Supplementary Online Information for

Maximizing detergent stability and functional expression of a GPCR by exhaustive recombination and evolution.

*Karola M. Schlinkmann[1], Matthias Hillenbrand[1], Alexander Rittner[1], Madeleine Künz[1,3], Ralf Strohner[2], and Andreas Plückthun[1]**

[1] *Department of Biochemistry, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland*

[2] *Sloning group at MorphoSys AG, Lena-Christ-Strasse 48, 82152 Martinsried/Planegg, Germany*

** Corresponding author*

[3] *Present address: Institute for Biochemistry and Molecular Biology, Laboratory for Structural Biology of Infection and Inflammation, University of Hamburg, c/o DESY Bldg. 22a, Notkestrasse 85, 22607 Hamburg, Germany*

Figure S1. The StEP process [1; 2] for *in vitro* shuffling of D03 and M30/ M303. Two different GPCR templates are used for *in vitro* DNA shuffling, either D03 (containing no additional mutation) and M30 (containing 30 additional mutations) or D03 and M303 (containing 33 additional mutations). The procedure is illustrated for M30 (1). By using high numbers of very short StEP-PCR cycles (125 cycles; 6 seconds each), the flanking primers are only extended by a few nucleotides (3a - 3c) until eventually a full-length and chimeric GPCR sequence is generated (3d). By template switching within the StEP-PCR cycles, mutations from the two templates are combined into one StEP-PCR product (3d). The StEP-PCR product is then purified from an agarose gel (4) and the flanking restriction sites are digested (5) for ligation into the expression vector (6).

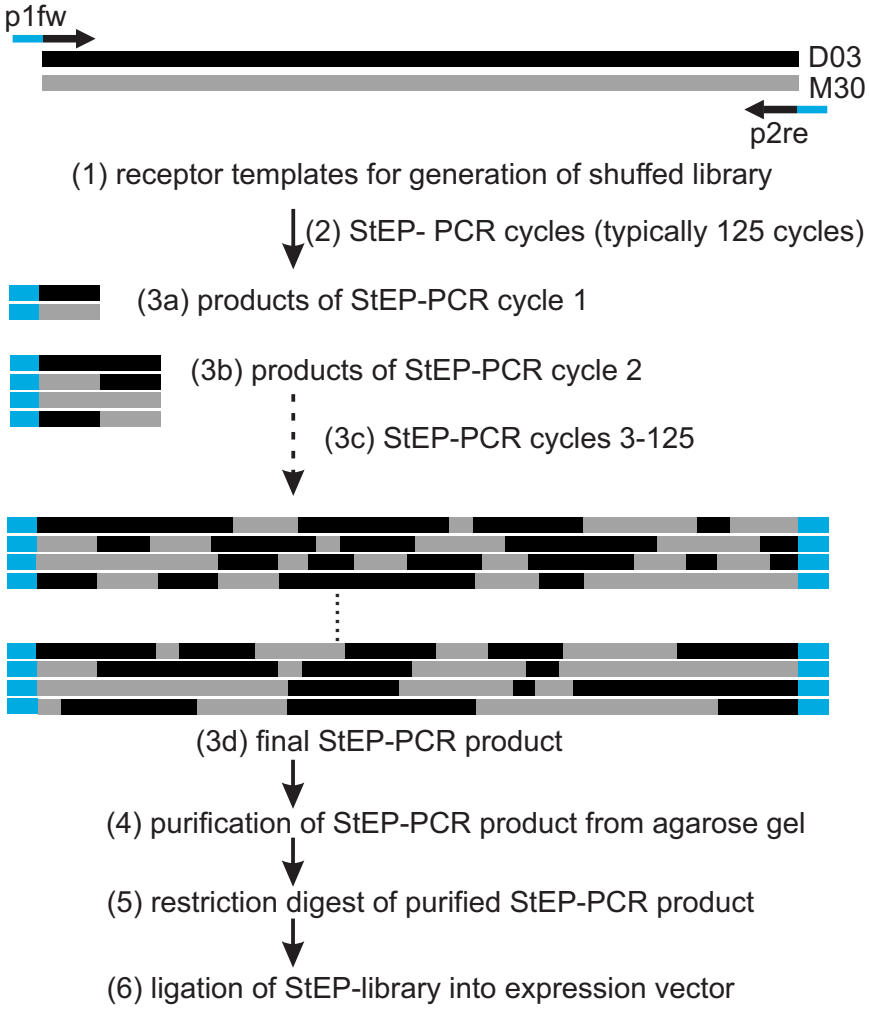
p1fw
D03
M30
p2re
(1) receptor templates for generation of shuffed library
(2) StEP- PCR cycles (typically 125 cycles)
(3a) products of StEP-PCR cycle 1
(3b) products of StEP-PCR cycle 2
(3c) StEP-PCR cycles 3-125
(3d) final StEP-PCR product
(4) purification of StEP-PCR product from agarose gel
(5) restriction digest of purified StEP-PCR product
(6) ligation of StEP-library into expression vector

