bio-protocol

Published: Jul 20, 2022

Gene Expression Analysis in Stem Cell-derived Cortical Neuronal Cultures Using Multi-well SYBR Green Quantitative PCR Arrays

Vasavi Nallur Srinivasaraghavan, Faria Zafar and Birgitt Schüle*

Department of Pathology, Stanford University School of Medicine, Stanford, USA
*For correspondence: bschuele@stanford.edu

Abstract

To optimize differentiation protocols for stem cell-based *in vitro* modeling applications, it is essential to assess the change in gene expression during the differentiation process. This allows controlling its differentiation efficiency into the target cell types. While RNA transcriptomics provides detail at a larger scale, timing and cost are prohibitive to include such analyses in the optimization process. In contrast, expression analysis of individual genes is cumbersome and lengthy.

Here, we developed a versatile and cost-efficient SYBR Green array of 27 markers along with two housekeeping genes to quickly screen for differentiation efficiency of human induced pluripotent stem cells (iPSCs) into excitatory cortical neurons. We first identified relevant pluripotency, neuroprogenitor, and neuronal markers for the array by literature search, and designed primers with a product size of 80-120 bp length, an annealing temperature of 60°C, and minimal predicted secondary structures. We spotted combined forward and reverse primers on 96-well plates and dried them out overnight. These plates can be prepared in advance in batches and stored at room temperature until use. Next, we added the SYBR Green master mix and complementary DNA (cDNA) to the plate in triplicates, ran quantitative PCR (qPCR) on a Quantstudio 6 Flex, and analyzed results with QuantStudio software.

We compared the expression of genes for pluripotency, neuroprogenitor cells, cortical neurons, and synaptic markers in a 96-well format at four different time points during the cortical differentiation. We found a sharp reduction of pluripotency genes within the first three days of pre-differentiation and a steady increase of neuronal markers and synaptic markers over time. In summary, we built a gene expression array that is customizable, fast, medium-throughput, and cost-efficient, ideally suited for optimization of differentiation protocols for stem cell-based *in vitro* modeling.

Keywords: Human iPSCs, Induced pluripotent stem cells, Cortical neurons, Neuronal differentiation, SYBR Green, Quantitative PCR, Multi-well qPCR, Primer design

This protocol was validated in: Stem Cell Res (2022), DOI: 10.1016/j.scr.2022.102733

Background

The real-time quantitative PCR (qPCR) technique detects amplification of target nucleic acid sequences, and it is considered sensitive, reproducible, and specific (Arya *et al*., 2005).

Here, we use multi-well qPCR assays to amplify multiple genes with SYBR Green technology (Arikawa *et al*., 2011). SYBR Green is a DNA binding dye that binds non-specifically to double-stranded DNA (dsDNA) (Boone *et al*., 2015).

Based on the RT^2 Profiler^{TM} array (Arikawa *et al*., 2011), we designed a multi-well SYBR Green qPCR panel to analyze the expression of genes involved in the differentiation of cortical neurons from the human iPSCs by forced expression of Neurogenin 2 (Ngn2), which is a neuronal transcription factor supporting neuronal differentiation of human embryonic stem cells or iPSCs into cortical-like neurons (Zhang *et al*., 2013). Here, we used a human iPSC line with a doxycycline-inducible mouse Ngn2 transgene engineered into a safe harbor locus (Wang *et al*., 2017). We collected cells at different time points (iPSCs, Day 0 pre-neurons, Day 15, and Day 30 cortical neurons) and analyzed genes that mark pluripotent stem cells, intermediate neuroprogenitors, and mature cortical neurons on a single 96-well plate. The amplicons/ primers were designed in the range of 80-120 bp, and the Tm was between 63°C and 66°C. The in-house preparation of the multi-well SYBR Green qPCR assay allows tailoring primers to specific experiments and assay modification as needed. This multi-well SYBR Green qPCR assay can be used for the quantitative analysis of any set of genes of interest. Different RT2 profiler arrays for pathway analysis are commercially available; however, none of them are optimized to follow the maturation of cortical neurons derived from iPSCs. We show that the multi-well SYBR Green qPCR is easily adaptable for customization in the laboratory. It is an economic platform and ideally suited for the optimization of differentiation protocols for *in vitro* stem cell modeling.

Materials and Reagents

A. RNA extraction

1. Homogenizer spin column (Thermo Fisher Scientific, Life Technologies, catalog number: 12183-026)
2. 2-mercaptoethanol (Sigma-Aldrich, Aldrich Chemistry, catalog number: M2650)
3. DNase I, amplification grade (Thermo Fisher Scientific, Invitrogen, catalog number: 18068015)
4. Ethanol, molecular grade (Thermo Fisher Scientific, catalog number: BP2818500)
5. PureLinkR RNA mini kit (Thermo Fisher Scientific, Life Technologies, catalog number: 12183025)
6. RNase away (Thermo Fisher Scientific, Life Technologies, catalog number: 10328011)

B. cDNA Reverse Transcription

1. MicroAmp 8-tube strip (Thermo Fisher Scientific, Applied Biosciences, catalog number: A30589)
2. High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Invitrogen, catalog number: 4368814)

C. Multi-well PCR reaction

1. MicroAmp Optical 96-well reaction plate (Thermo Fisher Scientific, Applied Biosystems, catalog number: N8010560)
2. MicroAmp Optical adhesive film (Thermo Fisher Scientific, Applied Biosystems, catalog number: 4311971)
3. Nuclease-free water (US Biological LifeSciences, catalog number: W0900)
4. PowerUp^{TM} SYBR^{TM} Green master mix (Thermo Fisher Scientific, Applied Biosystems, catalog number: A25780)

D. Consumables

1. 1.5 mL and 0.6 mL RNase-free microcentrifuge tubes
2. RNase-free filter pipette tips (P1000, P200, P20, and P2)

E. Others

1. 10× PBS, molecular grade (Fisher Scientific, catalog number: J75889K2
2. Accutase (Thermo Fisher Scientific, catalog number: NC9464543)
3. Bucket with wet ice
4. Personal protective equipment (gloves, lab coat, goggles)

Equipment

1. Revco Ultima II ultra low temperature -86°C freezer (Thermo Scientific, catalog number: ULT2586-9)
2. Microcentrifuge 5415C (Eppendorf, catalog number: M7282)
3. Refrigerated centrifuge (Beckman Coulter, catalog number: GS6 Allegra)
4. Mini centrifuge (Fisher Scientific, catalog number: 05-090-100)
5. Water bath (Thermo Fisher Scientific, Cole Parmer, catalog number: TSGP20)
6. MiniAmp™ thermal cycler (Applied Biosystems, Thermo Fisher Scientific, catalog number: A37834)
7. NanoDrop spectrophotometer (Thermo Fisher Scientific, catalog number: 13-400-525)
8. QuantStudio 6 Flex (Applied Biosystems, Thermo Fisher Scientific, catalog number: 4485691)
9. Optional: PlateR visual pipetting aid tablet (Biosistemika, catalog number: P-10)

