**Protocol for PLK4 Gene KO Using Crispr Cloning Method**

**Design gRNA**

* Use Benchling program
* After design, order the oligo F & R. Takes 1-2 days to reach.

**Inserting Plasmid into Bacteria**

* Take out TLCV2 plasmid from -20C fridge (Nick).
* Take out one plate agar from the 4C small plate fridge
* Take out competent cell
* Withdraw 1 uL TLCV2 into the
* Heatshock the mixture (using hand for 10 secs) then put back into ice. Leave it for 10 mins
* Put the agar plate on bench at room temperature or 37C bacti-incubator. Monitor for any colony growth.
* Put in LB solution (100ml) + Ampicilin 2000x 50uL + swab single colony bacteria into 1 coneflask 400ml or 500mL.
* Put in bacti shaker (in the special room) at 37C over 16 hours.
* Next day, pour out the bacti solution (~100mL) into two 50mL falcon. Withdraw 500uL from the falcon and add 500uL steril 50% glycerol into eppendorf. Keep at -80C. Another 50mL falcon spin down at 4C 3000rpm 10mins (use the big centrifuge. DO NOT USE our centrifuge as is bacteria based) and withdraw the supernatant to keep the pellet.

# when withdraw sterile glycerol, do it near the fire to avoid any contamination. Heat up the cap of falcon tubes and the pipette tips.

# DO NOT VORTEX Plasmid!

* Miniprep

# **TLCV2 (#87360)-** Inducible Cas9 plasmid for ML2 preparation.

# U6 promoter more stable.

**Lentiviral vector digestion (Digestion, Annealing & Ligation do in 1 day)**

* On the Thermocycler machine in the first morning.
* Take out LRT2B from -20C fridge (from Nelson)
* Withdraw 12uL of LRT2B plasmid to small eppendorf. (\*12uL because the conc of LRT2B was 324.7ng/uL. We need 4000ng@4ug. Thus ~12uL. So the volume depends on the plasmid concentration)
* Mix with BsmBI, 3.1 and top up with H2O accordingly:

For 2 hours digestion:

|  |  |
| --- | --- |
| **Solution** | **Amount** |
| BsmBI (-20C fridge)-enzyme | 3uL |
| 3.1 (-20C fridge) | 3uL |
| H2O (Ultrapure water) | 12uL |
| **Total** | **30uL** |

#Take out TLCV2 from -20C fridge.

#Withdraw 5uL of TLCV2 plasmid (Nanodrop = 800ng/uL) to SMALL Eppendorf. If Use TLCV2 need a lot sorting. (Not recommended)

* Digest the mixture at 55C for 2 hours then 80C for 15mins (use white colour machine)
* Purification 0.6% Gel, 45mins 120V..Go to gel purification steps:

**Phosphorylation and Annealing of each pair of Oligos**:

* Prepare Oligo 1 & 2 (100uM)- Spin down Max rpm for 1-2 mins. Then add in x10 of weight eg 20ng->200uL Ultrapure water
* Prepare the mixture below:

|  |  |
| --- | --- |
| **Solution** | **Amount/ Volume** |
| Oligo 1 (100uM)-F | 1uL |
| Oligo 2 (100uM)-R | 1uL |
| 10 x T4 Ligation Buffer (NEB) | 1uL |
| ddH2O (Ultrapure water) | 6.5uL |
| T4 PNK (NEB M0201S) | 0.5uL |
| **Total** | **10uL** |

**\*Must make sure the buffer is fully thaw!**

* Put the mixture in thermocycler using the following parameters:

37C for 30mins

95C for 5 mins then ramp down to 25C at 5C/min (use blue colour Machine-Nelson protocol sgRNA). May put aside at 4C O/N

* Dilute annealed oligo to 1:200 (1uL:200uL. Use ultrapure water)

**Set Agarose Gel**

* Prepare the gel rack with the comb. Make sure are clean. Dark bottom facing top.
* Use 50ml falcon to withdraw 30ml of TAE Solution.
* Weigh 0.6% of 30ml TAE = 0.18g ~0.2g of Agarose powder
* Mix both in a conical flask and heat up to 2 mins. Make sure the solution is fully dissolved and no bubble.
* Add 3uL of 10000x GelRed Solution into the heated solution. (1:50000) eg 1uL for 50,000uL solution.
* Pour the final solution into the gel rack and let it solidify.

