RT-qPCR Protocol for ioCells

ioCells™

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1.0

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THE CELL CODING COMPANY

1. Introduction

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This quantitative reverse transcription PCR (RT-qPCR) protocol has been optimised for bit.bio's portfolio of human iPSC-derived cells. RT-qPCR is a powerful and highly sensitive technique that uses fluorescent dyes or probes for the specific detection and quantification of RNA transcripts in a biological sample. As such, RT-qPCR can provide precise insights into the gene expression profiles and transcriptional activity of target genes.

For characterising iPSC-derived cells at the transcriptional level, scientists rely on tracking the expression of specific gene markers to confirm cell identity or assess cellular states. For example, the downregulation of pluripotency markers, such as *POU5F1* (OCT4) and *NANOG*, alongside the robust upregulation of lineage-specific genes, provides a reliable molecular signature of cellular identity and maturity.

While RT-qPCR of iPSC-derived cells is a highly established process, it does require careful design, optimisation and selection of housekeeping and reference genes to ensure accurate and reproducible quantification across different cell types and differentiation stages.

The protocol outlines recommended reagents and conditions for cell lysis, RNA extraction, cDNA synthesis, and the subsequent qPCR amplification of key marker genes relevant to our range of ioCells products. While specific probes are recommended by cell type, the core protocol serves as a foundation that can be readily adapted by scientists for use with any target genes of interest.

This document focuses on the RT-qPCR procedure itself. It assumes that the thawing, culturing, and harvesting of the cells have been performed according to the instructions in the specific User Manual provided with the chosen product.

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2. Materials and equipment

2.1. Cells

- ioAstrocytes and derivatives
- ioGABAergic Neurons and derivatives
- ioGlutamatergic Neurons and derivatives
- ioMotor Neurons and derivatives
- ioOligodendrocyte-like cells and derivatives
- ioOPC-like cells and derivatives
- ioSensory Neurons and derivatives
- ioSkeletal Myocytes and derivatives

2.2. Equipment

- -80°C freezer
- Nanophotometer
- Thermocycler
- qPCR Instrument
- Benchtop spinner
- Micro-centrifuge capable of spinning >21,000 x g
- 1000 µL, 200 µL, 20 µL and 10 µL micropipettes
- Multi-dispense pipette
- 10 µL Multichannel pipette
- 1.5 mL microcentrifuge tubes
- Eppendorf tubes 1.5 mL
- 96-well plate (non-skirted)
- Optical qPCR 384-well plate
- Adhesive optical seals
- DNA Low bind 1.5 mL microcentrifuge tubes
- Optical qPCR plates (200 µL)
- PCR reaction tube strips

For required reagents, kits and probes, please refer to section 6.

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This protocol is split into 2 main parts:

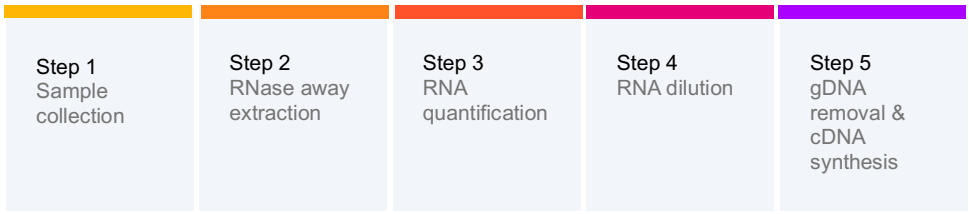
- Protocol for RNA extraction | Section 3
- Protocol for RT-qPCR | Section 4

Before starting

Refer to the specific product User Manual for complete details on cell coating, cell thawing, and cell culture.

If you need assistance, visit www.bit.bio/support-hub.

3. Protocol for RNA extraction



3.1. Reagent preparation

3.1.1. Clean the workstation with RNase AWAY™ or similar RNase decontaminating buffer.
3.1.2. Ensure 100% ethanol has been added to buffer RPE, following the RNeasy kit instructions.
3.1.3. Calculate the volume of 70% ethanol required based on the number of samples to be extracted.
3.1.4. Prepare 70% ethanol by adding 100% molecular grade ethanol to the required volume of nuclease-free water.