Figure S1.

Figure S2. **Detergent-stability in DDM (a) and DM (b) in the ligand-free state.** Receptors were solubilized in DM, and then detergent-exchanged before incubation at elevated temperatures. Remaining agonist-binding activity was determined after incubation with 15 nM [^{3}H]-NT. Time-dependent detergent-stability is measured at 4°C in DDM (c) and DM (d). NTS1-7m was constructed according to [3].

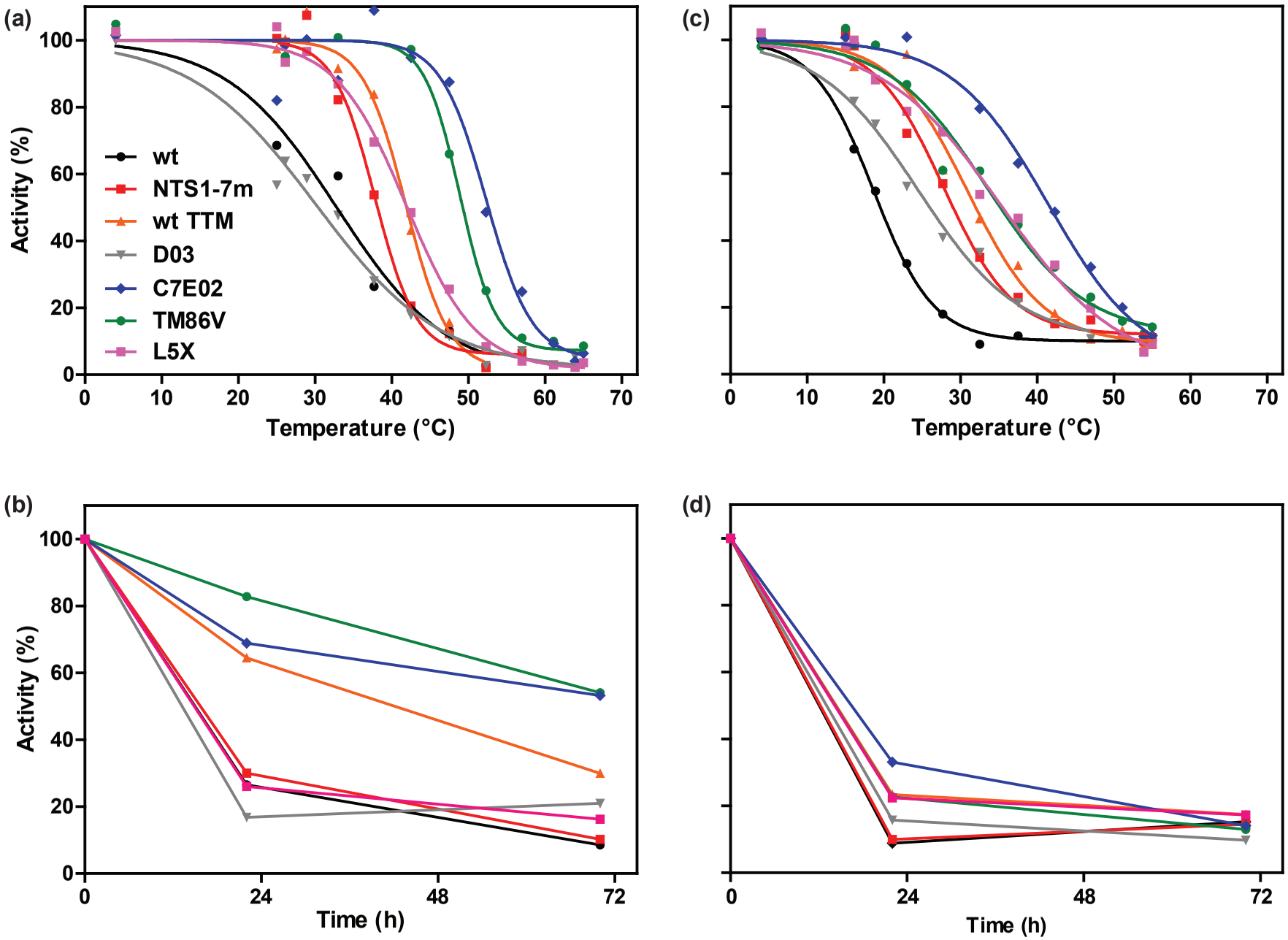
(a)
Activity (%)
Temperature (°C)
wt
NTS1-7m
wt TTM
D03
C7E02
TM86V
L5X
(c)
Temperature (°C)
(b)
Activity (%)
Time (h)
(d)
Time (h)

