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WORKS FOR ME 2

CRISPR-Cas9 screen in NIH-3T3 cells to identify modulators of LRRK2 function

COMMENTS 1

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ABSTRACT

Activating mutations in the LRRK2 gene represent the most common cause of inherited Parkinson's disease, and modifiers of LRRK2 kinase activity are of broad interest in terms of identification of novel drug targets and for helping us understand the molecular events underlying pathogenic LRRK2-driven Parkinson's disease. We describe here a pooled, CRISPR Cas9 screen to identify modulators of LRRK2 activity. LRRK2 phosphorylates a subset of Rab GTPases, thus LRRK2 activity can be monitored easily using phosphoRab-specific antibodies.

Our CRISPR-Cas9 based screen is carried out in mouse cells using a ready-to-use pooled guide RNA (gRNA) mouse library consisting of 78,637 gRNAs targeting 19,674 genes and an extra 1,000 control gRNAs. This library is known as the Mouse Brie CRISPR knockout pooled library, and is a kind gift from David Root and John Doench (Addgene #73633). The protocols below are modifications of the approach described by Feng Zhang and colleagues (https://doi.org/10.1038/nprot.2017.016).

We use commercial antibodies that specifically and sensitively detect phospho-Rab10 in conjunction with flow cytometry to separate cells based on pRab10 content. A pooled library of cells is first generated, each cell harboring a different gene knock-out. Genes encoding negative regulators of the LRRK2-pRab10 pathway will increase pRab10 when knocked out, and genes encoding positive regulators will decrease pRab10 when knocked-out. Fluorescence activated cell sorting permits enrichment of cells with higher or lower than normal phosphoRab levels; the gene knockouts responsible for changes in phosphoRab10 levels are then identified by gene sequencing.

This method involves these steps:

1. Create Cas9-expressing 3T3 cells
2. Obtain large scale plasmid preparation of the gRNA library
3. Make large scale lentiviruses that carry sgRNAs
4. Titrate the Lentiviruses
5. Infect 3T3-Cas9 cells and select infected cells
6. Fix, stain and sort cells
7. Isolate genomic DNA from sorted cells (dx.doi.org/10.17504/protocols.io.eq2lynm9qvx9/v1)
8. Carry out PCR of the genomic DNA
9. Purify PCR products and pool for sequencing

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MATERIALS TEXT

Cell culture reagents:
Low passage NIH-3T3-flpin cells (Life Technologies)
DMEM medium with Glutamate (Hyclone)
100X Non essential amino acids (Hyclone)
100X Penicillin-Streptomycin (Hyclone)
100X Sodium Pyruvate (Hyclone)
Fetal bovine Serum (FBS, Sigma)
Blasticidin (Invivogen)
Puromycin (Invivogen)

Molecular Biology reagents:
BRIE gRNA library (Addgene #73633)
Endura Duo electro-competent cells (Lucigen)
Gene Pulser II for electroporation (Biorad #165-2105)
Luria broth (Sigma)
Machery-Nagel kit (Maxi-prep for plasmids)
Electroporation Cuvette 0.1mm (Biorad)
Cell sorter (Sony)
Rabbit anti-pRab10 (Clone MJF-R-22-5)
Donkey anti-rabbit Alexa-647H+L (Thermo Fisher)
Qiagen genomic DNA mini-kit
Proteinase K 20mg/ml (Zymo)
RNase H (Zymo)
DNA mini-columns (Econospin)
Titanium-Taq (Takara)
10mM dNTPS (Thermo Fisher)
AMPure XP reagent (Beckman Coulter)
Nuclease free water (Thermo Fisher)
Sterilized centrifuge flasks
PsPax2 (Addgene 12260)
VSV-G (Addgene 12259)
PCR library amplification primers

CRISPR-Cas9 screen in NIH-3T3 cells to identify modulators of LRRK2

2h

1 Large scale plasmid prep of the gRNA library

The Mouse gRNA library cloned into the pLenti-guide-puro vector named BRIE was obtained from Addgene 73633.

2.1 Fifty Bioassay 25x25cm plates need to be washed, cleaned and sterilized before filling with Luria-Broth agar for bacterial selection.

- Dip plates in a 20% bleach-filled bucket for 2-3 mins and then rinse under clean running tap water for a few minutes; dip in a bucket of water and allow to air dry for 02:00:00
- Once semi-dry, spray with 70% ethanol and then wipe with kimwipes; keep plates closed in a cool, dry, clean location (lab bench suffices).

