

Submission ID #: 71804

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Title: Generation of GABAergic Neurons from Human Induced Pluripotent Stem Cells Using Doxycycline-Inducible *ASCL1*/*DLX2* Expression at *AAVS1*

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

If **Yes**, can you record movies/images using your own microscope camera?

No

Scope shots: 5.4.1, 5.5.2, 5.5.3

Videographer: Please film the above-mentioned scope shots using a scope kit.

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

3. Filming location: Will the filming need to take place in multiple locations? **No**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

Current Protocol Length

Number of Steps: 38

Number of Shots: 58

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Théo Rabin:** Our research focuses on generating pure GABAergic neuron populations from hiPSCs to model human neuronal diversity and disease.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Théo Rabin:** Existing protocols require repeated viral transduction for every differentiation. This protocol addresses this limitation using an AAVS1 knock-in line.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Théo Rabin:** These neurons enable electrophysiological recordings and integration into MEA co-cultures to study inhibitory network activity.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Théo Rabin:** Additionally, electrophysiology, MEA recordings, or single-cell RNA sequencing can be performed following this procedure.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Théo Rabin:** Future studies building on this protocol can analyze the effects of different mutations linked to GABAergic dysfunction in epilepsy.

- 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

What value do you expect this video article to have for other researchers working in your field?

1.6. **Théo Rabin**: (authors will present their testimonial statements live)

1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

How does the video format of your article help you to share your work with the broader scientific community?

1.7. **Théo Rabin**: (authors will present their testimonial statements live)

1.7.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Preparation of Freezing Medium and BME-Coated Culture Plates

Demonstrator: Théo Rabin

- 2.1. To begin, prepare the freezing medium by combining 40 milliliters of knock-out serum replacement or KOSR (*K-O-S-R*) and 10 milliliters of DMSO (*D-M-S-O*) in a 50-milliliter conical tube [1].
 - 2.1.1. WIDE: Talent adding knock-out serum replacement and dimethyl sulfoxide into a 50-milliliter conical tube. ***Videographer:** Please obtain CU shot as well for this shot.*
- 2.2. Homogenize the freezing medium by pipetting up and down 10 times [1] and store the solution at 4 degrees Celsius [2].
 - 2.2.1. Talent pipetting the freezing medium up and down repeatedly in the 50-milliliter conical tube.
 - 2.2.2. Talent placing the freezing medium in the refrigerator
- 2.3. Next, dilute the basement membrane extract or BME (*B-M-E*) in DMEM and F12 medium to a final concentration of 80 micrograms per milliliter [1].
 - 2.3.1. Talent diluting the basement membrane extract with Dulbecco's Modified Eagle Medium/F12 medium.
- 2.4. Add 1 milliliter of the diluted BME to each well of a 6-well plate [1].
 - 2.4.1. Talent dispensing diluted basement membrane extract into the wells of a 6-well plate.
- 2.5. Tilt the plate to distribute the solution evenly across the entire surface of the wells, ensuring there are no uncoated areas [1], and incubate the plate for a minimum of 30 minutes at 37 degrees Celsius [2].
 - 2.5.1. Shot of tilting the 6-well plate and the solution being spread across all the wells.
 - 2.5.2. Talent placing the plate into the incubator.

3. Maintenance and Passaging of hiPSCs

- 3.1. Passage human induced pluripotent stem cells or hiPSC (*H-I-P-S-C*) approximately every 4 to 6 days when the cells reach 60% confluence [1].

