Protocol for Lab work:

Setting up:

Make sure you have: Liquid Nitrogen, forceps, bleach and ethanol solutions, vials for legs with pollen, 70% ethanol.

**SETUP:**

1. Choose specimen to be processed. Mark in Excel sheet and give a lab ID number.
2. Write in lab notebook:
   1. Procedure to be done
   2. Date
   3. List of specimens to be processed with Lab ID number
3. Place specimen on liquid nitrogen
4. Get the following materials ready:
   * 1. Pestles
     2. GITC buffer
     3. Labeled 1.5 tubes (2 for each specimen) plus one for a negative control
     4. Labeled small tubes for pollen/legs (1 for each specimen)

**Making bleach and ethanol for forceps**

**PREPARING SPECIMENS AND HOMOGENIZING TO CREATE CRUDE EXTRACTIONS:**

1. Using disinfected forceps, pull of legs of bees and pollen and place into the labeled small tubes. Place in freezer.
2. Place the rest of bee into a 1.5 tube.
3. Use pestle to grind bee for 30 sec.
4. Add 600ul GITC buffer to tube
5. Homogenize with pestle for 1.5 minutes.
6. Put in ice
7. Repeat for all specimens
8. Centrifuge to push bee specimen to bottom of tube for 3 minutes

**BEGINNING RNA ISOLATION**

1. Add 600 ul of buffer/mercap solution to the tube
2. Pipette out 100 ul of bee juice from the crude extraction sample into a new 1.5 tube
3. Create a negative control here (600 ul RLT buffer + 100 ul GITC buffer)
4. Add 1 volume (700 ul) of 70% ethanol to all tubes

**\*\*\*Making 70% ethanol**: 700 ul 100% ethanol + 300 ul of RNAse free water. Mix with pipette.

\*\*\*Proceed with Qiagen protocol for RNA extraction

**NANODROP INSTRUCTIONS:**

\*\*\*Bring smallest pipette and tips to nanodrop room

Click on Nanodrop icon

Click nucleic acid

Put 2 ul distilled H2O on spec

Initialize the spec

Make sure system is set to RNA-40

Add 2 ul distilled H20

Click ‘blank’

Wipe clean and load 2 ul of the first sample

Enter in sample ID (Lab ID)

Click measure

Results of Nanodrop:

Quality:

Good if between 2.0 & 2.2

Quantity:

**WHAT’S THE LOWEST ng/ul that can be used?**

*TROUBLESHOOTING: If the nanodrop results show low yield, 2 options are possible:*

1. *reduce qty of GITC buffer from 600 to maybe ½ or 1/3*
2. *reduce quanity of RLT buffer + mercap*

**GETTING READY FOR RT-QPCR: NANODROP ANALYSIS**

Export report in a txt format **(IS THIS POSSIBLE?)** upload into excel doc (Nanodrop analysis)

Import Nanodrop results into “bumble bee sample dilutions” sheet in Nanodrop Analysis excel workbook.”

Increase “RNA for Dilution” cells by 5 until the ‘final volume’ is over **40 (??)**

Fill out the plate setup tab

| Well | **LAB ID #** | Date | ng/ul | 260/280 | 260/230 | Constant | RNA for Dilution | ***final vol*** | **H2O** | **dil.factor** | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 1,4,7 | 17 | 2/19/15 | 44.26 | 2.18 | 0.49 | 40 | 20 | *44.3* | 24.3 | | 2.2 |
| 2,5,8 | 18 | 2/19/15 | 210.64 | 2.16 | 1.35 | 40 | 5 | *52.7* | 47.7 | | 10.5 |
| not run | 19 | 2/19/15 | 17.63 | 2.11 | 0.18 | 40 | 50 | *44.1* | -5.9 | | 0.9 |
| 3,6,9 | 20 | 2/19/15 | 105.34 | 2.17 | 0.58 | 40 | 10 | *52.7* | 42.7 | | 5.3 |

**Creating master mixes:**

Using word doc: “plate setup and Master Mix”, calculate Master Mix. Count number of rxns for each primer and add 5 because of measurement error.

Worksheet for Bumble Bee Survey 2014 virus assays

Target: B-ACTIN, Am-Actin2, Actin

|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| A | 17 | 18 | 19 | 17 | 18 | 19 | 17 | 18 | 19 |  |  |  |
| B | 17 | 18 | 19 | 17 | 18 | 19 | 17 | 18 | 19 |  |  |  |
| C | 17 | 18 | 19 | 17 | 18 | 19 | 17 | 18 | 19 |  |  |  |
| D |  |  |  | B-act NTC | ACT-Am NTC | ACTIN NTC |  |  |  |  |  |  |
| E |  |  |  |  |  |  |  |  |  |  |  |  |
| F |  |  |  |  |  |  |  |  |  |  |  |  |
| G |  |  |  |  |  |  |  |  |  |  |  |  |
| H |  |  |  |  |  |  |  |  |  |  |  |  |

NHBS 2014 qPCR: Samples

Master mix: 10 reactions for each primer plus 5 because of measure error. (15 rxns)

Create 3 Master mixes (1 for each primer)

|  | **Stock** | **Final konc** | **20 µl** | **10 µl** |  | **X 15** |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **H2O** |  |  |  | 1.475 µl |  | 22.125 |  |  |
| **iTaq Universal SYBR Green mix** | 2x | 1x |  | 5 µl |  | 75 |  |  |
| **Primer 1** | 10 μM | 0.2 μM |  | 0.2 µl |  | 3 |  |  |
| **Primer 2** | 10 μM | 0.2 μM |  | 0.2 µl |  | 3 |  |  |
| **iScript reverse transcriptase** |  | 1x |  | 0.125 µl |  | 1.875 |  |  |
| **Template RNA** |  |  |  | 3 µl |  | 3 |  |  |

Use 7 ul for each rns + 3 ul RNA = 10 ul/rxn

**Creating Master Mix**

Create Master Mix for each target (1 for each primer)

Take out of freezer:

Primers (dilute and mix)

SYBR Green mix (mix well)

RNAse free water.

Keep transcriptase in freezer and add last. Do not move around too much because it is an enzyme.

**RNA dilutions:**

Using “nanodrop analysis excel workbook” in tiny tubes: add H20 & RNA to dilute to 20 ng/ul.

before adding RNA, defrost in ice

**Getting ready for qPCR:**

Add 3 ul of each RNA rxn and 7 ul of Master mix to 96 well plate.

Put in qPCR machine.

**Creating serial dilutions:**

**From qPCR machine: Dilute with nuclease free water. 1:1000 and 1:100. Use Nanodrop to check cDNA concentrations. Then create dilutions.**