Dr. Kerry Kriger's qPCR Instructions for detecting *B. dendrobatidis*. *Method for doing 93 samples at a time,* in <u>singlicate</u>). Avoid contamination! 1-Oct-09 savethefrogs.com/chytrid

<u>Extracting DNA from Samples</u>

1. With blue marker, label the right side of 93 swabs A1 thru H12 (*don't label E10, E11 or E12*). Label 94 1.5mL microtubes A1 thru H12 (*don't label E10 or E12*; *Note: you will need a microtube for the E11 pos ctrl.*) Write the actual swab ID (e.g. 732) in its corresponding cell (e.g. D7) on the "Chart with Swab Numbers" printout. *Photocopy and keep extra in safe place*!!!

2. Fill 94 1.5mL microfuge tubes with 30-40mg of 0.5mm zirconium/silica beads (*weigh, don't guess*). Next, add 50μL PrepMan Ultra (**gets stored at room temp!**). Last, break off each swab into its corresponding tube. *Overall order is: 1) beads 2) PrepMan 3) swabs*

3. **Homogenize** using Mini Beadbeater 8:
   - Beadbeat 45 seconds. Microfuge 30 seconds (at ~13,000g).
   - Now beadbeat 45 seconds again, and microfuge 30 seconds again.
   Verify tubes are closed after every step. Stick tubes into ice bucket for a few seconds after beadbeating. When microfuging, put hinge parts of lids toward outside.

4. **Pierce lid of microtubes with 22 or 23 gauge needle** (can use same needle if flamed in a Bunsen burner between samples.) Discard if contamination with liquid from any tube is suspected. *Use 4 needles on rotation, so that one has always cooled.*

5. Place microtubes in floating holder and **boil sample 10 minutes** in pan of boiling water. *Verify tubes are closed! Don't use a lid on pan.* If running a positive control, boil last (separately) so as to avoid potentially contaminating real samples.

6. *Dab Kleenex on top of tubes to remove any condensation.* Cool 2 min at room temperature then **microfuge for 3 minutes at full speed (**~13,000g).

7. **Collect and store as much supernatant as possible – usually 20 to 40μL.** *20 to 40μL of supernatant is needed so that 5μL can easily be gotten for the dilution step.*
   - Label plate like: "2009-Jan-18 V-Bottom Plate #1 (samples)".
   - All 93 samples (*and* pos ctrl) go into V-Bottom plate #1's correspondingly labeled well. (microtube A1→well A1).

8. **Dilution step**:
   - Label plate like: "2009-Jan-18 V-Bottom Plate #2 (Dilutions)".
   - Put 45μL of DEPC H_2O into each of V-Bottom #2's 94 usable wells (<u>not</u> *E10 or E12)*.
   - Now take 5 μL supernatant from each of 94 wells of V-Bottom Plate #1, and put in its respective well of V-Bottom Plate #2. Now all samples are diluted 1/10.

Harvested supernatants can be stored for a week at 4°C if the assay is being done in that time other wise store frozen at −20°C (*normal freezer*). **The plate needs to be sealed to prevent evaporation of sample.**

Getting Samples, Standard and NTC ready for Singlicate PCR plate

20mike reactions!!!! (15 µL master mix + 5 µL sample); 93 samples per plate.

Each reaction will contain 5 µL of 1/10 diluted sample, and 15 µL *master* mix:
10 µl   ABsolute QPCR ROX 500nM Mix
0.8 µl   18µM ITS1-3 Chytr   5'- CCTTGATATAATACAGTGTGCCATATGTC-3'
0.8 µl   18µM 5.8S Chytr   5'- AGCCAAGAGATCCGTTGTCAAA -3'
1 µl   5µM ChytrMGB2   5' - 6FAM CGAGTCGAACAAAAT MGBNFQ – 3'
2 µl   10x Exo IPC mix
0.4 µl   50x Exo IPC DNA

1) Prepare the *master* mix:

Make 105 wells worth to be on the safe side, since we NEED 96 wells worth. Dump any excess when finished. **Always vortex**, and centrifuge tubes/mixes as necessary.
So add these in this order to a 2mL microtube labeled "MM":

- 1,050 µL ABsolute QPCR ROX 500nM Mix   (105*10 µL = 1,050 µL)
- 84   µL ITS1 primer   (105 * 0.8 µL = 84 µL)
- 84   µL 5.8S primer   (105 * 0.8 µL = 84 µL)
- 105   µL MGB2 probe   (105 * 1 µL = 105 µL)
- 210   µL IPC mix   (105 * 2µL = 210µL)
- 42   µL IPC DNA   (105 * 0.4µL = 42µL)

2) Put the Samples, 100 Zoo standard, Pos Ctrl, and NTC in the PCR plate:

- *-Always change pipette tip between samples! Keep PCR plate in its holder, to keep bottom clean!*
- **Add** 15 µL *master* to all of the PCR plate's wells.
- **Add** 5 µL of diluted samples from V-Bottom Plate #2 to respective wells of the PCR plate's wells (excluding E10, E11, and E12). Mix well**.**
- **Add** 5 µL of 100 zoospore standard to E10.
- **Add** 5 µL of diluted positive control from V-Bottom Plate #2's E11 to PCR Plate's E11.
- **Add** 5 µL of DEPC H$_2$O (NTC) to E12.
- **Cover PCR plate with plate sealer.**
- Seal the V-Bottom Plate and freeze.

