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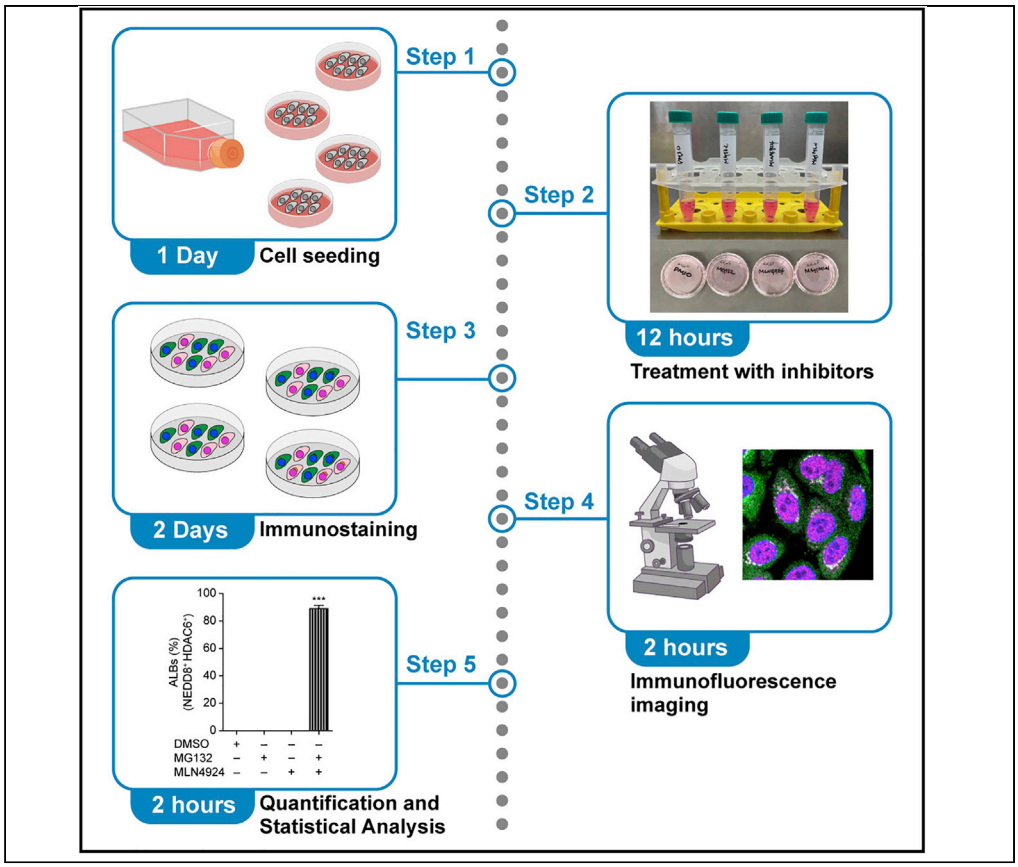
A protocol to visualize cytosolic aggresome-like bodies using confocal microscopy



Ubiquitin stress-induced NEDDylation leads to the formation of aggresome-like bodies (ALBs) in the perinuclear region of cells. Therefore, imaging analysis is essential for characterizing the biological phenotypes of ALBs. Here, we describe a protocol to monitor ALBs induced by ubiquitin stress using immunocytochemistry and to quantify cells containing ALBs. This optimized protocol details the use of readily available materials and reagents and can be applied to explore diverse molecules involved in stress-induced ALBs.

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Highlights

ALBs induced by ubiquitin stress can be monitored using immunocytochemistry

Diverse types of aggresomes in cells can be quantified

Kim et al., STAR Protocols 2, 100674
September 17, 2021
https://doi.org/10.1016/j.xpro.2021.100674

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A protocol to visualize cytosolic aggresome-like bodies using confocal microscopy

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https://doi.org/10.1016/j.xpro.2021.100674

SUMMARY

Ubiquitin stress-induced NEDDylation leads to the formation of aggresome-like bodies (ALBs) in the perinuclear region of cells. Therefore, imaging analysis is essential for characterizing the biological phenotypes of ALBs. Here, we describe a protocol to monitor ALBs induced by ubiquitin stress using immunocytochemistry and to quantify cells containing ALBs. This optimized protocol details the use of readily available materials and reagents and can be applied to explore diverse molecules involved in stress-induced ALBs.

