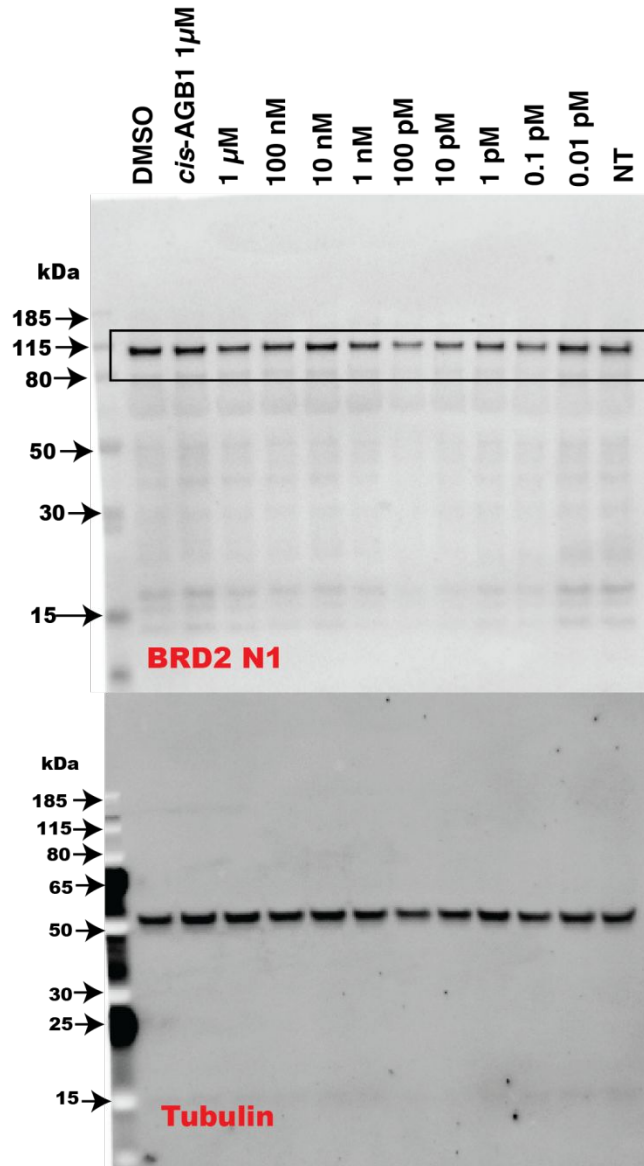


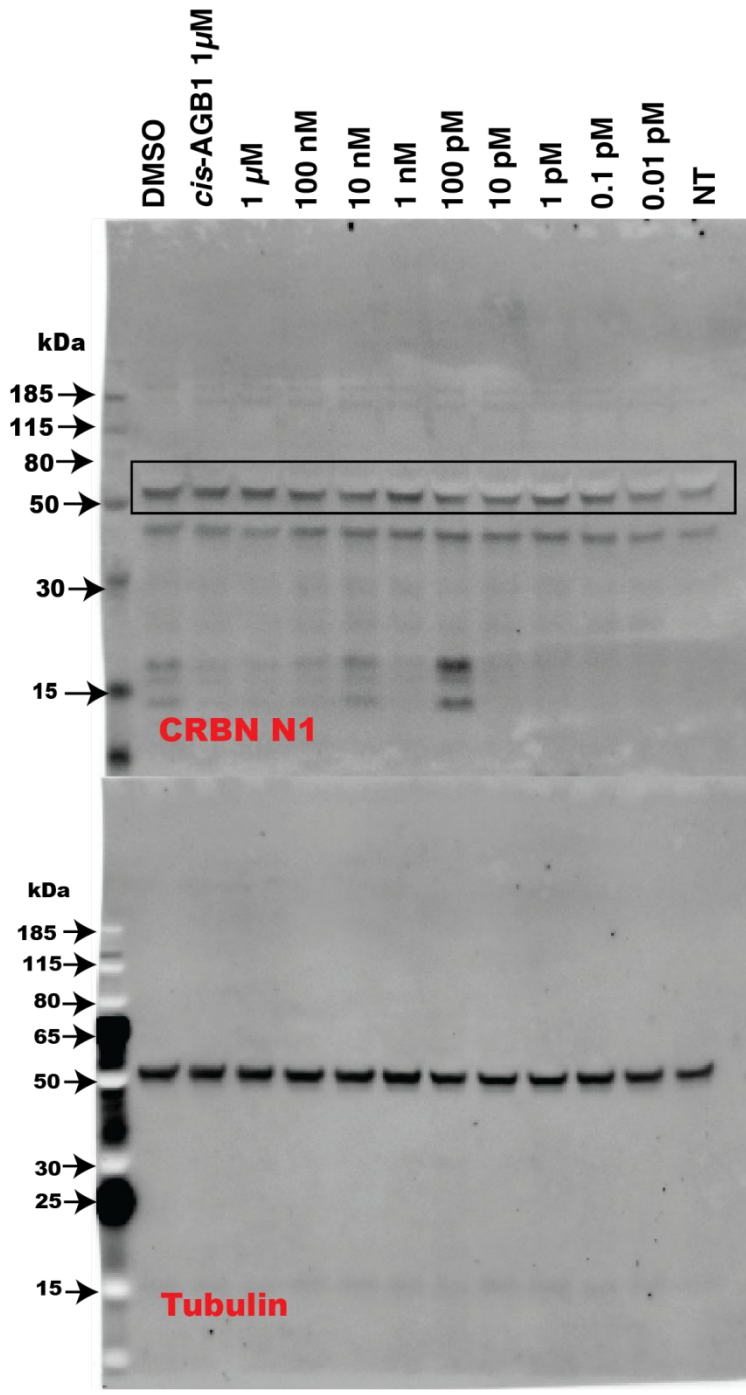




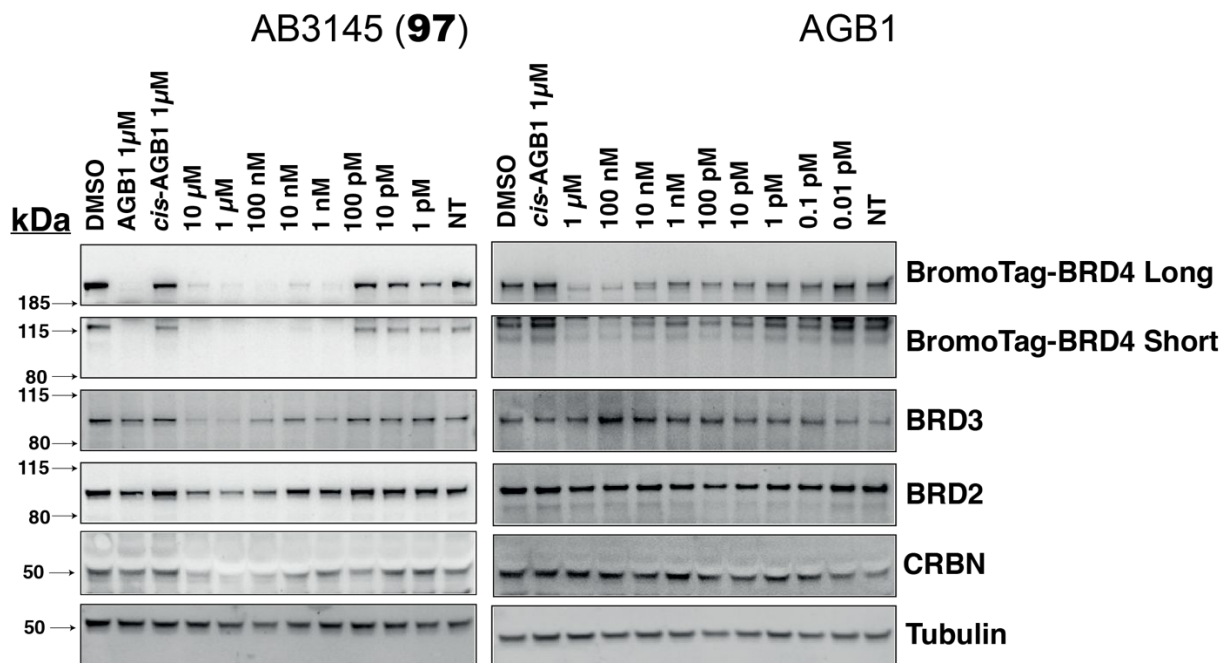








Supplementary Figure 11 (related to Figure 12). Cropped western blotting images of the evaluation of heterotrivalent AB3145 (97) and AGB1 in homozygous BromoTag-BRD4 HEK293 cells.



