

# Generation of Insulin-Producing Alpha TC1-6 Cells Using EpiCRISPR System for Targeted DNA Methylation

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## Abstract

Diabetes lacks concrete curative strategies due to diverse aetiologies and, therefore, represents the perfect candidate for cell replacement therapy, since it is caused by either an absolute (type 1 diabetes) or relative (type 2 diabetes) defect in the insulin-producing beta cells of the pancreas. Pancreatic alpha cells are a promising source for transdifferentiation into insulin-producing cells as they share a common developmental origin with beta cells and exhibit a certain degree of cellular plasticity. Furthermore, impairment of glucagon signaling in diabetes leads to a marked increase in alpha cell mass, raising the possibility that such alpha cell hyperplasia provides an increased supply of alpha cells for their transdifferentiation into new beta cells.

In this protocol, we used the modular epigenetic CRISPR/dCas9 toolbox for targeted DNA methylation (EpiCRISPR) and silencing of the *Arx* gene (Aristaless Related Homeobox, *Arx*), which is essential for the maintenance of alpha cell identity. Methylation-based silencing of *Arx* initiates the reprogramming of pancreatic alpha cells into insulin-producing cells. As a key novelty, this protocol provides a direct route for epigenetically induced transdifferentiation of mouse pancreatic alpha TC1-6 cells into insulin-producing cells and thereby confirms a proof of concept of reversible cellular epigenetic reprogramming in vitro. In addition, this streamlined workflow addresses the inherent challenges of transfecting clustered alpha TC1-6 cells by optimizing their dissociation into single-cell suspensions, thereby improving uptake and reproducibility. In summary, this approach for cell transdifferentiation involves precise epigenetic editing of a lineage-specific marker gene, thereby enabling direct lineage conversion in a safe and versatile strategy to generate insulin-producing cells by epigenetic reprogramming. In contrast to approaches that rely on viral vectors or permanent genome editing, this method reduces the risk of off-target effects and immunogenic responses while ensuring reproducibility. The combination of efficiency and precision makes it a valuable tool to advance regenerative approaches for diabetes therapy and to explore the epigenetic regulation of cell identity.

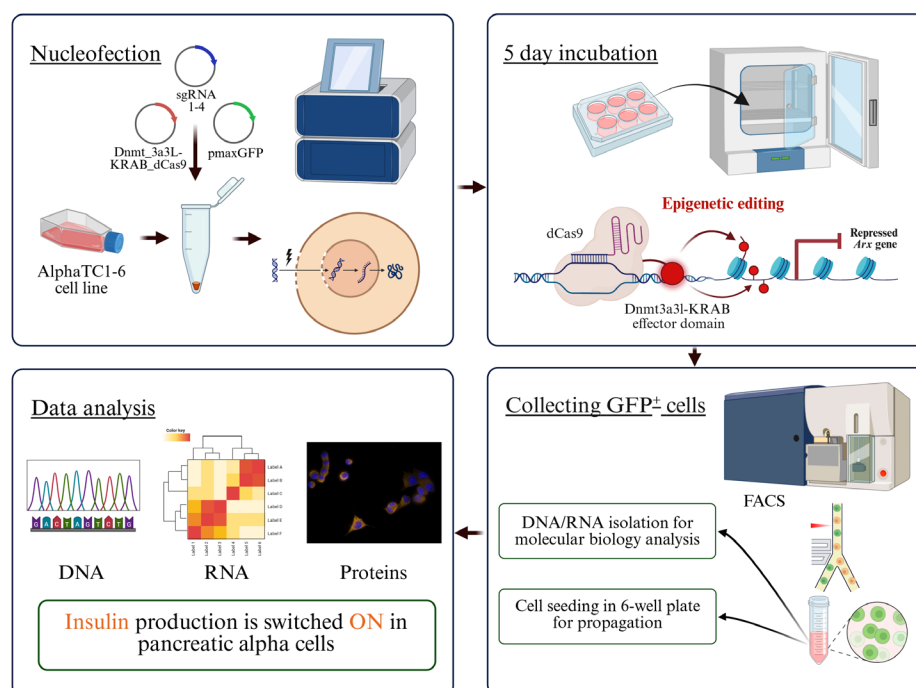
## Key features

- This protocol describes a reprogramming of alpha TC1-6 cells into insulin-producing cells by inducing transdifferentiation through targeted epigenetic modulation of a single gene.
- Targeted repression of *Arx* reduces off-target effects while maintaining genomic integrity.
- This protocol requires at least five days to complete and includes cell preparation, nucleofection, and cell sorting.
- This approach avoids agents like viral vectors or nonspecific demethylating compounds that could limit its potential for therapeutic translation.

