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HIGHLIGHTS
A protocol for reprogramming human MnSCs to iPSC using an oocyte combination (AOX15)

Detailed protocols for MnSCs isolation, viral preparation, and expected outcomes

AOX15-iPSCs generated using this method of derivation reach a pluripotent state
Protocol to Reprogram Human Menstrual Blood-Derived Stromal Cells to Generate AOX15-iPSCs

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SUMMARY
Cell reprogramming has revolutionized the fields of cell and regenerative biology. However, human induced pluripotent stem cell (iPSC) derivation remains inefficient and variable. Here, we present a protocol that uses human menstrual blood-derived stromal cells (MnSCs), which are susceptible to reprogramming, as a source of somatic cells. We describe an oocyte-based reprogramming combination to generate AOX15-iPSCs that can be used to study different states of pluripotency. For complete details on the use and execution of this protocol, please refer to Lopez-Caraballo et al. (2020).

BEFORE YOU BEGIN
Prepare the media below. Pre-warm the media intended for cell culture at 37°C at least 30 min prior to beginning each section of this protocol. Refer to the Key Resources Table for a complete list of materials.

1. Human Menstrual derived stromal cells (MnSCs) culture Medium: Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% FBS, 1x Glutamax, 1x non-essential amino acids (NEAA) and 1% penicillin/streptomycin (P/S).
2. Pluripotent cells culture Medium (hES): Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 20% KnockOut Serum Replacement (KSR), 1x Glutamax, 1x non-essential amino acids (NEAA), 1% penicillin/streptomycin (P/S), 50 μM-mercaptopetoethanol and 8 ng/mL bFGF.
3. Mouse embryonic fibroblast (MEF) and 293T culture Medium: Dulbecco’s Modification of Eagle Medium (DMEM) supplemented with 10% FBS, 1x non-essential amino acids (NEAA) and 1% penicillin/streptomycin (P/S).
4. All cell types should be cultured in an incubator at 37°C, 5% CO2, 85% humidity.
5. MEF inactivation medium: 9.5 mL of MEF medium + 0.5 mL mitomycin stock solution (200 μg/mL). Prepare fresh before use.
6. Menstrual blood collection medium: 5 mL of calcium/magnesium-free PBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 5 mM EDTA. Keep at 4°C.
7. MnSC freezing media: FBS with 10% DMSO. Prepare fresh before use. Keep at 4°C.
**Preparation of Transfection Reagent PEI**

© Timing: 2 h

8. Warm 10 mL of MilliQ water to 80°C.
9. Dissolve 10 mg of PEI into pre-warmed MilliQ water.
10. Let the solution reach 25°C.
11. Measure pH and adjust to pH 7.
12. Filter solution using a 0.22 μm syringe filter.
13. Aliquot into 1 mL sterile Eppendorf tubes and freeze (−20°C) until use (up to 1 year).

⚠ CRITICAL: It is important to adjust pH to exactly 7; higher or lower pH will lead to poor transfection efficiency. Do not perform more than two freeze-thaw cycles.

**Mouse Embryonic Fibroblasts (MEF) Thawing, Culturing, and Inactivation**

© Timing: 4 days

14. Day 0. MEF thawing.
   a. Thaw one frozen vial of murine embryonic fibroblasts (MEFs) (≈ 1–3 × 10^6 cells) in a 37°C water bath. MEFs may be obtained from a number of qualified vendors, see Key Resources Table for specific strain used in this protocol.
   b. Transfer the content of the vial into a 15 mL tube containing 10 mL of MEF medium.
   c. Centrifuge at 700 × g for 4 min to pellet cells.
   d. Remove supernatant.
   e. Resuspend cell pellet in 1 mL of MEF medium using a p1000 micropipette to a single cell suspension pipetting up and down 3–5 times.
   f. Add 14 mL of MEF medium.
   g. Transfer the cell suspension into one 75 cm² flask (T-75).
   h. Place the MEFs in an incubator at 37°C, 5% CO₂, 86% humidity.

⚠ CRITICAL: Every frozen MEF preparation thaws a little differently. It is important for the MEFs to be thawed and maintained at a relatively high density (over 50% confluent on day 1 after thawing and after passaging).

