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Utilizing split-NanoLuc luciferase fragments as luminescent probes for protein solubility in living cells

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Abstract

Protein misfolding and aggregation is now recognized as a hallmark of numerous human diseases. Standard bioanalytical techniques for monitoring protein aggregation generally rely on small molecules that provide an optical readout of fibril formation. While these methods have been useful for mechanistic studies, additional approaches are required to probe the equilibrium between soluble and insoluble protein within living systems. Such approaches could provide platforms for the identification of inhibitors of protein aggregation as well as a means to investigate the effect of mutations on protein aggregation in model systems. In this chapter, we provide detailed protocols for employing split-NanoLuc luciferase (Nluc) fragments to monitor changes in protein solubility in bacterial and mammalian cells. This sensitive luminesce-based assay can report upon changes in protein solubility induced by inhibitors and disease-relevant mutations.

1. Introduction

Protein aggregation is now recognized as a key phenotype of numerous human diseases (Chiti & Dobson, 2006, 2017), sparking interest in the development of methods for detecting changes in protein solubility. Such methods have the potential to provide mechanistic insights into protein misfolding as well as platforms for the identification of inhibitors. Initial efforts to monitor the formation of protein aggregates *in vitro* utilized small molecules that bind to protein fibrils and produce a change in optical signal (LeVine, 1993; Westermarck, Johnson, & Westermarck, 1999). While these probes are powerful tools for staining protein aggregates *in vitro*, issues associated with sensitivity and selectivity have hindered their use in living systems. In addition, assays that report on changes in the amount of soluble protein, as opposed to protein aggregates, could provide screens for the identification of inhibitors (Kim et al., 2006; Stains, Mondal, & Ghosh, 2007). To address these issues, efforts have focused on the development of cell-based approaches for monitoring protein solubility (Maxwell, Mittermaier, Forman-Kay, & Davidson, 1999; Waldo, Standish, Berendzen, & Terwilliger, 1999; Wigley, Stidham, Smith, Hunt, &

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Thomas, 2001). These strategies leverage genetically encodable reporters that can be easily delivered into cells, producing a colorimetric or fluorescence-based readout of protein solubility. Despite their clear utility, these methods are hindered by the inherent detection limits of colorimetric- and fluorescence-based assays as well as the potential for false positives due to autofluorescent compounds.

In an alternative strategy, our laboratory has recently described a generalizable protein solubility assay that relies on the spontaneous reassembly of Nluc fragments (Zhao, Nelson, Vu, Truong, & Stains, 2016; Zhao, Vu, & Stains, 2016). Nluc is a small (19.1kDa), engineered luciferase capable of generating bioluminescence with its native substrate (coelenterazine, CTZ) as well as an optimized analog (furimazine, FMZ, Fig. 1A and B) (Hall et al., 2012; Tomabechi et al., 2016). To construct a Nluc-based probe for protein solubility with low background, we sought to identify fragments of Nluc with virtually no activity prior to spontaneous reassembly into an active enzyme. During screening efforts, we identified several fragment pairs capable of spontaneous reassembly. Using the brightest pair from our screen, termed N65 (residues 1–65) and 66C (residues 66–171) we have developed a generalizable luminescent platform for monitoring protein solubility in bacteria as well as mammalian cells (Fig. 1C). This methodology has been applied to amyloid-beta, amylin, and huntingtin and can be used in vitro as well as cell-based assays (Zhao, Nelson, et al., 2016; Zhao, Vu, et al., 2016). This sensitive, luminescence-based assay platform can be used to probe the effects of mutations and inhibitors on protein solubility. In this chapter, we present a generalized protocol for designing and assaying protein solubility using this split-Nluc system.

