Berger Lab Protocol: Quantitative PCR from Human Cell RNA
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RNA Preparation
RNA can be prepared using various kits. For primary human cells or other mammalian cell culture, the Berger Lab uses the RNeasy Mini – QIAShredder kit (Qiagen, Cat# 79654). Once prepared, RNA can be stored at -20 °C for short term and -80 °C for long term. It is highly recommended that the RNA is aliquoted into multiple tubes to prevent unnecessary freeze thaw.

cDNA Preparation
cDNA can be prepared using various kits and using different priming sequences. The Berger Lab utilizes the TaqMan Reverse Transcriptase Kit (Applied Biosystems, Cat# N808-0234), which provides both random hexamers and oligo dT primers. In the case of most Pol II-mediated transcripts from mammalian cells, oligo dT should be sufficient for priming reverse transcription; however, depending on the quality of the RNA and the method for RNA preparation, it is recommended to try both the random hexamer and oligo dT to determine which works most efficiently.

50uL cDNA Reaction with Oligo dT

1ug RNA (volume adjusted to 5uL with RNase-free water)
5uL 10x TaqMan RT Buffer (final 1x)
11uL 25mM MgCl (final 5.5mM)
10uL dNTP Mix (final 500uM each dNTP)
2.5uL 50mM oligo $d(T)_{16}$ (final 2.5uM)
1uL Rnase Inhibitor (final 0.4U/uL)
1.25uL MultiScribe Reverse Transcriptase (final 1.25U/uL)
14.25uL RNase-free Water

50uL cDNA Reaction with Random Hexamers

1ug RNA (volume adjusted to 5uL with RNase-free water)
5uL 10x TaqMan RT Buffer (final 1x)
11uL 25mM MgCl (final 5.5mM)
10uL dNTP Mix (final 500uM each dNTP)
2.5uL 50mM Random Hexamers (final 2.5uM)
1uL Rnase Inhibitor (final 0.4U/uL)
1.25uL MultiScribe Reverse Transcriptase (final 1.25U/uL)
14.25uL RNase-free Water

Prepare cDNA reactions according to protocols above. It is highly recommended that a master mix is made for multiple samples. Also, a set of reactions without reverse transcriptase (no RT control) is highly recommended. Once prepared, samples should be run in a PCR machine using the following protocol:

PCR Machine Protocol for RT Reaction

25°C, 10min (necessary for optimal oligo d(T) binding)

48°C, 45min (increased from 30min, as recommended by TaqMan protocol, based on increased reaction efficiency when using longer extension time)

95°C, 5min (inactivation of RT)

Once run, cDNA can be stored at -20°C. It is recommended that the cDNA is aliquoted into multiple tubes to prevent unnecessary freeze thaw.

qPCR Reaction

cDNA is often diluted for use in qPCR assays, but the dilution factor is limited by the abundance of the transcript being assayed. To begin most qPCR protocols, it is recommended to perform a 10-fold dilution of the cDNA; depending on the results, cDNA can be further or less diluted in future qPCR assays. Note: the qPCR protocol given here is optimized for 10uL reactions in 384 well plate format (plates: Applied Biosystems, Cat# 4309849; covers: Applied Biosystems, Cat# 4311971) using a 2x SyBr Green PCR Mix (Applied Biosystems, Cat# 4367659) on an ABI qPCR Machine. Adjust volumes accordingly if utilizing a different platform.

pPCR Reaction Mix (10uL)

2uL 1:10 diluted cDNA

5uL 2x SyBr Green PCR Mix

0.05uL 10uM forward primer

0.05uL 10uM reverse primer

2.9uL water

It is highly recommended that a 384-well template is used to plan the plate setup and to calculate the number of wells being run per mastermix. Also, each set of primers being used for qPCR will require a standard curve run on the same plate. Finally, it is recommended that all samples are run in triplicate.

The plate is run using the following protocol:

qPCR Protocol

Stage I: 50°C, 2min

Stage II: 95°C, 10min

Stage III: (40 cycles)

- Step 1: 95°C, 15sec
- Step 2: 60°C, 1min

Data are analyzed using the SDS Software Program, provided with the ABI Machine. Contact Berger Lab member for assistance and further information.