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2.2. Pour 125-150 mL of LB agar containing 100µg/ml Carbenicillin per plate:

- Prepare 6.5 L, LB Agar using 8, 2L flasks: 25g LB agar /L water is autoclaved, allowed to cool, and some bottles are held under cold running water and then rapidly poured into plates. Hotter flasks are then poured as they cool or subsequently cooled. Note that antibiotics can be temperature sensitive.

- Allow plates to cool at RT for 4h, and when solidified transfer to 4°C; store wrapped in plastic as bunches of 5 plates until needed

- Endura electrocompetent cells are used for electroporating. Generally, 100ng/µl of library plasmid is used for bacterial electroporation.

- Perform 8 electroporation transformations for the 80,000 guides (1 electroporation per 10,000-15,000 gRNA); 6 bioassay large format plates are needed per electroporation

- Thaw four vials of Lucigen Endura Duo containing 50µl electro-competent cells; pool the 4 vials into one vial to achieve 200µl total volume

- Add 800ng of library plasmid and tap gently. Use this mix for 8 X 25µl sequential electroporations

- For each electroporation, 25µl of the competent cell mix is added to a 0.1mm Biorad E Coli gene pulser cuvette and electroporated using EC1 setting (1.8KV). Immediately add 200µl SOC medium from the kit and transfer to a 1.5ml Eppendorf tube. Repeat this addition of 200µl medium 5 times to recover all bacteria and achieve a final suspension of 1ml of SOC medium.

- Repeat this 8 times using 8 electroporation cuvettes; the overall procedure yields 8ml SOC medium containing electroporated bacteria

- After electroporation, cells allow cells to recover for 1 hour at 37°C in SOC medium

- In the meantime, the bioassay plates are pre-warmed to 37° for 4h in the 37° room (or non-humidified incubator) prior to use so that they are completely dry. The lids of the plates also need to be dry and can be gently wiped with 70% ethanol

- After recovery, all electroporated bacteria are pooled into a 50ml conical tube and 0.5ml plated per

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bioassay plate (50 plates total).

- Some cells are diluted 1000x, 20000x and 50,000x and plated onto a 10cm LB agar petri dish to allow back-calculation of how many colonies were actually plated. This provides an estimate of total single colonies that have been transformed

- Incubate the plates at 37°C for 12-13 hr

- Limit the growth to <14hr. Too much growth may lead to inter-colony competition, slowed growth rates and skewed distribution of the gRNA containing plasmids

- Discrete bacterial colonies should be observed on the large bioassay plates; this step enriches for transformed bacterial cells that survive carbenicillin

- Collect the cells from each plate using cell scrapers into 25ml medium per plate

- Some agar may come along with the bacteria but it is not a problem

- Spin pooled bacterial cultures in sterile centrifuge bottles at 3500rpm for 10min

- Wash cell pellets with 25ml, 37°C pre-warmed PBS and spin again at 3500 rpm for 10min

- Bacterial pellets can be frozen at this point at -20°C for short term or -80°C long term for future plasmid purification

- Use Machery-Nagel Giga-kit for plasmid purification according to the manufacturer: manufacturer suggests : 2.25g wet bacterial pellet = 1 maxi-prep

- Resuspend cells in 12ml Resuspension buffer RES-EF + RNAse (kept at 4°C)

- Add 12ml lysis buffer; invert gently 5 times and incubate for 5 min (not more)

- Meanwhile, equilibrate two columns by adding 35ml Equilibration buffer EQU-EF and let it drip by gravity. Add buffer gently along the tube edge using a circular motion

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- Neutralize the lysed cells using 12ml Neutralization buffer NEU-EF. Neutralization is complete when the initial color disappears

- Chill the neutralized lysate on ice for 5 min

- Mix by inverting the tubes 3X gently and pass the solution through the column

This step usually takes 45-60 min as the filter can become clogged

- Wash the column filter with 10ml of Filter Wash buffer FIL-EF. Apply the buffer to the funnel-shaped rim of the filter and not directly onto the filter.