For complete details on the use and execution of this protocol, please refer to Kim et al. (2021).

BEFORE YOU BEGIN

The formation of stress-induced cytosolic aggresome-like bodies (ALBs) is controlled by NEDD8, HDAC6, and p62 (Kim et al., 2021). Analysis of cells containing ubiquitin stress-induced ALBs is important for understanding how cells respond to diverse stresses via protein aggregation. To monitor this phenomenon in cells, endogenous proteins are detected by immunostaining, which can be used to quantify ALB-containing cells. Therefore, this protocol provides simplified methods to examine the proteins involved in the formation of stress-induced ALBs.

A list of all reagents used and buffer recipes for this protocol are described in the Key Resources Table and Materials and Equipment sections. All buffers required for the experiments were prepared in advance. We also suggest using HeLa cells to clearly observe this phenomenon.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-NEDD8 antibody [Y297] (ab81264)	Abcam	ab81264
HDAC6 antibody [4C5]	GeneTex	GTX84377
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 647 conjugate	Thermo Fisher Scientific	A-21245
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 546 conjugate	Thermo Fisher Scientific	A-11030

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dimethyl Sulfoxide	Sigma-Aldrich	D2650
Tubacin	Selleckchem	S2239
MLN-4924 (Pevonedistat)	Active Biochem	A1139
Z-Leu-Leu-Leu-al ≥90% (HPLC) (MG132)	Sigma-Aldrich	C2211
4′,6-Diamidino-2-phenylindole (DAPI)	Sigma-Aldrich	D9542
MEM (Minimum Essential Medium)	Gibco	11095080
Fetal Bovine Serum, certified, United States	Gibco	16000044
PBS, pH 7.4	Gibco	10010023
Triton X-100	Sigma-Aldrich	T8787
Bovine Serum Albumin	Bovostar	BSAS-NZ
VECTASHIELD Antifade Mounting Medium	Vector Labs	H-1000
Paraformaldehyde	Sigma-Aldrich	P6148
Sodium chloride, NaCl (Optional)	Sigma-Aldrich	S3014
Potassium chloride, KCl (Optional)	Sigma-Aldrich	P9541
Sodium hydrogen phosphate, Na_2HPO_4 (Optional)	Sigma-Aldrich	S5136
Potassium dihydrogen phosphate, KH_2PO_4 (Optional)	Sigma-Aldrich	P0662
Experimental models: Cell lines		
Human: HeLa cells	ATCC	CCL-2
Software and algorithms		
NIS-Element AR Version 5.01	Nikon	http://www.microscope.healthcare.nikon.com/products/software/nis-elements/nis-elements-advanced-research
GraphPad Prism8	GraphPad	https://www.graphpad.com/scientific-software/prism/
Other		
Confocal Dish (Optional)	SPL	200350
Cover glasses circular (12 mm Ø)	Marienfeld	0111520
Eclipse A1Rsi and Eclipse Ti-E (Optional)	Nikon	N/A

MATERIALS AND EQUIPMENT

10× phosphate buffered saline (PBS)

Timing: ~30 min

Reagent	Final concentration	Amount
NaCl	1.4 M	81.8 g
KCl	27 mM	2.01 g
Na_2HPO_4	100 mM	14.2 g
KH_2PO_4	18 mM	2.45 g
ddH_2O	n/a	Add to 1 L
The pH should be adjusted using 5 mol/L NaOH until obtaining a stable pH of 7.3.		
Total	n/a	1 L

Note: Final 10× PBS solution should be filtered using a 0.22 μm filter paper prior to use. Store at 25°C and verify the lack of phosphate precipitates prior to use.

