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Real-time quantitative RT-PCR (Taqman)

This is performed as a 2-step reaction:
1. cDNA synthesis from DNase 1-treated total RNA
2. PCR

1. cDNA synthesis (Advantage ä RT-for-PCR Kit - Clontech)

All reagents listed are provided with the kit

a) Quickly thaw each tube in kit and place on ice
b) Spin each tube briefly in a tabletop microcentrifuge and return to ice
c) In a sterile 0.5ml microcentrifuge tube, add purified DNase 1-treated RNA preparation to a volume of DEPC-treated H_2O that will give a total volume of 12.5 ul. (Can use 0.2-1ug of total RNA, but recommend 1ug where possible). Use the same amount of RNA for each sample.
d) Add 1ul of either the random hexamer or the oligo$(dT)_{18}$ primer (both are provided with the kit). (Oligo(dT) priming is the method of choice; however, if the 5′ gene-specific Taqman primer is located greater than about 2-3 kb from the poly-A tail would recommend use of random hexamer)
e) Heat total RNA at 70°C for 2 min and *RAPIDLY* place heated RNA in ice
f) Add the components listed in the table below according to the volumes given

Reagent	Volume
5X reaction buffer	4.0 ul
dNTP mix (10 mM) each	1.0 ul
Recombinant RNase inhibitor	0.5 ul
MMLV reverse transcriptase	1.0 ul

N.B. If more than one RNA sample is being used for RT-PCR, it is recommended to prepare a master reagent mix.
Some reactions may also be included substituting DEPC-treated H_20 for reverse transcriptase, and later amplified in a separate PCR reaction in order to detect any genomic DNA contamination that may be present within the RNA samples.

g) Mix the contents of the tube by pipetting up and down
h) Incubate the reaction at 42°C for 1 hour
i) Heat at 94°C for 5 min to stop the cDNA synthesis reaction and to destroy any DNase activity; then spin down the contents of the tube
j) Dilute the reaction to a final volume of 100ul by adding 80ul of DEPC-treated H_20. Vortex and spin again.

2. PCR protocol (multiplex reaction)

Before the PCR can be performed on the newly-synthesised cDNA in 1., a number of steps must be carried out:

a) Selection of a suitable internal standard
b) Design of primers and probes to target genes
c) Validation of equal efficiency of amplification of target gene and internal standard
d) Optimisation of primers

a) Internal standard selection

An appropriate gene for use as an internal standard and normalisation of data must be selected (this may be already designed and available from Applied Biosystems or can design own using PrimerExpress software)

N.B. For accurate quantitation, the internal standard must:

- be amplified simultaneously with the target
- be expressed at a constant level and be unaffected by the experimental treatment
- should be expressed at roughly the same level (preferably slightly more abundant – see primer optimisation below) as the RNA under investigation, to avoid competition of the more abundant target for PCR reagents

b) Primer and probe design

Primers and probes may be designed for selected target sequence using Applied Biosystems PrimerExpress software.
The following must be considered:

- primer and probe selection is based on estimated T_m, the desire for small amplicon size and location
- must be designed to fit many specifications– *Primer Express* software
- primers should bind to separate exons to avoid false positive results arising from amplification of contaminating genomic DNA
- if intron/exon boundaries are unknown, or when targeting an intron-less gene, RNA should be treated with DNase I
- When ordering target gene and internal standard probes to be used in multiplex reactions, ensure they are labelled with different reporter dyes
- on arrival, primers should be resuspended in DEPC-H_2O to a concentration of 100 pmol/ul; probes arrive in solution at 100 pmol/ul

c) Amplification efficiency validation

A validation experiment must be performed to ensure that amplification efficiencies of the target gene and internal standard are approximately equal. This is necessary in order to determine if any competition may occur within the multiplex reaction.

- A sensitive method for assessing if two amplicons have the same efficiency is to look at how ΔCt (CT target – CT internal standard) varies with template dilution.
- This may be carried out by amplification of the target gene and internal standard over a range of template dilutions. (Recommend serial 5-fold dilutions of orignal cDNA template).

