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Validation in mammalian NanoLuc Two-Hybrid

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We are still developing and optimizing this protocol

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Abstract

Assessment of the impact of missense variants on protein-protein interactions

Cloning into N2H vectors

1 **LR reaction**

- Put all plasticware (PCR plates, reservoir, tubes) On ice before starting.
- All reagents should be thawed On ice, thoroughly vortexed and briefly spun to remove any liquid from the cap.
- Assemble the following master mix:

	Reagent	Stock concentration	Final concentration	Volume (uL)/reaction	Volume (uL)/100 reactions
	TE buffer, pH 8.0	/	/	1	100
	Gateway™ LP Clonase™ II Enzyme mix	10x	1x	1	100
	Destination vector	100 ng/uL	20 ng/uL	1	100

LR master mix.

- Vortex the master and transfer into a pre-chilled reservoir.
- Aliquot 3 µL of the master mix into a new PCR plate using a automatic multichannel pipette.
- Using a multichannel pipette, add 2 µL of the purified entry products to the plates preloaded with the LR master mix.
- Seal the plates and spin 00:05:00 at 1000 rpm.
- Incubate the plates at 25 °C Overnight. After incubation, BP reaction plates can be stored at -20 °C.

2 **Transformation into DH5a cells**

- Put all plasticware (PCR plates, reservoir, tubes) On ice before starting.
- Thaw *E. coli* DH5a cells On ice.
- Gently mix tube by inverting (do not vortex!) to homogenize the suspension.
- Transfer cells to a pre-chilled reservoir and aliquot 10 µL of cells into each well of a pre-chilled empty PCR plate using an automatic multichannel pipette.
- Add 3 µL of BP product into the cell suspension using a multichannel pipette.
- Mix the cells by swirling the pipette tips in the wells after dispensing.

- Incubate On ice for 00:30:00 .
- Using a 96well heating block, pre-heated at 42 °C , heat-shock the cells for 00:00:45 .
- Immediately return the cells On ice for 00:02:00 .
- Carefully add 90 µL of complete SOC media to each well using an automatic multichannel pipette.
- Seal the plate with an airpore tape and incubate at 37 °C for 01:00:00 .
- Fill a costar plate with 120 µL of LB media containing [M] 100 µg/mL ampicillin.
- Transfer 10 µL of the recovered cells into the costar plates and seal with airpore tape.
- Incubate at 37 °C Overnight .

3 **Plating and spreading of bacterial culture**

Liquid bacterial cultures are spread onto solid selective media to isolate single colonies. This was conducted on 15 cm diameter petri dishes with a Tecan Evo robot, using a custom program.
Importantly, glycerol stocks of the liquid cultures are generated, in two copies, by combining 40 µL of [M] 40 Mass Percent sterile glycerol and 40 µL of overnight culture. These glycerol stocks are sealed with aluminum foil and stored at -80 °C .

4 **Colony picking and isolation**

- Fill costar plates with 150 µL LB media supplemented with [M] 100 µg/mL ampicillin.
- One colony is picked and isolated by hand with sterile wooden toothpicks from each spot on solid media and transferred into the costar plates prefilled with LB+ampicillin.
- Seal plates with airpore tape and incubate at 37 °C Overnight .
- The next day, make glycerol stocks, in three copies, by combining 40 µL of [M] 40 Mass Percent sterile glycerol and 40 µL of overnight culture.
- Seal plates with aluminum foil and store at -80 °C .

5 **DNA purification**

- Fill deep well plates with 1.2 mL LB media supplemented with [M] 100 µg/mL ampicillin.
- Inoculate 10 µL glycerol stocks.
- Seal plates with airpore tape and incubate at 37 °C Overnight .
- The next day, spin for 00:15:00 at 2000 rpm , or until cells form a pellet.

- Discard supernatant.
- DNA is extracted using a QIAGEN BioRobot 8000.