2. Assay design

Our assay system is comprised of two genetically encodable Nluc fragments (N65 and 66C) that spontaneously reassemble to form active Nluc. These two fragments can be expressed from separate plasmids or using co-expression vectors (such as pET-Duet or pRSF-Duet). The assay operates by reporting on the concentration of the N65 fragment available for reassembly. Fusion of a protein of interest (POI) to the N-terminus of N65 results in a modulation of the solubility of the POI-N65 fusion that is proportional to the solubility of the POI (Fig. 1C). We have found that fusion of a POI to the N-terminus of 66C results in poor luminescence recovery, potentially due to inhibition of reassembly (Zhao, Nelson, et al., 2016). Therefore, fusions to the non-native termini of N65 and 66C should be avoided. Additionally, we have shown that 66C expresses at relatively low concentrations. Thus, we recommend fusion of a POI to the N-terminus of N65 since this design has been repeatedly shown to produce changes in N65 solubility that are proportional to the solubility of the POI (Zhao, Nelson, et al., 2016; Zhao, Vu, et al., 2016). If purification of the fragments is desired, affinity tags can be added. Lastly, we include a short amino acid linker of GGGSSGGG between the POI and N65 to allow for productive reassembly (Table 1). Vectors for expression of N65 and 66C are available upon request from our laboratory.

3. General assay procedures

Below we provide our optimized procedures for using the Split-Nluc assay to probe protein solubility in cell lysates or intact cells. Procedures are given for experiments using bacterial as well as mammalian cells. Assays can be performed using live cells or cell lysates and signal can be readout using a standard luminescence plate reader or Western blot imager with luminescence capability.

3.1 Equipment and reagents

1. Electroporator (or water bath)
2. Sterilized water
3. Incubator
4. Orbital shaker with cooling capability
5. UV-Vis spectrophotometer
6. Bench-top centrifuge
7. Plate reader capable of monitoring luminescence
8. Western blot imaging system with luminescence capability
9. Nitrocellulose membrane
10. Corning assay plates (white)
 - a. 384-well assay plates (3824) [flat bottom, 40 μ L reaction volume]
 - b. 96-well assay plates (3359) [half area, round-bottom, 120 μ L reaction volume]
11. LB-agar plates
12. Terrific Broth (TB) liquid medium (RPI Corp., T15000)
13. Antibiotics, such as: ampicillin/carbenicillin (1000 $\times$ stock solution: 100mg/mL) and/or kanamycin (1000 $\times$ stock solution: 50mg/mL)
14. Isopropyl β -D-1-thiogalactopyranoside (IPTG, 500 stock solution: 100mM)
15. 2 $\times$ Nluc assay buffer (100mM MES pH 6.0, 1mM EDTA, 150mM KCl, 2mM β -mercaptoethanol, 35mM thiourea)
16. Nluc substrate
 - a. Nano-Glo Assay System (FMZ-based; Promega, N1110 [lytic] or Promega, N2011 [nonlytic])
 - b. CTZ in acidified ethanol (2.5mM)
17. B-PER lysis reagent (Thermo Fisher Scientific, 78243)
18. Lipofectamine 3000 (Life Technologies, L3000001) or other transfection reagent

19. Mammalian cell culture media (DMEM, 10% FBS, 1% penicillin-streptomycin), with and without phenol red
20. TrypLE(Thermo Fisher Scientific, 12605036)or other trypsinization agent
21. Dulbecco's phosphate-buffered saline (DPBS), no calcium, no magnesium (Thermo Fisher Scientific, 14190)
22. T-25 cell culture flask
23. 96-well cell culture plate

3.2 Bacterial cell culture

1. Prepare LB-agar plates with antibiotics as dictated by the expression vector(s) to be used. Prepare TB without antibiotics.
2. Mix a bacterial stock with the desired DNA and transform using electroporation or heatshock. Recover the transformed cells in 500 μ L TB and incubate in an orbital shaker (200–250rpm) at 37°C for 45min. Spread 100–150 μ L of cells on an LB-agar plate and incubate overnight at 37°C.

