Biotinylation method to purify ssDNA

Introduction

Method to prepare ssDNA as a donor for CRISPR..via PCR and streptavidin magnetic beads... Leads to very pure ssDNA (can be long) and possiple to isolate both ssDNA strands if needed (here shown only first part to generate the reverse ssDNA)... But compared to IVT based method, the yield is much lower.

Forward primer has to be with modification (if neccessary, it can be upgraded to make it general/cheaper ... see Bennett, H., E. Aguilar-Martinez, and A.D. Adamson. 2021. CRISPR-mediated knock-in in the mouse embryo using long single stranded DNA donors synthesised by biotinylated PCR. *Methods*. 191:3–14. doi:10.1016/J.YMETH.2020.10.012.):

dual-biotin tag (/52-bio/) from IDT... (HPLC..)
Ideally you will have at the end of the protocol 30 ul of 100 ng/ul of highly pure full leght ssDNA. Empiric rule: ssDNA max 10 ng/ul in 30 ul = 300 ng per initial 1 ug of PCR

Materials

- Forward (dual-biotin tag) + Reverse primer
- gel extraction columns (DNA)
- Dynabeads Streptavidin Trial Kit-4 x 1 mL,4 x 1ml .... All works (C1 one best) except M-270... it is very cheap!!!
- AmpureXP beads + magnetic holder
- ice cold Tween buffer (5mM Tris pH8, 0.5mM EDTA, 1M NaCl, 0.05% Tween)
- ice cold 2x BB (10mM Tris pH8, 1mM EDTA, 2M NaCl)
- 1x BB (5mM Tris pH8, 0.5mM EDTA, 1M NaCl)
- SyberGreen/Agarsose/RNA loading dye with formamide (Thermo; #R0641)
- EDTA...NaoH... HCl

Procedure

Commercial dsDNA fragment

1. Prepare dsDNA template containing the template sequence (For CRISPR knock in gene + ideally 300 pb flanking homology sequences) using gBlock (KRD/IDT) or GeneArt (Thermo)

PCR

2. PCR as in table with these distinctions: 600 ul of total reaction (50 ul per 12 well strip), 20-100 ng template (plasmid or PCR product with corresponding homologous arms), final 0.1-0.2 uM F +R primers (to minimise non-incorporated biotinylated primer dimers which will compete for the streptavidin binding sites when purifying). Use high fidelity enzyme such as Phusion or Pfu-X7 or Q7.

Component	20 µl Reaction	50 µl Reaction	Final Concentration
Nuclease-free water	to 20 µl	to 50 µl	
5X Phusion HF or GC Buffer	4 µl	10 µl	1X
10 mM dNTPs	0.4 µl	1 µl	200 µM
10 µM Forward Primer	1 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 250 ng
DMSO (optional)	(0.6 µl)	(1.5 µl)	3%
Phusion DNA Polymerase	0.2 µl	0.5 µl	1.0 units/50 µl PCR

3. Set PCR programm as you need... Tm based on primers: Rab11a douB_168_F+ 06_R (Tm 59) ; Rab11b (186dBioF + R_7d (short Tm59) or 186dBioF + R_**7e** (long Tm 69); Rab25 douB_169_F+ 09_R(Tm 59)..... extension 1:40 min ...*(do not use douBio170F primer for Rab11b as it is unspecific)*

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25-35 Cycles	98°C 45-72°C 72°C	5-10 seconds 10-30 seconds 15-30 seconds per kb
Final Extension	72°C	5-10 minutes
Hold	4-10°C	

4. RUN 5-10 ul on gel... Make sure there is only one product of the correct size, if any spurious bands then purify the correct product via gel.

5. **Concentrate PCR volume by DNA clean up Columns** (ratio 3:1 gel solubil. buffer).... USE 2 columns in total... ALL TO ONE FIRST (repeatadly one by one), then flowthrough to second ... wash 2x with kit wash buffer (has 70% ethanol)... centrifuge max speed 2 min to remove any ethanol... elute first column by 40 ul (ultra pure RNAse free H2O heated to 70C to increase efficiency of elution, 5 min incubation before spining in centrifuge), second column by 20 µl and combine. Then add again extra 20 ul of ultra-pure H2O to the FIRST COLUMN, centrifuge and combine = total 100 ul...