**Keywords:** Alpha cells, Nucleofection, *Arx* gene, EpiCRISPR, Epigenetic editing, DNA Methylation

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## Graphical overview



**Workflow for the generation of insulin-producing pancreatic alpha TC1-6 cells using the EpiCRISPR system**

## Background

Cell transdifferentiation, which refers to the direct conversion of one mature cell type into another without passing through a pluripotent state, has emerged as a powerful strategy in regenerative biology [1]. Epigenetic modifications, such as DNA methylation, histone modifications, higher-order chromatin structures, or noncoding RNAs, play a regulatory role in gene expression, determining the developmental trajectories and the maintenance of cell identity and function [2].

Platforms for epigenetic editing, such as CRISPR/dCas9, which utilize catalytically inactive dCas9 fused to epigenetic effectors (e.g., DNMT3A, TET1, or KRAB domains), possess wide applicability across diverse biological systems and pathological conditions [3]. The EpiCRISPR toolbox that we used utilizes an engineered dCas9 that can specifically recognize a unique sequence at a target site, coupled with an effector domain capable of altering the methylation status of that site (DNA methyltransferases) and also repressing it due to the presence of the KRAB repressor (Figure 1C) [4]. Retargeting of the dCas9 effector complex to another locus is achieved by introducing a new single-guide RNA (sgRNA) sequence that is complementary to the desired target site. Regenerative medicine, which focuses on the repair and restoration of tissues and organs damaged by disease or injury, could significantly benefit from rapid progress in epigenetic reprogramming technologies. The initial steps for manipulating gene expression levels include target gene(s) identification, effector domain selection, and sgRNA design. The precision and efficiency of targeted reprogramming approaches, particularly those based on single-gene induced transdifferentiation, make them especially attractive for therapeutic applications, e.g., in monogenic disorders, reactivation of tumor suppressor genes, or oncogene silencing [5].

The strategy to use pancreatic alpha cells from the same organ (pancreas) for transdifferentiation was based on the fact that alpha-to-beta cell transdifferentiation can lead to restoration of beta cell mass while simultaneously reducing alpha cell mass, thus restoring the balance between pancreatic hormones (insulin and glucagon), which is perturbed in diabetes (bihormonal hypothesis of diabetes) [6]. Increasing the number of insulin-producing beta cells while decreasing the number of glucagon-

producing alpha cells turns out to be a promising therapeutic avenue. Furthermore, impairment of glucagon signaling in diabetes leads to a marked increase in alpha cell mass, raising the possibility that such alpha cell hyperplasia provides an increased supply of alpha cells for their transdifferentiation into new beta cells [7]. In addition, the advantages of using pancreatic alpha cells for transdifferentiation into insulin-producing cells with this particular method for gene repression are numerous. Targeted epigenetic silencing of the lineage-specific marker gene for pancreatic alpha cells, *Arx*, combines cellular plasticity with precision control in targeting, offering a promising strategy to restore insulin production from the patient's own pancreas (endogenously). Since other pancreatic endocrine and exocrine cell types already display silenced *Arx* expression through promoter methylation [8], this approach minimizes off-target effects and ensures specificity to alpha cells, which are resilient, numerous, and remain intact in most diabetic patients. The strength of the protocol described here lies in its distinction from previous approaches to alpha cell transdifferentiation, such as direct *Arx* knockout [9], which causes a permanent DNA alteration, or drug-induced *Arx* inhibition, which represents a non-invasive and scalable, but less specific, alternative, potentially affecting off-target GABA signaling pathways [10]. Directed epigenome editing avoids double-stranded DNA breaks and offers reversible, adjustable effects with a profile that is safer than permanent gene knockouts.

Despite the promise of reprogramming pancreatic alpha cells into insulin-producing beta cells, some challenges remain. Achieving high reprogramming efficiency without compromising cell viability still remains an important limitation. A major impediment of this approach is the low reprogramming efficiency, emphasizing the tremendous need for further optimization of EpiCRISPR systems to achieve robust and durable *Arx* promoter methylation and sustained insulin expression (Figure 2) [4]. In addition, the immunogenicity of reprogrammed alpha cells remains undefined. It is yet to be determined whether these cells are susceptible to autoimmune attack in the context of type 1 diabetes or if parallel immunomodulatory strategies are required. Besides, this approach is confronted by in vivo delivery challenges, as achieving specific and efficient targeting of pancreatic alpha cells is critical for experimental reproducibility and remains technically demanding. Therefore, the development of advanced delivery platforms, such as cell type-selective lipid nanoparticles, will be essential to ensure targeted, long-term, and non-toxic expression of epigenetic editing components [11]. Overcoming these challenges is crucial to developing safe and effective epigenome-based reprogramming strategies for diabetes therapy.