15. Day 2–4 (or when cells reach 100% confluency). Perform MEF inactivation to generate inactivated MEF (iMEF).
   a. Remove the growth medium from the flask and replace it with 10 mL per T75 of freshly prepared inactivation medium (MEF media [9.5 mL] + 0.5 mL mitomycin stock solution [200 μg/ml]) to cover the monolayer.
   b. Place the flask in the incubator (37°C, 5% CO₂, 86% humidity) for 3 h.
   c. Aspirate inactivation medium from the flask.
   d. Wash the monolayer of cells twice with 10 mL of DPBS.
   e. Dissociate cells with 0.05% trypsin/EDTA for 5 min.
   f. Add 5 mL of MEF medium and break up cell aggregates by pipetting up and down with a 5 mL serological pipette.
   g. Count cells and dilute to 3.0 × 10^5 cells/mL in MEF medium.
   h. Plate 1.0 × 10^5 cells/cm². (For example, for a 6-well plate we will plate 150,000–200,000 iMEF/well).
   i. Incubate for 12–16 h and use the cells as feeder layers the next day. Feeders can be used up to 5 days after preparation. Renew with fresh MEF medium every other day.
# KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| goat anti-OCT4      | Santa Cruz Biotechnology | Cat#sc-8628 |
| rabbit anti-NANOG   | Santa Cruz Biotechnology | Cat#sc-33760 |
| rabbit anti-LIN-28  | Santa Cruz Biotechnology | Cat#sc-67266 |
| rabbit anti-SOX2    | Abcam  | Cat#AB5603 |
| mouse anti-TRA-1-60 | Chemicon/Millipore | Cat#MA84360 |
| mouse anti-SSEA4    | Developmental Studies Hybridoma bank (Iowa) | Cat#MC-813-70 |
| Alexa Fluor 488 donkey anti-mouse IgG (H+L) | Life Technologies | Cat#A21202 |
| Alexa Fluor 488 donkey anti-rabbit IgG (H+L) | Life Technologies | Cat#A21206 |
| Alexa Fluor 555 donkey anti-rabbit IgG (H+L) | Life Technologies | Cat#A31572 |
| Alexa Fluor 555 donkey anti-mouse IgG (H+L) | Life Technologies | Cat#A31570 |
| Alexa Fluor 488 donkey anti-goat IgG (H+L) | Life Technologies | Cat#A11055 |
| **Bacterial and Virus Strains** |        |            |
| pMXs-GFP            | Cell Biolabs | Cat#RTV-053 |
| pMXs-hOCT4          | (Takahashi et al., 2007) | Addgene Cat#17217 |
| pMXs-hASF1A         | (Gonzalez-Munoz et al., 2014) | Supplied after request |
| pMXs-SOX15          | (Lopez-Caraballo et al., 2020) | Supplied after request |
| Gag/Pol Retroviral vector | (Reya et al., 2003) | Addgene Cat#14887 |
| pCMV-VSV-G          | (Stewart et al., 2003) | Addgene Cat#8454 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| DMEM/F12            | Thermo Fisher Scientific | Cat#11320-082 |
| MEM Non-Essential Amino Acids Solution (100x) | Thermo Fisher Scientific | Cat#11140068 |
| Opti-MEM I Reduced Serum Media | Thermo Fisher Scientific | Cat#31985062 |
| L-Glutamine         | Thermo Fisher Scientific | Cat# 21051024 |
| Penicillin-Streptomycin | Thermo Fisher Scientific | Cat# 15070063 |
| 2-mercaptoethanol   | Thermo Fisher Scientific | Cat# 21985023 |
| Fetal Bovine Serum, Regular (Heat Inactivated) | Corning | Cat# 35-011-CV |
| KO-Serum Replacement | Thermo Fisher Scientific | Cat# 10828028 |
| 2-mercaptoethanol   | Thermo Fisher Scientific | Cat# 21985023 |
| Recombinant human basic FGF-premium grade | MACS-Milenyi Biotec | Cat#130-093-843 |
| 0.05% Trypsin/EDTA  | Thermo Fisher Scientific | Cat# 25300054 |
| Mitomycin C from Streptomyces caesporosus | Sigma-Aldrich | Cat# M4287-2MG |
| Gelatin Solution    | Sigma-Aldrich | Cat#G1393 |
| Polybrene           | Sigma-Aldrich | Cat#H9268-10G |