1× PBS

Timing: ~5 min

Reagent	Final concentration	Amount
10× PBS	n/a	100 mL
ddH_2O	n/a	900 mL
Total	**n/a**	**1 L**

4% Paraformaldehyde solution (PFA)

Timing: ~1 day

Reagent	Final concentration	Amount
Paraformaldehyde	4% (w/v)	40 g
1× PBS	n/a	Add to 1 L
The pH should be adjusted using 5 mol/L NaOH until obtaining a stable pH of 7.3.		
Total	**n/a**	**1 L**

Note: To dissolve PFA powder, stir the mixture at 60°C in a ventilation hood (DO NOT boil). PFA powder does not dissolve instantly, so the pH of the mixture needs to be raised by adding 5 mol/L NaOH drop by drop until a clear solution is formed. PFA solution can be stored at 4°C for a short-term (up to 1 month) storage or at –20°C for a long-term storage.

⚠ CRITICAL: PFA is a toxic irritant to the skin, eye, and respiratory tract and should be handled while wearing gloves, safety goggles, and a mask. Researchers must follow all laboratory safety guidelines.

Permeabilization buffer

Timing: ~5 min

Reagent	Final concentration	Amount
10% TritonX-100	0.25% (v/v)	1.25 mL
1× PBS	n/a	Add to 50 mL
Total	**n/a**	**50 mL**

Note: Permeabilization buffer has to be filtered using a 0.22 μm filter paper prior to use. The filtered permeabilization buffer can be stored at 25°C for 1 month.

Alternatives: The final concentration of TritonX-100 can be changed to 0.1%–0.5% depending on the requirements of the cell types.

Blocking buffer

Timing: ~30 min

Reagent	Final concentration	Amount
BSA	1% (v/v)	0.5 g
10% TritonX-100	0.25% (v/v)	1.25 mL
1× PBS	n/a	Add to 50 mL
Total	**n/a**	**50 mL**

Note: Blocking buffer should be freshly prepared before each use.

STEP-BY-STEP METHOD DETAILS

This protocol is divided into four main sections.

Cell seeding

Timing: ~24–30 h

1. Approximately 2 × 10^5 HeLa cells in 2 mL of Minimum Essential Medium (MEM) are seeded in a confocal glass bottom dish (recommended glass thickness: 0.13–0.16 mm) and allowed to attach for 20–24 h (troubleshooting 1).
   a. HeLa cells are cultured in MEM supplemented with 10% fetal bovine serum (FBS) in the absence of antibiotics. Dulbecco's modified Eagle's medium (DMEM) with low glucose can also be used for the culture of HeLa cells.

Note: We suggest that cell confluency in each dish should reach at least 70% at 24 h post-seeding. A low cell confluency may be insufficient for this experiment. Seeding with low cell numbers is recommended if you plan to culture for several days before use. Dishes seeded with higher cell numbers will be ready earlier for use. Standard HeLa cell culture guidelines often indicate to use media supplemented with antibiotics to prevent cell contamination. As previously reported, however, antibiotics can affect gene expression and regulation, so culture of HeLa cells without antibiotics is recommended for this experiment (Ryu et al., 2017). Please stick to the time of trypsinization to avoid generation of clumping cells, which inhibit even distribution of cells in the dish.

Optional: For studies using siRNA knockdown or overexpression of genes of interest, cells should be seeded using at least 1.2 × 10^5–1.8 × 10^5 cells in each confocal dish and then allowed to attach for 24 h prior to transfection.

Treatment with inhibitors

Timing: 12 h

2. For pretreatment with Tubacin, dilute Tubacin to a final concentration of 10 μM in 2 mL of MEM supplemented with 10% FBS (MEM-Tubacin).
3. Change to medium with MEM-Tubacin.
4. Further incubate cells for 12 h at 37°C in a 5% CO_2 atmosphere.
5. To treat the cells with inhibitors, dilute inhibitors in 2 mL of MEM-Tubacin (MEM-Tubacin/inhibitor).
   a. Dilute MG132 to a final concentration of 5 μM, MLN4924 to a final concentration of 3 μM, or use a combination of MG132 and MLN4924 in 2 mL of MEM-Tubacin.

⚠ CRITICAL: Care should be taken to avoid freeze-thaw cycles of inhibitors (troubleshooting 2).

6. Remove the media from dishes.
7. Change to medium with MEM-Tubacin/inhibitors.
8. Incubate inhibitor-treated cells for 12 h at 37°C in a 5% CO_2 atmosphere.