## Materials and reagents

### Biological materials

1. Alpha TC1 clone 6 cell line,  $\alpha$ TC1-6 (American Type Culture Collection, Manassas, VA, USA, CRL-2934; origin: pancreas of a mouse with adenoma)
2. TOP10 *Escherichia coli* cells [Thermo Fisher Scientific, Invitrogen, C404003; origin: *E. coli* (K12)]

### Reagents

1. Fetal bovine serum (FBS) (Sigma-Aldrich, catalog number: F9665) (-20 °C)
2. Bovine serum albumin (BSA) (SERVA Electrophoresis GmbH, catalog number: 11930.03) (4 °C)
3. DMEM, high glucose, GlutaMAX™ Supplement, HEPES (Gibco, Thermo Fisher Scientific, catalog number: 32430027) (4 °C)
4. DMEM, low glucose (Sigma-Aldrich, catalog number: D6046) (4 °C)
5. Cell dissociation buffer, enzyme-free, PBS (Gibco, Thermo Fisher Scientific, catalog number: 13151014) (4 °C)
6. RPMI 1640 medium (Gibco, Thermo Fisher Scientific, catalog number: 52400025) (4 °C)
7. Penicillin/streptomycin solution (Sigma-Aldrich, catalog number: P4333) (-20 °C)
8. Kanamycin sulfate from *Streptomyces kanamyceticus* (Sigma-Aldrich, catalog number: K1377-1G) (4 °C)
9. Trypan blue solution, 0.4% (Gibco, Thermo Fisher Scientific, catalog number: 15250-061) [room temperature (RT)]
10. SF Cell Line 4D-Nucleofector X kit L (Lonza Solution and Supplement, catalog number: V4XC-2024); solution box (catalog number: PBC2-02250), supplement 1 (catalog number: S-08983), pmaxGFP 1.0 (lot. no. D-00068) (4 °C)
11. Plasmid Plus Midi kit (25) (Qiagen, catalog number: 12943); components: buffer P1(4 °C), buffer P2 (RT), buffer P3 (RT), buffer BB (RT), buffer ETR (RT), and buffer EB (RT)
12. Ethanol 100% (ZORKA Pharma, catalog number: 56/238) (RT)
13. HBSS w/o calcium, w/o magnesium, w/o phenol red (Biowest, catalog number: L0605) (4 °C)
14. Dimethyl sulfoxide (DMSO) (Santa Cruz Biotechnology, catalog number: sc-358801) (RT)

15. Tryptone (Difco, catalog number: 0123-01) (RT)
16. Yeast extract (Difco, catalog number: 0127-01) (RT)
17. KCl (Alkaloid Skopje, catalog number: 06743) (RT)
18.  $\text{NaH}_2\text{PO}_4 \times 12 \text{ H}_2\text{O}$  (Merck, catalog number: 6579) (RT)
19.  $\text{KH}_2\text{PO}_4$  (Moss Hemos, catalog number: 369K2) (RT)
20. NaCl (ZORKA Pharma, catalog number: 7647-14-5) (RT)
21. T4 DNA ligase (Thermo Fisher Scientific, catalog number: EL0011) (-20 °C)
22. 10× T4 ligase buffer (Thermo Fisher Scientific, catalog number: B69) (-20 °C)
23. BbsI enzyme (New England Biolabs, U.S.A, catalog number: R0539S) (-20 °C)

## Solutions

1. DMEM high glucose full medium (see Recipes)
2. DMEM low glucose full medium (see Recipes)
3. PBS (see Recipes)
4.  $\alpha$  final medium (see Recipes)
5. Buffer for cell transfection (see Recipes)
6. Buffer for cell sorting (see Recipes)
7. Lysogeny broth (see Recipes)

## Recipes

### 1. DMEM high glucose full medium

Reagent	Final concentration	Volume
DMEM high glucose	89%	445 mL
Penicillin/streptomycin	1×	5 mL
FBS	10%	50 mL
Total	100%	500 mL

- a. Perform all the steps in sterile conditions.
- b. Pour out the 55 mL of clean medium from the 500 mL bottle; then, add FBS and penicillin/streptomycin solution to the bottle and mix by inverting several times.
- c. Store at 4 °C until use.

### 2. DMEM low glucose full medium

Reagent	Final concentration	Volume
DMEM low glucose	89%	445 mL
Penicillin/streptomycin	1×	5 mL
FBS	10%	50 mL
Total	100%	500 mL

- a. Perform all the steps in sterile conditions.
- b. Pour out the 55 mL of clean medium from the bottle; then, add FBS and penicillin/streptomycin solution and mix by inverting several times.
- c. Store at 4 °C until use.

### 3. PBS (1×, pH 7.4)

Reagent	Final concentration	Quantity or volume
NaCl	137 mM	8 g
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	10 mM	3.6 g
KCl	2.7 mM	0.2 g
$\text{KH}_2\text{PO}_4$	1.8 mM	0.24 g
$\text{H}_2\text{O}$		1 L
Total	1×	1 L

- a. Prepare 800 mL of distilled water in a 1 L glass cup.
- b. Add 8 g of NaCl, 0.2 g of KCl, 3.6 g of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , and 0.24 g of  $\text{KH}_2\text{PO}_4$  to the solution.