Figure S2.

Figure S3. Agonist-bound detergent stability of different mutants in DDM (a), DM (b), NM (c) and OG (d). Receptors were solubilized in DM, saturated with agonist [^{3}H]-neurotensin and then detergent-exchanged. Samples were incubated at elevated temperatures and the remaining agonist-binding activity was determined. NTS1-7m was constructed according to ref [3].

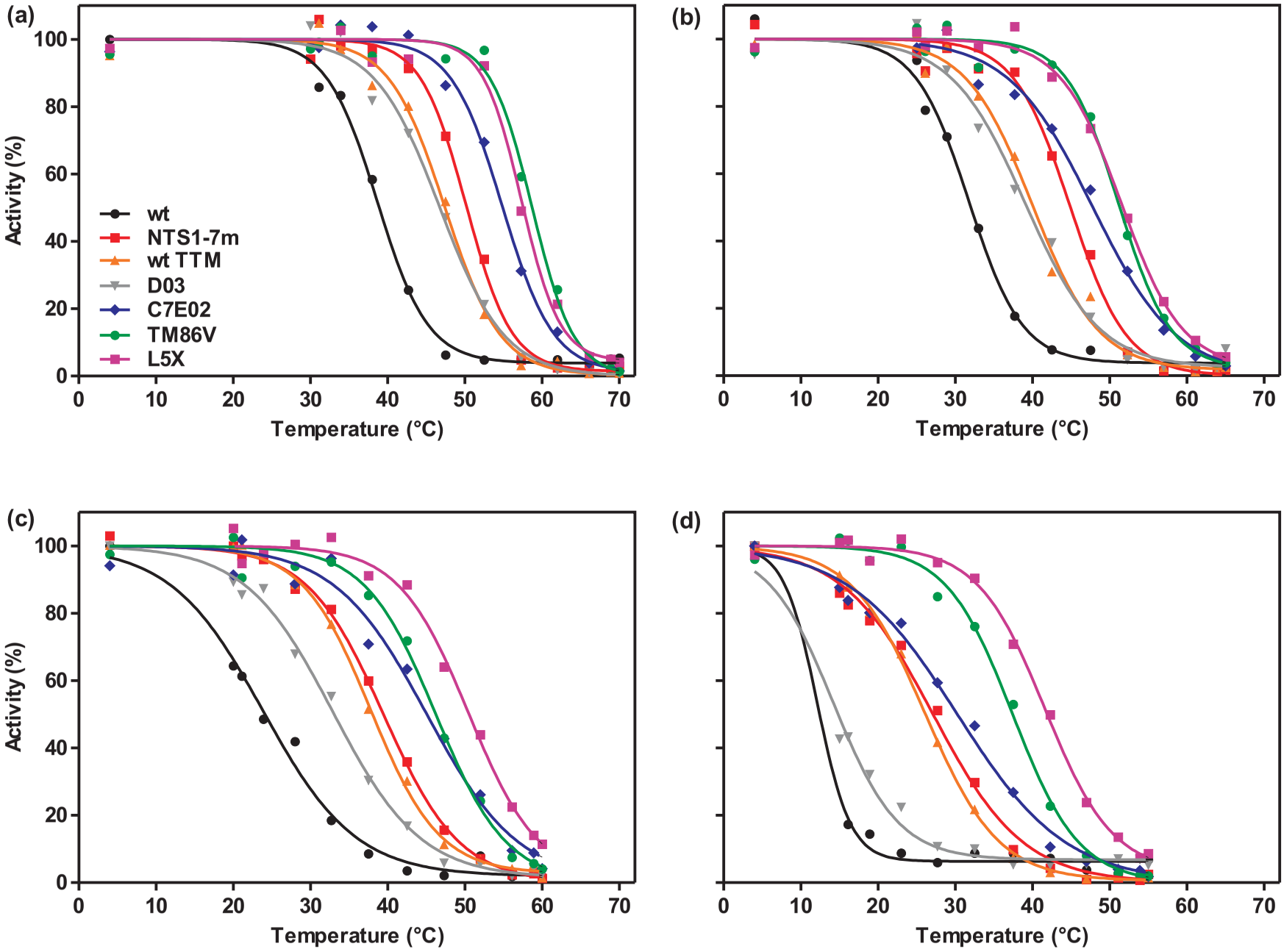
(a)
(b)
(c)
(d)
Activity (%)
Temperature (°C)
wt
NTS1-7m
wt TTM
D03
C7E02
TM86V
L5X
0
20
40
60
80
100
0
10
20
30
40
50
60
70

Figure S3.

Figure S4. **Slonomics® library design**. 33 positions of D03 are randomized and both the D03 and the shift amino acid are represented equally by their degenerate codons (yellow). All positions adjacent to a randomized position were also represented by all degenerate codons (brown). For illustration, the codons used for positions 353 to 359 are shown in detail.

rNTR1 position	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92
																			TM1																															
