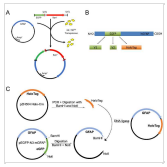

Feb 03, 2021

Cloning and mutagenesis

Federico Herrera[1]

[1]Faculdade de Ciências, Universidade de Lisboa

1 Works for me dx.doi.org/10.17504/protocols.io.77fhrjn

Federico Herrera

ABSTRACT

We generated a human-GFAP-EGFP fusion construct by randomly inserting the coding region of EFGP into the coding region of hGFAP (A). Briefly, in the presence of a transposase protein, a 1902 bp transposon containing the coding region for EGFP and kanamycin resistance (Kan^r) was inserted randomly into a pcDNA3.1/Ampicillin(Amp^r) resistant target plasmid containing the coding region for human GFAP. Successfully transposed plasmids carried both Amp^r and Kan^r as selection markers. The transposon insertion was confirmed by PCR-based colony screening using primers specific for GFAP. Positive colonies were then digested with Srf1 to remove the Kan^r region, and the resulting plasmids were transfected into U251 cells and tested for fluorescence by means of flow cytometry and fluorescence microscopy. Once confirmed the efficacy of the EGFP insert, we tried unsuccessfully to exchange it for a Halo tag and bimolecular fluorescence complementation (BiFC) tags Venus 1 (amino acids 1-158), Venus 2 (amino acids 158-238)(B).

For the mGFAP-Halo construct, the host vector was obtained by substitution of the EGFP tag from a pEGFP-N3-mGFAP plasmid for a HaloTag using *BamHI* and *NotI* restriction sites (C). The HaloTag was obtained by PCR-amplification of the pZH504Halo-Cro plasmid using specific primers and carrying *BamHI* and *NotI* restriction sites. PCRs were done using 20 ng of template DNA, 0,2 µM of cloning primers and 200 µM of dNTPs in a total volume of 50 µl containing 1 unit of Phusion DNA polymerase and the corresponding amplification buffer. The thermocycling conditions were the following: denaturation at 98ºC for 30 sec followed by 30 cycles at 98ºC for 10sec; 65ºC for 30s; 72ºC for 30 sec (30s/kb, as specified by the manufacturer) and a final extension at 72ºC for 10 min.

Single p.Arg236His(R236H) and p.Arg239Cys(R239C) mutations were individually inserted by site-directed mutagenesis into the wild-type mGFAP and hGFAP constructs, respectively. PCRs were carried out using 25 ng of template DNA, 0,2 µM of mutagenesis primers and 250 µM of dNTPs in a total volume of 50 µl containing 2,5 units of *Pfuturbo* DNA polymerase and the corresponding amplification buffer. The following thermocycling conditions were used: denaturation at 95ºC followed by 12 cycles at 95ºC for 30 sec; 55ºC for 1 min; 68ºC for 8 min (1 min/kb, as specified by the manufacturer) and a final extension at 68ºC for 5 min.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Letra-Vilela, R., Quiteres, R., Murtinheira, F., Crevenna, A., Hensel, Z., & Herrera, F. (2020). New tools for the visualization of glial fibrillary acidic protein in living cells. *Experimental Results, 1*, E4. doi:10.1017/exp.2020.1

DOI

dx.doi.org/10.17504/protocols.io.77fhrjn

PROTOCOL CITATION

Federico Herrera 2021. Cloning and mutagenesis. **protocols.io** https://dx.doi.org/10.17504/protocols.io.77fhrjn

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Letra-Vilela, R., Quiteres, R., Murtinheira, F., Crevenna, A., Hensel, Z., & Herrera, F. (2020). New tools for the visualization of glial fibrillary acidic protein in living cells. *Experimental Results, 1*, E4. doi:10.1017/exp.2020.1

CREATED

Oct 14, 2019

LAST MODIFIED

Feb 03, 2021

PROTOCOL INTEGER ID

28615

MATERIALS TEXT

Cloning enzymes were purchased from Thermo Scientific (Waltham, MA, USA), unless otherwise indicated. Exceptions include: *SrfI* (New England Biolabs, Ipswich, MA, USA), PfuTurbo DNA polymerase (Agilent, SantaClara, CA, USA) and *DpnI* and alkaline phosphatase (NZYTech, Lisbon, Portugal). The pEGFP-N3-mGFAP plasmid was a kind gift from Dr. Cécile Leduc (Institut Pasteur, Paris, France), the pZH504 Halo-Cro plasmid was kindly gifted by Dr. Zach Hensel (ITQB, Oeiras, Portugal), the pcDNA-hGFAP construct was a kind gift by Dr. Michael Brenner (University of Alabama, Birmingham, USA), and the template transposon construct with kanamycin resistance and EGFP was kindly provided by Dr. Mika Ruonala. PCR primer synthesis and DNA sequencing were performed by StabVida (Caparica, Portugal). The primers used for this set of experiments were:

mGFAP - HaloTag	Forward: AATGGATCCATGGCAGAATCGGTACTGGC
	Reverse: TATGCGGCCGCTTAGCCGGAAATCTCGAG
Arg236His (R236H)	Forward: CTGAGAGAGATTCACACTCAATACGAG
	Reverse: CTCGTATTGAGTGTGAATCTCTCTCAG
Arg239Cys (R239C)	Forward: CTGAAAGAGATCTGCACGCAGTATG
	Reverse: CATACTGCGTGCAGATCTCTTTCAG

Table I. Primers used for cloning and mutagenesis.

Citation: Federico Herrera (02/03/2021). Cloning and mutagenesis. https://dx.doi.org/10.17504/protocols.io.77fhrjn