Note: For dilution of inhibitors, culture media supplemented with 10% FBS in the absence of antibiotics should be used. All inhibitors used in this protocol were dissolved in DMSO. In the

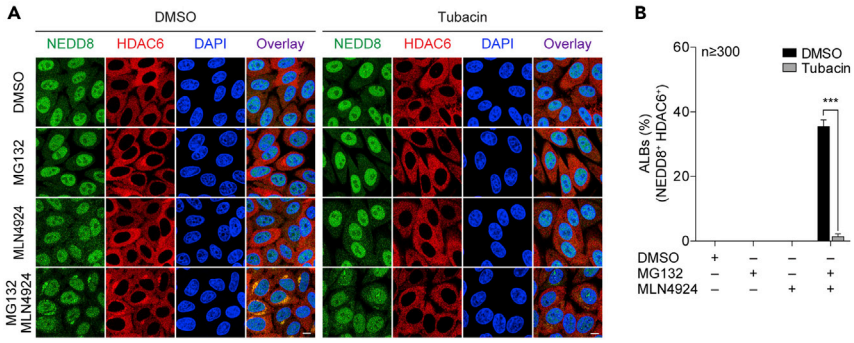

Figure 1. Immunofluorescence imaging of endogenous NEDD8 and HDAC6 in HeLa cells

(A) HeLa cells were treated with 10 μM Tubacin for 12 h, followed by treatment with each reagent for 12 h. The cells were fixed and stained with anti-NEDD8 and anti-HDAC6 antibodies. Nuclei were counterstained with DAPI. Scale bars, 10 μm.

Figure adapted from (Kim et al., 2021).

(B) The cells containing ALBs in (A) were quantified as indicated. + sign denotes positive cells showing ALB formation with indicated markers. Data represent mean ± SEM of three independent experiments. ***P ≤ 0.01.

Figure adapted from (Kim et al., 2021).

experiment treating with inhibitors, the same volume of DMSO should be used for the negative control. Cells should be passage at least once after thaw and the use of low-passage cells is recommended.

Immunostaining of endogenous proteins

Timing: ~2 days

This section describes the immunostaining method for the endogenous proteins. This is a critical step in the observation of ALBs using confocal microscopy and quantification of cells containing ALBs (Figure 1A). (troubleshooting 3)

Note: In our study, we used anti-NEDD8 and anti-HDAC6 antibodies to detect stress-induced ALBs.

9. After a 12 h treatment of cells with inhibitors, carefully remove the medium from dishes.
10. Wash cells once with 1× PBS at 25°C and remove the residual PBS.
11. Fix the cells with 1 mL of 4% PFA for 15 min at 25°C.
12. After 15 min of fixation, wash the cells three times with 1× PBS for 5 min each.

Pause point: Cells fixed in PFA can be stored in 1× PBS at 4°C up to 1 week for the next step.

13. Add 1 mL of permeabilization buffer to the cells for 15 min at 25°C to permeabilize the cell membrane.

Note: Methanol fixation method can be alternatively used for this permeabilization step. Other cell fixation methods were not tested in this protocol.

14. Add 1 mL of blocking buffer for 30 min at 25°C to block non-specific binding of the antibody.
15. During blocking, dilute primary antibodies in blocking buffer.
    a. Dilute the NEDD8 and HDAC6 antibodies at a ratio of 1:500 in blocking buffer.
16. Mix the diluted antibodies by inverting or vortexing, and spin down for a second.

17. After the 30 min blocking period, remove the blocking buffer from the dishes and then wash cells once with 1 × PBS to remove residual blocking buffer.
18. Add 100 μL of the diluted primary antibody solution to the cells and cover with both clear wrap and foil to prevent drying of the antibody solution.
19. Dishes are then left stationary for 12–16 h at 4°C.
20. After incubation, wash cells three times with 1× PBS for 5 min each.
21. Dilute the Alexa Fluor-conjugated secondary antibodies at a ratio of 1:500 using blocking buffer.
22. Mix the diluted secondary antibodies by inverting or vortexing, and spin down for a second.
23. Add 100 μL of the diluted secondary antibodies solution to the cells.
    a. Incubation should be performed in the dark. Cover the dishes with a foil to protect them from light and prevent photobleaching.
24. Incubate for 1–2 h at 25°C.

