**qPCR primer design guidelines:**

• For highly homologous gene family members, software design is not recommended.  Pick the unique region for each gene, especially 3’-region of primers.
• Manage the melting temperature (Tm) between 58-60°C, and primers can be 20-30 bases in length.
• The run of an identical nucleotide should be avoided, especially G. Avoid consecutive Gs and Cs in the last five nucleotides at the 3' end.
• It’s critical that the maximum amplicon size should not exceed 400 bp (ideally 75-150 bases).

– PCR is more efficient and more tolerant of reaction

• Choose exon-exon junctions in the cDNA to avoid false-positive amplifications.

 **Conducting qPCR**
Check points:
• After reverse transcription reaction, do “normal” PCR amplification and check the product purity and size by electrophoresis

– Test primer hybridization specificity and cDNA quality

• Prepare no-reverse transcriptase cDNA samples as negative controls and run qPCR with each primer, for at least once.

– Positive with 18S primer indicates genomic DNA contamination.

– Positive with specific gene indicates cDNA contamination from that gene.

• After the qPCR reaction finishes, check “dissociation stage” of the results

– One peak indicates that primers are specific
– If several primers give different types of single peak, choose the one with sharper peak and higher dissociation temperature.

**Step 1:  RNA Extraction (QIAgen plant mini kit)2**

* 1. Autoclave motor pestle, eppendroff tubes and  tips and dry.
	2. Weight around 100 mg plant material, grind it in liquid N2 and make powder in mortar pistil.
	3. Add 450 µl buffer RLT (add beta merceptoethanol (ß-ME) before using (10 µl in 1 ml RLT buffer) and vertex vigorously.
	4. Pipett the lysate directly into a QIA shredader spin column (purple color) in 2 ml collection tube.
	5. Centrifuge  for 2 min at maximum speed (~14000 rpm)
	6. Transfer the supernatant carefully (without disturbing the green pellet) into a new 2ml centrifuge tube.
	7. Add (0.5 vol) 225 ml ethanol (96-100%) to the cleared lysate and mix immediately by pipetting. (DO NOT VORTEX)
	8. Add 650 ml sample (including any precipitation to an RNeasy mini column placed in a 2 ml collection tube, close it gently.
	9. Centrifuge for 15 sec at 10,000 rpm. Discard the flow through.

**If you are doing DNAse treatment (DNase treatment is optional, read the manual for more details)**

1. Pipett 350 µl RW1 buffer into RNAse column and centrifuge for 15 sec at 10,000 rpm. Discard the flow through and collection tube.
2. Add 10 µl DNAse I (stock) and 70 µl RDD buffer (supplied) and mix gently by inverting the tube. Pipett this mix on silica gel  membrane, place on bench top for 15-20 min.
3. Pipett 350 µl RW1 buffer into RNeasy mini column.
4. Centrifuge for 15 sec at 10,000 rpm. Discard flow through and collection tube.
5. Transfer the RNAse mini column into a 2 ml collection tube (supplied).
6. Pipett 500 µl RPE buffer onto RNeasy column, close it gently.
7. Centrifuge it for 15 sec at 10,000 rpm, Discard the flow through
8. Add again 500 µl RPE buffer, close it gently.
9. Centrifuge it for 2 min at 10,000 rpm. Discard the flow through and collection tube.
10. To elute, transfer the RNAeasy column to new 1.5 ml collection tube, pipett 30-50 µl RNAase free water directly onto RNAeasy column silica gel membrane, close it gently
11. Centrifuge it for 1 min at 10,000 rpm.
12. Quantify the RNA by spectrophotometer at 260 and 280 nm.
13. **Step 2:  c DNA preparation (when you are using Random Hexamer Primer)**

|  |  |
| --- | --- |
| RNA (2 µg-5 µg) | x µl |
| Primer (0.3 µg/ µl) | 1 µl |
| 10 mM dNTPs | 1 µl |
| DW | y µl |
| Total | 13 µl |

1. Keep this reaction mix on 65°C for 5min (The program already feeded in Mastercyler Gradiant).
Chill on ice for 2-5 min, brief centrifuge the reaction mix.

Add-

|  |  |
| --- | --- |
| 5x First strand buffer | 4 µl |
| 0.1M DTT | 1 µl |
| RNAse inhibitor | 1 µl |
| Reverse transcriptase (SSIII) | 1 µl |
| Total | 20 µl |

1. Pipetting it gently, Incubate at 25°C for 5 min.
Incubate at 50°C for 50 min. Inactive the reaction heating at 70°C for 15 min.
Hold at 4°C. cDNA is ready.

**Step3 : qPCR reaction set up**

1. Dilute cDNA  to 0.02 µg/original RNA µl
2. Prepare a labeled tubes for each c DNA samples (For example: prepare 3 tubes for 3 cDNA samples)

1. cDNA+ dye mix

|  |  |  |
| --- | --- | --- |
| cDNA | 1.95x No of primers | (3) |
| DW | 13.8 x No.of primers | (3) |
| Dye Mix | 15x No. of primers | (3) |

1. 3. First mix c DNA and DW then dye mix through pipetting gently and keep immediately on ice Centrifuge the tubes in cold condition now all the mixing and pipetting will done on ice
4. Aliquot the each sample (30 µl) in separate 0.5 ml tubes already lebelled and kept on ice

|  |  |  |  |
| --- | --- | --- | --- |
| cDNA+dye Mix | Primer A | primer B | primerC (Ribosomal) |
| Sample 1 | 30 | 30 | 30 |
| Sample 2  | 30 | 30 | 30 |
| Sample 3  | 30 | 30 | 30 |