- 3.1.1. LABMEDIA: Show hiPSC colonies at approximately 60% confluence **NOTE: Use the missing media card**
- 3.2. Using an aspirating pipette, aspirate the culture medium from the wells [1].
 - 3.2.1. Talent aspirating culture medium from the wells with an aspirating pipette.
- 3.3. Add 1 milliliter of ReLeSR (*Re-le-ser*) to the well and incubate for 30 seconds at room temperature [1].
 - 3.3.1. Talent adding ReLeSR to the well and keeping it aside for incubation.
- 3.4. Aspirate the dissociation reagent [1] and incubate the plate for 8 minutes at 37 degrees Celsius [2].
 - 3.4.1. Talent aspirating the dissociation reagent from the well.
 - 3.4.2. *Reuse 2.5.2*
- 3.5. Using a 5-milliliter pipette, gently add 1 milliliter of hiPSC maintenance medium to the well [1]. Collect the cell suspension in a 50-milliliter tube [2].
 - 3.5.1. Talent adding hiPSC maintenance medium to the well with a 5-milliliter pipette.
 - 3.5.2. Talent transferring the cell suspension into a 50-milliliter tube.
- 3.6. Mix 10 microliters of the cell suspension with 10 microliters of trypan blue in a microcentrifuge tube [1]. Load 10 microliters of the mixture onto a cell counter slide [2] and determine the viable cell concentration [3].
 - 3.6.1. Talent adding the cell suspension and trypan blue into a microcentrifuge tube.
 - 3.6.2. Talent loading the mixture onto a cell counter slide.
 - 3.6.3. SCREEN: cell-counter-results.JPEG
Videographer: Please film the computer screen as a back up
- 3.7. After aspirating the BME coating from a previously prepared well, add 2 milliliters of hiPSC maintenance medium supplemented with Rho-associated coiled-coil kinase or ROCK (*ROCK*) inhibitor [1-TXT].
 - 3.7.1. Talent adding supplemented human induced pluripotent stem cell maintenance medium to the well. **TXT: ROCK inhibitor: Y-27632**
- 3.8. Seed 100,000 hiPSC into the well, aiming for greater than 90% viability [1]. Move the plate in a figure-eight motion to ensure a homogeneous distribution of cells [2] and incubate the cells at 37 degrees Celsius with 5% carbon dioxide [3].
 - 3.8.1. Talent adding hiPSC into the well.
 - 3.8.2. Talent moving the plate in a figure-eight motion.
 - 3.8.3. Talent placing the plate into the incubator

3.9. After 24 hours, replace the medium with 2 milliliters of fresh mTeSR (*m-te-ser*) Plus medium without ROCK inhibitor [1].

3.9.1. Talent replacing the existing medium with fresh mTeSR Plus medium.

4. Nucleofection of hiPSCs

4.1. Pass the cells when the confluence reaches approximately 60% [1]. After turning on the nucleofection device, add the nucleofection supplement to nucleofection solution P3 (*P-three*) [2].

4.1.1. **LABMEDIA**: Show hiPSC culture at approximately 60% confluence.

4.1.2. Talent adding the nucleofection supplement to nucleofection solution P3.

4.2. Add 110 microliters of nucleofection solution P3 to a microcentrifuge tube placed on ice [1].

4.2.1. Talent dispensing nucleofection solution P3 into a microcentrifuge tube on ice.

4.3. Next, add 1 microgram of the CRISPR (*KRIS-per*) associated protein 9 plasmid [1] and 1 microgram of the donor plasmid to the tube and mix gently without introducing bubbles [2]. Aspirate BME from two coated wells, add 2 milliliters of hiPSC maintenance medium containing a ROCK inhibitor to each well, and incubate the plate at 37 degrees Celsius [3]. After dissociating hiPSC at 60% confluence from a well, as previously demonstrated, transfer the cell suspension to a sterile tube [4]. Centrifuge for 5 minutes at 500 g at room temperature [5].

4.3.1. Talent adding the CRISPR/Cas9 plasmid to the microcentrifuge tube.

4.3.2. TEXT ON A PLAIN BACKGROUND:

Donor Plasmid Components:

ASCL1-P2A-DLX2 cassette (doxycycline-inducible)

Fluorescent reporter

Puromycin-resistance cassette

4.3.3. Talent aspirating BME from two coated wells and adding maintenance medium containing the ROCK inhibitor to each well.

4.3.4. Talent transferring the cell suspension into a sterile tube.

4.3.5. Talent placing the tube into the centrifuge.

4.4. Once the supernatant is discarded, resuspend the cell pellet in 110 microliters of nucleofection solution P3 containing the plasmids and pipette gently to avoid bubble formation [1].

4.4.1. Talent adding the nucleofection solution containing the plasmids to the cell pellet and pipetting the cell suspension.