Software

1. Beacon Designer (Premier Biosoft, http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1)
2. In silico PCR prediction (UCSC Genome Browser, https://genome.ucsc.edu/cgi-bin/hgPcr)
3. Primer3 software (Whitehead Institute for Biomedical Research, Steve Rozen, Maido Remm, Triinu Koressaar, and Helen Skaletsky, https://bioinfo.ut.ee/primer3-0.4.0/)
4. QuantStudio Flex 6-v1.7.1 (Applied Biosystems, ThermoFisher Scientific, https://www.thermofisher.com/us/en/home/global/forms/life-science/quantstudio-6-7-flex-software.html)
5. UNAFold (Integrated DNA technology, https://www.idtdna.com/UNAFold). Create an account to use the software

Procedure

A. Ngn2-guided cortical neuron sample collection

The iPSCs seeded at 1.5×10^5 cells, were differentiated in a 12-well plate (~40,000 cells per cm^2) according to the protocol of Wang *et al*. (2017) using an Ngn2-inducible cell line. The media was changed every other day. As shown in Figure 1, cells were collected at day -3 (3 days before doxycycline induction), day 0, day 15, and day 30 for iPSCs, pre-neurons, day 15, and day 30 cortical neurons, respectively, for the illustration of this SYBR Green multi-well array. A confluent well of a 12-well plate of iPSCs yields 3×10^6–4×10^6 cells, and the RNA yield will be approximately 8–10 µg total RNA. For pre-neurons, the RNA yield ranges between 5–6 µg from 2×10^6–3×10^6 cells, and for neurons (days 15 and 30), RNA yield ranges between 1–3 µg from 1×10^6–2×10^6 cells.

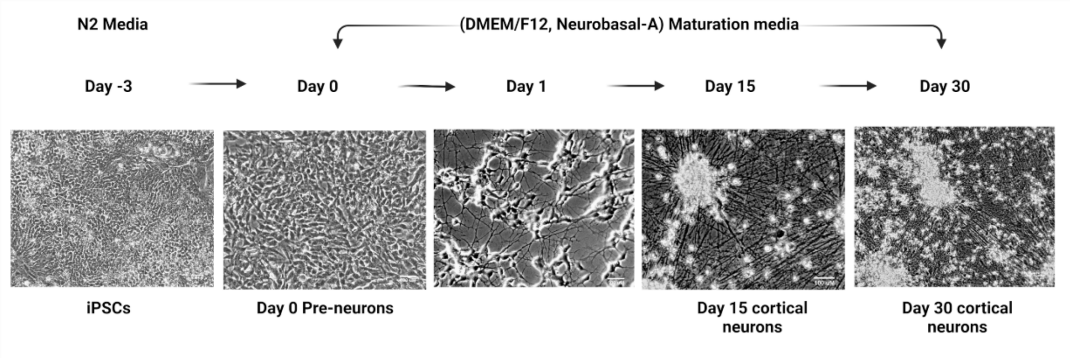

Figure 1. Timeline for the Ngn2-guided cortical neuronal differentiation and collection of samples for RNA extraction. Scale bar: 100 μm.

1. To dissociate the cells for sample collection, aspirate the old media, directly add 400 μL of Accutase per well of a 12-well plate, and incubate the cells at 37°C for 3–5 min to lift them off.
2. Add 400 μL of 1× cold PBS to Accutase, pipette the cell solution up and down to dissociate, and transfer the cell suspension to a 1.5 mL Eppendorf tube. Wash the well with additional 400 μL of 1× cold PBS to collect the remaining cells and add to the same Eppendorf tube.
3. Centrifuge the cell suspension at 15,000 × *g* at 4°C for 5 min.
4. Aspirate and discard the supernatant.
5. Wash the cell pellet with 1 mL of 1× cold PBS by pipetting up and down several times, and centrifuge again at 15,000 × *g* at 4°C for 5 min.
6. Aspirate the supernatant while keeping it on wet ice.
7. Continue with RNA extraction or store the cell pellet at -80°C.

B. RNA extraction and purification (PureLinkR RNA Mini Kit, Thermo Fisher, Life Technologies, 12183025)

Preparation:

1. Clean the bench with 70% ethanol and RNase Away.
2. Use RNase-free barrier filter tips for RNA extraction.
3. Before using Wash Buffer II for the first time:
   a. Add 60 mL of 96–100% ethanol directly to the Wash buffer bottle.
   b. Check the box on the Wash Buffer II label to indicate that ethanol was added.
   c. Store Wash Buffer II with ethanol at room temperature.
4. Prepare fresh Lysis Buffer containing 1% 2-mercaptoethanol for each purification. Under the chemical hood, add 3 μL of 2-mercaptoethanol per 300 μL of Lysis Buffer.

Procedure:

1. Take the cell pellet from the -80°C freezer and place it on ice (perform subsequent steps at room temperature).
2. Add 300 μL of Lysis Buffer to the cell pellet.
3. Vortex for 10 s at high speed until the cell pellet is thoroughly mixed.
4. Transfer the lysate to a homogenizer spin column inserted in an RNase-free Eppendorf tube and centrifuge at 12,000 × *g* for 2 min. Remove and discard the homogenizer cartridge after centrifugation.
5. Add 300 μL of 70% ethanol to the cell homogenate.
6. Vortex to mix thoroughly and to disperse any visible precipitate that may form after adding ethanol.
7. Transfer up to 700 μL of the sample to a spin cartridge (with a collection tube).