- c. Adjust the solution to pH  $\approx$  7.4.
- d. Add distilled water to a final volume of 1 L.
- e. After autoclaving, store the buffer at 4 °C (maximum 12 months).
- f. Warm to 37 °C before use.

#### 4. $\alpha$ final medium

Reagent	Volume
DMEM high glucose full medium	24.5 mL
DMEM low glucose full medium	24.5 mL
BSA (1% in PBS)	1 mL
Total	50 mL

- a. Dissolve 0.5 g of BSA in 50 mL of PBS (1% BSA) and then filter the solution through a syringe filter (pore size 0.2  $\mu$ m).
- b. Mix DMEM high and low glucose full media and 1% BSA solution in a 50 mL conical tube by inverting several times.
- c. Store at 4 °C until use.
- d. Warm to 37 °C before use.

#### 5. Buffer for cell transfection

Reagent	Final concentration	Quantity or volume
SF Nucleofector™ X solution	77.52%	82 $\mu$ L
Supplement 1	17.02%	18 $\mu$ L
	pMax eGFP	0.47%
	dCas9-Dnmt3a3L-KRAB	0.99%
Plasmids	1	0.99%
	2	0.99%
	3	0.99%
	4	0.99%
Arx sgRNA		3.99%
		1.406 $\mu$ g
		1.406 $\mu$ g
		1.406 $\mu$ g
		1.406 $\mu$ g
		5.625 $\mu$ g
Total	n/a	100 $\mu$ L

- a. First, warm the SF solution and supplement to RT.
- b. Mix all the components of the buffer for cell transfection freshly prior to use.
- c. The volume of added plasmids should not exceed 10  $\mu$ L.

#### 6. Buffer for cell sorting

Reagent	Final concentration	Quantity or volume
HBSS	97.6%	48.8 mL
EDTA	2 mM	0.2 mL (0.5 M)
FBS	2%	1 mL
Total	n/a	50 mL

- a. Mix the HBSS and EDTA solution with FBS freshly prior to use.
- b. Warm to 37 °C before use.
- c. Store the HBSS at 4 °C until use.

#### 7. Lysogeny broth (LB)

Reagent	Final concentration	Quantity or volume
Tryptone	1%	10 g
Yeast extract	0.5%	5 g
NaCl	1%	10 g
Distilled water		1 L

- a. Dissolve all the components in 0.8 L of water on a mixer.
- b. After component dissolving, adjust the pH to 7.2 and add water up to 1 L.
- c. Sterilize by autoclaving and store at 4 °C for up to 6 months.

## Laboratory supplies

1. Cell culture flasks, 25 cm<sup>2</sup> (T-25 adherent flask) (Thermo Fisher Scientific, catalog number: 130189)
2. 6-well sterile culture plates (Thermo Fisher Scientific, catalog number: 130184)
3. 10 mL conical tubes (Sarstedt, catalog number: 62.9924.284)
4. 50 mL conical tube (Sarstedt, catalog number: 62.547.254)
5. Cell counting chamber slide, Neubauer Pattern (Blaubrand, catalog number: BR718605-1EA)
6. Sterile syringes 50 mL (Weigao, catalog number: SP503PLS)
7. Syringe filter, pore size: 0.2 µm (Sarstedt, catalog number: 83.1826.102)
8. Reaction tube, 1.5 mL, PP (Sarstedt, catalog number: 72.690.001)
9. Reaction tube, 2 mL, PP (Sarstedt, catalog number: 72.691)
10. 10 µL pipette tips (Eppendorf, catalog number: 0030 000.811)
11. 200 µL pipette tips (Sarstedt, catalog number: 70.3030)
12. 1,000 µL pipette tips (Sarstedt, catalog number: 70.3050)
13. FACS tube, 3.5 mL, L×Ø: 55 × 12 mm, PS (Sarstedt, catalog number: 55.484)
14. Glass cup, 1 L (LLG, Pirex catalog number: 9014005\*LLG)

