Supplementary Information

Design and development of stapled transmembrane peptides that disrupt the activity of G-protein coupled receptor oligomers

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Running Title: *Stapled TM peptides modulate GPCR oligomers*

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Keywords: G protein-coupled receptor (GPCR), cannabinoid receptor type 1 (CB_1), dimerization cell-penetrating peptide (CPP), peptide chemical synthesis, serotonin receptor type 2A ($5HT_{2A}$), NanoLuc binary technology (NanoBiT), hydrocarbon stapling, transmembrane peptide, bimolecular fluorescence complementation

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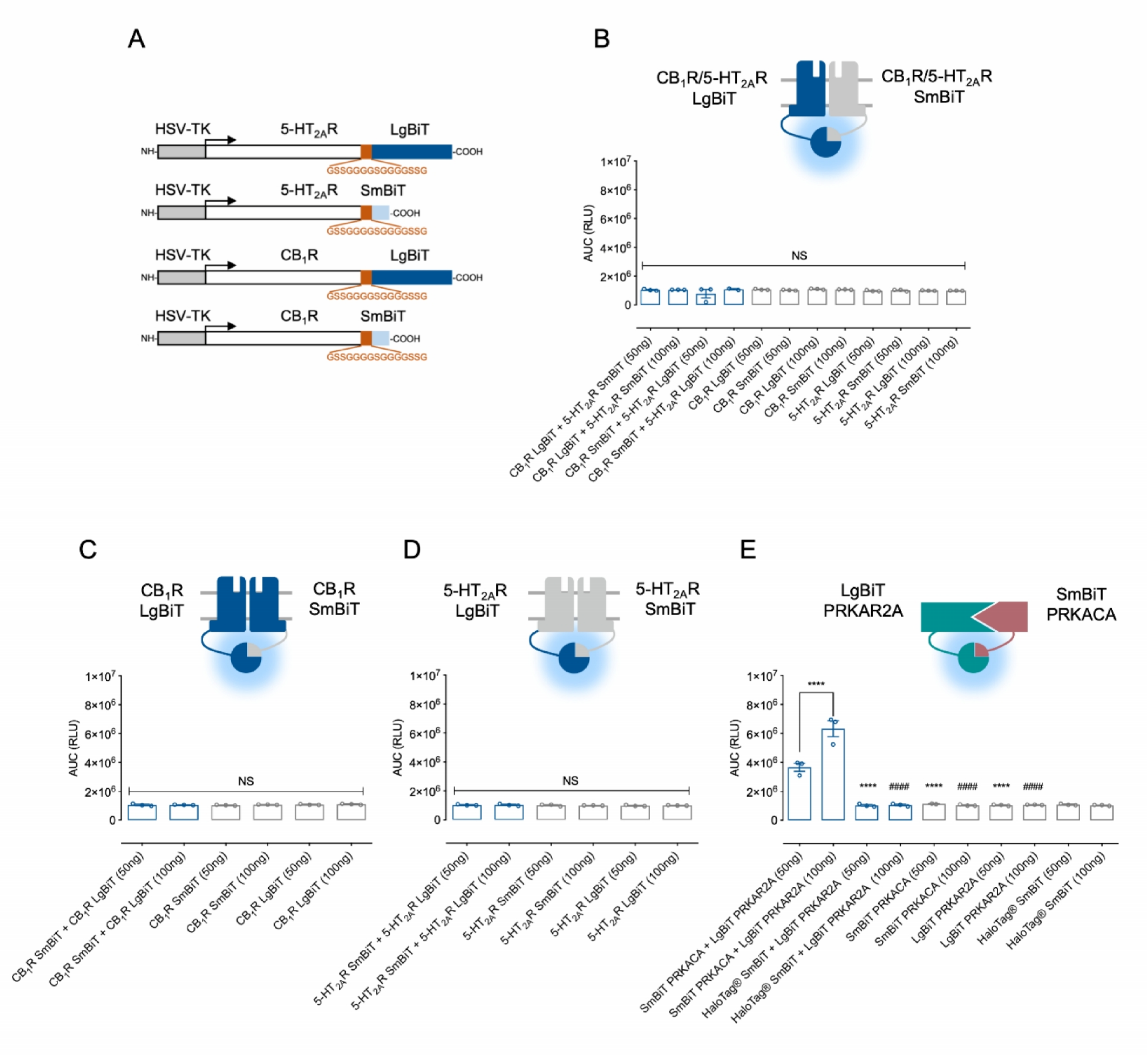
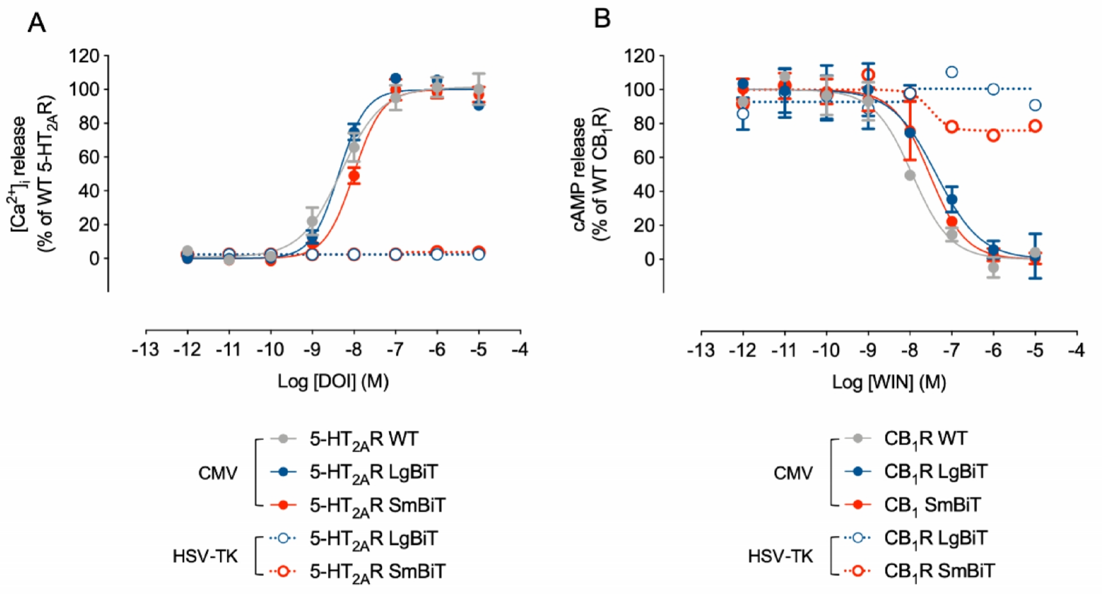
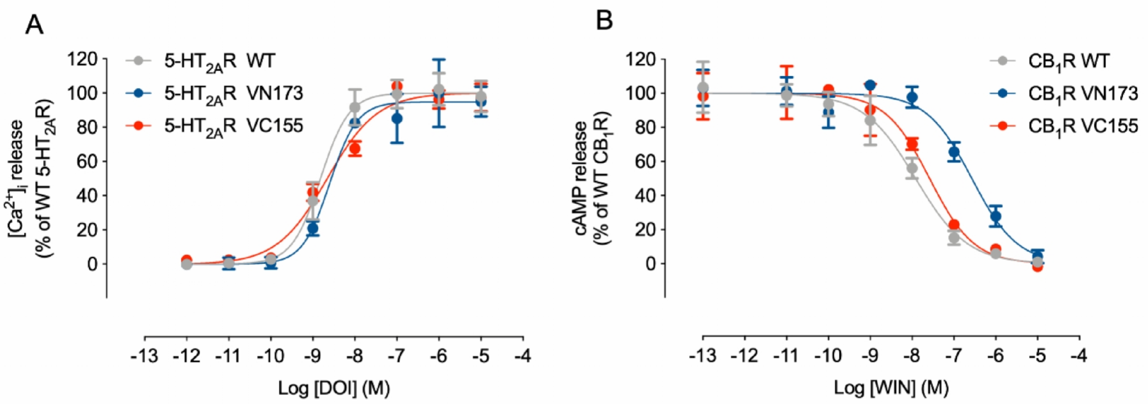


