Assaying Protein Kinase A activity using a FRET-based sensor purified from mammalian cells

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Abstract

Protein Kinase A (PKA) is the major intracellular receptor for cAMP. Research into this prototype kinase is supported by kinase assays that are typically performed *in vitro* using radio-labelled ATP. For *in vivo* studies, genetically-encoded FRET-based sensors have become popular for monitoring PKA activity. Here, we show that it is also possible to apply such reporters *in vitro*. We describe how to express and purify milligram quantities of a FRET-based PKA activity reporter using cultured human embryonic kidney cells. We demonstrate how to utilize the purified reporter in a plate reader to determine the IC_{50} for the widely-utilized PKA inhibitor H89 in the presence of a physiologically-relevant concentration of ATP. The protocol takes advantage of the economical transfection reagent polyethylenimine, and can be performed in a standard cell culture facility. Whereas assays based on radiolabelling are more sensitive, the approach presented here has several advantages: it enables continuous measurement of changes in substrate phosphorylation; a single preparation produces enough reporter for thousands of recordings; the reporter has a long shelf life; and it avoids the safety considerations that arise when working with radioactive material.

1. Introduction

cAMP-dependent protein kinase, also known as protein kinase A (PKA), is the major intracellular receptor for cAMP. The enzyme is critical for cellular responses including sympathetic regulation of the heart [1], control of water reuptake in the kidney [2], and long-lasting changes in synaptic strength [3], to name only a few. Mutations affecting PKA activity [4] or localisation [5] are pathogenic. PKA also serves as a prototype for understanding the broader protein kinase family, and accordingly its catalytic mechanism and the structural basis of its function have been scrutinized in great detail. Methods to assay PKA activity have of course been fundamental to this research effort. For example, PKA activity assays have been utilized in structure-function studies focused on understanding the phospho-transfer mechanism of PKA [6], its mode of activation by cAMP [7], the molecular determinants of substrate recognition by PKA [8], and regulation of the kinase by different metal ions [9]. Kinase assays are frequently performed with protein immuno-precipitates to identify signalling complexes that contain PKA [10]. Furthermore, PKA assays have supported identification and characterisation of small molecule PKA inhibitors including H89 [11], and there is ongoing demand for new assay formats that facilitate high-throughput kinase inhibitor development [12].

The most common method for measuring PKA kinase activity is to monitor transfer of radio-labelled ^{32}P from the γ position of ATP to substrates. PKA assays are typically performed using this approach with basic peptides such as Kemptide [13] that will bind to p81 phosphocellulose paper. This enables the peptide to be recovered and separated from free phosphate and ATP once the reaction has been terminated with, e.g., phosphoric acid [14]. The extent of ^{32}P incorporation is then measured in a scintillation counter. ^{32}P-labelled protein substrates can be recovered after reaction termination using mixed cellulose ester membrane filters [9]. Radioactive kinase assays have also been adapted for 96-well plates [15]. Although ^{32}P-based kinase assays are highly sensitive and accurate, only a single time-point can be collected per reaction. Furthermore, ^{32}P-ATP has a limited shelf life since the half-life or ^{32}P is only ~ 14 days, and there are additional safety and logistical considerations associated with working with radioactive material. Non-radioactive methods have become popular for assaying protein phosphatase activity, including assays with the chromogenic substrate p-nitrophenyl phosphate [16] and application of molybdate/malachite-based reagents for colorimetric quantitation of free phosphate [17], but equivalent methods are yet to catch on in the kinase field. Kemptide labelled with either rhodamine or carboxytetramethylrhodamine (TAMRA) enables phospho-peptide formation to be monitored by separating terminated kinase reactions using high performance liquid chromatography [18]. This method avoids radioactivity, but running HPLC separations is time consuming and it is only possible to perform end-point assays using this approach.

Genetically-encoded reporters are in wide use for monitoring PKA activity in live cells. A popular reporter is A-kinase activity reporter 4 (AKAR4), which contains cpVenus and Cerulean fluorescent proteins on either side of an FHA domain and PKA phosphorylation motif (**Fig. 1A**). Phosphorylation of the central motif by PKA leads to association of the central elements that increases FRET efficiency between the terminal fluorophores. In theory, sensors of this type could be used for *in vitro* assays, but it is typically not possible to express these reporters using conventional bacterial and insect cell approaches. In this protocol, we describe how to express and purify a modified version of AKAR4 that contains a C-terminal octa-histidine (8His) tag by transfecting cultured HEK293T cells. Our protocol takes advantage of the economical transfection reagent polyethyleneimine (PEI) [19]. It enables large-scale expression of AKAR4 in a standard tissue culture facility and yields enough purified reporter for thousands of *in vitro* PKA assays. Although assaying PKA activity with purified AKAR4 is less sensitive than monitoring ^{32}P incorporation, it has several advantages. It is possible to monitor the full time-course of a single reaction; it is economical; the reporter is stable for > 12 months in a – 80 °C freezer; measurements can be performed in high-throughput in 96-well plates; there are no concerns about radiation safety; and assays can be performed quickly with no need for wash steps after reaction termination. In this protocol, we first set out a standard procedure for optimizing expression of AKAR4 in HEK293T cells (Subheading **3.1**) – this step may also be applied to other available FRET-based sensors of kinase activity [20] and to other genetically-encoded reporters including cAMP sensors [21]. We next explain how to purify AKAR4 (Subheading **3.2**), and how to calibrate the purified reporter for plate reader assays (Subheading **3.3**). Finally, we provide an example application by detailing how to determine the IC_{50} for H89 inhibition of PKA catalytic (C) subunits in the presence of 1 mM ATP (Subheading **3.4**). Common pitfalls, potential modifications, and theoretical insights are highlighted in 'Notes' (Section **4**).

2. Materials

Prepare solutions using ultrapure water (e.g., by purifying deionized water with a MilliQ system to a resistivity of > 18 MΩ), and analytical grade reagents.

