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Cloning protocols for Kroon et al., 2024

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We use this protocol and it's working

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Abstract

Here you find the detailed protocols that describe the cloning procedures for the making of plasmids in the publication Kroon et al., 2024. All generated plasmids are in pCAX backbone (CAG promoter) and have a C-terminal tag (Flag tag separated with a flexible GSGGGSG-linker, His tag with a VDGRP-linker).

Troubleshooting

General cloning protocol

1 PCR was performed using KOD Hot Start DNA polymerase kit (#71086-3, Merck Millipore)
- denaturation at 95°C for 2.5 minutes
- 33 cycles at 95°C for 30 seconds
- annealing at 63°C for 20 seconds
- elongation at 70°C for 50 seconds
- A final elongation for 3 minutes at 70°C

NB! To amplify GC-rich BASP1 DNA, 4 µl of DMSO was added into 50 µl PCR reaction to decrease thermostability, and annealing was done at 50°C.

2 PCR products and their intended vector were digested with restriction enzymes (FastDigest, Thermo Scientific) for 1 hour at 37°C according to manufacturer's protocol

3 PCR products and their intended vector were run on an 1-2% agarose gel and purified using the NucleoSpin Gel and PCR Clean-up kit (#740609.25, Macherey-Nagel)

4 PCR products were ligated with the vector using T4 DNA ligase (#M0202L, NEB) for 5 hours at room temperature according to manufacturer's protocol

5 Ligation products were transformed into competent E.coli (DH5α):
- competent bacteria were thawn on ice for 10 minutes
- ligation mix was added to bacteria, gently stirred with the pipet tip and incubated on ice for 10 minutes
- heat shock for 40 seconds at 42°C, followed by 5 minutes on ice
- incubation at 37°C with 900 rpm for 30-45 minutes
- transformed bacteria were plated on agar with selection antibiotic and grown overnight at 37°C

6 Individual colonies were picked and grown in 5 ml LB medium with selection antibiotic overnight, and plasmid DNA was purified using the NucleoSpin Plasmid Mini kit (#740588.50, Macherey-Nagel) according to the manufacturer’s protocol.
Control digests with restriction enzymes cutting within the insert were performed whenever possible and all clones were sent for sequencing at LGC.
1 µg of DNA from the correct clones were transformed into E.coli (see above), the transformation mix was added directly into 200 ml LB medium with selection antibiotic and grown overnight.

7 The final plasmid was purified with NucleoBond Xtra Maxi Plus kit (#740416.50, Macherey-Nagel) according to the manufacturer’s protocol. DNA concentration was

measured using ND-1000 Spectrophotometer (Thermo Fisher Scientific). All final plasmids were validated by control digest and sequencing.

Insertion of membrane/cytosol tag to PLPPR3 intracellular domain by oligo annealing

8 pMT4-PLPPR3-ICD-His vector (Fatih Ipek) was digested with EcoRI and PstI for 1 hour at 37°C to excise a short N-terminal sequence

9 Digested vector was purified via gel extraction (see step 3) and dephosphorylated with Antarctic phosphatase (#M0289L, NEB) according to manufacturer´s protocol

10 Oligonucleotides (10 µM) carrying the membrane/cytosol tag were annealed in T4 ligase buffer by
heating to 95°C for 5 minutes and gradually cooling to room temperature over 30 minutes.
Annealed oligonucleotides were phosphorylated with T4 PNK (#M0201L, NEB) according to manufacturer´s protocol

11 Dephosphorylated vector (step 9) and phosphorylated annealed oligos (step 10) were ligated (see step 4) and steps 5-7 were carried out.