Supplementary figure 1. Conformational screening of the optimal orientation for CB_1, $5\text{-}HT_{2A}$ and $5\text{-}HT_{2A}\text{-}CB_1$ receptor oligomers. HSV-TK promoter studies. (A) Schematic representation of the NanoLuc fusion proteins. HEK-293 cells were transiently transfected with all possible orientations of LgBiT and SmBiT C-terminal fusions at two different DNA ratios (50 or 100 ng receptor/well) to assess $5\text{-}HT_{2A}R\text{-}CB_1R$ heteromers **(B)** or CB_1R **(C)** and $5\text{-}HT_{2A}R$ **(D)** homomers. In **(E)**, the SmBiT-PRKACA and LgBiT-PRKAR2A positive interacting control was transfected under the same conditions. The LgBiT-PRKAR2A plus HaloTag®-SmBiT combination was used as a non-interacting negative control. Grey bars represent the RLU of each individual construct expressed alone. Data are mean AUC (RLU) ± SEM (n = 3). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests showing significant effects over SmBiT PRKACA + LgBiT PRKAR2A (50 ng) (****$p \leq 0.0001$) or over SmBiT PRKACA + LgBiT PRKAR2A (100 ng) (####$p \leq 0.0001$). HSV-TK, herpes simplex virus thymidine kinase gene promoter; NS, non-statistical significant.



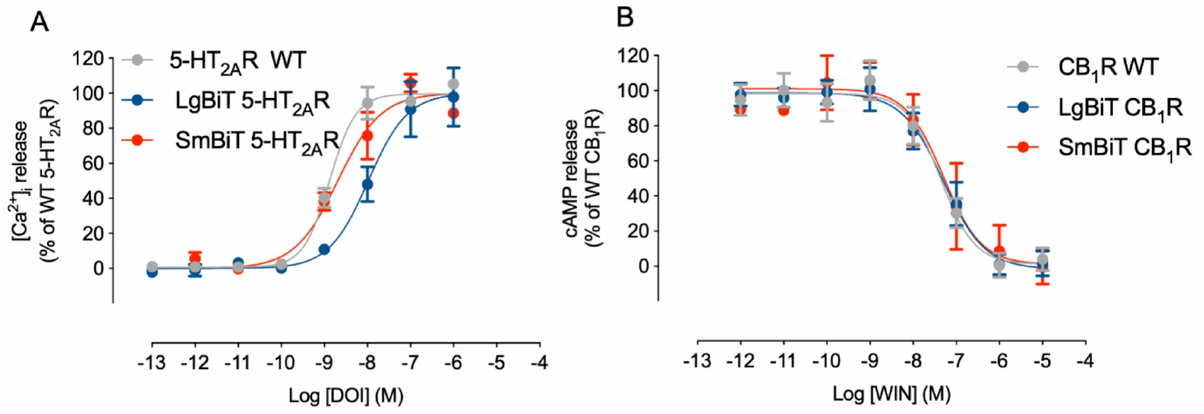
	CMV promoter	**HSV-TK promoter**
5-HT$_{2A}$R WT	8.31 ± 0.13	-
5-HT$_{2A}$R LgBiT	8.37 ± 0.06	N.D.
5-HT$_{2A}$R SmBiT	8.01 ± 0.06	N.D.
CB$_1$R WT	7.95 ± 0.14	-
CB$_1$R LgBiT	7.37 ± 0.16	N.D.
CB$_1$R SmBiT	7.52 ± 0.09	7.51 ± 0.35

Supplementary figure 2. Functional validation of NanoBiT C-terminal fusions. In **(A)**, dose-response intracellular Ca^{2+} release curves for 5-HT$_{2A}$R constructs. In **(B)**, forskolin-induced (7.5μM) cAMP release inhibition dose-response curves for CB$_1$R. Data are mean ± SD (n≥2) percentage of activation normalised to each wild type receptor maximal response. The bottom table represents the mean pEC$_{50}$/pIC50 ± SD (n≥2). HSV-TK, herpes simplex virus thymidine kinase gene promoter; CMV, human cytomegalovirus immediate-early promoter. ND, non-determined, indicates non-convergent curve fits.