~~4.5. Transfer 100 microliters of the cell suspension into a nucleofection cuvette placed on ice [1]. After transferring 100 microliters of the cell suspension into the cuvette and placing it into the nucleofector device, run the CB-150 (CB-one-fifty) program for hiPSCs [2].~~ **NOTE: The VO is edited for the deleted shot**

~~4.5.1. Talent transferring the cell suspension into a nucleofection cuvette on ice.~~ **NOTE: 4.5.1. and 4.5.2 are not filmed**

~~4.5.2. Talent inserting the nucleofection cuvette into the nucleofector device.~~

4.5.3. SCREEN: nucleofector-interface-with-CB-150-program.JPG.

~~4.6. Using a plastic Pasteur pipette, immediately retrieve the cells and transfer equal volumes of the cell suspension into the two previously prepared wells [1].~~ **NOTE: The VO is deleted, and the information is added in the next shot**

~~4.6.1. Talent retrieving the cells from the nucleofection cuvette using a plastic Pasteur pipette and dispensing equal volumes of the cell suspension into the two prepared wells.~~ **NOTE: Not filmed**

4.7. Once the cells are retrieved, distribute the cells using a figure-eight motion into the two previously prepared wells [1] and incubate at 37 degrees Celsius [2].

4.7.1. Talent moving the plate in a figure-eight motion.

4.7.2. *Reuse 2.5.2*

4.8. After 24 hours, replace the medium with mTeSR Plus medium lacking ROCK inhibitor and verify nucleofection efficiency by observing fluorescence [1].

4.8.1. Talent replacing the culture medium with fresh mTeSR Plus medium.

5. Selection and Validation of Genome-Edited hiPSCs

5.1. Wait for 3 passages post-nucleofection, typically approximately 1 to 2 weeks depending on recovery and growth rate, to allow the loss of non-integrated plasmids before initiating selection [1].

5.1.1. Talent performing passaging of the recovered hiPSC culture.

5.2. Seed 100,000 cells to each well [1].

5.2.1. Talent adding 100,000 cells into each well.

5.3. Add puromycin at 0.4 to 0.6 micrograms per milliliter after 24 hours and maintain selection for about 5 days [1].

5.3.1. Talent adding puromycin to the culture medium.

5.4. Validate the selected population by fluorescence microscopy to estimate the proportion of reporter-positive cells [1].

5.4.1. SCOPE: Reporter-positive-cells-under-fluorescent-microscopy.mp4: 00:08-00:20

5.5. Confirm that the selected hiPSC population remains mycoplasma-negative [1] and maintains robust growth after puromycin selection [2].

5.5.1. SCREEN: Mycoplasma-test-results.png

5.5.2. SCOPE: View-of-healthy-expanding-human-induced-pluripotent-stem-cell-colonies-after-puromycin-selection.mp4: 00:06-00:20

6. Direct Differentiation of hiPSCs into GABAergic Neurons

6.1. Prepare 6.9 milliliters of doxycycline-supplemented neuro-induction medium per BME-coated well, adding 15 percent extra to account for evaporation or pipetting errors [1].

6.1.1. Talent adding doxycycline to the neuro-induction medium.

6.2. After dissociating the engineered hiPSCs on day 0, seed 200,000 cells into the prepared BME-coated wells [1] and incubate at 37 degrees Celsius [2].

6.2.1. Talent adding cells into the prepared wells.

6.2.2. [Reuse 2.5.2](#)

6.3. Once the medium is aspirated on days 1, 2, and 3, add 2 milliliters of fresh neuro-induction medium containing doxycycline [1]. On day 4, prepare neuro-maturation medium [2]. After aspirating the neuro-induction medium, carefully add 2 milliliters of neuro-maturation medium, drop by drop, along the well wall to avoid disturbing the newly formed neurons [3].

6.3.1. Talent adding fresh neuro-induction medium containing doxycycline to the wells.

6.3.2. TEXT ON A PLAIN BACKGROUND:

Neuro-maturation Medium

BDNF: 10 ng/mL

GDNF: 10 ng/mL

NT-3: 10 ng/mL

Doxycycline: 1 µg/mL

PluriSin: 20 µM

6.3.3. Talent adding neuro-maturation medium drop-by-drop along the wall of the well.

6.4. On Day 7, once the medium is aspirated, gently add 2 milliliters of neuro-maturation medium containing the neurotrophins and doxycycline, while omitting the pluripotent cell survival inhibitor [1].

- 6.4.1. Talent gently adding neuro-maturation medium containing neurotrophins and doxycycline.
- 6.5. For long-term maintenance, replace the neuro-maturation medium completely every 3 to 4 days, adapting the frequency based on cell density [1].
 - 6.5.1. Talent replacing the neuro-maturation medium in the neuronal cultures.
- 6.6. Maintain cultures beyond Day 21 for extended maturation if the networks remain attached, viable, and free of proliferating hiPSC-like colonies [1]. After Day 21, perform half-medium changes every 3 to 4 days using fresh neuro-maturation medium containing doxycycline and neurotrophic factors [2].
 - 6.6.1. LABMEDIA: Mature neuronal networks remaining attached and viable in culture
NOTE: Use the missing media card
 - 6.6.2. Talent performing a half-medium change using fresh neuro-maturation medium.

