**Protocols for assembly of a serine integrase-based platform for functional validation of genetic switch controllers in eukaryotic cells**

**Marco A. de Oliveira1,2, Lilian H. Florentino1,2,3, Thais T. Sales1,2,3, Rayane N. Lima2,3, Luciana R. C. Barros4, Cintia G. Limia5, Mariana S. M. Almeida2,3, Maria L. Robledo5, Leila M. G. Barros2,3, Eduardo O. Melo2,3, Daniela M. Bittencourt2,3, Stevens K. Rehen6,7, Martín H. Bonamino8,9, Elibio Rech2,3, \***

#

1 Department of Cell Biology, Institute of Biological Science, University of Brasília, Brasília, Distrito Federal, Brazil

2 National Institute of Science and Technology in Synthetic Biology (INCT BioSyn), Brasília, Distrito Federal, Brazil

3 Embrapa Genetic Resources and Biotechnology, Brasília, Distrito Federal, Brazil

4 Center for Translational Research in Oncology, Instituto do Câncer do Estado de São Paulo, Hospital das Clínicas da Faculdade de Medicina de Universidade de São Paulo, São Paulo, São Paulo, Brazil

5 Molecular Carcinogenesis Program, Research Coordination, National Cancer Institute (INCA), Rio de Janeiro, Rio de Janeiro, Brazil

6 D’Or Institute for Research and Education (IDOR), Rio de Janeiro, Rio de Janeiro, Brazil

7 Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Rio de Janeiro, Rio de Janeiro, Brazil

8 Cell and Gene Therapy Program, Research Coordination, National Cancer Institute (INCA), Rio de Janeiro, Rio de Janeiro, Brazil

9 Vice-Presidency of Research and Biological Collections (VPPCB), FIOCRUZ – Oswaldo Cruz Foundation Institute, Rio de Janeiro, Rio de Janeiro, Brazil

# \*Corresponding author

E-mail: elibio.rech@embrapa.br (ER)

PROTOCOL

# Timing

**HUMAN**

**PBMC**

Steps 1-9, cell isolation: 1.5 h

Steps 10-14, cell electroporation: 1 h

Steps 15-21, integrase activity evaluation by flow cytometry: 2h

**HEK293T cells**

Steps 22-26, cell transfection: 1 h

Step 27, integrase activity evaluation by flow cytometry: 2h

**hES cells**

Steps 28-, cell expansion: 12-15 d

 Steps 28-45, passage cells: 30 min

 Steps 46-49, maintenance of cell culture: 5 min daily

Steps 50, cells preparation for electroporation: 6 d

Steps 51-76, hES cell electroporation: 1 h

Step 77, postelectroporation: 2 d

Steps 78-93, integrase activity evaluation by flow cytometry: 2 h

**NSC**

Steps 94-117, cell expansion: 14-20 d

Steps 94-113, passage cells: 30 min weekly

Steps 114-117, maintenance of cell culture: 5 min daily

Step 118, cells preparation for electroporation: 6 d

Steps 119-143, cell electroporation: 1 h

Steps 144, postelectroporation: 2 d

Steps 145-159, integrase activity evaluation by flow cytometry: 2 h

**ANIMAL**

**Bovine fibroblast**

Steps 1-16, cell passage: 30 min

Steps 17-20, cell transfection: 1h

Steps 21-29, integrase activity evaluation by flow cytometry: 2.5h

Steps 30-34, cell viability assays: 7h

**PLANT**

**Protoplast**

Step 1, plant growth: 4–6 weeks

Steps 2-21, protoplast isolation: 4-6 hours

Steps 22-30, protoplast transformation: 1-2 hours

Steps 31-36, flow cytometry: 30 min by sample

**MOLECULAR ANALYSES**

Steps 1-3, primer design: 1d

Steps 4-10, target sequence amplification: 5h

Steps 11-26, amplicon sequencing: 5d

 Steps 11-14, amplicon purification and ligation: 2d

Steps 15-26, pgem-T-easy plasmid cloning: 3d

##

##

# Step-by-step

## HUMAN

# **Materials**

### ***Biological materials***

Obtaining nonactivated human T lymphocytes from PBMCs

This protocol describes the isolation of human peripheral blood mononuclear cells (PBMCs) from leukocyte filters. Usually, 5 x 107 to 3 x 108 cells are obtained from a leukocyte filter from a healthy blood donor donation.

**! CAUTION** Universal precautions must be taken, experiments must be carried out in (at least) category 2 biological safety cabinets, and appropriate personal protection equipment should be used. **! CAUTION** Informed consent must be obtained for the use of human blood samples.

**! CAUTION** Experiments with human materials must conform to all relevant institutional and governmental ethics regulations, and appropriate informed consent must be obtained for the use of human blood or patient-derived materials.

### ***hES/NSC cell lines***

Human embryonic stem (hES) cells called BR1 and human induced pluripotent stem (hiPS) cell-derived neural stem cell (NSC) lines (the cell lines were supplied by Dr. Stevens Rehen of D´Or Institute for Research and Education (iDOR), Rio de Janeiro, Brazil)

▲**CRITICAL** Use the BR1 cell line up to 40 passages of cells and NSCs line using up to 20 passages cells.

# **Reagents**

### ***Medium and supplements for PBMC***

RPMI 1640 media (Gibco, cat. no. 11875101).

FBS (Fetal bovine serum) (Gibco, cat. no. 12657)

Penicillin-Streptomycin 10,000U/mL(Gibco, cat. no. 15140122)

L-Glutamine 200mM (Gibco, cat. no. 25030081)

IL-2 human (R&D Systems, cat no. 202-GMP-010)

### ***PBMC separation reagents***

PBS (Phosphate Buffered Saline), pH 7.4, (Gibco, cat. no. 003002)

Ficoll-Paque PLUS density gradient media (GE Healthcare, cat. no. GE17-1440-02)

Trypan Blue (Sigma-Aldrich, 72-57-1)

Alcohol 70% for material sterilization

7-AAD Viability Staining Solution (eBioscience, cat. no. 00-6993-50)

***Medium and supplements for HEK293T cell line***

DMEM media powder (Gibco, cat. no. 12100046)

FBS (Fetal bovine serum) (Gibco, cat. no. 12657)

Penicillin-Streptomycin 10,000U/mL (Gibco, cat. no. 15140122)

Sodium Pyruvate 100 mM (Gibco, cat. no. 11360070)

HEPES 1M, (Gibco, cat. no. 15630080)

MEM Amino Acids Solution (50X) (Gibco, cat. no. 11130051)

MEM Non-Essential Amino Acids Solution (100X) (Gibco, cat. no. 11140050)

MEM Vitamin Solution (100X) (Gibco, cat. no. 11120052)

2-Mercaptoethanol (Gibco, cat. no. 21985023)

### ***Growth medium and supplements for hECs***

mTeSR™1 hES medium (Stem Cell Technologies, cat. no. 85850)

Advanced™ DMEM⁄F-12 (Thermo Fisher Scientific, cat. no. 12634)

Neurobasal® Medium and Neural Induction Supplement (NIS) (Gibco, cat. no. 12634)

DMEM/F-12, GlutaMAX™ Supplement medium (Thermo Fisher Scientific, cat. no. 10565)

### ***Enzymes, growth factors and chemicals for hECs***

Accutase™ Cell detachment solution (Stem Cells Technologies, cat. no. 07920)

Rho-associated protein kinase (ROCK) Inhibitor (Y-27632, Sigma-Aldrich, cat. no. SCM075)

### ***Other reagents and chemicals***

HEK293T transfection reagents using HBS and CaCl2

HEPES (Sigma-Aldrich, cat. no. H3375)

CaCl2 - Calcium chloride dihydrate (Sigma-Aldrich, cat. no. C5080)

Na2HPO4 - Sodium Phosphate dibasic heptahydrate (Sigma-Aldrich, cat. no. 30413)

NaCl – Sodium Chloride (Sigma-Aldrich, cat. no. S3014)

1S electroporation buffer:

5mM KCl (Merck, cat. no. 1.04936)

15mM MgCl2 (Merck, cat. no. 1.05833)

120mM Na2HPO4 (Merck, cat. no. 1.06579)

 120mM NaH2PO4 (VETEC, cat. no. 001236)

50mM Sodium Succinate (Merck, cat. no. s6638601)

For 1SM electroporation buffer add

25mM Manitol (VETEC, cat. no. c000197)

▲**CRITICAL** Before preparing electroporation buffers, mix Na2HPO4/NaH2PO4 (phosphate buffer) and adjust the pH to 7.2 using 1 M NaOH or 1 M HCl. Use the electroporation buffer aliquots only once. Electroporation buffer aliquots (1 mL) can be stored at -20 °C for up to 3 months.

Vectors for transfection: The pT2/CAGGS-GFP plasmid was kindly provided by Dr. Sang Wang Han (UNIFESP, Brazil), and the pT3-Neo-EF1a-GFP plasmid was ordered from Addgene (Addgene, no. 69134)

Geltrex™ Matrix solution (GIBCO, cat. no. A1413302)

# **Equipment**

Centrifuge for 50 mL tubes and microtubes (Thermo Centrifuge C3Ri, cat. no. 1115774)

Multifunction Centrifuge (Thermo Centrifuge, Jouan B4i, cat. no.11175671)

Biological Safety Cabinet Class 2

Nucleofector IIb (Lonza, cat. no. AAB-1001)

Tissue culture incubator at 5% CO2, 21% O2 at 37°C.

FACSCalibur® (BD Bioscience)

Evos XL Cell Imaging System (Thermo Fisher Scientific).

Cell culture flask, 75 cm2 (Corning®, cat. no. CLS430720)

12-well cell culture plate (Corning®, cat. no. 3512)

Sterile Syringe 5 mL, 10 mL, 25 mL (Greiner Bio-One 606107, cat. no. 607180, 760180)

Conical Sterile Polypropylene Centrifuge Tubes, 50 mL (Greiner Bio-One, cat. no. 210261)

Sterile Polypropylene Microtubes, 1.5 mL (Greiner Bio-One, cat. no. 616275)

Barrier Pipette tips, 10 µL, 20 µL, 200 µL, 1000 µL (KASVI cat. no. K8-10F-1, K8-20F-1K8-200F-1, K8-1000F-1)

Micropipettes, 2 µL, 10 µL, 200 µL, 1000 µL (Gilson, FA10001M, FA10002M, FA10005M, FA10006M)

**PBMC electroporation**

Electroporation Cuvette 0.2 cm (Mirus Biotech®, cat. no. MIR 50121)

### ***For hES/NSC cells***

Tissue-culture plates, 24 wells, flat bottom (Corning®, cat. no. CLS3527)

Tissue-culture treated culture dishes D×H 60mm×15mm (Corning®, cat. no. CLS430166)

Tissue-culture treated culture dishes D×H 100mm×20 mm (Corning®, cat. no. CLS430167)

Cell scraper (Corning®, cat. no. CLS3010)

### ***Software***

TreestarFlowJo Version 10 (http://www.flowjo.com/)

# **Reagent Setup**

**hEC/NSC cell lines**

Thaw Geltrex™ Matrix solution undiluted vial at 4 °C overnight on ice. Transfer aliquots of adequate volume into Eppendorf tubes. The aliquots can be stored at −20 °C until the expiration date. Thaw aliquots on ice and dilute with 1% DMEM/F-12 and GlutaMAX™ Supplement medium.

Neural expansion medium (NEM): 50% v/v Advanced™ DMEM/F-12, 50% v/v Neurobasal® Medium, 2% Neural Induction Supplement (NIS). NEM medium can be stored at 4 °C for up to 1 month.