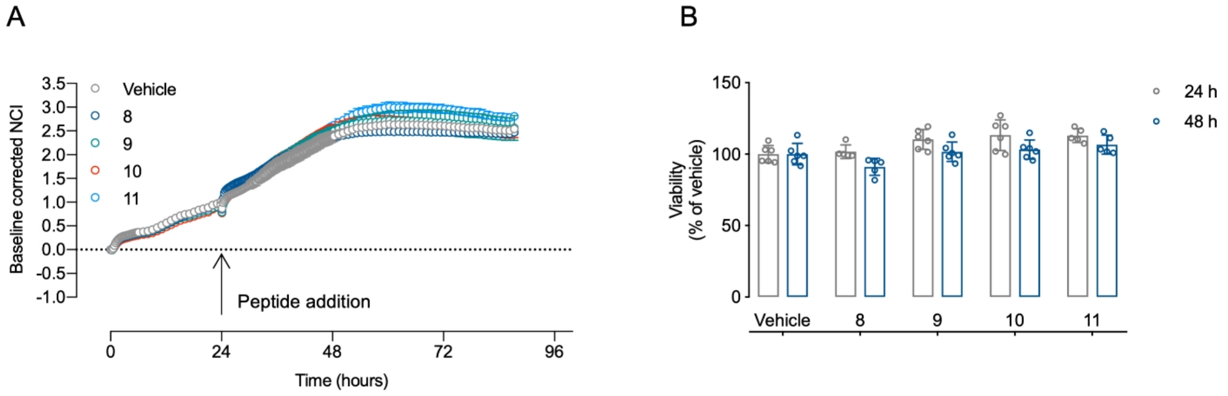
	pEC/IC$_{50}$
5-HT_{2A}R WT	8.82 ± 0.11
5-HT_{2A}R VN173	8.60 ± 0.16
5-HT_{2A}R VC155	8.66 ± 0.13
CB_1R WT	7.92 ± 0.13
CB_1R VN173	6.57 ± 0.16**
CB_1R VC155	7.58 ± 0.20

Supplementary figure 3. Functional validation of split Venus YFP C-terminal fusions. In **(A)**, dose-response intracellular Ca^{2+} release curves for 5-HT_{2A}R constructs. In **(B)**, forskolin-induced (7.5µM) cAMP release inhibition dose-response curves for CB_1R. Data are mean ± SD (n≥2) percentage of activation normalised to each wild type receptor maximal response. The bottom table represents the mean pEC_{50}/pIC50 ± SD (n≥2). Statistical significance was evaluated by one-way ANOVA followed by Bonferroni post hoc tests indicating significant differences over its respective WT receptor (**p ≤0.01).



	pEC/IC$_{50}$
5-HT$_{2A}$R WT	8.88 ± 0.09
LgBiT 5-HT$_{2A}$R	7.98 ± 0.16*
SmBiT 5-HT$_{2A}$R	8.69 ± 0.16
CB$_1$R WT	7.36 ± 0.18
LgBiT CB$_1$R	7.30 ± 0.15
SmBiT CB$_1$R	7.30 ± 0.17

Supplementary figure 4. Functional validation of NanoBiT N-terminal fusions. In **(A)**, dose-response intracellular Ca^{2+} release curves for 5-HT$_{2A}$R constructs. In **(B)**, forskolin-induced (7.5μM) cAMP release inhibition dose-response curves for CB$_1$R. Data are mean ± SD (n≥2) percentage of activation normalised to each wild type receptor maximal response. The bottom table represents the mean pEC$_{50}$/pIC50 ± SD (n≥2). Statistical significance was evaluated by one-way ANOVA followed by Bonferroni post hoc tests indicating significant differences over its respective WT receptor (*p ≤0.05).



Supplementary figure 5. Label-free real-time evaluation of peptide toxicity. (A) Representative real-time iCELLigence RTCA impedance traces monitoring cell growth after peptide (4 µM) addition. Data are shown as the mean baseline corrected NCI ± SD (n=2-3). In **(B)**, bar graphs plotting cell index values 24- and 48-hours post-peptide addition. Data are mean ± SEM (n≥5) normalised to vehicle-treated cells. Statistical significance was evaluated by two-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests showing no statistically significant reduction in viability.

Supplementary table 1.

