This protocol describes how to clone the target flanking regions in a vector to then do a genomic insertion in yeast. It uses Phusion Hot Start II DNA polymerase and NEBuilder® HiFi DNA Assembly Master Mix (NEB #E2621).

1. **Genomic amplification**: HR\_primers are used to amplify genomic regions flanking the target. Use ~20 ng of genomic template and 0.2 µM primers in 100 ul reactions, and perform touch-down PCR:

98°C 45s, 12x (98°C 10s, *68>54*°C 20s, 72°C 20s), 28x (98°C 10s, 72°C 35s), 72°C 5min (see note a)

2. PCR product purification and elution in water. If amplicon molarity (roughly band intensity) is similar, you may pool flanking regions for the same gene together (see note b).

3. **Vector amplification**: backbone\_ and insert\_primers are used to amplify the vector into two modules for the Gibson reaction. Use ~10 ng of plasmid template and 0.2 µM primers in in 100 ul reactions, and perform touch-down PCR:

98 45s, 35x (98 15s, 72 Xs + 5s/cycle from cycle 25) 72 7 min (see note c)

X = 15s / Kb of amplicon

4. PCR product purification and elution in water.

5. Concentrate PCR eluates to 30-50 ng/ul for flanking regions and to 100-250 ng/ul for vector modules (see note d)

6. **Gibson reaction**. Use ~0.017 pmol of each of the two vector modules, and ~0.10 pmol of each flanking region, to have approximately a 3:1 ratio flanking region:vector and a total ~0.22-0.27 pmol reaction (see note e).

(a) HR\_primers are designed minimising appearance of hairpins, so a wider range of Tm's can be expected. The touch-down *68>54*°C (as per Phusion recommendations, the minimum Ta = Tm + 3°C, Tm being the lowest Tm of the primer set) will give specificity, so if you are cloning several genes you can do all reactions together with a high rate of success. If the primer Tm is much lower, just go lower than 54°C. If there is a hairpin raise the temperature; also, heating the primers at 95°C for 3 min, cooling them immediately on ice and keeping the PCR reaction on ice until the PCR machine is at 98°C can help fixing problems with hairpins.

The two-step part of the PCR assumes that primers with tails (therefore with very high Tm) are used, so if you were using non-tailed primers you should do a regular three-step PCR.

The extension times of 20 and 35s work well for 600-900 bp amplicons, otherwise adjust them accordingly.

(b) Use your method of choice, as long as it gets rid of primers and you obtain single bands to set up clean Gibson reactions. E.g. silica column purification, paramagnetic beads or gel extraction, the latter being less recommended due to lower recovery.

(c) Adding 5s/cycle in the extension increases final yield. For these big amplicons (3-8Kb) it is key to make sure that primers are in good condition. If extra bands appear after PCR, try and reduce the reaction volume to 25 ul to favour thermal exchange and keep primer annealing specificity and efficiency.

(d) This will allow you to keep the Gibson reaction volume low. You need to have roughly equivalent molar amounts of modules in the Gibson reaction: the bigger the amplicon the higher the concentration needed. You can use a speedvac at 30-50°C, freeze-dry or in the previous steps just use low-elution volume kits or paramagnetic beads.

(e) 5 ul Gibson reactions in PCR tubes work well: aim for 0.21 pmol total modules (flanking regions + insert + backbone) and for a 3:1 ratio inserts:vector (0.09 pmol for each of two inserts at 3:1 insert:vector, plus 0.03 pmol of vector). If you prefer 20 ul Gibson reactions, aim for 0.84 pmol total modules.

moles dsDNA (mol) = mass of dsDNA (g)/((length of dsDNA (bp) x 617.96 g/mol) + 36.04 g/mol)

moles of dsDNA ends = moles dsDNA (mol) x 2

DNA copy number = moles of dsDNA x 6.022e23 molecules/mol

HTML (SHINY) VERSION

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