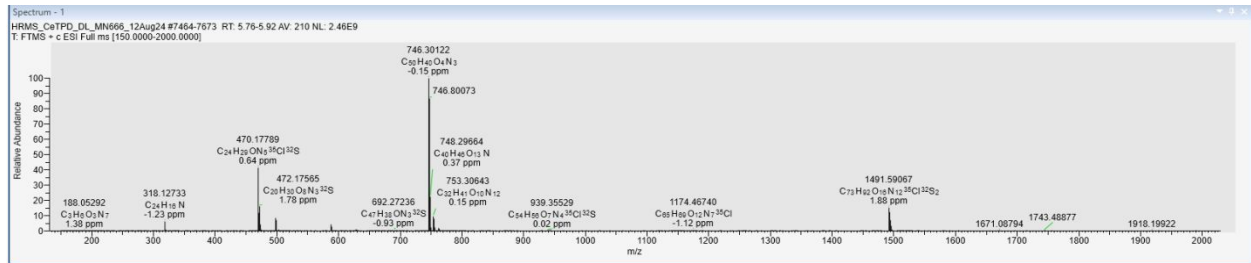
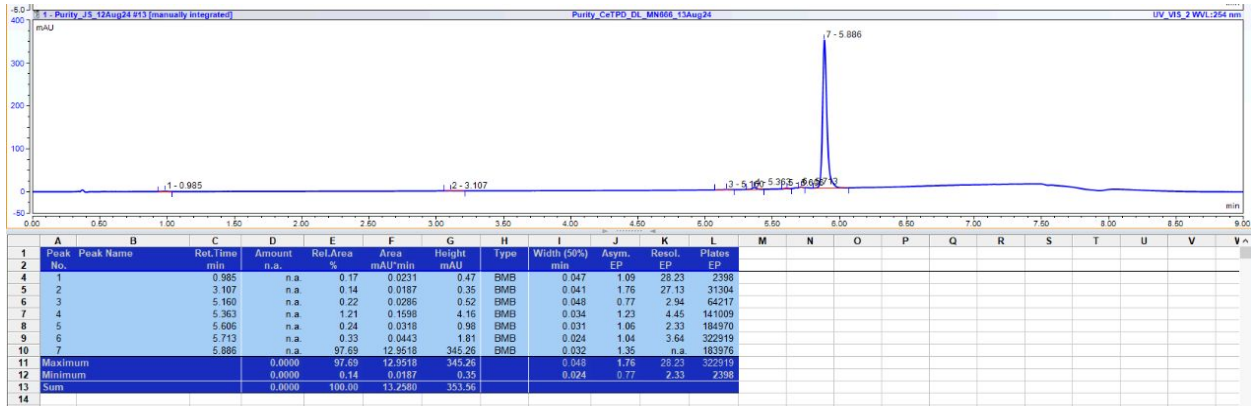
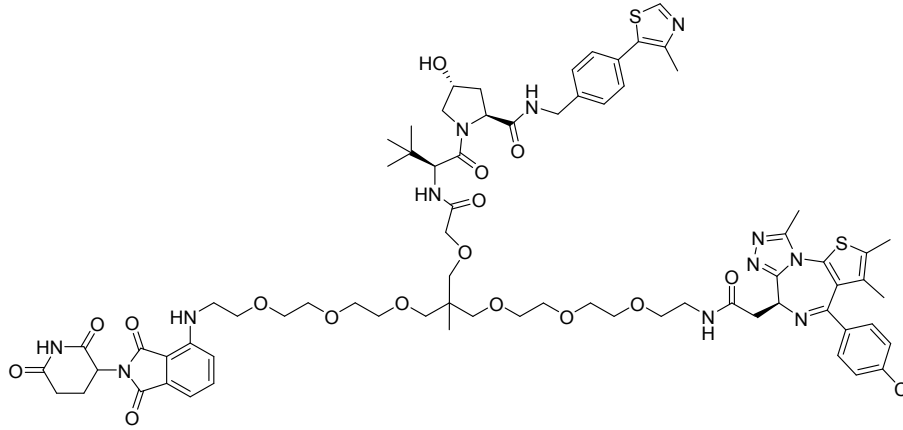
Supplementary Table 8 (related to Figure 12). DC<sub>50</sub> values of AB3145 (97) and AGB1 in homozygous BromoTag-BRD4 HEK293 cells.

