RNA Extraction, Reverse Transcription, and qPCR Analysis of Adherent Mammalian Cell lines

Zorya: 2019 iGEM at

University of California, Davis

https://2019.igem.org/Team:UC_Davis

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It is our goal to expand student experience with Mammalian-based systems in iGEM through the creation of a series of protocols. We hope that these instruction manuals ease the transition for future teams so they do not have to experience the same hardships we did.

-Team Zorya UCD iGEM 2019

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RNA Work

RNA Extraction

Ready RNA work space: Apply RNaseZap solution directly to RNA only work surface. Wipe thoroughly with paper towel, rinse with water then dry with clean kimwipe. Ensure that all tools, tips, and other equipment is for RNA use only.

🚫 ***WARNING!!!***

Trizol ("TRI reagent") is a phenol, which must be specially treated as hazardous waste! Use separate (disposable) liquid waste container for anything containing TRI and separate (disposable) solid waste container for any tips, tubes, pipettes, and even gloves that come into contact with phenols.

Once done with procedure, pour liquids into sealed secondary container and leave in fume hood for disposal. Double bag all solid waste in sealable bags for disposal.

All RNA Extractions were done with Direct-zol RNA Miniprep Plus kit (Zymo Research Cat. No. R2072)

Centrifuge at 10,000 - 16,000 g for 30 seconds for all steps, except the last step.

1. Clean the RNA station with RNAseZap and ethanol. The fume hood does not need this treatment.
2. While the cells are still in theirs T75 flasks, transport them to the fume hood. **(see above warning!)** Prepare Zymo-Spin IIICG Columns and collection tubes inside the fume hood.
3. Resuspend <10^7 cells in 600 ul of ***TRI Reagent***. Swirl it around the bottom of the flask.
4. Add an equal volume of 95%-100% ethanol. And swirl to mix thoroughly.
5. Transfer up to 700ul into a Zymo-Spin IIICG Column and collection tube at a time.
6. After centrifuging, bring it straight **back to the fume hood**. Discard flow through into a waste container **inside the fume hood**.
7. Bring it over to the RNA station, then change gloves. Add 400 ul RNA wash buffer to column and centrifuge.
8. Prepare the DNAse I treatment. Make a master mix. Incorporate well.

reagent	1 rxn	# rxns	5 rxns with 10% extra
DNase	5 ul	5* #	27.5 ul
DNA Digestion Buffer	75 ul	75 * #	412.5 ul

Add 80 ul to each spin column. Incubate at room temperature for 15 min.

9. Add 400 ul Direct-zol RNA PreWash to the column and centrifuge. Discard flow through and repeat this step.
10. Add 700 ul RNA Wash Buffer to the column and centrifuge for 2 min. Transfer to an RNAse-free tube.
11. Elute RNA by adding 30 ul DNase/RNAse-Free water directly to the column and centrifuge.
12. Nanodrop: aliquot ~3ul into PCR tubes and transport in ice. Choose the RNA setting in the program and blank using nuclease free water!
13. Use RNA immediately or aliquot and store frozen in 80C.

Reverse Transcription

Protocol from Thermo Fisher **(Goal:** turn RNA into cDNA for use in PCRs)
Thermo Fisher Cat. No. 18090200

Stage1: anneal primer to template RNA

1. Combine 11 uL (10 pg-5ug) purified RNA with 1 uL 10mM dNTP mix and 1 uL 50 uM random hexamers for a total reaction volume of 13 uL.
2. Mix and briefly centrifuge tubes
3. Heat RNA-primer mix at 65C for 5 minutes in thermocycler, then incubate on ice for at least one minute

Stage 2: Prepare RT reaction mix

4. Vortex and briefly centrifuge 5x SSIV buffer from kit
5. Create reaction mix by combining the following per reaction
    a. 4ul 5x SSIV Buffer
    b. 1ul 100mM DTT
    c. 1ul RNaseOUT Recombinant RNase Inhibitor
    d. 0.5 uL SuperScript IV Reverse Transcriptase
6. Cap tube, mix, and briefly centrifuge contents