Insertion of point mutations to generate phospho-mutants

12 Standard PCR (see step 1) was used to generate DNA fragments from the N-terminus to the point mutation and from the point mutation to the C-terminus

13 The two DNA fragments were purified via gel extraction (see step 3)

14 Splice-overhang extension PCR was used to merge the two fragments into one insert. In this PCR reaction, the first 10 cycles were run without primers to achieve annealing between the two overlapping fragments. After adding N- and C-terminal primers, the reaction was run another 30 cycles to amplify the full-length insert. To generate the PLPPR3-N-S351A/S379A/T380A
triple mutant, PLPPR3-N-S379A/T380A mutant was used as a template to introduce the additional S351A mutation. Subcloning of PLPPR3-S351A/D-Flag into lentiviral vectors was achieved by PCR with forward and reverse primers covering the entire insert.

15 Steps 2-7 were carried out.

Plasmids and their primers

16

	A	B	C
	Plasmid	**Source or reference**	**Cloning primers**
	ICDm-His	this work	**fw:** aattcgCTAGCgccaccAtgggctgcgtgcagtgcaaagataaagaagcgcaggcaccacctgca **rev:** ggtggtgcctgcgcttctttatctttgcactgcacgcagcccaTggtggcGCTAGcg
	ICDm-S351A-His	this work	Mutagenesis primers: **fw:** CTGAAGCGAGCCgcCGTGGATGTGGAC **rev:** GTCCACATCCACGgcGGCTCGCTTCAG Extension primers: **fw:** same as for PLPPR3-ICDm-His **rev:** gtgatggggccggccgtcgacgtcctggtacctcctggcc
	ICDc-His	this work	**fw:** aattcgCTAGCgccaccAtggCGtgcgtgcagtgcaaagataaagaagcgcaggcaccacctgca **rev:** ggtggtgcctgcgcttctttatctttgcactgcacgcaCGccaTggtggcGCTAGcg
	N-S351A-Flag	this work	Mutagenesis primers: **fw:** CTGAAGCGAGCCgcCGTGGATGTGGAC **rev:** GTCCACATCCACGgcGGCTCGCTTCAG Extension primers: **fw:** GCTAGCgtcaccATGCTTGCTATG **rev:** gtcgcggccgctTTACTTGTCATCGTCATCC
	N-S379A-Flag	this work	Mutagenesis primers: **fw:** CTGCCCCGGGTCgcCACGCCCTCGCTG **rev:** CAGCGAGGGCGTGgcGACCCGGGGCAG Extension primers: Same as above
	N-T380A-Flag	this work	Mutagenesis primers: **fw:** CCCCGGGTCAGCgCGCCCTCGCTG **rev:** CAGCGAGGGCGcGCTGACCCGGGG Extension primers: Same as above

	A	B	C
	N-S379A/T380A-Flag	this work	Mutagenesis primers: **fw:** CTGCCCCGGGTCgcCgCGCCCTCGCTG **rev:** CAGCGAGGGCGcGgcGACCCGGGGCAG Extension primers: Same as above
	N-S351A/S379A/T380A-Flag	this work	Same primers as for S351A and S379A/T380A mutants
	PLPPR3-S351A-Flag	this work	Same primers as for N-S351A-Flag
	PLPPR3-S351D-Flag	this work	Same primers as for N-S351D-Flag
	BASP1-Flag	this work	**fw:** tagagCTAGCgccaccATGGGAGGCAAGCTGAGC **rev:** ccaccggatccCTCTTTGACGGCCACGCTTTGCTCG GAG
	pPAL_ICD-His	Fatih Ipek	NA
	pCAX_N-Flag	Dr. Joachim Fuchs/ Dr. George Leondaritis (Brosig et al., 2019)	NA
	pCAX_Cm-Flag		
	pCAX_Cc-Flag		
	pCMV6_BASP1-tGFP	#MG217147, Origene	NA

	A	B	C
	pCAX_PLPPR3-Flag	Brosig et al., 2019	NA
	pN1_GFP-F	Jiang & Hunter, 1998	NA
	f(syn)-Syp-GFP-w	Viral Core Facility, Charité (Sampathkumar et al., 2016)	NA