Prepare Y-27632 stock solution in aliquots of 10 mM in PBS. Stored at −20 °C for 1 year.

Buffer FACS for hECs/NSCs: DPBS without CaCl2 and MgCl2, 1% fetal bovine serum, fresh.

PBST (0.2% Tween 20-PBS)

Add 0.2% (vol/vol) Tween 20 to PBS. Store the buffer at room temperature for 1 year.

▲**CRITICAL** For a high transfection efficiency, at the electroporation step, the final volume of buffer + plasmid should be 100 µL. Do not mix less than 85 µL of buffer because it drops transfection efficiency.

▲**CRITICAL** Ficoll-Paque and PBS for PBMC separation must be at room temperature before use.

▲**CRITICAL** Antibiotic addition immediately after electroporation will cause intense cell death.

# **PROCEDURE**

**PBMC isolation ● Timing 1.5 h**

1. After performing the sterilization procedure in the safety cabinet, couple the syringe containing 20 mL PBS to the leukocyte filter, cut the filter tube extremity, and put it into a 50 mL tube.

**! CAUTION** Blood can spill over from the tube, always maintain hypochlorite solution to clean up the drops.

1. Press the syringe to wash the leukocyte filter. Repeat this step if more cells are needed.
2. With a 25 mL serological pipette, add ~35 mL of blood at the top of the 10 mL tube containing Ficoll-Paque at RT.

▲**CRITICAL STEP** Add the blood on top of the Hystopaque very carefully. Do not take more than 20 minutes with blood on top of Ficoll; otherwise, the red blood cells will start to fall into the gradient. If the blood shakes and mixes with Ficoll-Paque, separation will not occur.

▲**CRITICAL** Do not shake or disturb the gradient between the Ficoll and blood.

1. Centrifuge at 800 g, 20 minutes, RT, in a swinging-bucket rotor, without breaking. ▲**CRITICAL STEP** Centrifuge must be at low acceleration and without a break setting; otherwise, the separation gradient will be lost.
2. With a 10 mL serological pipette, very carefully remove the PBMC “white ring” on top of the Ficoll-Paque and put it into a fresh tube.

**! CAUTION** Avoid removing and placing the pipette several times as it will disturb the gradient and could contaminate the leukocytes with other fractions.

▲**CRITICAL STEP** Remove only the white ring containing the leukocytes without disturbing the red blood cell at the bottom. Additionally, avoid the solution above the white ring because it contains platelets and soluble factors that can change culture conditions. If the person is not experienced, add more than 10 mL of Ficoll-Paque to increase the distance between the leukocytes and the red blood cells.

1. To wash the cells, complete the volume to 50 mL with PBS and centrifuge at 400 × g for 5 minutes at 10 °C. Remove the supernatant.
2. Repeat the wash step twice.
3. Resuspend the cells in 5-10 mL PBS and maintain them on ice.
4. Count the cells with a Neubauer chamber by adding Trypan blue solution at 4% for cell viability staining.

PAUSE POINT The cells can be placed on ice for up to 2-3 hours.

**PBMC electroporation ● Timing 1 h**

1. Add 10x106 cells per electroporation reaction separating in individual microtubes.
2. Centrifuge the tubes at 400 × g for 5 minutes at 10 °C.
3. Remove all supernatant with a pipette and resuspend the cells in a total of 100 µL of 1SM electroporation buffer + plasmids (integrase + target) per reaction.
4. Immediately insert the cells in the cuvette and electroporate using program U-014 on Nucleofector 2b.

▲**CRITICAL** Electroporate and remove the cells from the cuvette quickly to avoid cell death. Wait 2 minutes for new cell electroporation.

1. Very carefully, resuspend the cells in warm RPMI/FBS 20% (no antibiotics) and plate them in total 500 µL in a 12-well plate. After 16 hours, add 500 µL RPMI/FBS 10% + P/S antibiotics + 50 U/mL IL2 + L-Glutamine.

▲**CRITICAL STEP** If T-cell activation is needed, it should be done at this moment.

**Integrase activity evaluation in PBMCs at 2 h**

Integrase activity was evaluated by flow cytometry 24 h after transfection. For optimization, several time points should be evaluated. In our hands, 72 h after electroporation, eGFP expression achieved its maximum (1-35% of positive cells).

1. Collect the cells and place them in a microtube.
2. Centrifuge at 400 × g for 5 minutes. Remove the supernatant.
3. Wash the cells with 1 mL of cold PBS. If DNA will be extracted, separate an aliquot at this time.
4. Repeat the centrifugation 2X to wash the cells. If antibody staining will be performed, it should take place at this moment.
5. Resuspend the cells in 300 µL of cold PBS and place them in the flow cytometer tub.

▲**CRITICAL** Fixation buffers based on formaldehyde should not be used because formaldehyde decreases GFP fluorescence.

**! CAUTION** Fixation buffers should not be used in case of cell viability staining with 7-AAD or PI (Propidium Iodate).

1. Add 5 µL of 7-AAD and incubate at 4 °C for 20 minutes.

▲**CRITICAL** Keep the cells in the dark after staining. Do not wash cells after the addition of the 7-AAD staining solution.

1. Acquire at least 10,000 cells at the viable gate to evaluate eGFP expression. For a higher sensitivity, acquire more cells.

▲**CRITICAL STEP** Acquire a nonstained GFP-negative tube as a negative control for the gating strategy. Acquire a 7-AAD -stained GFP negative tube as a negative control for GFP expression for the gating strategy. Acquire a 7-AAD -negative and GFP-positive tube (with the GFP control plasmid as pT2-GFP) for GFP-positive staining and gating strategies.

**HEK293T transfection with Calcium Phosphate ● Timing 1 h**

1. Plate 4x106 HEK293T cells per well of a 75 cm2 plate at least 16 h before transfection in DMEM complete medium. ▲**CRITICAL** Cells should be less than 80% confluent, or transfection efficiency will decrease.
2. Replace the medium before transfection, adding only 10 mL of DMEM complete medium.
3. Mix the plasmids (integrase + targets, 5 µg each) to 500 µL of 2X CaCl2 **! CAUTION** CaCl2 must be freshly prepared or maintained frozen in single-use aliquots.
4. Next, agitate CaCl2 using a vortex at full speed and add 500 µL of the HBS solution drop-by-drop very slowly. Make bubbles on the solution with a Pasteur pipette. Let it rest for an instant. ▲**CRITICAL STEP** HBS solution should be at pH 7.1 at the transfection of HEK293T cells. Any small change will have a great impact on transfection efficiency.
5. Very carefully add drop-by-drop at the cells covering all the flask with circular movements avoiding two drops at the same spot. Mix the solution with ∞ movement and place the cells in the incubator.

**Integrase activity evaluation in HEK293T cells.● Timing 2h**

1. Integrase activity was evaluated by flow cytometry 24 h after transfection. For optimization, several time points should be evaluated. In our hands, 72 h after electroporation, eGFP expression achieved its maximum (1-35% of positive cells).

**Cell expansion of the hES cell line ● Timing 12-15 d**

**Passage cells ● Timing 30 min**

1. Culture hES cells in a 100-mm culture dish until the cells cover the dish area at 60-70% confluence. This occurs at approximately 5-6 days.

▲**CRITICAL** Start the enzymatic dissociation with high-quality hES colonies. The spontaneous differentiation of the colonies should be removed before the split.

**! CAUTION** For 60-70% confluence, consider a split ratio of 1:5.

1. Prior to starting the dissociation, coat the fresh 100-mm culture dishes with 6 mL/dish of cold Geltrex™ and place it into the incubator at 37 °C for at least 30 minutes.
2. Remove Geltrex™, and immediately add 9 mL/dish of fresh growth mTeSR™1 medium to prevent drying.
3. Place the fresh culture dishes into the incubator at 37 °C
4. Carefully aspirate the old growth medium from the cell culture dish
5. Wash the dish with 4 mL of DPBS without calcium and magnesium (DPBS-/-) and remove immediately
6. Add 4 mL of Accutase™ into the 100-mm culture dish, ensure that cover of dish area
7. Incubate the cell suspension at 37 °C for 5 minutes

**! CAUTION** Monitoring the cell viability and morphology changes, it should be observed cells round up into the colonies while remaining attached to the surface of the dish. If necessary, repeat step 35.

**! CAUTION** Dissociate hES cells into small clusters rather than single-cell suspensions at each passage.

1. Add 3 mL of DMEM/F12 and gently triturate the clusters across the dish surface
2. Scrape the attached cells using a cell scraper
3. Transfer the unattached cell suspension into a 15 mL conical tube containing 3 mL of DMEM/F12 using a 5 mL serological pipette.
4. Spin down at 100 × g for 4 minutes at 25 °C
5. Aspirate and discard the supernatant
6. Resuspend the cell pellet with 5 mL of fresh growth mTeSR™1 Medium using a 5 mL serological pipette
7. Add the cell suspension 1 mL/dish into fresh 100-mm culture dishes prepared previously at sep 28-31
8. Incubate the cells at 37 °C, 95% O2, 5% CO2 in humidified air.
9. After 24 h, replace the old growth medium with fresh mTeSR™1 medium
10. Split the cells every 5-6 days.

 **Maintenance of hES cell culture ● Timing 5 min daily**

1. Carefully aspirate the old growth medium from the cell culture dish
2. Replace with 10 mL of fresh growth mTeSR™1 Medium
3. Incubate the cells at 37 °C, 95% O2, and 5% CO2 in humidified air until 60-70% confluent.
4. Replace the medium daily

**! CAUTION** After 10 passages to ensure the pluripotency status and genetic integrity of hES cells

**Cells preparation for electroporation** **● Timing** **6 d**

1. Replace the old growth mTeSR™1 Medium for 6 days.

**! CAUTION** After 80% confluence, the number of cells/dish should be increased to approximately 8-10 x 106, sufficient for 8-10 electroporation reactions.

**hES cell electroporation** **● Timing** **1 h**

1. (Optional) Prior to starting dissociation, add iROCK (10 µM) in the 100-mm cell culture dish for 1 h.
2. Coat the fresh 12-well plate with 6 mL of cold Geltrex™ (0.5 µL/well) as described in steps 29-31.
3. Aspirate Geltrex™ and add 0.5 µL/well of antibiotic-free fresh mTeSR™1 Medium with iROCK (10 µM)
4. Carefully aspirate the old growth medium from the cell 100-mm culture dishes
5. Wash with 4 mL/dish of DPBS-/- and remove immediately
6. Add 4 mL/dish of Accutase™, ensure that cover of dish area
7. Incubate the cell suspension at 37 °C for 5-10 minutes

**! CAUTION** The spontaneous differentiation of the colonies should be removed before the split.

**! CAUTION** During the incubation with Accutase™ monitoring the morphology of colonies, it should be observed separated round cells by microscopy. Dissociate hES cells into single-cell suspensions and avoid clusters. If necessary, repeat step 30.

▲**CRITICAL STEP** High transfection efficiency is dependent on high-quality hES colonies as well as efficient single-cell enzymatic dissociation.

1. Add 3 mL/dish of DMEM/F12 and scrape the attached cells using a cell scraper
2. Gently triturate the clusters across dish surface
3. Transfer the unattached cell suspension using a 5 mL serological pipette into a 50 mL conical tube containing 10 mL of DMEM/F12
4. Spin down at 100 × g for 4 minutes at 25 °C
5. Remove the supernatant and wash with 10 mL of DPBS-/-
6. Count the cells with Trypan blue dye exclusion

▲**CRITICAL STEP** An ineffective transfection is observed using lower cell density or low cell viability.

