

Aug 15, 2025

High-throughput cell-based 384-well format screening assay to identify small-molecule inhibitors of ALPK1 disease-causing mutants

DOI

<https://dx.doi.org/10.17504/protocols.io.n2bvjn74wgk5/v1>

Tom Snelling¹

¹MRC Protein Phosphorylation and Ubiquitylation Unit, School of Life Sciences, University of Dundee, DD1 5EH, Scotland, UK



Tom Snelling

University of Dundee

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.n2bvjn74wgk5/v1>

Protocol Citation: Tom Snelling 2025. High-throughput cell-based 384-well format screening assay to identify small-molecule inhibitors of ALPK1 disease-causing mutants. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.n2bvjn74wgk5/v1>

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

I developed this protocol and it is working for screening up to 20x384-well plates at once.

Created: October 08, 2024

Last Modified: August 15, 2025

Protocol Integer ID: 109363

Keywords: ALPK1, TIFA, 384-well, ROSAH, Spiradenoma, Screening, Semi-automated, Inhibitor, molecule inhibitors of alpk1 disease, applicable to other alpk1 mutant, other alpk1 mutant, constitutive activity of alpk1 disease, alpk1 disease, screening assay, reporter assay, kb, molecule inhibitor, assay, reporter system activation, mutants this protocol, throughput screening, causing mutant, molecule library, suitable for the screening, screening

Funders Acknowledgements:

Medical Research Council (PhD studentship)

Grant ID: 2087974

Abstract

This protocol outlines a semi-automated 384-well format NF- κ B/AP-1 reporter assay designed to quantify the constitutive activity of ALPK1 disease-causing mutants using only 8000 transfected HEK-Blue cells per well. The assay is optimised for high-throughput screening, can be used to analyse up to 20×384-well plates at once and uses NG-25 as a positive control compound that blocks reporter system activation. While the protocol is tailored for the ALPK1[V1092A] mutant, it is also applicable to other ALPK1 mutants (such as T237M, Y254C, and S277F), all of which exhibit constitutive activity when expressed in cells. The assay is suitable for the screening of various small-molecule libraries, supporting single time points, time-course studies, and dose-response analyses.

Guidelines

Key Features

1. Highly sensitive reporter assay allowing detection of low level activity arising from ALPK1 mutants
2. Optimised in 384-well plate format, requiring only 8000 transfected HEK-Blue cells per well
3. Suitable for compound screening to identify inhibitors of ALPK1 signalling driven by mutant ALPK1 in cells
4. Semi-automated, requiring only a few hours of hand-on time to complete



Materials

Laboratory Equipment

Pipetman 4-Pipette Kit (Gilson, #F167360)
Stripettor Ultra Pipet Controller (Corning, #4099)
CB150 Cell Culture Incubator (Binder)
BioMAT 2-SF Cell Culture Hood (Conditioned Air Solutions)
Cellometer Auto 2000 (Nexcelom Bioscience)
Allegra X-12 benchtop centrifuge (Beckman Coulter, #392474)
Large Bore 8 Channel Cassette For VIAFILL (Integra Biosciences, #5724)
VIAFILL Reagent Dispenser (Integra Biosciences)

Laboratory Supplies

15 cm Nunc cell culture dishes (ThermoFisher, #168381)
384-well cell culture plates (ThermoFisher, #164688)
500 ml sterile PET storage bottles (Sigma-Aldrich, #CLS431532)
50 ml conical centrifuge tubes (Greiner, #227261)
Serological pipettes (ThermoFisher, #10710810)
Safe-Lock 5.0 ml tubes (Eppendorf, #30119401)
Safe-Lock 1.5 ml microcentrifuge tubes (Eppendorf, #30123611)
Cellometer counting chamber (Nexcelom, #11522186)
Cellometer counting chambers (Nexcelom, #11522186)

Biological Material

ALPK1 knockout (KO) HEK-Blue cells (Invivogen, #hkb-koalpk) (liquid nitrogen)