2.1. Mammalian cell expression

1. HEK293T cells (ATCC, reference CRL-3216; *see* **Note *1***)
2. High-serum culture media: DMEM containing pyruvate and high glucose, mixture of 100 U/mL penicillin and 100 µg/mL streptomycin, 1 % (v/v) GlutaMAX, 10 % heat-inactivated horse serum.
3. Low-serum culture media: DMEM containing pyruvate and high glucose, 1 % (v/v) GlutaMAX, 2 % heat-inactivated horse serum (*see* **Note *2***).
4. DMEM containing pyruvate and high glucose.
5. Phosphate-buffered saline.
6. Plasticware including 10-cm diameter tissue culture dishes and 6-well plates.
7. pcDNA3-AKAR4-8His-NES expression vector, ~ 500 µg prepared at > 0.5 µg/µL using, e.g., a Plasmid Maxiprep kit (Qiagen) after production in TOP10 cells (Thermo Fisher Scientific). The vector was assembled by introducing an 8His tag prior to the nuclear export signal in AKAR4-NES (Addgene clone 64727) using the restriction enzymes EcoRI and XbaI to insert the following annealed primer pair: EcoI_8HisNLS_XbaI (5'AATTCGCCGGCCACCACCACCACCACCACCACCACGGCGCCCTGCCCCCCCCTG GAGCGCCTGACCCTGTAAT) and XbaI_8HisNLS_EcoRI (5'CTAGATTACAGGGTCAGGCGCTCCAGGGGGGGCAGGGCGCCGTGGTGGTGGT GGTGGTGGTGGTGGCCGGCG).
8. 1mg/ml PEI (linear, MW25000, transfection grade): dispense 100 mg powder into a beaker and suspend in 90 mL H_2O. Stir while adding HCl dropwise until the pH is just below 2, then cover and stir for approximately 10 minutes until the powder has completely dissolved. Carefully adjust to pH 7 with NaOH and bring up to 100 mL with H_2O. Sterilize with a 0.2 µm filter, and divide into 1 mL aliquots for long-term storage at – 20 °C (*see* **Note *3***).
9. A tissue culture facility equipped with a tissue culture hood, 37 °C incubators maintained in a humidified atmosphere with 5 % CO_2 in air, and a microscope for determining cell confluence.
10. A ChemiDoc gel imaging system (bio-rad) operated using ImageLab software (*see* **Note *4***).
11. ImageJ software (NIH) for quantifying AKAR4 fluorescence in images of 6-well plates.

2.2. Reporter purification

All buffers should be filter sterilized and stored at 4 °C for use within one week.

1. Lysis buffer: 30 mM TrisHCl pH 8, 500 mM NaCl, 10 mM imidazole, 1 mM benzamidine, 0.5 % Igepal CA-630. Supplement with one cOmplete Protease Inhibitor Cocktail (Roche) per 100 mL immediately prior to cell lysis.
2. Nickel buffer A: 30 mM TrisHCl pH 8, 500 mM NaCl, 10 mM imidazole, 1 mM benzamidine.
3. Nickel buffer B: 30 mM TrisHCl pH 7.5, 500 mM NaCl, 300 mM imidazole 1 mM benzamidine.
4. Storage buffer: 25 mM Na Hepes pH 7.5, 100 mM NaCl.
5. Sonicator such as a Q500 sonicator (Fisher Scientific).
6. HisTrap HP, 5 mL column (GE Healthcare).
7. HiPrep 26/10 Desalting column (GE Healthcare).
8. AKTA Start Chromatography System (*see* **Note 5**) controlled using Unicorn Start 1.2 software (GE Healthcare).
9. Vivaspin Turbo 15, 10 kDa MWCO PES centrifugal protein concentrators (Sartorius)
10. For running SDS-PAGE gels: NuPAGE 4 to 12%, Bis-Tris Mini Protein Gels, NuPAGE MES running buffer (20x), NuPAGE LDS sample buffer (4x), Novex Sharp Pre-stained protein standards (all Thermo Fisher Scientific).
11. Coomassie blue stain (10 % ethanol, 30 % acetic acid, 0.125 % w/v Coomassie R-250) and destain (10 % ethanol, 10 % acetic acid) solutions.
12. BCA protein assay kit.

2.3. Ratiometric plate reader assays

1. Reaction buffer: 20 mM Na Hepes pH 7.5, 100 mM NaCl, 10 mM DTT, 20 mM $MgCl_2$, 0.5 % Igepal CA-630. Prepare 20 mL and divide into aliquots of 500 µL and store at – 80 °C for use within six months.
2. Injection buffer: 20 mM Na Hepes pH 7.5, 100 mM NaCl, 5 mM ATP. Prepare 50 mL, filter sterilize and divide into aliquots of 1.5 mL. Store aliquots at – 80 °C for use within 6 months.
3. Dilution buffer: 20 mM Na Hepes pH 7.5, 100 mM NaCl. Store at 4 °C for use within six months.
4. Purified PKA Cβ subunit (0.3 mg/mL in storage buffer), purified after transgenic expression in bacteria [22] **(*see* Note *6***).
5. H89, Dihydrochloride. Prepare a 10 mM solution by dissolving 1 mg in 193 µL DMSO. Store at 4 °C for use within 4 months.

6. FLUOstar Omega microplate reader (BMG Labtech) equipped with a 430 nm excitation filter, an emission filter wheel containing both 485 nm and 520 nm emission filters, and an injector (*see* **Note *7***).
7. 96-well black-walled microplates for fluorescence-based assays.
8. Data analysis software: MARS (BMG Labtech), Microsoft Excel, and Origin (OriginLab).

3. Methods

3.1 Optimising reporter expression by PEI transfection

The following procedures should be carried out in a sterile tissue culture hood using aseptic technique unless otherwise stated. This protocol may be applied to optimize expression of other fluorescent reporter besides AKAR4 including the latest PKA activity reporters (*see* **Note *8***).