1. Spin down at 100 × g for 4 minutes at 25 °C
2. Aspirate and discard the supernatant

▲**CRITICAL STEP** Calculate the total volume needed to resuspend the hES cells. A single electroporation reaction requires 1x106 viable hES cells. It should be transferred 2 mL per electroporation reaction (i.e., For 3 electroporation reactions, add a total volume of 6 mL into a tube.)

1. Resuspend the cells with ice-cold DPBS-/-
2. Gently mix the suspension cells and transfer 2 mL/15 mL conical tube (1x106 cells per tube)
3. Spin down at 100 × g for 4 minutes at 4 °C
4. Remove the supernatant and resuspend the pellet with 1SM electroporation buffer mix with the plasmids in a total volume of 100 µL.

▲**CRITICAL STEP** For transient expression, hES cells can be electroporated with 12 µg of pIE plasmids and 8 µg of pSG.

1. Immediately transfer the suspension cells into the 0.2 cm electroporation cuvette, preventing bubbles
2. Electroporate the cells by using the Nucleofector 2B program A-23

▲**CRITICAL STEP** Electroporation frequently leaves considerable cell death.

1. Immediately add 1 mL of fresh mTeSR™1 Medium with iROCK (10 µM) into the electroporation cuvette using a micropipette with a 1000 µL tip to avoid low cell viability
2. Immediately transfer the cell suspension into a 15 mL conical tube containing 0.5 mL of antibiotic-free fresh mTeSR™1 Medium with iROCK (10 µM)

▲**CRITICAL STEP** Repeat steps 69-72 for each single electroporation reaction. It is important to transfect one reaction at a time. A long period of cell incubation with the electroporation buffer can affect cell viability.

1. Gently mix the cell suspension and transfer into three wells (0.5 mL/well) of the coated Geltrex™ 12-well plate previously prepared in steps 51-52.
2. Incubate the cells at 37 °C, 95% O2, 5% CO2 in humidified air
3. After 24 h, replace the old growth medium with antibiotic-free fresh mTeSR™1 medium.

### **Postelectroporation ● Timing 2 d**

1. After 48 h of transfection, the integrase activity was evaluated by flow cytometry.

### **Preparation of sample and flow cytometry ● Timing 2 h**

1. Harvest cells using Accutase™ as described in step 24
2. Remove the old growth medium and wash with 0.5 mL/well of DPBS-/-, remove immediately
3. Add 0.4 mL/well of Accutase™
4. Incubate the cells in the incubator at 37 °C for 5-10 minutes

**! CAUTION** Monitoring the morphology of colonies, it should be observed individual round cells by microscopy. Dissociate hES cells into single-cell suspensions, avoiding clusters. If necessary, repeat step 81.

1. Add 0.5 mL/well of DMEM/F12 into a 15 mL conical tube containing 5 mL of DMEM/F12
2. Spin down at 100 × g for 4 minutes at 25 °C
3. Aspirate and discard the supernatant
4. Wash the cells with 2 mL of ice-cold DPBS-/-
5. Spin at 100 × g for 4 minutes at 4 °C
6. Remove the supernatant and resuspend the cells in 2 mL of ice-cold DPBS-/-
7. Count the cells with Trypan blue dye exclusion
8. Transfer 1 x 105 cells/tube for flow cytometry analysis
9. Keep the cell suspension in the original 15 mL conical tube and collect the pellet for DNA extraction
10. Spin at 100 × g for 4 minutes at 4 °C
11. Resuspend the cells (1 x 105 cells/tube) in 300 µL of ice-cold buffer for FACS.
12. Add 5 µL of 7-AAD into a tube with the cells and incubate at RT for 10 minutes

▲**CRITICAL STEP** After incubation, store the tubes at 4 °C protected from light prior to analysis.

▲**CRITICAL STEP** Acquire at least 10,000 events at the viable gate to evaluate eGFP expression.

### **Cell expansion of the NSC cell line ● Timing 14-20 d**

**Passage cells ● Timing 30 min**

1. Culture NSC cells into a 60-mm culture dish until the cells cover the dish area, at 85-90% confluence. It is approximately 6-7 days.

▲**CRITICAL STEP** Start enzymatic dissociation with high-quality NSC cells.

▲**CRITICAL STEP** For over confluence consider a split ratio of 1:8 to 1:10. NSCs can be seeded at a density of 0.5x105 cells/cm2. For the 60-mm culture dish, consider an area of 20 cm2.

1. Prior to starting the dissociation, coat the fresh 60-mm culture dishes with 3 mL/dish of cold Geltrex™ and place it into the incubator at 37 °C for at least 30 minutes.
2. Remove Geltrex™, and immediately add 2 mL/dish of fresh NEM medium to prevent drying.
3. Place fresh culture dishes into the incubator at 37 °C
4. Carefully aspirate the old medium from the cell culture dish
5. Wash the dish with 2 mL of DPBS-/-, remove immediately
6. Add 2 mL of Accutase™ into the 60-mm culture dish, ensure that cover of dish area
7. Incubate the cell suspension at 37 °C for 3-5 minutes

▲**CRITICAL STEP** Monitoring the cell viability and morphology changes, it should be observed that cells round up while remaining attached to the surface of the dish. If necessary, repeat step 101.

▲**CRITICAL STEP** Dissociation of NSC cells into single-cell suspensions is required at each passage.

1. Add 3 mL of DMEM/F12 and gently triturate the clusters across the dish surface
2. Transfer the unattached cell suspension into a 15 mL conical tube containing 3 mL of DMEM/F12 using a 5 mL serological pipette.
3. Spin down at 300 × g for 4 minutes at 25 °C
4. Aspirate and discard the supernatant
5. Wash with 5 mL of DMEM/F12 using a 5 mL serological pipette
6. Count the cells with Trypan blue dye exclusion
7. Spin down at 300 × g for 4 minutes at 25 °C
8. Resuspend the cell pellet with fresh growth NEM medium using a 5 mL serological pipette

**! CAUTION** Calculate the total volume needed to resuspend the NSC cells. Consider seed 1x106 cells/dish in 1 mL.

1. Add the cell suspension at 1 mL/dish into fresh 60-mm culture dishes previously prepared at steps 94-97. Should have a total volume of 3 mL/dish.
2. Incubate the cells at 37 °C, 95% O2, 5% CO2 in humidified air
3. After 24 h, replace the old growth medium with 3 mL of fresh NEM medium.
4. Split the cells every 6 days.

**Maintenance of NSC cell culture for 5 min daily**

1. Carefully aspirate the old growth medium from the cell culture dish
2. Replace with 4 mL of fresh growth NEM medium.
3. Incubate the cells at 37 °C, 95% O2, and 5% CO2 in humidified air until they reach 80-90% confluent.
4. Change the growth medium every 2 days.

▲**CRITICAL STEP** After 10 passages to ensure the pluripotency status and genetic integrity of NSC cells

**Cells preparation for electroporation ● Timing 6 d**

1. Replace the old growth NEM media for 6 days.

**! CAUTION** After 90-95% confluence, the number of cells/dish should be increased to approximately 8-12 x 106, sufficient for 8-12 electroporation reactions.

### **NSC cell electroporation● Timing *1 h***

1. (Optional) Prior to starting dissociation, add iROCK (10 µM) into 60-mm culture cells dish for 1 h
2. Coat the fresh 12-well plate with 6 mL of cold Geltrex™ (0.5 µL/well) as described in steps 95-97.
3. Aspirate Geltrex™ and add 0.5 µL/well of antibiotic-free fresh NEM medium with iROCK (10 µM)
4. Carefully aspirate the old growth medium from the cell 60-mm culture dishes
5. Wash with 2 mL/dish of DPBS-/-, and remove immediately
6. Add 2 mL/dish of Accutase™, ensure that cover of dish area
7. Incubate the cell suspension at 37° for 3-5 minutes

**! CAUTION** Discard the cells if spontaneous differentiation is observed.

▲**CRITICAL STEP** High transfection efficiency is dependent on high-quality NSCs as well as efficient single-cell enzymatic dissociation.

▲**CRITICAL STEP** During incubation with Accutase™ monitor the morphology of colonies by microscopy. Cells must be rounded. Dissociate NSC cells into single-cell suspensions. If necessary, repeat step 125.

▲**CRITICAL STEP** Ineffective transfection is observed in the presence of cluster cells.

1. Add 2 mL/dish of DMEM/F12 and gently triturate the clusters across the dish surface
2. Remove unattached cells using a 5 mL serological pipette
3. Transfer the cell suspension into a 50 mL conical tube containing 10 mL of DMEM/F12
4. Spin down at 300 × g for 4 minutes at 25 °C
5. Remove the supernatant and wash with 5 mL of DPBS-/-
6. Count the cells with Trypan blue dye exclusion
7. Spin down at 300 × g for 4 minutes at 25 °C

**! CAUTION** Calculate the total volume needed to resuspend the NSC cells. A single electroporation reaction requires 1x106 viable NSCs. It should be transferred 2 mL per electroporation reaction (i.e., For 3 electroporation reactions, add a total volume of 6 mL into a tube.)

▲**CRITICAL STEP** An ineffective transfection is observed using lower cell density or low cell viability.

1. Resuspend the cells with ice-cold DPBS-/-
2. Gently mix the suspension cells and transfer 2 mL/15 mL conical tube
3. Spin down at 300 × g for 4 minutes at 4 °C
4. Remove the supernatant and resuspend the cells with 1S electroporation buffer mix with the plasmids in a total volume of 100 µL.

▲**CRITICAL STEP** For transient expression, NSC cells can be electroporated with 12 µg of pIE plasmids and 8 µg of pSG.

1. Immediately transfer the suspension cells into the 0.2 cm electroporation cuvette, avoid bubbles
2. Electroporate the cells by using the Nucleofector 2D program A-33

**! CAUTION** Cell death is frequently observed after electroporation.

1. Immediately add 1 mL of fresh NEM media with iROCK (10 µM) into the electroporation cuvette using a micropipette with a 1000 µL tip to avoid low cell viability
2. Immediately transfer the cell suspension into a 15 mL conical tube containing 0.5 mL of antibiotic-free fresh NEM media with iROCK (10 µM)

▲**CRITICAL STEP** Repeat steps 136-138 for each single electroporation reaction. It is important to transfect one reaction for a specified time. A long period of cell incubation with the electroporation buffer can affect cell viability.

1. Gently mix the cell suspension and transfer into three wells (0.5 mL/well) of the coated Geltrex™ 12-well plate previously prepared in step 28.
2. Incubate the cells at 37 °C, 95% O2, 5% CO2 in humidified air
3. After 24 h, replace the old growth medium with antibiotic-free fresh NEM medium.

**? TROUBLESHOOTING**

### **Postelectroporation ● Timing 2 d**

1. After 48 h of transfection, the integrase activity can be evaluated by using flow cytometry.

**Preparation of sample and flow cytometry ● Timing 2 h**

1. Harvest cells using Accutase™
2. Remove the old growth medium and wash with 0.5 mL/well of DPBS-/-, remove immediately
3. Add 0.4 mL/well of Accutase™
4. Incubate the cells in the incubator at 37 °C for 5-10 minutes

▲**CRITICAL STEP** Monitor the morphology of colonies by microscopy. Rounded cells must be observed.