Tip: Genetically encodable inhibitors can be co-transformed with a co-expression plasmid encoding the assay system. An empty vector corresponding to the plasmid encoding the inhibitor should be used as a negative control in a separate co-transformation.

3. Select a colony from the plate and add to a tube containing 5mL of TB and the appropriate antibiotic. Grow overnight in an orbital shaker at 37°C.
4. Quantify bacterial growth using optical density at 600nm (OD₆₀₀).
5. Dilute cells to an OD₆₀₀ of 0.1 in 5mL TB containing the appropriate antibiotics.
6. Grow cells in an orbital shaker at 37°C until an OD₆₀₀=0.6–0.8 is reached.
7. To induce protein expression, add IPTG to a final concentration of 0.2mM. Incubate in an orbital shaker at 16°C overnight.

Tip: If investigating small molecule inhibitors, they should be added to the culture at this time at the desired concentration(s).

8. The resulting cells can either be used directly in luminescence assays (Section 3.3), lysate assays (Section 3.4), or for imaging on a nitrocellulose membrane (Section 3.5).

3.3 Bacterial cell assay

1. Measure the OD₆₀₀ of the cell cultures from Section 3.2 and pipette enough cells to obtain an OD₆₀₀ = 3.0 in 1mL into a 1.5-mL Eppendorf tube.

Tip: If assay signal is too high, adjust the amount of cells used in the assay.

2. Centrifuge samples at 700 g for 10–15min at 4°C.

3. Remove the supernatant with a pipette and resuspend cells in 200 μ L of 1 $\times$ Nluc buffer.
4. Prepare the substrate by diluting the CTZ stock to 50 μ M in 1 $\times$ Nluc buffer. Cover the Eppendorf tube with foil to protect from light. Each sample will require an equal volume (200 μ L) of substrate.
5. Add 200 μ L of substrate to each sample tube (reaching a final volume of 400 μ L) and mix thoroughly. Aliquot samples into a 384-well white Corning assay plate, using 40 μ L per well.
6. Record luminescence at 460nm for 1h.
7. For data analysis see Section 3.7.

3.4 Bacterial lysate assay

Testing or validating small molecule inhibitors of aggregation can be confounded by toxicity or issues associated with membrane permeability. However, such compounds can produce valuable leads for further optimization. To circumvent issues associated with toxicity and membrane permeability in cell-based assays, lysates be generated and used to test inhibitor efficacy. To perform this procedure, separate transformants expressing the POI-N65 fusion or 66C should be obtained using the procedure outlined in Section 3.2.

1. Transfer the cell cultures from Section 3.2 to a 15-mL conical tube and centrifuge at 17,000 $\times$ g for 10min at 4°C. Remove and discard media.
2. Weigh each pellet and resuspend the pellet in 4mL of B-PER reagent per gram of cells.
3. Incubate for 15min to ensure complete lysis.
4. Centrifuge at 17,000 $\times$ g for 5min at 4°C. Transfer 100 μ L of the POI-N65 supernatant to an Eppendorf tube. Likewise, aliquot 50 μ L of the 66C supernatant.
5. Add an inhibitor, dissolved in phosphate-buffered saline (PBS) or similar, to the POI-N65 supernatant and adjust the total volume to 150 μ L using PBS. Separate assays containing the inhibitor solvent can be used as negative controls. Incubate the inhibitor with the lysate for 3h at 37°C.
6. Prepare the substrate by diluting the CTZ stock to 50 μ M in 2 Nluc buffer (final will be 1 $\times$ after mixing with lysates). Cover the Eppendorf tube with foil to protect from light. Each sample will require an equal volume (200 μ L) of substrate.
7. Mix 50 μ L of 66C supernatant with the POI-N65 sample, reaching a total volume of 200 μ L. Incubate samples at room temperature for 5min.
8. Add 200 μ L of the substrate to each sample tube and mix, reaching a final volume of 400 μ L. Aliquot samples into a 384-well white Corning assay plate, using 40 μ L per well.