3.2. Sample collection

3.2.1. Initial wash: Aspirate media from the required wells of a 6-well plate gently wash each well with 1 mL DPBS, taking care not to disturb the cells. Remove and discard the DPBS.
3.2.2. Lysis buffer addition: Add 700 µL of Buffer RLT to the first well. Pipette up and down to detach and lyse the cells, avoiding the introduction of air bubbles.
3.2.3. Sequential harvesting – max 2 wells: Transfer the lysate from the first well into the second well. Pipette up and down gently to harvest the cells, avoiding the introduction of air bubbles. Note: to maintain optimal lysate viscosity and RNA concentration, do not pool more than two wells into a single 700 µL volume of Buffer RLT.
3.2.4. Final homogenisation and scaling: Transfer the lysate to a labelled 1.5 mL microcentrifuge tube, pipette up and down to ensure complete lysis, and vortex for 5 seconds. If more than two wells are required for a single sample, repeat steps 3.2.2 and 3.2.3 using a fresh 700 µL aliquot of Buffer RLT, then pool the resulting lysates or process them as separate tubes as required.

3.2.5. Storage: Immediately place the sample tube(s) on dry ice.
3.2.6. Repeat steps 3.2.1 to 3.2.5 for all remaining experimental samples, ensuring fresh tips and fresh Buffer RLT are used for each unique sample set.

If not performing RNA extraction immediately, store samples at -80°C for up to 1 year until RNA extraction is to be performed.

3.3. RNA extraction

3.3.1. Thaw lysates on wet ice.
3.3.2. For each sample to be extracted, label 1x RNeasy mini spin column with the sample name.
3.3.3. Transfer each sample lysate to its associated spin column.
3.3.4. Centrifuge the RNeasy mini spin column at ≥8000 x g for 30 seconds at room temperature (RT).
3.3.5. Discard the column(s) and add 700 µL of 70% ethanol (refer to step 5.1) to each eluate.
3.3.6. For each sample to be extracted, label a RNeasy mini spin column with sample name and place in the 2 mL collection tube.
3.3.7. Transfer each sample (including any precipitate) to the associated RNeasy mini column.
3.3.8. Close the lid(s) of the column(s) and centrifuge at ≥8000 x g for 30 seconds at RT.
3.3.9. Carefully remove the RNeasy column from its collection tube and discard the flow through. Ensure the last drop of liquid is removed by inverting the tube and touching the edge on a piece of tissue.
3.3.10. Replace the RNeasy column in its collection tube.
3.3.11. Add 700 µL Buffer AW1 to each RNeasy mini spin column.
3.3.12. Close the lid(s) and centrifuge at ≥8000 x g for 15 seconds at RT.
3.3.13. Repeat step 3.3.9 to discard the flow through for each sample.
3.3.14. Add 500 µL Buffer RPE to the RNeasy mini spin column(s).
3.3.15. Close the spin column lid and centrifuge the samples at ≥8000 x g for 15 seconds at RT.
3.3.16. Repeat step 3.3.9 to discard the flow through for each sample.
3.3.17. Add 500 µL Buffer RPE to the RNeasy mini spin column(s).
3.3.18. Close the lid(s) and centrifuge at ≥8000 x g for 2 minutes at RT.
3.3.19. Replace the RNeasy column in a new collection tube.
3.3.20. Close the lid(s) and centrifuge at full speed for 1 minute at RT.
3.3.21. Remove from the centrifuge and relocate to a PCR workstation. Place each RNeasy mini spin column in a new appropriately labelled 1.5 mL microcentrifuge tube.
3.3.22. Add 30 µL of RNase-free water directly to the centre of the spin column (take care to not touch the membrane with the pipette tip) and incubate for 2 minutes at RT.
3.3.23. Close the column lid and centrifuge at ≥8000 x g for 1 minute at RT to elute the RNA.
3.3.24. Place RNeasy spin columns to one side and immediately transfer eluted RNA to ice.
3.3.25. Proceed to RNA quantification using the Nanophotometer.