8. Centrifuge at 12,000 × *g* for 15 s at room temperature. Discard the flow-through and reinsert the spin cartridge into the same collection tube.
9. Add 700 µL of Wash Buffer I to the spin cartridge. Centrifuge at 12,000 × *g* for 15 s at room temperature. Discard the flow-through and the collection tube. Place the spin cartridge into a new collection tube.
10. Add 500 µL of Wash Buffer II (supplemented with ethanol) to the spin cartridge.
11. Centrifuge at 12,000 × *g* for 15 s at room temperature. Discard the flow-through and reinsert the spin cartridge into the same collection tube.
12. Repeat steps 10-11 once.
13. Centrifuge the spin cartridge at 12,000 × *g* for 2 min to dry the membrane containing the RNA.
14. Discard the collection tube and insert the spin cartridge into a new recovery tube.
15. Add 30 µL of RNase-free water to the center of the spin cartridge.
16. Incubate at room temperature for 1 min.
17. Centrifuge the spin cartridge for 2 min at ≥12,000 × *g* at room temperature to elute the RNA from the membrane into the recovery tube.
18. Add the eluted RNA sample to the same spin cartridge again and repeat steps 16–17 to increase the yield of the RNA sample.
19. The concentration of the eluted RNA is determined using a NanoDrop spectrophotometer.
20. The concentration of RNA varies between cell types as mentioned in Figure 2.
21. **Critical step!** Aliquot the RNA into RNase-free tubes, each having 1 µg of RNA, to avoid freeze-thaw cycles and degradation of RNA. The aliquot volume for 1 µg of RNA is directly used for the DNase I treatment.
    Note: The aliquots are treated with DNase I before storage.
22. Proceed with DNase I treatment directly after RNA purification or freeze aliquots at -80°C.
23. It is absolutely critical to aliquot RNA and cDNA samples to avoid freeze-thaw cycles and avoid degradation of the sample.

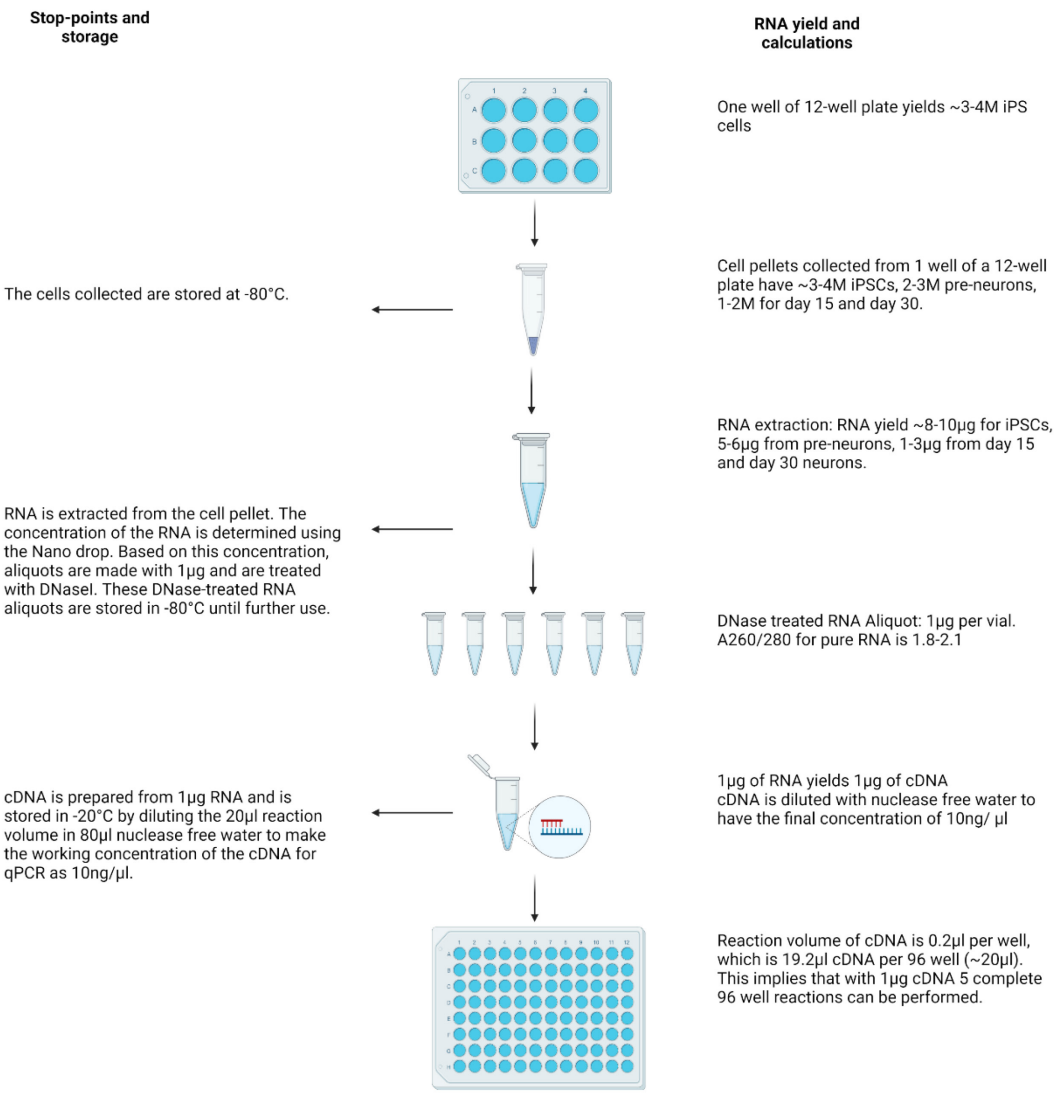

Figure 2. Workflow and calculations for the different cell types through the cortical neuron differentiation with stopping points and storage.

C. DNase I Treatment (Thermo Fisher Scientific, Invitrogen, catalog number: 18068015)

1. Combine the items shown in Table 1 in RNase-free tubes:

Table 1. Reaction mix for DNase I treatment of eluted RNA

Component	Volume
RNA (1 µg)	up to 8 µL
10× DNase I Buffer	1 µL
RNase-free Water	add up to 8 µL
DNase I, Amplification Grade	1 µL
Final Volume	10 µL

2. Mix and incubate for 15 min at room temperature.
3. Heat-inactivate the DNase I by adding 1 µL of 25mM EDTA to the DNase I treated RNA sample and place it in a water bath at 65°C for 3 min.
4. Once the RNA samples are DNase I heat-inactivated, place them on ice.
5. Proceed with the reverse transcription or store aliquots at -80°C.

D. cDNA Reverse Transcription (High-Capacity cDNA Reverse Transcription Kit, Thermo Fisher, Invitrogen, catalog number: 4368814)

The extracted RNA is converted to cDNA for qPCR amplification. As a control, prepare a reverse transcription control with no addition of the reverse transcriptase in the reaction mix.
The reaction mixture (RT mix) is prepared according to Table 2. The 1 µg RNA (10 µL volume) from the previous step is mixed with the reaction mix (Table 2) for a final volume of 20 µL for the cDNA reaction.

Table 2. Reaction mix for the cDNA reverse transcription reaction.

