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QPCR Protocol

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Protocol status: Working
We use this protocol and it's working

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Abstract

Collecting gene data from various cells - seeing what sort of gene is being expressed

Materials

cells, QPCR tubes, RNAse free H2O,
SYBER green master mix (Sigma Aldrich 4913850001)
SuperScript VILO
Direct-Zol RNA Miniprep - Zymo Research Cat No R2050

Cell Collection

1 Remove cells from the incubator. This protocol is for a 24-well plate.

2 Aspirate the cell media and wash the cells gently once with DPBS.

3 Aspirate the DPBS and add 350ul Trizol (Cat No.R2051, Zymo Research) onto the cells directly. Increase the volume of trizol if you have a well plate larger than a 24 well plate.

4 Use P1000 to pipette up and down to collect all the cell lysis. Make sure to go through all the corners The bottom should become clear if all cells have been detached. At this point, the cell lysis can be frozen down in 1.5mL Eppendorf tubes at -80°C, and stored up to 3 days.

RNA extraction from cells

5 Once the cell lysis has been collected in 1.5mL Eppendorf tubes, the rest of the RNA extraction is preformed using the Direct-zol RNA Miniprep kit (Cat No. R2051) and protocol provided by Zymo Research.

6 Use a P1000 pipette to add an equal volume (350uL) of 100% ethanol to each sample. Pipette up and down multiple times to thoroughly mix the ethanol and trizol, and transfer the entire mixture into a Zymo-Spin IICR column. Centrifuge the columns at x10,000g for 1 minute at room temperature. In the meantime, take out DNAse 1 to thaw.

7 Transfer the columns into a new collection tube, and discard the flow-through. Add 400uL of RNA Wash Buffer (with ethanol already added) to each column, and centrifuge at x10,000g for 1 minute at room temperature.

8 In a separate 1.5mL Eppendorf tube, mix 5uL of DNase 1 with 75uL of DNA digestion buffer (per sample). Mix by gentle inversion/pipetting, and add 80uL of this mixture to each sample column. Incubate for 15-20 minutes at room temperature.

9 After incubation, add 400uL RNA Pre-Wash Buffer to each sample, and centrifuge at same conditions. Discard the flow through.

10 Add 700uL of RNA Wash buffer to each column and centrifuge again at same conditions. Discard the flow-through and centrifuge again to ensure complete removal of ethanol and washing buffer.

11 Transfer the columns to pre-labeled RNAse-free Eppendorf tubes. Add 50uL of DNAse/RNAse-free water to each column, and let sit for one minute.

12 Centrifuge at same conditions for one minute. Discard flow-through and Nanodrop the RNA concentrations. At this point, RNA can be stored long-term in -80°C.

CDNA Processing

13 Calculate volume of RNA and UPH_2O required for cDNA synthesis. In this, protocol we use 1000ng of RNA per sample.

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	A	B	C	D	E	F
	name of cell	RNA CONCENTRATION	1000/rna concentration = CDNA	total volume - superscript volume - cdna = water	5X SUPERSCRIPT	TOTAL VOLUME
	2037	126.6	7.9	24.1	8uL	40uL
	2052	88.7	11.3	20.7	8uL	40uL

Example calculation for a total of 40uL of cDNA.

15 Add calculated volumes of UPH_2O and RNA to PCR tubes and add 8µL (or 1:5 ratio compared to total volume) of SuperScript™ VILO™ MasterMix to each reaction (40µL final vol). Vortex and spin down the PCR tubes.

16 Transfer the PCR tubes to a thermocycler, and run under the following conditions.

	25°C	10 min
	42°C	60 min
	85°C	5 min
	4°C	Hold

THERMOCYCLER SETTINGS

Wait until the run has finished and then proceed to the next section (75 minutes).