Note: Incubation time should not exceed 2 h.

25. Wash cells three times with 1× PBS for 5 min each.
26. Dilute DAPI in 1× PBS to a final concentration of 1 μg/mL, from a stock solution of 1 mg/mL.
27. Add 200 μL of diluted DAPI solution.
28. Incubate for 15–30 min at 25°C.
29. Wash cells three times with 1× PBS for 5 min each.
30. Remove the PBS from dishes.
31. Add 50 μL of mounting solution.
32. Cover the cells with a 12-mm cover glass.

Note: A large volume of the mounting solution will help to avoid bubble formation in step 32.

Pause point: Stained cells can be stored at 4°C up to 1 week for the next step.

Immunofluorescence imaging

Timing: ~2 h

This section describes the visualization of stained cells to analyze endogenous proteins. All images are acquired by confocal microscopy. To use NIS-Element AR software, researchers need to refer to the manufacturer's manual for the Nikon A1R instrument.

33. The samples are observed using a confocal microscope equipped with a 60× 1.4 NA oil immersion objective in a 35 mm chamber.
34. The channel is set to 546-nm laser, 647-nm laser, and DAPI using NIS-Element AR software.
    a. Adjust the pinhole, laser power, PMT gain (HV), and offset.
    b. For this study, the variables were adjusted as follows: pinhole to 1.2 A.U., laser power to at least "5.0," PMT gain (HV) to "100–120," and offset to "−20."
    c. Researchers should optimize the settings for optimal image intensity.
    d. After capturing, images can be processed using NIS-Element AR software to adjust their intensity.
35. Scan field cells stained with DAPI.
36. Capture images of at least 5 fields for quantification of ALB-containing cells.
    a. Acquired images are saved as ".nd2" files and can be opened using NIS-Element AR software. Images can then be exported to the Tagged image file format.

Pause point: Quantification of ALBs can be performed at any time.

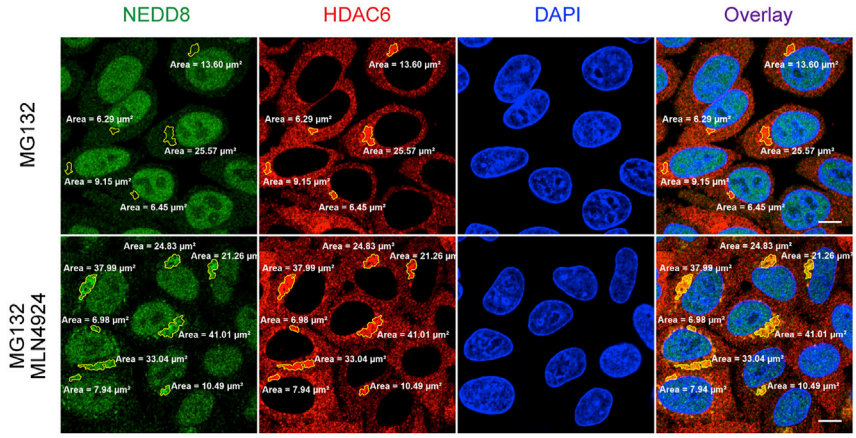

Figure 2. Measurement of ALB size from the acquired image

The above images show the sizes of ALBs measured by our protocol. Scale bars, 10 μm.

EXPECTED OUTCOMES

Analysis of cells containing ALBs is important for understanding how cells control aggregate responses to diverse stresses. In this regard, this protocol provides a useful method for monitoring diverse types of aggresomes in cells. HDAC6 controls aggresome formation in response to misfolded proteins in the presence of MG132, which is a proteasome inhibitor (Kawaguchi et al., 2003). Moreover, MLN4924 is a NAE1 inhibitor that induces UBA-dependent NEDDylation, following ubiquitin stress (Hjerpe et al., 2012; Kim et al., 2021; Leidecker et al., 2012). Consistent with this, we observed that MG132-treated cells contained only HDAC6-positive aggregates. Co-administration of MG132 and MLN4924 led to the colocalization of NEDD8 and HDAC6 (Figure 1A). This colocalization disappeared after pretreatment with Tubacin, which is a specific HDAC6 inhibitor (Haggarty et al., 2003). This phenomenon is termed "ALBs," and cells containing these ALBs can be quantified and plotted on a graph (Figure 1B). In addition, various types of aggresomes can be generated as a result of diverse intrinsic or extrinsic stimuli. Thus, we expect that our protocol will provide a feasible method to monitor and quantify diverse types of aggresomes in cells.