1. Seed 3 x 6-well plates (labelled low, medium, and high DNA) with HEK293T at a confluence of ~ 35 % in 2 mL high-serum media per well, and incubate the plates at 37 °C with 5 % CO_2.
2. On the following day when the cells have reached a confluence of ~ 70 %, replace the media with 2 mL low-serum media per well an hour before addition of the transfection mixtures and return to the incubator.
3. The aim of this optimization experiment is to compare AKAR4 expression at the following five different PEI:DNA ratios (2:1, 3:1, 4:1, 5:1, 6:1), and at three different concentrations of DNA per well (0.67, 1.67, 3.33 μg per well, *see* **Note *9***). Accordingly, add either 0 (control), 20, 30, 40, 50, or 60 μL of 1 mg/mL PEI solution to six 1.5 mL Eppendorf tubes, and bring the volume in each tube to 250 μL with DMEM. Vortex each tube for ~3 seconds.
4. Add 60μg pcDNA3-AKAR4-8His-NES DNA to a separate 15 mL falcon tube, and bring the volume to 1.5 mL with DMEM.
5. After 20 minutes, divide the DNA solution between the five Eppendorf tubes containing different PEI concentrations to yield the five desired PEI:DNA ratios and a control solution. Vortex each mixture for 3 seconds, and leave to stand for 20 minutes at room temperature.
6. For each PEI-DNA mixture, add either 36 μL/well (low DNA plate), 90 μL/well (medium DNA plate), or 180 μL DNA/well (high DNA plate). Swirl the plate while adding the solutions dropwise, and return the plates to the incubator.
7. On the following morning, replace the media with high-serum culture media (2 mL/well).

8. 24 hours after transfection, image AKAR4 fluorescence in the three plates in a ChemiDoc imager using blue epi illumination for excitation and a 530/28 emission filter to detect fluorescence emission. Image using a range of exposure times including very short times (0.05 s) to ensure that day-to-day comparisons can be made without signal intensity rising out of range on the latter days.
9. On each subsequent day up to 5 days after transfection, carefully substitute in fresh media and re-image the plates (**Figure 1B**).
10. Quantify the signal intensity of each well on each day using ImageJ to yield time courses for the five different PEI:DNA ratios with either 0.67 μg DNA/well (**Figure 1C**), 1.33 μg DNA/well (**Figure 1D**), or 3.33 μg DNA/well (**Figure 1E**). For AKAR4-8His-NES, high expression can be achieved three days after transfection with 3.33 μg and 20 μL PEI per well (light blue, **Figure 1E**).

3.2 Reporter purification following scaled up expression in HEK293T cells

All protein purification steps should be performed on ice or in a refrigerated cabinet.

1. Seed 20 x 10 cm diameter tissue culture dishes with HEK293T cells at a confluence of ~ 35 % in 10 mL high-serum cell culture media per dish, and return to a 37 °C incubator with 5 % CO_2.
2. On the following morning when the cells have reached a confluence of ~ 70 %, substitute in 10 mL low-serum media one hour before addition of the transfection mixtures and return to the incubator.
3. Pipette 400 μg DNA into a 15 mL falcon tube and bring up to 10 mL with DMEM.
4. Mix 2.4 mL PEI (1 mg/mL) with 7.6 mL DMEM in a separate 50 mL falcon tube, vortex for 3 seconds, and leave to stand at room temperature.
5. After 20 minutes, add the PEI solution to the DNA solution, vortex for 3 seconds, and then leave to stand for a further 20 minutes at room temperature.
6. Pipette 1 ml of the PEI-DNA mixture onto each plate dropwise while swirling the plate gently, and return the plates to the incubator.
7. The following morning, replace the media with 10 mL high-serum media per plate.
8. Two days after transfection, substitute in fresh high-serum media again.
9. Three days after transfection, aspirate the media, wash each plate with 10 mL PBS taking care not to detach the cells, aspirate the PBS, and transfer the plates into a – 80 °C freezer (see **Note *10***).

10. Thaw plates on ice, then add 1 mL lysis buffer per plate and leave to incubate for 10 minutes. Resuspend cells using a 1 mL pipette, and collect all of the material (~ 25 mL) in a 50 mL falcon tube. Sonicate for 3 x 10 seconds before clarifying the lysate by centrifugation at 48, 000 x *g* for 30 minutes.
11. Exchange the supernatant into Nickel Buffer A using 2 HiPrep 26/10 Desalting columns connected in series (*see* **Note *11***) using the AKTA start.
12. Apply the protein solution to a 5 mL HisTrap HP column pre-equilibrated in Nickel Buffer A using the AKTA start, then wash the column for 30 mL after stepping up to 7% Nickel Buffer B (30 mM imidazole final concentration).
13. Elute the protein with a gradient over 30 mL up to 100 % Nickel Buffer B (black triangle, **Figure 2A**), collecting 2 mL fractions throughout the elution.
14. To detect fractions containing AKAR4-8His-NES, dispense 30 µL from each fraction into a black-walled 96-well plate and measure emission at 520 nm following excitation at 430 nm in the FLUOstar Omega plate reader (*see* **Note *12***). The reporter elutes towards the end of the imidazole gradient (green line, **Figure 2A**).
15. Take 20 µL from each fraction and mix with 1 µL 1 M DTT, and 7 µL 4x NuPAGE sample buffer. Without heating (see **Note *13***), separate the samples using a 4-12 % NuPAGE gel alongside Novex Sharp Pre-stained protein standards.
16. After the run has completed, transfer the gel into distilled water and immediately image AKAR4 fluorescence using the ChemiDoc system (**Figure 2B**).
17. Transfer the same gel into 50 mL Coomassie stain solution and incubate overnight on a rocker, then destain the following day with 3 x 1-hour incubations in 100 mL destain solution (**Figure 2C**).
18. Pool peak fractions containing pure AKAR4 (fractions 12-14 in **Figure 2**), exchange into storage buffer using a HiPrep 26/10 desalting column, and concentrate to ~ 4 mL using a Vivaspin Turbo 15 centrifugal concentrator.
19. Determine the protein concentration by BCA assay. This protocol yields ~ 2.5 mg purified AKAR4-8His-NES. Dilute the protein to 0.381 mg/mL for a 5 µM working solution, and divide into aliquots of 100 µL for storage at – 80 °C.