Component	Volume/reaction (µL)
10× RT Buffer	2.0
25× dNTP Mix (100mM)	0.8
10× RT Random Primers	2.0
Multiscribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free water	3.2
Total volume per reaction mix (RT mix)	10.0

1. Preparation of cDNA RT reaction:
   a. The RT mix is prepared, mixed gently, and placed on ice.
   b. Pipette 10 µL RT master mix into MicroAmp 8-tube strip.
   c. Add 10 µL of DNase I treated RNA sample (1 µg) to the reaction mix and mix by pipetting up and down several times.
      Notes:
      i. One tube has to be prepared containing the RNA input but not the Multiscribe™ Reverse Transcriptase. This is used as reverse transcriptase control (RTC), which can confirm that no genomic DNA is amplified.
      ii. Do not introduce bubbles while pipetting.
   d. The tubes are sealed and centrifuged to spin down the contents and eliminate air bubbles.
   e. Place the tubes on ice and load them into the MiniAmp™ Thermal Cycler.
2. Reverse transcription thermal cycling conditions (Table 3):
   a. The reaction volume is set to 20 µL.
   b. Load the reaction tubes into the thermal cycler.
   c. The thermal cycler program is set to RUN.
   d. Once the cycle is complete, add 80 µL of nuclease-free water to the 20 µL cDNA for a working concentration of 10 ng/µL, store the cDNA at -80°C.
   e. **Critical step!** Aliquot the sample to avoid freeze-thaw cycles and degradation of cDNA.

Table 3. Thermal cycler conditions of cDNA reaction.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

E. Primer Design

We designed SYBR Green primers using an established protocol (Thornton and Basu, 2011). The primer parameters of this section are: Product size: 80–120 bp, product melting temperature (Tm): 63–66°C, secondary structure ΔG: not more than -3.5 and the GC percentage: 35–80%, with optimal at 65%. The primers were designed in an intron-spanning fashion, so that the forward and the reverse primer were not placed in the same exon. This can avoid contamination from genomic DNA.

1. Step 1: To obtain the sequence of the gene of interest in FASTA format from the National Center for Biotechnology Information (NCBI) website: http://www.ncbi.nlm.nih.gov.
   a. Select the Nucleotide option from the dropdown menu in search "All Databases".
   b. Enter the gene name or the sequence ID of interest in the search box and click on Search.
   c. Since the cortical neurons are differentiated from the human iPSCs, either mention homo sapiens along with the gene name in the search bar, or select homo sapiens from the species filter on the top left of the webpage after clicking on search.
   d. Click on the RefSeq transcripts and select the FASTA option, then click apply.
2. Step 2: After obtaining the FASTA sequence for the selected gene, design the primers using Primer3 software: https://bioinfo.ut.ee/primer3-0.4.0/ (SantaLucia, 1998), Figure 3.
   Note: The explanation for the default values is given in the webpage: https://bioinfo.ut.ee/primer3-0.4.0/input-help.htm.

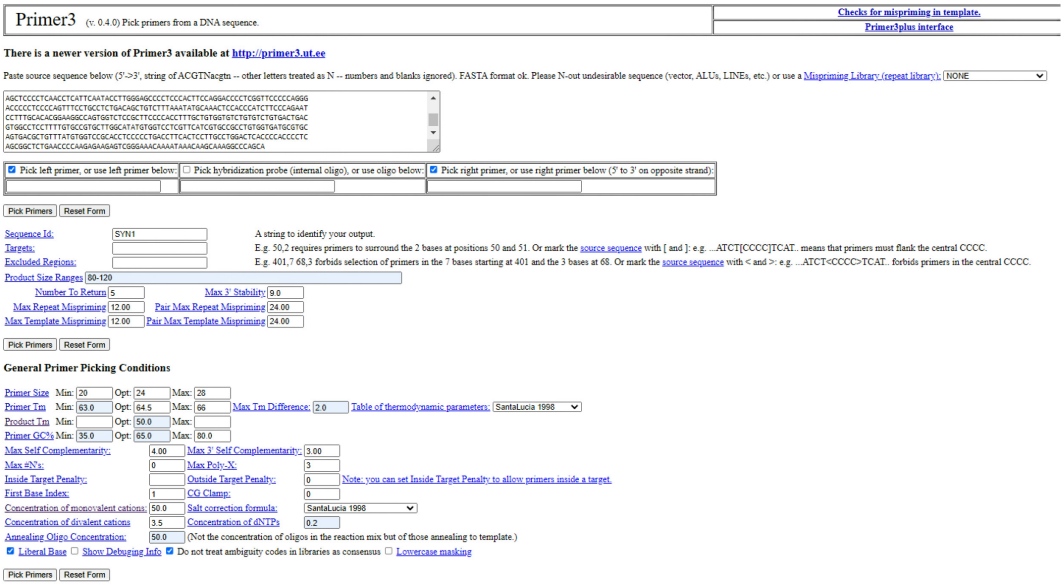

Figure 3. Screenshot of Primer3 webpage indicating the parameters and conditions for designing primers of the *SYNAPSIN1* (*SYN1*) gene.

   a. Copy and paste the FASTA format sequence of the gene interest into the box provided on the Primer3 primer design page.
   b. Pick left primer: This option was left blank for the software to pick the primers.
   c. Pick hybridization probe: This option was left blank (not required with this experiment).
   d. Pick right primer: If this option is left blank, the Primer3 program will choose the right primer.
   e. Sequence Id (Name of the gene): This is to identify the primers for the sequence. A proper name is set corresponding to the sequence.
   f. Targets (region of the sequence of the gene of interest): After looking into the CDS and exon regions (shown in Figure 4) the specific nucleotide position in the sequence is entered against the target,

following with the number of nucleotides along to be flanked for the primers to design surrounding that particular region. An example, on how to enter the target region is shown in Figure 4.