(Continued on next page)
## STEP-BY-STEP METHOD DETAILS

### Isolation of Menstrual Blood-Derived Stromal Cells (MnSCs)

**TIMING: 5–10 days**

MnSCs are obtained from the menstrual blood of volunteers at the peak of flow (first or second day of menses). The samples are collected in 5 mL of calcium/magnesium-free PBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 5 mM EDTA (collection medium). The cells are harvested with the informed consent of the donor as approved by an institutional review board. All cell lines must be regularly tested to ensure that they are free of mycoplasma prior to reprogramming and every 5–10 passages (we use a PCR-based test, see Key Resources Table for specific reagent used in this protocol).

1. Prepare 15 mL tubes containing 3 mL of Ficol l (Histopaque 1077-Sigma) and bring to 25°C.
2. Carefully layer the menstrual blood sample onto the Ficoll Histopaque-1077 (Figures 1A–1C).
   **CRITICAL:** When transferring the menstrual blood sample into Ficoll-containing tubes it is important to add the blood drop by drop on the tube wall to avoid mixing with the Ficoll phase.
3. Centrifuge at 400 × g for 30 min at 25°C to pellet red blood cells.
4. Recover supernatant containing MnSCs (Figure 1D) and transfer into a clean 50 mL tube.
5. Wash the cells by adding 10 mL of DPBS with 1% penicillin/streptomycin (P/S) and mix by gently drawing in and out of a 10 mL pipette.
6. Centrifuge at 250 × g for 10 min to pellet stromal cells.
7. Discard supernatant.
8. Repeat steps 5–7 twice.
9. Resuspend cell pellet with 1 mL of MnSC medium using a p1000 micropipette.
10. Add 6 mL of MnSC and transfer cell suspension into a T25 cell culture flask.
11. Place in a 5% CO₂, 37°C incubator.
12. Change MnSC medium every other day.
13. When the cells reach 80%–90% confluency (usually after 5–7 days) (Figure 2) (see Troubleshooting 1), dissociate them by adding 1 mL of 0.05% trypsin-EDTA to the T25 flask after PBS washing and incubate for 5–10 min at 37°C.

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### Continued

## REAGENT or RESOURCE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Polyethylenimine, Linear, MW 25000, Transfection Grade (PEI 25K) | Polysciences | Cat#23966 |
| Vener GeM Classic mycoplasm test | Minerva Biolabs | Cat#11-1050 |
| Histopaque 1077 | Sigma-Aldrich | Cat#10771-100ML |
| GlutaMAX Supplement | Gibco-Thermo Fisher | Cat# 35050061 |
| Paraformaldehyde | Electron Microscopy Sciences | Cat# 15710 |
| DPBS | Coming | Cat#.21-031-CM |
| Donkey Serum | Equitech-Bio | Cat# SD30-0500 |
| Triton X-100 | Fisher Scientific | Cat# BP151-500 |
| Normal Donkey Serum | Sigma-Aldrich | Cat#D9663 |
| Human: HEK293T/17 cells ATCC Cat# CRL | 11268; RRID: CVCL_1926 |
| EmbryoMax Primary Mouse Embryonic Fibroblasts | Merk-Millipore | PMEF-CFL |

### Experimental Models: Cell Lines

| EXPERIMENTAL MODEL | SOURCE | IDENTIFIER |
|-------------------|--------|------------|
| Human: HEK293T/17 cells ATCC Cat# CRL | 11268; RRID: CVCL_1926 |
| EmbryoMax Primary Mouse Embryonic Fibroblasts | Merk-Millipore | PMEF-CFL |
14. Add 5 mL of MnSC medium to inactivate the trypsin and mix well by gently pipetting up and down using a 5 mL pipette and plate at $10^4$ cells/cm².