Results

7. Results

- 7.1. Upon doxycycline induction, hiPSCs underwent a rapid morphological transformation [1].
 - 7.1.1. LAB MEDIA: Figure 1C. *Video editor: Emphasize the images with doxycycline at Day 0 and Day 3.*
- 7.2. Cell division ceased by Day 3 [1], and neurite outgrowths subsequently emerged [2].
 - 7.2.1. LAB MEDIA: Figure 1C. *Video editor: Emphasize the Day 3 image with doxycycline.*
 - 7.2.2. LAB MEDIA: Figure 1C. *Video editor: Emphasize the long, thread-like extensions growing out from the cells in the Day 3 image with doxycycline.*
- 7.3. By Day 20, successful differentiation was confirmed by extensive axonal networks [1], and high expression of interneuron markers DLX1 (*D-L-X-one*) [2], GAD67 (*Gad-sixty-seven*) [3], and GAD2 (*Gad-two*) was observed on day 21 [4].
 - 7.3.1. LAB MEDIA: Figure 1D. *Video editor: Emphasize the Day 20 image.*
 - 7.3.2. LAB MEDIA: Figure 1E. *Video editor: Emphasize the Day 21 data point on the DLX1 graph.*
 - 7.3.3. LAB MEDIA: Figure 1F. *Video editor: Emphasize the Day 21 bar for GAD67.*
 - 7.3.4. LAB MEDIA: Figure 1F. *Video editor: Emphasize the Day 21 bar for GAD2.*
- 7.4. Glutamatergic marker SLC17A6 (*S-L-C-seventeen-A-six*) [1] and SLC17A7 were negative [2] compared to the NGN2 (*N-G-N-two*) mediated differentiation protocol [3].
 - 7.4.1. LAB MEDIA: Figure 1G. *Video editor: Emphasize the ASCL1-D12 bar in the SLC17A6 graph.*
 - 7.4.2. LAB MEDIA: Figure 1G. *Video editor: Emphasize the ASCL1-D12 bar in the SLC17A7 graph.*
 - 7.4.3. LAB MEDIA: Figure 1G. *Video editor: Emphasize the NGN2 D21 bar in the SLC17A6 and SLC17A7 graph.*

1. Knock-out serum replacement
Pronunciation link: No confirmed link found

- IPA: /'nɑ:k, aʊt 'sɪrəm rɪ'pleɪsmənt/
Phonetic Spelling: NOK·out SEER·uhm rih·PLAYS·muhnt
2. Dimethyl sulfoxide
Pronunciation link: <https://www.merriam-webster.com/dictionary/dimethyl%20sulfoxide>
IPA: /daɪ'mɛθəl sʌl'fɑ:ksaɪd/
Phonetic Spelling: dye·METH·uhl sul·FOK·side
 3. Homogenize
Pronunciation link: <https://www.merriam-webster.com/dictionary/homogenize>
IPA: /hə'mɑ:dʒəˌnaɪz/
Phonetic Spelling: huh·MOJ·uh·nyze
 4. Basement membrane extract
Pronunciation link: No confirmed link found
IPA: /'beɪsmənt 'mɛmˌbrɛn 'ɛkstrækt/
Phonetic Spelling: BASE·muhnt MEM·brayn EK·strakt
 5. Dulbecco's Modified Eagle Medium
Pronunciation link: No confirmed link found
IPA: /dʌl'bekoʊz 'mɑ:dəˌfaɪd 'i:ɡəl 'mi:diəm/
Phonetic Spelling: dul·BEK·ohz MOD·uh·fyd EE·guhl MEE·dee·uhm
 6. Pluripotent
Pronunciation link: <https://www.merriam-webster.com/dictionary/pluripotent>
IPA: /plʊ'ripətənt/
Phonetic Spelling: ploo·RIP·uh·tuht
 7. Confluence
Pronunciation link: <https://www.merriam-webster.com/dictionary/confluence>
IPA: /'kɑ:nfluəns/
Phonetic Spelling: KON·floo·uhns
 8. ReLeSR
Pronunciation link: No confirmed link found
IPA: /rɪ'li:sər/
Phonetic Spelling: rih·LEE·ser
 9. Dissociation
Pronunciation link: <https://www.merriam-webster.com/dictionary/dissociation>
IPA: /dɪˌsoʊsi'eɪʃən/
Phonetic Spelling: dih·soh·see·AY·shuhn
 10. Trypan blue
Pronunciation link: <https://www.merriam-webster.com/medical/trypan%20blue>
IPA: /'traɪpæn blu:/
Phonetic Spelling: TRY·pan bloo
 11. Microcentrifuge
Pronunciation link: No confirmed link found
IPA: /ˌmaɪkroʊ'sentrɪˌfju:dʒ/
Phonetic Spelling: my·kroh·SEN·trih·fyooj
 12. Rho-associated coiled-coil kinase
Pronunciation link: No confirmed link found

