**Gene Expression Analysis by RT-qPCR**

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**Introduction:**

General Steps of gene expression analysis by RT-qPCR includes

1. Total RNA Isolation
2. DNase I Treatment
3. Reverse Transcription
4. Real time qPCR

**1. Total RNA Isolation:**

Single-step RNA isolation method was developed by Chomczynski, P. and Sacchi, N. in 1987 (1). The name of the method is Guanidinium thiocyanate-phenol-chloroform extraction. The isolation of total RNA from cells and tissues can be achieved by **TriZol Reagent**, a commercial reagent (Sigma Cat. No. 15596-018). The reagent contains a mono-phasic solution of phenol and guanidine isothiocyanate. During sample homogenization or lysis, TriZol Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an upper aqueous phase and a lower organic phase (mainly chloroform). RNA remains exclusively in the aqueous phase, while DNA and protein partition in the interphase and organic phase, respectively. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. After removal of the aqueous phase, the DNA and proteins in the sample can be recovered by sequential precipitation. Precipitation with ethanol yields DNA from the interphase, and an additional precipitation with isopropyl alcohol yields proteins from the organic phase.

Here you can find the step-by-step descriptions of **home-made TriZol Reagent** preparation and total RNA isolation by using TriZol Reagent.

Preparation for Isolation:

1. Prepare DEPC-treated water (DEPC-dH2O).
2. Keep all pipette tips (blue, yellow and white), eppendorf tubes, graduated cylinders and bottles in DEPC-dH2O in hood for at least 10 hours (can be done overnight).
3. Next day, remove all the liquid from plastic-ware.
4. Cover mortars and rap pestles with aluminum foil.
5. Autoclave covered mortars, pestles and plastic-ware at 121⁰C for 40 minutes (liquid cycle).
6. Let everything dry overnight in the oven.

Caution! Always wear gloves to eliminate any RNase contamination coming from your hands.

 Use mask while preparing the TriZol (if you have any allergies).

Preparation of DEPC-treated distilled water (DEPC-dH2O):

DEPC – Diethylpyrocarbonate (Sigma, Cat. No. D-5758). Stock solution is 10% (V/V) DEPC in ethanol. Store at 4⁰C (Lab location - R2 D1).

Danger

DEPC is carcinogenic. Prepare and use in hood.

For DEPC-dH2O, Add DEPC stock solution to dH2O to a final concentration of 0.1% (V/V) such that add 1mL DEPC into 1L dH2O. Then, mix vigorously until all the droplets are dissolved in solution. Incubate overnight in hood while the lid is half-closed. The next day, autoclave the solution for 25 minutes at 121⁰C (liquid cycle).

**Note:** Tris solutions cannot be DEPC treated. Solutions should be DEPC treated and autoclaved before adding Tris. After addition of Tris, the solution should be autoclaved again.

**Home-made TriZol Reagent Preparation**

Required chemicals:

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Name of the chemical** | **Supplier** | **Cat. No.** | **Lab Location** | **Caution** | **Usage Purpose** |
| Guanidine isothiocyanate | EMD | 5120 | A26-197 | Toxic, carcinogen | Denatures proteins, RNases, and separates rRNA from ribosomes |
| N-Lauryl-sarcosine sodium salt (sarcosyl) | Fisher | BP234 | Cobb’s Lab | Irritant | Detergent |
| Sodium citrate (C6H5Na3O7.2H2O) | Sigma | S-4641 | Cobb’s Lab |  | Buffers the solution |
| Potassium acetate (KC2H3O2) | Fisher | 350175 | A7-126 | Irritant | Buffers the solution |
| Water-saturated phenol | EMD | PX0511-1 | Liquid Storage Cabinet | Irritant, Carcinogen, Toxic, Mutagenic, Corrosive | Dissolves proteins |
| β-mercaptoethanol (2-mercaptoethanol) | EMD | 6010 | Fridge R2 | Toxic, Irritant | Denatures proteins by breakage of S- bonds |
|  |  |  |  |  |  |

Danger

 TriZol is carcinogenic.