Plasmid pool sequencing

6 To confirm the successful transfer of the desired DNA insert from the entry vector into the destination vector, all purified DNA samples are sequenced. A pooled sequencing strategy is employed on the Illumina platform, where each pool contains one clone from different genes to ensure reliable identification.

6.1 **Preparation of DNA pools**

- Measure the concentration of each 96-well (containing DNA pool).

*For this step, a Qubit dsDNA BR Assay Kit (catalog: Q32851) suffices.

- For each well, take an aliquot and dilute with PCR grade water in a new 96-well plate (label “sample plate”) to the required concentration of [M] 15 ng/µL

6.2 **Nextera Oligo Setup**

Pre-mix i5 and i7 oligos for a concentration of [M] 10 micromolar (µM) (see attached sheets)

6.3 **Illumina Tagment MasterMix prep**

	Reagent	1X (single reaction)	100X (one full plate)
	Tagment DNA (TD) buffer	2µL	200µL
	PCR grade water	6.5µL	650µL
	Tagment DNA Enzyme (TDE1; catalog: 20034197) *	0.25µL	25µL

*Keep @ -20 °C, or on ice when in use!

- Use a multichannel pipette to add 8.75 µL MasterMix to each well of a 96-well plate (label “reaction plate”)
- Use a multichannel pipette to add 1.25 µL DNA ([M] 15 ng/µL ; from the sample plate) to each corresponding well of the reaction plate
- Gently pipette to mix
- Cover the reaction plate with a BioRad PCR plate seal
- Incubate the reaction plate for 00:10:00 @ 55 °C in a thermal cycler
- After, immediately place the reaction plate on ice
- Proceed immediately with the PCR

6.4 **PCR amplification of tagmented DNA**

Using a new 96-well plate (labeled amplification), add the following:

	A	B
	Reagent	1X (per well)
	2X Phusion MasterMix (catalog: M0530S)	10µL
	i5 & i7 oligo (10uM; see above/attached sheet)	2µL
	Tagmented DNA	8µL

- Using a multichannel pipette, pipette gently to mix
- Cover with a BioRad plate seal and placed in a thermal cycler

	Cycle(s)	Time	Temp
	1	3min	72°C
	10	10sec	98°C
		30sec	56°C
		3min	72°C

Thermal cycler Program

- Check 10 random wells confirming that the PCR worked by running 2 µL of each on a 1% EX e-gel along with a 1kb ladder.
- See a smear from ~100bp to ~5kb and each lane should have roughly equal amounts of product.

6.5 **Pool amplified DNA**

Using a multichannel, pool all wells (10 µL from each well)

6.6 **Purifying for sequencing**

- Vortex to mix the pooled, amplified DNA
- Load all wells of a single 1% EX e-gel from ThermoScientific (one plate per gel; catalog: A42100)
- Run using 50V until the 100bp band (1kb+ DNA ladder, catalog: N0469S) reaches the bottom of the gel.
- Cut the smear from 500bp-800bp
- Purified using the Qiagen gel purification kit (catalog: 28604)
- Elute the library in 15 µL
- Measure concentration using Qubit dsDNA HS Assay Kit (catalog: Q33230)

mN2H

2d 0h 30m 2s

7 **Cell plating** 1d

- Seed HEK293T cells at 54,000 cells in 100 µL in a 96-well white Greiner plate
- Incubate at 37 °C 24:00:00

8 **Transfection** 1d 0h 30m

- Prepare a DNA mix of 100 ng of each partner and up to 12 µL of DMEM per well
- Prepare a PEI mix with 0.6 µL of PEI per well and 7.4 µL of DMEM per well
- Mix PEI and DNA mixes and incubate at Room temperature for 00:30:00
- Add the 20 µL of DNA/PEI mix to each well
- Incubate at 37 °C for 24:00:00

9 **NanoLuciferase Reading** 2s

- Prepare a mix of 1/100 dilution of Furimazine in 1X homemade NanoGlow buffer
- Remove cellular supernatant from the 96-well culture plate
- Add 50 µL of diluted substrate to each well
- Measure activity on CentroXS Berthold for 00:00:02 per well