15. Split the cells every four – five days to maintain 80% confluence. See Troubleshooting 2 and 3.

⚠️ CRITICAL: Do not dilute cells below $5 \times 10^3$ cells/cm². Low confluency yields into poor cell proliferation and early senescence.

 Пауза: Cells may be stored at $-80 \degree$ C for short-term storage or in liquid nitrogen for long-term storage. Freeze cells in 10% DMSO/FBS.

### Preparation of the Reprogramming Viruses

⏱ Timing: 5–8 days

Retroviral particles are prepared from pMX-backbone based vectors (from Cell Biolabs, find information in Key Resources Table) encoding reprogramming factors ASF1A, SOX15 and OCT4 (AOX15) (please find pMX-ASF1A, pMX-SOX15 and pMX-OCT4 vector description in Lopez-Carballeiro et al., 2020) (Figure 3). Retroviral particles will be used to over express AOX15 combination in MnSCs.

16. Seed $5 \times 10^4$ cells of HEK293T cells onto a 100 mm tissue culture plate and culture for 24–48 h in 293T medium.

17. Day 0: When the cells reach 100% confluency split cells at a 1:4 ratio. Allow cells to reach 80%–90% confluency (Figure 4). See Troubleshooting 4 and 5.

18. Day 1: Remove medium and replace with 10 mL fresh pre-warmed growth medium 1–2 h before transfection.

⚠️ CRITICAL: Be careful not to dislodge the cells when adding the medium.
19. Prepare the two solutions listed below in 15 mL Eppendorf tubes for each of the retroviruses:

| 5 µg Gag/Pol          |
|-----------------------|
| 3 µg VSV-G            |
| 7 µg pMX (transgene vector: pMX-ASF1A, pMX-SOX15 or pMX-OCT4) |
| 500 µL of pre-warmed Optimem medium |

20. Add 60 µL PEI (1 µg/µL in water, pH7) to the mixture (4:1 v/w ratio of PEI:DNA), drop by drop while vortexing at medium speed (Methods Video S1).

△ CRITICAL: When adding PEI to the DNA mix, it is important to add it drop by drop while vortexing.

21. Vortex briefly (30 s) and leave for 10 min at 25°C.
22. Add the mixture dropwise in a circular motion, to the 293T cells.
23. Gently rock the plate in a back and forth and side-to-side motion to ensure even distribution of the transfection mixture. Incubate the plate in a 37°C, 5% CO₂ tissue culture incubator for 8 h.
24. After 8 h aspirate the medium and add 6 mL of 293T medium.

△ CRITICAL: Do not exceed 12 h post-transfection.

25. Virus Collection Point 1: Approximately 36 h after transfection (on day 3), collect the supernatant using a disposable syringe and filter the supernatant using a 0.45 µm syringe filter, into a 50 mL conical tube. Use this supernatant for cell transduction or store this tube for 17–18 h at 4°C. For short term storage freeze supernatants down at −20°C (up to 1 month) or at −80°C for long-term storage (up to 6 months).
26. Gently and slowly add 6 mL of fresh 293T medium down the side of the culture plate and incubate in a 37°C, 5% CO₂ tissue culture incubator for 24 h.

△ CRITICAL: Be careful not to dislodge the cells when removing or adding the medium.

27. Virus Collection Point 2: On day 4, repeat step one from day 3, by filtering the additional 8 mL of medium into a 50 mL conical tube and use for cell transduction or store as in virus collection point 1.

28. Virus titration using 293T transduction and flow cytometric analysis:
   a. The day before transduction (day 0), plate 6 × 10⁵ 293T cells in a 6-well plate (10⁵ cells/well).
b. On day 1, add 1 mL or 3 mL of pMX-SOX15, pMX-ASF1A or pMX-OCT4 viral supernatant to each well (6 total). pMX-SOX15 and pMX-ASF1A will allow expression of GFP as they are bicistronic vectors, while pMX-OCT4 will not express GFP (Figure 3) and thus it will be the GFP negative control.