D03 residue	T	S	E	S	D	T	A	G	P	N	S	D	L	D	V	N	T	D	I	Y	S	K	V	L	V	T	A	I	Y	L	A	L	F	V	V	G	T	V	G	N	S	V	T	A	F	T	L	A	R	K
shift residue															I	R										S		L					L								G			L						

rNTR1 position	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142
									TM2																																									
D03 residue	K	S	L	Q	S	L	Q	S	T	V	D	Y	Y	L	G	S	L	A	L	S	D	L	L	I	L	L	L	A	M	P	V	E	L	Y	N	F	I	W	V	H	H	P	W	A	F	G	D	A	G	C
shift residue									R									L			S						F		L			D	V																	

rNTR1 position	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192
D03 residue	R	G	Y	Y	F	L	R	D	A	C	T	Y	A	T	A	L	N	V	V	S	L	S	V	E	L	Y	L	A	I	C	H	P	F	K	A	K	T	L	M	S	R	S	R	T	K	K	F	I	S	A
shift residue	K							E																						R					H															

rNTR1 position	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242
		TM4																																																	
D03 residue	I	W	L	A	S	A	L	L	A	I	P	M	L	F	T	M	G	L	Q	N	L	S	G	D	G	T	H	P	G	G	L	V	C	T	P	I	V	D	T	A	T	L	K	V	V	I	Q	V	N	T
shift residue									S	L						V																											R					L		

