



Determination of Nutrient Ligand-Sensor Binding Affinity

Xin Gu

Abstract

Cells contain dedicated mechanisms to sense nutrient levels in the environment to regulate their growth by balancing anabolism and catabolism [1, 2]. The mechanistic Target of Rapamycin Complex 1 (mTORC1), a multi-protein kinase complex, serves as an essential growth regulator that integrates various upstream inputs including growth factors and nutrients like amino acids [1, 2]. Nutrient sensors upstream of mTORC1 directly bind cognate nutrient ligands to convey their availability and thereby regulate mTORC1 signaling [1, 2]. A reliable method is needed to quantitatively determine the binding affinity (K_d) of the nutrient sensor to its ligand. In parallel, quantitative metabolomic analysis can reveal metabolite levels in fed and starved cells; which represent the physiological range of the nutrient of interest. Whether or not the binding affinity is within the physiological range serves as an indicator to determine the physiological relevance of the sensing mechanism. This chapter describes a generalizable protocol that allows reproducible determination of nutrient ligand-nutrient sensor binding affinity. Here, the S-adenosylmethionine (nutrient ligand)-SAMTOR (nutrient sensor) pair is used as an example [3]. Nutrient sensor purification, radioactive nutrient ligand incubation, and eventual scintillation counting are included, along with a description of the mathematical equation that is used to calculate the binding affinity.

Key words mTORC1, Nutrients, Nutrient sensors, Radioactive ligands, Protein purification, Affinity beads, Scintillation, Competitive binding assays

1 Introduction

The mTORC1 protein kinase complex is an essential component of a pathway that regulates anabolic and catabolic processes in response to various environmental cues including growth factors and nutrients like amino acids [1, 2, 4]. Nutrients activate mTORC1 by promoting its translocation to the lysosomal surface where its activator, Rheb GTPase, resides [4]. This localization is dependent on the nucleotide state of the heterodimeric Rag GTPases, which are comprised of RagA or RagB bound to RagC

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or RagD [5, 6]. Two stable Rag GTPase nucleotide states exist, one is inactive under nutrient deprivation where RagA/B is GDP bound and RagC/D is GTP bound, the other is active under nutrient repletion where RagA/B is GTP bound and RagC/D is GDP bound. Multiple protein complexes serve as nutrient-sensing core machinery to regulate the nucleotide states of the Rag GTPases, each probably conveying a distinct nutrient input [7–12].

Among all proteogenic amino acids, leucine, arginine, and methionine have established cytosolic nutrient sensors to convey their availability to regulate mTORC1: Sestrin proteins for cytosolic leucine sensing [13, 14], Castor proteins for cytosolic arginine sensing [15, 16], and SAMTOR for cytosolic methionine sensing [3]. Cognate nutrient ligands regulate the interaction between these sensor proteins and the nutrient-sensing core machinery complexes [3, 14, 16]. For example, S-adenosylmethionine (SAM, a direct downstream metabolite of methionine) is used by all methyltransferases in cells to achieve a universal post-translational modification, methylation; it disrupts the interaction between SAMTOR and the GATOR1-KICSTOR complex, likely by causing a conformational change of SAMTOR that precludes it from binding with GATOR1-KICSTOR, leading to mTORC1 activation (Fig. 1) [3].

To determine whether the sensing mechanism through a given nutrient sensor is physiologically relevant, it is important to reliably measure the binding affinity between the nutrient sensor and the cognate nutrient ligand. Then the binding affinity can be compared with the metabolomic data from fed versus starved cells or animals that indicate the dynamic range of fluctuation for the nutrient

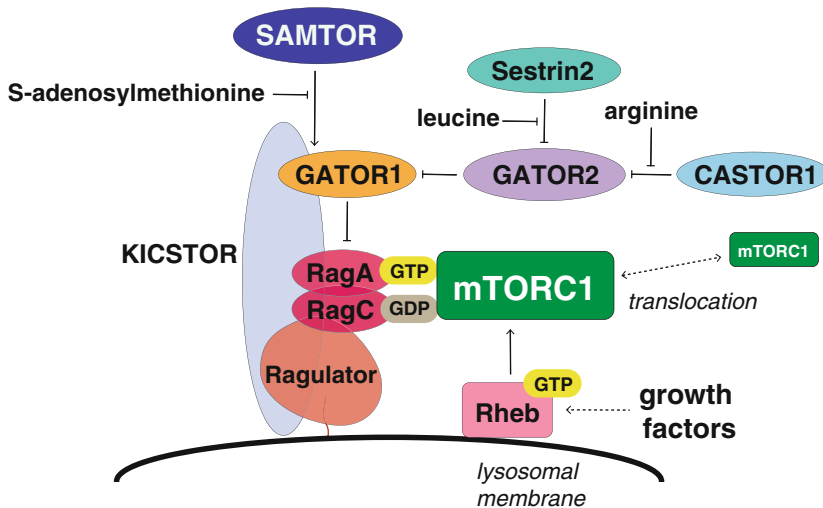


Fig. 1 Model depicting the nutrient-sensing pathway upstream of mTORC1. (Adapted from [3])

ligand. If the binding affinity lands within the fluctuation range, it is consistent with the sensor-ligand interaction being regulated by the physiological fluctuations of the ligand levels.