1. Add 0.5 mL/well of DMEM/F12 into a 15 mL conical tube containing 5 mL of DMEM/F12
2. Spin down at 300 × g for 4 minutes at 25 °C
3. Wash the cells with ice-cold DPBS-/-
4. Spin at 300 × g for 4 minutes at 4 °C
5. Remove the supernatant and resuspend the cells in 2 mL of ice-cold DPBS-/-
6. Count the cells with Trypan blue dye exclusion
7. Transfer 1 x 105 cells/tube by flow cytometry analyses
8. Keep the cell suspension in the original 15 mL conical tube and collect the pellet for DNA extraction.
9. Spin at 300 × g for 4 minutes at 4 °C.
10. Resuspend the cells (1 x 105 cells/tube) in 300 µL of ice-cold buffer for FACS.
11. Add 5 µL of 7-AAD into a tube with the cells and incubate at RT for 10 minutes

▲**CRITICAL STEP** After incubation, store the tubes at 4 °C protected from light prior to analysis.

▲**CRITICAL STEP** Acquire at least 10,000 events at the viable gate to evaluate eGFP expression.

## ANIMAL

# **Materials**

### ***Biological materials***

Bovine fibroblast (see ‘Reagent setup’) cells were isolated according to the protocol described by Freshney[51](https://www.zotero.org/google-docs/?E20RRg), with modifications.

# **Reagents**

### ***Growth medium and supplements***

distilled water, dH2O, sterile

DMEM (Dulbecco's Modified Eagle Medium,) powder, low glucose (Gibco, cat. no.3600-034)

Sodium bicarbonate powder (Sigma-Aldrich, cat. no. S5761)

D-(+)-Glucose (Sigma-Aldrich, cat. no. G7021)

L-glutamine 200mM, (Sigma-Aldrich, cat. no. G6392)

Sodium Pyruvate (Sigma-Aldrich, cat. no. P5280)

FBS (Fetal Bovine Serum), (Gibco, cat. no. 12657-029)

Penicillin G sodium salt (Sigma-Aldrich, cat. no. P3032)

Streptomycin sulfate salt (Sigma-Aldrich, cat. no. S9137)

PBS (Phosphate Buffered Saline), tablet, pH 7.4 (Sigma-Aldrich, cat. no. P4417-50TAB)

0.05% Trypsin-EDTA (1X), (Gibco, cat. no. 25300-062)

Trypan blue solution (Sigma-Aldrich, cat. no. T8154)

Alcohol 70% for material sterilization (any brand) **! CAUTION** alcohol is flammable. Handle it in a fume hood with proper PPE.

### ***Transfection***

Lipofectamine LTX and Plus Reagent (Invitrogen, cat. no. 15338-100)

Opti-MEM I (1X), Reduced Serum Media, (Gibco, cat. no. 31985-070)

### ***Flow cytometry***

Rinse: Deionized water

Sheath solution: PBS (Phosphate Buffered Saline), tablet, pH 7.4 (Sigma-Aldrich, cat. no. P4417-50TAB)

Debubbler solution: Isopropyl alcohol 70% (MilliporeSigma, cat. no. 563935)

Sterilizer solution: 0.4-0.7%Hypochlorite

Cleanser solution: CoulterClenz® (BeckmanCoulter cat. no. 8546929)

Flow Sight Calibration Beads (Amnis, cat. no. 400300)

### ***Viability assay***

PBS (Phosphate Buffered Saline), tablet, pH 7.4 (Sigma-Aldrich, cat. no. P4417-50TAB)

Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. D2650-5X10ML)

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Invitrogen, cat. no. M-6494)

### ***Plasmid extraction and cloning/PCR***

LB Broth medium (Sigma-Aldrich, cat. no. L3022 or equivalent)

One Shot™ MAX Efficiency™ DH10B T1 Phage-Resistant Cells (Invitrogen, cat. no.12331013)

dH 2 O, sterile

Ampicillin sodium salt (Sigma-Aldrich, cat. no.A0166 )

Kanamycin sulfate (Sigma-Aldrich, cat. no. K1377 )

QIAGEN Plasmid Maxi Kit (QIAGEN, cat. no. 12163 )

pGEM®-T Easy Vector System I (Promega, cat. no. A1360)

EcoRI, Restriction Endonuclease (New England Biolabs, cat. no. R0101S)

*Platinum* Taq DNA Polymerase (Invitrogen, cat. no. 10966018)

PureYield™ Plasmid Miniprep System (Promega, cat. no. A1222 or equivalent)

ReliaPrep™ DNA Clean-Up and Concentration System (Promega, cat. no.A2893 or equivalent)

Agarose (Sigma-Aldrich, cat. no.9539 or equivalent)

SYBR Safe DNA gel stain (Invitrogen, cat. no. S33102)

1 Kb Plus DNA Ladder (Invitrogen, cat. no.10787018)

DNeasy Blood & Tissue Kits (Qiagen, cat. no.69504)

# **Equipment**

### ***Equipment for bovine cell isolation, culture and analysis***

Biological Safety Cabinet Class 2 (VecoFlow LTDA)

Stericup Quick Release-GP Sterile Vacuum Filtration System 500 mL (Millipore, cat. no.S2GPU05RE)

0.22 μm PES membrane (KASVI, cat. no. K18-230 or equivalent)

Vacuum pump (Millipore, model WP6111560)

Bottles with screw cap Boro 3.3 500 mL and 1L (Boeco Germany, cat. no. BOE5080445, BOE5080545)

Graduated cylinder 500 mL (any brand)

Beaker 500 mL (any brand)

Pipette tips, 10 µL, 200 µL, 1000 µL (any brand)

Micropipettes, 2 µL, 10 µL, 200µL, 1000µL (Gilson, FA10001M, FA10002M, FA10005M, FA10006M)

Semi-Micro Analytical Balance (Shimadzu, model AUW220D)

OrionStar™ A211 benchtop pH meter (Thermo Scientific, cat. no.STARA2119)

Water bath (Ultronic, model Q3.0/040A or equivalent)

Cell culture flask, 25 and 75 cm2 (TPP, cat. no. 90025, 90075)

Flat-bottom cell culture plates, 24 and 96-well (TPP, cat. no. 92024, 92096)

LoBind Microcentrifuge Tubes, 1.5 mL and 2.0 mL (Axygen, cat. no. MCT-150-L-C, MCT-200-L-C)

Cell incubator (37°C, 5% CO2) (Thermo Scientific)

Axiovert 135M fluorescence microscope (Carl Zeiss)

Neubauer chamber (KASVI, cat. no. K5-0111)

NanoDrop 2000c Spectrophotometer (Thermo Scientific)

Conical centrifuge tube, 15 mL and 50 mL (Kasvi, cat. no. K19-0015 and K19-0050 respectively)

Mini spin mini centrifuge for microtubes (Eppendorf, MFG Part Number, 22620100)

Erlenmeyer flask, glass 250 and 500 mL (any brand)

Parafilm® M (Bemis, cat. no. P6543)

Thermocycler with programmable temperature control, 96 wells T100™ Thermal Cycler (Bio-Rad, cat. no.1861096)

Electrophoresis PowerPac (Bio-Rad)

SmartView Pro 1100 Imager System (Major Science, cat. no. UVCI-1100)

Sunrise Microplate Reader (Tecan)

Amnis® brand FlowSight® Imaging Flow Cytometer (Merck Millipore, cat. no. 100300 or equivalent)

Ultrospec10 cell density meter spectrophotometer (Amersham Biosciences)

### ***Software***

Magellan <https://www.selectscience.net/products/magellan-data-analysis-software/?prodID=20443>

 IDEAS software <https://www.merckmillipore.com/BR/pt/20150121_204850?ReferrerURL=https%3A%2F%2Fwww.google.com%2F>

SnapGene

<https://www.snapgene.com/support/downloads>

# **Reagent Setup**

### ***Bovine fibroblast isolation***

Bovine fibroblast cells were isolated according to the protocol described by Freshney[51](https://www.zotero.org/google-docs/?mz8E7Z) with modifications. The cells were removed from 14-month-old Nelore (Bos indicus) bull oxtail by biopsies and washed three times in 0.05% trypsin (Gibco). The cells were then transferred to 25-cm2 cell culture flasks and incubated in DMEM (Gibco) supplemented with 10% FCS (Gibco) and penicillin‒streptomycin at 37 °C in a 5% CO2 atmosphere. After three passages or when the fibroblast cultures showed homogeneity, the cells were ready for transfection. Cell cultures with 60% to 70% confluence were picked. **! CAUTION** The use of bovine cells was performed under ethical guidelines and was approved by the Ethics Committee on the Use of Animals (CEUA) of Embrapa Genetic Resources and Biotechnology in March 2013 approval reference no. 001/2013.

### ***Culture medium stock***

For 1 L of DMEM, measure approximately 90% of the volume to be prepared with sterile distilled water (15-20 °C), and slowly add the 13.5 g of DMEM powder under constant agitation at room temperature. After completely dissolving the DMEM powder, add 3.7 g of sodium bicarbonate (NaHCO3) per liter, complete with sterile distilled water up to 1 L and store at 4 °C for up to 6 months.

### ***Culture medium (working solution)***

For each 400 mL of DMEM solution, add 40 mL of FBS (10% (vol/vol) final), 0.4 mL of penicillin/streptomycin (1% (vol/vol) final), 44 mg of 100X sodium pyruvate, xx mg of D-(+)-glucose, and 116 mg of L-glutamine 200 mM. Filter through a 0.22 μm Vacuum Filtration System and store at 4 °C for up to 2 months.

### ***MTT stock solution***

Dissolve 500 mg of MTT powder in 10 mL of 1X PBS (pH 3.7), stir the solution for approximately 1 hour in the dark, sterilize it by filtration with a 0.22 µm filter ▲**CRITICAL STEP** store 10 mL aliquots (50 mg/mL) at -20 °C in the dark.

# **PROCEDURE**

### **Cell Passage ● Timing 30 min**

1. Cultivate bovine fibroblast cells at 5.0x105 cells per culture flask with a 75 cm2 surface area. Well area coverage, between 60-80% confluence. It takes approximately 8-10 days.
2. Before starting dissociation, place the working aliquot of 0.05% trypsin and EDTA solution in the incubator at 37°C for at least 10 minutes.
3. Remove the DMEM culture medium and wash with Dulbecco's Phosphate-Buffered Saline (DPBS, GIBCO). Discard the DPBS and immediately add 2 mL of 0.05% trypsin and EDTA. Make sure to cover the plate area.
4. Place the culture flask in the incubator at 37°C for 5 minutes

▲**CRITICAL STEP** Monitoring cell viability and morphology changes, round cells should be observed while remaining adhered to the plate surface. If necessary, repeat step 4.

▲**CRITICAL STEP** Cells need to be dissociated in each passage.

1. Add 2 mL of DMEM
2. Transfer the loose cell suspension to a 1.5 or 2 mL centrifugation tube
3. Spin at 1300 rpm for 5 minutes at room temperature
4. Aspirate and discard the supernatant
5. Wash with 5 mL of DPBS using a serological pipette
6. Spin at 1300 rpm for 5 minutes at room temperature
7. Aspirate and discard the supernatant
8. Resuspend the cell pellet with fresh DMEM medium using a serological pipette
9. Add 1 mL of cells/flask culture to new culture flasks with a 75 cm2 surface area that have been previously prepared. Should have a total volume of 10 mL
10. Incubate the cells at 37°C, 95% humidity, 5% CO2
11. After 24h, replace the old culture medium with 10 mL of fresh DMEM medium.

▲**CRITICAL STEP** Regularly observe the cells to ensure they are growing properly and that there is no contamination.

▲**CRITICAL STEP** When the cells reach confluence, subculturing is necessary to avoid high cell density.