3.3 Calibrating the reporter for plate reader activity assays

The aim of this section is to determine a suitable PKA C subunit concentration and time range to use for assaying PKA activity with the purified reporter. It is written in such a way that it could be adapted to a different PKA activity reporter (**Note *14***).

1. Prepare serial dilutions of PKA C subunits in dilution buffer at the following nM concentrations: 667, 333, 167, 66.7, 33.3, 1.67, and 0. Addition of 15 μL of each solution into total reaction volumes of 50 μL will achieve the desired final concentrations of 200, 100, 50, 20, 10, and 0 nM C subunits.
2. Prepare 250 μL reaction master mix comprising 180 μL dilution buffer, 50 μL reaction buffer (10x), and 20 μL purified AKAR4 (2 μM stock).
3. Dispense 25 μL master mix into six wells of a black-walled 96-wall plate. To each well, add 15 μL of the appropriate C subunit dilution. To a seventh well, add 35 μL dilution buffer and 5 μL reaction buffer (10x) to serve as a control well enabling subtraction of background fluorescence.
4. While the plates are equilibrating to room temperature for 15 minutes, thaw an aliquot of injection solution to 22 °C using a heat block, then prime the injector of the plate reader with this solution (*see* **Note 15**).
5. Insert the plate into the plate reader, and determine a suitable gain for both 520 and 485 nm emission upon excitation at 430 nm using one of the experimental wells.
6. Start the run with the following parameters: measure 520 and 485 nm emission for each well at 5 second intervals for a total of 31 minutes (*see* **Note *16***). In place of the fourth recording at 15 seconds, initiate PKA phosphorylation by injecting 10 μL of injection solution into each of the seven wells (*see* **Note *17***).
7. Process the data by first subtracting background fluorescence from each of the six experimental wells according to the value of the control well in which the reporter was omitted. Average the three readings before ATP injection to determine the starting emission ratio. Once background-subtracted values for both channels have been obtained for each datapoint, calculate the 520/485 nm emission ratios. This process can be automated using MARS data analysis software.
8. Plot the data (**Figure 3A**), and calculate the rates (expressed as % change in 520 nm/485 nm emission ratio over time) during the initial period of linear change. Slopes can be determined using, for example, the SLOPE function in Excel.
9. Repeat the experiment twice more and plot the initial rates – averaged from three runs – against PKA C subunit concentration. For AKAR-8His-NES, the initial rate follows a linear relationship to PKA concentration up to 200 nM C (**Figure 3B**). This data indicates that recording over 20 minutes with C subunits in the 10-50 nM range would be appropriate for determining the IC_{50} of H89.

3.4 Determining the IC_{50} of a PKA inhibitor

The small molecule H89 inhibits PKA with a K_i = 48 nM by binding competitively to the ATP-binding pocket of the C subunit [11]. Since H89 competes with ATP, its half-maximal inhibitory concentration (IC_{50}) should theoretically be substantially higher at physiological concentrations of ATP. One of the advantages of using AKAR4 to monitor PKA activity is that elevated concentrations of ATP do not dilute out the tracer as would happen in an assay based on ^{32}P. Therefore, in a proof-of-concept experiment, here we describe determination of the IC_{50} for H89 inhibition of PKA in the presence of 1 mM ATP. This is a much higher concentration of ATP than used in previous studies [11] and is close to physiological levels [23] .

1. Prepare serial dilutions of H89 in dilution buffer at the following μM concentrations: 66.7, 33.3, 16.7, 6.67, 3.33, 1.67, 0.667, 0.333, 0. Addition of 15 μL of each solution into total reaction volumes of 50 μL will achieve the desired final concentrations of 20, 10, 5, 2, 1, 0.5, 0.2, 0.1, and 0 μM H89.
2. Prepare 300 μL of the following master mix: 60 μL reaction buffer (10x), 36 μL dilution buffer, 24 μL AKAR4, 180 μL PKA C subunit (from 83.3 nM stock for a final concentration of 25 nM)
3. Dispense 25 μL of the master mix to each of nine wells in a black-walled 96-well plate. Add 15 μL of the appropriate H89 dilution to each well. To a tenth background fluorescence control well add 35 μL dilution buffer and 5 μL reaction buffer (10x).
4. While the plates are equilibrating to room temperature for 15 minutes, thaw an aliquot of injection solution to 22 °C using a heat block, then prime the plate reader injector.
5. Insert the plate into the FLUOstar Omega plate reader and determine a suitable gain for both 520 and 485 nm emission channels upon excitation at 430 nm using one of the experimental wells.
6. Start the run with the following parameters: measure 520 and 485 nm emission for each well at 10 second intervals for a total of 21 minutes. In place of the fourth recording at 30 seconds, initiate PKA phosphorylation by injecting 10 μL of injection solution into each of the seven wells.
7. Process the data to obtain background-subtracted 520/485 nm emission ratios. As shown in **figure 4A**, higher H89 concentrations noticeably reduce PKA activity. Calculate the rate of change in 520 nm/485 nm emission ratio between 10-310 seconds using the SLOPE function in Excel.
8. Repeat the experiment twice more before plotting the initial rates against H89 concentration on a logarithmic scale. Determine the IC_{50} by curve fitting using a four-parameter function such as the Hill1 function in Origin (**Figure 4B**). Using this approach, we determined an

IC_{50} = 1.53± 0.06μM for H89 inhibition of PKA, confirming that a physiological ATP concentration elevates the IC_{50} of H89 into the low micromolar range (*see* **Note 18**).