## Equipment

1. Cell culture incubator (37 °C, 5% CO<sub>2</sub>) (Thermo Fisher Scientific, BB15, catalog number: 51023121)
2. Water bath (Thermo Fisher Scientific, Precision GP 20, catalog number: TSGP20)
3. Light microscope (Nikon, model: ECLIPSE Ts2)
4. Axio Observer Z1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany)
5. High-speed centrifuge (Eppendorf, model: 5804)
6. Swing bucket rotor (Eppendorf, model: A-4-44)
7. Mini centrifuge with standard rotor (Carl Roth, model: SD 220 VC)
8. Amaxa<sup>TM</sup> 4D-Nucleofector<sup>TM</sup> Core Unit (Lonza, 100-240, catalog number: 817B1102)
9. Amaxa<sup>TM</sup> 4D-Nucleofector<sup>TM</sup> X Unit (Lonza, catalog number: 817X0291)
10. Flow cytometer and cell sorter (FACS) (BD Biosciences, San Diego, USA, BD FACSAria III)
11. Vortex mixer (Neuhaus I Swix, model: TA1707AA0301)
12. Vertical laminar flow bench (Technology. Science. Life, Telstar, model: AV-100)
13. Ultracentrifuge (Beckman Coulter, model: Optima L-100 KSP)
14. Titanium rotor (Beckman Coulter, Type 70 Ti Fixed-Angle, catalog number: 06E 2591)
15. Incubated/refrigerated stackable shaker [Thermo Scientific MaxQ, model: SHKE6000-1CE (4352)]
16. NanoPhotometer MicroVolume spectrophotometer (Implen, Germany, catalog number: N60 UV/Vis)
17. Thermomixer (Eppendorf, Thermomixer comfort with 2 mL Thermoblock, catalog number: 5355)
18. Laboratory vacuum pump (Sartorius, Microstart Mini-Vac, catalog number: 025250)
19. Refrigerator (2–8 °C)
20. Freezer (-20 °C)
21. Deep freezer (-80 °C)

## Software and datasets

1. FACS Diva software v6.1.3, 2011
2. BioRender (<https://biorender.com>)
3. SnapGene Viewer Version 7.0.1
4. GraphPad's Prism8.0.1 (263) software for statistical analyses

## Procedure

### A. Preparation of sgRNA expression vectors

See General note 1.

#### 1. Ligation cloning

##### a. Preparation of sgRNA oligos

- i. Design and order complementary oligonucleotides encoding the desired sgRNA sequence.
- ii. Resuspend oligos in nuclease-free water to a concentration of 100  $\mu$ M.

##### b. Oligo annealing

- i. Mix 1  $\mu$ L each of forward and reverse sgRNA oligos (100  $\mu$ M), 1  $\mu$ L of 10 $\times$  T4 ligase buffer, and 7  $\mu$ L of water.
- ii. Heat to 95  $^{\circ}$ C for 5 min and allow to cool slowly to RT ( $\sim$ 30 min) to anneal the oligos.

##### c. Vector preparation

- i. Digest 2  $\mu$ g of sgRNA plasmid (with dual BbsI sites) using BbsI at 37  $^{\circ}$ C for 1 h.
- ii. Purify the digested vector using a gel extraction cleanup kit.

##### d. Ligation reaction

- i. Combine 50 ng of BbsI-digested plasmid, annealed oligos (3:1 molar ratio), 1  $\mu$ L of 10 $\times$  T4 ligase buffer, 1  $\mu$ L of T4 DNA ligase, and water to 10  $\mu$ L.
- ii. Incubate at 16  $^{\circ}$ C overnight.

#### 2. Heat shock transformation of bacteria

- a. Add 10 ng of ligation reaction to competent *E. coli* TOP10 cells (50  $\mu$ L) that have just begun to thaw and mix by tapping gently.
- b. Incubate the tubes with cells on ice (4  $^{\circ}$ C) for 30 min.
- c. Transfer the tubes to 42  $^{\circ}$ C for 45 s.
- d. Pour 900  $\mu$ L of prewarmed LB medium on the cells and incubate for 1 h at 37  $^{\circ}$ C in a shaking incubator (250 rpm).
- e. Centrifuge the tubes for 3 min at 0,8 $\times$  g (RT).
- f. Discard half of the supernatant (SN). Resuspend the cells in the rest of the SN and sow on a prepared solid medium with kanamycin as a selection antibiotic.
- g. Incubate the plates at 37  $^{\circ}$ C overnight.
- h. Store plates with bacteria at 4  $^{\circ}$ C for 1–2 weeks.

#### 3. Plasmid isolation

- a. Transfer a single bacterial colony from a solid substrate to a liquid LB medium with a selective antibiotic and incubate overnight (12–16 h) at 37  $^{\circ}$ C on a shaker.
- b. Centrifuge the overnight culture at 7,000 $\times$  g for 15 min at 4  $^{\circ}$ C.
- c. Resuspend the bacterial pellets in 4 mL of buffer P1.
- d. Add 4 mL of P2 lysis buffer to the cell suspensions. Mix by gently inverting the 50 mL tube until the lysate becomes viscous.
- e. Incubate the lysates for 3 min at RT.
- f. Add 4 mL of P3 neutralization buffer to the lysate and mix immediately by vigorously inverting the tube 4–6 times.
- g. Transfer the lysate to the QIAfilter and incubate for 10 min at RT.
- h. Filter the lysate through the QIAfilter, add 2 mL of BB buffer to the filtrate, and mix by inverting 4–6 times.
- i. Transfer the lysate to QIAGEN Plasmid Plus Midi spin columns and apply a vacuum of  $\sim$ 300 mbar until all the liquid has passed through the column.
- j. Wash the column 2 $\times$  with 700  $\mu$ L of ETR buffer each time using a vacuum.
- k. Centrifuge the columns at 10,000 $\times$  g for 1 min to eliminate residual wash buffer.
- l. Transfer the columns to 1.5 mL tubes.
- m. Add 200  $\mu$ L of EB buffer and incubate for  $\geq$ 1 min at room temperature.
- n. Centrifuge at 10,000 $\times$  g for 1 min to obtain dissolved plasmid DNA in the eluate.