Preparation Procedure:

The procedure consists of 4 separate steps of chemical preparation & mixture:

**1. 100mM Sodium citrate (pH:7.0):** (For 20mL)

Dissolve **0.588 g** of sodium citrate in 15mL DEPC-dH2O & Adjust pH to 7.0 by using 10N HCl. Finally, complete the volume to 20mL by adding DEPC-dH2O.

**2. 2M Potassium acetate (pH: 4.8):** (For 20mL)

 Dissolve **3.92563 g** of potassium acetate in **10mL** DEPC-dH2O & Adjust pH to 4.8 by using **acetic acid**. Finally, complete the volume to 20mL by adding DEPC-dH2O.

**3. Mixing Chemicals (part1):**

Add & weigh & mix following chemicals & solutions as given for each total volume of TriZol Reagent.

Caution! β mercaptoethanol is toxic; add it in hood. Mix the solution while the bottle lid is closed

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Chemical** | **For 110mL** | **For 100mL** | **For 50mL** | **For 30mL** |
| Weigh out guanidine thiocyanide | 23.7 g | 21.545 g | 11.85 g | 6.493 g |
| Add 100mM Na-citrate (pH: 7.0) | 12.5 mL | 11.364 mL | 6.25 mL | 3.4 mL |
| Add 35% sarcosyl | 0.25025 g | 0.2275 g | 0.11375 g | 0.06825 g |
| Add β-mercaptoethanol | 340 µL | 309.1 µL | 170 µL | 93 µL |
| Bring volume to given amount with DEPC-dH2O | 50 mL | 45.5 mL | 22.75 mL | 13.6 mL |

\* Autoclave all after mixing & dissolving solid materials (at 121⁰C for 20 mins).

Danger

**TriZol is light sensitive**; bottle should be covered with aluminum foil before

phenol adition.

**4. Mixing Chemicals (part2):**

Caution! Phenol is toxic; Add in hood.

Continue to add phenol and potassium acetate

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Chemical** | **For 110mL** | **For 100mL** | **For 50mL** | **For 30mL** |
| Mix with phenol | 50 mL | 45.5 mL | 22.75 mL | 13.6 mL |
| Add 2M KOAc (pH: 4.8) | 10 mL | 9 mL | 4.5 mL | 2.8 mL |

Caution! TriZol should be stored at **+4⁰C**.

**Total RNA Isolation:**

Caution! Mortal and pestle should be pre-cooled by liquid nitrogen before placing the frozen tissue in mortar.

1. Cool mortar and pestle in liquid nitrogen (N2). Then, keep some amount of N2 in mortar before placing the plant material.
2. Put plant material into N2-containing cold mortar and disrupt the material grinding in N2 until the material becomes unified without any observable big particles.
3. Add some N2 onto disrupted material and adjust the mortar in an inclined angle such that all the liquid and materials may be collected at the bottom of mortar. (**Figure 1-A**)
4. Label the 1.5mL eppendorf tube. Open the tip of the tube without touching inside of the tip. Cool it in N2 for a few seconds (Be careful not to submerge the tube all the way into N2) (**Figure 1-B**)
5. Without touching inside of the tip collect all the material into the eppendorf tube by using it like a hoe as shown in **Figure 1-C**.
6. Immediately store the eppendorf tube **lid open** on a small rack kept in N2.



 **Figure1-A Figure1-B Figure1-C**

**Figure 1.** First steps of sample grinding and RNA extraction.

1. Add 1mL of TriZol Reagent into eppendorf tube.
2. Immediately vortex the tube as quickly as possible to dissolve the plant material. This process may take up to 2 mins per tube until the mixture is homogenous.
3. Repeat steps 7 and 8 for other samples.
4. Cool the micro-centrifuge to +4⁰C.
5. Centrifuge the tubes for 5 mins at +4⁰C at 15000 RPM.

(Personally, you can centrifuge the tube for an additional 3 mins by converting the tube in reverse direction)

1. Transfer 900-950µL of supernatant into new tube (Don’t touch to pellet).
2. Add 200µL of chloroform & Shake vigorously for 15 secs. & incubate at room temperature (RT) for 5 mins.
3. Centrifuge the tube for 20 mins at +4⁰C at 15000 RPM.