QUANTIFICATION AND STATISTICAL ANALYSIS

Timing: ~2 h

1. Load ".nd2" image files into NIS-Element AR software.

   Note: This software was used for this protocol. If you have other types of confocal microscope such as Zeiss LSM780 and Fuji LSA400, please follow the manufacturer's protocol for quantification of protein aggregates.

2. To classify ALB-containing cells, measure aggregate sizes larger than 5 μm^2.
   a. Use the Measure > Manual measurement > Area command to measure aggregate size (Figure 2).

   Note: There is no difference between manual and automatic measuring of aggregates sizes larger than 5 μm^2.

3. Count cells stained with DAPI and then count cells stained with NEDD8 or HDAC6.
   a. Cells with an aggregate size larger than 5 μm^2 should be counted.

b. The number of DAPI-stained cells can represent the total number of cells.
c. Exclude cells with nuclei that appear to have a crushed shape.

4. Export the number of counted cells to an excel file.
5. Quantify the percentage of cells containing ALBs.
   a. The number of stained cells with an aggregate size larger than 5 μm^2 should be divided by the total number of cells.
      i. (Number of cells stained with antibody/total number of cells) × 100 = Cells containing ALBs (%)
6. Perform statistical analysis using GraphPad Prism.
   a. The results can be plotted on a graph using GraphPad Prism.

Note: More than 100 cells should be counted. Three independent experiments should be performed on different days.

LIMITATIONS

Protein aggregates are classified into different types according to several properties, such as size, composition, and dissociation ability. Among these, size is the most often used characteristic to classify aggregates. Therefore, protein aggregates should be quantified using at least two different methods depending on the size of the aggregates. Characterizing protein aggregates using orthogonal methods is important because there are no protein aggregate standards; therefore, different methods, based on different detection principles, that cover a wide range of characteristics should be used. For example, transmission electron microscopy can be used as an orthogonal method to characterize protein aggregates. Therefore, if possible, it is recommended that different methods for aggregate characterization should be used to obtain a better understanding of the biological phenotypes of protein aggregates. It should be noted that this protocol was only tested in HeLa cells, but not in other adherent or suspension cell types with different size and shape. This protocol should thus be optimized for target cell lines.

TROUBLESHOOTING

Problem 1

Cell death after treatment with inhibitors.

Potential solution

Cell confluency should reach at least 70% on a glass bottom dish. A low cell confluency is not suitable for this experiment.

Problem 2

Low percentage of cells containing ALBs induced by MG132 or MG132/MLN4924 treatment.

Potential solution

ALB formation can be affected by inhibitor activity. Therefore, freeze-thaw cycles of inhibitors should be avoided. All inhibitors used in this protocol should be dissolved in DMSO, and then aliquoted. We suggest that the stock solution is stored at –20°C or –80°C to prevent reduction of the inhibitor activity.

Problem 3

Low signal intensity of stained antibodies.

Potential solution

The concentration of primary antibodies should be optimized to allow sufficient signal intensity.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ho Chul Kang (hckang@ajou.ac.kr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets or code.

ACKNOWLEDGMENTS

This work was supported by a National Research Foundation of Korea grant funded by the Korean government (MSIP) (no. 2020R1A2C2004988). This research was also supported by a grant from the Korea Health Technology R&D Project of the Korea Health Industry Development Institute (KHIDI) funded by the Ministry of Health & Welfare, Republic of Korea (grant no. HI16C0992).

AUTHOR CONTRIBUTIONS

Conceptualization, S.K. and H.C.K.; investigation, S.K. and Y.H.; writing – original draft, S.K.; writing – review & editing, H.C.K.; supervision, H.C.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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