Jun 23, 2021

Accelerated High-Yield Generation of Limb-Innervating Motor Neurons from Human Stem Cells

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 dx.doi.org/10.17504/protocols.io.burmnv46

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ABSTRACT

Human pluripotent stem cells are a promising source of differentiated cells for developmental studies, cell transplantation, disease modeling, and drug testing. However, their widespread use even for intensely studied cell types like spinal motor neurons is hindered by the long duration and low yields of existing protocols for in vitro differentiation and by the molecular heterogeneity of the populations generated. We report a combination of small molecules that within 3 weeks induce motor neurons at up to 50% abundance and with defined subtype identities of relevance to neurodegenerative disease. Despite their accelerated differentiation, motor neurons expressed combinations of HB9, ISL1, and column-specific markers that mirror those observed in vivo in human embryonic spinal cord. They also exhibited spontaneous and induced activity, and projected axons toward muscles when grafted into developing chick spinal cord. Strikingly, this novel protocol preferentially generates motor neurons expressing markers of limb-innervating lateral motor column motor neurons ($FOXP1^{+}/LHX3^{-}$). Access to high-yield cultures of human limb-innervating motor neuron subtypes will facilitate in-depth study of motor neuron subtype-specific properties, disease modeling, and development of large-scale cell-based screening assays.

ATTACHMENTS

dfqvbiqa7.pdf

DOI

dx.doi.org/10.17504/protocols.io.burmnv46

PROTOCOL CITATION

Mackenzie W. Amoroso, Gist F. Croft, Damian J. Williams, Sean O'Keeffe, Monica A. Carrasco, Anne R. Davis, Laurent Roybon, Derek H. Oakley, Tom Maniatis, Christopher E. Henderson 2021. Accelerated High-Yield Generation of Limb-Innervating Motor Neurons from Human Stem Cells. **protocols.io** https://dx.doi.org/10.17504/protocols.io.burmnv46

KEYWORDS

Limb-Innervating Motor Neurons, Human Stem Cells, Neurodegenerative disease

CREATED

May 05, 2021

LAST MODIFIED

Jun 23, 2021

OWNERSHIP HISTORY

May 05, 2021 Urmilas

May 11, 2021 hendersa

PROTOCOL INTEGER ID

49677

MATERIALS TEXT

GFP Polyclonal Antibody **Invitrogen - Thermo Fisher Catalog #A10262**

Anti-GFP antibody (ab290) **Abcam Catalog #Ab290**

Mouse anti-Islet 1 (ISL1) **Developmental Studies Hybridoma Bank Catalog #39.4D5**

Mouse anti-HB9 **Developmental Studies Hybridoma Bank Catalog #815.c10-s/ 815.c10-c**

(ab14545) Anti-beta III Tubulin antibody [TUJ-1] **Abcam Catalog #Ab14545**

Anti-FOXP1 antibody (ab16645) **Abcam Catalog #Ab16645**

Anti-Brn-3a/BRN3A/POU4F1 Antibody (14A6): sc-8429 **Santa Cruz Biotechnology Catalog #sc-8429**

Primary antibodies:

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A	B	C	D
Antibodies	**Dilution**	**Supplier**	**Catalog no.**
Chicken anti-GFP	1:1000	Invitrogen	A10262
Rabbit anti-GFP	1:5000	Abcam	Ab290
Mouse anti-HB9	1:50/1:100	DSHB	815.c10-s/ 815.c10-c
Guinea pig anti-HB9	1:40,000	Thomas Jessell/Susan Morton	Columbia University #1633 Human Hb9 n-TERM: NH2 MEKSKNFRIDALLAVDPPRAAS(C) COOH
Guinea pig anti-Islet 1 (ISL1)	1:20,000	Thomas Jessell/Susan Morton	Columbia University # 1278
Mouse anti-Islet 1 (ISL1)	1:100	DSHB	39.4D5
Mouse-anti beta-III tubulin (TUJ1)	1:2000	Abcam	Ab14545
Guinea pig anti-FOXP1	1:40,000	Thomas Jessell/Susan Morton	Columbia University # 1636ENSIPLYTTASMGNPT(C)
Rabbit anti-FOXP1	1:20,000	Abcam	Ab16645
Mouse anti-LHX3	1:200	DSHB	4E12
mouse anti-NF-H (nonphosphorylated; SMI-32)	1:5000	Santa CruzTechnology	sc-58554
Rabbit anti-RALDH2	1:20,000	Thomas Jessell/Susan Morton	Columbia University # 1676
BRN3A	1:1000	Santa CruzTechnology	sc-8429