1. Divide the cells every 5 days.

### **Bovine fibroblast cell transfection with Lipofectamine LTX and Plus Reagent. ● Timing 1h**

1. Prepare the cells for transfection: the cells should be at a specific stage of culture and in the appropriate medium for transfection.
2. Prepare the transfection solution: mix the LTX Plus with the desired plasmid following the manufacturer's instructions.
3. Add the transfection solution to the cells: add the transfection solution to cells grown in culture plate or to the medium for suspended cells.
4. Incubate the cells: incubate the cells with the transfection solution for a specific period of time, usually 4-6 hours.

▲**CRITICAL** Add new culture medium: after the incubation period, change the culture medium to remove the transfection solution and allow the cells to continue developing.

▲**CRITICAL STEP** Integrase activity can be evaluated by flow cytometry 24 hours after transfection. For optimization, several time points should be evaluated. In our hands, 72 hours after transfection eGFP expression was reduced.

▲**CRITICAL STEP** The results were analyzed 48 h after transient transfection by flow cytometry.

### **Analysis of integrase expression in bovine fibroblast cells by flow cytometry. ● Timing 2.5h**

Dissociating cells using 0.05% Trypsin-EDTA (1X):

1. Remove the old growth medium from each well and wash with 0.5 mL/well of DPBS, remove immediately
2. Add 0.5 mL/well of 0.05% Trypsin-EDTA
3. Incubate the cells in the incubator at 37°C for 5 minutes

▲**CRITICAL STEP** Monitoring colony morphology, rounded cells should be observed by microscopy.

1. Add 0.5 mL/well of DMEM in a microcentrifuge and 1.5 or 2 mL tube containing DMEM
2. Spin at 1300 g for 5 minutes
3. Wash the cells with DPBS
4. Spin at 1300g for 5 minutes
5. Remove the supernatant and resuspend the cells with DPBS
6. Transfer the cells to a 1.5 mL centrifugation tube for flow cytometry analysis

▲**CRITICAL STEP** Store the tubes at 37°C protected from light before analysis.

▲**CRITICAL STEP** Acquire at least 10,000 events in the viable gate to evaluate eGFP expression.

###

### **Cell viability assays. ● Timing 7h**

1. Count the cells with a Neubauer chamber and cultivate bovine fibroblasts and HEK 293T cells in 96-well plates at a density of 1x105 cells/well in triplicate and grown for 24 hours at 37°C in a 5% CO2 atmosphere.
2. After 48 hours of transient cotransfection, incubate the cells with 15 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Thermo Fisher Scientific) (5 mg/mL). ▲**CRITICAL**  Protect from light!

▲**CRITICAL** For the negative control, use 20 μL of dimethyl sulfoxide (DMSO) directly on the cells, in a final volume of 200 μL per well.

1. Incubate for 4 hours at 37°C.
2. Remove the MTT solution and add 150 μl of DMSO to each well to dissolve formazan crystals. ▲**CRITICAL**  perform the procedure in the absence of light and do not aspirate the crystals.
3. Read the absorbance at 595 nm in a plate reader or spectrophotometer.

## PLANT

# **Materials**

### ***Biological materials***

Arabidopsis thaliana plants at 60 days after germination. ▲**CRITICAL** The age of the plant is essential for good results in the isolation of protoplasts. Older plants produce a lower quality and quantity of protoplast.

# **Reagents**

### ***Plant Grow***

Substrate for greenery (Carolina Soil, Bioplant, PlantMax or equivalent)

DECIS 25 EC® (Bayer, Active principle: deltamethrin 25 g.L-1)

0.5 X MS liquid medium (see “Reagent Setup”) when necessary for fertilization

### ***Protoplast Isolation***

4-Morpholineethanesulfonic acid, MES (Sigma Aldrich, cat. no. M3671 or equivalent)

Bovine Serum Albumin, BSA (Sigma-Aldrich, cat. no. A7906 or equivalent)

Calcium chloride, CaCl2 (Sigma-Aldrich, cat. no. C2661 or equivalent)

Cellulase from Trichoderma sp. (Sigma-Aldrich, cat. no. C1794)

Driselase (Sigma-Aldrich, cat . no. D9515)

Ethanol 70% (JFeres, cat. no. 50731 or equivalent)

Magnesium chloride, MgCl2 (Vetec, cat. no. V000149 or equivalent)

Mannitol, D-Mannitol (Sigma-Aldrich, cat. no. M1902 or equivalent)

Pectolyase from *Aspergillus japonicus* (Sigma-Aldrich, cat. no. P5936)

Potassium chloride, KCl (Sigma-Aldrich, cat. no. P9541 or equivalent)

Potassium hydroxide, KOH (J.T.Baker, cat. no. 3140-19 or equivalent)

Sodium chloride, NaCl (Sigma-Aldrich, cat. no. S3014 or equivalent)

Distilled water, dH2O, sterile

Ice

### ***Protoplast transfection***

1 µg.µL-1 plasmid DNA

4-Morpholineethanesulfonic acid, MES (Sigma-Aldrich, cat. no. M8250)

Calcium chloride, CaCl2 (Sigma-Aldrich, cat. no. C2661)

Distilled water, dH2O, sterile

Mannitol, D-Mannitol (Sigma-Aldrich, cat. no. M1902)

Polyethylene glycol, PEG4000 (Sigma-Aldrich, cat. no. 81240)

Potassium chloride, KCl (Sigma-Aldrich, cat. no. P9541 or equivalent)

Potassium hydroxide, KOH (J.T.Baker, cat. no. 3140-19or equivalent)

Ice

### ***Protoplast Flow Cytometry***

Cleanser solution: CoulterClenz® (BeckmanCoulter cat. no. 8546929)

Debubbler solution: Isopropyl alcohol 70% (Fisher Scientific, cat. no. A459)

Flow Sight Calibration Beads (Amnis, cat. no. 400300)

Rinse: Milli-Q deionized water, at least 0.22 µm filtered

Sheath solution: PBS (Phosphate Buffered Saline), tablet, pH 7.4 (Merck, cat. no. P4417-50TAB)

Sterilizer solution: 0.4-0.7% sodium hypochlorite (VWR, cat. no. JT9416-1)

### ***Protoplast Viability assay***

Acetone (Sigma-Aldrich, cat. no. 904082 or equivalent)

Aluminum foil UV clean (any brand)

Dimethyl sulfoxide, DMSO (Sigma-Aldrich, cat. no. D8418 or equivalent)

Fluorescein diacetate, FDA (Sigma-Aldrich, cat. no. F7378 or equivalent)

### ***Plasmid extraction and Cloning***

Agarose (Sigma-Aldrich, cat. no.9539 or equivalent)

Ampicillin sodium salt (Sigma-Aldrich, cat. no. A9518 or equivalent)

Distilled water, dH2O, sterile

DNeasy Plant Mini Kit (Qiagen, cat. no. 69104)

dNTP Set (100 mM) (Invitrogen, cat. no. 10297018)

EcoRI, Restriction Endonuclease (New England Biolabs, cat. no. R0101S or equivalent)

Kanamycin (Sigma-Aldrich, cat. no. K1377 or equivalent)

LB broth (Sigma-Aldrich, cat. no. L3022 or equivalent)

One Shot™ MAX Efficiency™ DH10B T1 Phage-Resistant Cells (Invitrogen, cat. no.12331013)

PCR entry vector, pGEM®-T Easy Vector System I (Promega, cat. no. A1360)

*Platinum* Taq DNA Polymerase (Invitrogen, cat. no. 10966018 or equivalent)

QIAGEN Plasmid Maxi Kit (Qiagen, cat. no. 12163 )

SYBR® Safe DNA Gel Stain (Invitrogen, cat. no. S33102)

Wizard® Plus SV Minipreps DNA Purification Systems (Promega, cat. no. A1340)

Wizard® SV Gel and PCR Clean-Up System (Promega, cat. no. A9282)

XL1-Blue *Escherichia coli* Supercompetent Cells (Agilent, cat. no. 200236)

#

# **Equipment**

Autoclave Vitale Class CD54 (Cristofoli or equivalent)

Axiovert 135M fluorescence microscope (Carl Zeiss)

Centrifuge MiniSpin® (Eppendorf, cat. no. 5452000816 or equivalent);

Centrifuge 5804R, refrigerated (Eppendorf, cat. no. EP022628146) with Rotor A-4-81, with 15-30 mL buckets and adapters

Ice Machine Super Ice (BenMax cat. no. BMGX26-07 or equivalent)

Incubator Shaker Series I26 (New Brunswick Scientific)

Laminar Flow Cabinet Sentinel Gold (ESCO cat. no. LHG-6CG-F8 or equivalent)

Midi Plus-1 Horizontal Electrophoresis System (MajorScience cat. no. ME10-7-10 or equivalent)

Millipore Milli-Q® Direct 8 Water Purification System (Marck ct. no. C85358)

Thermomixer Confort (Eppendorf cat. no. EXT 20441 or equivalent)

Water bath (Ultronic, model Q3.0/040A or equivalent)

Vacuum chamber - Biobalistic Particle Delivery System PDS-1000/He System (Bio-Rad cat. no. 1652257 or equivalent)

### ***Protoplast transfection***

Eppendorf® Centrifuge 5804R, refrigerated (Eppendorf, cat. no. EP022628146) with Rotor A-4-81, with 15-30 mL buckets and adapters

Plate incubator (TECNAL, model TE-420 or equivalent)

Whatman® UNIFLO® 25 syringe filters (Whatman cat. no. WHA9913-2502)

### ***Flow cytometry***

Amnis® brand FlowSight® Imaging Flow Cytometer (Merck Millipore, cat. no. 100300 or equivalent)

Eppendorf® Centrifuge 5804/5804R, refrigerated (Eppendorf, cat. no. EP022628146) with Rotor A-4-81, with 15-30 mL buckets and adapters

### ***Solutions, Plasmid extraction and Cloning***

Centrifuge MiniSpin® (Eppendorf, cat. no. 5452000816)

Gel Documentation System SmartView Pro 1100 Imager System, (Major Science, cat. no.UVCI-1100 or equivalent)

Plate incubator (TECNAL, model TE-420 or equivalent)

Magnetic stirrer with hot plate (any brand)

NanoDrop 2000C Spectrophotometer (Thermo Scientific)

PowerPac™ Basic Power Supply horizontal electrophoresis system (Bio-Rad, cat. no.1645050 or equivalent)

Precision scale (any brand)

Incubator Shaker Series I26 (New Brunswick Scientific)

Stir bars (any brand)

scalpel (any brand)

Surgical Blade nº 11 (any brand)

Thermocycler with programmable temperature control, 96 wells T100™ Thermal Cycler (Bio-Rad, cat. no.1861096)

### ***Glassware and disposable instruments***

150 mL plastic cup (any brand)

10 μl pipette tips (any brand)

100 mL volumetric flask (any brand)

1 mL pipette tips WB (Axygen, cat. no.T-1005-WB-C or equivalent)

200 μl pipette tips WB (Axygen, cat. no.T-205-WB-C or equivalent)

0.2-2 μl single channel pipette, micropipette (Gilson, cat. no.F144054M or equivalent)

1-10 μl single channel pipette, micropipette (Gilson, cat. no.F144055M or equivalent)

10-100 μl single channel pipette, micropipette (Gilson, cat. no.F144057M or equivalent)

100-1000 μl single channel pipette, micropipette (Gilson, cat. no.F144059M or equivalent)

500-5000 μl single channel pipette, micropipette (Gilson, cat. no.F144066 or equivalent)

44-µm metal mesh (any brand)

500 mL volumetric flask (any brand)

Beaker, 1000 mL, 500 mL 100 mL, 50 mL (any brand)

Cell culture plate, 12-wells, flat bottom (Kasvi, cat. no. K12-012 or equivalent)