Figure 4. Image of NCBI webpage indicating the position of exons

g. Primer Tm: This is the temperature at which 50% of the primer is hybridized to the DNA template. For this experiment, all primers are designed in the range of 63–66°C Tm.

h. Maximum Tm difference: Enter the value as 2.

i. Table of Thermodynamic parameters: Primer3 uses these formulas to calculate the melting temperature. Set the method to SantaLucia (1998).

j. Product Tm: This is the temperature at which 50% of the amplicon is ssDNA. Set the optimal value to 50.

k. Primer GC: This is the minimum and maximum percentage of guanine and cytosine (GC) allowed. The GC content of primers is used to determine the melting temperature of the primer, which can be used to predict the annealing temperature. Set the values to Minimum: 35, Optimum: 65, Maximum: 80.

l. Max Self-complementary: Primers should not be self-complementary or complementary to each other. Primers that are self-complementary form self-dimers or hairpin structures. Enter the value as 4.

m. Max 3’ Self-complementary: As polymerases add bases at the 3’ end of the oligonucleotide, the 3’ ends of primers should not be complementary to each other, as primer dimers will occur. Enter the value as 3.

n. Max #N: This is the maximum number of unknown bases which Primer3 could consider for designing primers. Set value at 0.

o. Max Poly-X: The maximum number of mononucleotides repeats to allow in the primer. Long mononucleotide repeats can promote mispriming. Enter the value as 3.

p. Inside target penalty and outside target penalty: Used if the primer needs to be designed to overlap a region. Leave as default.

q. First Base Index: This parameter tells Primer3 which programming index type the first base in the input sequence is. Leave as default.

r. GC Clamp: Defines the specific numbers of Gs and Cs at the 3’ end of both the left and right primers. Leave the value as 0.

s. Conc. of monovalent cations: This is the millimolar concentration of KCl salt in the PCR. Enter the value as 50 µM.

Note: According to the SantaLucia (1998), the concentration for monovalent cations is assumed at 50 µM and at 3.5 mM for divalent cations. Other literature suggests a range for monovalent cations between 20 to 100 µM and divalent cations between 1.5 to 5 mM.

t. Salt correction formula: Factors such as ΔG and Tm affect PCR performance and alter the efficiency of primer pairs. The SantaLucia (1998) salt formula is preferred by Primer3. This formula is designed to accommodate the salt correction independent of sequence but dependent on oligonucleotide length.
u. Conc. of divalent cations: This is the concentration of divalent salts present in the PCR mix. Set value at 3.5 mM.
v. Conc. of dNTPs: A dNTP concentration of 200 µM is usually recommended for Taq polymerase to function efficiently in a conventional PCR. Some SYBR Green master mixes come with Taq, KCl, $MgCl_2$, and dNTP. These mixes tested in laboratories give maximum performance. Enter the value as 0.20 mM
w. Annealing Oligo Concentration: Used to calculate the oligo melting temperature, this is the nanomolar concentration of annealing oligos in the PCR. Leave at default.
x. Objective function penalty weights for Primers: The penalty weights section allows Primer3 users to modify the criteria that Primer3 uses to select the best sets of primers.
Objective Function Penalty Weights for Primers:
• Tm Lt = 1; Gt = 1
• Size Lt = 1; Gt =1
• Self complementary = 3
• 3' Self complementary = 3
• #N's = 2
• All other values = 0
Objective Function Penalty Weights for Primer Pairs:
• Product Tm: Lt = 1; Gt = 1
• Tm difference = 2
• Any complementary = 3
• 3' complementary = 3
• Primer Penalty weight = 1
• All other values = 0

3. Analyzing primers: Once all the Primer3 parameters are set as mentioned above, Primer3 will design primer pair options.
a. Once all options are entered, press 'Pick Primers'.
b. The sequence will be displayed under the primers with the details on the primers generated, and the location of the primers within the sequence is indicated by >>>>>>> for the forward primer and <<<<<< for the reverse primer. The first primer that has the correct product length and Tm is analyzed for the secondary structure using Beacon Designer™ free edition. Select the primer pair based on the 3′ value, which should not be more than 3.00. The 3′ value is the measurement of primer dimers formed within the primer pair (Figure 5).

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3'  seq
LEFT PRIMER        642   20   65.51   65.00  2.00  0.00 GGTGAAGGTCGTGCGGTCTC
RIGHT PRIMER       723   21   65.46   57.14  3.00  2.00 GTAGTCTCCGTTGCGTGCCAT
SEQUENCE SIZE: 3210
INCLUDED REGION SIZE: 3210