qPCR primer sequences:

A	B	C
Primers	Forward	Reverse
RALDH2	TTTTGCTGATGCTGACTTGG	GCAGCACTGACCTTGATTGA
FOXP1	TGACCTTTTGAGGTGACTATAACTG	GCAGCACTGACCTTGATTGA
LHX3	GTTCAGGAGGGGCAGGAC	CCCAAGCTCCCGTAGAGG
CHT1	AAGCCATCATAGTTGGTGGCCGAG	CCAAGCTAGGCCATAACCTGGTAC
HOXA5	CAGCACCCACATCA	CGGAGAGGCAAAGA
HOXC6	CCAGGACCAGAAAGCCAGTA	GTTAGGTAGCGATTGAAGTGAAA
HOXC8	CTTCGCTGTTTGATTTCTATTCTG	TACGCTGGAGGTTTCTTTCTTT
HOXD9	TCGCTGAAGGAGGAGGAGA	CAAACACCCACAAAGGAAAAC

hESC medium:

A	B
DMEM: nutrient mixture F-12 (DMEM/F:12; Invitrogen) with 20% Knockout Serum Replacer (Invitrogen)	
Rho-associated kinase inhibitor Y27632 (Ascent Scientific)	10 uM
Beta-mercaptoethanol (BME; Sigma)	110 uM
L-glutamine	
Nonessential amino acids (NEAA; Invitrogen)	
bFGF (Invitrogen)	20 ng/ml
SB435142	10 uM
LDN193189	0.2 uM

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Neural induction medium:

A	B
DMEM/F:12 with L-glutamine	
NEAA	
Penicillin/streptomycin	
Heparin	2 ug/ml
N2 supplement; Invitrogen	

Neurobasal medium:

A	B
DMEM/F:12 with L-glutamine	
NEAA	
Penicillin/streptomycin	
Heparin	2 ug/ml
N2 supplement; Invitrogen	
Insulin-like growth factor 1 (IGF-1)	10 ng/ml
Glial cell line-derived neurotrophic factor (GDNF)	10 ng/ml
Ciliary neurotrophic factor (CNTF) (R&D)	10 ng/ml
B27 (Invitrogen)	10 ng/ml

Cell culture maintenance 1d 0h 45m

1 Maintain all cell cultures at **37 °C** , 5% CO_2.

2 Maintain hES and iPS cells [HUES3 (control), male; H9 (control), female; HS001 (ALS-SOD1 N139K), male; LWM002 (ALS-SOD1 A4V), female; MBN007 (ALSSOD1 A4V), female; TM008 (ALS-SOD1 A4V), female; DCM009 (ALSSOD1 V148G), male; 10013.13 (control), male)] on gelatinized tissue-culture plastic on a monolayer of irradiated CF-1 mouse embryonic fibroblasts (GlobalStem), in hESC media, consisting of DMEM: nutrient mixture F-12 (DMEM/F:12; Invitrogen) with 20% Knockout Serum Replacer (Invitrogen), 110 uM -mercaptoethanol (BME; Sigma), L-glutamine and nonessential amino acids (NEAA; Invitrogen), and 20 ng/ml basic fibroblast growth factor (bFGF; Invitrogen) (Cowan et al., 2004).