**Note1:** This step gives phase separation as shown in **Figure 2**.



**Figure 2.** Phase separation after centrifugation. Phase separation by centrifugation of a mix of the aqueous sample and a solution containing water-saturated phenol, chloroform and a chaotropic denaturing solution (guanidinium thiocyanate) results in an upper aqueous phase and a lower organic phase (mainly chloroform). Nearly all of the RNA is present in the aqueous phase, while DNA and protein partition in the interphase and organic phase, respectively. In a last step, RNA is recovered from the aqueous phase by precipitation with 2-propanol.

**Note2:** If you want to isolate **genomic DNA**, you can collect the white, sticky interphase layer by help of a tip or toothpick. The cleaning and precipitation procedures are given in **Appendix A**.

1. Take 300-400µL from upper phase into new tube. (Use yellow tips and collect the upper phase in two steps of 150-200µL each.)

Caution! Do not touch to white interphase (DNA).

 In case you touch to the interphase, re-centrifuge the tube for 5mins and try to collect the upper phase.

1. Add equal volume of pre-cooled isopropanol onto recovered upper phase. (Example: If you recover 300 µL upper phase, add 300µL of cold isopropanol)
2. Mix by inverting the tube several times & Incubate the tube at -20⁰C for 10mins.
3. Pellet the RNA by centrifuging at 15000 RPM for 10 mins at +4⁰C.
4. Take off the supernatant.
5. Add 1mL of pre-cooled 75% Ethanol & Vortex the tube briefly to wash the pellet. Leave tubes for 3 mins at RT.
6. Centrifuge the tube for 5 mins at 15000 RPM.
7. Take off supernatant.
8. Centrifuge the tube for an additional 15secs to collect the pellet at the bottom of the the & Remove any visible liquid by using yellow tips.
9. Dry the pellet for 3 mins in speedvac. (Alternatively, you may air dry the pellet as the lid open in clean bench for 10 mins).
10. Add 40 µL of DEPC- dH2O & Incubate the tube at 65⁰C water bath for 15 mins. Vortex the tube once in every 5 mins.
11. Spin down the tube for 5 secs.

Storage of RNA Samples:

At -20⁰C for short term storage

At -80⁰C for long term storage

Determination of Total RNA Quality & Quantity:

RNA quality and quantity can be determined by spectrophotometric analysis of nucleic acids. For this purpose, 10mM Tris-HCl (pH: 8.0) is used.

Spectrophotometric Analysis:

**Note:** Keep quartz cuvettes in 100% ethanol for 30 minutes.

1. In an eppendorf tube, add 598 µL of 10mM Tris-HCl (pH: 8.0) and 2 µL of total RNA sample. By this way, RNA sample will be 300 times diluted (DF=300).

2. Measure absorbance at 260, 280 and 230 nm against 10mM Tris-HCl (pH: 8.0)(as blank).

3. Calculate total RNA concentration according to the formula

**C= A260 x 40/103 x DF** where DF is dilution factor (300).

(1 absorbance unit is equal to 40 µg/mL RNA)

**Quality of RNA:**

Ratio of A260/A280 should be between 1.8 – 2.1. If it is over 2.1, sample is contaminated with DNA; if the ratio is below 1.8, sample is contaminated with organic solution (phenol).

Ratio of A230/A230 should be above 2.0. If it is below 2.0, then sample is contaminated with protein.

**Note:** RNA quantification can also be done by using NanoDrop, in which no dilution is necessary! Only 2 µL of RNA sample is placed on tip of the detecting probe in NanoDrop, and the measurement is taken. The probe should be cleaned with dH2O before measurement and between each samples.

For RNA quality analysis, you can run total RNA samples on 1% Agarose gel.

**Agarose Gel Electrophoresis:**

Before gel preparation, the electrophoresis tank, comb and cassette should be cleaned with 3% hydrogen peroxide (H2O2) for 30 mins.