- IPA: /rou ə'souʃi, ɛɪtɪd 'kɔɪld, kɔɪl 'kaɪnɪs/
Phonetic Spelling: roh uh·SOH·see·ay·tid koyld·koyl KYE·nays
13. Nucleofection
Pronunciation link: No confirmed link found
IPA: /,nu:kliʊʊ'fɛkʃən/
Phonetic Spelling: noo·klee·oh·FEK·shuhn
14. CRISPR
Pronunciation link: <https://www.merriam-webster.com/dictionary/CRISPR>
IPA: /'krɪspər/
Phonetic Spelling: KRIS·per
15. Plasmid
Pronunciation link: <https://www.merriam-webster.com/dictionary/plasmid>
IPA: /'plæzmɪd/
Phonetic Spelling: PLAZ·mid
16. ASCL1
Pronunciation link: No confirmed link found
IPA: /,ɛɪ ɛs si: ɛl 'wʌn/
Phonetic Spelling: A·S·C·L·one
17. Doxycycline
Pronunciation link: <https://www.merriam-webster.com/dictionary/doxycycline>
IPA: /,dɔːksɪ'saɪkli:n/
Phonetic Spelling: dok·see·SYE·kleen
18. Puromycin
Pronunciation link: <https://www.merriam-webster.com/medical/puromycin>
IPA: /,pjʊərə'maɪsɪn/
Phonetic Spelling: pyur·uh·MY·sin
19. Nucleofector
Pronunciation link: No confirmed link found
IPA: /,nu:kliʊʊ'fɛktər/
Phonetic Spelling: noo·klee·oh·FEK·ter
20. Mycoplasma
Pronunciation link: <https://www.merriam-webster.com/dictionary/mycoplasma>
IPA: /,maɪkoʊ'plæzmə/
Phonetic Spelling: my·koh·PLAZ·muh
21. GABAergic
Pronunciation link: No confirmed link found
IPA: /,gæbə'ɜːrdʒɪk/
Phonetic Spelling: gab·uh·UR·jik
22. Neuro-induction
Pronunciation link: No confirmed link found
IPA: /,nʊroʊ ɪn'dʌkʃən/
Phonetic Spelling: NUR·oh in·DUK·shuhn
23. Neuro-maturation
Pronunciation link: No confirmed link found

IPA: /ˌnʊroʊ mətʃəˈreɪʃən/

Phonetic Spelling: NUR·oh mach·uh·RAY·shuhn

24. Neurotrophins

Pronunciation link: <https://www.merriam-webster.com/medical/neurotrophin>

IPA: /ˌnʊrəˈtroʊfɪnz/

Phonetic Spelling: nur·uh·TROH·finz

25. Neurite

Pronunciation link: <https://www.merriam-webster.com/medical/neurite>

IPA: /ˈnʊr aɪt/

Phonetic Spelling: NUR·yte

26. Axonal

Pronunciation link: <https://www.merriam-webster.com/dictionary/axonal>

IPA: /ˈæksənəl/

Phonetic Spelling: AK·suh·nuhl

27. Interneuron

Pronunciation link: <https://www.merriam-webster.com/dictionary/interneuron>

IPA: /ˌɪntərˈnʊrɑːn/

Phonetic Spelling: in·ter·NUR·on

28. Glutamatergic

Pronunciation link: No confirmed link found

IPA: /ˌɡluːtəməˈnɜːrdʒɪk/

Phonetic Spelling: gloo·tuh·mih·NUR·jik