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3.4. RNA quantification

3.4.1. Wipe down the Nanophotometer with 70% Ethanol, followed by water.
3.4.2. Perform BLANKING: Pipette 2 µL of nuclease-free water onto the sampling positions of the Nanophotometer.

Perform the blank measurement using the same water as the RNA is eluted in.

3.4.3. Pipette the first sample up and down to mix and transfer 2 µL to a sampling well of the Nanophotometer, repeat in the next well to perform a duplicate measurement.
3.4.4. Repeat the step above, transferring 2 µL of each sample (in duplicate) to the sampling wells of the Nanophotometer.
3.4.5. Measure the RNA and record the concentration and purity.
3.4.6. Based on the average concentration (ng/µL) calculations in the RNA Quantification table, calculate the volume of RNA and nuclease-free water needed to add 500 ng of each RNA to its corresponding strip tube or PCR tube used for the cDNA reaction.

For optimal results, check that the OD260:280 ratio is between 1.9 and 2.1. A value over 2 indicates a purer RNA population in the eluted sample

3.5. RNA dilution

3.5.1. Dilute the RNA to 62.5 ng/µL, so that the final amount of RNA in the cDNA reaction is 500 ng.

At this stage, RNA samples can be stored at -20°C for 24 hours or at -80°C for 1 year. RNA samples should then be thawed on ice.

3.6. gDNA Removal and cDNA Synthesis

3.6.1. Briefly spin the samples in a benchtop spinner for around 5 seconds to settle the contents and immediately place back on ice.
3.6.2. Thaw the cDNA kit on ice. Briefly spin the tubes in a benchtop spinner to settle the contents and immediately place back on ice.
3.6.3. For each RNA sample prepare the gDNA removal reaction according to Table 1, adding up to 500 ng total RNA.

If the concentration of RNA is below 62.5 µg/µL then no pre-dilution needs to be performed.

3.6.4. Prepare a no template control (NTC) and no reverse transcriptase (-RT) control, which should be RNA from a pool of all the test article samples (refer to table 1).

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Table 1 Required volumes for gDNA removal step (per sample and controls). *X indicates the volume of RNA required to ensure 500 ng are added to the reaction.*

Component	RNA (Test) Sample	No template control (NTC)	No reverse transcriptase (-RT) control
		Volume (µL)	
RNA (500 ng)	X	n/a	X
Nuclease-Free Water	(8-X)	8	(8-X)
10x dsDNase buffer	1	1	1
dsDNase Enzyme	1	1	1
Total volume	10	10	10

3.6.5. Briefly spin the samples in a benchtop spinner to settle the contents and immediately place back on ice.
3.6.6. Incubate the gDNA reactions for 2 minutes at 37°C in the thermocycler
3.6.7. Following incubation, prepare the final cDNA reaction mixtures according to Table 2. For multiple samples, master mixes can be prepared as below, multiplying the values by the total number of samples, plus a 20% overage.

Table 2 Required volumes for cDNA synthesis (per sample and controls).

Component	RNA (Test) Sample	No template control (NTC)	No reverse transcriptase (-RT) control
		Volume per Rxn (µL)	
dsDNase-treated sample	10	10	10
Nuclease-Free Water	4	4	6
5x Rxn mix	4	4	4
RT Enzyme	2	2	n/a
Total volume	20	20	20

3.6.8. Gently pipette up and down to mix the samples and briefly spin the samples in a benchtop spinner to settle the contents
3.6.9. Place the samples in a thermocycler and run the temperature cycles for cDNA synthesis according to Table 3.

Table 3 Temperature cycles for cDNA synthesis.

Temperature	Time
25°C	10 minutes
50°C	30 minutes
85°C	5 minutes 30 seconds
4°C	Infinite Hold

3.6.10. Remove tubes from the thermocycler and chill on ice for 2 minutes.
3.6.11. Briefly spin the samples in a benchtop spinner to settle the contents.