General Reagents

Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, #11960-085) (4°C)
200 mM L-Glutamine (Gibco, #25030024) (-20°C)
Penicillin-Streptomycin 100X stock (Gibco, #15140122) (-20 °C)
Opti-MEM I Reduced Serum Medium (Gibco, #31985062) (4 °C)
Lipofectamine 2000 (ThermoFisher, #11668019) (4 °C)
Phosphate-buffered saline (PBS) (Gibco, #10010023) (4 °C)
Foetal Bovine Serum (FBS) (Sigma-Aldrich, #F7524) (-20 °C)
Trypsin-EDTA solution (Gibco, #25200056) (4 °C)
Trypan Blue solution (Gibco, #11538886) (room temp)
Quanti-BLUE solution 100× stock (Invivogen, #rep-qbs) (-20 °C)
Sterile-filtered water (Sigma-Aldrich, #W3500) (4 °C)
NG25 trihydrochloride (Sigma-Aldrich, #SML1332) (-20 °C)

Before start

Before starting the procedure, a confluent 15 cm cell culture dish of ALPK1 knockout HEK-Blue cells is required (cultured in 20 ml of culture media), sufficient for an experiment using up to 10×384-well plates (if required, double the number of cells used in the protocol to plate up to 20×384-well plates which is the recommended upper limit). The cells should be cultured by incubation at 37 °C with 5% CO₂ and tested regularly for mycoplasma using a MycoAlert Mycoplasma Detection Kit (Lonza, #LT07-318). The cells should be passaged once confluent at a ratio of 1:10 (v/v), and not used beyond 30 passages.

Culture media (1 bottle)

Reagent	Final concentration	Quantity to add
DMEM	Not applicable	500 mL
FBS	10% (v/v)	50 mL
200 mM L-Glutamine	2 mM	5.6 mL
Penicillin-streptomycin 100×	1×	5.6 mL

(Recipe for preparing a 500 ml bottle of Culture Media)



Plate ALPK1 knockout HEK-Blue cells into 2×15 cm culture dishes for transfection (Day 1)

18m

- 1 At approximately 4 pm, aspirate the culture media from a confluent 15 cm cell culture dish of ALPK1 knockout HEK-Blue cells and replace with 10 ml of PBS. 2m
- 2 Aspirate the PBS and add 3 ml of trypsin-EDTA solution. Return the dish to the incubator until the cells have visually detached. 5m
- 3 Add 12 ml of culture media and pipette up and down with a serological pipette until a single-cell suspension has been produced (the total volume should be 15 ml). 2m
- 4 Transfer the cell suspension to a 50 ml conical centrifuge tube and add 35 ml of culture media, bringing the total volume to 50 ml. 1m
- 5 Transfer 20 µl of the cell suspension to a 1.5 ml microcentrifuge tube and dilute 1:5 (v/v) by adding 80 µl of culture media. Mix 20 µl of the diluted cell suspension with 20 µl of trypan blue solution and count the number of cells, ensuring that the cell viability is at least 90%. 5m
- 6 Seed 14 million ALPK1 KO HEK-Blue cells into each of the two 15 cm cell culture dishes and add culture media up to a total volume of 20 ml. Ensure that cells are evenly distributed by moving the plates in a figure-of-8 motion prior to returning them to the incubator for approximately 24 h. 5m 
- 7 Seed an additional 15 cm cell culture dish with 2 million ALPK1 KO HEK-Blue cells to maintain the cell line, which should become confluent in approximately 4 days. 1m

Transfect ALPK1 knockout HEK-Blue cells with FLAG-ALPK1[V1092A] (Day 2)

14h 17m

- 8 Note: Transfection should be performed at approximately 5 pm, with a media change at 8 pm and the cells plated into 384-well plates at 8 am the following day.
- 8.1 Confirm that the confluency of the ALPK1 KO HEK-Blue cells is approximately 90%. 1m
- 8.2 Each transfected plate requires 150 µl of lipofectamine 2000 diluted in 500 µl of OptiMEM within a 1.5 ml microcentrifuge tube. Prepare two tubes of diluted lipofectamine 2000 (one for each dish) and invert them five times to mix. 1m



- 8.3 In two separate 1.5 ml microcentrifuge tubes (one for each dish), dilute 60 µg of ALPK1[V1092A] plasmid in 500 µl of Opti-MEM per tube. Invert five times to mix. 1m
- 8.4 Add the contents of one tube of diluted lipofectamine 2000 to one tube of diluted plasmid and repeat for the second pair of tubes. Invert five times to mix and incubate for 10 min at room temp. 11m
- 8.5 Add the transection mixture dropwise to each dish of cells using a P1000 pipette and return them to the incubator. 1m
- 8.6 After 3 h, carefully aspirate the culture media and add 20 ml of fresh culture media by pipetting slowly against the side of the plate to minimise cell detachment. This step removes the DNA-lipid complexes, which is observed to minimise cell toxicity. 2m
- 8.7 Return the plates to the incubator for 15 h. 14h