Protein	AB3145 (97)		AGB1	
	pDC <sub>50</sub>	D <sub>max</sub> %	pDC <sub>50</sub>	D <sub>max</sub> %
BRD4 <sup>Long</sup>	9.9 ± 0.6	14	8.0 ± 0.2	4
BRD4 <sup>Short</sup>	9.9 ± 0.1	14	8.6 ± 0.1	11
BRD3	7.5 ± 0.4	21	N.A.	N.A.
BRD2	6.5 <sup>a</sup>	56	N.A.	N.A.
CRBN	7.7 <sup>b</sup>	23	N.A.	N.A.

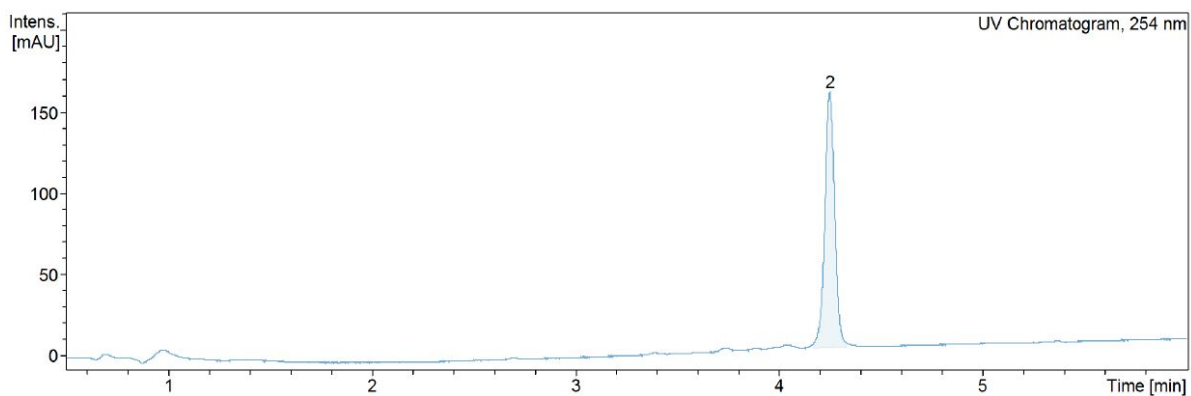
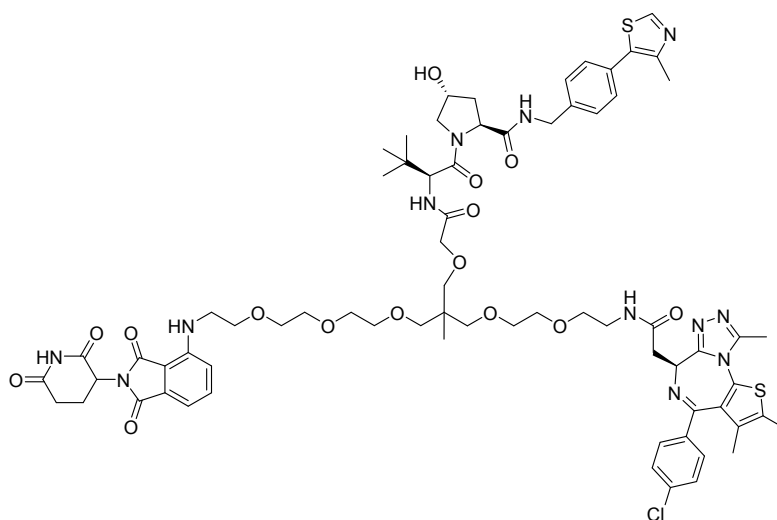
<sup>a</sup> Unable to produce a meaningful S.E. due to an incomplete curve for one independent repeat. <sup>b</sup> S.E. unavailable due to N=1 repeat.

# HPLC/HRMS Traces for Compounds 1, 2, 23 – 32, 86, 93 – 95 and 97.

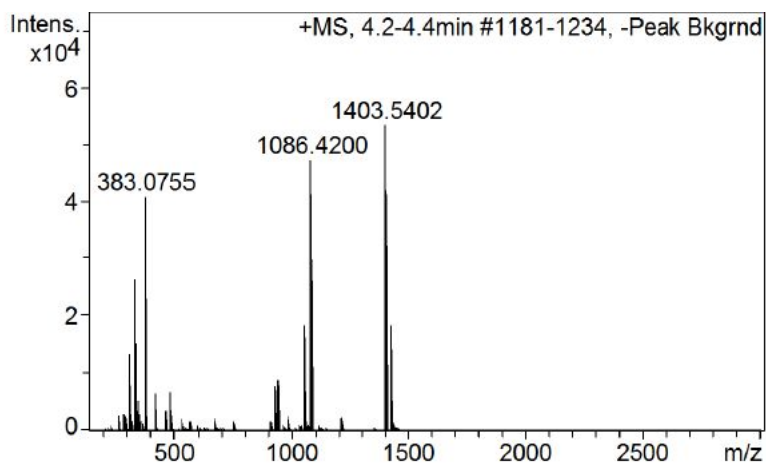
## MN666 (1)



MN675 (2)



#	RT [min]	Area Frac. %	Chromatogram
1	4.1	9.80	BPC 149.0000-3000.0000 +, Masses excluded
2	4.2	100.00	UV Chromatogram, 254 nm
3	4.3	90.20	BPC 149.0000-3000.0000 +, Masses excluded