Do not open the tubes before allowing the thermocycler temperature to reach the final 4ºC hold, and centrifugation step, as condensation in the tube may result in sample loss and/or cross contamination.

3.6.12. Store cDNA at -20°C until used. If qPCR will be run the following day cDNA can be refrigerated at 4°C instead overnight.

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4. Protocol for RT-qPCR

Step 1
Collect RLT pellets

Step 2
RNA extraction

Step 3
cDNA synthesis

Step 4
RT-qPCR

4.1. qPCR plate sample requirements

4.1.1. Alongside the genes of interest, an iPSC control, NTC and -RT control should be run. These can be prepared in bulk and stored at -20°C for up to 1 year.

4.2. qPCR master mix preparation

4.2.1. Referencing the reagent volumes in Table 4, prepare a master mix for each target gene. Ensure the total volume is sufficient for all samples to be run in triplicate.

When calculating required volume, add 20% overage to allow for volume loss during pipetting.

Table 4 Reagent volumes for qPCR reaction mixes. For the gene of interest, use one probe per qPCR reaction mix. Once the sample is added, the total reaction volume will be 10 µL.

Reagent	Volume per reaction (µL)
Nuclease-Free Water	2.5
TaqMan® Master Mix	5
TaqMan Assay (Gene of interest)	0.5
Total volume	**8**

4.2.2. Label the required number of 1.5mL DNA Low-bind tubes with the gene of interest (TaqMan assay name) and place on ice (or suitable cold block).
4.2.3. Add the required volume of nuclease-free water to each tube as calculated in step 4.2.1.
4.2.4. Mix the TaqMan Fast Advanced Master Mix by gently pipetting, avoiding the introduction of air bubbles, and add the required volume to each tube as calculated in step 4.2.1.
4.2.5. Confirm TaqMan assay is completely thawed and briefly vortex the tubes to mix. Briefly spin down in a benchtop spinner to settle the contents.
4.2.6. Add the required volume of TaqMan assay to its corresponding master mix as calculated in step 4.2.1.
4.2.7. Protect the master mixes from light and keep on ice or suitable cold block until ready to use.
4.2.8. Prepare cDNA strip tubes for each sample, including the controls listed in section 4.1.1.
4.2.9. Dilute each cDNA sample 1 in 20 by adding 6 µL cDNA to 114 µL nuclease-free water.
4.2.10. Calculate the volume of cDNA required per sample based on the number of genes (TaqMan assays) to be tested.

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4.3. qPCR plate preparation

4.3.1. Set a multi-dispenser pipette to dispense 8 µL and set the appropriate number of steps (equals the number of reactions per gene target)

If using an electronic multi-dispenser pipette, discard the first dispense volume as indicated on the pipette display screen.

4.3.2. Using a multi-dispenser pipette, aspirate the master mix for each gene target and dispense 8 µL into the designated wells according to the plate map. Repeat for all remaining gene targets.

4.3.3. Once all the master mixes have been transferred, gently tap the plate to enable the solution to settle to the bottom of the plate.

Use a dark background beneath the plate to enhance the visibility of the liquid in the wells.

4.3.4. Place cDNA strip tubes in a plate rack in the same order as the plate layout.

4.3.5. Using a multichannel pipette, transfer 2 µL of cDNA into the designated wells. Visually verify that all tips are loaded before dispensing and confirm they have fully emptied before discarding.

4.3.6. Repeat step 4.3.5 for each of the samples, including the -RT control and NTC as designated on your plate map.

4.3.7. Cover the plate with optical adhesive cover and spin at 2000 x g for 2 minutes to distribute reactions at the bottom of the well and eliminate air bubbles.

4.3.8. Store the excess diluted cDNA overnight at 4°C in case any immediate repeats are required.

Once confirmed that no repeats are required cDNA can be stored at -20°C for up to a year

If processing multiple plates in a day, plates can be stored in the fridge for up to 12 hours after cDNA addition. After removing from fridge centrifuge as per step 4.3.7 to settle plate contents.

