Q5 Site-directed mutagenesis

To insert the tags before the sfGFP a Q5 site directed mutagenesis was performed. Therefore, non-overlapping primers were designed using the NEBase Changer tool. Annealing temperatures were calculated using the NEB Tm Calculator. The reaction composition is as follows in table 1.

Table 1: Q5 Site-directed mutagenesis reaction composition.

Component	50 µL Reaction	Final concentration
5x Q5 Reaction buffer	10 µL	1x
10 mM dNTPs	1 µL	200 µM
10 µM Forward Primer	2.5 µL	0.5 µM
10 µM Reverse Primer	2.5 µL	0.5 µM
Template DNA	variable	1 pg–10 ng
Q5 High-Fidelity DNA Polymerase	0.5 µL	0.02 U/µL
Nuclease-Free Water	To 50 µL	

Table 2: Thermocycling conditions for Q5 site-directed mutagenesis

Step	Temperature	Time
Initial Denaturation	98 °C	30 seconds
	98°C	10 seconds
25 cycles	50-72°C	30 seconds
	72°C	20-30 seconds/kb
Hold	4 °C	

After PCR, the product was phosphorylated, then ligated and finally digested with DpnI. The reaction composition for the non-radioactive phosphorylation is as follows. Since ATP is already present in the T4 ligase buffer, the same approach was used for both reactions. The T4 polynucleotide kinase exhibits 100% activity in this buffer.

Table 3: T4 PNK and ligase reaction composition.

Component	50 µL Reaction
T4 DNA Ligase Buffer (10x)	5 µL
T4 PNK	1 µl (10 units)
T4 Ligase	1 µL (10 units)
DNA	Up to 300 pmol
Nuclease-Free Water	To 50 µL

The approach was then digested with DpnI. 1 µL DpnI was added to the entire reaction mixture and incubated overnight at 37°C to ensure complete digestion. Afterwards 1 µL was transformed into competent Dh5a cells. After successful transformation, the DNA was purified and the mutagenesis was verified by Sanger sequencing.