Conical centrifuge tube, 15 mL and 50 mL (Kasvi, cat. no. K19-0015 and K19-0050 respectively or equivalent)

Round bottom Corex glass tubes, 15mL, 30 mL (no. 8441 and 8445 respectively, or equivalent)

Glass bottle with a screw lid, 500 mL and 300 mL (any brand)

Glass cell culture dishes ø90 mm and ø120 mm (any brand)

Glass Coverslips (24 x 50 mm) (Olen, cat. no. K5-245)

Glass Microscope Slides (26 x 76 mm) (Olen, cat. no. K5-710)

Graduated syringe without needle 20 mL and 50 mL (any brand)

Graduated cylinder, 100 mL, 1000 mL (any brand)

Membrane Filter, 0.22 µm pore size (KASVI, cat. no. K18-230 or equivalent)

Membrane Filter, 0.45 µm pore size (KASVI, cat. no. K18-430 or equivalent)

Metal spatula (any brand)

Metal tweezer (any brand)

Microtubes, 1.5 mL (Axygen Scientific, cat. no. MCT -150)

PCR tubes 0.2 mL (Axygen Scientific, cat. no. PCR-02-C or equivalent)

Plant vessels (any brand)

Steritop® vacuum filtration system 0.22 µm (Millipore, cat. no.SCGPS05RE)

Whatman® UNIFLO® 25 syringe filters (Whatman cat. no. WHA9913-2502)

#

# **Reagent Setup**

Prepare stock solutions (Tables X1-X3) in advance and follow the instructions outlined below. ▲**CRITICAL** The volumetric flask is the most suitable glassware for preparing solutions. It is used to measure accurate amounts of liquid. Try to use a volumetric flask whenever possible; otherwise, use a graduated cylinder. After preparing the solution and adjusting to the final volume, transfer the solution to a beaker and filter or transfer the solution to a glass bottle with a screw lid to autoclave it. **! CAUTION** Do not screw the glass bottle lid on completely as it may explode inside the autoclave. Store the solutions as recommended.

### ***Protoplast Isolation***

***Stock Solutions***

### ***KOH 2.5 N***

Use a 100 mL beaker. Carefully dissolve 7.01 g of KOH in approximately 30 mL of distilled water. Mix well, then adjust the final volume to 50 mL with dH2O. Transfer to a 50 mL glass bottle with a screw lid. **! CAUTION** Hazardous. KOH is corrosive and irritant. Wear proper personal protective equipment (PPE). ▲**CRITICAL** KOH is a base and slowly dissolves glass. Store at 25 °C in a polycarbonate conical centrifuge tube for up to 2 years . Label it properly as corrosive.

### ***MES (pH 5,7) 0.1 M***

Use a 250 mL beaker. Dissolve 3,91 g of MES in approximately 150 mL of distilled water. Add a stir bar and put in a magnetic stirrer. Adjust pH with KOH to pH 5,7. Mix well, then adjust the final volume to 200 mL with dH2O. Filter it with Steritop® vacuum filtration system 0.22 µm in a 500 mL sterile glass bottle with screw lid. Label it properly. Store at 25 °C for up to 3 months.

### ***Mannitol 0.8 M***

Use a 500 mL beaker. Dissolve 58,30 g of mannitol in approximately 300 mL of distilled water. Mix well, then adjust the final volume to 400 mL with dH2O and filter it with a Steritop® vacuum filtration system 0.22 µm in a 500 mL sterile glass bottle with a screw lid. Label it properly. Store at 25 °C for up to 3 months. ▲**CRITICAL** Mannitol shows low solubility at this concentration. Gently pour under constant stirring until the complete dissolution.

### ***NaCl 5 M***

Use a 250 mL beaker. Dissolve 58.44 g of NaCl in approximately 150 mL of distilled water. Mix well, then adjust the final volume to 200 mL with dH2O and filter it with a Steritop® vacuum filtration system 0.22 µm in a 250 mL sterile glass bottle with a screw lid. Label it properly. Store at 25 °C for up to 3 months.

### ***MgCl2 1 M***

Use a 100 mL beaker. Dissolve 4.76 g of MgCl2 in approximately 30 mL of distilled water. Mix well, then adjust the final volume to 50 mL with dH2O and filter it by using a 50 mL syringe with a 0.22 µm filter in a 50 mL sterile glass bottle with a screw lid. Label it properly. Store at 25 °C for up to 3 months.

### ***CaCl2 1 M***

Use a 250 mL beaker. Dissolve 22.2 g of CaCl2 in approximately 150 mL of distilled water. Mix well, then adjust the final volume to 200 mL with dH2O and filter it with a Steritop® vacuum filtration system 0.22 µm in a 250 mL sterile glass bottle with a screw lid. Label it properly. Store at 25 °C for up to 3 months.

### ***KCl 1 M***

Use a 100 mL beaker. Dissolve 3.73 g of KCl in approximately 30 mL of distilled water. Mix well, then adjust the final volume to 50 mL with dH2O and filter it by using a 50 mL syringe with a 0.22 µm filter in a 50 mL sterile glass bottle with a screw lid. Label it properly. Store at 25 °C for up to 3 months.

***Working Solutions*** (reagents and respective final concentrations are listed in **Table 2**)

### ***W5 Solution***

Use a 500 mL beaker. Mix 12.32 mL of 5 M NaCl, 50 mL of 1 M CaCl2, 2 mL of 1 M KCl, and 8 mL of 0.1 M MES (pH 5.7). Adjust the final volume to 400 mL with dH2O and filter it with a Steritop® vacuum filtration system 0.22 µm in a 500 mL sterile glass bottle with a screw lid. Label it properly. Store at 4 °C for up to 3 months.

### ***Enzymatic Solution***

### ***Step 1***

Use a 200 mL beaker. Mix 62.5 mL of 0.8 M mannitol, 2 mL of 1 M KCl and 20 mL of 0.1 M MES (pH 5.7), and then dissolve 1.5 g of cellulase, 0.5 g of Driselase and 0.2 g of Pectolyase. ▲**CRITICAL** Stir slowly with heat at 55 °C for 10 minutes. ▲**CRITICAL** Cool the solution before proceeding to step 2.

### ***Step 2***

Add 100 mg of BSA and 1 mL of 1 M CaCl2 and mix gently. Adjust the final volume to 100 mL with dH2O and filter it by using a 50 mL syringe with a 0.22 µm filter. ▲**CRITICAL** Divide into 10 mL aliquots in 15 mL conical tubes. Label it properly. Store at -20 °C for up to 3 months.

### ***MMg Solution***

Use a 50 mL beaker. Add 25 mL of 0.8 M mannitol, 0.75 mL of 1 M MgCl2 and 2 mL of 0.1 M MES (pH 5.7). Adjust the final volume to 50 mL with dH2O. Filter it by using a 50 mL syringe with a 0.22 µm filter in a glass bottle with a screw lid. Store at 4 °C. Label it properly.

**Table 2**. Reagents and final concentration for protoplast isolation working solutions

|  |  |  |
| --- | --- | --- |
| **Component** | **Stock solution** | **Final concentration** |
| **W5 Solution** |
| NaCl | 5 M | 154 mM |
| CaCl2 | 1 M | 125 mM |
| KCl | 1 M | 5 mM |
| MES (pH 5.7)\* | 0.1 M | 2 mM |
| **Enzymatic Solution** |
| Mannitol | 0.8 M | 500 mM |
| KCl | 1 M | 20 mM |
| MES (pH 5.7)\* | 0.1 M | 2 mM |
| Cellulase |  | 1.5% wt/vol |
| Driselase |  | 0.5% wt/vol |
| Pectolyase |  | 0.2% wt/vol |
| CaCl2 | 1 M | 20 mM |
| BSA |  | 1 mg/mL |
| **MMg Solution** |
| Mannitol | 0.8 M | 400 mM |
| MgCl2 | 1 M | 15 |
| MES (pH 5.7)\* | 0.1 M | 4 mM |

\* Use a KOH solution to adjust the MES stock solution pH.

### ***Protoplast transfection*** (reagents and respective final concentrations are listed in **Table 3**)

**40% PEG Solution**

Use a 50 mL conical centrifuge tube. Dissolve 8 g of PEG4000 in 10 mL of dH2O. Add 5 mL of 0.8 M mannitol and 2 mL of 1 M CaCl2. Adjust the final volume to 20 mL with dH2O and mix gently. ▲**CRITICAL** Prepare fresh and cut off sterile 1 mL plastic tips to pipette it. Do not store the 40% PEG solution. Label it properly.

### ***WI Solution***

Use a 100 mL beaker. Add 31.25 mL of 0.8 M mannitol, 1 mL of 1 M KCl and 2 mL of 0.1 M MES (pH 5.7). Adjust the final volume to 50 mL with dH2O. Filter it by using a 50 mL syringe with 0.22 µm in a 50 mL glass bottle with screw lid. Label it properly. Store at 4 °C for up to 3 months.

**Table 3**. Reagents and final concentration for protoplast transfection working solutions.

|  |  |  |
| --- | --- | --- |
| **Component** | **Stock solution** | **Final concentration** |
| **40% PEG Solution** |
| PEG4000 |  | 40% wt/vol |
| Mannitol | 0.8 M | 200 mM |
| CaCl2 | 1 M | 100 mM |
| **W1 Solution** |
| Mannitol | 0.8 M | 500 mM |
| KCl | 1 M | 20 mM |
| MES (pH 5.7)\* | 0.1 M | 4 mM |

\* Use a KOH solution to adjust the MES stock solution pH.

### ***Viability assay*** (reagents and respective final concentrations are listed in **Table 4**)

### ***Fluorescein diacetate (FDA) solution***

Prepare a stock solution by dissolving 5 mg of FDA in 1 mL of acetone. Store at 4 °C for up to 7 days. Label it properly. **! CAUTION** Hazardous. Acetone is toxic, flammable and irritating. Wear proper personal protective equipment (PPE). Add 10 µL of FDA stock solution in 2.5 mL of W1 solution. ▲**CRITICAL** Prepare fresh and cover with aluminum foil.

**Table 4**. Reagents and final concentration for FDA viability assay working solutions

|  |  |  |
| --- | --- | --- |
| **Component** | **Stock solution** | **Final concentration** |
| **Fluorescein diacetate (FDA) Solution** |
| FDA | 5 mg.mL-1 | 20 .mL-1l |

# **PROCEDURE**

### **Plant growth ● Timing 4–6 weeks**

1. Grow Arabidopsis thaliana ecotype Columbia plants in a 150 mL plastic cup with aerated, moist, fertilized and autoclaved soil in an environmentally controlled chamber with a medium photoperiod (12 h light/12 h dark at 22 °C) under low light (optimum light is approximately 150 µE.m-2.s-1) and 50-60% relative humidity. ▲**CRITICAL STEP** Pierce the base of the plastic cup to drain excess water and prevent root rot. ▲**CRITICAL STEP** Always maintain seedling production. Remember to transplant new plants every 2 weeks to ensure that the experiments can be repeated. Label each batch of plants properly. ▲**CRITICAL STEP** Due to laboratory logistics, we used a photoperiod of 12 h light/12 h dark, but the recommended photoperiod condition to optimize the production of the plant's vegetative area is 8 h light/16 h dark. **? TROUBLESHOOTING**

### **Protoplast isolation. ● Timing 4-6 hours**

1. Choose two to four healthy plants and take 20 leaves (3-4 cm long) by cutting each petiole vertically with the help of a scalpel. **! CAUTION** Scalpel blade is sharp: use it with extreme caution. ▲**CRITICAL STEP** Leaf selection will directly impact protoplast production. Watch the development of the plant aerial part. ▲**CRITICAL STEP** Place the leaves immediately in a 100 mL beaker with 50 mL of sterile distilled water. This step is necessary to I) prevent the formation of air bubbles in the leaf, which could hamper the infiltration of the enzyme solution, and II) wash away any remnants of soil and dust from the leaves.