3) Centrifuge the Plate

- Counterbalance centrifuge by adding in the dummy plate/plate holder.
- Centrifuge PCR plate at 4000 RPM for 2 minutes. *NB: Donna does 1500 RPM for 30sec. Either way is fine.*

*If using a BioRad machine to analyze samples, you may need to add fluorescein to master mix at 1.0 µl Fluorescein dye per 20 µL reaction (for 20x fluorescein; precise amount can vary slightly.)

Thus, instead of using 210 µL DEPC H$_2$O (if making 105 wells worth!), do
105 µL Flourescein   (105 * 1.0 µL = 105 µL)
105 µL DEPC H$_2$O   (105 * 2µL = 210µL; 210µL - 105µL = 105µL)
NB: water is added at whatever amount brings the total volume to 210 µl)

Using ABI Prism 7700 Sequence Detection System: Singlicate

If using an ABI Prism 7700 Sequence Detection System, the default amplification conditions (2 min 50°C, 15 min 95°C followed by 15 s 95°C, 1 min 60°C for 50 cycles) are used.   (Note well: Applied Bioystems' 2x Taqman Master Mix uses only 10 min at 95°C).

- Never turn off the machine.
- Quit program if already open, then open SDS 1.9.1 software, or just open "Singlicate Template".
- Save As a new name (i.e. 2005May19 which is the date of the extraction always!!!)
- If necessary:
  o Highlight appropriate cells and give samples designation: "unknown".
  o Highlight appropriate cells and give standards info: i.e. sample name STD100; quantity 100.
  o Highlight appropriate cells and give NTC sample name: NTC.
  o If some wells are not being used, highlight those wells, and put sample type as "not in use".
- Can toggle between screens by hitting "Show Analysis" button, or "Show Setup" button.
- Give comments box a name and date if you want.
- In Sample Type drop down menu, choose 'Sample Type Setup' and make IPC+ reporter be VIC. Also, change 'Dye Layer' to VIC and make any well with IPC in it be set to 'Sample Type' IPC+.
- *Gently* wiggle your PCR plate out of its holder, then **stick in the machine in the correct orientation**.
- Extra seal the plate using Kevin's plate sealer thing (which will simultaneously push the plate firmly into the machine). Pull big thing back, hold firmly while tightening the knob, then once it "hits", give it ¼ or so turn more.
- Where NTC vs Standard vs Unknown is, choose sample type setup; the window under Quencher should say None, not Tamra (the default).
- **ALWAYS** check default conditions from the sheet taped to the machine:
- o Instruments→Diagnostics→Advanced Options
- o **My exposure time for plates is 10   (if using caps it is 25!)**
- o "Use spectral compensation for Real time" should be left UNCHECKED. (not too important though.)
- o Check the "Use background in "spectral components" folder, and check the "Use pure spectra in "spectral components" folder (as shown on sheet taped to machine).
- o **All 3 boxes in Analysis→Options should be ticked!**
- Lastly, at top of window, **verify status is idle**, then hit run.
- Wait for beep (30secs).
- Don't touch computer now.

NB: If you consider it unimportant to add IPC to the singlicate reactions, then make the 15 μL *master* mix as follows (this method was published in my thesis):

10 μl ABsolute QPCR ROX 500nM Mix
1 μl   18μM ITS1-3 Chytr   5'- CCTTGATATAATACAGTGTGCCATATGTC-3'
1 μl   18μM 5.8S Chytr   5'- AGCCAAGAGATCCGTTGTCAAA -3'
1 μl   5μM ChytrMGB2   5' - 6FAM CGAGTCGAACAAAAT MGBNFQ – 3'
2 μl   DEPC H$_2$O