Radioactive molecules have been commonly used to measure ligand binding to corresponding sensors [17]. A radioactive ligand binding assay enables relatively accurate detection and measurement of low-binding-affinity ligand-sensor pairs, and it can be performed with relatively small quantities of protein [18].

There are three major types of radioactive ligand binding assay: kinetic, saturation, and competitive [17]. Kinetic radioactive binding assays are used to determine both the association and dissociation rates of a given ligand for a sensor [17]. Saturation radioactive binding assays are used to measure two parameters: the number of binding sites and the dissociation rate [19]. Competitive radioactive binding assays allow determination of the relative affinities of test ligands to a given sensor. This is done by incubating sensor with unlabeled test ligands at a range of concentrations and a radioactive ligand at a fixed concentration. This allows the measurement of the IC50 value of test ligands to inhibit the interaction between the radioactive ligand and the sensor competitively [20].

Technically, two types of competitive radioactive ligand binding assays are commonly used: filtration [21] and on-beads [22]. Filtration assays are usually utilized to determine affinities for membrane-bound sensors, because the membrane fractions in the mixture are easily trapped to the glass fiber filters. This type of assay typically incubates membrane homogenates with radioactive ligands and unlabeled test ligands. Once reaching equilibrium, sensor-bound radioactive molecules are trapped on the filter while the unbound radioactive ligands flow through. After scintillation counting to determine the amount of radioactivity present on the filter, the results can be used to obtain IC50 and K_d/K_i values. The on-beads assay bypasses the need to use filters for separating bound vs. unbound radioactive ligands. Instead, this type of assay uses beads to bind the sensor, then the beads-sensor mixture is incubated with radioactive ligands and unlabeled test ligands. After reaching equilibrium, radioactive ligands that are bound to the sensor on the beads elicit light emission which can be counted using a scintillation counter. This method can be used for both cytosolic sensors and membrane-bound sensors.

Here I describe an on-beads protocol for the radioactive ligand-binding scintillation assay implemented to quantitatively measure the interaction between SAMTOR and SAM (Fig. 2) [3]. The same method has been successfully used to measure the binding affinity between the cytosolic leucine sensor Sestrin1/2 and leucine [14], as well as the cytosolic arginine sensor Castor1 and arginine [16] previously. Two subtypes of protein isolation methods are described: the first one outlines larger scale isolation