4. Notes

1. It is also possible to perform PEI transfection of suspension mammalian cells such as Freestyle 293F cells (Thermo Fisher Scientific) where suitable facilities are available (an orbital shaker with a humidified atmosphere of 8% CO_2 in air). This will reduce plastic waste.

2. Higher transfection efficiency can be achieved by initially omitting antibiotics and reducing the serum concentration in the culture media.

3. Standard transfection reagents such as Lipofectamine-2000 (ThermoFisher) or FuGENE (Promega) may be used in place of PEI if cost is less of a consideration.

4. Alternative imaging systems include the ImageQuant series (Cytiva). A qualitative comparison can be made on a standard long-wavelength UV transilluminator.

5. If an FPLC is not available, the procedure may be performed in batch by diluting the initial clarified lysate into 200 mL Nickel Buffer A, and incubating with 5 mL Ni-NTA agarose (Qiagen) for one hour in a roller bottle. After washing with buffer containing 100 mM imidazole, elute the reporter with Nickel Buffer B. The final buffer exchange step into storage buffer may be performed with a PD-10 desalting column packed with Sephadex G-25 resin (Cytiva).

6. Purified PKA C subunit is also commercially available (e.g., Promega, catalogue no. V516A).

7. Many alternative microplate readers are available. The key requirements are that the reader can rapidly switch emission filters for ratiometric fluorescence measurements, and that the reader is equipped with at least one injector for initiating reactions with ATP.

8. The most advanced PKA activity reporters include ExRai-AKAR2 [24] and tAKARα [25].

9. These numbers are equivalent to 4, 10, and 20 μg DNA per 10 cm-diameter cell culture dish.

10. The plates can be left in storage to continue the protocol at a later date, if convenient.

11. Alternatively, divide the supernatant and run two batches of equal volume through a single column.

12. This step is not essential – the SDS-PAGE analysis (**Figure 2B & C**) is sufficient to show which fractions contain purified AKAR4-8His-NES.

13. Heating the samples prior to electrophoresis would reduce subsequent in-gel fluorescence of AKAR4.

14. If adapting the assay for a different reporter, the first step is to determine a suitable concentration of reporter. This can be achieved by reading a serial dilution of the reporter – for AKAR4, the signal is approximately 10-fold above background in both channels with the reporter at 0.2 μM.

15. A fresh vial of injection solution should be thawed before each plate reader run otherwise ATP hydrolysis in the solution will lead to reduced apparent PKA activity in later runs.

16. For recordings with the FLUOstar Omega, when reading 7 wells in parallel in 'plate' mode, the shortest possible spacing between readings is approximately 5 seconds.

17: If a second injector is available, this can be exploited to vary the concentration of injected components such as metal ions, nucleotides, or small molecule inhibitors.

18. The elevated IC_{50} of H89 for PKA at physiological ATP concentrations undermines the applicability of the inhibitor given that it is known to trigger off-target effects in the low micromolar range [26].

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Figure Legends

Figure 1. Optimization of PKA activity reporter expression. (A) An octa-histidine (8His, purple) affinity tag was inserted at the C-terminus of the genetically-encoded reporter AKAR4 immediately prior to the terminal nuclear export signal (NES, green). Phosphorylation of the reporter at a central PKA consensus site (blue) triggers a conformational change in which the central FHA domain associates with the phosphorylated site and thereby brings cerulean and cpVenus into closer proximity. In this way, increased FRET serves as a readout of PKA phosphorylation. (B) AKAR4-8His-NES expression was monitored in 6-well plates using GFP fluorescence comparing five ratios of PEI:DNA, and three different amounts of DNA per well. GFP fluorescence intensity is shown in wells three days after transfection. (C-E) Quantification of AKAR4 expression in transfected HEK293T cells as indicated by fluorescence emission at 530 nm following excitation with blue light. Measurements are shown for different PEI:DNA ratios up to 5 days after transfection using either (c) 0.67 **µ**g, (d) 1.67 **µ**g, or (e) 3.33 **µ**g DNA per well.

Figure 2. PKA activity reporter purification. (A) The trace shows UV absorbance (blue) throughout purification of AKAR4-8His-NES following expression in HEK293T cells. Fluorescence emission at 520 nm following excitation at 430 nm was also measured for each fraction and is overlaid on the trace in green. The first datapoint for FRET emission corresponds to the starting lysate. AKAR4-8His-NES was eluted using the indicated imidazole gradient. Fractions including the starting lysate (lys) and flow-through (f/t) were subjected to SDS-PAGE on a 4-12 % gel, and the fluorescent reporter was initially detected by in-gel fluorescence emission at 530 nm following excitation with blue light (B). Coomassie staining of the same gel confirmed that AKAR4-8His-NES was present at high purity in fractions 12-14 (C).

Figure 3. Calibration of purified PKA activity reporter measurements in plate reader. (A) Changes in the ratio of emission at 520 (yellow, Y) and 485 (cyan, C) nm emission, upon excitation at 430 nm, were monitored over time in an FLUOstar Omega microplate reader. Time series were collected in triplicate either in the absence of PKA C subunit (green), or with 10 (light blue), 20 (gold), 50 (grey), 100 (orange), or 200 (dark blue) nM C subunit. All reactions were initiated by injection of ATP to a final concentration of 1 mM. (B) Plot of initial rate of change in Y/C emission ratio vs C subunit concentration. Initial rates were calculated between 10 and 50 seconds for 50, 100, and 200 nM C subunits; and between 10 and 310 seconds for 0, 10, and 20 nM C subunits. The two variables follow a linear relationship (red line). All data is shown as mean ± standard error.