6. Measure DNA concentrentration (best/wanted 100 ng/ul...total 10 ug). Seqencing is reccomended, but not obligatory as long as the template is sequenced completely (final ssDNA can be sequenced partially only from one end).

Biotin PCR purification protocol

7. Pipette myOne c1 Strep beads 100 ul of 50% slurry per 10 ug of purified double biotinylated PCR product is enough (capacity of binding)!!! Do not vortex beads!... put to 2 ml tube and incubate on magnet 2 min..discard the supernatant by pipetting!!!! (always...do not aspirate with Strep beads!!!)... you can combine more beads here for more ssDNA purifications...

8. Add 600ul ice cold Tween buffer (5mM Tris pH8, 0.5mM EDTA, 1M NaCl, 0.05% Tween) to the beads, gently resuspend (if some of the beads flick into the lid then extremely briefly spin to bring the solution down without pelleting the beads) and place on magnetic rack for 2 minutes. Discard supernatant. Repeat.

9. Add 600ul ice cold 2x BB (10mM Tris pH8, 1mM EDTA, 2M NaCl) to the beads, gently resuspend and place on magnetic rack for 2 minutes. Discard supernatant. Repeat.

10. Add 50-100 ul of ice cold 2 xBB to the beads (TOTAL 100 ul of 50% slurry = check), then 100 ul (10ug biotinylated PCR product) = TOTAL 200ul (1x BB). Incubate at RT on whirligig for 60 minutes. Then place on the magnetic rack and discard supernatant by pipetting.

11. Add 600ul ice cold 1x BB (5mM Tris pH8, 0.5mM EDTA, 1M NaCl) to the beads, gently resuspend and place on magnetic rack for 2 minutes. Discard supernatant. Repeat a further three times (DO NOT NEED TO RESUSPEND after first time...keep on magnet for wash...it is faster...)

12. Add 20 ul freshly made 20mM NaOH (5ul 1M NaOH + 245ul H2O) to the beads = elution. Incubate at 22C with 600rpm shaking for 10 minutes.

13. Place on magnetic beads and remove the supernatant (20ul) and put into an eppendorf containing 5 ul 80 mM HCl to neutralise the NaOH, then add 0.75 ul 1M Tris pH 8.0. This is your bottom strand ssDNA.

    The top strand can be also purified if modified biotinilated forward primer with rescriction site used. See Bennett, H., E. Aguilar-Martinez, and A.D. Adamson. 2021. CRISPR-mediated knock-in in the mouse embryo using long single stranded DNA donors synthesised by biotinylated PCR. *Methods*. 191:3–14. doi:10.1016/J.YMETH.2020.10.012.

14. Use AMPureXp beads to remove salts and contaminants (ratio 1:1 ... 27 ul of beads).... wash 3 times with clean 70 % ethanol/...dry and elute using RNA-free water 27 ul...

15. Measure DNA concentration (as ssDNA!!!)... Ideally you will have now concentration around 100 ng/ul of ssDNA.....

16. Run agarose gel (use RNA loading dye and denature ssDNA by 5 min at 70 °C) if plenty...

    To be able to see ssDNA by SyberGreen, you need to run 200 ng.. (SyberGreen binds ssDNA less efficiently than dsDNA)... 1% agarose is usually the best

17. SEQUENCE AT THE END USING correct forward primer! before approaching CRISPR based knock in...

    if ssDNA concentration is very very low (option to add 1 M Hepes and 5 M NaCl (50x) to final 20 mM Hepes, 100 mM Nacl... to make Cas9 happy)... but **10 ng/ul is ok!!! (96 well CRISPR knock in with 15 ng ssDNA isolated by this approach = 100 ng prepared by IVT approach)**