Sybr Green Real Time qRT-PCR

Purpose: To assay transcript levels using SYBR® Green.

Reagents:

- SYBR® Green PCR Master Mix (Applied Biosystems®) Catalog Number 4309155 (Size 1 x 5 mL)

- TaqMan® Reverse Transcription Reagents (Invitrogen™) Catalog Number N8080234

(Size 200 reactions)

- MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems®) Catalog Number N8010560 (Size10 plates)

- MicroAmp® Optical Adhesive Film (Applied Biosystems®) Catalog Number4360954

Size25 covers.

Protocol:

Step 1. Reverse Transcription (From TaqMan® Universal PCR Master Mix)

A 100-µL RT reaction efficiently converts a maximum of 2 µg total RNA to cDNA. Perform multiple RT reactions in multiple wells if you are using more than 2 µg total RNA. For a 10-µL RT reaction you can use up to 500ng of RNA in ≤ 3.85µl of water.

RT Reaction Mix

Component	Volume (µL) Per Sample	Final Concentration
RNase-free water	See below[a]	—
10X TaqMan RT Buffer	1.0	1X
25 mM Magnesium Chloride	2.2	5.5 mM
deoxyNTPs Mixture	2.0	500 µM per dNTP
Random Hexamers[b]	0.5	2.5 µM
RNase Inhibitor	0.2	0.4 U/µL
MultiScribe Reverse Transcriptase (50 U/µL)	0.25	1.25 U/µL
Total	6.15[c]	—

a. The volume of RNase-free water (µL) is 3.85–RNA sample volume in a 10-µL reaction.

b. Random hexamers, oligo $d(T)_{16}$, or sequence-specific reverse primers can be used for primers of cDNA synthesis.

c. If changing the reaction volume, make sure the final proportions are consistent with the recommended values above.

Note The RT volume can vary from 10 µL to 100 µL. Increasing the RT volume will reduce the total number of reactions.

Thermal Cycling To conduct RT thermal cycling:

Step	Action
1	Load the reactions into a thermal cycler.
2	Program the thermal cycler or Sequence Detection System with the following conditions:

Step	Incubation[a]	RT	Reverse Transcriptase Inactivation
	HOLD	HOLD	HOLD
Temp	25 °C	48 °C	95 °C
Time	10 min	30 min	5 min
Volume	10 µL		

a. If using random hexamers or oligo $d(T)_{16}$ primers for first-strand cDNA synthesis, a primer incubation step (25 °C for 10 min) is necessary to maximize primer-RNA template binding.

3	Begin RT. **IMPORTANT** After thermal cycling, store all cDNA samples at –15 to –25 °C.

Step 2. qRT-PCR using sybr green.

- Dilute your cDNA 1/10 in water.

- Prepare master mix for each primer set. Final volume per well= 25 µl.

Reagent	Volume
2X SYBR	12.5 µl
Water	10.5 µl
10µM Primer 1	0.5 µl
10µM Primer 2	0.5 µl
Template (1-5ng)	1 µl

Step 3. Assay and Analysis.

A. Load Optical 96 Well Reaction Plate

- Pipet 1 µl from each template into appropriate well.

- Pipet 24 µl from master mix into appropriate well.

- Load 25 µl from leftover Master Mix tubes as No-Template-Controls (NTC). Load at least one NTC for each primer pair.

- Cover the plate with optical adhesive cover film. Handle only by the edges and do not touch the surface of the film

- Spin the plate for 2 min. in the benchtop swing bucket centrifuge (1000 rpm for one minute).

- Run standard Real Time PCR in the QuantStudio 6 system.