HUMAN EMBRYONIC STEM CELL (hESC) ASSESSMENT BY REAL-TIME POLYMERASE CHAIN REACTION (PCR)

OBJECTIVE: Real-Time Polymerase Chain Reaction (PCR) is performed to analyze gene expression of human Embryonic Stem Cells (hESCs) in culture. Real-Time PCR is used to monitor whether cells are pluripotent at a messenger ribonucleic acid (mRNA) level. This Standard Operating Procedure (SOP) describes how to perform Real-Time PCR.

SCOPE: This procedure applies to all Massachusetts Human Stem Cell Bank laboratory personnel responsible for assessing gene expression by Real-Time PCR.

RESPONSIBILITY: It is the responsibility of the Laboratory Operations Manager and Quality Assurance Officer to ensure all required personnel are properly trained in and follow this SOP for Real-Time PCR.

SAFETY: All laboratory personnel should be in compliance with UMASS Employee Health and Safety regulations when working in the laboratory. Specifically, wear personal protective equipment (lab coat and disposable gloves) while working in the lab.

ABBREVIATIONS AND DEFINITIONS
RNA: Ribonucleic Acid
DNA: Deoxyribonucleic Acid
PCR: Polymerase Chain Reaction
RT-PCR: Reverse Transcription Polymerase Chain Reaction
Real-Time PCR: Real-Time Polymerase Chain Reaction
Real-Time RT-PCR: Real-time Reverse Transcription Polymerase Chain Reaction
hESC: Human Embryonic Stem Cell
AB: Applied Biosystems
SOP: Standard Operating Procedure
UMASS: University of Massachusetts Medical School
mRNA: Messenger Ribonucleic Acid
cDNA: Complementary Deoxyribonucleic Acid
Assay Master Mix: Solution consisting of TaqMan® Gene Expression Master mix and a gene specific primer/probe.

REFERENCES
Mastercycler® ep Realplex Instruction Manual, Eppendorf.

1. MATERIALS REQUIRED

1.1 EQUIPMENT
- Microcentrifuge with adapters for 96 well plates
- Timer (Fisher S90861)
- Micropipettors
  - P2 (0.2 µl to 2 µl)
- P20 (0.5 to 20 µl)
- P200 (20 to 200 µl)
- P1000 (200 to 1000 µl)

- Mastercycler® ep Realplex thermal cycler (Eppendorf 5345)

1.2 Supplies
- Kimwipes (Fisher 06666A-C)
- Disposable nitrile gloves (World Wide Medical Supplies 71011000-3)
- Sterile micropipettor filtered tips to fit P2, P20, P200 and P1000 micropipettors (Corning 4807, 4821, 4823, 4809)
- RNase-free microcentrifuge tubes (1.5 ml) (Fisher 07-200-183)
- TwinTec Real-Time PCR Plate with PCR film (Eppendorf, 951022003 and 951023019)

1.3 Reagents
- 70% ethanol (Diluted from 95% ethanol, Fisher NC9608803)
- Spray bottle of 70% ethanol (Fisher 03-438-12A and Fisher NC9608803)
- RNase-free water (Fisher BP24701)
- TaqMan® Gene Expression Master Mix (Applied Biosystems 4369016)
- Primers: (AB, product number)
  - DNMT3B (Hs01003416_m1)
  - NANOG (Hs02387400_g1)
  - POU5F1 (Hs01895061_u1)
  - SOX2 (Hs01053049_s1)
  - ZFP42 (Hs00399279_m1)
  - TDGF1 (Hs02339499_g1)
  - AFP (Hs00173490_m1)
  - CD9 (Hs00233521_m1)
  - FGF9 (Hs00173564_m1)
  - FOXD3 (Hs00255287_s1)
  - TERT (Hs99999022_m1)
  - AC133 (Hs00195682_m1)
  - GATA4 (Hs01034629_m1)
  - PAX6 (Hs00240871_m1)
  - ACTC (Hs01109515_m1)
  - DDX4 (Hs00251859_m1)
  - GATA6 (Hs00232018_m1)
  - BMP2 (Hs01055564_m1)
  - CDX2 (Hs00230919_m1)
  - ACTB (Hs99999903_m1)
  - GAPDH (Hs99999905_m1)
**Note 1:** Listed are primers that are currently used or may be used for characterization for bank cell lines.