rNTR1 position	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292
				TM5																																														
D03 residue	F	M	S	F	L	F	P	M	L	V	I	S	I	L	N	T	V	I	A	N	K	L	T	V	M	V	H	Q	A	A	E	Q	G	R	V	C	T	V	G	T	H	N	G	L	E	H	S	T	F	N
shift residue											A							A		R	R																													

rNTR1 position	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342
D03 residue	M	T	I	E	P	G	R	V	Q	A	L	R	R	G	V	L	V	L	R	A	V	V	I	A	F	V	V	C	W	L	P	Y	H	V	R	R	L	M	F	C	Y	I	S	D	E	Q	W	T	T	F
shift residue																												L				L								V										A

rNTR1 position	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392
D03 residue	L	F	D	F	Y	H	Y	F	Y	M	L	T	N	A	L	F	Y	V	S	A	A	I	N	P	I	L	Y	N	L	V	S	A	N	F	R	Q	V	F	L	S	T	L	A	C	L	C	P	G	W	R
shift residue												S				V																																		

rNTR1 position	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424
D03 residue	H	R	R	K	K	R	P	T	F	S	R	K	P	N	S	M	S	S	N	H	A	F	S	T	C	A	T	R	E	N	L	Y
shift residue																																

rNTR1 position	353	354	355	356	357	358	359
residue 1 (D03)	L	T	N	A	L	F	Y
residue 2 (shift)		S				V	
codon 1	CTA	ACC	AAC	GCT	CTT	TTC	TAC
codon 2	CTT	ACT	AAT		CTA	TTT	TAT
codon 3	CTC	ACA			CTG	GTT	
codon 4	CTG	ACG			TTA	GTC	
codon 5	TTA	TCT			TTG	GTA	
codon 6	TTG	TCC				GTG	
codon 7		TCA					
codon 8		TCG					
codon 9		AGT					
codon 10		AGC					

Figure S4.

Figure S5. Time-dependence of detergent stability of different mutants with bound agonist in DDM (a), DM (b), NM (c) and OG (d). After detergent exchange, samples were incubated at 4°C for the indicated time and the remaining agonist-binding activity was determined. NTS1-7m was constructed according to ref [3].

