Real Time PCR protocol

Reverse Transcription for Standard RNA (AB Taqman)

Total RNA (best from liver) (2.0 µg)	Xµl
10X Buffer	10.0µl
25mM $MgCl_2$	22.0µl
dNTP Mixed (10mM)	20.0µl
Oligo dT or Random Hexamer (50µM)	5.0µl
RNase Inhibitor (20U/µl)	2.0µl
Reverse Transcriptase (50U/µl)	2.5µl
RNase free H_2O	(38.5-X)µl

Total Volume ***100 µl***

Note*: A 100µl RT reaction efficiently converts a maximum of 2.0µg total RNA to cDNA. Multiple RT reactions should beperformed if more than 2.0µg total RNA is used.*

Incubate @ 25°C for 10 min
RT @ 48°C for 30 min
Inactivate @ 95°C for 5 min

Note*:*
1.The incubation step is necessary to maximize primer-RNA template binding.
2.Store all cDNA samples at -15 to -25ºC

Make Standard Curve

Note*:*

1. *The ideal standard curve range is between 10^2~10^7 copy of cDNA.*
2. *average: 2.28 x 10^8 copy = 1ng cDNA*

Use **SPECTRAmax PLUS** plate reader to read the cDNA concentration.
Use the following protocol: Under menu → Assays → Nucleic Acids → Pre-read Plates with Dilutions
Note*: At least do duplicates, usually do triplicates.*

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1. Add 98µl of H_2O to each well
2. Read plates
3. Add 2µl of cDNA prepared by previous RT reaction.
4. Read plates.

***Note**: Make sure dilution factor is 50. The concentration unit is in µg/mL.*

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From the reading concentration (usually several hundred µg/mL or ng/µl), try to dilute down the concentration to 1x 10^7 copy of cDNA/µl. That is, if in 100µl of final volume, 4.39ng of cDNA should be added. Make a serial dilution to achieve this concentration instead of one simple dilution (less accurate because of small vol- ume pipeting).

Calculations: (*eg*. **550**ng/µl →(*10µl in 90µl*)→ **55**ng/µl →(*10µl in 90µl*)→ **5.5**ng/µl →(*10µl in 45µl*)→ **1** ng/µl →(*4.39µl in 95.61µl*)→ **10^7** copy/µl)

Once 1x10^7 copy of cDNA was made. Make a serial dilution range from 1x10^2 ~ 1x10^7 (6 different concentrations). These will be used as a standard curve purpose in later real time PCR reactions.

***Note**: It might be more accurate to make 2-fold standard curve instead of 10-fold. But for totally unknown sample, make 10-fold standard curve usually is the case.*

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Real time PCR Reactions (SYBR Green 2x Master from AB)

1. Normalize the primer concentrations and mix gene-specific forward and reverse primer pair. Each primer concentration in the mixture is 0.4 pmol/µl.
2. Make the following mixture/reaction.

SYBR Green Mix (2x)	12.5µl
Sample cDNA or 10-fold dilutions standard cDNA	1µl
Primer pair mix (0.4 µM)	1µl
H2O	up to 25µl
Total volume	**25µl**

Note: *Make triplicates for all samples and standard cDNA!*

3. The conditions for the amplification were as follows:

1. 50°C	2 min
2. 95°C	10 min
3. 95°C	15 sec
4. 60°C	30 sec
5. 72ºC	30 sec
5. go to step 3 for 39 times	
6. 72°C	10 min

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4. After PCR is finished, remove the tubes from the machine. The PCR specificity can be examined by 3% agarose gel using 5µl from each reaction.
5. Analyze the data.

Note: *Each primer set needs to have each standard curve. A control primer set is usually required.*

Tips: *Small volume pipeting: Always pipet small volume on the plate first, then pipet the large amount. Here, try pipet 1µl ofcDNA sample in each 96-well first, then add the reaction mixture on top of it.*

Real-time PCR Run

1. Use the white 96-well plates (located in the drawer of realtime PCR machine); caps in the same drawer. Donot try to recycle the plate, as potential mistakes can be made.
2. Open the opticon program, select user, load the main protocol that has same cycles as described earlier but also included a melting curve calculation following the final protocol: 65ºC to 95ºC in 0.2ºC increments withdata acquisition at each increment for 0.1 s.
3. The entire plates will be read no matter how the plate setup were looked like, therefore, it's not critical to have plate setup right at the beginning. However, it is highly recommended to use the template attached to thisprotocol to plan ahead the plate.