Figure 4. Determination of IC_{50} for H89 using purified PKA activity reporter. (A) Changes in 520/485 nm emission ratio were measured in a FLUOstar Omega microplate reader. Wells contained

25 nM C subunits mixed with variable concentrations of H89. Reactions were initiated by addition of ATP to a final concentration of 1 mM, and fluorescence readings were collected at 10-second intervals for 20 minutes as shown in the example recordings (B) Rates of change in Y/C emission ratio were calculated between 10-310 seconds for each H89 concentration (n=3), and are shown as mean ± standard error plotted against H89 concentration. An IC_{50} value for H89 was determined by fitting the data to a Hill function.

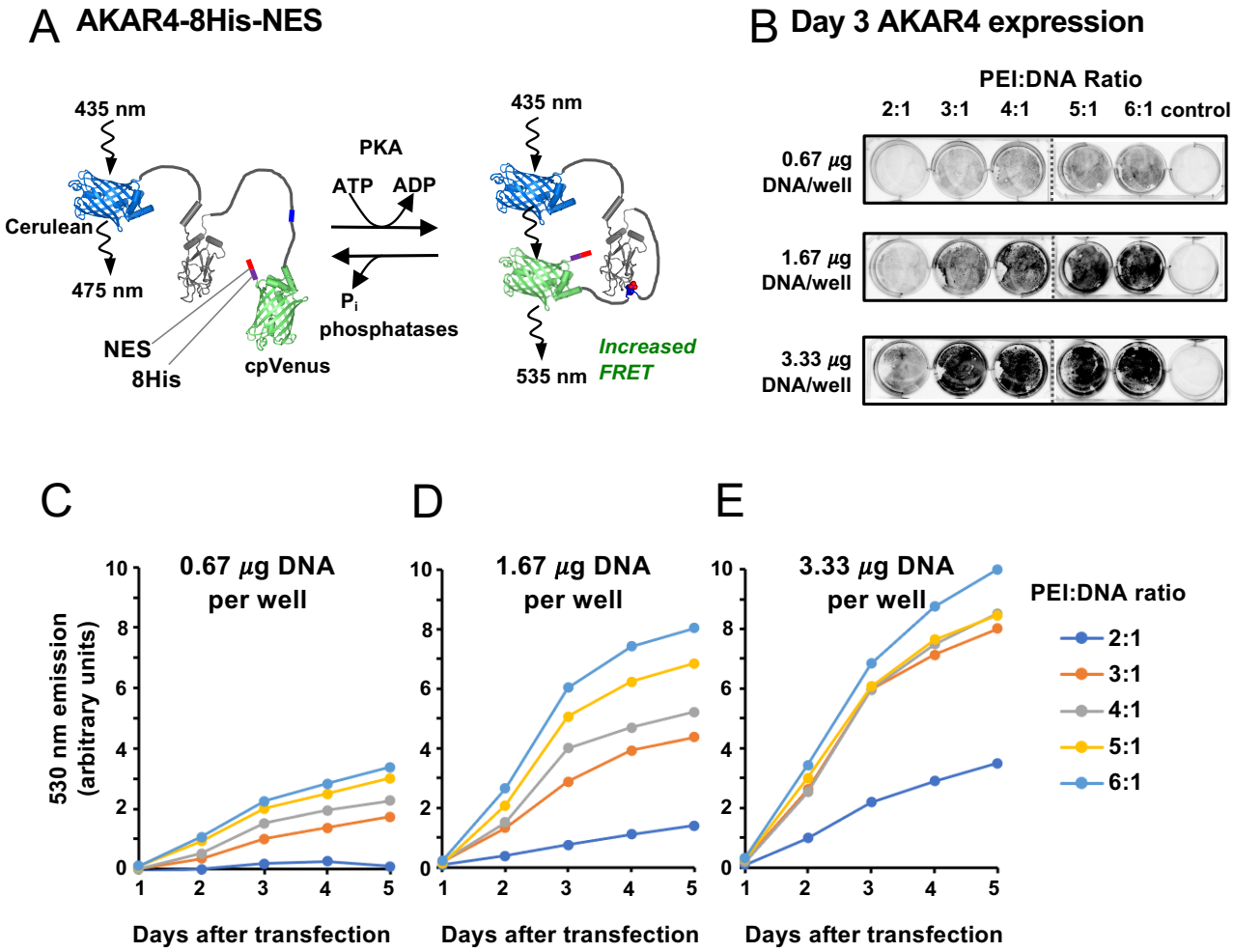

A AKAR4-8His-NES
435 nm
Cerulean
475 nm
NES
8His
cpVenus
PKA
ATP ADP
Pi
phosphatases
435 nm
535 nm
Increased FRET
B Day 3 AKAR4 expression
PEI:DNA Ratio
2:1 3:1 4:1 5:1 6:1 control
0.67 µg DNA/well
1.67 µg DNA/well
3.33 µg DNA/well
C
D
E
0.67 µg DNA per well
1.67 µg DNA per well
3.33 µg DNA per well
530 nm emission (arbitrary units)
Days after transfection
PEI:DNA ratio
2:1
3:1
4:1
5:1
6:1