Stage 3: Combine and Incubate

7. Add 6.5ul of master mix to each PCR tube
8. Incubate combined reaction mix in thermocycler at 23C for 10 minutes, 55C for 10 minutes, then heat inactivate at 80C for 10 minutes

Storage: either use RT reaction immediately for PCR amplification or store at -20C.

qPCR

Objective:

qPCR is a test that allows for the quantification of relative cDNA via amplification cycles of PCR. The SYBR FAST dye is activated when bound to double stranded DNA formed during the PCR. When activated, the dye fluoresces and this

fluorescence is monitored by the qPCR machine. The number of cycles it requires to reach a threshold level of fluorescence is indicative of how much “template” was present in the cell to begin with.

Following protocol from KAPA SYBR® FAST qPCR Master Mix (2X) Kit (KAPA BioSystems Cat. No. KR0389)

- We will be doing 2-step qPCR which involves reverse transcription of the extracted RNA into cDNA followed by qPCR as a separate step

Stage 1: plan your plate layout

Controls to include in your qPCR text batch:

1. Reverse Transcriptase Positive HeLa control - one per 24-well plate of samples
    - Provided with reverse transcriptase kit (see above) designed and tested to be successful most of the time
2. Reverse Transcriptase Negative HeLa (provided in reverse transcriptase kit) control - one per 24-well plate of samples
    - Provided with reverse transcriptase kit (see above) run without reverse Transcriptase, only RNA
3. No template control - run for each qPCR primer pair
    - Follow the same protocol below but do not add template cDNA as the final step negative reverse transcriptase control with HeLA cells provided with the kit

Stage 2: Prepare Master Mix

1. Based on the table below (for a single qPCR reaction) combine all reagents common for a subset of the reaction set as a master mix (ie. all reagents sans cDNA) in a microcentrifuge tube

Reagent	**Volume** (total=10ul)
PCR-grade water (use fresh MillQ H2O)	Up to 10ul total
KAPA SYBR FAST qPCR master mix (2X) Universal	5uL
10uM forward primer	0.2uL
10uM reverse primer	0.2uL
Template DNA (RT-cDNA)	0.5ul undiluted cDNA

2. Add 9.5 ul of the master mix to each well of your 96-well plate, then add 0.5 ul of cDNA. Gently pipette to mix

For Roche LightCycler®

Detection Format	Block Type	Reaction Volume	
SYBR Green	96 well	10 – 25 µL	
	384 well	3 – 20 µL	
Program Name	**Cycles**	**Analysis Mode**	
Pre-incubation	1	None	
Amplification	40[1]	Quantification	
Melting curve	1	Melting curves	
Cooling	1	None	
Program Name	**Target (°C)**	**Acquisition Mode**	**Hold (hh:mm:ss)**
Pre-incubation	95	None	00:03:00[2]
Amplification	95	None	00:00:10
	Primer dependent[3]	None	00:00:20[4]
	72	Single	00:00:01[5]
Melting curve	95	None	00:00:05
	65	None	00:01:00
	97	Continuous	5 –10 acq/°C
Cooling	40	None	00:00:10

[1]40 cycles are suitable for most assays; however, this may be reduced depending on initial target concentration.

[2]20 sec at 95°C is sufficient time for enzyme activation; however, optimal denaturation of complex targets may require up to 3 min denaturation.

[3]qPCR primers are typically designed for optimal annealing at 60°C; however, optimal annealing temperatures may differ from calculated values.

[4]It is not recommended to use <20 sec for primer annealing.

[5]Due to the high processivity of the engineered KAPA SYBR FAST DNA Polymerase, 1 sec at 72°C is sufficient time for extension of amplicons <400 bp.

Stage 3: Run on LightCycler

Run plate in qPCR machine (we use Roche LightCycler) under the following protocol