Preparation of 3% H2O2: (For 300mL)

Main stock of H2O2 is 30% (V/V) and is kept at +4⁰C (Lab location – R2) (Producer is Mallinchrodt Chem. Cat. No. 5240). Take 30mL from 30% H2O2 stock and add 270mL DEPC- dH2O.

After cleaning with H2O2, remove it and fill the tank with 1X TAE.

Note: Dilute 50X TAE stock solution to obtain 1X TAE running solution.

Preparation of 1% Agarose Gel: (For 30mL)

Dissolve 0.3g agarose in 30mL 1X TAE solution by heating the solution in microwave oven will it starts to boil.

Then, add 2µL of Ethidium Bromide into hot solution, mix it and pour into cassette. Place the comb carefully without making any bubbles and remove the forming ones by help of a pipette tip. Solidify the gel for 30 minutes (This may be done in hood).

 Remove the comb and place the cassette into 1X TAE running buffer in the tank.

Mix 2µL of RNA sample, 2µL of 5X loading dye and 6µL of DEPC- dH2O in a small tube or on a piece of parafilm wrap. Then, load 10µL of mixture into each well (Should you want, you can load 3 µL of Marker – Invitrogen 1 Kb Plus DNA Ladder – into a well).

Run the gel at 100V for 15-20 mins. Expose the gel to UV light and take the image of the gel.

Expect to see band formations such as in **Figure 3**.



**Figure 3. Quality assessment of total RNAs run on 1% Agarose gel electrophoresis.** Lane 1, Degraded RNA; Lane 2 and Lane 3, good quality RNA. The 28S band contains approximately 4100 bp and the 18S band contains approximately 1900 bp. Good quality RNA shows that the 28S band is about 2.5 times as intense as the 18S band is. Any DNA contamination will show up on upper parts of the gel as a smear and an intensive band formation in the wells.

**2. DNase I Treatment of Total RNA Samples:**

The DNase treatment of total RNA samples is critical for the removal of DNA from RNA samples prior to applications such as RT-PCR. We use Promega RQ1 (RNA Qualified) RNase-Free DNase (Cat. No. M6101), which is a DNase I that degrades both double-stranded and single-stranded DNA endonucleolytically, producing 3´-OH oligonucleotides. In the presence of Mg2+, DNase I attacks each strand of DNA independently and cleaves both strands of DNA at approximately the same site to yield fragments that are blunt ended or have protruding termini only one or two nucleotides in length. Then, the enzyme is inhibited by heat treatment in the presence of Mg2+ chelator such as EDTA.

Procedure for this step includes two sub-titles: DNase I treatment, and sodium acetate precipitation of RNA samples.

**Note:** Promega RQ1 (RNA Qualified) RNase-Free DNase comes with 3 vials, namely RNase-Free DNase, RNase-Free DNase 10X Reaction Buffer and DNase Stop Solution. Store all at -20⁰C.

DNase I Treatment:

**Note:** The final volume of digestion reaction is given as 10µL for 1µg RNA in manufacturer’s protocol. We scale up the final volume of the reaction to 200µL for 20µg RNA; Hence, everything is multiplied by 20 as given below.

Caution! Work on ice.

 Keep RNase-Free DNase at -20⁰C until you add it.

1. Set up the DNase digestion reaction as follows:

|  |  |  |
| --- | --- | --- |
|  | **For 1µg RNA (Total V= 10µL)** | **For 20µg RNA (Total V= 200µL)** |
| Total RNA sample | Equivalent volume of 1 µg | Equivalent volume of 20 µg |
| RQ1 RNase-Free DNase 10X Reaction Buffer | 1 µL | 20 µL |
| RQ1 RNase-Free DNase | 1 µL | 20 µL |

\* Nuclease-free water is added to final volume of 10 µL or 20 µL for 1 µg or 20 µg RNA, respectively.

Caution! Mix all by pipetting several times. Do not vortex!

2. Incubate at 37⁰C for at least 30 mins (up to 1h). Chill tubes on ice.

3. Add 1 µL or 20 µL of RQ1 DNase Stop Solution (which contains EDTA) for 1 µg or 20 µg RNA, respectively, to terminate the reaction. Vortex eppendorf tubes.