PRODUCT SIZE: 82, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00
```

Figure 5. Image of Primer3 output indicating the 3′ value, for the primer dimers in SYN1

4. Use Beacon Designer™ free edition to check for primer secondary structures. The acceptable ΔG value for the primers should not be more than -3.5.
a. Go to http://free.premierbiosoft.com. Click on Beacon Designer [Free Edition]. Then click on launch Beacon Designer™ Free Edition.

b. Click the SYBR Green option and enter the left primer sequence in the box for 'Sense primer.' Enter the right primer sequence in the box for 'Anti-sense primer.' Click 'Analyze.'

c. Beacon Designer free edition allows you to visualize secondary structures that can form between primers or primer pairs. An example of the secondary structure analysis is shown in Figure 6.
Note: If self-dimers or cross dimers cannot be avoided, choose primers with the highest -ΔG (meaning the least negative number, the one closest to zero). Redesign primers with ΔGs more negative than -3.5 kcal/mol. If hairpins cannot be avoided, steer clear of hairpins that involve a 3' end, and use UNAFold software to determine the melting temperature of the structure.

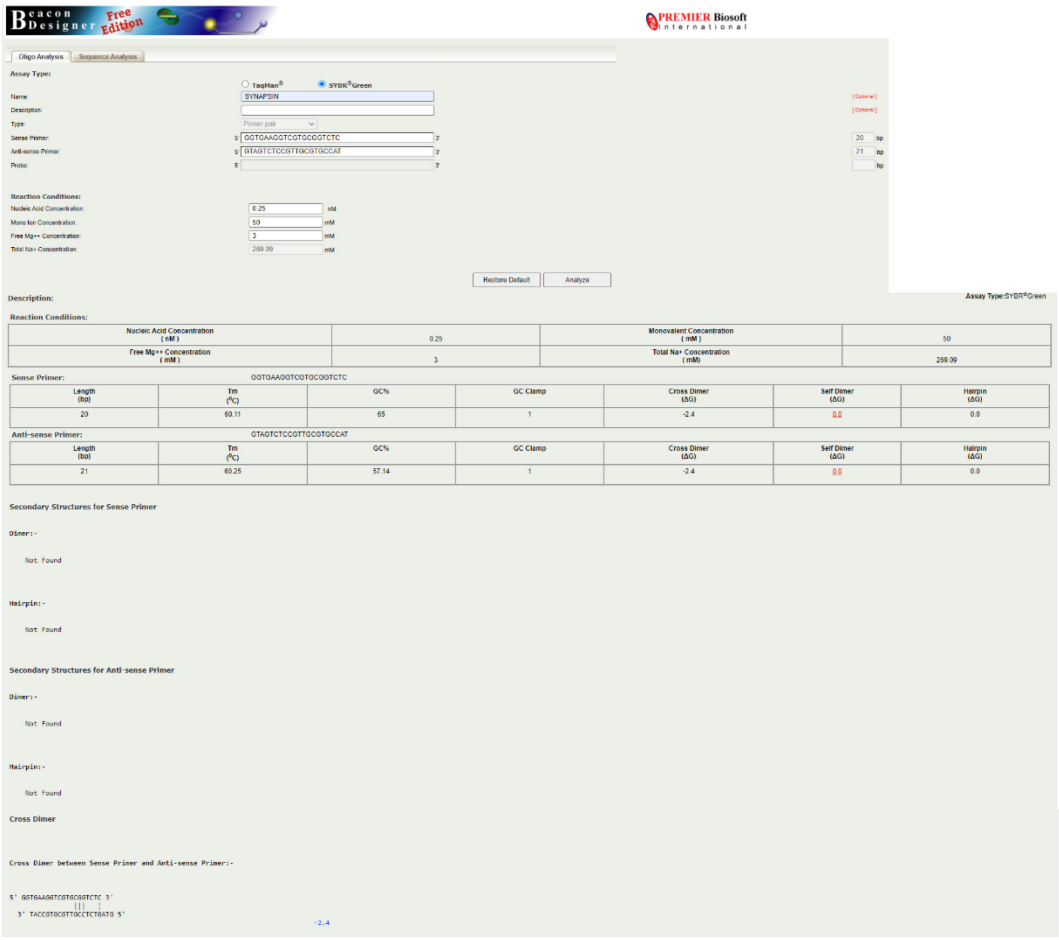

Figure 6. Images of Beacon Designer webpage for analyzing the secondary structure of SYN1 primer.

5. Use UNAFold software to check amplicon secondary structures.
   a. Once the primers have been checked for secondary structures, an additional QC step is needed to verify the amplicon's secondary structures using UNAFold software by Integrated DNA Technologies (IDT).
   b. Go to: https://www.idtdna.com/UNAFold. Copy the amplicon (include both forward and reverse primers) into the sequence box. Change the annealing temperature to 60°C and the magnesium concentration to 3 mM. Click submit.
   c. Evaluate the structures that are displayed for the amplicon by checking for the ΔG values, which should not be -3.5.
6. In-silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr): This is a QC step for verification of the exon spanning of the designed forward and reverse primers and also to cross verify the Tm and product length of the primer sets (Figure 7).

a. Insert the forward and the reverse primer for each gene into the blank spaces.
b. It is also important to set the target to GENCODE, which shows cDNA sequence, whereas genome assembly shows genomic DNA and allows to verify of intron spanning primer sequences. https://genome.ucsc.edu/FAQ/FAQgenes.html#ens.
c. Select submit, to study results of in-silico PCR.

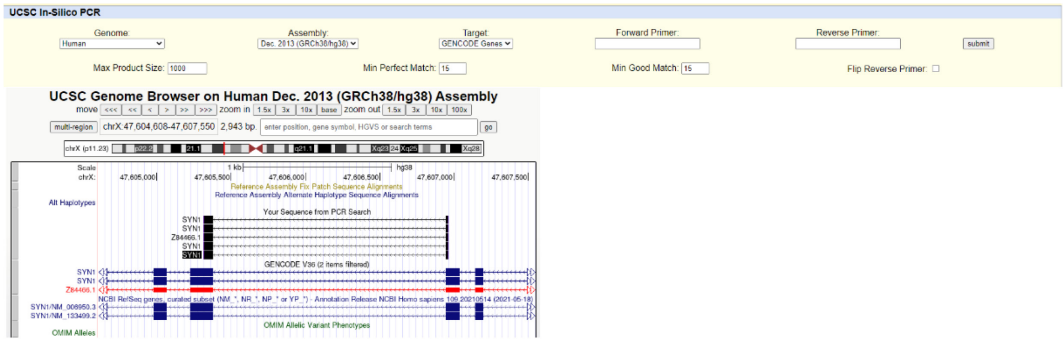

Figure 7. In-Silico PCR analysis and result for SYN1 in UCSC browser.

7. All the primers are designed and ordered at a 40 nmol scale. Primer stocks are re-constituted at 100 µM in nuclease-free water and dilutions at 10 µM are the working concentration.

F. Test primer efficiency

It is important to test primer efficiency for each primer pair by creating a standard curve with five serial dilutions of your cDNA template, *e.g.*, 1:5 dilution. The primer efficiency should lie between 90–110%. The standard curve should cover the Ct value of the experimental value (Figure 8).

1. The primer (forward and reverse) concentrations are 300 nM and the cDNA concentration is set for a 5-fold serial dilution (12.5 ng, 2.5 ng, 0.5 ng, 0.1 ng, 0.02 ng) for a 5 µL reaction volume as triplicates in a 384-well plate.
2. The primer efficiency is calculated using the Excel template available at https://toptipbio.com/calculate-primer-efficiencies/.

OCT4- 300nM						
Tube	Ct 1	Ct 2	Ct 3	Avg Ct	Sample Quantity	Log (sample quantity)
A	18.343	18.721	18.356	18.47333	1.0000	0.00
B	21.087	21.105	21.107	21.09967	0.2000	-0.70
C	23.408	22.591	22.603	22.86733	0.0400	-1.40
D	25.686	25.315	25.403	25.468	0.0080	-2.10
E	27.177	28.038	27.858	27.691	0.0016	-2.80
					Dilution Factor	5
					Slope	-3.262467134
					R Squared	0.9972
					Efficiency (%)	102.54

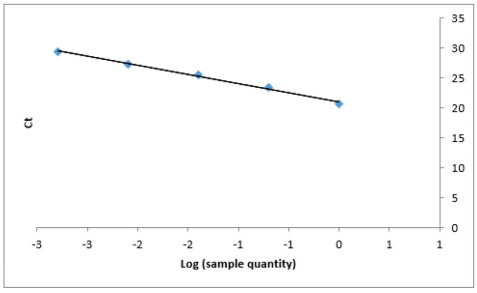

Figure 8. Primer efficiency calculation of genes involved in different timepoints of Ngn2 cortical neuron differentiation.
OCT4 is tested for its efficiency with iPS cells.

G. Plating of primers for multi-well SYBR Green qPCR array for cortical neuron differentiation

For this array, we designed SYBR Green primers for 27 genes with two housekeeping genes. We wanted to capture pluripotency genes, neuro-precursor genes, and genes that are upregulated in cortical neurons. We also looked for synaptic markers and astrocyte gene expression. We used triplicates for each gene in a 96-well optical plate to study the expression (ΔΔCt) for each gene along with negative control (NC) and reverse transcriptase control (RTC). The working concentration of the primers diluted in nuclease-free water was 10 µM.

1. Prepare the arrays for the PCR run at least a day in advance for the primers to dry out overnight.
2. Plate primers according to the design of the 96-well, in triplicates (Figure 9). Pre-mix the forward and reverse primer for each gene and add to the corresponding well. The calculation for the amount of primer to be added to each well is shown in Step 3.