This step also takes 30-45 mins for the buffer to pass through

- Remove the filter by turning it upside down

Flow can be accelerated using low vacuum pressure

- Wash the column alone (anion resin) with 90 ml Wash buffer ENDO-EF

This wash passes through quickly, in 10 min

- Wash the column with 45 ml Wash buffer WASH-EF

This wash buffer passes through in 15 min
Flow can be accelerated using low vacuum pressure

- Elute with 15ml of elution buffer ELU-EF

The elution can take 15 min with gravity but gives high yield

- Determine concentration using a Nano-drop with 1:5 diluted eluate

- To further purify the plasmid prep, add 10.5ml room temperature isopropanol and vortex vigorously

- Centrifuge at 15000 x g for 30 min at 4°C using 50ml conical tubes in a conical specific f15b 50*8 (Fiberlite) fixed angle rotor

- Carefully discard the supernatant; the pellet will be located on one side of the conical bottom

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- Add 5ml molecular biology grade 70% ethanol at room temperature
- Centrifuge at 15000 x g for 5 min at RT
- Carefully discard all the ethanol without disturbing the DNA pellet

Extra drops can be removed using 2-20µl pipettes

- Invert tube at RT for 20 min to air dry
- Resuspend the pellet in the elution buffer Buffer TE-EF (or nuclease free water) depending on the earlier Nanodrop concentration
- Usually 500µl to 1ml is sufficient - and it can take a few hours for the pellet to completely re-suspend at 37°C
- Dilute to 500ng/µl and store multiple aliquots at -80°C

• Amplify 50ng plasmid library using illumina sequencing primers and sequence using a Miseq kit to obtain an initial gRNA distribution.

2 See our related protocol entitled, **Creating pooled CRISPR-Cas9 knock-outs in NIH-3T3 cells** (dx.doi.org/10.17504/protocols.io.eq2ly7wpmlx9/v1)

Prepare large scale lentiviruses carrying the cloned sgRNAs

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- Thaw a vial of low passage 293T cells in a 10cm culture dish with fresh DMEM medium containing 7.5% FBS
- Expand cells by splitting 1:4 every 3 days to obtain 5 x 15cm dishes
- For transfection, low passage 293T cells should be ~50% confluent
- In a 15ml tube, use 1ml of Optimem to make a DNA:PEI mix

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- Per 15cm dish, use 1ml of Optimem containing 1) psPAX2, 20µg, 2) pMD2.G-VSVG, 7.5µg, 3) pLenti-guide-puro BRIE plasmid prep, 15µg
- Add 212.5µl PEI (1mg/ml stock) drop-wise to the DNA solution in Optimem
- Allow the PEI and DNA to form a complex at RT for 20 min
- Add to cells in a total volume of 20ml medium per 15 cm dish
- 48 h after transfection, collect the supernatant in 50ml conical tubes (this supernatant contains a lot of virus)
- Centrifuge in a swinging bucket rotor at 300 x g for 5 min to remove cells and cell debris. This “first batch” virus collection can be stored at 4°C
- Add fresh, complete DMEM medium to the cells to enable collection of a second batch of virus
- After 24h, collect and pool the supernatants again from the 15cm dishes and centrifuge as above. This is the “second batch” of virus from the same cells
- Pool first and second batches of lentivirus supernatants and filter through a 0.45µm filter
- Freeze down the lentivirus supernatant as 5ml and 10ml aliquots in 15ml conical tubes at -80°C for long term storage

Titration of the gRNA lentiviruses

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- Plate 2 x 105 3T3-Cas9 expressing cells into 24 wells of four 6-well plates at 9AM and place them in the CO2 incubator (16 wells are used for virus titration and the others are used as puromycin controls or to count cells at the time of infection)
- Gently thaw frozen 5ml lentivirus stock in a warm water bath
- While the cells are attaching, perform 2-fold serial dilutions of the lentivirus stock in 2ml microfuge tubes:

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- To each 2ml of virus dilution, add polybrene to achieve a final concentration of 5µg/ml
- By 4PM, cells will have attached. Add each of the virus dilutions to one well of each well in a 6 well plate. (This will require 8 wells total; 4 wells do not receive virus and are used as controls.)
- After 48 h, lentivirus infected cells should be ~70-80% confluent and ready to split
- Split the cells from each well of a 6-well plate into 2 x 6cm dishes (this is equivalent to a 1:4 split)
- Add 1µg/ml puromycin to one set of 6cm dishes and use the other set of 6cm dishes as controls
- Cells infected with the virus will be resistant to puromycin and should survive 1µg/ml puromycin; the rest of the cells will die
- After 48 h puromycin selection, uninfected cells should show 0% survival
- In no-puromycin control dishes, 100% cells should survive
- At this point, cells from each condition - with or without virus, with or without puromycin, should be counted
- Use a hemocytometer or automated cell counter to count of number of surviving cells
- Virus per µl quantitation is estimated as follows:

For “V” volume of the virus infected into “N” cells,

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[(Number of cells surviving puromycin) / (Number of cells surviving without puromycin) * N]/ V
For eg. 0.25ml (V) virus was added to 2 x 105 (N) cells. After 48h of splitting that well into 2 x 6cm dishes, there were 5 x 105 cells with puromycin and 15 x 105 cells without puromycin
[(500000) / (1500000) * 200000] / 250 = 266 cells survived per µl virus

- Determine estimates for every dilution and take the mean of the number of cells that can be infected per µl of the virus per condition

- For the next steps, the virus should be diluted such that the number of virus particles is 1/5th of the number of cells to be infected.