3 Change media every **24:00:00** and passage lines with dispase (Invitrogen, **1 mg/ml**) in hESC media for 1d 0h 45m **00:15:00** – **00:30:00** at **37 °C**).

Motor neurons generation 3h

4 To generate motor neurons, passage undifferentiated hESCs using dispase (**1 mg/ml**) and triturated into small, 50- to 100-cell clumps and placed into ultralow adherent culture dishes (Corning).

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5 For the first 3 d, keep cells in suspension in hESC medium, supplemented with [M]**10 Micromolar (µM)** Rho-associated kinase inhibitor Y27632 (Ascent Scientific) to enhance single cell survival (Watanabe et al., 2007), [M]**20 ng/ml** bFGF (Invitrogen) to enhance growth, and [M]**10 Micromolar (µM)** SB435142 (SB; Sigma) and [M]**0.2 Micromolar (µM)** LDN193189 (LDN; Stemgent) for neuralization.

6 At day 3, switch embryoid bodies (EBs) to neural induction medium (DMEM/F:12 with L-glutamine; NEAA; penicillin/streptomycin; heparin, [M]**2 µg/ml** ; N2 supplement; Invitrogen).

7 

At day 5, add all-trans retinoic acid (RA; [M]**0.1 Micromolar (µM)** or [M]**1 Micromolar (µM)** ; Sigma), ascorbic acid ([M]**0.4 µg/ml** ; Sigma), and brain-derived neurotrophic factor ([M]**10 ng/ml** ; R&D).

8 Pursue dual ALK inhibition (SB + LDN) until day 7.

9 Initiate Hedgehog signaling on day 7 by application of C25II modified SHH (R&D), at the standard concentration of [M]**200 ng/ml** ; a human Smo agonist (HAG, [M]**1 Micromolar (µM)** ; gift from Lee Rubin; Dimos et al., 2008; Boulting et al., 2011); mouse Smo agonist 1.3 (SAG; [M]**1 Micromolar (µM)** ; Frank-Kamenetsky et al., 2002; Wichterle et al., 2002; Wada et al., 2009; Boulting et al., 2011); or purmorphamine (PUR, [M]**1 Micromolar (µM)** ; Stemgent; Sinha and Chen, 2006; Li et al., 2008).

10 At day 17, change basal medium to Neurobasal (Invitrogen), containing all previous factors and with the addition of [M]**10 ng/ml** each of insulin-like growth factor 1 (IGF-1), glial cell line-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF) (R&D), plus B27 (Invitrogen).

11 At day 20 or 30, disassociate EBs with 0.05% trypsin (Invitrogen) and plate onto poly-lysine/laminin-coated 8-well chamber slides (BD Biosciences) at 0.2-0.5.10 6 cells/well and/or **15 mm** coverslips at $0.5.10^6$.

12 Culture plated neurons in the same medium with the addition of [M]**25 Micromolar (µM)** BME and [M]**25 Micromolar (µM)** glutamic acid (Sigma) and fix 1 d later.

Immunocytochemistry assay 3h

13  50m

Fix cultures for **00:30:00** with 4% paraformaldehyde (PFA) in PBS at **4 °C** , wash three times for **00:05:00** in PBS, quench, and permeabilize in wash buffer (PBS, 0.1% Triton X-100) plus [M]**50 Milimolar (mM)** glycine for **00:15:00** .

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14 For the EB outgrowth RALDH2 staining, fix samples for **00:10:00** at **Room temperature** with 4% PFA/10% sucrose prewarmed to **37 °C** . 10m

15 2h

Block samples with wash buffer plus 10% normal donkey serum for **01:00:00** and incubate with primary antibody (Table 1) **Overnight** .