 (Optional) Sterilize the leaves by washing once in 70% ethanol, for 5 minutes in a 1% sodium hypochlorite solution, and five times in sterile dH2O. **! CAUTION** Sodium hypochlorite is hazardous: wear proper personal protective equipment (PPE).

1. Using a metal tweezer, transfer one leaf with the adaxial side facing upward to a glass cell culture dish (90 mm x 15 mm) containing 25 mL of the W5 solution. Using the scalpel, extract and discard the remaining petiole and make sequential cross-sections (1-2 mm thick) from the midrib to the leaf margin. It is not necessary to cut the leaves completely, only to make little “scratches” on the leaf surface. **! CAUTION** Scalpel blade is sharp, use it with extreme caution. ▲**CRITICAL STEP** Leaf cuts are made in W5 solution to prevent air bubble formation in the leaf, which could hamper the infiltration of the enzyme solution, thus influencing the achievement of a satisfactory number of viable protoplasts.
2. Using a metal tweezer, carefully pass the chopped leaf on the cell culture dish´s edge to remove excess W5 solution and transfer the leaf with the adaxial side down to another glass cell culture dish (60 mm x 15 mm) containing 5 mL of the enzyme solution. ▲**CRITICAL STEP** Repeat steps 3 and 4 one leaf at a time. ▲**CRITICAL STEP** Place the aliquot of stock enzyme solution on ice to thaw slowly before use.
3. Transfer the cell culture dish with the enzyme solution and chopped leaves into the vacuum chamber and vacuum infiltrate 3 times for approximately 5 seconds under 500 mm Hg pump pressure. **! CAUTION** With each vacuum repetition, release the vacuum very carefully. ▲**CRITICAL STEP** Vacuum is essential for enzyme solution leaf infiltration. Vacuum until air bubbles emerge from the leaves.
4. Cover the cell culture dish with aluminum foil and then incubate in the platform shaker with gentle swirling (up to 40 rpm) for 3 hours at room temperature. ▲**CRITICAL STEP** Digestion should be performed in the dark to prevent oxidation and to reduce the photosynthetic pathway.
5. After 3 hours of incubation, release protoplasts by swirling the cell culture dish for 1 minute or until the solution turns green. **? TROUBLESHOOTING**
6. Filter the digested sample through a 74-µm cell mesh and carefully transfer it into a 30 mL glass round bottom centrifugation tube. ▲**CRITICAL STEP** Place bottle of W5 on ice thirty minutes before use (steps 4, 10,12). **? TROUBLESHOOTING**
7. Wash the mesh with up to 10 mL of ice-cold W5 solution to remove all remaining protoplasts.
8. Centrifuge the sample at 100 x g for 2 minutes at 4 °C. ▲**CRITICAL STEP** Use a refrigerated centrifuge for all centrifugations. Turn on and program the centrifuge to refrigerate 30 minutes before use. ▲**CRITICAL STEP** Use a swing-bucket rotor. The swing-type rotor allows the pellet to be positioned exactly at the bottom of the tube, which facilitates the discard of the supernatant. Set up the refrigerated centrifuge to slow acceleration and deceleration. Follow these critical instructions for steps 13, 15, 25, and 32 as well..
9. Carefully remove supernatant, leaving enough solution to cover the protoplasts (green pellet). ▲**CRITICAL STEP** Use a 5000 µl pipette with a cut end tip to gently pipette out the supernatant. The pellet is very fragile. Also apply this method in steps 16 and 20.
10. Carefully resuspend the protoplasts in 20 mL of ice-cold W5 solution and gently rock the tube until the protoplasts are resuspended. ▲**CRITICAL STEP** Do not resuspend the pellet by pipetting to prevent rupturing of intact protoplasts. Just gently swirling the tube. Follow these critical instructions in steps 14 and 17.
11. Centrifuge 100 x g for 2 minutes at 4 °C. Remove the supernatant with a 5000 µl pipette, again leaving enough to cover the protoplasts.
12. Carefully resuspend protoplasts in 20 mL of ice-cold W5 solution and then incubate protoplasts on ice for 30 minutes. ▲**CRITICAL STEP** Use this time to prepare the 40% PEG solution, as this can take some time to fully dissolve.
13. Swirling the protoplast tube gently until the protoplast pellet is completely resuspended and centrifuge at 100 x g for 2 minutes at 4 °C.
14. Carefully remove the supernatant with a 5000 µl micropipette, again leaving enough to cover the protoplasts. ▲**CRITICAL STEP** Place bottle of MMg solution on ice 30 minutes before use.
15. Resuspend the protoplasts to bring the total volume in the 30 mL tube to 1 mL with ice-cold MMg solution. ▲**CRITICAL STEP** Use another tube with the same volume for comparison. Swirling gently to resuspend.
16. Count protoplasts and adjust the concentration to 4 x 105 protoplasts/mL with MMg solution (see steps 19-21).

 Counting the protoplasts:

1. In a 1.5 mL microcentrifuge tube, dilute 5 µl of the protoplast solution obtained in step 17 above into 1 mL of MMg solution, then pipet 2 µl of the diluted protoplasts on a microscope slide and count the total number of protoplasts in that 2 µl drop. ▲**CRITICAL STEP** Only count whole, circular protoplasts that have no cell wall remaining. Count from 5 different drops, one drop at a time to avoid inaccuracies and average them.
2. To dilute to 4 x 105 protoplasts/mL, apply the formula:

Final volume of MMg solution (mL)= (D\_(A)× V\_S×100)/(4×〖10〗^5)

where DA is the average number of protoplasts from the five 2 µl drops and VS is the exact volume of resuspended protoplasts in microliters. ▲**CRITICAL STEP** Measure the exact volume of resuspended protoplasts using a 5000 µl micropipette.

1. Add MMg solution to bring the original protoplast solution to the final volume calculated in the previous step.

### **Protoplast transformation. ● Timing 1-2 hours**

1. In a 15 mL glass round bottom centrifugation tube, mix by gently swirling the protoplast solution, DNA solution, and 40% PEG solution, according to **Table 5**. Add the components in this order. Increase the number of reactions as needed. Use one tube for each reaction. ▲**CRITICAL STEP** Do not vortex, pipette up-and-down or invert tubes to mix the solution. ▲**CRITICAL STEP** Do not produce bubbles while mixing, which can cause protoplasts to explode.

**Table 5** - Reaction mix for protoplast transformation

|  |  |  |
| --- | --- | --- |
| **Order** | **Component** | **Instructions** |
| 01 | 100 µL of protoplast solution (concentration 4 x 105 protoplasts/mL) | Swirl the tube with protoplast solution gently and thoroughly to make sure that no pellet is formed. Use a wide-bore pipette tip to prevent damage to the protoplasts. Do not make bubbles. |
| 02 | 10 µL of each plasmid DNA(concentration 1 µg x mL-1) | Use a filter pipette tip to prevent contamination. |
| 03 | 110 µL 40% PEG solution | The solution is very thick; pipette very carefully. Gently swirl the tube until the solution is well mixed and layers can no longer be seen in the solution. Do not introduce bubbles. |
| 320 µL final volume | The final volume will vary according to the number of plasmids used in the transformation (up to 5). |

1. Incubate the 15 mL tubes for 15 minutes at room temperature.
2. To stop the reaction, add 2 volumes of ice-cold W5 Solution. Gently swirl the tubes to mix.
3. Centrifuge at 100 x g for 2 minutes at 4 °C. ▲**CRITICAL STEP** The pellet is very delicate: handle the tubes carefully. ▲**CRITICAL STEP** Place bottle of W1 solution on ice 30 minutes before use.
4. The supernatant was carefully removed, leaving enough solution to cover the protoplast.
5. Resuspend the protoplast in 500 µL of ice-cold W1 Solution. Gently swirling the tube to mix. This volume corresponds to one single replica transformation reaction. ▲**CRITICAL STEP** Completely and carefully resuspend the pellet.
6. Transfer the protoplast solution to a 12-well cell culture plate. If you perform multiple transformation reactions, transfer each reaction to a single and labeled well of the plate. ▲**CRITICAL STEP** If you are working with multiple reactions and/or plates, make sure that you labeled the wells/plates correctly.
7. Place the lid on the plate and seal with Parafilm. Set the plate in a wet chamber and incubate for 24 h in the dark under gentle shaking (up to 40 rpm) at 25-30 °C. To make a dark wet chamber, put some paper towels in a plastic box/tray, wet with distilled water, place the plates and cover with aluminum foil. ▲**CRITICAL STEP** The wet chamber prevents the samples from trying to dry out. ▲**CRITICAL STEP** The plates should be maintained in the dark. ▲**CRITICAL STEP** The time of incubation is enough to observe the accumulation of GFP in these conditions. For other situations, different times of incubation should be tested.
8. After 24 h of incubation, put the sealed 12-well cell culture plate in an Axiovert 135 M fluorescence microscope under UV light with filter set 15 (Carl Zeiss). Excitation: BP 546; beam splitter: FT 580; emission: LP 590. Capture images of GFP emission with attached DS-Ri1 digital camera (Nikon).

### **Flow cytometry ● Timing 30 min by sample**

1. Transfer all of the well contents to a labeled 1.5 mL microcentrifuge tube. ▲**CRITICAL STEP** Use a wide-bore 1000 µL pipette tip to prevent damage to the protoplasts.
2. Centrifuge at 100 x g for 1 minute at 4 °C in a swing-bucket rotor for plates with 1.5-2 mL microcentrifuge tubes block adapter. ▲**CRITICAL STEP** Do not use a microcentrifuge: the pellet will stay in the well of the microtube and prejudice the quality of the readings.
3. Carefully remove the supernatant, leaving approximately 50 µL of the solution.
4. Flick the tube gently to thoroughly resuspend the pellet. ▲**CRITICAL STEP** Carefully resuspend the pellet thoroughly. Prevent bubbles from forming in the bottom of the tube.
5. Analyze the presence and intensity of GFP fluorescence in the Amnis® brand FlowSight® Imaging Flow Cytometer (see Equipment Setup).
6. Repeat steps 31-35 for each well/sample.