**Note 2:** Aseptic technique must be followed when performing Real-Time PCR. Clean RNase/DNase-free pipette tips must be used. Always pipette carefully and slowly.

### 2. General Overview

#### 2.1 Real Time RT-PCR Flow Chart

Reverse Transcription Step:
- For each RNA sample there are three reactions: +RT (reaction includes both reverse transcriptase enzymes and RNA), -RT (reaction includes RNA but no reverse transcriptase enzymes), No RNA (reaction includes reverse transcriptase enzymes but no RNA).

PCR Step (all reactions run in duplicate):
- Each +RT sample will be assayed with primer/probes for both genes of interest and housekeeping genes.
- Each –RT sample will be assayed with primer/probes for housekeeping genes.
- Choose one No RNA sample to be assayed with primer/probes for one housekeeping gene.
Note: A housekeeping gene (e.g. GAPDH, ACTB) is expressed in the cell type that is being analyzed at a baseline level.

2.2 Procedure Overview
- Assay master mix will be made per primer/probe and will consist of TaqMan® Gene Expression Master Mix (2x) and TaqMan® primer/probe. Each well will be loaded with 11 µl of assay master mix.
- 9 µl of diluted template (cDNA, water or control reactions) will then be added to each well.

<table>
<thead>
<tr>
<th>Final volume of each reaction</th>
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<tr>
<td>10 µl TaqMan® Gene Expression Master Mix (2x)</td>
</tr>
<tr>
<td>1 µl TaqMan® Primer/Probe</td>
</tr>
<tr>
<td>9 µl 100 ng of cDNA</td>
</tr>
<tr>
<td>20 µl Final Reaction Volume</td>
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</tbody>
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2.3 Sample 96-well Plate Set Up
- Below is an example of a Real Time PCR layout. It is a sample of 6 genes of interest and 2 housekeeping genes. Any genes can be substituted.

Lanes 1-12 are actual wells of plate
S1 (+RT) = cDNA from RNA sample #1
S1 (-RT) = RNA sample #1 without reverse transcriptase
S4 (RT with no RNA) = Reverse transcriptase reaction without RNA
no cDNA = Negative control for PCR reaction where cDNA is substituted with water

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<td>S3 (+RT)</td>
<td>S4 (+RT)</td>
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<tr>
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<td>S1 (-RT)</td>
<td>GAPDH</td>
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<tr>
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<td>S4 (+RT)</td>
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<td>S1 (-RT)</td>
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<tr>
<td>S3 (+RT)</td>
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<td>no cDNA</td>
<td>ACTB</td>
<td>S4 (-RT)</td>
<td>GAPDH</td>
</tr>
</tbody>
</table>
2.4 Calculate Assay Master Mixes

Note: For each cDNA template and primer/probe combination, run samples in duplicate or triplicate. (The sample 96-well plate set-up is to run each sample in duplicate.)

Calculate assay master mixes for gene of interest:

<table>
<thead>
<tr>
<th>1 cDNA sample</th>
<th>Q number of cDNA samples</th>
<th>Volume of 1 cDNA x Q x 2 (each reaction is run in duplicate)</th>
<th>+ 1 negative control run in duplicate (PCR with no cDNA)</th>
</tr>
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<tbody>
<tr>
<td>TaqMan® Master Mix (2x)</td>
<td>10 µl</td>
<td>(10 µl x 2Q)</td>
<td>+ 20 µl</td>
</tr>
<tr>
<td>Primers (ex.OCT4)</td>
<td>1 µl</td>
<td>(1 µl x 2Q)</td>
<td>+ 2 µl</td>
</tr>
<tr>
<td>100 ng of cDNA (+RT)</td>
<td>9 µl</td>
<td>(9 µl x 2Q)</td>
<td>+ 18 µl</td>
</tr>
</tbody>
</table>

Q = 4 cDNA samples

Volume of each reagent needed for 4 cDNA samples:
(10 µl x 8) + 20 µl = 100 µl
(1 µl x 8) + 2 µl = 10 µl
(9 µl x 8) + 18 µl = 90 µl

Calculate assay master mixes for housekeeping genes:

<table>
<thead>
<tr>
<th>1 cDNA sample</th>
<th>Q number of cDNA samples</th>
<th>Volume of 1 cDNA (+RT) x Q x 2 (each reaction is run in duplicate)</th>
<th>+ 1 negative control run in duplicate (PCR with no cDNA)</th>
<th>Volume of 1 cDNA x (-RT) Q x 2 (each reaction is run in duplicate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan® Master Mix (2x)</td>
<td>10 µl</td>
<td>(10 µl x 2Q)</td>
<td>+ 20 µl</td>
<td>(10 µl x 2Q)</td>
</tr>
<tr>
<td>Primers for ACTB</td>
<td>1 µl</td>
<td>(1 µl x 2Q)</td>
<td>+ 2 µl</td>
<td>(1 µl x 2Q)</td>
</tr>
<tr>
<td>100 ng of cDNA (+RT)</td>
<td>9 µl</td>
<td>(9 µl x 2Q)</td>
<td>+ 18 µl</td>
<td>(9 µl x 2Q)</td>
</tr>
</tbody>
</table>

Q = 4 cDNA samples

Volume of each reagent needed for 4 cDNA samples:
(10 µl x 16) + 20 µl + (10 µl x 16) = 340 µl
(1 µl x 16) + 2 µl + (1 µl x 16) = 34 µl
(9 µl x 16) + 18 µl + (9 µl x 16) = 306 µl

- Calculate the amount of TaqMan® Gene Expression Master Mix and primer/probe needed for each assay master mix. For every 10 reactions, make enough master mix for 11 reactions, in order to account for pipette error.
2.5 **CALCULATE CDNA DILUTIONS**

1. Calculate the total number of reactions that are needed per cDNA sample. For every 10 reactions, make enough for 11, in order to account for pipette error.

2. For each cDNA sample, use 100ng of cDNA per reaction in a 9 µl volume. Using the quantities calculated in [SOP CH006 Quantifying Complementary Deoxyribonucleic Acid (cDNA)](https://example.com) and the amount of reactions needed, calculate cDNA dilutions.

**Note:** Calculate cDNA dilutions for both the +RT and –RT samples according to the +RT concentration for each. (–RT should have had no amplification). Because –RT reactions are negative controls there should not be any products, use an equivalent volume of the +RT reaction for each corresponding –RT sample.

3. **PREPARATION**

3.1 **PREPARE LABORATORY BENCH AND EQUIPMENT**

1. Wash hands thoroughly with soap.
2. Rinse them completely with warm tap water.
3. Dry hands with paper towel.
4. Put on appropriate-sized gloves.
5. Spray workspace on laboratory bench thoroughly with 70% ethanol and wipe dry with a Kimwipe.
6. Spray a Kimwipe with 70% ethanol and wipe pipettes that may be used. Allow to dry.
7. Spray gloves with 70% ethanol. Allow to dry.

3.2 **PREPARE LABORATORY EQUIPMENT**

1. At least 10 minutes prior to use, turn on Realplex cycler using the switch in the back of the instrument next to the power cord. The LED on the cycler lid will blink yellow. Then turn on the computer by pressing the power button on the front.

3.3 **PREPARE TUBES**

- Label one sterile RNase free microcentrifuge tube per cDNA template.
- Label one sterile RNase free microcentrifuge tube for each control (–RT and No RNA).
- Label one sterile RNase free microcentrifuge tube per primer/probe. This will be for each gene specific assay master mix.

3.4 **THAW REAGENTS**

- To thaw reagents, place TaqMan® Gene Expression Master Mix and all primer/probes on ice. While thawing, check the tube of primer/probes and vortex occasionally to aid thawing.

3.5 **PREPARE ASSAY MASTER MIXES**

- In each of the tubes that labeled in Section 2.3, transfer the calculated amounts of TaqMan® Gene Expression Master Mix and primer/probe into the appropriately labeled microcentrifuge tubes. Mix with a pipette. Centrifuge briefly.
Note: The gene specific primer/probe mixture is now prepared and will be referred to as Assay Master mix in this SOP.

3.6 PREPARE CDNA DILUTIONS
- Dilute the calculated quantity of cDNA into RNase Free water. Transfer into the appropriately labeled microcentrifuge tubes. Mix with a pipette. Centrifuge briefly.