<table>
<tr><th>Plasmid name</th><th>Backbone</th><th colspan="2">Backbone primers (5’ 3’)</th><th>Insert</th><th colspan="2">Insert primers (5’ 3’)</th></tr>
<tr><td rowspan="2">HSV-TK-3xHA-5-HT$_{2A}$R-LgBiT</td><td rowspan="2">pBiT1.1-C [TK/LgBiT]</td><td>FW</td><td>CTGTGTGGGCTCGAGCGGTGGTGGC</td><td rowspan="2">pcDNA3.1(+)-3xHA-5-HT$_{2A}$R</td><td>FW</td><td>GGAATTCTGGTACCACCATGTACCCATACGA</td></tr>
<tr><td>RV</td><td>GGTGGTACCAGAATTCCCCTGAGCTCC</td><td>RV</td><td>CTCGAGCCCACACAGCTCACCTTTTCATTCACTCC</td></tr>
<tr><td colspan="7">MYPYDVPDYAYPYDVPDYAYPYDVPDYADDILCEENTSLSSTTNSLMQLNDDTRLYSNDFNSGEANTSDAFNWTVDSENRTNLSCEGCLSPSCLSLLHLQEKNWSALLTAVVIILTIAGNILVIMAVSLEKKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAVWIYLDVLFSTASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDDSKVFKEGSCLLADDNFVLIGSFVSFFIPLTIMVITYFLTIKSLQKEATLCVSDLGTRAKLASFSFLPQSSLSSEKLFQRSIHREPGSYTGRRTMQSISNEQKACKVLGIVFFLFVVMWCPFFITNIMAVICKESCNEDVIGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFSRYIQCQYKENKKPLQLILVNTIPALAYKSSQLQMGQKKNSKQDAKTTDNDCSMVALGKQHSEEASKDNSDGVNEKVSCVGSSGGGGSGGGGSSGVFTLEDFVGDWEQTAAYNLDQVLEQGGVSSLLQNLAVSVTPIQRIVRSGENALKIDIHVIIPYEGLSADQMAQIEEVFKVVYPVDDHHFKVILPYGTLVIDGVTPNMLNYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLITPDGSMLFRVTINS</td></tr>
<tr><td rowspan="2">HSV-TK-3xHA-5-HT$_{2A}$R-SmBiT</td><td rowspan="2">pBiT2.1-C [TK/SmBiT]</td><td>FW</td><td>CTGTGTGGGCTCGAGCGGTGGTGGC</td><td rowspan="2">pcDNA3.1(+)-3xHA-5-HT$_{2A}$R</td><td>FW</td><td>GGAATTCTGGTACCACCATGTACCCATACGA</td></tr>
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<tr><td colspan="7">MYPYDVPDYAYPYDVPDYAYPYDVPDYADDILCEENTSLSSTTNSLMQLNDDTRLYSNDFNSGEANTSDAFNWTVDSENRTNLSCEGCLSPSCLSLLHLQEKNWSALLTAVVIILTIAGNILVIMAVSLEKKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAVWIYLDVLFSTASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDDSKVFKEGSCLLADDNFVLIGSFVSFFIPLTIMVITYFLTIKSLQKEATLCVSDLGTRAKLASFSFLPQSSLSSEKLFQRSIHREPGSYTGRRTMQSISNEQKACKVLGIVFFLFVVMWCPFFITNIMAVICKESCNEDVIGALLNVFVWIGYLSSAVNPLVYTLFNKTYRSAFSRYIQCQYKENKKPLQLILVNTIPALAYKSSQLQMGQKKNSKQDAKTTDNDCSMVALGKQHSEEASKDNSDGVNEKVSCVGSSGGGGSGGGGSSGVTGYRLFEEIL</td></tr>
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<tr><td>RV</td><td>GTACATGGTGGTACCAGAATTCCCCTGAGCTCC</td><td>RV</td><td>ACCACCGCTCGAGCCCAGAGCCTCGGCAGA</td></tr>
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</table>