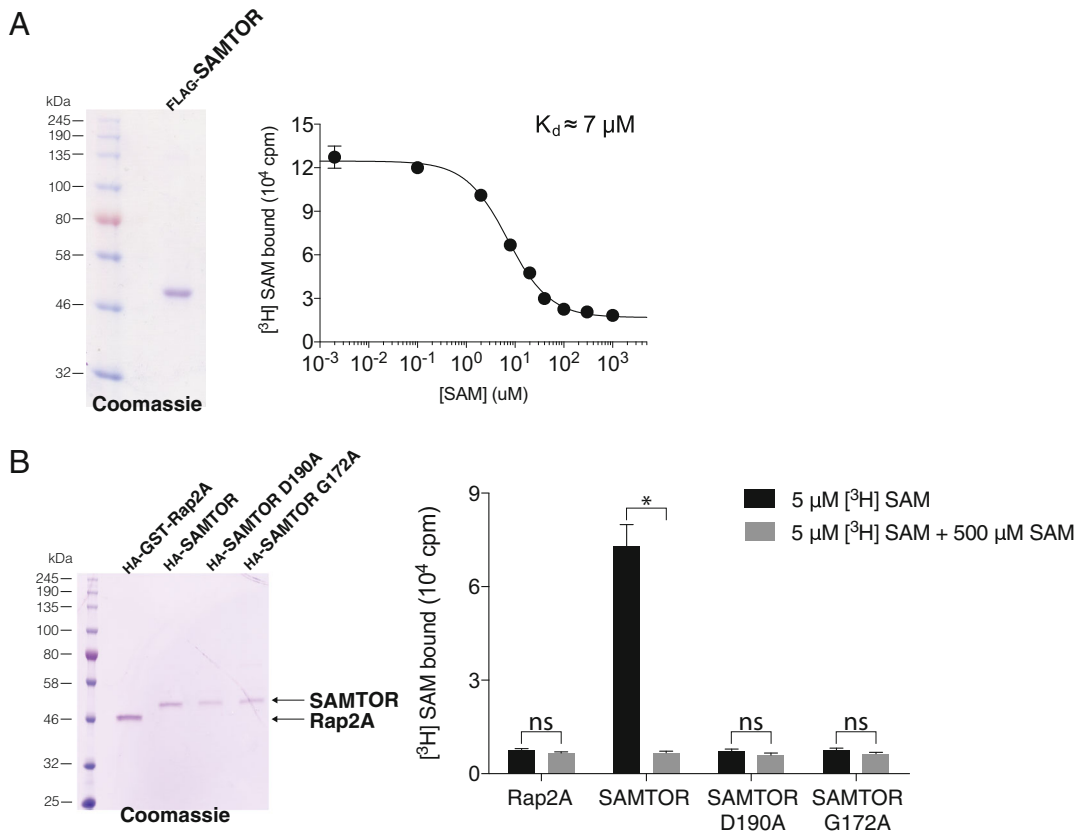


Fig. 2 The radioactive binding assay indicates that S-adenosylmethionine (SAM) binds SAMTOR. (a) SAMTOR binds SAM with a $K_d \sim 7 \mu\text{M}$. Left shows the SDS-polyacrylamide gel electrophoresis followed by Coomassie staining. Right are the results of the binding assays using the FLAG-SAMTOR protein incubated with indicated concentrations of radiolabeled SAM ($5 \mu\text{M}$) as well as unlabeled SAM (a range of concentrations: 0.002, 0.1, 2, 8, 20, 40, 100, 300, 1000 μM). Values are means \pm SD of three technical replicates from one representative experiment. (b) Wild-type SAMTOR but not G172A or D190A SAMTOR binds SAM. Left shows the SDS-polyacrylamide gel electrophoresis followed by Coomassie staining. Right is the result of the binding assay using the HA-tagged SAMTOR proteins, either the wild-type or the point mutants. Values are means \pm SD of three technical replicates from one representative experiment. (Adapted from [3])

suitable for K_d measurement, and the second one is suitable for testing whether multiple mutants of the protein of interest interact similarly to the wild-type protein without the need of large-scale protein purification.

2 Materials

2.1 Cell Culture and Transfection

1. HEK-293F cells (FreeStyle™ 293 Expression medium).
2. HEK-293 T cells (High-glucose Dulbecco's modified Eagle's medium, 1% penicillin-streptomycin antibiotics, 10% fetal bovine serum).

3. 0.4% trypan blue stain.
4. Mammalian expression plasmid for FLAG-tagged wild-type sensor of interest, e.g., pRK5-FLAG-SAMTOR for Subheading 3.1.1.
5. Mammalian expression plasmid for FLAG-tagged Rap2A (negative control), wild-type and mutant sensors of interest, e.g., pRK5 empty backbone vector (for normalization of total plasmid amount per condition), pRK5-FLAG-Rap2A, pRK5-FLAG-SAMTOR, pRK5-FLAG-SAMTOR G172A, and pRK5-FLAG-SAMTOR D190A for Subheading 3.1.2.
6. Polyethylenimine (PEI, 1 mg/mL stock solution, pH 7.0, filtered through 0.22 μ m filter, aliquot and store in -20°C).
7. Cell culture flasks and plates.
8. Cell counter.
9. Orbital shaker.
10. Cell incubator.

2.2 SAMTOR Protein Isolation

2.2.1 FLAG-Tagged Protein Isolation from HEK-293F Cells

1. 250 mL centrifuge bottles.
2. Centrifuge with rotor for 250 mL centrifuge bottles.
3. $1\times$ phosphate-buffered saline (PBS), sterile.
4. 15 mL & 50 mL falcon tubes.
5. Lysis buffer: 1% Triton \times -100, 40 mM Hepes pH 7.4, 150 mM NaCl, 2.5 mM MgCl_2 and EDTA-free protease inhibitor tablet from Roche (1 tablet per 50 mL lysis buffer).
6. High speed chilled centrifuge.
7. High-speed PPCO centrifuge tubes with sealing cap (50 mL).
8. M2 anti-FLAG agarose beads.
9. FLAG-peptide elution buffer: 40 mM Hepes pH 7.4, 150 mM NaCl, 2.5 mM MgCl_2 and 0.5 mg/mL $3\times$ FLAG peptide.
10. 10 kDa MWCO centrifugal filter.
11. Fast protein liquid chromatography (FPLC) Superdex S75 10/300 column.
12. Size exclusion chromatography running buffer: 20 mM Hepes pH 7.4, 150 mM NaCl, 1 mM DTT.
13. 4–12% Tris-Glycine polyacrylamide gel.
14. Tris-Glycine SDS running buffer.
15. Coomassie stains.
16. 2 mL centrifuge tubes.
17. BSA (molecular biology grade).