4. Incubate at 65⁰C for 10 mins to inavtivate the DNase. Chill tubes on ice.

Sodium Acetate Precipitation of RNA Samples :

**3M Sodium acetate (pH: 5.2):** (For 20mL)

Weigh 8.1648 g of NaOAc.3H2O (Producer is Mallinchrodt Chem. Cat. No. 7364 – Lab Location – A8 138) and dissolve in 10mL DEPC- dH2O. Adjust the pH to 5.2 by addition of acetic acid. Autoclave the solution at 121⁰C for 20 mins. Store at room temperature.

Following DNase I treatment, RNA samples are precipitated by 3M sodium acetate solution:

Note: Total volume of mixture in eppendorf tube is 220µL after DNase I treatment.

1. Add 1/10 Volumes of 3M NOAc (pH: 5.2) [ i.e. Add 22 µL 3M NOAc]

2. Vortex the tube.

3. Add 3 Volumes of pre-cooled 100% Ethanol [ i.e. Add 660 µL 100% Ethanol].

4. Mix by inverting several times.

5. Incubate a. at -20⁰C overnight or

 b. at -80⁰C for 2-3 hours (in this case, precipitation will be less efficient)

6. Next day, centrifuge at 15000 RPM at +4⁰C for 30 mins.

7. Carefully remove ethanol.

8. Wash pellet with 1mL of pre-cooled 75% Ethanol. Incubate 3 mins at RT & Centrifuge at 15000 RPM at +4⁰C for 5 mins.

9. Remove ethanol. Dry the samples in speed vac for 3 minutes.

10. Dissolve RNA in 20-30 µL DEPC- dH2O & Keep the tube at 65⁰C for 5 mins.

11. Cool the tube on a rack at RT & Spin down the tube to collect all the solution at the bottom of the tube.

12. Store RNA samples at -20⁰C for future use.

\* Determine the quality and quantity of RNA samples by using spectrophotometric analysis. Measure absorbance at 260, 280 and 230 nm against 10mM Tris-HCl (pH: 8.0)(as blank). Use DF=300.

**3. Reverse Transcription:**

1st strand cDNA synthesis from mRNA is performed using reverse transcriptase enzyme. For this purpose, we use Invitrogen SuperScript III Reverse Transcriptase (Cat. No. 18080).

**Note:** Incubate components of Invitrogen SuperScript III Reverse Transcriptase at -20⁰C.

 RNasin® Ribonuclease Inhibitor doesn’t come with the kit. Buy it separately from Promega (Cat. No. N2511).

 Random hexamers doesn’t come with the kit. Order them from IDT DNA Technologies ([http://www.idtdna.com/](http://www.idtdna.com/Home/Home.aspx)). In order to order them, select ReadyMade Primers, then Random hexamers.

**Note:** For more detailed explanation of primer selection in cDNA synthesis go to **Appendix B**.

Caution! Work on ice.

 Keep SuperScript III Reverse Transcriptase and RNasin® Ribonuclease Inhibitor at -20⁰C until you add it. Add them last!

Procedure:

Note: RT reaction can be set up for 10pg-5µg total RNA o 10pg-500ng mRNA. In our case, RT reaction is always set up for **2µg total RNA** and calculations are done accordingly.

1. Add the following components to a nuclease-free microcentrifuge tube (Oxygen):

Total RNA volume equals to 2µg total RNA (calculated from spectroscopic analysis)

Random primers 1µL

10mM dNTP Mix 1µL

Sterile DEPC- dH2O volume to make the total volume 13µL.

2. Mix everything by pipetting.

3. Incubate the mixture at 65⁰C for 5 mins. (**Program “RT”**). After that immediately incubate on ice for at least 1 min.

4. Collect the contents of the tube by brief centrifugation and add:

**Note:** You can multiply the below quantities if you prepare multiple cDNA samples from different RNA samples). Then, distribute this master mix into individual sample tubes in equal (7 µL) volumes.