Figure 1

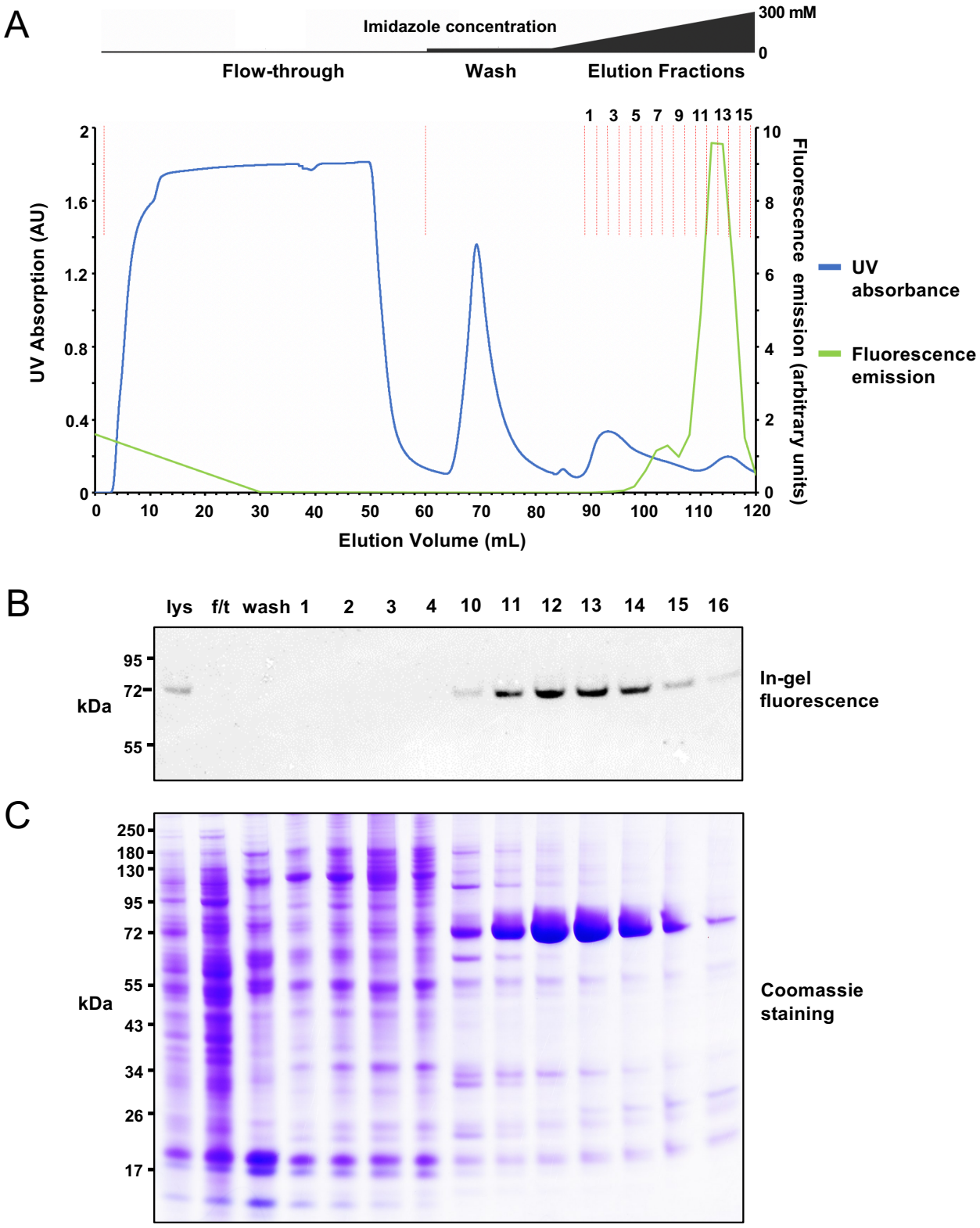
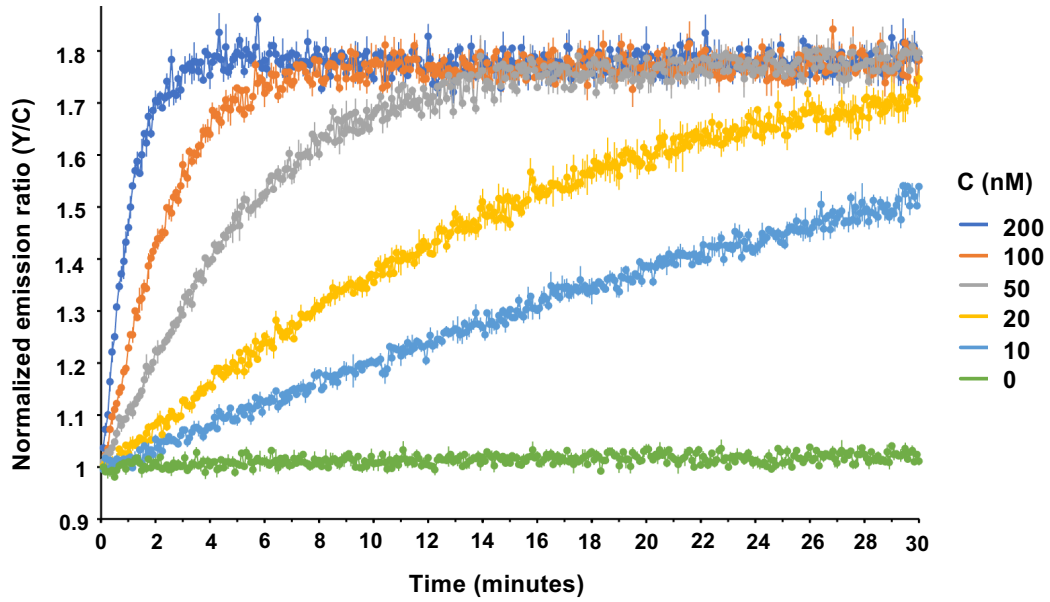

A
Imidazole concentration
300 mM
0
Flow-through
Wash
Elution Fractions
1 3 5 7 9 11 13 15
UV Absorption (AU)
Fluorescence emission (arbitrary units)
Elution Volume (mL)
UV absorbance
Fluorescence emission
B
lys f/t wash 1 2 3 4 10 11 12 13 14 15 16
kDa
95
72
55
In-gel fluorescence
C
250
180
130
95
72
55
43
34
26
17
kDa
Coomassie staining