Note: This is the optimal time point for re-plating of cells into 384-well plates for downstream steps, because the expression of ALPK1[V1092A] is minimal and therefore activation of the reporter assay is not yet detectable, reducing background signal in screening assays where compounds are pre-plated.



Plating of cells into 384-well plates (Day 3)

49m

- 9 The following morning (15 h post-transfection), aspirate the culture media, add 20 ml PBS, aspirate the PBS and add 3 ml of trypsin-EDTA solution. Return the dishes to the incubator until the cells have visually detached. 5m
- 10 Add 12 ml of culture media and pipette up and down with a serological pipette until a single-cell suspension has been produced (the total volume should be 15 ml). 2m
- 11 Transfer the cell suspension to a 50 ml conical centrifuge tube and add 35 ml of culture media, bringing the total volume to 50 ml. 2m
- 12 Transfer 20 µl of the cell suspension to a 1.5 ml microcentrifuge tube and dilute 1:5 (v/v) by adding 80 µl of culture media. Mix 20 µl of the diluted cell suspension with 20 µl of trypan blue solution and count the number of cells, ensuring that the cell viability is at least 90%. 5m
- 13 Dilute the cell suspension to 160,000 cells/ml in a 500 ml sterile bottle. 5m
- 14 Plate cells into 384-well culture plates using a VIAFILL on the "medium" setting, set to a volume of 50 µl (i.e. 8000 cells per well). Return the 384-well plates to the incubator for 24 h. 30m



Detection of ALPK1[V1092A] activity (Day 4)

1h 5m

- 15 The following day (24 h after plating), add 50 µl of detection solution (1x QUANTI-BLUE) to each well using a ViaFill on the "medium" setting (see below for recipe). 5m
- 16 Return the plates to the incubator for 30 min and then read the absorbance values at 645 nm using a PheraSTAR with a plate stacker attachment. 30m

Protocol references

Zhou, P., She, Y., Dong, N., Li, P., He, H., Borio, A., Wu, Q., Lu, S., Ding, X., Cao, Y., et al. (2018). Alpha-kinase 1 is a cytosolic innate immune receptor for bacterial ADP-heptose. *Nature*. 561(7721): 122–126.

<https://doi.org/10.1038/s41586-018-0433-3>

Williams, L. B., Javed, A., Sabri, A., Morgan, D. J., Huff, C. D., Grigg, J. R., Heng, X. T., Khng, A. J., Hollink, I. H., Morrison, M. A., et al. (2019). ALPK1 missense pathogenic variant in five families leads to ROSAH syndrome, an ocular multisystem autosomal dominant disorder. *Genet Med*. 21(9): 2103–2115. <https://doi.org/10.1038/s41436-019-0476-3>

Rashid, M., van der Horst, M., Mentzel, T., Butera, F., Ferreira, I., Pance, A., Rütten, A., Luzar, B., Marusic, Z., de Saint Aubain, N., et al. (2019). ALPK1 hotspot mutation as a driver of human spiradenoma and spiradenocarcinoma. *Nat Commun*. 10(1): e1038/s41467-019-09979-0. <https://doi.org/10.1038/s41467-019-09979-0>

Snelling, T., Saalfrank, A., Wood, N. T. and Cohen, P. (2023). ALPK1 mutants causing ROSAH syndrome or Spiradenoma are activated by human nucleotide sugars. *Proc Natl Acad Sci USA*. 120(50): e2313148120. <https://doi.org/10.1073/pnas.2313148120>

Snelling, T., Garnotel, L. O., Jeru, I., Tusseau, M., Cuisset, L., Perlat, A., Minard, G., Benquey, T., Maucourant, Y., Wood, N. T., et al. (2024). Discovery and Functional analysis of a novel ALPK1 variant in ROSAH syndrome. *bioRxiv*: e612837. <https://doi.org/10.1101/2024.09.13.612837>