2.2.2 HA-Tagged Protein Isolation from HEK-293 T Cells

1. $1 \times$ phosphate-buffered saline (PBS), sterile.
2. 15 mL & 50 mL falcon tubes.
3. Lysis buffer: 1% Triton \times -100, 40 mM Hepes pH 7.4, 150 mM NaCl, 2.5 mM $MgCl_2$ and EDTA-free protease inhibitor tablet from Roche (1 tablet per 50 mL lysis buffer).
4. Plastic cell lifter.
5. High speed chilled centrifuge.
6. Anti-HA dynabeads.
7. BSA (molecular biology grade).
8. Magnetic rack.
9. HA synthetic peptide.
10. 4–12% Tris-Glycine polyacrylamide gel.
11. Tris-Glycine SDS running buffer.
12. Coomassie stains.

2.3 Radioactive S-Adenosylmethionine Binding Assay

1. Cytosolic buffer: 0.1% Triton \times -100, 40 mM HEPES pH 7.4, 10 mM NaCl, 150 mM KCl, 2.5 mM $MgCl_2$.
2. Binding wash buffer: 0.1% Triton \times -100, 40 mM HEPES pH 7.4, 300 mM NaCl, 2.5 mM $MgCl_2$.
3. Tritiated S-adenosylmethionine (SAM) ligand (e.g., American Radiolabeled Chemicals).
4. Unlabeled S-adenosylmethionine (SAM) ligand.
5. Opti-Fluor scintillation fluid.
6. Scintillation vials (6.5 mL, with push-on/twist-off caps).
7. TriCarb scintillation counter.

2.4 Calculating the K_d

1. GraphPad Prism software.

3 Methods

3.1 Cell Culture and Transfection

Two separate methods are included under Subheadings 3.1 and 3.2 for different purposes. Subheadings 3.1.1 and 3.2.1 are for expression and purification of FLAG-tagged proteins in HEK-293F cells for large-scale protein preparation, which is needed for an accurate K_d measurement. Subheadings 3.1.2 and 3.2.2 are for small-scale expression and isolation of HA-tagged wild-type and mutant proteins in HEK-293 T cells, representing a scalable and simple protocol to quickly test whether mutated sensor proteins still bind the nutrient ligand or not. Both assays need to be performed to demonstrate that a sensor protein indeed binds a nutrient ligand.

3.1.1 *Expression of FLAG-Tagged Proteins for Purification from HEK-293F Cells*

1. Thaw a frozen vial of suspension HEK-293F cells into cell culture flasks with culture medium and grow them at 37 °C, in a humidified atmosphere with 8% CO₂, 125 rpm in orbital shakers. Passage the cells as needed to obtain the desired quantity for seeding for further transfection (*see Note 1*). Here we will use 1.25×10^9 cells.
2. On the day of the experiment, prepare 500 mL of fresh medium and dilute 1.25×10^9 cells to 2.5×10^6 cells/mL. Keep culturing the cells in a 3 L culture flask to ensure enough room for efficient shaking.
3. To prepare the transfection mixture, pipette 500 µg of pRK5-FLAG-SAMTOR cDNA plasmid into a 50 mL falcon tube.
4. In the sterile TC hood, add 25 mL of DMEM (no serum) medium.
5. Pipette up and down several times to mix.
6. Add 1.5 mL PEI directly to the middle of the plasmid-containing DMEM medium; avoid touching the side of the plastic tube (*see Note 2*).
7. Briefly vortex for 5 s, and then incubate at room temperature for 15 min.
8. Bring the culture flask that contains the 500 mL of HEK-293F cells to the sterile TC hood (*see Note 3*).
9. Pipette the plasmid-PEI-DMEM mixture into the flask.
10. Shake the flask well to ensure homogenous mixing, then put it back to the shaking incubator.
11. Proceed to Subheading 3.2.1 for protein purification instructions.