## B. Cultivation and propagation of $\alpha$ TC1-6 cells

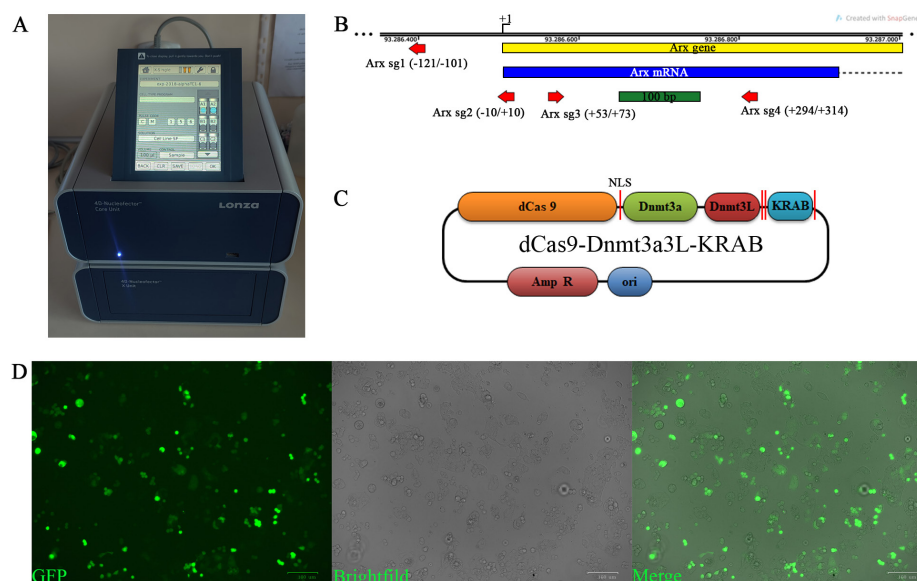
*Note: Warm all solutions to 37 °C prior to use.*

1. Incubate a mouse pancreatic  $\alpha$ TC1 clone 6 cell line at 37 °C in humidified air containing 5% CO<sub>2</sub> in  $\alpha$  final medium, which contains 15.25 mM glucose. Change the medium every second day, without PBS washing. When cells reach 85% confluency (T-25 flask), transfer half of the cells to a new T-25 flask for further cell propagation:
  - a. Wash the cells with 5 mL of PBS.
  - b. Add 1 mL of cell dissociation buffer and gently rock the flask to immerse the cells at RT for 2 min.
  - c. Leave the flask for an additional 3 min at RT in the laminar flow hood.
  - d. Detach the cells with 4 mL of  $\alpha$  final medium and transfer the cell suspension to the 10 mL centrifuge tube.
  - e. Centrifuge the tube with the cell suspension at 120× g for 5 min. Remove medium and resuspend the cell pellet in fresh  $\alpha$  final medium.
  - f. Add half an aliquot of cell suspension to a new T-25 flask.
  - g. Incubate cells at 37 °C.

## C. Nucleofection of $\alpha$ TC1-6 cells

*Note: All steps for nucleofection include gently handling the cells.*

1. Prewarm the 6-well plate with 1,800  $\mu$ L/well of  $\alpha$  final medium at 37 °C.
2. After reaching 70% cell confluency (see General note 2), aspirate the medium from the flask and rinse the cells with 5 mL of PBS. Detach the cells using cell dissociation buffer (as described in **step B1a–d**).
3. Mix the cell suspension with Trypan Blue solution (1:1), load onto a hemocytometer, and count viable cells to determine the concentration per unit volume.
4. Centrifuge  $5 \times 10^6$  cells per one 100  $\mu$ L single Nucleocuvette™ at 90× g for 10 min at RT.
5. Pour out the medium and rest the centrifuge tube on filter paper to absorb residual liquid to complete removal of media without drying the pellet.
6. Add 100  $\mu$ L of transfection buffer (4D-Nucleofector™ SF Solution with Supplement and 7.5  $\mu$ g of plasmid DNA) to the dry cell pellet. Mix gently by pipetting 3–4×.
7. Transfer the cell suspension into a 100  $\mu$ L single Nucleocuvette™.
8. Insert the Nucleocuvette™ into the retainer of the 4D-Nucleofector™ X Unit (Figure 1A). Initiate the CM-156 nucleofection program.



