**Appendix A.**

Example of a student-written experimental plan to determine an optimal reference gene for use in quantitative PCR (qPCR) gene expression studies of muscle cells. Highlighted on the right side of the example is each required component for the experimental plan. Students were provided the Instruction Manual for the SSoAdvanced™ Universal SYBR Green Supermix and the guidelines as outlined in the “Instructions to Students”

# 

Student Example

Required Component

# Optimal reference gene

We must ensure that the reference gene we are choosing to normalize against has approximately equivalent (or relatively equivalent) amplification during qPCR at different time points. Our reference gene must be determined with our cell type and experimental setup. The output of this experiment will help determine which normalization protocol we will choose:

Purpose of experiment

1. ΔΔCq
2. Pfaffl
3. Multiple reference genes

## RNA isolation, validation, and reverse transcription

**NOTE:** materials list is the same in previous protocol.

1. Isolate RNA from one myoblast (MB) and one myotube (MT; e.g. day 7) sample as per protocol.

Identification of sample processing required to perform this experiment \*note that the students had already wrote experimental plans for these experiments and were told to link those protocols were appropriate.

**NOTE:** We need to ensure that everyone is doing a consistent protocol.

1. Perform RNA validation experiments (quantification, quality, contamination) as per protocol.
2. Perform reverse transcriptase reaction [as per protocol](https://docs.google.com/document/d/1c_gIq363nqMFozxdEms7vE_Dtqk2PoHc5_6jO2L8OiY/edit) without the serial dilution of RNA step. Use a consistent amount of RNA for both the MB and MT samples added to reaction (e.g. 1 µg).

RT mix prep for number of reactions: 6 (+1 residual)

|  |  |  |
| --- | --- | --- |
| **Component** | **Volumes per rxn (uL)** | **Volume (uL)** |
| 10X RT buffer | 2 | 14 |
| 25X dNTP mix (100 mM) | 0.8 | 5.6 |
| 10X RT random primers | 2 | 14 |
| MultiScribe reverse transcriptase | 1 | 7 |
| RNase inhibitor | 1 | 7 |
| Nuclease-free water | 3.2 | 22.4 |
| Total per reaction | 10 | 70 |

Calculations to prepare reverse transcriptase reaction \*note that students were given manufacturer user information

Student identifies how the amount of input RNA will be determined

**NOTE:** We need to get the results from the linear dynamic range experiment before we know what this template amount will be.

## qPCR

**NOTE:** materials list is the same in [previous protocol](https://docs.google.com/document/d/1c_gIq363nqMFozxdEms7vE_Dtqk2PoHc5_6jO2L8OiY/edit)

1. Thaw SsoAdvanced and universal SYBR Green (supermix) and other frozen reaction components to room temperature. Mix thoroughly, centrifuge briefly to collect solutions at the bottom of tubes, and then store on ice protected from light.
2. Prepare (on ice or at room temperature) enough master mix for all qPCR reactions (+ a little extra) by adding all required components except the template.

**NOTE:** I am suspecting that only one NTC among the 5 reference genes tested?

qPCR setup

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Control (10% FBS) | | | Experimental (2% HS) | | |
| MB | MB | MB | MB | MB | MB |
| MT | MT | MT | MT | MT | MT |
| NTC | NTC | NTC | NTC | NTC | NTC |

MB=myoblast, MT=myotube, NTC=no template control

|  |  |  |
| --- | --- | --- |
| Number of samples | Residual and triplicate modifier | Total reactions |
| 4 | (4+0.5)\*3.5 | 16 |

Preparation of qPCR reactions includes correct calculations and controls

|  |  |  |
| --- | --- | --- |
| **Component** | **Volume per rxn (uL)** | **Total MM prepared (uL)** |
| Supermix | 10 | 160 |
| Forward primers | 1 | 16 |
| Reverse primers | 1 | 16 |
| Nuclease free water | 6 | 96 |
| Total reaction mix | 18 | 288 |

1. Thoroughly vortex the master mix to ensure homogeneity and dispense equal aliquots into each PCR tube.

\*\*Student is missing identification of the primers to be used

1. From the mastermix, create 3 minimixes.

**NOTE:** We are using 7 uL of template because it is a 1:10 dilution of a 100% assumed RT reaction of 1 ug → 100 ng, which is at the upper recommended final amount in the Sso protocol.

|  |  |  |  |
| --- | --- | --- | --- |
|  | MB sample (uL) | MT sample (uL) | NTC |
| MM | 63 | 63 | 63 |
| cDNA template | 7 | 7 | 0 |
| DNase free water | 0 | 0 | 7 |
| Total | 70 | 70 | 70 |

1. Add 20 µL of the minimixes from their respectives tubes into each of the PCR tube of the triplicates. Cap the tubes and vortex for 30 seconds or more to ensure thorough mixing of the reaction components. Centrifuge the tubes to remove any air bubbles and collect the reaction mixture in the vessel bottom - ensure NO bubbles. Visually confirm that each tube has the entire sample collected at the bottom.
2. Label the tubes, but not on the top\* :)
3. Program the thermal cycling protocol according to the table below.
4. Load tubes and start the PCR run, depending on which system.

## Analysis

How data will be analyzed to determine the optimal reference gene

1. Calculate standard deviation (SD) for the MB and MT Cq values.
2. Determine which reference gene has the lowest SD.

**NOTE:** a good rule is to ignore a SD > 0.5

Grading by the instructor can be done using a spreadsheet and then copied and pasted into each student’s experimental plan document.

|  |  |  |  |
| --- | --- | --- | --- |
| **Required Component** | **Worth** | **Student 1** | **Student 2** |
| Purpose | 1 |  |  |
| RNA validation of samples mentioned (protocol should be linked) | 1 |  |  |
| Input RNA determined by dynamic range of the reverse transcriptase validation experiment |  |  |  |
| qPCR protocol includes number of reactions, controls and correct calculations |  |  |  |
| Identification of reference gene primers to be tested |  |  |  |
| How the data will be analyzed to determine the optimal reference gene |  |  |  |

**Appendix B.**

Example of an experimental plan with comments from another student. Students should prepare and submit their experimental plans prior to lab. At the beginning of the lab, students share (google drive) their experimental plan with another student. The instructor then goes through the marking breakdown and the required components of the experimental plan. Students use the commenting function to identify each required component or missing information.

Graphical user interface, text, application

Description automatically generated

**Appendix C.**

Example of a student-written data submission for the data obtained from the optimal reference gene experiment. The grading rubric also identifies “general feedback” for all students at the bottom of the marking rubric.

Graphical user interface, application, table, Excel

Description automatically generated