CMV-3xHA-5-HT$_{2A}$R-LgBiT	pcDNA3.1(+)-3xHA-5-HT$_{2A}$R	FW	GTAACCATCAACAGCTGACTCGAGTCTAGAGGG	pBiT1.1-C [TK/LgBiT]	FW	AAGGTGAGCTGTGTGGGCTCGAGCGGTGGT
		RV	ACCACCGCTCGAGCCCACACAGCTCACCTTTTC		RV	TCTAGACTCGAGTCAGCTGTTGATGGTTACTCGG
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CMV-3xHA-5-HT$_{2A}$R-SmBiT	pcDNA3.1(+)-3xHA-5-HT$_{2A}$R	FW	TTCGAGGAGATTCTGTGACTCGAGTCTAGAGGG	pBiT2.1-C [TK/SmBiT]	FW	AAGGTGAGCTGTGTGGGCTCGAGCGGTGGT
		RV	ACCACCGCTCGAGCCCACACAGCTCACCTTTTC		RV	TCTAGACTCGAGTCACAGAATCTCCTCGAACAGC
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CMV-3xHA-CB$_1$R-LgBiT	pcDNA3.1(+)-3xHA-CB$_1$R	FW	GTAACCATCAACAGCTGACTCGAGTCTAGAGGG	pBiT1.1-C [TK/LgBiT]	FW	TCTGCCGAGGCTCTGGGCTCGAGCGGTGGT
		RV	ACCACCGCTCGAGCCCAGAGCCTCGGCAGA		RV	TCTAGACTCGAGTCAGCTGTTGATGGTTACTCGG
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CMV-3xHA-CB$_1$R-SmBiT	pcDNA3.1(+)-3xHA-CB$_1$R	FW	TTCGAGGAGATTCTGTGACTCGAGTCTAGAGGG	pBiT2.1-C [TK/SmBiT]	FW	TCTGCCGAGGCTCTGGGCTCGAGCGGTGGT
		RV	ACCACCGCTCGAGCCCAGAGCCTCGGCAGA		RV	TCTAGACTCGAGTCACAGAATCTCCTCGAACAGC
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CMV-LgBiT-5-HT$_{2A}$R	pcDNA3.1(+)-3xHA-5-HT$_{2A}$R	FW	GGAGGCTCGAGCGGTGATATTCTTTGTGAAGAAATACTT	pBiT1.1-N [TK/LgBiT]	FW	AAGCTTGGTACCACCATGGTCTTCACACTCGAAG
		RV	GAGTGTGAAGACCATGG		RV	TTCACAAAGAATATCACCGCT