9. Record luminescence at 460nm for 20min.
10. For data analysis see Section 3.7.

3.5 Imaging bacterial cells on nitrocellulose membranes

1. Measure the OD₆₀₀ of cell cultures and transfer an amount of cells corresponding to an OD₆₀₀ = 3.0 in 1mL into a 1.5-mL Eppendorf tube.
2. Centrifuge samples at 700 × g for 10–15min at 4°C and remove supernatant.
3. Prepare a 50μM working solution of CTZ in 1 × Nluc buffer. Resuspend each sample of cells in 15μL of substrate solution.
4. Spot equal volumes of the samples (e.g., 5–10μL) onto a nitrocellulose membrane and image using the chemiluminescence function of a Western blot imager according to manufacturer recommendations.
5. The resulting image intensities can be used as an estimate of relative protein solubility.

3.6 Mammalian cell assay

While bacterial assays are advantageous for applications such as high-throughput screening, mammalian cell lines can provide a more relevant context for investigating protein solubility. To this end, we have utilized CMV-based promoter systems to drive expression of split-Nluc fragments in mammalian cells. This assay has been used in NIH-3T3 and HEK293 cell lines in our laboratory.

1. Grow cells in cell culture media at 37°C and 5% CO₂ in a T-25 or similar flask. Perform trypsinization when cells are confluent and seed cells at 50% confluency in 96-well cell culture plates.
2. Allow cells to grow to ~70–80% confluency. Transfect cells with a mammalian expression vector(s) encoding for the split-Nluc assay components, following manufacture protocols. We find that 100–600ng of plasmid is generally sufficient for transfection. A control transfection without DNA should also be performed as a negative control.
3. Allow cells to grow at 37°C and 5% CO₂ for 24–48h. Aspirate media and wash with DPBS to remove residual phenol red. Remove DPBS and replace with growth media containing no phenol red.
4. Assay using the Nano-Glo Assay System following the manufacturer's protocol.
5. Record luminescence at 460nm for 1h.
6. For data analysis see Section 3.7.

3.7 Data analysis

Analysis of luminescence data from assays can be performed using a spreadsheet application such as Microsoft Excel. Arbitrary luminance units (ALU) are averaged and standard deviations are calculated. When working with living bacterial cells, we observe a peak in

luminescence at approximately 20min followed by a gradual decay (Fig. 2). This phenomenon is attributed to the time required for diffusion of substrate across the cell membrane. In support of this hypothesis, no lag phase in luminescence is observed when using lysates or purified proteins. Based on these observations we pick timepoints at which substrate equilibrium has been reached for data analysis purposes, generally 15–30min for intact bacterial cell assays and 0min for lysates and purified protein. For comparison purposes, the same timepoints should be used for each sample run under identical conditions. Relative luminescence units (RLU) can be calculated by dividing the ALU of each sample by the ALU corresponding to the POI without inhibitor. RLU can be compared for experiments performed on different days. Negative controls (such as nontransformed cells) can be used for background subtraction. Data is plotted in bar graph form in order to compare relative protein solubilities across samples (Fig. 3).

4. Validation of assay results by Western blotting

Upon identification of a molecule or mutation that influences solubility, secondary conformation by Western blotting is used to verify assay results. A validated primary antibody for the POI or an anti-6X His-tag antibody can be used. Levels of soluble protein are compared to total protein expression in order to confirm changes in solubility. Combining this low-throughput validation approach with the high-throughput split-Nluc assay can provide a means to identify and validate the effects of inhibitors and mutations on protein solubility (Zhao, Nelson, et al., 2016; Zhao, Vu, et al., 2016).

4.1 Initial sample preparation

1. Prepare and induce expression of the assay system with IPTG using the protocol in Section 3.2.
2. After induction, normalize two samples of cells to an OD₆₀₀ of 3 in 1mL. Pellet cells by centrifugation at $700 \times g$ for 30min and remove the supernatant. Soluble (Section 4.2) and total (Section 4.3) protein fractions are generated using these pellets.