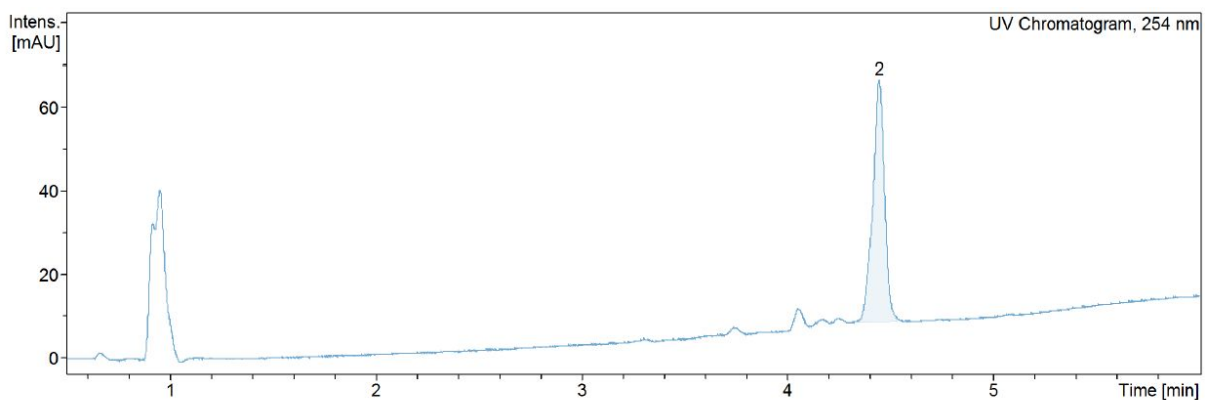
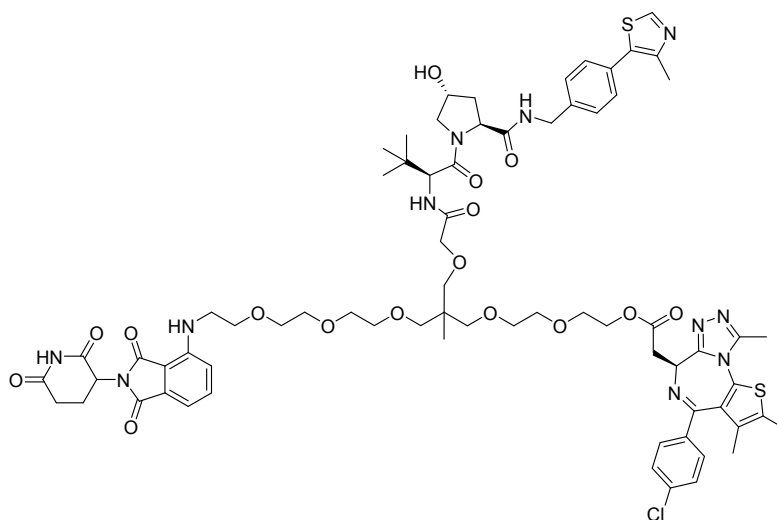




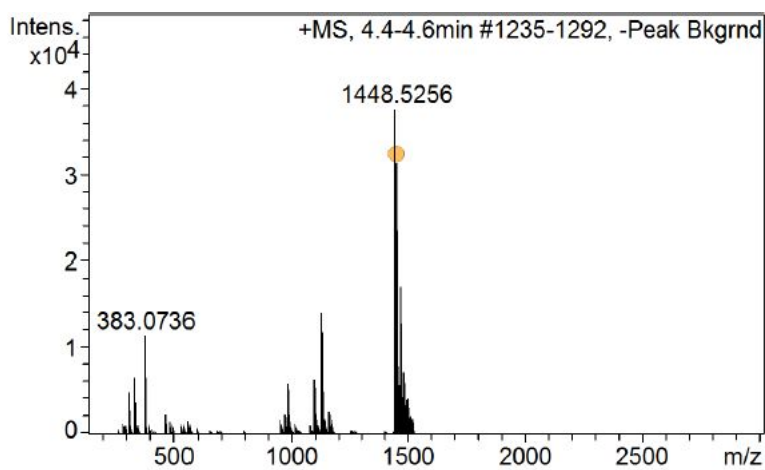




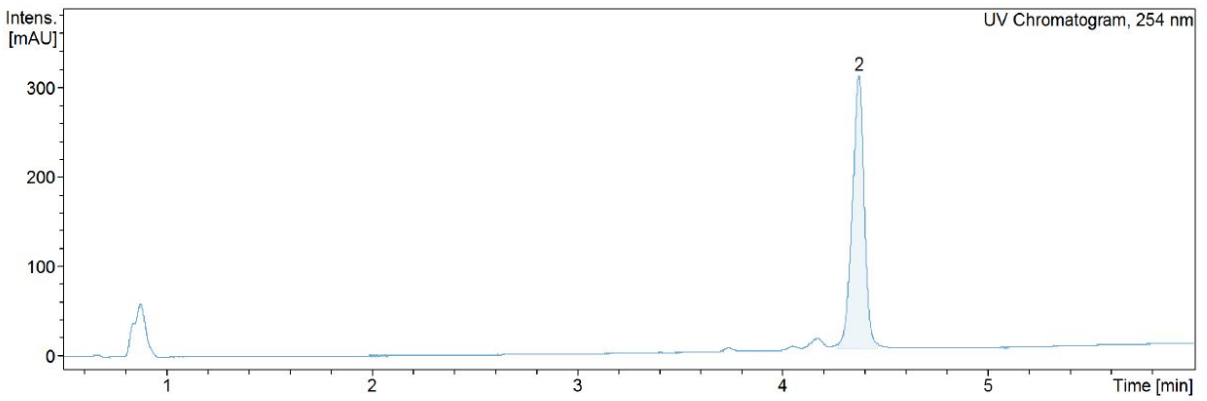
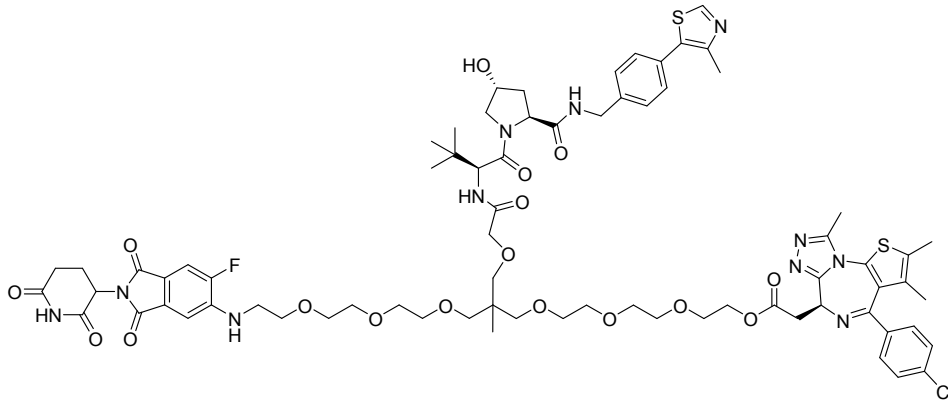
AB3064 (25)



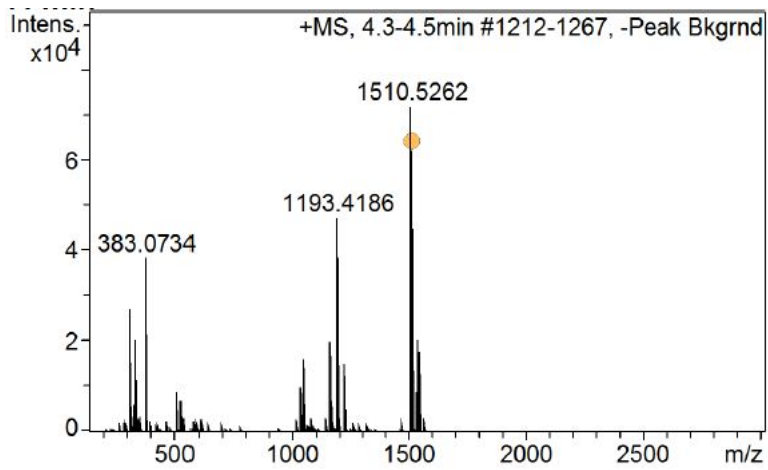
#	RT [min]	Area Frac. %	Chromatogram
1	4.1	27.74	BPC 149.0000-3001.0000 +, Masses excluded
2	4.4	100.00	UV Chromatogram, 254 nm
3	4.5	72.26	BPC 149.0000-3001.0000 +, Masses excluded