	1	2	3	4	5	6	7	8	9	10	11	12
A	OCT4	OCT4	OCT4	NANOG	NANOG	NANOG	SOX1	SOX1	SOX1	SOX2	SOX2	SOX2
B	PAX6	PAX6	PAX6	NEUROD1	NEUROD1	NEUROD1	BCL11B	BCL11B	BCL11B	CUX1	CUX1	CUX1
C	EMX1	EMX1	EMX1	EMX2	EMX2	EMX2	FEZF2	FEZF2	FEZF2	FOXG1	FOXG1	FOXG1
D	HOXB4	HOXB4	HOXB4	POU3F2	POU3F2	POU3F2	SATB2	SATB2	SATB2	SIX6	SIX6	SIX6
E	SLC17A7	SLC17A7	SLC17A7	TBR1	TBR1	TBR1	TBR2	TBR2	TBR2	GFAP	GFAP	GFAP
F	S100B	S100B	S100B	NESTIN	NESTIN	NESTIN	DCX	DCX	DCX	ALDH1	ALDH1	ALDH1
G	Ki-67	Ki-67	Ki-67	BSN	BSN	BSN	SYN	SYN	SYN	GAPDH	GAPDH	GAPDH
H	B-ACTIN	B-ACTIN	B-ACTIN	NC	NC	NC	RTC	RTC	RTC			

Figure 9. 96-well plate template design for the multi-well qPCR reaction.

3. Primer volume:
   a. Primer concentration in 20 µL qPCR reaction volume.
   b. Total reaction volume of qPCR reaction per well of 96 well plates: 20 µL.
   c. Primer volume (from 10 µM working solution): 0.6 µL (forward primer) + 0.6 µL (reverse primer) to reach 300 nM per reaction.
   d. Total: 1.2 µL of pre-mixed primers per gene per well.
   e. The primer mix is prepared in triplicates per gene. The amount of primer mix is (0.6 µL + 0.6 µL) × 4 times (includes 1 extra reaction for pipetting error). Prepare mix depending on the number of plates that are prepared.

Optional: For plating of 96- or 384-well plates, pipetting can be facilitated by using PlateR (Biosistemika), a tablet-based visual support to pipette samples into wells.

4. **CRITICAL**. Dispense the primer mix at the bottom of the well and spin down the plate for the primer mix to settle at the bottom of the well.
5. Let the primer-coated plates dry overnight at room temperature in a Tupperware container to reduce the contamination.
6. Time consideration: Coating of primers for a single 96-well plate takes about 20–30 min.
   Note: Do the primer coating for the array design after optimization in large batches. Plates can be stored at room temperature for several months, e.g., in sealed plastic bags.
7. The concentration of primers and cDNA was determined as 300 nM and 2 ng (calculated for 96-well) based on the primer efficiency experiments (done in a 384-well plate) for each gene primer pair.

H. qPCR Analysis (PowerUp™ SYBR™ Green Master Mix [Thermo Fisher, catalog number: A25780])

PCR reaction mix:

1. Since the primers are already pre-coated onto the plates, only the SYBR Green master mix, cDNA template, and nuclease-free water are combined as a master mix and added to each well. Since the pre-coated primers dry out, the master mix is made only with reagents shown in Table 4, which would be total volume of 20 µL.
2. Before adding to the plate, the reaction mix is mixed thoroughly and spun down to avoid air bubbles. The plate should be kept on ice while adding the reaction mix. Calculate 1–2 extra reactions to account for pipetting errors. For a 96-well plate reaction, *e.g.*, prepare a 98× reaction mix (Table 4).

Table 4. Reaction mix for the multi-well Qpcr.

Components	Volume (each well)
SYBR Green master mix	10 µl
cDNA template	2 ng
Nuclease free water	variable
Total reaction mix	20 µl

3. Once the reaction mix is added, tightly seal the 96-well plate with the MicroAmp Optical adhesive film and centrifuge them briefly to remove any air bubbles and to bring all of the reaction mix to the bottom of the well.

I. Plate set-up in QuantStudio 6 Flex and link to the software

1. Once the plate is ready for the qPCR run, open the QuantStudio software v1.7.1 (link mentioned in the software section).
2. PCR reaction set-up (for Tm of Primers more than 60°C):
   a. Spin down the plate after adding the reaction mix and then place it in the QuantStudio 6 Flex.
   b. The thermal cycling settings are set according to the Table 5 below for the Tm of primers between 63–66°C. Annealing temperature should be approximately 3°C lower than Tm.

Table 5. Thermal cycling settings for the PCR reaction.

Step	Temperature	Duration	Cycles
UDG activation	50°C	2 min	Hold
Dual-Lock™ Taq DNA polymerase	95°C	2 min	Hold
Denature	95°C	15 s	40
Anneal/ extend	60°C	1 min	

   c. The instrument should be set for the default dissociation step as shown in Table 6.

Table 6. Setting for the dissociation step.

Step	Ramp rate	Temperature	Time
Denature	1.6°C/s	95°C	15 s
Anneal	1.6°C/s	60°C	1 min
Dissociation	0.15°C/s	95°C	15 s

   d. Once the run is complete the Ct data can be analyzed by clicking on analyze and the data can be exported as an excel file by clicking on the excel sheet.

e. The plate setup in Quantstudio 6 is illustrated in Figure 10. For detailed plate set up, run parameters, and analysis, follow the User Guide for the QuantStudio software attached: https://tools.thermofisher.com/content/sfs/manuals/4489822.pdf.

Figure 10. Workflow representation of setting up the plate in a QuantStudio 6 Flex software.

Data analysis

For the data analysis, we used the QuantStudio software to analyze the Ct values of each gene and the variability of replicates. The acceptable cycle difference should not be more than 0.5 cycles. The housekeeping genes B-ACTIN or GAPDH are used as the loading controls for ΔΔCt calculation (Livak and Schmittgen, 2001) of genes comparing the Day 0, Day 15, and Day 30 cortical neurons Ct values to the iPSCs Ct value. The negative template control should not show amplification (The link for the calculation of ΔΔCt in Excel is https://1drv.ms/x/s!AgUabBW4Y2yQgZEon-ZKuwMJ4PhaLQ).