1. Prepare a mastermix of the reaction mix below so that there is enough for all samples to be investigated.

	Reagent	Volume (for 1 sample)
	Taqman universal PCR mix	10.0 ul
Target gene	Forward primer	0.2 ul
	Reverse primer	0.2 ul
	Probe	0.05 ul
Internal standard	Forward primer	0.2 ul
	Reverse primer	0.2 ul
	Probe	0.05 ul
	H_20	4.1 ul

2. Prepare serial 5-fold dilutions of original cDNA template

3. Add 15 ul of reaction mix above to each well in a 96-well plate.

4. Add 5 ul of each cDNA sample to separate wells in the plate.

5. Subject plate to the following cycling on the ABI Prism 7700 sequence detector:

   50°C, 2 mins
   95°C, 10 mins
   40 cycles of the following:
   95°C, 15 sec
   60°C, 1 min

4. Following amplification, record CT values for target gene and internal standard.

If efficiency of amplification of the target gene and internal standard are the same, DCT should remain the same for all template dilutions. However, if the amplification efficiency of the target gene and internal standard are not equal, this may indicate that competition may occur in the mutiplex reaction. If this is the case, a new internal standard may be selected, or reactions may be carried out in separate wells.

d) Primer optimisation

Reactions to amplify two different segments in the same tube share common reagents. If the two segments have different initial copy numbers, it is possible for the more abundant species to use up these common reagents, impairing amplification of the rarer species. For accurate quantitation, it is important that the two reactions do not compete. Competition can be avoided by limiting the concentration of primers used in the amplification reactions. Primer concentrations are therefore adjusted such that accurate CTs are obtained, but soon after that, the exhaustion of primers defines the end of the reaction. The desired concentrations are those that show a reduction in DRn but little effect on CT. In this way, amplification of the majority species is stopped before it can limit the common reactants available for amplification of the minority species. It is preferable for the selected internal standard to be slightly more abundant than the target gene, as then only the concentrations of the internal standard need be limited.

Limiting primer concentrations may be defined by running a matrix of forward and reverse primer concentrations:

1. Make up enough mastermix for 76 samples as follows:

Reagent	Volume (for 1 sample)
Taqman universal PCR mix	10.0 ul
Probe	0.05 ul
cDNA	5.0 ul
H_20	0.95 ul

2. Prepare a separate dilution series for each of the forward and reverse primers.
   First dilute primers (as resuspended, see 2b) to 10 uM (i.e. a 1:10 dilution)

32ul 10uM primer	+	368 ul H20	⇒	800nM
75ul 800nM primer	+	25 ul H2O	⇒	600nM
62.5ul 800nM primer	+	37.5 ul H20	⇒	500nM
50ul 800nM primer	+	50 ul H20	⇒	400nM
37.5ul 800nM primer	+	62.5 ul H20	⇒	300nM
25ul 800nM primer	+	75 ul H20	⇒	200nM

3. Add to wells in the PCR plate

   2ul of 800nM forward primer to A1-12
   2ul of 600nM forward primer to B1-12
   2ul of 500nM forward primer to C1-12
   2ul of 400nM forward primer to D1-12

2ul of 300nM forward primer to E1-12
2ul of 200nM forward primer to F1-12

4. Add to wells in the PCR plate

   2ul of 800nM reverse primer to 1, 2 A-F
   2ul of 600nM reverse primer to 3,4 A-F
   2ul of 500nM reverse primer to 5,6 A-F
   2ul of 400nM reverse primer to 7,8 A-F
   2ul of 300nM reverse primer to 9, 10 A-F
   2ul of 200nM reverse primer to 11, 12 A-F

5. Add 16 ul of master mix to each tube

6. Set up the thermal cycling conditions for the ABI Prism 7700 sequence detector as described previously:

   50°C, 2 mins
   95°C, 10 mins
   40 cycles of the following:
   95°C, 15 sec
   60°C, 1 min

7. Following amplification, select the concentration of primers that show a reduction in ΔRn, but little effect on CT.

N.B. If limiting primer concentrations cannot be defined, or if the internal standard is less abundant than the target gene, amplification of individual genes may be carried out in separate wells or primers should be redesigned.

e) Real-time quantitative PCR

Once each of the steps a-d have been carried out, the quantitative PCR reaction may then be performed.

1. Prepare a mastermix of the reaction mix below so that there is enough for all samples to be investigated.

	Reagent	Volume (for 1 sample)
	Taqman universal PCR mix	10.0 ul
Target gene	Forward primer (optimised conc.)	0.2 ul
	Reverse primer (optimised conc.)	0.2 ul
	Probe	0.05 ul
Internal standard	Forward primer (optimised conc.)	0.2 ul
	Reverse primer (optimised conc.)	0.2 ul
	Probe	0.05 ul
	H_20	4.1 ul

2. Pipette 15 ul of the PCR mix above into individual wells on a 96-well plate

3. Add 5ul of each cDNA sample to separate wells in the plate.

4. Subject the plate to the following cycling on the ABI Prism 7700 sequence detector:

50°C, 2 mins
95°C, 10 mins
40 cycles of the following:
95°C, 15 sec
60°C, 1 min

5. Following amplification, compare CT values of samples (normalised to internal standard) in order to assess fold differences in mRNA levels of the target genes.