**Figure 1. Alpha TC1-6 transfection.** (A) 4D-Nucleofector X unit with set parameters for transfection of alpha TC1-6 cells in the Nucleocuvette. (B) The map illustrates the positions of the four sgRNAs (indicated by red arrows; the sequences are presented in Table 1) used for transfection and targeting of the EpiCRISPR fusion construct. The *Arx* coding region is

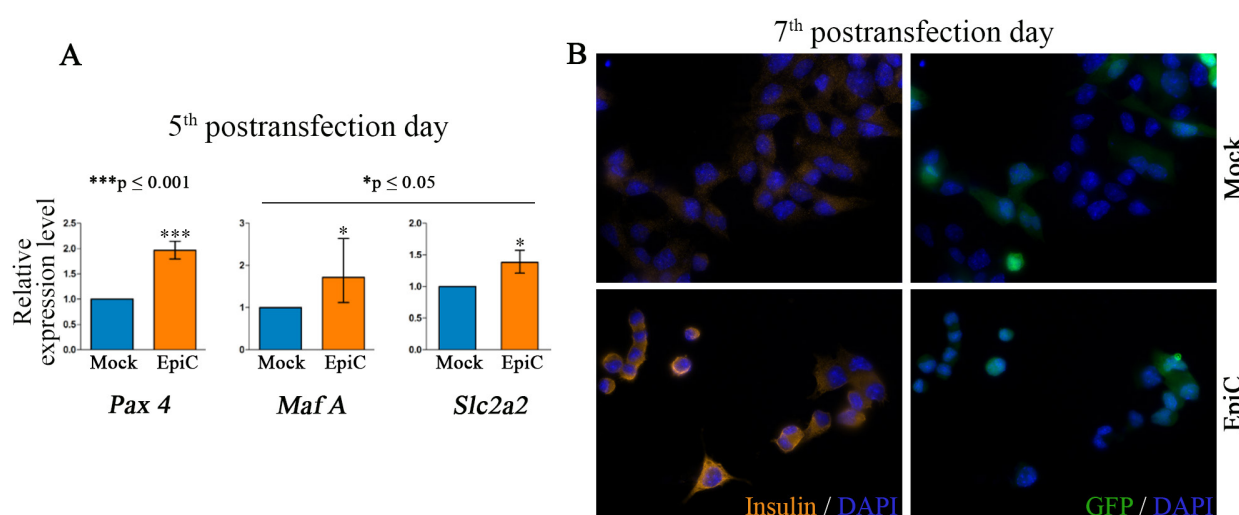


represented as a blue box, while the intron is depicted as a dashed line. NLS, nuclear localization signal. (C) Schema of the dCas9-Dnmt3a3L-KRAB plasmid that was used to transfect the alpha TC1-6 cells for silencing the expression of the *Arx* gene. (D) Alpha TC1-6 cells at day 5 post-transfection. Images were taken with an AxioCam digital camera attached to the Axio Observer Z1 microscope. Scale bars, 100  $\mu$ m.

**Table 1. sgRNA targeting sequences**

<i>Arx</i> sgRNA	sgRNA targeting sequence
sgRNA 1	GCAAAGCGCAAAGCGCGAAC
sgRNA 2	TAACAAGTGTAAGTGAGCCGC
sgRNA 3	AGGGTGGGAGCCCGCAACCG
sgRNA 4	GATGCTGTCGATGCAGTAGG

9. Upon completion of the program for nucleofection, add 400  $\mu$ L of prewarmed RPMI medium to each Nucleocuvette™ slowly down the cuvette wall and incubate for 10 min at 37 °C.
10. **(Critical)** Gently resuspend the cells by pipetting up and down 2–3 times. The application of the electrical pulse creates temporary pores in the cell membrane, making the cells highly sensitive to mechanical stress.
11. Transfer the 200  $\mu$ L of resuspended cells from one Nucleocuvette™ into one well of a pre-incubated 6-well plate containing  $\alpha$  final medium (two wells per sample in total).
12. Cultivate the transfected cells under standard conditions until further analysis (Figure 2).



**Figure 2. Alpha TC1-6 cells exhibit a beta-cell-like phenotype.** (A) Relative expression at the mRNA level of pancreatic beta cell key marker genes (*Pax 4*, *Maf A*, and *Slc2a2*) in Mock (cells transfected with a mix of plasmid with empty sgRNA instead of the four listed sgRNAs for *Arx*) and EpiC transfected and sorted  $\alpha$ TC1-6 at day 5 post-transfection. The error bars denote SD from three biological replicates. A one-sample t-test was used for determining statistical significance, \* $p \leq 0.05$ , \*\*\* $p \leq 0.001$ . The figure is modified from Đorđević et al [4]. (B) Immunocytochemistry (ICC) analysis of Mock and EpiC-transfected  $\alpha$ TC1-6 at day 7 post-transfection shows that insulin is present in the EpiC cells at the protein level, while it was not detected in Mock-transfected cells.