4.4. Perform qPCR

4.4.1. Load the qPCR plate into the instrument ensuring the correct orientation. If necessary, consult the manufacturer's manual for instrument-specific loading instructions.

4.4.2. Select the correct qPCR conditions, ensuring chemistry, plate formats, and cycling conditions are correctly set up.

4.4.3. Ensure your qPCR plate map aligns with the plate setup, assigning the -RT and NTC samples as negative controls.

4.4.4. Configure thermal cycling parameters: Program the instrument using the settings in Table 5. If you are using alternative master mix or primer/probe sets, optimisation of the thermal cycling conditions may be required.

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Table 5 Temperature cycles for cDNA synthesis.

Condition	Temperature	Time	Cycles
UNG Digestion	50°C	2 minutes	1
Enzyme activation	95°C	2 minutes	1
Denaturation	95°C	1 second	40
Anneal/extension	60°C	20 seconds	40

4.4.5. Run the qPCR protocol.
4.4.6. Once the run is complete, verify that all data has been saved and ensure that the correct analysis settings, as well as any baseline or threshold settings are saved and applied against the run correctly.

4.5. Data Analysis

Review the plate layout for correct well assignments and screen for outliers or technical artifacts before proceeding with the final analysis.

4.5.1. Calculate the gene expression using the formulas outlined in section 5.

- Calculate the delta-Ct for each gene and convert to fold difference in gene expression: subtract the average Ct for each target gene from the Ct for reference gene (*HMBS*) to calculate the delta-Ct (dCt). Convert this value to fold difference in gene expression via the formula 2^-dCT.
- Optional: If normalising to a reference/control sample (e.g. another cell type, iPSCs, wild type product, etc), calculate the fold difference from the delta-delta Ct using the formula 2^-ddCT.

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5. Calculations

The following are useful calculations for gene expression analysis.

5.1. Option A: Fold change over housekeeping gene

5.1.1. Delta CT

$$\boldsymbol{Delta\ Ct = Ct(Target\ Gene) - Ct(Housekeeping)}$$

5.1.2. Fold difference

$$\boldsymbol{Fold\ Change\ from\ Housekeeping\ Gene = 2^{-\Delta Ct}}$$

5.2. Option B: Fold change over a reference sample

5.2.1. Delta CT

$$\boldsymbol{Delta\ Ct = Ct(Target\ Gene) - Ct(Housekeeping)}$$

5.2.2. Delta Delta CT

$$\boldsymbol{Delta - Delta\ Ct\ (\Delta\Delta Ct) = \Delta Ct(Reference\ Sample) - \Delta Ct(Test\ Sample)}$$

5.2.3. Fold difference

$$\boldsymbol{Fold\ Change\ from\ Reference\ Sample = 2^{-\Delta\Delta Ct}}$$

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6. Reagents

6.1. Recommended reagents

Reagent	Supplier	Cat no	Storage
RNase AWAY	ThermoFisher	7002	Room temperature
DPBS, no calcium, no magnesium	ThermoFisher	14190169	Room temperature
Ethanol (molecular biology grade)	ThermoFisher	16606002	Room temperature
RNeasy Mini Kit	Qiagen	74134	Room temperature
Nuclease-Free Water	ThermoFisher	AM9937	Room temperature
Maxima First Strand cDNA Synthesis Kit for RT-qPCR (with DNase)	ThermoFisher	K1671	-20°C
TaqMan Fast Advanced Master Mix (with UNG)	ThermoFisher	4444965	+4ºC

6.2. RNA controls for use with ioCells

ioCells	RNA source	Supplier	Cat no
ioAstrocytes	Human brain	ThermoFisher	AM7962
ioGABAergic Neurons	Human brain	ThermoFisher	AM7962
ioGlutamatergic Neurons	Human brain	ThermoFisher	AM7962
ioOligodendrocyte-like cells	Human brain	ThermoFisher	AM7962
ioOPC-like cells	Human brain	ThermoFisher	AM7962
ioMotor Neurons	Human brain	ThermoFisher	AM7962
ioSensory Neurons	Human brain	ThermoFisher	AM7962
ioSkeletal Myocytes	Skeletal muscle	AMSBIO	R1234171-50