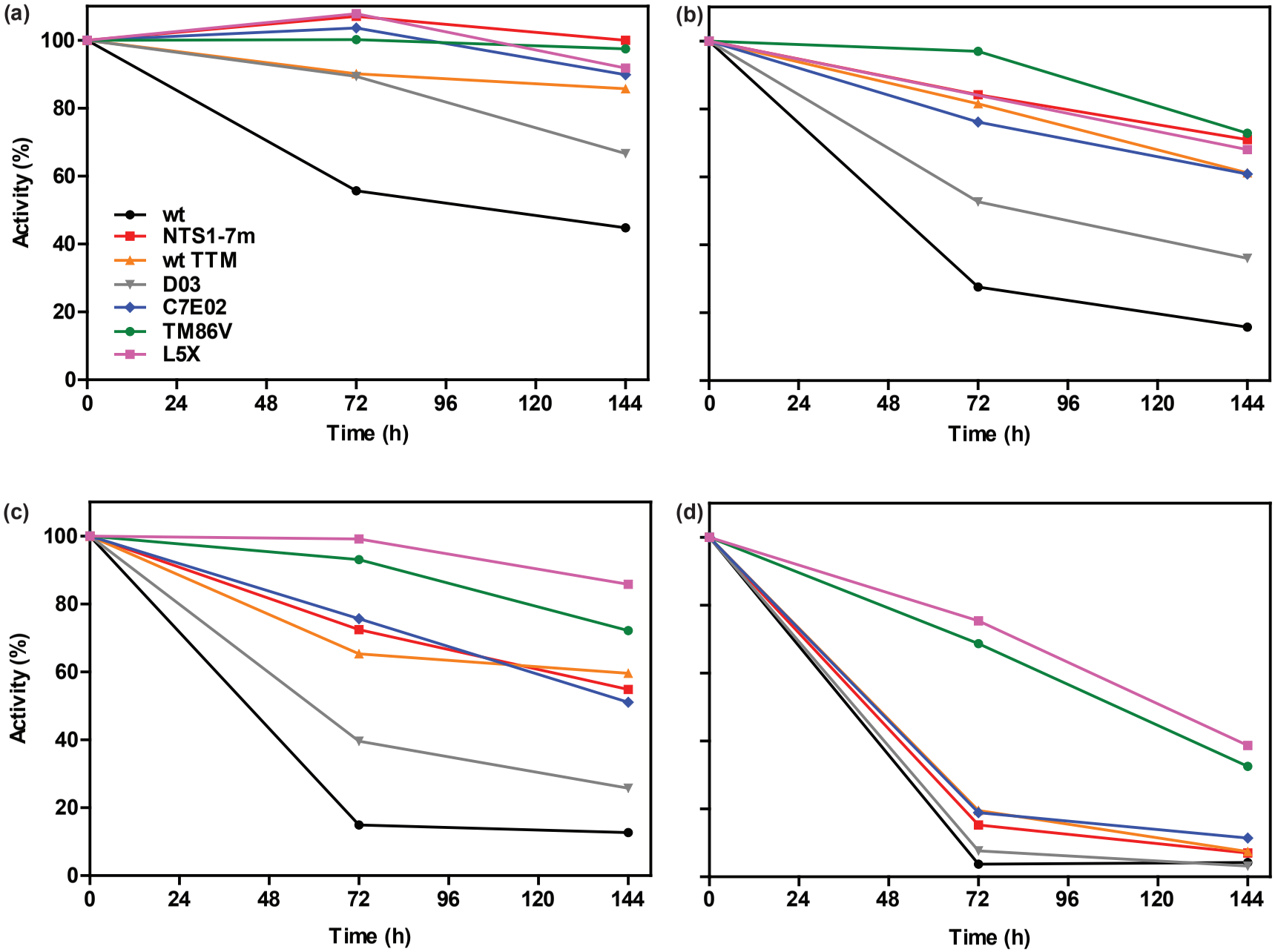
(a)
(b)
(c)
(d)
Activity (%)
Time (h)
0
20
40
60
80
100
0
24
48
72
96
120
144
wt
NTS1-7m
wt TTM
D03
C7E02
TM86V
L5X

Figure S5.

Figure S6. Functional expression levels of rNTR1-wt, wt-TTM and NTS1-7m in *E. coli*. An average of three independent expressions (20 h at 20°C) is shown.

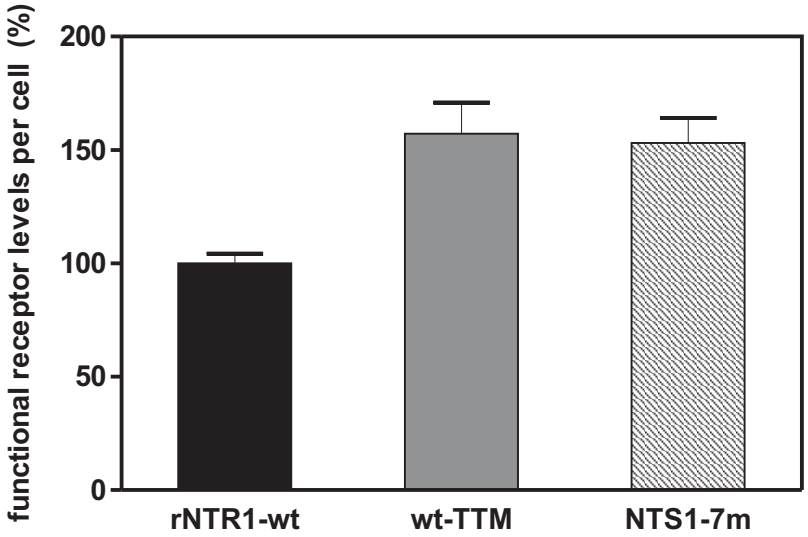
functional receptor levels per cell (%)
200
150
100
50
0
rNTR1-wt
wt-TTM
NTS1-7m

Figure S6.