Table 1. Primary antibodies

	Dilution	Supplier	Catalog no.
Chicken anti-GFP	1:1000	Invitrogen	A10262
Rabbit anti-GFP	1:5000	Abcam	Ab290
Mouse anti-HB9	1:50/1:100	DSHB	815.c10-s/ 815.c10-c
Guinea pig anti-HB9	1:40,000	Thomas Jessell/Susan Morton	Columbia University #1633 Human Hb9 n-TERM: NH2 MEKSKNFRIDALLAVDPPRAAS(C) COOH
Guinea pig anti-Islet 1 (ISL1)	1:20,000	Thomas Jessell/Susan Morton	Columbia University # 1278
Mouse anti-Islet 1 (ISL1)	1:100	DSHB	39.4D5
Mouse-anti β-III tubulin (TUJ1)	1:2000	Abcam	Ab14545
Guinea pig anti-FOXP1	1:40,000	Thomas Jessell/Susan Morton	Columbia University # 1636 ENSIPLYTTASMGNPT(C)
Rabbit anti-FOXP1	1:20,000	Abcam	Ab16645
Mouse anti-LHX3	1:200	DSHB	4E12
mouse anti-NF-H (nonphosphorylated; SMI-32)	1:5000	Santa Cruz Technology	sc-58554
Rabbit anti-RALDH2	1:20,000	Thomas Jessell/Susan Morton	Columbia University # 1676
BRN3A	1:1000	Santa Cruz Technology	sc-8429

16 Wash cells, incubate with DyLight coupled donkey primary anti-secondary antibodies (Jackson ImmunoResearch, 1:1000).

17 Finally, wash cells and counterstain with DAPI (Invitrogen).

Quantitative image analysis of differentiated neuronal cultures 1w 0d 3h 1m

18 Perform quantitative image analysis of differentiated neuronal cultures using the Multi-Wavelength Cell Scoring module in MetaMorph software (Molecular Devices).

19 Briefly, dissociate EBs enzymatically and plate in the presence of neurotrophic factors at densities for which cell overlap was minimal.

20 Following immunostaining, capture images of at least nine randomly selected fields (15,000 cells in total) for each condition using a preprogrammed automated microscope stage.

21 Analyze images using the Multi-Wavelength Cell Scoring module of the MetaMorph software, using parameters predefined to count only unambiguous bright labeling for each antigen.

22 Set intensity thresholds while blinded to sample identity to selectively identify positive cells that displayed unambiguous signal intensity above local background.

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These parameters are used on all samples in a given experiment, and only minimally adjusted for different staining batches as necessary. Script and Parameter files are available upon request (typically, a cell is approximately 5000 gray levels above background to be called positive for any nuclear marker and is approximately 10,000 for cytoplasmic markers).

23 Analyze a minimum of 15,000 cells per sample.

24 Image all samples using 10× or 20× objectives on a Zeiss AxioObserver with a Coolsnap HQ2 camera (Photometrics).

25 Acquire some images using a structured illumination technique using an Apotome module (Zeiss) to achieve **1.9 µM** optical sections to ensure colocalization of labeling.

26 For the figures, adjust the brightness and contrast of each channel of an image in an appropriate manner to improve clarity.

27 For Ca^{2+} imaging experiments using the Hb9::GFP reporter, differentiate stem cells under the motor neuron differentiation protocol described above, dissociate at day 21 or day 31, and sort FACS based on green fluorescent protein (GFP) intensity with a five laser ARIA-IIu ROU Cell Sorter configured with a **100 µM** ceramic nozzle and operating at 20 psi (BD BioSciences).

28 Comprise the H9 assays of mixed neuronal cultures, for which a parallel coverslip is stained and quantified to have 53% $HB9/ISL1^{+}$ motor neurons.

29 Plate all cultures onto **15 mm** – **25 mm** diameter coverslips at a density of 100,000–150,000 cells per coverslip in day 17+ Neurobasal media with factors described above, with the addition of **0.5 Micromolar (µM)** EdU, and mature 6 d before Ca^{2+} imaging.