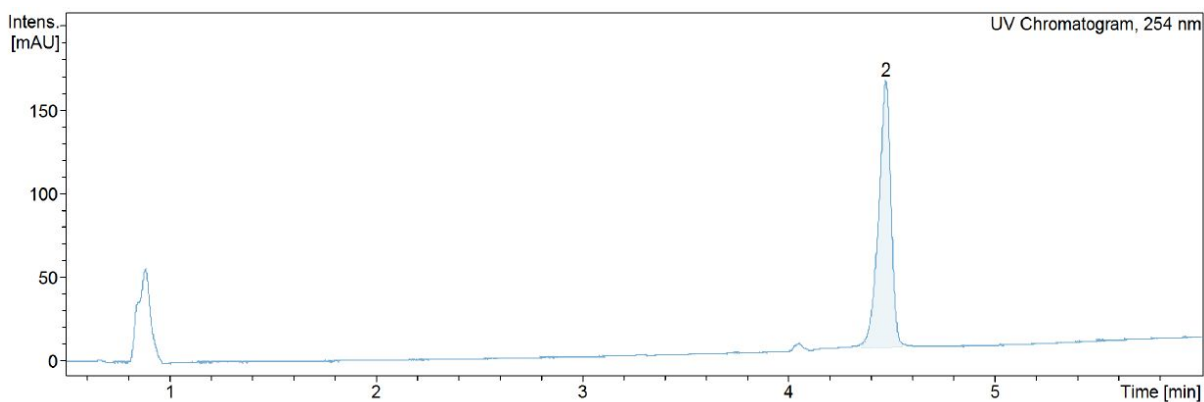
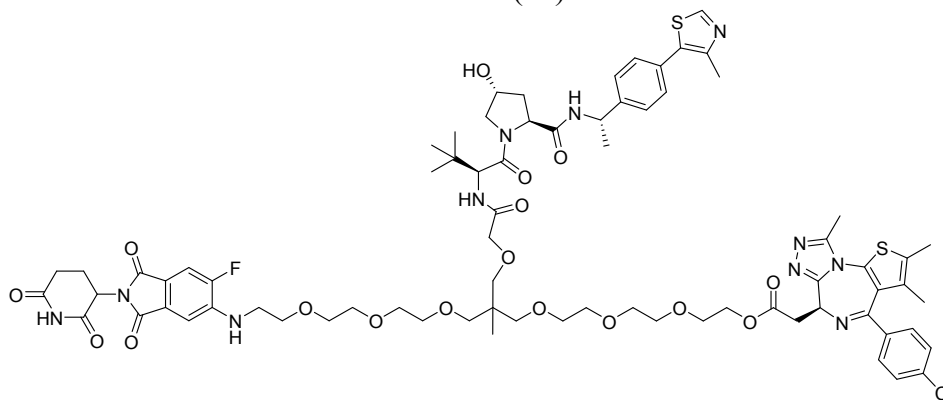
AB3063 (26)



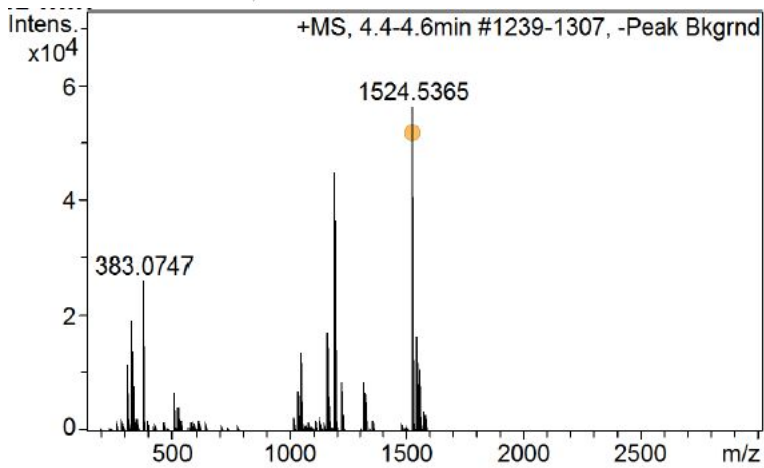
#	RT [min]	Area Frac. %	Chromatogram
1	4.1	10.77	BPC 149.0000-3001.0000 +, Masses excluded
2	4.4	100.00	UV Chromatogram, 254 nm
3	4.4	89.23	BPC 149.0000-3001.0000 +, Masses excluded



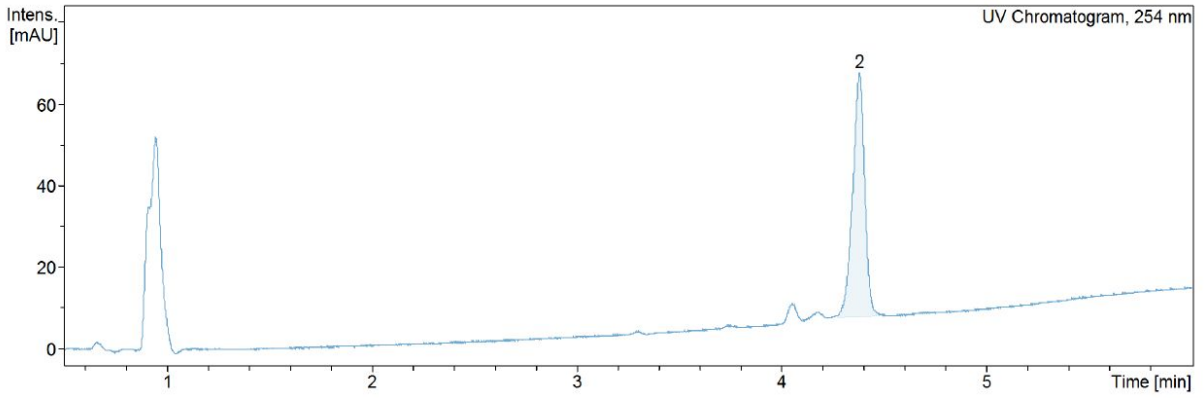
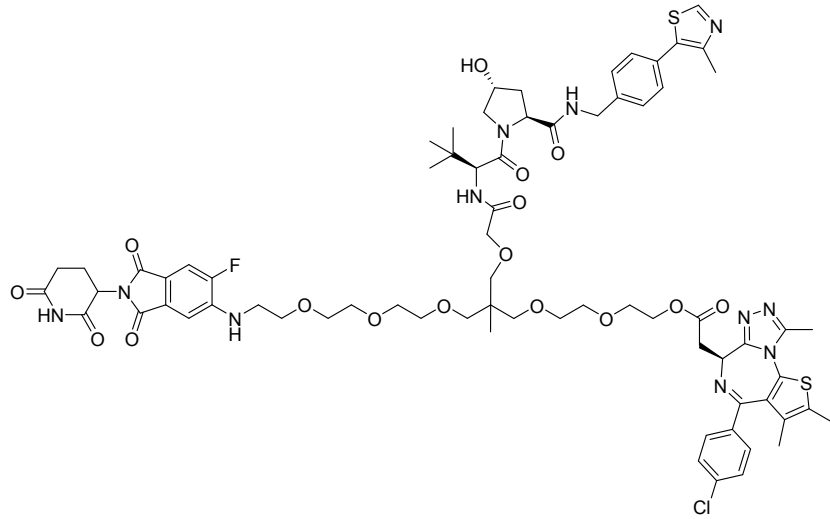
AB3067 (27)