5X First-strand Buffer 4 µL

0.1M DTT 1 µL

RNasin® Ribonuclease Inhibitor 1 µL

SuperScript III RT (200 U/µL) 1 µL

 **Total Volume** 20 µL

 5. Mix by pipetting. Incubate tube at 25⁰C for 5 mins; then 50⁰C for 60 mins; then, 70⁰C for 15 mins. This is pre-set as **Program “RT1”.**

Caution! Prepare one tube of 1st RT-PCR reaction without SuperScript III RT for each RNA sample. This will be used as negative control for each RNA samples in RT-qPCR to check any genomic DNA contaminations.

**4. Real time (RT)-qPCR:**

RT-qPCR consists of two separate steps: Real time PCR and Data Analysis.

**Real time PCR:**

Caution! Work on ice.

 Keep SYBR Master Mix away from light. Use it immediately and return to refrigerator.

Before starting the procedure, make a chart on your notebook that will reflect the wells on PCR plate (**Figure 4**).



**Figure 4.** Working Chart on Lab Notebook for RT-qPCR.

Also, dilute gene specific primers (both forward and reverse) into 10mM. Take 10 µL from 100mM main stock and add 90 µL of dH2O. Then, take equal volumes from each forward and reverse primer and mix tem in a new tube so that the final concentration of the primer mixture will be 5mM.

Caution! Whenever you start setting up RT-qPCR, pay attention not to make any air bubbles. To eliminate the bubble formation, never pipette all the solution out of the tip. Push the operating button till the 1st stop (never go to 2nd stop).

1. Set up the following master reaction mixture for each RNA sample in an eppendorf tube: (30.75µL of reaction) (Multiply if you are preparing multiple genes)

dh2O 13.8 µL

SYBR Green 15 µL

cDNA 1.95 µL

2. Mix by pipetting. Spin down to collect the solution at the bottom of the tube.

Caution! Do not vortex!

3. Order the small PCR tubes on ice such a way that each tube will represent three adjacent wells (from left to right) on PCR plate.

4. Distribute 30 µL of master reaction mixture into each small PCR tubes.

5. Add 1.2 µL of primer mixture. Mix well by pipetting.

6. Place PCR plate on ice. Add 10 µL of master reaction mixture into each well.

7. Cover the top of PCR plate with supplied cover. Use caution not to touch the cover with bare hands as the machine reads from the top (through the cover).

8. Secure the cover by using elastic and spin the plate 15 secs in small centrifuge placed next to Roche LightCycler 480. While spinning, pay attention to place the plate the bottom looking outward away from the center.

9. Turn the Roche LightCycler 480 and the computer on. The machine initiation takes place for 5-10 minutes. When the two lights found in front of the machine turn to yellow, the machine is ready to operate.

**Note:** You may want to turn the Roche LightCycler 480 on earlier than you spin the plate down. If you forget to turn it on, always keep the plate at dark on ice until the initiation is completed.

10. After initiation, open the door of Roche LightCycler 480 and place the PCR plate as the upper left corner touching to the plate holder of the machine.

11. To operate the computer,

A. Go to the program Roche LightCycler 480 SW1.5 and sign in by using your own user name and password.

B. In main menu, select “New Experiment from Template“. Then, from the newly opened window, select “SYBR Green I 384-I”.

C. In next window, change the reaction volume to 10 µL.

**Note:** The reaction protocol will be

|  |  |  |  |
| --- | --- | --- | --- |
| Program Name | Temperature(⁰C) | Incubation time  | Cycle number |
| Pre-Incubation | 95 | 5 minutes | 1 |
| Amplification | 95 | 10 seconds | 45 |
|  | 60 | 10 seconds |
|  | 72 | 10 seconds |
| Melting Curve | 95 | 5 seconds | 1 |
|  | 65 | 1 minute |
|  | 97 | Continuous |
| Cooling | 40 | 30 seconds | 1 |

\* The program can be changed depending upon the amplification and melting curves, length of the amplicon etc.

D. Then, start the reaction by pushing “Start Run” button on lower right-hand side.

While the reaction runs, you can continue to select the wells you are using on the PCR plate and then name the samples, genes and select which well represent internal control genes etc.

E. Then, from the left hand-side of the window, select “Subset Editor”. This window will allow you to select the subsets of wells on PCR plate you are using for this reaction.