c. Incubate in a 37°C, 5% CO₂ tissue culture incubator for 24 h.
d. On day 2, replace medium with 2 mL/well of 293T medium.
e. Incubate for 72 h (replacing the medium 48 h after transduction, on day 4).
f. On day 5, dissociate transduced and control (non-transduced) 293T cells with 0.05% Trypsin/EDTA, and resuspend pellet with 1 mL of 4% Paraformaldehyde (PFA).
g. Incubate 20 min at 25°C to fix the cells.
h. Wash cells adding 10 mL of DPBS, centrifuge at 1,000 × g for 5 min. Repeat this step once again.
i. Resuspend cells with 0.5–1 mL PBS-0.1% BSA-150 mM EDTA-0.02% sodium azide and analyze cells by flow cytometry.

Pause Point: or store cells at 4°C for up to 1 week.
j. Analyze GFP positive cells by flow cytometry (Figure 5).

Note: Only viral supernatant reaching an efficiency of 80% GFP positive cells in 1 mL supernatant (and over 95% in 3 mL) should be used for cell reprogramming.

AOX15 Reprogramming of MnSCs

© Timing: 3–4 weeks

MnSCs are reprogrammed into iPSC using the oocyte-based factor combination ASF1A, SOX15 and OCT4 (AOX15). Transduction of the reprogramming factors must be carried out using low passage MnSC cells (ideally passage 3–5).

29. The day before reprogramming (day 0) plate 10⁵ MnSCs into 35 mm cell culture plates.
30. 24 h later (day 1), add 5 mL of each pMX-SO15, pMX-ASF1A and pMX-OCT4 retroviral supernatant (it should be a MOI= 5 for each vector), and 15 µL of 8 µg/mL polybrene per plate and return plate to incubator.
Note: When using frozen viral supernatant, thaw them on ice.

31. On day 2, replace medium with 5 mL of MnSC medium and return plate to incubator. See Troubleshooting 6.
32. On day 4, dissociate cells and plate $5 \times 10^4$ cells/well (6-well plate) containing iMEFs using hES medium as the final medium and return plate to incubator.
33. Change hES medium daily.
34. On day 14–21, the reprogrammed cells should now transform from fibroblast morphology to a round shaped morphology (Figure 6) and can be collected for characterization of pluripotency markers (Figure 7) and reprogramming efficiency and can be further analyzed for iPSC phenotype and pluripotent state characterization as needed.

Note: If several colonies arise from one well during the reprogramming process, every single clone should be independently expanded for characterization and freezing.

EXPECTED OUTCOMES
AOX15-iPSCs can be generated and passaged within 3–4 weeks. IPSC clones can be expanded for characterization. We recommend the following characterization assays: Immunocytochemistry for the visual detection of markers indicative of pluripotency, flow cytometry for a quantitative estimate of reprogramming efficiency (NANOG, OCT4, TRA-1-60, TRA-1-81, SSEA4, LIN28). We expect over 80% of the cells to be positive for mentioned pluripotency markers. Additionally, qRT-PCR can be performed to confirm the expression of pluripotency genes (including OCT4, SOX2, hTERT, DNM3TB, KLF4, NANOG, ZFP42, among others) (Lopez-Caraballo et al., 2020).

Pluripotency is not a single state. Further characterization of the functional status of reprogrammed cells’ pluripotency, including differentiation capacity, efficiency, and other molecular signatures can be performed (Liu et al., 2017; Lopez-Caraballo et al., 2020; Mishra et al., 2018). To this end, a list of recommended antibodies for immunocytochemistry is provided in the Key Resources Table.

We recommend dedicating at least three to four wells to each reprogramming quality control assay and isolating and characterizing at least three to four iPSC clones to allow pluripotency acquisition efficiency analysis and further pluripotent characteristics studies.

Quality Control: Measure of Viral Packaging
When performing the viral packaging of pMXs encoded reprogramming factors, it is important to measure the viral transduction efficiency using pMX-ASF1A and pMX-SOX15 bicistronic vectors.
that express GFP. Only viral supernatant reaching a concentration of 80% GFP positive cells with 1 mL supernatant (and over 95% with 3 mL) should be used for cell reprogramming. These plasmids and flow cytometric analyses can also be used to estimate transfection efficiency and troubleshoot any problems with viral packaging.