30 Load cells with **3 Micromolar (µM)** Fluo-4 AM (Invitrogen) dissolved in 0.2% dimethylsulfoxide/0.04% pluronic acid (Sigma) in HEPES-buffered physiological salt solution (PSS) for **01:00:00** at **Room temperature** .[1h]

PSS contained the following (in mM): 145 NaCl, 5 KCl, 10 HEPES, 2 $CaCl_2$, 2 $MgCl_2$, and 5.5 glucose (pH 7.4).

31 Superfuse cultures continuously with PSS at a rate of approximately **0.5 ml/min** .

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32 Image the cultures using a 10× objective on an inverted epifluorescent Zeiss AxioObserver microscope, equipped with a Coolsnap HQ2 camera (Photometrics).

33 For imaging spontaneous Ca 2^+ transients, acquire single sets of 200–300 images at a rate of approximately 2 Hz from each coverslip.

34 For the kainate (KA) experiments, acquire 36 images at a rate of 0.033 Hz and replace the superfusing PSS with PSS-containing KA ([M]**100 Micromolar (µM)**) for **00:01:00** . 1m

35 Perform image analysis using ImageJ (http://rsb.info.nih.gov/ij/) or AxioVision 4.7 (Zeiss).

36 Determine Ca $^{2+}$ transients from regions of interest encompassing the soma of individual cells.

37 Obtain a minimum of two cultures from a single differentiation of each cell line and use each time point for the KA and all Ca $^{2+}$ imaging experiments.

Whole-cell patch-clamp recordings 1w 0d 3h 1m

38 For whole-cell patch-clamp recordings, Plate S + P differentiated HUES3 Hb9::GFP cells on polyornithine/laminin-coated **25 mm** diameter coverglass at density of 50,000 per coverslip and culture for **168:00:00** in the presence of [M]**0.5 Micromolar (µM)** EdU before recording (i.e., 21 + 7 days in vitro; DIV). 1w

39 Perform current-clamp recordings using an Axopatch 2B amplifier.

40 Digitize data using a Digidata 1322A digital to analog converter and record at a 10 kHz sample rate using pClamp 10 software (all equipment from Molecular Devices).

41 Fabricate patch pipettes using a P-97 pipette puller (Sutter Instruments).

The external recording solution contained the following (in mM): 145 NaCl, 5 KCl, 10 HEPES, 10 glucose, 2 $CaCl_2$, and 2 $MgCl_2$.

42 Adjust the [pH]**7.3** using NaOH and adjust the osmolality to **325 mOSm** with sucrose.

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The pipette solution contained the following (in mM): 130 CH_3KO_3S, 10 CH_3NaO_3S, 1 $CaCl_2$, 10 EGTA, 10 HEPES, 5 MgATP, and 0.5 Na_2GTP (pH 7.3, 305 mOsm).

Whole-cell patch-clamp recordings 1w 0d 3h 1m

43 Perform experiments at **Room temperature** (**21 °C** – **23 °C**).

44 During recordings, inject current to hold the cells at approximately -60 mV.

45 Evoke action potentials (APs) using incrementally increasing current steps 1 s in duration.

46 Calculate the maximum amplitude of the current step (20–50 pA) and the size of the increment based on the input resistance of the cell.

Xenotransplantations 1w 0d 3h 1m

47 To perform xenotransplantations day 21, collect EBs from HUES3 Hb9::GFP under ventralization with SAG + PUR and place into L-15 media (Invitrogen) containing penicillin/streptomycin (Invitrogen).

48 Perform transplantation as previously described (Wichterle et al., 2002).

49 Briefly, load lightly triturated EBs into a handheld micro-injector, after a small suction lesion at the prospective intraspinal site was created in a chick embryo at stage 15–18 at somites 15–20.