Figure 2

A


Normalized emission ratio (Y/C)
Time (minutes)
C (nM)
200
100
50
20
10
0

B

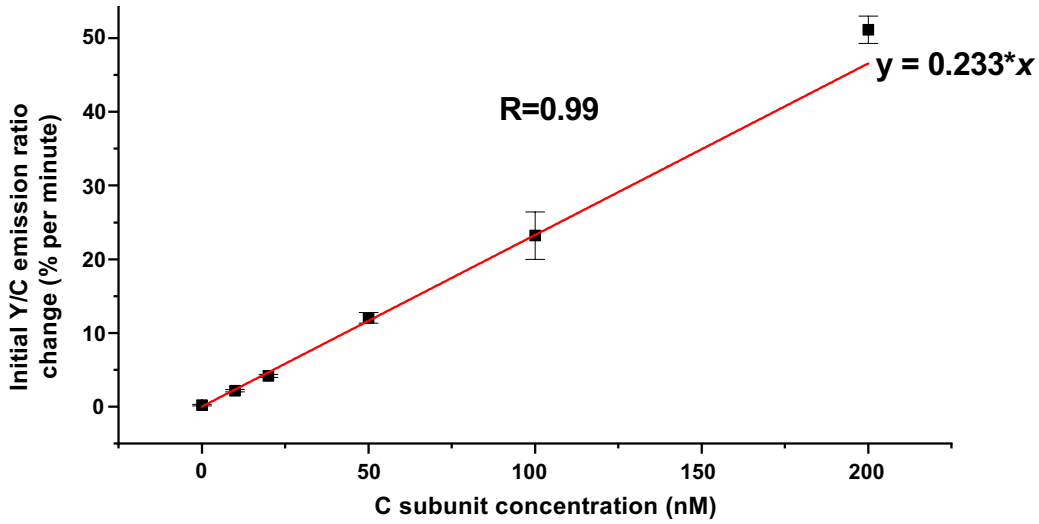

Initial Y/C emission ratio change (% per minute)
C subunit concentration (nM)
R=0.99
y = 0.233*x

Figure 3

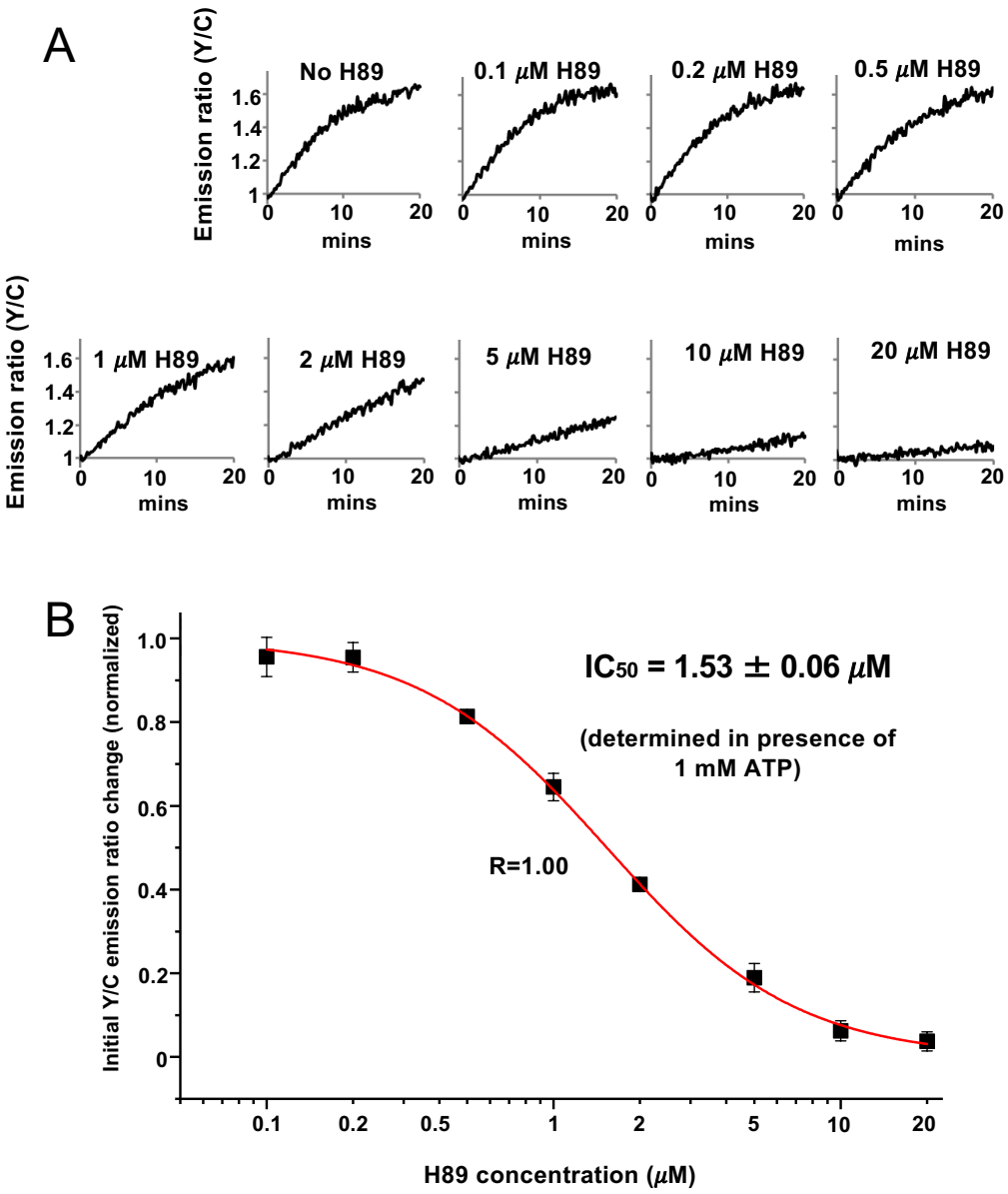

A
Emission ratio (Y/C)
No H89
0.1 μM H89
0.2 μM H89
0.5 μM H89
1 μM H89
2 μM H89
5 μM H89
10 μM H89
20 μM H89
mins
B
Initial Y/C emission ratio change (normalized)
IC50 = 1.53 ± 0.06 μM
(determined in presence of
1 mM ATP)
R=1.00
H89 concentration (μM)

Figure 4