RNA Isolation and qRT-PCR:

The protocol below was used for RNA isolation, cDNA synthesis and qRT-PCR.

RNA Isolation and cDNA Synthesis:

1. RNA was isolated using the RNeasy Kit (Qiagen, Cat#74014) according to manufacturer instructions.
2. Total of 2µg RNA was used for cDNA synthesis and prepared using high capacity reverse transcriptase kit (ThermoFisher Scientific Cat#4368814) as per the manufacturer's protocol below.

Components	Volume per reaction, µL (1X)
10x RT buffer	2
10X Random Primer	2
100mM dNTPs	0.8
RNAse Inhibitor	1
Multiscribe Reverse Transcriptase	1
Nuclease free water	3.2

3. 2µg of RNA was used in the reaction mix and made total volume upto 20 µl with nuclease free water. The total reaction volume was 20µl. The below program was used for cDNA synthesis on the standard thermocycler.

Temperature	Time (min)
25℃	10 min
37℃	120 min
85℃	5 min
4℃	Hold

4. The cDNA samples were diluted at 1:5 with nuclease free water before using the samples for qRT-PCR and 2µl of the cDNA sample was used per reaction/well to check the expression of specific genes, for example, POU5F1, NANOG, TH, KCNJ6 etc..

qRT-PCR:

1. Real time qRT-PCR was performed using PowerUP SYBR green (ThermoFisher Scientific, Cat#A25741) on a QuantStudio 6 flex PCR machine with 384 well plate format. Sample reactions were prepared as per reaction master mix below.

Components	Volume per reaction, µL (1X)
2x SYBR green	5
Forward primer (10µM)	1
Reverse Primer (10µM)	1
cDNA	2
Nuclease free water	1

2. Reaction mix was vortexed and briefly spun down before loading 10ul of master mix per well.
3. Standard 40 cycle program was used on the QuantStudio 6 flex for the thermocycler reaction.
4. Results (ct values) were normalized to *GAPDH.*

qRT-PCR primers used in the study are found in the table below:

Gene Name	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
POU5F1	ATTCTCCAGGTTGCCTCTCA	GTGGAGGAAGCTGACAACAA
NANOG	AGTCCCAAAGGCAAACAACCCACTTC	TGCTGGAGGCTGAGGTATTTCTGTC
SOX2	TTCACATGTCCCCGAACTACCAGA	TCACATGTGTGAGAGGGGCAGTGTGC

FOXA2	GGGGTAGTGCATCACCTGTT	CCGTTCTCCATCAACAACCT
LMX1A	ACGTCCGAGAACCATCTTGAC	CACCACCGTTTGTCTGAGC
NR4A2	GCTGGACTCCCCATTGCTTT	CGGAGCTGTATTCTCCCGAA
KCNJ6	GCTACCGGGTCATCACAGAT	ACTGCATGGGTGGGAAAAGAC
PITX3	CCAACCTTAGTCCGTGCCAG	TGTGTAGGGCCTAGTCCACC
TH	CCGAGCTGTGAAGGTGTTTGA	CGGGCCGGGTCTCTAGAT
EN2	AGGAGCTGAGCCTCAACGAGTC	CTTGGCTGTGGTGGAGTGGTTG
AADC	GAACAGACTTAACGGGAGCCTTT	AATGCCCGGTAGTCAGTGATAAGC
SYN1	GCAAGGACGGAAGGGATCACATCA	CCTGAGCCATCTTGTTGACCACGA
GAPDH	TCGGAGTCAACGGATTTGGT	CCTGGAAGATGGTGATGGGA