50 Place the EBs into the lesion.

51 After 48 h, sacrifices the chicks, fix with 4% PFA for **02:00:00** at **4 °C** , and access neurite outgrowth and cell body placement by cutting **200 µM** vibratome sections (n = 2) or by cutting **30 µM** sections along the spinal cord (n = 5). 2h

52 Collect human embryonic spinal cords in accordance with the national guidelines of the United States (National Institutes of Health, U.S. Food and Drug Administration) and the State of New York and under Columbia University institutionally approved ethical guidelines relating to anonymous tissue.

53 Obtain the material after elective abortions, and classify on the basis of external morphology according to the Carnegie stages.

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54 Determine gestational age by last menstrual period of the patient or by ultrasound, if the ultrasound estimate differed by >1 week as indicated by the obstetrician.

55 Remove the spinal cord as intact as possible before fixation with fresh, cold 4% PFA for **01:30:00** **On ice** . 1h 30m

56 Postfixation, measure the cord and cut into three anatomical sections to accommodate embedding in OCT Compound (Tissue-Tek) and store at **-80 °C** before cutting on a microtome.

57 Cut sections (**12 µM**) along the full length of the cord, taking care to have all three sections on each slide in seven independent sections.

> This allowed for full analysis and internal staining controls since each slide had cervical, brachial, thoracic, and lumbar sections that clearly showed staining within the various otor columns present at different rostrocaudal levels of the spinal cord.

58 Obtain cDNA from 50,000 FACS-purified motor neurons from either day 21 S + P (methods described above), or from RA/SHH motor neurons at day 31.

59 Perform cDNA preparation using commercially available kits following the manufacturer's instructions: RNA isolation (Trizol LS; Invitrogen) and cDNA by Brilliant II SYBR green (Stratagene) without amplification.

60 Process all samples in parallel on the same quantitative PCR (qPCR) plate (Table 2).

Table 2. qPCR primer sequences

Primers	Forward	Reverse
RALDH2	TTTTGCTGATGCTGACTTGG	GCAGCACTGACCTTGATTGA
FOXP1	TGACCTTTTGAGGTGACTATAACTG	TGGCTGAACCGTTACTTTTTG
LHX3	GTTCAGGAGGGGCAGGAC	CCCAAGCTCCCGTAGAGG
CHT1	AAGCCATCATAGTTGGTGGCCGAG	CCAAGCTAGGCCATAACCTGGTAC
HOXA5	CAGCACCCACATCA	CGGAGAGGCAAAGA
HOXC6	CCAGGACCAGAAAGCCAGTA	GTTAGGTAGCGATTGAAGTGAAA
HOXC8	CTTCGCTGTTTGATTTCTATTCTG	TACGCTGGAGGTTTCTTTCTTT
HOXD9	TCGCTGAAGGAGGAGGAGA	CAAACACCCACAAAGGAAAAC

STD qPCR amplification: 95°-30″, 55°-60″, 72°-45″.

Paired-end RNA-Seq experiments

61 For paired-end RNA-Seq experiments, Prepare **400 ng** of total RNA after FACS purification of 500,000 GFP^+ or GFP^-

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cells.

62 Amplify the RNA samples using a NuGEN RNA kit for genomic sample amplification, and sequence to a depth of 21 (S + P) and 35 (SHH) million paired-end reads on an Illumina HiSeq instrument at the HudsonAlpha Institute of Biotechnology.

63 Align the reads to the reference transcriptome as well as a library of exon junctions using Bowtie (Version 1) (Langmead et al., 2009).

64 Analyze data using Expression Plot (Friedman and Maniatis, 2011) using a p value of 0.001 and a two fold change threshold.

65 Perform gene ontology using DAVID (Huang da et al., 2009a, b) with enrichment sets from Expression Plot.

The RNA-seq data are available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE41795.

66 Analyze all quantitative data using Sigma Plot 11 or Microsoft Excel.

67 Subject sample groups to Student's t-test or where appropriate a one-way ANOVA with Holm–Sidak post hoc pairwise comparisons was performed.

All experimental data passed an equal variance and normality test (Shapiro–Wilk).

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