QPCR

16m 15s

17 Once the cycling has finished, transfer the 40uL cDNA from each sample to a new Eppendorf tube. Calculate how much water must be added to each sample tube. Each well needs 5uL of water + 1uL of cDNA (6uL total), and so if you have 15 genes per sample, each sample needs 15 genes x 3 replicates = 45 wells (so around 45uL of cDNA + 225uL of water). It is recommended to make extra (for instance, 50 wells in this case). Make sure to thoroughly pipette the water and cDNA mixture, and then disperse 6uL of the mixture into each well using a repeating pipette.

	A	B	C	D
	18 (round up to 22)	25 ul	107 ul	22 × 6 = 132
	48 (round up to 55)	75 ul	255 ul	55 × 6 = 330

Example of cDNA calculation for QPCR

18 In another separate tube, prepare the SYBER Green and primer mixture. You need a separate Eppendorf tube for each gene you are looking at. Again, note that you want to prepare for more wells then needed. For example, if you have 8 samples, you will need 8 samples x 3 replicates = 24 wells per gene, so it recommended to prepare for 30 wells. For each gene tube, add 7.5uL of SYBER Green with 1.5uL of Primer per well. So, for 30 wells, this would be 225uL SYBER Green + 45uL of Primer for each gene tube. Make sure to thoroughly pipette the SYBER Green and primer mixture, and then disperse 9uL of the mixture into each well using a repeating pipette.

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	A	B	C	D	E	F	G	H	I	J	K	L
	cell sample 1 + gene 1	cell sample 2 + gene 1	cell sample 3 + gene 1	cell sample 4 + gene 1	cell sample 5 + gene 1							
	cell sample 1+ gene 1	cell sample 2 + gene 1	cell sample 3 + gene 1	cell sample 4 + gene 1	cell sample 5 + gene 1							
	cell sample 1	cell sample	cell sample	cell sample	cell sample							

	A	B	C	D	E	F	G	H	I	J	K	L
	+ gen e 1	2 + gen e 1	3 + gen e 1	4+ gen e 1	5 + gen e 1							
	cell sam ple 1+ gen e 2	cell sam ple 2 + gen e 2	cell sam ple 3 + gen e 2	cell sam ple 4 + gen e 2	cell sam ple 5 + gen e 2							
	cell sam ple 1 + gen e 2	cell sam ple 2 + gen e 2	cell sam ple 3 + gen e 2	cell sam ple 4 + gen e 2	cell sam ple 5 + gen e 2							
	cell sam ple 1 + gen e 2	cell sam ple 2 + gen e 2	cell sam ple 3 + gen e 2	cell sam ple 4 + gen e 2	cell sam ple 5 + gen e 2							

Example QPCR plate

20 After the plate is all finished, add the BioRad microseal to the top of the plate. Line up the dashed lines to be at the edges to ensure that all of the wells are covered. Seal the plate tightly, and then spin down the plate in a PCR plate spinner, ensuring that the wells are pointing inwards.

21 Transfer the prepared plate to a QPCR machine (we used CFX Opus 384). Input the well plate, and choose the relevant protocol. Our protocol was the following since we used SYBER green master mix:

21.1 95 degrees for 10 minutes 10m

21.2 95 degrees for 15 seconds 15s

21.3 58 degrees for 1 minute 1m

21.4 Plate Read

21.5 Go to step 2 39 times

21.6 65-95 degree gradient for 5 minutes at a rate of 0.5 degrees celsius/cycle 5m

21.7 Plate Read

21.8 Then plate is kept at 4 degree until you are ready to remove it

22 When machine is finished running, the data can be exported to a USB for further analysis.

QPCR ANALYSIS

23 The machine will give an output of the qPCR data. Copy the relevant rows and transpose them in excel.
Line up each data in the same way the well plate was prepared, you should end up with a matrix which exactly matches the well plate inputs.

24 Check each set of 3 replicates and check to ensure there are no outliers. if there are Δ > 1.00 then this data point must be ignored.

25 Average the data to get one number per gene per sample.

26 Use the formula antilog (average # of gene A - average # of housekeeping (control)) note that housekeeping after this formula should always equal 1.000.