Infect 3T3-Cas9 cells and select infected cells

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- When cells are 70% confluent, trypsinize, resuspend and pool cells from all 10 x 15cm dishes

- Expand 3T3-Cas9 cells by plating 5 x 106 cells / plate onto 40 x 15 cm dishes in DMEM medium with 10% FBS (total of 200 million cells)

- Mix virus volume equivalent to 40 x 106 virus particles with polybrene to a final concentration of 5µg/ml and distribute this in equal volume to the 40 plates

- Ensure that cells are evenly plated as single cells to avoid clumps during infection

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- 48h after adding the virus, cells will have grown to >80% confluency and virus infection is complete

- Trypsinize, resuspend and split cells into 80 x 15 cm dishes with 1µg/ml puromycin to ensure efficient puromycin selection

- Along with the infected 40 plates, plate a control dish of cells not infected with lentivirus

- 48-72h later, the control plate should be 100% dead. The 80 x 15cm dishes should be 30% confluent

- Refresh medium with fresh 1µg/ml puromycin and leave for another 3 days

- By day 4 post puromycin selection, cells should be growing with some additional cell death and ~60% confluency

- On day 5, cells should be >80% confluent with cell death; at this point puromycin is no longer needed

- Freeze down all Puromycin resistant cells in cryo-vials (10-20 million cells per vial)

Freezing medium contains DMEM, 20% serum and 10% DMSO

Fix, stain and sort cells

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- Thaw 40 x 106 cells and plate onto 14 x 15 cm dishes and culture in DMEM with 10% FBS without puromycin (and one extra 6cm dish as well)

For 80,000 gRNAs, 40 million cells equates to a theoretical 500 fold representation of each guide in the original library

- After 24 h, change medium to remove floating and dying cells

There is ~20% cell death due to freezing, reducing the library fold representation down to ~320 fold.

- 72 h after plating cells, treat the extra 6cm dish with 200nM MLi-2 for 4 hours

- Trypsinize cells from all 15 cm dishes and pool (total volume ~100ml)

- Separately trypsinize the 6cm dish with MLi-2 as a no-pRab10 signal control

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It is very useful to get an accurate cell count

- Expect ~90-100 million cells from 14 dishes after 72 h post plating
- MLi-2 treated cells should also be trypsinized, resuspended and processed in parallel while maintaining them in 200nM MLi-2
- After resuspending trypsinized cells in complete medium, pellet and wash the cells once with 25ml 1X HBSS and resuspend the pooled cells to 4 million cells per ml
- Fix the cells by adding 10ml of 6% PFA in HBSS to the 10ml of cell suspension (final concentration 3% from a 16% methanol-free paraformaldehyde)
- Mix cells gently and incubate on a rotator for 30 min at room temperature
- Centrifuge the cells for 5 min at 300 x g in a swinging bucket rotor at room temperature
- Discard the supernatant and wash the pellet 3X with 20ml HBSS
- Permeabilize the cells with 0.2% Saponin, 2% BSA and 5% Bovine calf serum for 30 min at room temperature
- Wash the cells by centrifuging at 350 x g for 5 min and resuspending them in 20ml HBSS containing 2% BSA and 5% Bovine calf serum
- Incubate cells with rabbit anti-pRab10 primary antibody diluted 1:1000 overnight at 4˚C on a rotator
- Wash cells twice with 10ml blocking solution by centrifuging cells at 350 x g for 5 min
- Incubate the cells with Donkey anti-rabbit Alexa 647 diluted 1:1000 for 2 hours at room temperature
- After 2h, wash cells twice with 25ml of 2% BSA in PBS
- Finally resuspend the cells in flow cytometry tubes at 2 million cells per ml

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- Check whether cells are stained, put 20µl on a slide with a coverslip and view under a microscope; if no staining is seen, it is still possible to add more second antibody

- Cells appear round and pRab10 signal is visualized as a peri-centriolar vesicle accumulation. Occasionally spots in the nuclei can be observed

- MLi-2 treated cells should have very little to no signal

- An aliquot of cells not treated with secondary antibody serves as a useful control “blank” during flow cytometry