**Gel Purification And Extraction of Digested Plasmid**

* Prepare items: 5x Loading Dye (4C fridge), DNA Ladder (4C fridge), the digested Plasmid from (4C fridge after thermocycler)
* Take out the gel from the rack
* Remove excess gel at the outer corners
* Prepare the TAE solution and pour into the big white rack.
* Add in 6uL of 5xloading dye into the digested plasmid (30uL).
* Insert max 20uL into the gel well and balance 16uL into another adjacent well.
* Skip another well and load in DNA ladder each left and right side.
* Cover back the gel purification rack
* Set 120V 45 mins for 0.6% gel
* Make sure DNA well site match with the black wire because DNA is charge ‘negative’ and red wire to the bottom of well.
* After finish. Can wash using tapwater in the tray.
* Then use the UV to identify the band location
* Cut the respective band gel and make sure around 100g+. Max is 400g gel. Put inside the 1.5mL Eppendorf tube. Prepare for gel extraction Kit.

**Gel Extraction Kit**

* Add in 3 volumes QG buffer to 1 volume gel (100mg gel~100uL) Maximum amount of gel per spin is 400mg.
* EB Buffer must be boiled to 60C 5 mins before use.

**Ligation**

|  |  |
| --- | --- |
| **Solution** | **Amount/ Volume** |
| Digested plasmid (50ug) | 2.5uL |
| Diluted oligo | 1uL |
| 10 x T4 Ligase Buffer | 1uL |
| ddH2O (Ultrapure water) | 6.5uL |
| T4 Ligase | 1uL |
| **Total** | **10uL** |

**Must have a pair of blank (without oligo)**

* 16C O/N (Black Machine)

**Day 2- Transformation**

* Take out competent cell (1 eppendorf tube) from -80C. Use Ice box to transport
* Then just wipe and flick the bottom and put back to ice box. Do not heat up or use the hand to warm.
* Prepare the PCR tube upfront and label them.
* 20 uL competent cell + 1 uL ligation product (those with oligo-5)
* Use finger to flick the bottom to mix them before on ice for 30 mins
* Transfer to waterbath 42C for 40secs. (NOT MORE THAN 1 MIN!!!)
* On ice for 5 mins.
* Add 180uL LB medium into the eppendorf tubes (total ~100-120uL) – (Must have total 10 fold of 21uL transformation product)

# For recover usually 30-60 mins (+900uL LB -> recover 30mins 37C 220rpm)

* Spread plate using 100uL and incubate at 37C O/N.

**###FOR EXPANSION OF PLASMID ONLY(TOITRANS)**

* Take out 1 eppendorf of competent cell. Thaw on ice. (Do it 3-4pm)t
* Withdraw 10uL into PCR tubes + [0.5@1uL](mailto:0.5@1uL) Plasmid PAX2@MD2G@whatever KO gene
* DO NOT Vortex. (Just tickle)
* On ice incubate 10mins
* Draw the plate segment and label them. Withdraw all the solution in pcr tube (10uL) one by one spread the plate. Use 10ul tips. MUST have blank section as a control on plate agar.
* **Incubate in 37C bacti incubator O/N. After O/N, put in 4C with parafilm in bacti fridge.**
* **On Day 2 at 4.30pm, pick single colonies**
* Put the tips in flask with 100mL LB with antibiotic (Ampicilin-get from the -20C fridge) (Dose is 1:1000) #How to prepare LB with Amp stock? -One bottle of 800ml LB add in 800uL Amp.
* SHAKe 37C O/N in bacti shaker.
* On Day 3, take out the flask and divide into two 50ml falcon tubes. Spin down bacti using the common centrifuge in 801 at 4C 3000rpm 10mins
* Use MIDI KIT to extract plasmid ~100ml/Rx. Resuspend 1st falcon with Buffer P1 and withdraw the solution and combine with the 2nd falcon tube. Follow the protocol for subsequent steps.
* Do Nanodrop.

