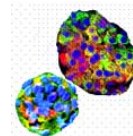


# SOP



Title:	<b>TaqMan based duplex PCR</b>				
Protocol #:	1.3	Submitted:	050510	Approved:	200610
Category:	MB	Author(s): <sup>1</sup>	MVJ, SNS	Checked by:	AAH

## Reagents:

1. Tri reagent (Sigma) / Trizol (Invitrogen) / RNeasy kit (Qiagen)
2. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, / Life Technologies, Foster City, CA)
3. TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Life Technologies, Foster City, CA)
4. Assay-on-Demand (Applied Biosystems, Life Technologies, Foster City, CA)

## Equipment

1. Eppendorf 5810R plate centrifuge or similar
2. 7500 FAST / 7900 HT real time pcr system

## Reagent Setup

N/A

## Procedure:

1. Isolate total RNA using Tri reagent (Sigma) / Trizol (Invitrogen) or RNeasy kit (Qiagen) as per the manufacturer's instructions.

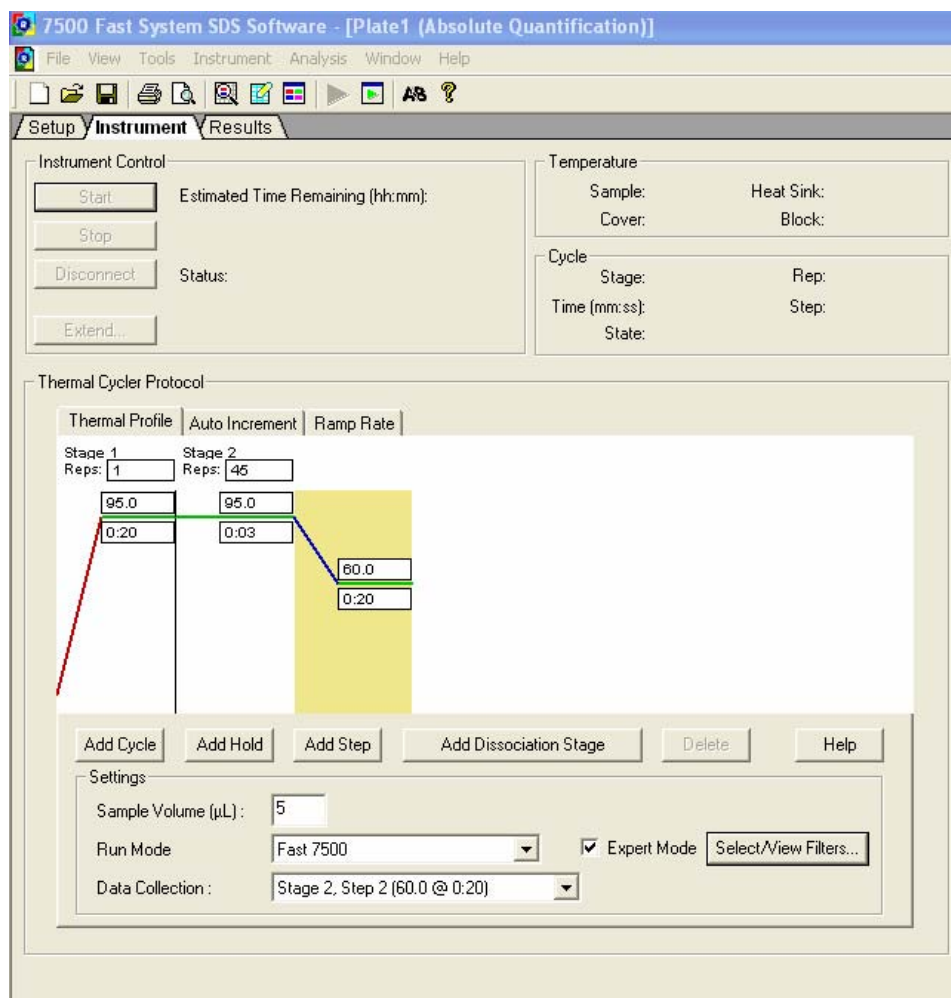
After RNA isolation, use the [Excel file](#) for calculations of master mix for reverse transcription and PCR (input the RNA concentrations and total volumes in the excel sheet). Once you have entered these values, all other calculations will be automatically populated in the sheet. Follow instructions on the sheet to prepare your reverse transcription and PCR mastermixes.

2. cDNA is prepared using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Foster City, CA).
3. PCR is performed in 5  $\mu$ l reactions in 96-well optical clear plates (AB barcoded plates) using 2 to 100ng cDNA input (prepared from 100 ng of total RNA) and TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Primers and probes are Assay-on-Demand (Applied Biosystems, Foster City, CA).  
Generally, use 100ng RNA in 10 $\mu$ l of reverse transcription reaction. After the RT reaction is over, make up this 10 $\mu$ l volume to 50 $\mu$ l by adding 40  $\mu$ l of nuclease-free water. Then, use 1  $\mu$ l of this (2ng) for real-time PCR in a 5 $\mu$ l total reaction volume for 7500 FAST /7900 HT real time PCR cycler. Primers and probes used here are Assay-on-

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Demand (Applied Biosystems / Life Technologies, Foster City, CA). For estimation of fold-changes by qRT-PCR when the initial transcript levels were undetectable, the initial Ct value is assigned to be 38, which would lead to a possible underestimation of the actual fold-change. All qRT-PCR results are normalized to 18S (VIC-labeled) ribosomal RNA carried out in duplex reaction (with FAM labeled target gene probes) to correct for any differences in RNA input. Alternatively, you can use 18s / Gapdh (FAM-labeled) in separate reaction.

After preparing the plate, centrifuge it in the 5810R plate centrifuge at 3000 rpm for 5 mins and then place it in the 7500 FAST real time pcr system next to it. Use the following program for running the plate.



4. qRT-PCR data are normalized to 18S rRNA carried out using VIC-labeled probe in duplex reaction in every well to correct for any differences in RNA input. Although we do not observe major differences in duplex Vs single plex data for 18S and GAPDH in pancreatic islet / islet-derived cells, it is advised to confirm this with your own cell samples. The template amount can be a limiting factor

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and at the same time, if you add in more of the cDNA, then this can actually lead to transferring a lot of inhibitors from your RT-reaction to the PCR. So try to keep the cDNA requirement low and work with 1ng (preferred) or a maximum of 100ng/reaction. The advantage of having duplex is really to get that additional information on the input in each well. However, always confirm your data in single-plex as well.

## Related reading:

Joglekar MV, Wei CJ and Hardikar AA (2010) Quantitative estimation of multiple miRNAs and mRNAs from a single cell. Cold Spring Harb Protocols, August issue