3.1.2 *Expression of HA-Tagged Proteins for Immunoprecipitation from HEK-293 T Cells*

1. Thaw a frozen vial of adherent HEK-293 T cells into cell culture dishes with culture medium and grow them at 37 °C, in a humidified atmosphere with 5% CO₂, in typical cell incubators. Passage the cells as needed to obtain desired quantity for seeding for further transfection (*see Note 4*).
2. Seed 6×10^6 HEK-293 T cells per plate for an appropriate number of 15 cm culture plates. For example, for the experiment to determine whether two SAM-binding mutants of SAMTOR still bind with SAM, a negative control plasmid expressing HA-tagged Rap2A, the plasmid expressing HA-tagged wild-type SAMTOR, and the plasmids expressing HA-tagged G172A or D190A mutant SAMTOR, were used. To obtain enough material for the radioactive binding assay, four 15 cm plates per type of construct (i.e., 16 plates in total for the above example) were seeded (*see Note 5*).

3. One day after seeding the cells, prepare the transfection mixture: pipette 12 μg Rap2A, or 15 μg SAMTOR wild-type or mutants, with empty pRK5 (backbone) vectors to make up to 20 μg plasmid per plate into a 1.5 mL Eppendorf tube (*see Note 6*).
4. In the sterile TC hood, add 700 μL of DMEM (no serum) medium.
5. Pipette up and down a couple of times to mix.
6. Add 60 μL PEI directly to the middle of the plasmid-containing DMEM medium, avoid touching the side of the plastic tube.
7. Briefly vortex for 5 s, and then incubate at room temperature for 15 min.
8. Bring the HEK-293 T cell plates to the sterile TC hood.
9. Pipette the plasmid-PEI-DMEM mixture onto the cells in a drop-wise manner (*see Note 7*).
10. Rock the culture plates back and forth well to ensure homogeneity, then put them back into the incubator.
11. Proceed to Subheading 3.2.2 for protein immunoprecipitation instructions.

3.2 SAMTOR Protein Isolation

3.2.1 FLAG-Tagged

SAMTOR Protein

Purification from HEK-293F Cells

1. 72 hours after transfection, transfer the cells to multiple 250 mL centrifuge bottles.
2. Spin at 300 g for 5 min at room temperature to pellet the transfected cells.
3. Aspirate the culture media without disturbing the cell pellets.
4. Resuspend the cell pellets in cold PBS and pool them together in two 50 mL falcon tubes.
5. Spin at 300 g for 5 min at 4 °C to pellet the cells.
6. Aspirate the PBS without perturbing the cell pellets.
7. Add 50 mL of lysis buffer. Pipette up and down until all cell pellets are resuspended into the lysis buffer, and no visible cell clumps remain.
8. Put the falcon tube on a shaker to continue lysing the cells for 30 min at 4 °C.
9. Transfer the cell lysates to 50 mL high-speed centrifugation compatible tubes.
10. Spin at 21,000 g for 20 min at 4 °C.
11. Transfer the clarified cell lysates to a 50 mL falcon tube without perturbing the bottom pellet (*see Note 8*).
12. During the centrifugation at **step 10**, place 2 mL of M2 anti-FLAG affinity bead slurry in a 15 mL falcon tube.

13. Centrifuge the tube at 2000 g, 4 °C to spin down the beads.
14. Aspirate the bead-storage buffer that contains glycerol. Add 8 mL of the cell lysis buffer to the falcon tube. Close the lid and mix the beads with the buffer by inverting 5 times (*see Note 9*).
15. Repeat **steps 13** and **14** two more times, then add lysis buffer to make the total volume 2 mL.
16. Add the 2 mL washed beads to the cleared cell lysate, and incubate with constant shaking at 4 °C for 2 h.
17. After the two-hour anti-FLAG immunoprecipitation, spin down the cell lysates with FLAG beads at 2000 g at 4 °C for 3 min.
18. Aspirate the cell lysates without perturbing the spun down beads.
19. Resuspend the beads with 1.8 mL lysis buffer containing 500 mM NaCl, transfer to a 2 mL Eppendorf tube.
20. Centrifuge the tube at 2000 g for 2 min to spin down the beads at 4 °C.
21. Aspirate the supernatant without perturbing the beads, then add 1.8 mL lysis buffer containing 500 mM NaCl again to resuspend the beads.
22. Repeat **steps 20** and **21** two more times for a total of four washes, then elute the bound proteins with 5 mL of the FLAG peptide-containing elution buffer for 1 h at 4 °C.
23. Spin down the elution mixture at 2000 g at 4 °C for 2 min, then pipette the liquid that contains the eluted protein without taking the beads or run the elution-beads mixture through a bead-trapping column at 4 °C to separate the elution from the beads.
24. Concentrate the elution through a 10 kDa cut-off centrifugal filter until 1 mL volume is reached (*see Note 10*).
25. Perform size-exclusion chromatography on the concentrated purified protein using a Superdex S75 10/300 column equilibrated in running buffer.
26. Only one peak that corresponds to ~46–50 kDa size should be observed for FLAG-tagged SAMTOR protein and all fractions that contain this peak should be collected and pooled.
27. Concentrate the pooled sample corresponding to the SAMTOR protein to approximately 1 mg/mL using the same type of 10 kDa cut-off centrifugal filter as **Step 24** (*see Note 11*).
28. Examine 1 µL of the purified protein by SDS-PAGE (4–12% Tris-Glycine gel, in Tris-Glycine running buffer) followed by Coomassie blue staining for purity analysis (Fig. 2a left) (*see Note 12*).
29. Proceed to Subheading 3.3.1 for further radioactive assay.