As shown in Table 7, to calculate the average of $2^{-\Delta\Delta Ct}$ fold change of genes, we initially compared the Ct of the gene of interest with the house-keeping gene (control), B-ACTIN, and the difference between them is the ΔCt. Next, to calculate the ΔΔCt, take an average of the ΔCt values of the iPSCs. When we subtract the ΔCt values of each sample from the average $(\Delta Ct)_{iPSCs}$, we get the ΔΔCt. With the ΔΔCt values, we calculate the $2^{-\Delta\Delta Ct}$ and the average of $2^{-\Delta\Delta Ct}$. This shows the fold change in the expression of genes in each sample relative to their expression in iPSCs. To visually illustrate the changes in gene expression, we plotted a heatmap for samples at different time points using GraphPad Prism. The pluripotency markers OCT4 and NANOG are highly expressed in the iPSCs (Figure 10) and expression is drastically reduced and, in some instances, undetectable with the assay at Day 15 and 30 of the cortical neuronal differentiation. Markers for early mature cortical, neuronal, and synaptic proteins show an increase in their expression on day 15 and 30 samples compared to the iPSCs (Figure 11). Both housekeeping genes B-ACTIN and

GAPDH showed comparable results.

Table 7. ΔΔCt calculation of *NANOG* gene.

NANOG							
Sample type	Ct of Gene of interest	Ct of B-ACTIN	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	Avg of $2^{-\Delta\Delta Ct}$	Avg $(\Delta Ct)_{iPSCs}$
iPSC	25.213	21.879	3.334	0.048	0.9674		
iPSC	25.117	21.886	3.231	-0.055	1.0391		3.286
iPSC	25.167	21.873	3.294	0.008	0.9947	1.0004	
Day 0	31.260	27.298	3.961	0.675	0.6262		
Day 0	31.160	27.069	4.091	0.805	0.5724		
Day 0	31.018	27.181	3.837	0.551	0.6825	0.6270	
Day 15	30.792	20.171	10.621	7.335	0.0062		
Day 15	30.730	20.160	10.570	7.284	0.0064		
Day 15	30.591	20.217	10.373	7.087	0.0074	0.0067	
Day 30	29.959	19.687	10.272	6.986	0.0079		
Day 30	30.306	19.743	10.563	7.277	0.0064		
Day 30	30.193	19.706	10.487	7.201	0.0068	0.0070	

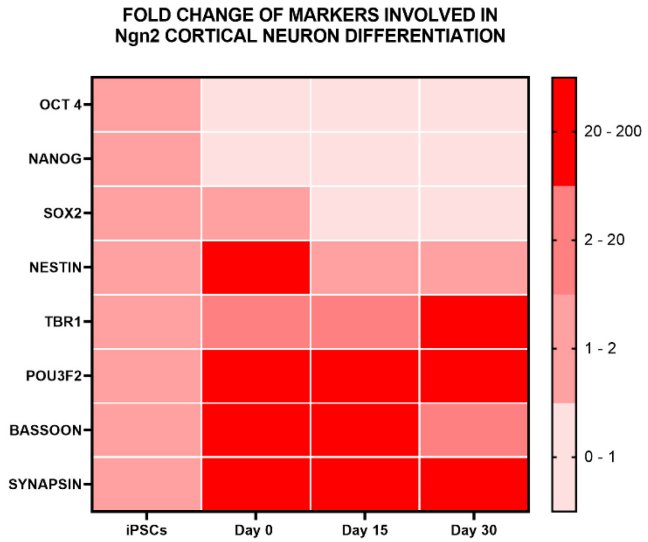

Figure 11. Fold change of gene expression using β-Actin as housekeeping gene comparing iPSCs with Day 0, Day 15, and Day 30 cortical neurons for pluripotency markers (OCT4 and NANOG), early neuronal markers (SOX2 and NESTIN), mature cortical neuronal markers (POU3F2 and TBR1) and synaptic markers (BASSOON and SYNAPSIN).

Summary:

In summary, we describe a comprehensive and economical protocol for a versatile multi-well SYBR Green qPCR protocol. Single-tube SYBR Green qPCR is a standard procedure in many labs to assess gene expression and there are also commercial platforms available for SYBR Green arrays. However, our protocol focusses on an efficient way to analyze multiple genes by building a multi-well qPCR array that can be customized and stored for several months at room temperature. These arrays are ideally suited to monitor iPSC differentiation protocols into various cell types and these arrays can be easily customized.

Acknowledgments

The study was supported by the California Institute for Regenerative Medicine (CIRM) Bridges program (TB1-01195).

Competing interests

Nothing to disclose.

Ethics

The work was approved under Stem Cell Research Oversight protocol SCRO-754 to use the WTC11 human-induced pluripotent stem cell for the Ngn2 differentiation.

References

Arya, M., Shergill, I. S., Williamson, M., Gommersall, L., Arya, N. and Patel, H. R. (2005). Basic principles of real-time quantitative PCR. *Expert Rev Mol Diagn* 5(2): 209-219.

Arikawa, E., Quellhorst, G., Ying, H., Pan, H. and Yang, J. (2011). RT2 Profiler™ PCR arrays: Pathway-focused gene expression profiling with qRT-PCR. *BioTechniques* 43(5).

Boone, D. R., Micci, M. A., Taglialatela, I. G., Hellmich, J. L., Weisz, H. A., Bi, M., Prough, D. S., DeWitt, D. S. and Hellmich, H. L. (2015). Pathway-focused PCR array profiling of enriched populations of laser capture microdissected hippocampal cells after traumatic brain injury. *PLoS One* 10(5): e0127287.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. *Methods* 25(4): 402-408.

SantaLucia, J., Jr. (1998). A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proc Natl Acad Sci U S A* 95(4): 1460-1465.

Thornton, B. and Basu, C. (2011). Real-time PCR (qPCR) primer design using free online software. *Biochem Mol Biol Educ* 39(2): 145-154.

Wang, C., Ward, M. E., Chen, R., Liu, K., Tracy, T. E., Chen, X., Xie, M., Sohn, P. D., Ludwig, C., Meyer-Franke, A., *et al*. (2017). Scalable Production of iPSC-Derived Human Neurons to Identify Tau-Lowering Compounds by High-Content Screening. *Stem Cell Reports* 9(4): 1221-1233.

Zhang, Y., Pak, C., Han, Y., Ahlenius, H., Zhang, Z., Chanda, S., Marro, S., Patzke, C., Acuna, C., Covy, J., Xu, W., Yang, N., Danko, T., Chen, L., Wernig, M. and Sudhof, T. C. (2013). Rapid single-step induction of functional neurons from human pluripotent stem cells. *Neuron* 78(5): 785-798.