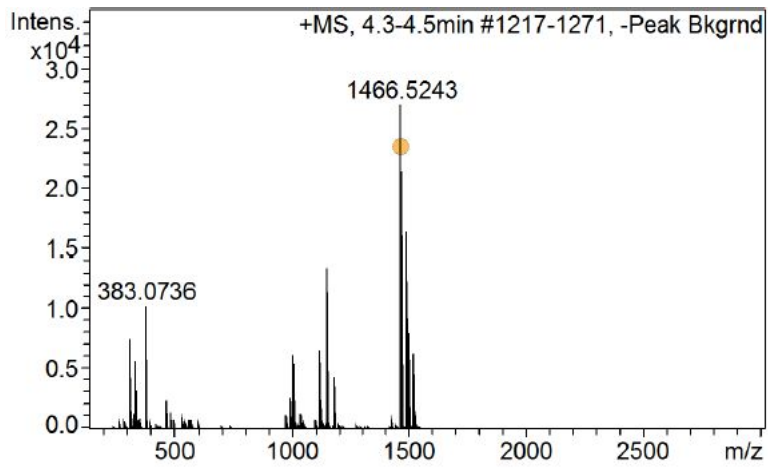
#	RT [min]	Area Frac. %	Chromatogram
1	4.1	16.07	BPC 149.0000-3001.0000 +, Masses excluded
2	4.5	100.00	UV Chromatogram, 254 nm
3	4.5	83.93	BPC 149.0000-3001.0000 +, Masses excluded



AB3065 (28)

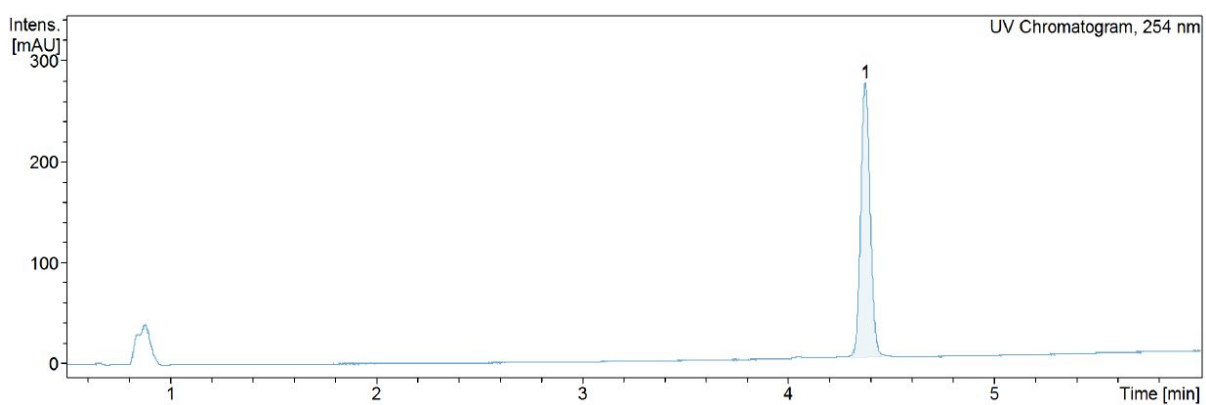
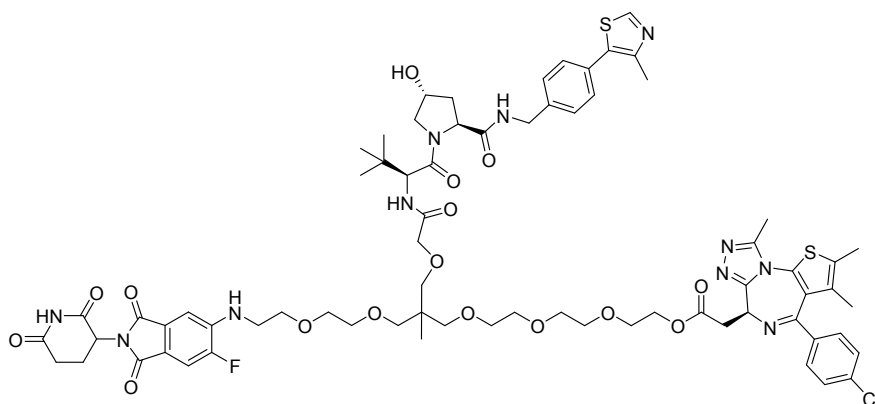


#	RT [min]	Area Frac. %	Chromatogram
1	4.1	29.94	BPC 149.0000-3001.0000 +, Masses excluded
2	4.4	100.00	UV Chromatogram, 254 nm
3	4.4	70.06	BPC 149.0000-3001.0000 +, Masses excluded