**PCR Colony**

* F and R – Forward use U6 promoter
* Set up the 1% agarose gel (0.3g agarose powder in 30mL TAE solution. If use more well, set higher volume)
* Withdraw 5uL of the PCR colony (from yesterday in 4C fridge) into the 17 well gel
* Pipette in 1-2uL pf DNA ladder (from the 4C)
* No need loading dye as the DreamTaq contains loading dye.
* Set 140 V 20 mins
* UV the gel and check the band. If present meanings got the respective colony wanted. Save and print.

**Inoculation**

* Make sure clean bench area. Lit up the fire awhile to make sure the surrounding area clean.
* Prepare LB solution (thru fire) ~50mL in Falcon tube
* Add in 25uL Ampicillin 1:2000(in eppendorf) (bacteria resistance growth) into the 50ml LB. Mix it (confirm the concentration of Ampicillin 50mg/mL or 100mg/mL?)
* One tube – for one colony for 1 exon.
* Add in 5ml of mixed LB solution into each bacteria tube. Label them
* Streak colony from the plate using tips into each falcon tube 15mL.
* Throw the whole tips into the falcon tube. Do not cap twice the tube as 2nd caping will lock the tube n prevent air entering.
* Incubate the tubes in bacterial shaker at 37C 12-16hrs. MAX 16 hrs.

**MiniPrep**

* Take out the pipette tip and CAP tightly (2nd click) before centrifuge. Pellet 1-5mL bacterial culture by centrifugation for 10mins 3000rpm. (use the Big common centrifuge). Discard supernatant by just pour out to the bacteria waste in 801. ENSURE cultures are not overgrown (12-16 hours is ideal).
* Resuspend pellet in 200uL plasmid resuspension Buffer (B1). Vortex or pipette up down to ensure cells are completely resuspended. There should be no visible clumps
* Add in 200uL Plasmid Lysis Buffer (B2)- gently invert tube 5-6 and incubate at RT for 1 min.
* Keep/store in -80C fridge.

#

\* If not enough time, do nanodrop the next day. Keep in 4C to avoid freeze and thaw

Setting NanoDrop:

* Pipette 1 uL H2O -then press blank @F3 then Measure (F1) before moving to samples plasmid
* Flick the eppendorf tube and stroke down.
* Finally wash using 1 uL water

Eg.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Conc (ng/uL)** | **260/280** | **260/230** | **To get 1.8mg** |
| **E1-1** | 506.3 | 1.90 | 2.37 | 3.5uL |
| **E1-2** | 448.7 | 1.91 | 2.39 | 4.0uL |
| **E2-1** | 553.6 | 1.88 | 2.16 | 3.25uL |
| **E2-2** | 545.7 | 1.89 | 2.35 | 3.3uL |
| **E3-1** | 536.5 | 1.89 | 2.33 | 3.35uL |
| **E3-2** | 802.1 | 1.89 | 2.31 | 2.24uL |
| **E6-1** | 512.8 | 1.88 | 2.29 | 3.51uL |
| **E6-2** | 824.6 | 1.91 | 2.39 | 2.18uL |

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **sgRNA** | **Conc (ng/uL)** | **260/280** | **260/230** | **To get 1.8mg** | **To get 13mg** |
| **E1-2 (1)** | 1390.5 |  |  | 1.3uL | 9.4uL |
| **E1-2 (2)** | 1472.1 |  |  | 1.2uL | 8.7uL |
| **E2-2** | 1972.5 |  |  | 0.9uL | 6.5uL |
| **E3-2** | 2705.3 |  |  | 0.67uL | 4.9uL |
| **E6-1** | 1812.1 |  |  | 1.0uL | 7.2uL |
| **PAX2** | 2506.4 |  |  | 0.72uL |  |
| **MD2G** | 1586.9 |  |  | 1.13uL |  |

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **shRNA** | **Conc (ng/uL)** | **260/280** | **260/230** | **To get 1.8mg** | **To get 13mg** |
| **E1-2 (1)** | 476 | 1.89 | 2.27 | 3.7uL | 26uL |
| **E1-2 (2)** | 431.1 | 1.89 | 2.28 | 4.2uL | 30 |
| **E2-1** | 468.6 | 1.89 | 2.25 | 3.8uL | 27 |
| **E4-1** | 441.3 | 1.89 | 2.25 | 4.1uL | 30 |
| **PAX2** | 2506.4 |  |  | 0.72uL | - |
| **MD2G** | 1586.9 |  |  | 1.13uL | - |

#MINIPREP- To use vacuum to withdraw the solution

#Use pipette tips without filter. Cut the tip area. Join the tube with the tips.