F. First, push to + sign in order to add a new subset. Then, name the subset (we use “Used” as the name). Next, select the wells you are using from the interactive figure. Selected ones will turn into white. Then, push to “Apply” button. The color of the well will become grey.

G. From the left hand-side of the window, select “Sample Editor”. First, in “Step 1”, select Relative Quantification. Then, in “Step 2”, select subset defined as “Used”. The wells you are using will be highlated.

H. Then, in the chart on the right-hand side, you can define which sample is replicate of which one, then give names to those RNA samples (such as control, treatment etc.).

I. From the drop down menu called as “Combined Sample and Target Type”, select

|  |  |  |
| --- | --- | --- |
| Idiom | Definition | Example |
| Target Unknown | Treatment + Gene of Interest | Exp of SOS1 gene in salt treated sample |
| Target PosCalibrator | Treatment + Internal control gene | Exp of TUB8/18S rRNA in salt treated sample  |
| Target Negative | No RT-cDNA sample of treatment  | No RT-cDNA sample from salt treated sample |
| Reference Unknown | Control + Gene of Interest | Exp of SOS1 gene in salt un-treated sample |
| Reference PosCalibrator | Control + Internal control gene | Exp of TUB8/18S rRNA in salt un-treated sample  |
| Reference Negative | No RT-cDNA sample of control  | No RT-cDNA sample from salt un-treated sample |

**Note:** For target or reference negative samples, you can use any gene you want. At the end of the reaction, you expect not to see any amplification as no RT-cDNA doesn not contain cDNA for RT-qPCR amplification. This is a critical check point for any genomic DNA contamination during 1st strand RT-PCR.

**Note:** You can also add the mixture of SYBR Green + water + primers (without any cDNA) into one well to check if any of the ingredients is contaminated with cDNA/ genomic DNA.

**Data Analysis**

After the run is complete, from the left hand-side of the window, select “Analysis”. Then, you can do several analyses.

**1. Melting Curve Analysis:**

This is performed to check the quality of gene specific primers used in RT-qPCR.

**2. Basic Relative Quantification:**

This is used for the comparative analysis of genes expression levels in treatment samples relative to control samples.

**3. Absolute Quantification/ 2nd Derivative Max:**

This is used to quantify the absolute amounts of RNA amplification of a given gene in each individual samples. This analysis requires a standard curve formed by using the known amounts of a RNA sample vs its amplification (Cp) value. It can also be used for obtaining Cp values of each individual samples.

**APPENDIX A**

**APPENDIX B**

The first-strand cDNA synthesis reaction can be primed using random hexamers, oligo(dT), or gene-specific primers (GSPs):

• Random hexamers are the most nonspecific priming method, and are typically used when the mRNA is difficult to copy in its entirety. With this method, all RNAs in a population are templates for first-strand cDNA synthesis, and PCR primers confer specificity during PCR. To maximize the size of cDNA, you should determine the ratio of random hexamers to RNA empirically for each RNA preparation.

• Oligo(dT), a more specific priming method, is used to hybridize to 3´ poly(A) tails, which are found in the vast majority of eukaryotic mRNAs. Since poly(A)+ RNA constitutes approximately 1% to 2% of total RNA, the amount and complexity of cDNA is considerably less than with random hexamers. For many reactions oligo(dT)20 usage is recommended.

• The most specific priming method uses a gene-specific primer for the sequence of interest. First-strand synthesis can be primed with the PCR primer that hybridizes nearest to the 3´ terminus of the mRNA. Note that some GSPs fail to prime cDNA synthesis even though they work in PCR on DNA templates. If gene-specific priming fails in RT-PCR, repeat first-strand synthesis using oligo(dT) as the primer.

According to the results of a RT-qPCR experiment conducted by Nucleic Acid Research Group, in 1st strand cDNA synthesis priming with an assay-specific primer resulted in the lowest Ct. The assay-specific primer was found the most effective priming strategy for low expressing gene, while oligo(dT) was second and random hexamer was third (<http://www.abrf.org/ResearchGroups/NucleicAcids/Studies/NARG06_DSG2_8.pdf>).