Quality Control: Measure of Reprogramming Efficiency
Three to four weeks after MnSC transduction with reprogramming factors, the reprogrammed cells should transform from fibroblast morphology to a round shaped morphology. The cells can be passaged for characterization of iPSC phenotype and reprogramming efficiency using immunofluorescence labeling of pluripotent markers (NANOG, OCT4, TRA-1-60, TRA-1-81, SSEA4, LIN28). The original somatic MnSC should be used as negative control of expression and also to produce the reference short tandem repeats (STR) profile for the iPSC lines generated.

LIMITATIONS
Although the efficiency of AOX15 reprogramming has been significantly improved by using MnSCs instead of dermal fibroblasts (Lopez-Caraballo et al., 2020), iPSC generation efficiency remains relatively low and reprogramming rates vary from 10% to 0.0001% (Gonzalez-Munoz et al., 2014; Gonzalez-Munoz and Cibelli, 2018). The following limitations should be mentioned specifically. First, this protocol renders efficient reprogramming when using MnSCs; other cell types may require further optimization. Second, based on our experience low passage MnSCs (passage 3–6) should be used for reprogramming. Enzymatic dissociation or passaging and long-term culture has been described to affect the epigenetic state of the cell and to hinder efficient reprogramming (Halley-Stott and Gurdon, 2013; Kim et al., 2010; Streckfuss-Bomeke et al., 2012). Third, this approach depends on the quality and quantity of viruses, and as explained in this protocol, virus titration using 293T transduction should be done after each production and only highly efficient supernatants should be used.

TROUBLESHOOTING
Problem 1
MnSCs do not reach 80%–100% confluency one week after isolation (related to step 13)

Potential Solution
Replate cells into smaller plates to have a cell density above $10^4$ cells/cm²

Problem 2
MnSCs do not proliferate properly (related to step 15)

Potential Solution
Do not dilute cells below $5 \times 10^3$ cells/cm². Low confluency gives rise to poor cell proliferation and early senescence. If cells do not reach confluency after 3–5 days of cell culture we recommend replating the cells into new dishes at higher cell density

Problem 3
MnSC sample population is not homogeneous (related to step 15)
Potential Solution
The user may choose to perform cell sorting for the mesenchymal markers CD90, CD105, CD73, CD44 and CD13 positive cells (and negative for CD45, CD34, and HLA-DR). However, it is not necessary. Following this isolation protocol, more than 99% of isolated cells display the mentioned markers profile

Problem 4
Low 293T transfection efficiency (related to step 17)

Potential Solution
Uneven distribution of cells may result in low 293T transfection and thus virus titer. Rock the plate back and forth when seeding the cells. The user may check cell distribution under the microscope prior to incubation. Also, the user should carefully swirl the plate after adding each respective plasmid

Problem 5
Low virus titer (related to step 17)

Potential Solution
Cells should reach 80%–90% confluency at the moment of transfection to ensure higher transfection efficiency and titer of viruses.

Problem 6
Excessive cell death after viral transduction (related to step 31)

Figure 7. Reprogrammed iPSCs
Representative images of immunofluorescence analysis of markers indicative of pluripotency in control somatic MnSCs and AOX15 reprogrammed iPSCs. Scale bar, 30 μm, for all images.
Potential Solution
Uneven distribution of cells may result in cell death after viral infection. Also, check the confluency of MnSCs at the moment of transduction, it should be over 50% for proper survival after transduction.

RESOURCE AVAILABILITY
Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Elena Gonzalez-Munoz egonmu@uma.es.

Materials Availability
All material used are listed in Key Resources Table and any further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact.

Data and Code Availability
This protocol does not include the generation of datasets.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100183.

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AUTHOR CONTRIBUTIONS
Conceived and Designed Experiments, E.G.-M.; Performed Experiments, E.G.-M., A.S.-M., and A.F.-G.; Analyzed the Data, E.G.-M.; Wrote the Manuscript, E.G.-M.; Funding Acquisition, E.G.-M.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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