4.2 Preparation of the soluble protein fraction

1. Lyse one pellet with 30 μ L B-PER reagent. Incubate at room temperature and centrifuge at $700 \times g$ for 30min at 4°C for.
2. Collect the resulting supernatant.
3. Add the appropriate volume of SDS-PAGE loading dye to 8 μ L of supernatant. Denature in a boiling water bath for 10min and then centrifuge at $17,000 \times g$ for 5min. Cool to room temperature.

4.3 Preparation of the total protein fraction

1. Resuspend the second pellet in 60 μ L of 4M urea containing 5% SDS. Denature in a boiling water bath for 10min followed by centrifugation at $17,000 \times g$ for 10min.

2. Collect the resulting supernatant.
3. Add the appropriate volume of SDS-PAGE loading dye to 8 μ L of supernatant. Denature in a boiling water bath for 10min and then centrifuge at 17,000 $\times$ g for 5min. Cool to room temperature.
4. Analyze both soluble and total protein fractions by Western blotting. A positive hit will display changes in soluble protein band intensities that correlate with split-Nluc luminescence assay data, with no change in the amount of total protein expression.

Tip: If issues with weak band intensities are encountered, protein concentrations can be increased through affinity purification with Ni-NTA resin. A small volume (10 μ L) of resin may be added to each fraction immediately after lysis. Resin is collected via centrifugation (500 $\times$ g for 10min) and washed with 200 μ L of PBS containing 10mM imidazole. After a second centrifugation step, the bound proteins are eluted by adding 10 μ L of PBS containing 1M imidazole. The resulting supernatant containing enriched protein is collected after centrifugation. For the total protein fraction, urea should be included throughout the procedure. These enriched samples can be used directly for Western blotting.

5. Conclusions

This self-assembling split-Nluc system provides a sensitive luminescence-based readout of protein solubility in bacterial and mammalian cells. The assay is general and has been used with several different proteins including amyloid-beta, amylin, and huntingtin (Zhao, Nelson, et al., 2016; Zhao, Vu, et al., 2016).

The effects of mutations and inhibitors on protein solubility can be rapidly assessed using the protocols outlined in this chapter, providing a platform for high-throughput screening efforts aimed at the identification of protein aggregation inhibitors.

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References

- Chiti F, & Dobson CM (2006). Protein misfolding, functional amyloid, and human disease. *Annual Review of Biochemistry*, 75, 333–366. 10.1146/annurev.biochem.75.101304.123901.
- Chiti F, & Dobson CM (2017). Protein misfolding, amyloid formation, and human disease: A summary of progress over the last decade. *Annual Review of Biochemistry*, 86, 27–68. 10.1146/annurev-biochem-061516-045115.
- Hall MP, Unch J, Binkowski BF, Valley MP, Butler BL, Wood MG, et al. (2012). Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chemical Biology*, 7(11), 1848–1857. 10.1021/cb3002478. [PubMed: 22894855]
- Kim W, Kim Y, Min J, Kim DJ, Chang YT, & Hecht MH (2006). A high-throughput screen for compounds that inhibit aggregation of the Alzheimer's peptide. *ACS Chemical Biology*, 1(7), 461–469. 10.1021/cb600135w. [PubMed: 17168524]