Figure S7. Expression (A) and detergent stability (B) of mutant M303 in comparison to D03. (a) Expression levels of D03 and M303 were analyzed by flow cytometry. The MFI of D03 and M303 are comparable. Nonspecific binding of BODIPY-NT to cells was measured in the presence of 10 µM unlabeled neurotensin. (b) Detergent stability of D03 (solid circles), M303 (open squares), and a randomly chosen StEP-variant MutR (open triangles) is compared in a buffer containing DDM, CHAPS and CHS (buffer SAB).

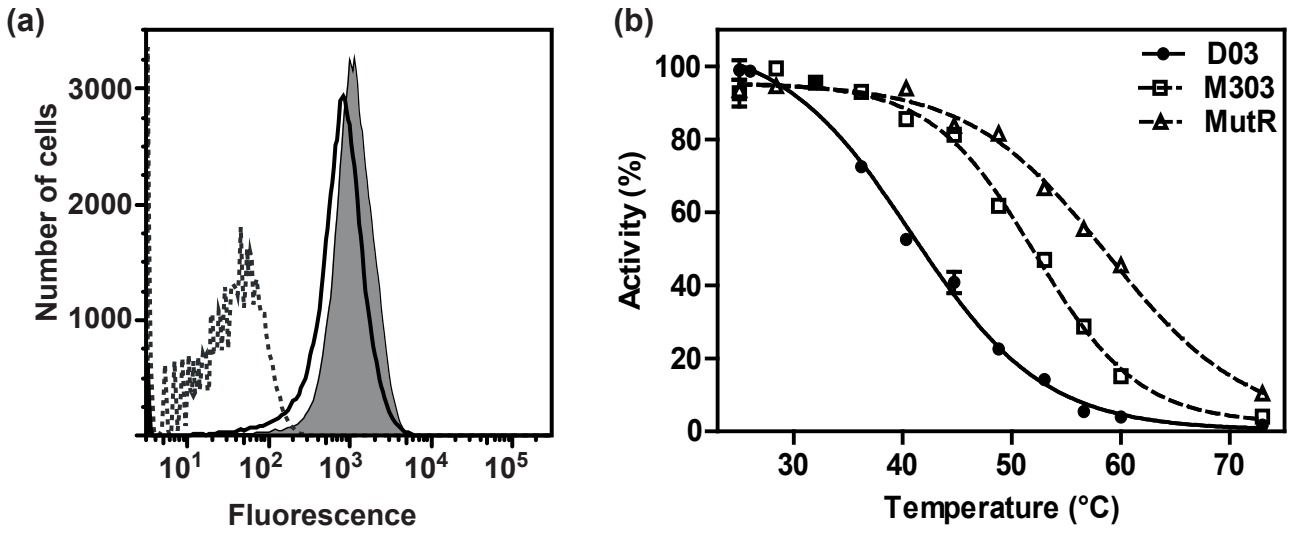
(a)
Number of cells
3000
2000
1000
10^1
10^2
10^3
10^4
10^5
Fluorescence
(b)
Activity (%)
100
80
60
40
20
0
30
40
50
60
70
Temperature (°C)
D03
M303
MutR

Figure S7.

References

1. Aguinaldo, A. M. & Arnold, F. (2002). Staggered extension process (StEP) *in vitro* recombination. *Methods Mol. Biol.* **192**, 235-239.
2. Zhao, H. & Zha, W. (2006). *In vitro* 'sexual' evolution through the PCR-based staggered extension process (StEP). *Nat. Protoc.* **1**, 1865-1871.
3. Shibata, Y., White, J. F., Serrano-Vega, M. J., Magnani, F., Aloia, A. L., Grisshammer, R. & Tate, C. G. (2009). Thermostabilization of the neurotensin receptor NTS1. *J Mol Biol* **390**, 262-277.