3.2.2 *HA-Tagged Control
or SAMTOR Protein
Immunoprecipitation from
HEK-293 T Cells*

1. 48 h after transfection, bring the culture plates to the bench and aspirate the media, quickly rinse the plate with 10 mL cold PBS, aspirate the PBS (*see Note 13*).
2. Lyse cells in 1 mL lysis buffer per plate.
3. Scrape cells from the plates, pool the lysate from plates transfected with the same plasmid, and transfer to a centrifuge-compatible tube.
4. Rock with end-to-end rotation at 4 °C for 20 min.
5. Centrifuge at 21000 g for 10 min at 4 °C to clear the cell lysate.
6. Transfer the cleared cell lysate to a 15 mL falcon tube (e.g., if 4 plates were pooled, the total volume will be between 5 and 8 mL; if only one plate is used, a 1.5 mL Eppendorf tube can be used instead).
7. During the centrifugation at **step 5**, add 15 µL/plate of the Pierce Anti-HA Dynabeads to a 2 mL Eppendorf tube (if multiple plates are used, add one more plate volume, 15 µL, to correct for pipetting error), put the tube on magnetic rack so the beads adhere to the magnet side of the tube.
8. Remove the storage buffer, and block the beads by cell lysis buffer containing bovine serum albumin (BSA) at 1 µg/µL in 1.5 mL total volume, and rotate the tube for 30 min at 4 °C. Longer incubation (1–2 h) works well too.
9. Put the tube on magnetic rack so the beads adhere to the magnet side of the tube.
10. Wait until almost all beads adhere, aspirate the top clear lysis buffer/BSA mixture. Add 1 mL of the cell lysis buffer to the tube. Mix the beads with the buffer by pipetting up-and-down 5 times.
11. Repeat **steps 9** and **10** two more times, then add lysis buffer to make the total volume up to the original volume of slurry (*see Note 14*).
12. Add 15 µL/plate of the washed Anti-HA beads to the lysate (s) from **step 6**.
13. Rock at 4 °C for 1.5–2 h.
14. The HA-tagged SAMTOR proteins are now captured on the Dynabeads.
15. Put the tubes on the magnetic rack and wait until the beads adhere.
16. Aspirate the supernatant, and resuspend the beads in 500 µL of lysis buffer.
17. Repeat **steps 15** and **16** two more times.
18. Take 20% of the beads, which is 100 µL of bead-containing lysis buffer, from each construct sample to a new tube, put the tubes on magnet and wait for the beads to adhere.

19. Aspirate the supernatant, and add HA synthetic peptide at 1 mg/mL concentration solubilized in the lysis buffer to elute off the HA-tagged proteins. The mixture should be incubated at 37 °C for 45 min.
20. After the incubation, put the tubes on magnet and wait for the beads to adhere, take the supernatant and analyze its protein content by SDS-PAGE (4–12% Tris-Glycine gel, in Tris-Glycine running buffer) followed by Coomassie blue staining for purity analysis (Fig. 2b left).
21. Take the rest 80% of the beads from **step 17**, after the last time of aspiration, proceed to Subheading 3.3.2 for further radioactive assay.

