Entry clone preparation for 2in1 cloning

Use only the gene-specific sequence of your Gateway primer for T_M calculation; design the primers that this lies in the range of 58-60°C and does not deviate more than 2°C between each primer pair. Please refer to our standard Gateway-PCR program below which usually allows easy amplification from cDNA.

1) Sense Primer for pDONR221-P1P4:

4 G's followed by attB1 (underlined), followed by 'NN' (here 'TA' to yield Leucine (TTA), ***do not*** choose 'GA' as this would result in a 'TGA' Stop codon), ATG of your gene-of-interest (GOI)-followed by 18-25bp of gene specific sequence (used for T_M calculation).

attB1-primer:

5'-GGGG-ACA AGT TTG TAC AAA AAA GCA GGC TTA-**ATG**[18-25bp of your GOI]

2) Antisense Primer for pDONR221-P1P4:

4 G's followed by attB4 (underlined), followed by one 'N' (here 'G' to yield Histidine (CAC)), followed by 18-25bp of gene specific sequence ***without*** its native STOP codon to allow for C-terminal fusions.

attB4-primer for C-terminal fusions to your GOI:

5'-GGGG-AC AAC TTT GTA TAG AAA AGT TGG GTG-[18-25bp of your GOI ***without*** Stop]

OR:

4 G's followed by attB4 (underlined), followed by 18-25bp of gene specific sequence ***including*** its native STOP codon to allow for the original C-terminus which is important if localisation signals are at the far C-terminus and should not be masked (e.g. PTS1 or HDEL/KDEL retention signal).

attB4-primer for native C-terminus of your GOI (C-terminal reading frame not important):

5'-GGGG-ACAACTTTGTATAGAAAAGTTGGGT-**STOP**-[18-25bp of your GOI ***including*** Stop]

3) Sense Primer for pDONR221-P3P2:

4 G's followed by attB3 (underlined), followed by 'NN' (here 'GA' to yield Glycine (GGA)), followed by ATG and 18-25bp of gene specific sequence of your GOI (used for T_M calculation).

attB3-primer:

5'-GGGG-ACA ACT TTG TAT AAT AAA GTT GGA-**ATG**[18-25bp of your GOI]

4) Antisense Primer for pDONR221-P3P2:

4 G's followed by attB2 (underlined), followed by one 'N' (here 'G' to yield Histidine (CAC)), followed by 18-25bp of gene specific sequence ***without*** its native STOP codon to allow for C-terminal fusions.

attB2-primer for C-terminal fusions to your GOI:

5'-GGGG-AC CAC TTT GTA CAA GAA AGC TGG GT**G**-[18-25bp of your GOI without Stop]

OR:

4 G's followed by attB2 (underlined), followed by 18-25bp of gene specific sequence ***including*** its native STOP codon to allow for the original C-terminus which is important if localisation signals are at the far C-terminus and should not be masked (e.g. PTS1 or HDEL/KDEL retention signal).

attB2-primer for native C-terminus of your GOI (C-terminal reading frame not important):

5'-GGGG-ACCACTTTGTACAAGAAAGCTGGGT-**STOP**-[18-25bp of your GOI ***including*** Stop]

Standard PCR protocol using KOD HotStart

1) 95°- 2'00
2) 95°- 0'20
3) 55°- 0'20
4) 70°- 0'15/kbp
5) Goto 2 for 5 (10) times
6) 95°- 0'20
7) 70°- 0'20 + 0'15/kbp
8) Goto 6 for 30 (35) times

(Note: we routinely add 5% DMSO in our PCR mix)

Reference:

Multisite Gateway Pro Manual, Thermo Fisher Life Technologies – available here:

https://tools.thermofisher.com/content/sfs/manuals/multisitegatewaypro_man.pdf