- LeVine H 3rd. (1993). Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: Detection of amyloid aggregation in solution. *Protein Science: A Publication of the Protein Society*, 2(3), 404–410. 10.1002/pro.5560020312. [PubMed: 8453378]
- Maxwell KL, Mittermaier AK, Forman-Kay JD, & Davidson AR (1999). A simple in vivo assay for increased protein solubility. *Protein Science: A Publication of the Protein Society*, 8(9), 1908–1911. 10.1110/ps.8.9.1908. [PubMed: 10493593]
- Stains CI, Mondal K, & Ghosh I (2007). Molecules that target beta-amyloid. *ChemMedChem*, 2(12), 1674–1692. 10.1002/cmdc.200700140. [PubMed: 17952881]
- Tomabechi Y, Hosoya T, Ehara H, Sekine SI, Shirouzu M, & Inouye S (2016). Crystal structure of nanoKAZ: The mutated 19 kDa component of Oplophorus luciferase catalyzing the bioluminescent reaction with coelenterazine. *Biochemical and Biophysical Research Communications*, 470(1), 88–93. 10.1016/j.bbrc.2015.12.123. [PubMed: 26746005]
- Waldo GS, Standish BM, Berendzen J, & Terwilliger TC (1999). Rapid protein-folding assay using green fluorescent protein. *Nature Biotechnology*, 17(7), 691–695. 10.1038/10904.
- Westermarck GT, Johnson KH, & Westermarck P (1999). Staining methods for identification of amyloid in tissue. *Methods in Enzymology*, 309, 3–25. [PubMed: 10507013]
- Wigley WC, Stidham RD, Smith NM, Hunt JF, & Thomas PJ (2001). Protein solubility and folding monitored in vivo by structural complementation of a genetic marker protein. *Nature Biotechnology*, 19(2), 131–136. 10.1038/84389.
- Zhao J, Nelson TJ, Vu Q, Truong T, & Stains CI (2016). Self-assembling NanoLuc luciferase fragments as probes for protein aggregation in living cells. *ACS Chemical Biology*, 11(1), 132–138. 10.1021/acscchembio.5b00758. [PubMed: 26492083]
- Zhao J, Vu Q, & Stains CI (2016). Luminescent platforms for monitoring changes in the solubility of amylin and huntingtin in living cells. *Molecular BioSystems*, 12(10), 2984–2987. 10.1039/c6mb00454g. [PubMed: 27483434]

Fig. 1.

Utilizing spontaneous reassembly of Nluc fragments for detection of protein solubility. (A) Nluc catalyzes the oxidative decarboxylation of CTZ, producing luminescence at 460nm.

(B) The structure of FMZ, an optimized CTZ analog for Nluc.(C) A generalizable platform for monitoring protein solubility using spontaneously reassembling Nluc fragments.

Changes in the solubility of a protein of interest (POI) result in a proportional change in the amount of N65 available for reassembly. Thus, luminescence of reassembled N65/66C (PDB: 5B0U) can be used to assess relative differences in the solubility of the POI under different conditions.

Fig. 2.
The temporal dynamics of a bacterial cell assay using the split-Nluc system. An initial lag phase in luminescence output is observed.

Fig. 3.

A bacterial cell assay indicating inhibition of protein aggregation. The split-Nluc system is expressed in bacterial cells and used to assay the solubility of a POI (left column). Addition of an inhibitor of protein aggregation increases the solubility of the POI, leading to an increase in luminescence (middle column). Blank cells, not expressing the split-Nluc system, display minimal background luminescence. * indicates a P -value of <0.05 , error bars represent the standard deviation of triplicate experiments.

Table 1

Examples of split-Nluc constructs for monitoring protein solubility.

Protein	Amino acid sequence
A β ₁₋₄₂ -N65	MAHHHHHHVGTGSNDDDDKSPDPAEFRHDS GYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVI AEISYASRGGSSGGGELMVFTLEDFVGDWRQT <u>AGYNLDQVLEQGGVSSLFQNLGVSVTPIQIRIVLS</u> <u>GENGLKIDIHVIIPYE</u>
66C	<u>MGGLSGDQMGQIEKIFKVVPVDDHHFKVILHY</u> <u>GTLVIDGVTPNMIDYFGRPYEGIAVFDGKKITVT</u> <u>GTLWNGNKIIDERLINPDGSLFRVTINGVTGW</u> <u>RLCERILALQGSELHHHHHH</u>

Sequences contain a 6 × -His Tag for purification, the Nluc amino acid sequence is underlined.