3.3 Radioactive S-Adenosylmethionine Binding Assay

3.3.1 Rebinding SAMTOR Protein from Subheading 3.2.1 to the Beads

1. Pipette 30 μ L/condition of Anti-FLAG bead slurry into an Eppendorf tube. For example, 9 samples were used for measuring SAM-binding K_d to SAMTOR, so $30 \times 9 = 270 \mu\text{L}$ of bead slurry was needed. To allow for pipetting errors, include one sample volume extra (300 μ L instead of 270 μ L).
2. Spin down the beads at 2000 g for 2 minutes at 4 °C, and remove the storage buffer, then add lysis buffer containing bovine serum albumin (BSA) at 1 $\mu\text{g}/\mu\text{L}$ in 1.5 mL total volume to block the beads, and rotate the tube for 30 min at 4 °C. Longer incubation, e.g., 1–2 h works well too.
3. Spin down the beads at 2000 g for 2 min at 4 °C.
4. Aspirate the supernatant in the tube without perturbing the beads, and resuspend the beads in lysis buffer, making the total volume up to the original volume of slurry (*see Note 14*).
5. Repeat **steps 3** and **4** two more times, then resuspend the beads in lysis buffer so the total volume reaches the initial slurry volume from **step 1**.
6. Add 5 μ L of purified SAMTOR protein (5 μg) to each empty Eppendorf tube and bring the volume up with lysis buffer to 500 μ L (*see Note 15*).
7. Add 30 μ L blocked and washed Anti-FLAG beads to each tube.
8. Incubate for 90 min at 4 °C with rotating to make sure that purified proteins efficiently bind to the beads.
9. Spin down the beads at 2000 g for 2 min at 4 °C.
10. Aspirate the supernatant without perturbing the beads, and resuspend the beads in 500 μ L of lysis buffer.
11. Repeat **steps 9** and **10** two more times, then dry the beads by removing all liquid, e.g., using a 27 Gauge/0.42 mm needle attached to an aspirator. Be careful not to aspirate any beads.

3.3.2 Radioactive Binding Assay Sample Preparation

1. Resuspend the beads in 20 μL of the cytosolic buffer with appropriate concentrations of [^3H]-labeled SAM and unlabeled SAM. Here, for measuring K_d , [^3H]-labeled SAM concentration is 5 μM , unlabeled SAM concentrations are: 0.002, 0.1, 2, 8, 20, 40, 100, 300, 1000 μM ; for testing SAMTOR mutants, [^3H]-labeled SAM concentration is 5 μM , unlabeled SAM concentration is 500 μM . For more information on concentration choices, *see* **Note 16**.
2. Incubate for 1 h on ice, and flick every 5 min because the beads settle after staying still (*see* **Note 17**).
3. At the end of 1 h, aspirate the FLAG-beads dry or put the HA-beads tubes on a magnetic rack and remove the supernatant after the beads adhere.
4. Quickly add 500 μL of the binding wash buffer, invert 3 times to mix.
5. Spin FLAG-beads at 2000 g for 30 s at 4 $^\circ\text{C}$ then remove supernatant from above the pelleted beads or place HA-beads tubes on a magnetic rack, and pipet out the liquid after the beads adhere.
6. Resuspend the beads in 500 μL of the binding wash buffer again and invert 3 times to mix.
7. Repeat **steps 4** and **5** one more time, then remove supernatant and resuspend the beads in 80 μL of the cytosolic buffer.
8. Mix each sample well, and aliquot 15 μL of the mixture into 5 mL of scintillation fluid pre-aliquoted in the scintillation tubes. For each sample, perform three technical replicates (for 9 samples, prepare $3 \times 9 = 27$ tubes).
9. Put the lids on tightly and mix well by inverting 5 times (*see* **Note 18**).
10. Measure the radioactivity of each sample using a scintillation counter (*see* **Note 19**).
11. The values for the HA-tagged proteins (negative control, wild type, and mutants) can be directly plotted and used to generate bar graphs (Fig. 2b right).

3.4 Calculate the K_d

1. The affinities of SAM for human FLAG-SAMTOR are determined by first normalizing the bound [^3H]-labeled SAM concentrations across three separate binding assays performed with varying amounts of cold SAM.
2. These values are plotted in GraphPad Prism software (or similar) and fitted to a hyperbolic equation (Cheng-Prusoff equation) to calculate the K_d value (Fig. 2a right). We assume that there is only one SAM molecule that binds with one SAMTOR protein due to what is already known about the

SAM binding domain (SAMTOR only has one SAM binding domain). The equation used is: $Y = B_{\max} * (1 - X / (K_d + X)) + \text{Offset}$, where X is the concentration of ligand, Y is the specific binding that comes from the scintillator counts directly, B_{max} is the maximum binding in the same units as Y, K_d is what is wanted and in the same units as X, Offset is background that corresponds to nonspecific binding (*see Note 20*).