## D. Sorting of transfected $\alpha$ TC1-6 cells

*Note: All steps for cell sorting should be done at RT.*

1. At day 5 post-transfection, aspirate medium and wash the cells with 1 mL of PBS and detach with 0.7 mL of cell dissociation buffer per well (as described in **step B1a–d**).
2. Centrifuge the cells at 90 $\times$  g for 10 min at RT in a FACS tube.
3. Pour out the medium from the tubes.
4. Resuspend the cell pellet in 1 mL of buffer for cell sorting.
5. Collect the GFP-positive  $\alpha$ TC1-6 cells using the FACS Aria III flow cytometer and cell sorter (cell sorting parameters are listed in **Table 2**).

6. Collect the cells into the 10 mL conical tubes containing 2 mL of  $\alpha$  final medium. Coat the inner walls of the tubes with  $\alpha$  final medium immediately before placing them in the holder.
7. After sorting and collection, centrifuge the cells at  $90\times g$  for 10 min at RT to pellet them.
8. The cell pellet can be used immediately for molecular biology analyses or further propagated in cell culture.

**Table 2. Cell sorting parameters**

Parameter	Value
Pressure	45 psi
Nozzle	85 $\mu$ m
Frequency	47 Hz
Gap	8
FSC voltage	20
SSC voltage	315
FITC/GFP	330
Purity mode	4-way purity

## Validation of protocol

This protocol has been used and validated in the following research articles:

- Đorđević et al. [14]. Nucleofection as an Efficient Method for Alpha TC1-6 Cell Line Transfection. *Applied Sciences* (Figure 9).
- Đorđević, M. B. [15]. Transdiferencijacija alfa ćelija pankreasa miša u ćelije koje proizvode insulin ciljanom metilacijom DNK primenom Epi-CRISPR sistema [Doctoral dissertation, University of Belgrade (Serbia)].
- Đorđević et al. [16]. Changes in up and down regulated gene expression after transient suppression of *Arx* gene in pancreatic alpha TC1-6 cell line. CoMBoS2-the Second Congress of Molecular Biologists of Serbia; 2023 Oct 6-8; Belgrade, Serbia (pp. 151–151). Belgrade: Institute of Molecular Genetics and Genetic Engineering, University of Belgrade.
- Đorđević et al. [17]. Uloga ciljanih (epi) genetičkih modifikacija u potencijalnoj terapiji dijabetesa. *Trendovi u molekularnoj Biologiji*.

## General notes and troubleshooting

### General notes

1. The *Arx* gene DNA sequences with NCBI ref. s. NC\_000086.7 [assembly: GRCm38.p6 (GCF\_000001635.26)] were obtained using the UCSC Genome Browser [12]. Potential binding sites for sgRNAs targeting the dCas9-3a3L-KRAB fusion protein to the *Arx* promoter were designed by the online E-CRISP platform [13]. The sgRNAs were chosen to target the *Arx* promoter region and CpG island, with a focus on high efficiency and low off-target effects (Figure 1B).
2. Transfection efficiency is strongly influenced by cell density. For optimal results, cells should reach 70%–75% confluency at the time of transfection, as this ensures they are actively dividing and most receptive to DNA uptake. Higher confluency levels can lead to reduced efficiency due to contact inhibition and changes in cellular physiology.
3. High plasmid concentration, good purity, and the absence of endotoxin are necessary for high transfection efficiency.

### Troubleshooting

Problem: Low transfection efficiency.

Possible causes:

1. The transfection protocol is not well optimized for cells other than alphaTC1-6.
2. Cells are of high density/confluency.
3. Low cell viability after nucleofection.
4. Low sorting recovery.

#### 5. Low plasmid purity.

##### Solutions:

1. Optimize the nucleofection program (pulse settings optimization), the amount of the DNA construct, and the number and viability of input cells. Expected efficiency ranges should be 70%–80% GFP-positive cells (Figure 1D).
2. Consider using a transfection method other than nucleofection (e.g., chemical transfection: lipid- or polymer-based reagents).
3. Adjust post-transfection recovery conditions, including medium composition, supplements, and handling during the first 24 h.
4. Adjust the buffers for collecting cells by adding FBS or BSA and HEPES (Ca/Mg-free).
5. Check endotoxin levels after plasmid isolation.

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- 3) Citation of the original research paper in which the protocol was described and validated: Đorđević, M., Stepper, P., Feuerstein-Akgoz, C., Gerhauser, C., Paunović, V., Tolić, A., Rajić, J., Dinić, S., Uskoković, A., Grdović, N., Mihailović, M., Jurkowska, R. Z., Jurkowski, T. P., Jovanović, J. A., & Vidaković, M. (2023). EpiCRISPR targeted methylation of *Arx* gene initiates transient switch of mouse pancreatic alpha to insulin-producing cells. *Frontiers in endocrinology*, 14, 1134478. <https://doi.org/10.3389/fendo.2023.1134478>.
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## Competing interests

The authors declare no conflicts of interest.

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