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qRT-PCR (RNAs including mRNA and 18S rRNA)

Supplies & Reagents:

Total RNA extracted by using Qiagen Plant RNeasy kit
TaqMan® Reverse Transcription Reagents (Applied Biosystems Part No. N808-0234)
FastStart Universal SYBR Green Master (Rox) (Roche, 04 913 850 001) or equivalent
RNase-free 0.2 ml microcentrifuge tube
96-well optical reaction plate (Applied Biosystems, Part No. 4306737) or equivalent
MicroAmp™ optical adhesive film PCR compatible, DNA/RNA/RNase free (Applied Biosystems, Part No. 4311971) equivalent
A desktop centrifuge
A thermal cycler, such as MJ PTC-100 (PCR) Thermal cycler PCR system
A real-time thermal cycler such as Applied Biosystems 7900HT sequence detection system

Important notes:

- Synthesis of cDNA from total RNA samples is the first step in the two-step RT-PCR gene expression quantification experiment. In this step, random hexamers from the TaqMan Reverse Transcription Reagents kit prime total RNA samples for reverse transcription using MultiScribe Reverse Transcriptase.
- Since we use 18S rRNA and inernal reference, therefore, we use reverse transcript protocol mainly follow the protocol for 18S amplicon using TaqMan® reverse transcript kit (ABI).
- As described by kit manual, a 100-uL RT reaction efficiently converts a maximum of 2 ug total RNA to cDNA. Perform multiple RT reactions in multiple wells if using more than 2 ug total RNA. For most of our case, we use 200ng total RNA in 10 ul RT reaction.

Reverse transcription protocol:

1. **Reaction assembling**: In a 0.2-mL microcentrifuge tube, prepare a reaction mix for all total RNA samples to be reverse transcribed. If preparing four reactions, follow the recommended volumes shown below.

10xRT buffer	1.0ul
25 mM $MgCl_2$	2.2ul
dNTPs	2.0ul
Random Hexamers	0.5ul
RNase Inhibitor	0.2ul
MultiScribe Reverse Transcriptase	0.625ul

Add 200ng RNA sample (e.g. 2ul 100ng/ul total RNA), then add RNase free water to final total volume of 10ul.

2. Cap the tubes and gently tap each to mix the diluted samples. Centrifuge the tubes briefly to eliminate air bubbles in the mixture. Load the reactions into a thermal cycler for RT reaction.
3. Program your thermal cycler with the following conditions: 25°C for 10 min, then 37°C for 60min followed 95°C 5min.
4. After thermal cycling, dilute cDNA samples and store at –15 to –25 °C.

qRT-PCR protocol:

1. **qRT-PCR**: Reaction is performed using SBRY green real time PCR reaction: Prepare PCR reaction mixture by adding 1 µl (diluted) template cDNA (equivalent to 0.1~1 ng total RNA), 5 pmol each of the forward and reverse primers, 10 µl 2×SYBR Green PCR Master Mix and added water to a final volume of 20 µl.
2. **PCR program**: we use standard protocol of the Applied Biosystems' 7900HT system for quantitation of gene transcript level and 18S rRNA level (reference for normalization): 10 min preheating followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C, followed by thermal denaturation to generate dissociation curves for verifying amplification specificity.
3. **Dissociation curve analysis:** Peak of product was examined following the protocol in the manual of the ABI 7900HT.In brief: in option window of dissociation curve, if there is a signal peak between 70 to 85°C, then PCR amplification is specific.
   Note: More peaks might result of unspecific amplification, and such PCR result will not reliable for further quantitation analysis.
4. **Quantitation**: In most cases, amplification efficiencies for most transcripts are near to the maximum (2). Thus, the quantity of test transcript relative to a reference transcript(18S rRNA), can be calculated using the formula $2^{-\Delta CT}$, where $\Delta C_T = (C_{T\ target\ RNA} - C_{T\ reference\ RNA})$[16]. To compare gene expression, such as the target transcript among different samples, their abundances can be calculated using a comparative C_T method[17] ($\Delta\Delta\ C_T$), and the relative gene expression can be quantified by using the formula of $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (C_{T\ target\ RNA} - C_{T\ reference\ RNA}) - (C_{T\ calibrator} - C_{T\ reference\ RNA})$. The wild type sample was usually selected as reference sample, or named as "calibrator", and its expression level represents 100% for normalization in each comparison