4 Notes

1. The size of the flasks depends on the total culture volume. Leave enough space in the flasks to ensure efficient shaking, and check the health of the cells frequently by measuring the death rate of your cells by trypan blue staining. Based on the suggestions from the manufacturer, antibiotics are not recommended, so be careful in every step to not contaminate the cells. Ideally the HEK-293F cells double every day; once they reach 5 million cells/mL, passage the cells. The death ratio should be less than 10%.
2. PEI is used to transfect cells for the purpose of low toxicity and optimal efficiency-cost ratio, especially for a large suspension culture like described here. For HEK-293F/T cells, PEI is very efficient and can reach >80% of transfection efficiency. Other transfection methods should also work as long as the efficiency is >80%.
3. Make sure to ethanol spray the flask (gently, avoiding the lid area that contains the air filter) thoroughly before bringing the flask to the TC hood to keep everything as sterile as possible.
4. Ensure the health of the cells is optimal before going ahead with transfection and the radioactive binding assay. Ideally the HEK-293 T cells double every day, and the death ratio (measured by trypan blue staining) should be less than 5%.
5. The number of 15 cm culture plates depends on two factors: expression levels of the protein, and the conditions one plans to test in the further radioactive binding assay. For example, typically for each type of protein, I test radioactive SAM alone (low concentration), radioactive SAM (low concentration) plus non-radioactive SAM or other SAM analogs (high concentration, 1:100–1:1000 radioactive:non-radioactive SAM). Four 15 cm plates were transfected for testing one construct (comparison: radioactive alone vs radioactive plus non-radioactive). Scale this number up if more conditions, either nonradioactive analogs or constructs, are needed.

6. The amount of transfected plasmids has been optimized. 20 μg per 15 cm culture plate is ideal. Depending on the expression level of a given construct, adjust the expression vector and empty backbone vector ratio.
7. Adding the transfection mixture to the cells in a drop-wise manner is important. Do not add the mixture all together to the media.
8. Simply pouring the cleared cell lysates into a falcon tube works well. Pipetting is not necessary.
9. A homogenous mixture of beads and buffer is important, make sure no clumps of beads can be seen, and this applies to all beads mixing steps in the later part of the protocol.
10. During the centrifugation step, pipette the liquid up and down gently a few times every 5–10 min to prevent a high protein concentration developing near the filter.
11. More generally, if a new sensor protein is purified using this method, the protein peak corresponding to the protein of interest needs to be tested by western blotting using specific antibodies prior to fraction collection and pooling to absolutely make sure the right fractions of proteins are used for further radioactive binding assay.
12. Examining the purity of the purified proteins is important. An ideal protein preparation should contain >95% of the protein of interest. If significant amount of contaminating protein exists, the protein purification protocol needs to be optimized further for customized cases.
13. HEK-293 T cells are very easy to detach from the bottom of the culture plates, so make sure to rinse the cells gently and avoid blasting liquid directly onto the cells.
14. A trick is to pre-label the volume of the initial beads by marking it using a permanent marker, and after the final wash, one just needs to add the buffer to the initial marked line.
15. The SAMTOR protein amount used for each sample has been optimized. If other proteins are used for testing, different amount of protein needs to be tested in advance before initiating a full K_d curve measurement.
16. For a K_d curve measurement, at least 8 concentrations of unlabeled ligands are needed. For novel sensor-ligand pairs whose affinity information is not available, try as low as 1 nM as the beginning point, then go as high as 1 mM to cover a decent dynamic range. For the radioactive ligand concentration, my default is 5 μM , but I do recommend trying couple concentrations between 1 μM and 20 μM . Each pair's affinity can be very different, so optimization of these concentrations is absolutely necessary. For example, if you see very little binding

signal when you use 5 μM radioactive ligand, try increasing the concentration. In reverse, if the signal is more than 1000 times higher than background, try lowering it.

17. Flicking every 5 min was done for all past assays performed to facilitate reaching binding equilibrium. But given the incubation time being relatively long (1 h), not flicking the tubes with the same frequency will likely not significantly affect the final results as long as the equilibrium has been reached.
18. The tritiated samples can be stored at 4 °C for a year or two due to the long half-life of the tritiated ligands. Once the mixture has been pipetted into the scintillation liquid, after mixing by multiple inversions, an immediate measurement by the scintillation counter is recommended.
19. For radioactive waste, both solid (pipette tips, etc.) and liquid (any liquid containing radioactive agents) need to be disposed in a specific sealed container labeled with radioactive tape depending on the institutional requirements. The liquid should be collected in a falcon tube or Eppendorf tube and the whole tube should be disposed in the container. It is important to note that the radioactive waste should not be mixed with normal biohazardous waste.
20. A prism template is provided with all equations and fitting built in (*See* Electronic Supplementary File 1). Only experimental values are needed for a K_d curve generation. If there is a chance that your sensor-ligand pair is not 1 to 1 ratio (one sensor protein binds 2 or more ligand molecules), other equations are needed to calculate the binding affinity (some examples can be found in GraphPad website: https://www.graphpad.com/guides/prism/latest/curve-fitting/reg_models_built-in_to_prism.htm).

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