Send for Sanger Sequencing

* Fill up the submission form and send softcopy and hardcopy.
* Put in 100-300ug of plasmid (from Miniprep)

|  |  |
| --- | --- |
| Solution | Amount Volume |
| Plasmid (Miniprep) | 0.5uL |
| U6 10uM Promoter Forward (sgRNA)  (For shRNA use PLKO.1 Tet On plasmid) | 0.5uL (Do Master mix) |
| ddH2O (ultrapure) | 14uL (Do Master Mix) |

* Put in PCR tubes and label A1-H1 (PLK4 E1-1, E1-2, …E6-1)
* Put in two transparent bags.

###########

Preparing 293FT cell line for next day transfection

**COAT PLATE**

* Add in 2mL PBS with 1:1000x Poly D-Lysine HBr(1mg/ml) in each well based on surface area (6 well plates)

|  |  |  |
| --- | --- | --- |
| ds | E1 | E2 |
|  | E1 | E2 |

|  |  |  |
| --- | --- | --- |
|  | E3 | E6 |
|  | E3 | E6 |

Qs3

* Put in 37C incubator for at least 30 mins
* Withdraw all the Poly D lysine solution (Withdraw by side of the well)
* Use 2ml PBS to wash each and every well. (*Use PBS instead HBSS-better)*
* Withdraw all the PBS out of the well.
* Put in solution that contain 293 (2ml each well)
* For transfection, prepare trypsin 0.05% and DMEM solution
* Use DMEM medium (add in 10% FBS + 5% PS) to culture cell.
* \* Trypsin to detach the adherent cell from the flask. Use 0.05% trypsin aliquot (get from the -20C fridge).
* Mix the whole trypsin solution with 45mL PBS solution (sterile).
* Withdraw the old medium from the flask. (Withdraw from side wall)
* Wash the flask with PBS solution. (Add in from the side wall)
* Decant the solution (containing dead cell).
* Pipette out 5ml of the mixture (trypsin/PBS) into the flask with cell. Pipette into the flask gently (side wall).
* Incubate 1-2 mins and look under microscope to ensure most of the cell become single cells. Swirl gently to detach the adhesion cells.
* Add in 5ml DMEM to the flask to stop the trypsin reaction (because DMEM has FBS)
* Pipette up and down -> centrifuge 5mins 1200rpm
* Withdraw supernatant -> finger tap
* Then dilute the 293 in the (falcon tube 15ml) with 1ml DMEM.
* Count cell using PBS.
* Add in the DMEM medium to a new flask and add in the sample (detached cells) (Friday)- standby for big lot usage.
  + 1: 5- Sun
  + 1: 10- Mon T175 flask (cell: medium)
  + 1:20- Tues <50mL:~30mL

**Transfection (Saturday)**

* Lentivirus packaging plasmid **PAX2 and** envelope plasmid **MD2.G** (from -20C fridge) to the bench. No need ice.

**#** pMD2.G (VSVG expressing envelope plasmid)

**#** psPAX2 (Contains Gag, Pol, Rev, and Tat; can be used with 2nd and 3rd generation of transfer plasmids)

* Calculate the total volume needed for 10 reaction (extra 1 reaction) - (using the excel Flask well surface)
  + Eg: psPAX2 concentration is 1671.9ng/uL (1490ng/uL). One well need 0.9ug. Thus, 10 wells need 5.4uL (6.04uL)
  + Eg MD2.G concentration is 653.4ng/uL. (1586.9ng/uL) One well need 0.45ug. Thus, 10 wells (10 reactions) need 6.88uL
* Calculate the plasmid E1-1, E1-2,… needed (1.8ug)
* Prepare mastermix of 500uL Optimem (1x) + 5.4uL PAX2 and 6.8uL MD2.G
* Withdraw 51uL into each Eppendorf tube.
* Add in plasmid E1-1, E1-2,… into each tube.
* Must have control (U6 dstomato p2). Add in 5.5uL (for 1 well)(1 well need 1.8ug)
* Add **PEI** (1mg/mL) (very toxic) (last to add) 63uL into 500uL OptiMEM (63mg for 10 reaction) in a new Eppendorf.
* VORTEX
* Add in 56uL into the main mixture.
* Vortex using the small vortex
* Wait 15mins at RT Outside bench. Then pipette the mixture into medium with 293 cells.
* Change medium (8hrs). Add in by the side of the well gently. Add DMEM gently at side