# **Troubleshooting**

**TABLE 6.** Troubleshooting for the Plant system stages

|  |  |  |  |
| --- | --- | --- | --- |
| **Step** | **Problem** | **Possible reason** | **Solution** |
| 1 | Early appearance of inflorescences | A long photoperiod | Keep a photoperiod of up to 12 hours of light  |
| 1 | Fungus-contaminated leaves | Fungi introduction via contaminated vessels, air, soil or water.Excess moisture. | Plants should only be irrigated with clean distilled water.Control air humidity and avoid damp soil.Readily dispose of symptomatic plants.For persistent events, apply fungicidal agents in soil. |
| 1 | Small, distorted, yellowish leaves | Nutritional deficiency | Use a good source of soil.If necessary, fertilizer should be enriched with macro- and micronutrients.Add 1X MS medium solution for the cultivation of Arabidopsis. |
| 1 |  Purplish leaves | Excessive light exposure. | Adjust for proper photoperiod and light source intensity. |
| 1 | Presence of fungi and algae in soil and/or trays | Excess of water or contaminated soil. | Water only when necessary to control humidity in the pots (once every two days must be sufficient).Autoclave soil mixture before planting |
| 1 | Presence of soil flies/ dark flies (Sciaridae) | Accumulation of organic matter | Remove excess water from the pots, and eliminate algae, slime and fungi that grow on the soil.Install yellow adhesive traps just above plants (replace weekly). |
| 1 | Presence of white flies or aphids | Accumulation of organic matter | Apply a 0.03% solution of DECIS 25E. to prevent and treat !CAUTION Hazardous. Use PEPInstall yellow adhesive traps just above plants (replace weekly). |
| 7 | Leaves do not digest | Slow infiltration of enzymatic cocktail in plant tissue. | Incubate for one additional hour |
| 7 | Leaves still not digested | Old enzymatic solution.Old or sick plants. | Discard the material and:Prepare a new batch of enzymatic solution.Check age and overall health of plants. |
| 8 | Solution retained in the mesh | Accumulation of leaf debris in the mesh | Use a 1 mL micropipette with a cut tip to press out any remaining enzyme solution from the sample.If necessary, pipette up to 2 mL of cold W5 solution to wash the mesh. |

## MOLECULAR ANALYSES **● Timing 6d**

### **Primer design ● Timing 1d**

1. Select approximately 20 nucleotides both upstream and downstream of the core region of each att site formed in the reporter plasmid after recombination takes place. ▲**CRITICAL STEP** forward primer must anneal to **attL**, while the reverse primer will anneal to the **attR** sequence.
2. Use an online oligo design tool to define the best forward primers annealing to promoter sequence and reverse primers annealing to terminator sequence present in the reporter plasmid.
3. Define oligo pairs to obtain two amplicons for each reporter plasmid. ▲**CRITICAL STEP** Primer pairs must consist of a forward oligo annealing to the attL site and a reverse oligo annealing to the terminator region for sequencing proper attR site formation in **amplicon I** and a forward oligo annealing to the promoter region and a reverse oligo annealing to the attR site for sequencing proper attL site formation in **amplicon II**. (**Figure 5**). The primers used in our studies are presented in **Table 7.

TABLE 7.** Oligonucleotides pair used in our studies. Highlighted in red are the core sequences of each respective att site.

|  |
| --- |
| **Oligonucleotides used for amplification of Amplicon I and sequencing of attL sites** |
| **Promoter** | **Forward primer (5’ -> 3’)** | **nt** | **Model** |
| EFa\_966F | TTCTCGAGCTTTTGGAGTACGTCGTCTTTAGGTTG | 35 | Mammal |
| 35S\_282F | ATTGATGTGATATCTCCACTGACGTAAGGGATGACGCAC | 39 | Plant |
| **attR** | **Reverse primer (5’-> 3’)** | **nt** | **Model** |
| attR \_Int2\_R | GTGTCTACGCGAGATTCTCGCCGGACCGTCGACATACTGC | 40 | All models used |
| attR \_Int4\_R | AGTTTTCAACCCTTGATTTGAATAAGACTGCTGCTTGTGT | 40 |
| attR \_Int5\_R | ATAACTCTCCTGGGAGCGCTACACGCTGTGGCTG | 34 |
| attR \_Int7\_R | CTGTGTGAGAGTTAAGTTTACATGGGCAAAGTTGATGAC | 39 |
| attR \_Int9\_R | TGGAAGTGTGTATCAGGTAACTGGATACCTCATC | 34 |
| attR \_Int13\_R | GTAGAACTTGACCAGTTGGTCCTGTAAATATAAGCAATCC | 40 |
| attR \_phiC\_R | CCAACTGGGGTAACCTTTGGGCTCC | 25 |
| attR \_Bxb1\_R | CTGGTCAACCACCGCGGTCTCCGTCGTCAGGATC | 34 |
| **Oligonucleotides used for amplification of Amplicon II and sequencing of attR sites** |
| **attL** | **Forward primer (5’-> 3’)** | **nt** | **Model** |
| attL\_Int2\_F | GGAGTAGCTCTTCGCCCGAGAACTTCTGCAAG | 32 | All models used |
| attL\_Int4\_F | CGACCTGAAATTTGAATTAGCGGTCAAATAATTTGTA | 37 |
| attL\_Int5\_F | GACGGCCTGGGAGCGTTGACAACTTGCGCACC | 32 |
| attL\_Int7\_F | GTCCGTCTGGGTCAGTTGCCTAACCTTAACTTTTAC | 36 |
| attL\_Int9\_F | ATAATTGGCGAACGAGGTATCTGCATAGTTATTCCGAAC | 39 |
| attL\_Int13\_F | TCCAGATCCAGTTGTTTTAGTAACATAAATACA | 33 |
| attL\_phiC\_F | TGCCAGGGCGTGCCCTTGAGTTCTCTCAGT | 30 |
| attL\_Bxb1\_F | TGTCGACGACGGCGGTCTCAGTGGTGTACGGT | 32 |
| **Terminator** | **Reverse primer (5’ -> 3’)** | **nt** | **Model** |
| TermiAni\_205R | AATGATTTGCCCTCCCATATGTCCTTCCGAGTG | 33 | Mammal |
| NOSt\_283R | ATAACAATTTCACACAGGAAACAGCTATGACATGATTACG | 40 | Plant |

###

### **Target sequence amplification by PCR ● Timing 5h**

1. Use a high-fidelity polymerase with non-template–dependent terminal transferase activity to insert a deoxyadenosine and the ends of generated amplicons. ▲**CRITICAL STEP** Amplicon modification is important for cloning into pGEM-t-Easy to be sequenced.
2. Prepare a PCR mix for all reactions plus one (n+1) to account for pipetting errors. Include a negative control with water instead of DNA; positive control will require a previous synthesis of the expected recombined reporter plasmid.
3. Combine the reagents in the order shown below in **Table 8**, mix well by vortexing and spin briefly:

**TABLE 8.** PCR reaction mix components

|  |  |
| --- | --- |
| **Component** | **Volume to add (µl)** |
| **dH2O nuclease free** | **18.65** |
| **Buffer 10x** | **2.5** |
| **MgCl2 [50 mM]** | **1.5** |
| **PCR Fw primer [10 μM]** | **0.75** |
| **PCR Rev primer [10 μM]** | **0.75** |
| **dNTP [10 mM]** | **0.75** |
| **Taq DNA polymerase** | **0.1** |

1. Add 24.5 μL of the PCR mix to 0.2 mL PCR tubes.
2. To each respective tube, add 20 ng of template DNA and adjust the final volume to 25 µl if the DNA is too concentrated. Negative controls were prepared first by adding an equivalent volume of nuclease-free water and closing lids before pipetting templates to minimize contamination risk.
3. Gently pipette each sample up and down ten times to mix thoroughly. Place the PCR microtubes into a thermal cycler, and run the following program listed in **Table 9** (volume = 25 μL)

**TABLE 9.** PCR cycling condition

|  |  |  |  |
| --- | --- | --- | --- |
| Cycle no. | Denature | Anneal | Extend |
| 1 | 94°C, 3min |   |   |
| 2-34 | 94°C, 30s | 65°C, 30s | 72°C, 60s |
| 35 |   |   | 72°C, 5min |

▲**CRITICAL STEP** Given the need for primers to align to a defined att site sequence, some parameter adjustments, such as Tm, GC content and 3’ end base composition, will be limited and can vary from one integrase reporter to another, requiring adjustments to PCR cycling conditions.

1. Resolve amplicons by electrophoresis in agarose gel following PCR. Run settings and gel density will depend on amplicon size according to the analyzed gene length and oligo pairs used. ▲**CRITICAL STEP** Load the same negative control in the every gel both technical (PCR without DNA) and biological (PCR using DNA from groups transformed with only either reporter plasmid or integrase plasmid) to ensure obtained bands indicate DNA inversion by Integrase activity. **? TROUBLESHOOTING**

### **Amplicon sequencing ● Timing 5d**

**Amplicon purification ● Timing 2d**

1. Excise the amplicon bands by cutting a square around them with the help of a scalpel on a UV light or blue light transilluminator. ▲**CRITICAL STEP** Use different scalpel blades for each band to avoid cross-contamination of samples. **! CAUTION** UV light can damage DNA, nicking and possibly removing DNA strand ends and interfering with downstream cloning steps. When available, blue light is highly recommended. If using UV, proceed quickly, turning the transilluminator off after making the cuts in the gel.
2. Proceed with amplicon purification using commercial DNA Clean-Up and Concentration kits, following the manufacturer’s recommendations.
3. Clone purified amplicons in an entry vector to ensure high-quality sequencing results. Although specifics may vary depending on the plasmid, we recommend a molar ratio of 1:3 (vector to amplicon) and 1.5 U of T4 ligase in 5 µl reactions with overnight incubation at 16 °C.
4. DH10b chemically competent cells were transformed with ligation products.

**Heatshock transformation of DH10b chemically competent cell ● Timing 3d**
5. Add 5 µl of the ligation reaction to 200 µl of cells thawed on ice.
6. Incubate cells on ice for 30 min
7. Subject cells to heat shock at 42 °C for 45 sec and return to ice for 2 min
8. Add 1 mL of LB or SOC medium
9. Incubate at 37 °C for 1 h and then plate different dilutions on LB plates with appropriate selecting agents. Incubate overnight at 37 °C.
10. Screening for positive transformants by colony PCR. A polymerase with less fidelity can be used in this step. Combine the reagents in the order listed in **Table 10** below, mix well by vortexing and spin briefly:

**TABLE 10.** PCR reaction mix components for colony screening.

|  |  |
| --- | --- |
| **Component** | **Volume to add (µl)** |
| **dH2O nuclease free** | **18.65** |
| **Buffer 10x** | **2.5** |
| **MgCl2 [50 mM]** | **1.5** |
| **PCR Fw primer [10 μM]** | **0.75** |
| **PCR Rev primer [10 μM]** | **0.75** |
| **dNTP [10 mM]** | **0.75** |
| **Taq DNA polymerase** | **0.1** |

1. Add 25 μL of the PCR mix to 0.2 mL PCR tubes.
2. With a sterile toothpick or 200 µl pipetting tip, pick approximately 1/3 of each colony and add it to their respective tubes containing the PCR mix.
3. Gently pipette each sample up and down ten times to mix thoroughly. Place the PCR microtubes into a thermal cycler, and run the following program listed in **Table 11** (volume = 25 μL)

**TABLE 11.** PCR cycling condition for colony screening

|  |  |  |  |
| --- | --- | --- | --- |
| **Cycle no.** | **Denature** | **Anneal** | **Extend** |
| **1** | **94°C, 10min** |  |  |
| **2-34** | **94°C, 30s** | **60°C, 30s** | **72°C, 90s** |
| **35** |  |  | **72°C, 5min** |

1. Resolve amplicons by electrophoresis in agarose gel following PCR.
2. Select multiple confirmed clones to isolate plasmids using commercial kits following the manufacturer’s recommendations and have the purified plasmids sequenced. ▲**CRITICAL STEP** Have your samples sequenced in both directions and in replicates to check for sequencing errors and identify possible mutations and DNA damage resulting from integrase activity.
3. Analyze sequencing electropherograms and alignment to expected sequences to confirm proper DNA recombination by Integrase activity.

# **Troubleshooting**

**TABLE 12.** Troubleshooting for the molecular analyses stage

|  |  |  |  |
| --- | --- | --- | --- |
| **Step** | **Problem** | **Possible reason** | **Solution** |
| 10 | Unspecific amplification and unexpected bands on agarose gel | Oligos annealing at att sites has a 3’ end complementarity to both original and recombined att sites | Increase the annealing temperature to more selective conditions |