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Microscale Thermophoresis determination of Rab29 binding to LRRK2 Armadillo Domain

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ABSTRACT

Several labs have shown that Rab29 GTPase can recruit LRRK2 to the surface of the Golgi complex. We describe here a method to monitor direct binding of Rab29 to the LRRK2 N-terminal Armadillo domain using Microscale Thermophoresis. This method utilizes purified, His-tagged LRRK2 Armadillo residues 1-552-labeled with NanoTemper Second Gen NHS-Red 647 dye and bacterially expressed Rab29 protein. Binding is detected using a Nanotemper Monolith NT-115 instrument.

ATTACHMENTS

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MATERIALS TEXT

Materials:

- Protein LoBind Tubes Protein LoBind® 0.5 mL PCR clean colorless 1 bag × 500 tubes **Eppendorf Catalog #0030108434**
- Eppendorf LoRetention tips 0.1-10uL **Catalog No. 022493018**
- pET21b HisSumo Rab29 full length wild type (tag is cleaved off)
- Labeled Armadillo should be used fresh or within ~2 days if kept on ice in cold room. From previous experience, freeze thaw cycles result in loss of protein amount and stability (varies depending on mutant/truncations)
- Every time any protein is thawed, spin hard at **14000 x g** for **00:10:00** at **4 °C** followed by measurement of A280 and protein concentration determination using extinction coefficients:
  WT Rab29 full length, Cys fully reduced 41940.00 M^{-1} cm^{-1}
  His LRRK2 Armadillo, Cys fully reduced 37930.00 M^{-1} cm^{-1}
- Range of unlabeled protein to titrate can be estimated if there is any estimation of KD expected. For example, for a **600 Nanomolar (nM)** KD, we use an unlabeled protein range of **19 Micromolar (µM)** - **40 Nanomolar (nM)** , 2:1 dilution series. There is software provided by Nanotemper to optimize the range.
- Unlabeled Rab29 is titrated against a fixed concentration of fluorescently labeled Armadillo (**100 Nanomolar (nM)** final in reaction); 16 serially diluted titrations of the unlabeled protein partner are prepared to generate one complete binding isotherm.

Labeling Buffer:

A	B
Hepes pH8	50 mM
NaCl	100 mM
MgCl2	5 mM
GTP	20 uM
TCEP	0.2 mM
Glycerol	5%

Binding Buffer:

A	B
Hepes pH8	50 mM
NaCl	100 mM
MgCl2	5 mM
GTP	20 µM
TCEP	0.2 mM
Glycerol	5%
BSA	5 µM
Tween-20	0.01%

Buffer exchange Proteins

1 Using either Nanotemper “A-Column” provided in labeling kit or (our preference) **0.5 mL** Zeba Spin column 7MWCO. Twist off bottom of column and loosen cap.

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2 1m

Prespin column: Centrifuge at **4 °C** **1500 x g** for **00:01:00** to remove storage solution.

3 1m

Apply **300 µl** of your labeling buffer to center of resin bed and centrifuge at **1500 x g** for **00:01:00** . Do this 4X.

4 2m

Place column in fresh collection tube. Apply **100 µl** of your sample to resin and spin at **1500 x g** for **00:02:00** .

5 Collect the protein flowthrough.

Labeling of LRRK2 Armadillo

6 Do the labeling with 2nd Generation NHS RED label from Nanotemper.

7 Resuspend the dye in **25 µl** DMSO as per protocol from Nanotemper to make it **600 Micromolar (µM)** final stock concentration.

8 Set up **100 µl** labeling reaction using a 3:1 ratio of dye to protein; use the dye at final concentration of **30 Micromolar (µM)** ; Buffer-exchanged Armadillo is used at a final concentration of **10 Micromolar (µM)** .

> Note: reducing reagent will interfere with labeling. Low concentration TCEP is acceptable

9

Add above buffer to bring volume to **100 µl** final volume, mix by flicking tube.

10 30m

Incubate for **00:30:00** in dark at **Room temperature** .

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11 Desalt excess dye using column B provided in kit or another Zeba Spin column as before.

12 

10m

Spin labeled sample hard **14000 x g** for **00:10:00** at **4 °C** to remove any aggregates.

13 Take absorbance **280 nm** of labeled protein and use extinction coefficient and the Nanotemper Degree of Labeling (DOL) calculator to determine concentration and DOL https://nanotempertech.com/dol-calculator/. DOL should be between 0.5-1.

Binding Reaction 35m

14 In **20 µl** , prepare **600 Nanomolar (nM)** Armadillo-647 from labeled stock, BSA to final concentration of **5 Micromolar (µM)** and Tween-20 to 0.01%.

15 18x **0.5 mL** Lobind tubes are set out in tube rack and label with final concentrations of Rab29 (#1-16), Test (initial fluorescence test #17), and **600 Nanomolar (nM)** Armadillo-647 (no Rab) (#18).

16 

Pipet **5 µl** binding buffer into bottom of Tubes 2-17. Make sure to cap, to prevent evaporation.

17 

To make 2:1 dilution series; Add **15 µl** of unlabeled Rab29 to Tube 1. Pipet up and down and then take **10 µl** from Tube 1 and add to Tube 2. Pipet up and down slowly 5 times, being careful to hold tip at the top of volume, raising and lowering it, to avoid any of solution sticking to outside of tip (try to keep trituration number consistent with all samples). Repeat by taking **10 µl** of Tube 2 and add to Tube 3. Continue until Tube 17. Use the same tip the whole way. Do best to avoid solution sticking to inner Lobind tube walls. At end, your dilution series should have **5 µl** in each sample.

18 

Add **1 µl** of **600 Nanomolar (nM)** Armadillo to each **5 µl** sample for a final volume of **6 µl** . Triturate each 5-10 (again be consistent across samples; change tips for each tube.

19 

30m

Quick spin each tube (1-16) and incubate in the dark for **00:30:00** at **Room temperature** to allow binding.

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20 While samples are incubating, take Test (tube 17), and load into a NT.115 premium treated capillary. Set into position 16 of monolith sample tray and hit “Start Cap Scan” to measure initial fluorescence. Adjust LED power to achieve a range of 800-1200.

21 Set up MST project settings, usually for a new binding experiment we do 2x 20% MST, 2x 40% MST, and 2x 60% MST. 5 sec before fluorescence before MST, 30 sec MST, 2 sec fluorescence after MST, and 2 sec delay. Enter protein titration concentrations with dilution ratio.

22  5m

After 30 min, hard spin tubes #1-16 at **14000 x g** for **00:05:00** at **4 °C** . Load into NT.115 premium treated capillaries (Nanotemper Technologies).

23 Place capillaries into machine and press “Start Cap Scan + MST measurements.

Data analysis is performed with NTAffinityAnalysis software (NanoTemper Technologies) in which the binding isotherms are derived from the raw fluorescence data and then fitted with both NanoTemper software and GraphPad Prism to determine the K_D using a non-linear regression method. The binding affinities determined by the two methods are similar.

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