**On Monday, Spinoculation**

**Collect lentivirus with PLK4 E1-1, E1-2, …into Eppendorf tubes**

* Prepare 9 Eppendorf tubes for the Lentivirus including the ‘ds’ as control and label PLK4 LV
* Use Satorius Minisart Syringe Filter 0.45uM (white base attach to syringe) and 3mL syringe Terumo.
* Keep in 4C fridge while waiting for Sanger Seq result.
* Doublecheck the sequencing using the downloaded Chromas software. Exclude those with weak band (see the colour bar on top of the peak). Throw way those plasmid (in -20C fridge) & lentivirus with weak band. Only spinoculate those wanted E1-2, E3-2 etc.
* Seed **0.5**-1M/ml ICas9 cell line (ML2, THP-1, MOLM-13 & Nomo-1). Lesser than 0.5M may not enough because spinning may lose some cells. Count cell.
* Top up to the falcon 15mL tube to 6.5mL using RPMI (because got 6 well for each cell lines).
* Add in 3uL Polybrene (use this range 1-10ug/mL) into the falcon tube.
* Dispense 1mL into each well in 6-well plate.
* Add in 300-400uL (depends on how much available in the eppendorf tubes) Lentivirus (E1-1, E2-2, E6-2 etc) in the each well plates of different cell lines (already have polybrene, ICas9 cell line). Eg ML2 PLK4 E1-2, THPPLK4 E1-2, Nomo-1 PLK4 E1-2, MOLM13 PLK4 E1-2.

|  |  |  |  |
| --- | --- | --- | --- |
| ML2 -ve | ML2 ds | THP -ve | THP ds |
| ML2 PLK4 1 | ML2 PLK4 2 | THP PLK4 1 | THP PLK4 2 |
| ML2 PLK4 3 | ML2 PLK4 6 | THP PLK4 3 | THP PLK4 6 |

* Spinoculate at 800g/800rcf 33C 99mins (suppose 1.5hrs but the machine only can set till 99 mins).
* Afterthat put it the 37C incubator.
* Before go back, transfer from the well to the Eppendorf. Spin down 5mins 1200rpm 25C
* Withdraw supernatant and replenish with new RPMI. (\*RPMI added to the well first to prevent cell dried n died. Using from the new RPMI in the well, dilute the pellet in the Eppendorf)
* Put back into the 37 incubators.

**On Tuesday (next day), Add in Blasticidin (10mg/mL) for Selection**

* Prepare a Mastermix (15mL RPMI +30uL Blasticidin 10)
* Add in 1mL master mix to a new 12-well plate
* Add in 800uL of the cell into each well.

|  |  |  |  |
| --- | --- | --- | --- |
| **ML2 -ve** | **Ds** | **THP1 -ve** | **ds** |
| **1** | **2** | **1** | **2** |
| **3** | **6** | **3** | **6** |

**# *How much Blasticidin to add -cell dependent***

***5ug/mL -All***

***10ug/mL- MOLM-13***

**Add in Doxycycline (5mg/ml @ 5ug/uL) 1:5000**

* Make sure enough cell before adding the doxycycline. (Allow few days 2-3 days after adding Blasticidin)
* Add in 1ug Doxycycline into each 1mL cell. (Transfer the cell and treat in 6 well plate-each well 3mL of RPMI if too much eg MOLM-13)

**b**

**VSVG-human**

**PECO-mouse**

**2nd generation packaging system**

**Plasmid PMSCV-IRES-GFP (Retrovirus backbone)**

**10ng/mL -sample PCR inside to it**

**[NRAS]**