			TGGTACCAAGCTTAAG			CGAGCCTCC
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CMV-SmBiT-5-HT$_{2A}$R	pcDNA3.1(+)-3xHA-5-HT$_{2A}$R	FW	GGAGGCTCGAGCGGTGATATTCTTTGTGAAGAAAATACTT	pBiT2.1-N [TK/SmBiT]	FW	AAGCTTGGTACCACCATGGTGACCGGCTACC
		RV	GTAGCCGGTCACCATGGTGGTACCAAGCTTAAG		RV	TTCACAAAGAATATCACCGCTCGAGCCTCC
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CMV-LgBiT-CB$_1$R	pcDNA3.1(+)-3xHA-CB$_1$R	FW	GGAGGCTCGAGCGGTAAGTCGATCCTAGATGGCC	pBiT1.1-N [TK/LgBiT]	FW	AAGCTTGGTACCACCATGGTCTTCACACTCGAAG
		RV	GAGTGTGAAGACCATGGTGGTACCAAGCTTAAGT		RV	ATCTAGGATCGACTTACCGCTCGAGCCTCC
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CMV-SmBiT-CB$_1$R	pcDNA3.1(+)-3xHA-CB$_1$R	FW	GGAGGCTCGAGCGGTAAGTCGATCCTAGATGGCC	pBiT2.1-N [TK/SmBiT]	FW	AAGCTTGGTACCACCATGGTGACCGGCTACC
		RV	GTAGCCGGTCACCATGGTGGTACCAAGCTTAAGT		RV	ATCTAGGATCGACTTACCGCTCGAGCCTCC
MVTGYRLFEEILGSSGGGGSGGGGSSGKSILDGLADTTFRTITTDLLYVGSNDIQYEDIKGDMASKLGYFPQKFPLTSFRGSPFQEKMTAGDNPQLVPADQVNITEFYNKSLSSFKENEENIQCGENFMDIECFMVLNPSQQLAIAVLSLTLGTFTVLENLLVLCVILHSRSLRCRPSYHFIGSLAVADLLGSVIFVYSFIDFHVFHRKDSRNVFLFKLGGVTASFTASVGSLFLTAIDRYISIHRPLAYKRIVTRPKAVVAFCLMWTIAIVIAVLPLLGWNCEKLQSVCSDIFPHIDETYLMFWIGVTSVLLLFIVYAYMYILWKAHSHAVRMIQRGTQKSIIIHTSEDGKVQVTRPDQARMDIRLAKTLVLILVVLIICWGPLLAIMVYDVFGKMNKLIKTVFAFCSMLCLLNSTVNPIIYALRSKDLRHAFRSMFPSCEGTAQPLDNSMGDSDCLHKHANNAASVHRAAESCIKSTVKIAKVTMSVSTDTSAEAL						
CMV-3xHA-5-HT$_{2A}$R-VN173	CMV-3xHA-5-HT$_{2A}$R-SmBiT	FW	CACAACATCGAGTAGCTCGAGTCTAGAGGGCC	pBiFC-bJunVN173	FW	GGAGGGTCGTCAGGTATGGTGAGCAAGGGCG
		RV	GCCCTTGCTCACCATACCTGACGACCCTCC		RV	CCCTCTAGACTCGAGCTACTCGATGTTGTGGCG
MYPYDVPDYAYPYDVPDYAYPYDVPDYADILCEENTSLSSTTNSLMQLNDDTRLYSNDFNSGEANTSDAFNWTVDSENRTNLSCEGCLSPSCLSLLHLQEKNWSALLTAVVIILTIAGNILVIMAVSLEKKLQNATNYFLMSLAIADMLLGFLVMPVSMLTILYGYRWPLPSKLCAVWIYLDVLFSTASIMHLCAISLDRYVAIQNPIHHSRFNSRTKAFLKIIAVWTISVGISMPIPVFGLQDDSKVFKEGSCLLADDNFVLIGSFVSFFIPLTIMVITYFLTIKSLQKEATLCVSDLGTRAKLASFSFLPQSSLSSEKLFQRSIHREPGSYTGRRTMQSISNEQKACKVLGIVFFLFV						

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<tr><td rowspan="2">CMV-3xHA-5-HT$_{2A}$R-VC155</td><td rowspan="2">CMV-3xHA-5-HT$_{2A}$R-SmBiT</td><td>FW</td><td>GAGCTGTACAAGTAACTCGAGTCTAGAGGGCC</td><td rowspan="2">pBiFC-bFosVC155</td><td>FW</td><td>GGAGGGTCGTCAGGTGACAAGCAGAAGAACGGC</td></tr>
<tr><td>RV</td><td>GTTCTTCTGCTTGTCACCTGACGACCCTCC</td><td>RV</td><td>CCCTCTAGACTCGAGTTACTTGTACAGCTCGTCC</td></tr>
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<tr><td rowspan="2">CMV-3xHA-CB$_1$R-VN173</td><td rowspan="2">CMV-3xHA-CB$_1$R-SmBiT</td><td>FW</td><td>CACAACATCGAGTAGCTCGAGTCTAGAGGGCC</td><td rowspan="2">pBiFC-bJunVN173</td><td>FW</td><td>GGAGGGTCGTCAGGTATGGTGAGCAAGGGCG</td></tr>
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<tr><td rowspan="2">CMV-3xHA-CB$_1$R-VC155</td><td rowspan="2">CMV-3xHA-CB$_1$R-SmBiT</td><td>FW</td><td>GAGCTGTACAAGTAACTCGAGTCTAGAGGGCC</td><td rowspan="2">pBiFC-bFosVC155</td><td>FW</td><td>GGAGGGTCGTCAGGTGACAAGCAGAAGAACGGC</td></tr>
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Primer sequences for Gibson cloning. The colour code corresponds to the translated sequences of: HA-Tag, 5-HT$_{2A}$R/CB$_1$R, G/S flexible Linker, LgBiT, SmBiT, VN173 Venus YFP and CV155 Venus YFP.