- On the Sony cell sorter, acquisition settings at FSC-1, BSC-25%, 45% EM-gain to detect pRab10 work well

- Make gates to identify a single cell population and highest 7.5% pRab10 signal (H-gate) and lowest 5% signal (L-gate) using a histogram with the Alexa-647 signal on the X-axis

- Pass cells at 3000 events per second, and sort cells from the H-gate and L-gate into two different 15ml tubes until 30 million cells have passed through the sorter

- Repeat the process with an independent second sample as a second replicate

- Keep ~ >6 million cells as “unsorted” from each sorting experiment

- Adjust the sample pressure and cell density to obtain at least 2 million sorted cells per condition for each gate

- Spin down the collected cells at 400 x g for 10 min, wrap the tubes with Parafilm and freeze down the cells for genomic DNA isolation

Genomic DNA isolation of fixed cells

7 See dx.doi.org/10.17504/protocols.io.eq2lynm9qvx9/v1

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PCR of the genomic DNA

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- Genomic DNA is next PCR amplified using primers designed for Illumina sequencing
- Make a “Forward primer mix” containing equimolar concentrations of all forward primers that have the P5 flow cell attachment sequence, Illumina read 1 sequence, stagger and the vector binding sequence
- Reverse primer contains the P7 flow cell attachment sequence, Illumina read 2 sequence, an 8mer Next-seq Index and the vector binding sequence
- For every sample, a unique reverse primer is used for PCR

- Number of PCRs performed per sample depends on the yield of the genomic DNA from that sample
- Perform as many PCRs as are needed to cover the entire genomic DNA yield. For example, for High-replicate-2, if 5µg gDNA is obtained, perform 25 PCRs in 50µl volume, each containing 200ng DNA to obtain complete coverage of the entire sample

- For the PCR cycling, these conditions are recommended:

- A single band at ~360bp should be visible with a hazy, lower primer band
- Lowering the annealing temperature <55˚C may yield multiple bands with this polymerase kit

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PCR purification and pooled for sequencing

9 PCR product clean up is performed using Ampure XP Reagent (Beckman Coulter) bead clean-up

- Dilute PCR product 1:1 with water prior to bead addition
- Mix the beads vigorously and bring to room temperature

The desired product is ~360bp. A two step purification is carried out to remove contaminants >500bp and <200bp.

>500bp band removal:

- Add the beads at a 0.55X vol/vol of the PCR products and mix by pipetting. Thus, for 100µl of the PCR product, add 55µl beads to bring total volume to 155µl
- Let the beads bind the DNA for 5 min
- Hold the tubes on a magnetic stand until the beads move to the side of the microfuge tube
- High molecular weight bands are bound to the beads and stick to the side of the wall because of the magnet
- Collect the supernatant and transfer to a fresh, clean microfuge tube

<200bp band removal: retain bead bound product:

- Bring the final vol/vol, beads to sample ratio to 0.9x. As an example, if we have ~150µl supernatant left after beads are removed in the first step, add 0.9x of 150µl = 135µl of beads
- Let the beads incubate with the sample for 5 min and place on the magnetic stand
- After 5 min, beads will bind to nucleic acids longer than 200bp while those lower than 200bp such as primers and primer-dimers will be left in the supernatant
- Discard the supernatant (containing smaller DNA species) and add 200µl of molecular biology grade 75% Ethanol to wash the beads 2X
- Place the samples on the magnetic stand, get rid of trace ethanol from the final wash and let the beads air-dry for 5 min
- Elute the desired PCR product bound to beads (>200bp and <500bp) by adding 25µl warm, nuclease free water
- After 5 min, place the beads with water on the magnetic stand to separate the beads and the liquid. This is the final purified and cleaned PCR product
- Run the samples on a 2% agarose gel to ensure that the PCR product is pure and concentrated
- Obtain the concentration of the DNA using a Qubit assay (Thermo Fisher Q33220) with a high-sensitivity kit
- Confirm the concentrations and the library sizes using an Agilent Bioanalyzer or tapestation capillary electrophoresis with a high-sensitivity kit
- Get accurate Qubit and Bioanalyzer data for all the barcoded PCR products
- Pool all the PCR products such that each barcoded sample is represented equally; all PCR libraries should be at an equimolar concentration (at least 2.5nM)
- This single pooled sample can now be sequenced using Illumina sequencing technologies such as Miseq or Hiseq to determine the genes that when knocked out, change phosphoRab10 levels in 3T3 cells.
- Sequence data was analyzed using MAGeCK software (2) for statistical analysis. Primers for

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amplification and sequencing are available (3).

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