4. PROCEDURE

4.1 LOAD THE REACTION PLATE
1. Using a new box of tips, carefully transfer 11 µl from the appropriate assay master mix (see Section 2.3) tube into the bottom of each well.

Note: Use the tip that corresponds to the same location on the reaction plate, which will help to keep track of pipetting and avoid plate-loading errors.

2. Next, using another new box of tips, pipette 9 µl of the appropriate cDNA into the bottom of each well. Follow the same procedure as above to keep track of pipetting. Add these cDNA dilutions directly to wells that have been preloaded with the appropriate Assay Master mix. To mix both solutions thoroughly, pipetting up and down a few times after adding cDNA to each well.
3. When finished, place a PCR film securely on top of the plate.
4. Place the plate into the appropriate adapter in the microcentrifuge. Spin for 2 minutes at 1000 x g.

4.2 RUN THE REAL-TIME PCR
1. Place the plate into the Mastercycler® ep Realplex thermal cycler. Close lid.
2. Open Realplex software, select a User, and enter login.
3. Select “Open Assay” and find “TaqMan® Assay”
4. Review the settings.
   - 50°C for 2 minutes
   - 95°C for 10 minutes
   - 40 cycles of 95°C for 15 seconds and 60°C for 1 minute
   - Final hold at 4°C
5. Select “Start PCR” and for background calibration, select twin.tec.skirted, 20 µl. Select “OK.”
6. In the plate layout, select all positions in the plate layout and define them as Unknowns. (Define them further once the run has begun.)
7. Select “Start RUN.” Lid will blink green, indicating that the machine is running.
8. While the run is proceeding, label the samples.
9. Name each sample by double clicking on it and typing in the “Name“ box. Indicate the primer/probe as well as cDNA sample. Indicate which samples are replicates.
10. For each housekeeping gene sample, designate by double clicking on the sample and selecting Housekeeping Gene. Be sure to indicate replicates.
4.3 ANALYZE REAL-TIME DATA

1. Once the run is complete, select “Start Analysis.”
2. In the “Plate View”, make sure the “Show all” box is checked.
4. Select “Text” format and click “OK.”
5. When the “Save as” window comes up, save in an appropriate file.
6. Open this exported program in Excel.
7. Copy the Sample Name, Ct (Threshold Cycle) and Mean value columns and paste in adjacent columns.
8. Create another data set from the Excel data set by copying the mean value of each sample only.
9. Next, divide each of the samples mean Ct by its corresponding housekeeping gene Ct.
10. Then plot this normalized data on a bar graph.
11. If using more than one housekeeping gene, repeat steps 7-10.
12. Also print out a report of the raw data by selecting “Edit” on the toolbar and then “Print Report.” Date and Initial report indicating that all of the data is correct.
13. Remove plate from cycler. If it is needed for further analysis, store in a 4°C refrigerator. Otherwise, discard plate.
14. Turn off Real-Time cycler and computer.
16. Place the dated and initialed quantification reports, raw data, and graphs in the binder specific to the cell line that has been analyzed.

4.4 DATA REPORTING AND OVERALL INTERPRETATION

In Progress
**REAL-TIME RT-PCR LOG SHEET**

- Cell line name and passage number(s): ________________________________
- Date hESCs seeded: ___________________ by ___________________.
- If more than one, list ________________________________
- Date Cells Harvested: ___________________ by ___________________.
- Comments: _______________________________________________________
- Date RNA purified: ___________________ by ___________________.
- RNA stored in ___________________ by ___________________.
- Comments: _______________________________________________________
- Reverse transcription performed on ____________ by ___________________.
- cDNA stored in ___________________ by ___________________.
- Comments: _______________________________________________________
- Real-Time PCR performed by _________________ on ___________________.
- Data file saved as: ___________________ Location: ___________________.
- Comments: _______________________________________________________
- Check once attached:
  - RNA quantification report
  - cDNA quantification report
  - Real-Time Report (Raw Data)
  - Real-Time graphs
- Comments: _______________________________________________________
- Decision: _______________________________________________________
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<th>Representation</th>
<th>Mean Ct</th>
<th>Mean Ct when normalized to GAPDH</th>
<th>Mean Ct when normalized to ACTB</th>
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