Note*: To enter the number greater than* 10^6*, use scientific citation, such as* 10^7 *= 1e7.*

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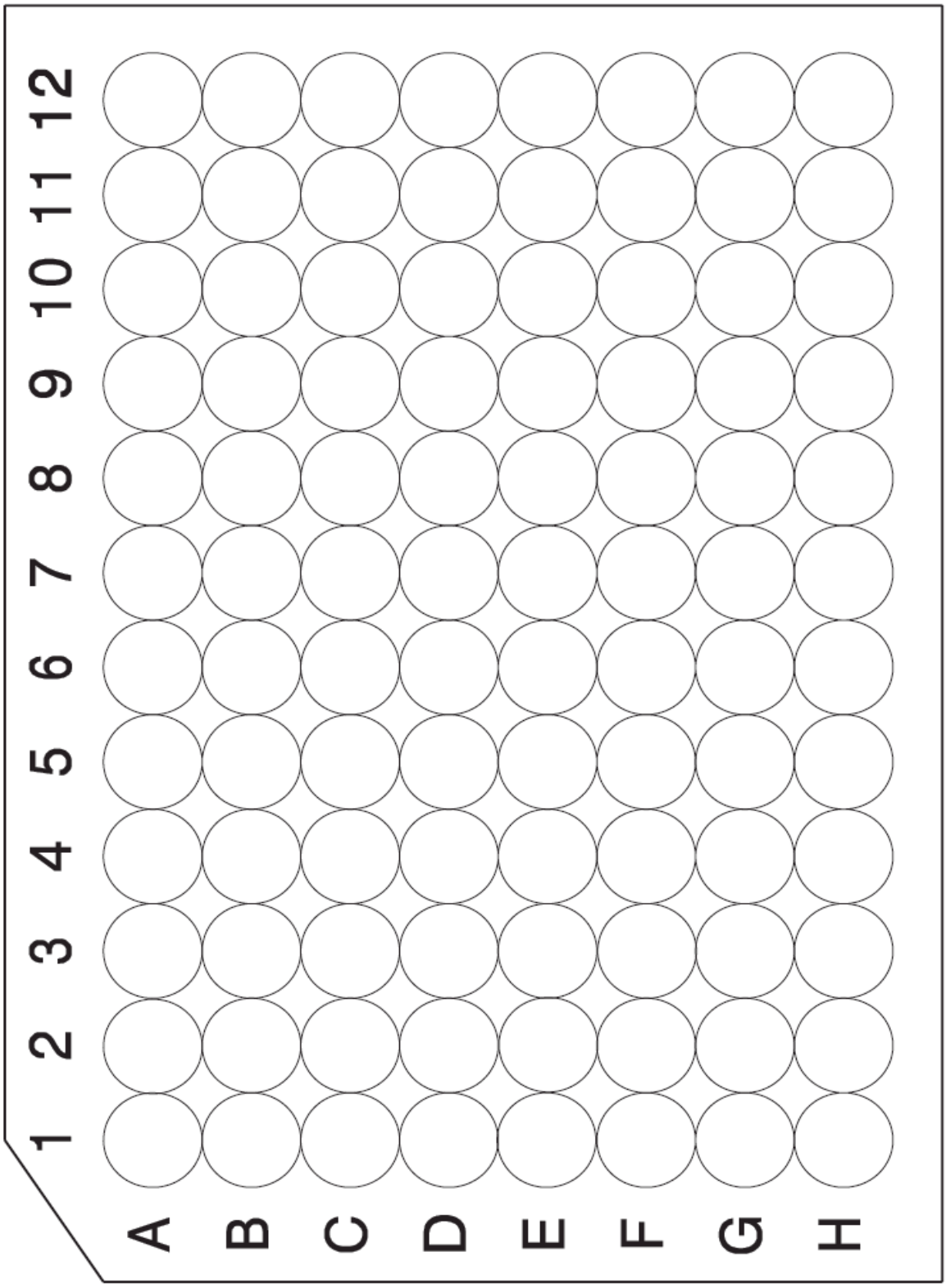

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