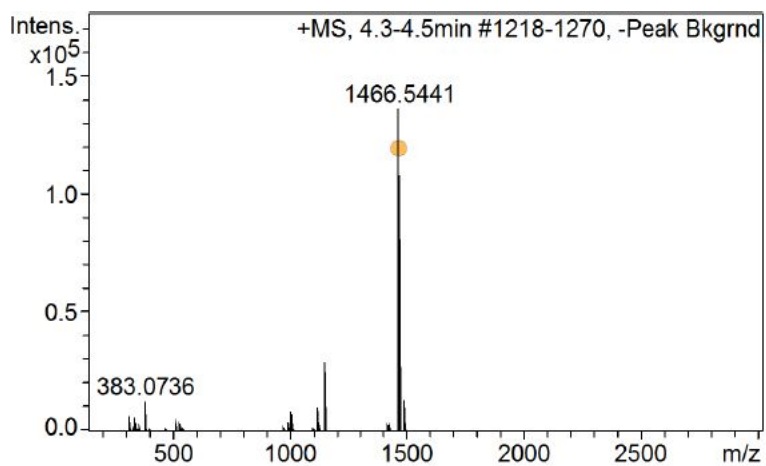




# AB3126 (29)



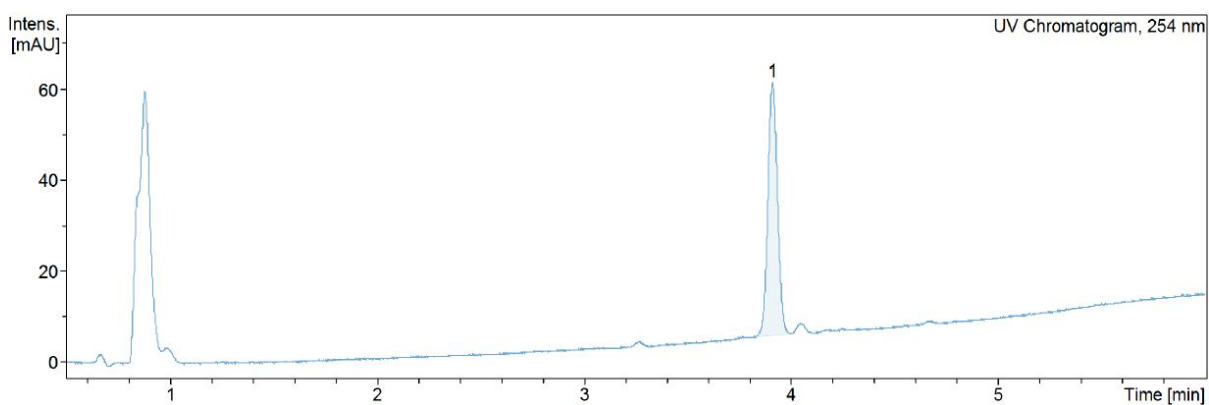
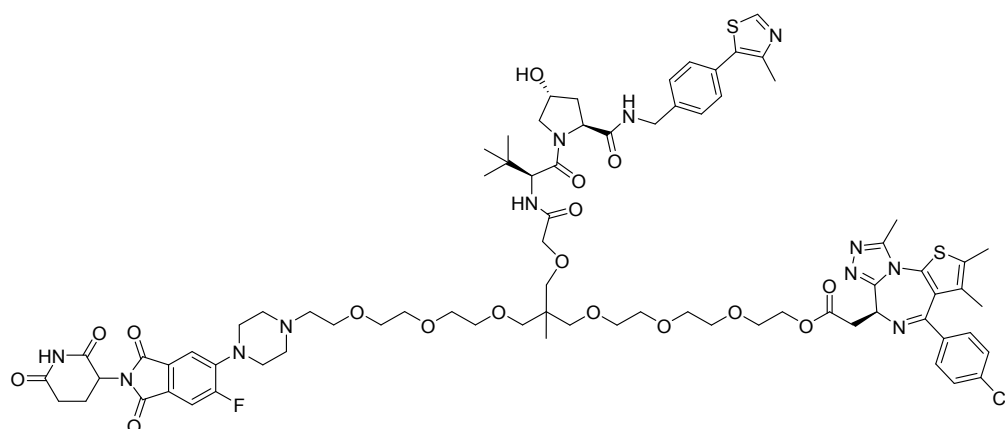
#	RT [min]	Area Frac. %	Chromatogram
1	4.4	100.00	UV Chromatogram, 254 nm
2	4.4	100.00	BPC 150.0000-3001.0000 +, Masses excluded