6.3. TaqMan gene expression assays

	Gene target name	HS code from ThermoFisher
Housekeeping gene	*HMBS*	Hs00609296_g1
Pluripotency marker	*POU5F1* (OCT-4)	Hs00999632_g1
ioAstrocytes	*VIM*	Hs00958111_m1
	S100b	Hs00902901_m1
	SLC1A3	Hs00904823_g1
ioGABAergic Neurons	*VGAT*	Hs00369773_m1
	DLX1	HS00698288_m1
	TUBB3	Hs00964962_g1
	DLX2	HS00269993_m1
	ASCL1	HS00269932_m1
	GAD1	HS01065893_m1
	GAD2	HS00609534_m1
ioGlutamatergic Neurons	*GRIA4*	Hs00898778_m1

	SLC17A7 (VGLUT1)	Hs00220404_m1
	SLC17A6 (VGLUT2)	Hs00220439_m1
	SYP	Hs00300531_m1
	TUBB3	Hs00964962_g1
ioOligodendrocyte-like cells	*PDGFRA*	Hs00998018_m1
	PLP1	Hs00166914_m1
	MBP	Hs00921945_m1
	MAG	Hs01114387_m1
ioOPC-like cells	*PDGFRA*	Hs00998018_m1
	CSPG4	Hs00361541_g1
	PLP1	Hs00166914_m1
	MBP	Hs00921945_m1
	CNP	Hs00263981_m1
ioMotor Neurons	*MAP2*	Hs00258900_m1
	TUBB3	hs00964962_g1
	ChAT	Hs00758143_m1
	VAChT	Hs00268179_S1
	ISL-2	Hs00377575_m1
	HB9	Hs00907365_m1
ioSensory Neurons	*MAP2*	Hs00258900_m1
	NTRK1	Hs01021011_m1
	PRPH	Hs00196608_m1
	TRPV1	Hs00218912_m1
ioSkeletal Myocytes	*DES* (Desmin)	Hs00157258_m1
	DMD (Dystrophin)	Hs00758098_m1
	MYH2	Hs00430042_m1
	MYH8	Hs00267293_m1
	MYOG (Myogenin)	Hs01072232_m1
	TNNT1	Hs00162848_m1

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7. Troubleshooting

Issue	Reason	Solution
Low amplification signal in results	Degraded primer/ probe set (assay mix)	Probes are sensitive to sunlight and freeze thaw cycles. For best practice, single use aliquot your assay mixes in PCR clean amber tubes.
	qPCR machine settings are incorrect	Check that your settings (chemistry, and thermal cycling settings) are correct for your run.
	RNA degradation	Ensure your RNA is always handled on ice and not subject to freeze/thaw cycles. If required add 2-Mercaptoethanol to the RLT buffer.
High amplification signal in negative controls	Cross contamination	Ensure your workstation is cleaned with a PCR compatible DNA/ RNase cleaning solution, or a weak bleach solution.
		Use filter tips and replace them after every sample addition.
		Ensure all reagents are stored correctly and handled with good molecular biology technique.
Inconsistent replicates	Pipetting errors	Ensure your sample additions follow to best practice advised through this document. If required, reverse pipette the sample into the wells.
	Plate preparation errors	Ensure plate was spun down before being run on the qPCR machine.
Low RNA yield	Incomplete cell lysis	Ensure the sample is properly mixed when lysing the samples with the RLT buffer.
	Extraction error	Follow the extraction kit steps exactly. Load the columns evenly and ensure the pipette tip does not make contact with the spin column membrane
gDNA contamination	dsDNase step incomplete	Follow the cDNA kit instructions exactly.

Our technical support team at bit.bio is available to answer any other questions you may have about either the protocol or the cells, contact us at technical@bit.bio.

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