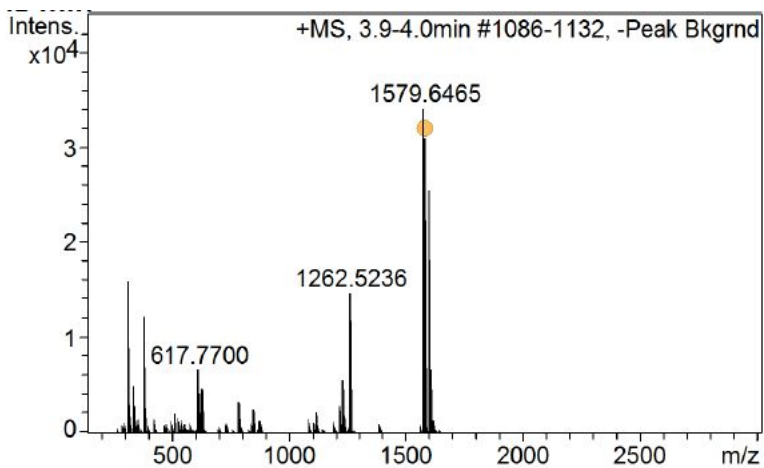




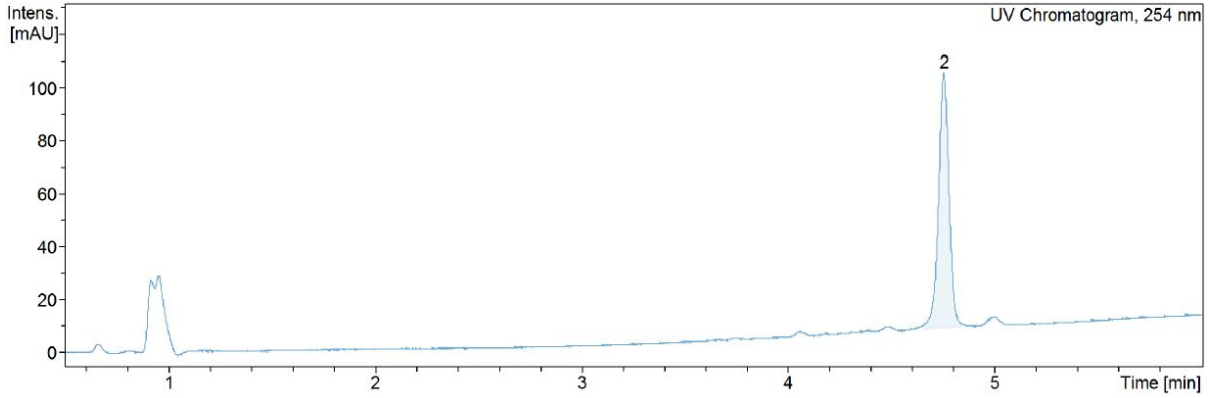
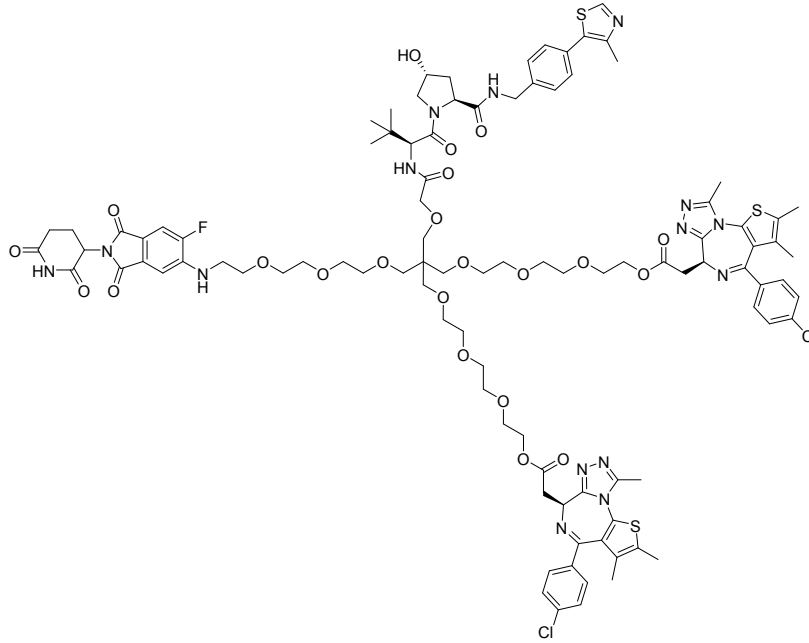
AB3030 (32)



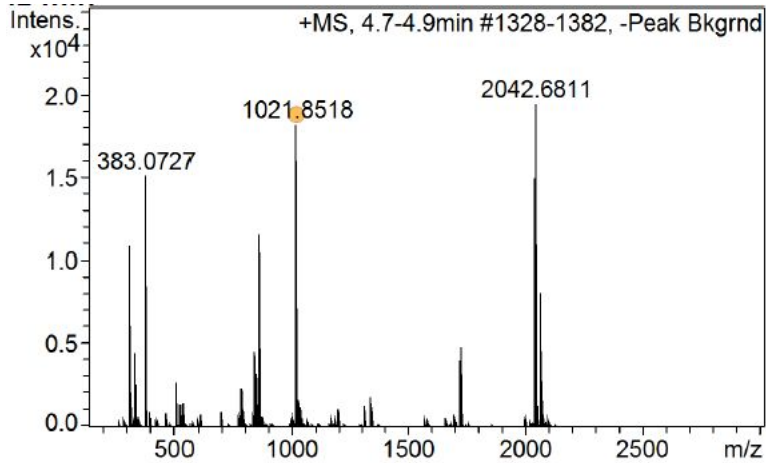
#	RT [min]	Area Frac. %	Chromatogram
1	3.9	100.00	UV Chromatogram, 254 nm



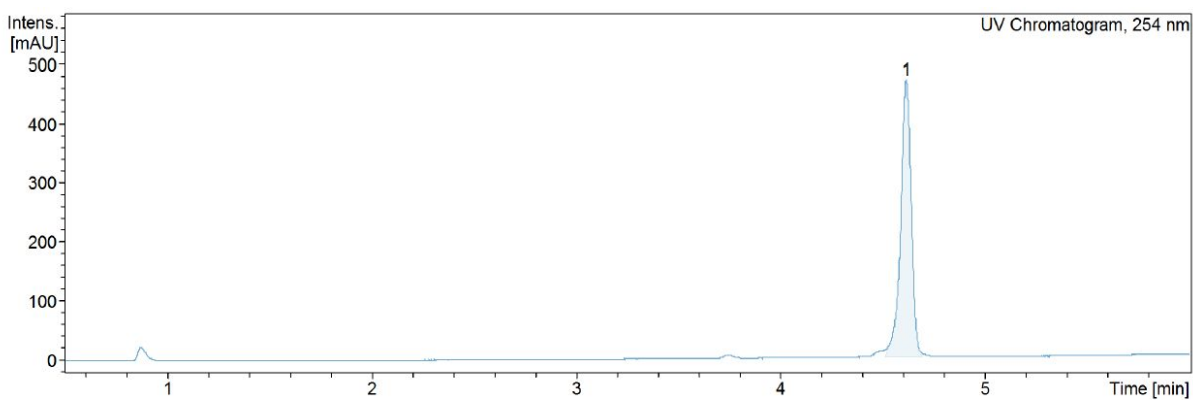
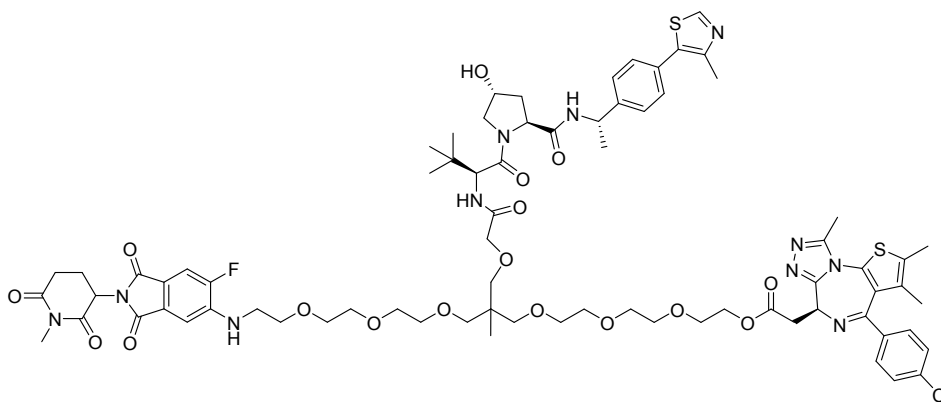
AB3124 (86)



#	RT [min]	Area Frac. %	Chromatogram
1	4.1	17.02	BPC 150.0000-3001.0000 +, Masses excluded
2	4.8	100.00	UV Chromatogram, 254 nm
3	4.8	82.98	BPC 150.0000-3001.0000 +, Masses excluded



*neg*-AB3067 (**93**)



#	RT [min]	Area Frac. %	Chromatogram
1	4.6	100.00	UV Chromatogram, 254 nm