Analyzing Singlicate Data

- When run is done, hit File→Save.
- Can pry used PCR plate out of machine with the tool there.
- If you ever clear any cell after this point, you **MUST** then SAVE AS a new name, or else original data will be lost!!! Use the original name plus "-ol" after it to signify outliers removed.
- Analysis→Analyze.
- If you need to see this amplification plot in the future, use Analysis→Amplification Plot, or Apple + G shortcut wih all cells highlighted.
- Set the Threshold: use the log scale (double-click the y-axis then choose "log scale" in display box), and move the horizontal bar about halfway up the linear portion of lines; just make sure it is BELOW high curves, and ABOVE the NTC lines.
  - NB: *Donna uses a delta Rn of 0.10 to standardize all assays.*
- If you ran a positive control, verify that it came up positive (Ct<50), then Edit→Clear that cell.
- NB: Any time you Edit→Clear a cell, you will need to re-analyze!
- Verify the standard came out positive (Ct<50), and the NTC came out negative (Ct=50).
- Set Baseline: the defaults of "Start: 3 and Stop: 15" are fine, but better is a Stop of 2 less than the earliest rise point (e.g. Stop: 22 perhaps). Use linear scale (double-click the y-axis then choose "linear scale" in display box) to visualize determine this.
- Hit "Update Calculations". Hit OK.
- NB: Anytime you make changes and re-analyze, you will have to reset threshold and baseline and Update Calculations again.
- Note any cells with Ct<50, they are chytrid positive.
- To test if IPC came out positive: in amplification plot, flip 'Reporter' drop down to be VIC, and see which wells came out Ct<50 (those are indeed IPC positive, implying no inhibition).
- NB: If data is lost, you can always retrieve it: Open your template, and then do File→import→labview→mac HD→runs f→choose your run by date and time.

Use 20μL reactions!!!! (15 μL master mix + 5 μL sample); 25 samples per plate. Use of IPC is unnecessary, as all singlicate reactions were inhibition free.

Each reaction will contain 5 μL of 1/10 diluted sample, and 15 μL *master* mix:
10 μl ABsolute QPCR ROX 500nM Mix
1 μl   18μM ITS1-3 Chytr   5'- CCTTGATATAATACAGTGTGCCATATGTC-3'
1 μl   18μM 5.8S Chytr   5'- AGCCAAGAGATCCGTTGTCAAA -3'
1 μl   5μM ChytrMGB2   5' - 6FAM CGAGTCGAACAAAAT MGBNFQ – 3'
2 μl   DEPC H_2O*

1) Prepare the *master* mix:
Make 112 wells worth to be on the safe side, since we NEED 95 wells worth. Dump any excess when finished. **Always vortex**, and centrifuge tubes/mixes as necessary.
So add these in this order to a 2mL microtube labeled "MM":

- 1,120 μL ABsolute QPCR ROX 500nM Mix (112 * 10 μL = 1,120 μL)
- 112 μL ITS1 primer   (18μM)   (112 * 1 μL = 112 μL)
- 112 μL 5.8S primer   (18μM)   (112 * 1 μL = 112 μL)
- 112 μL MGB2 probe (5μM)   (112 * 1 μL = 112 μL)
- 224 μL DEPC H_2O (112 * 2μL = 224 μL)
                                                (Adds up to 1,680 μL)

2) If you have not yet done so today, make your stock standards.
- If running 1 plate today, make 50 μL of each standard; 2 plates 60 μL; 3 plates 90 μL.
- i.e. serial dilutions of 45 μL H_2O + 5 μL DNA; 54 μL H_2O + 6 μL DNA, or 81 μL H_2O + 9 μL DNA.

3) Prepare the samples as one well with a triplicate's worth:
- **Add** 49.5 μL *master* to a V-Bottom plate's A1-A12 and F1-F12 cells (and D10!)
- **Add** 16.5 μL of positive diluted sample to its respective A1-A12 or F1-F12 cell (and D10!).
- Seal the diluted samples' plate and re-freeze.

--Always change pipette tip between samples!!!
--This is 66 μL of each sample: enough for a triplicate (three 20 μL reactions) with 6 μL spare.
-- NB: 15 μL *master* mix * 3.3 wells = 49.5 μL; 5 μL sample * 3.3wells = 16.5 μL).

4) Put the Samples in the PCR plate
Always put PCR plate in its holder!!! Keep bottom clean! *Do NOT write on the PCR plate!!!*
- Take 20 μL from A1 of V-Bottom plate, and add as triplicate to the PCR plate's three Sample 1 cells (A1-C1). Change pipette tip. Continue thru 20 μL from F12 of V-Bottom plate being added as triplicate to the PCR plate's three "sample24" cells (F12-H12). (and D10-D12!)

5) Prepare the Standards/NTC mixes:

- Line up five 1.5mL microtubes. Label them <u>100</u>, <u>10</u>, <u>1</u>, and <u>0.1</u>, and NTC to differentiate them from the stock tubes that are *just* standards (versus these, which will be standards plus *master* mix).
  Use new tips each time; start with weakest standard
- ➢ Add 64.5 μL *master* mix to each of the four **standards** tubes and to the **NTC** tube.
- ➢ Add 21.5 μL of each **standard** to its respective tube, and then 21.5 μL of DEPC H_2O to the **NTC** tube.

  Now there is:
  -- 86 μL of each standard mix and NTC mix: enough for a quadruplicate (four 20 μL reactions) with 6 μL to spare. The math: 15 μL *master* mix * 4.3wells = 64.5 μL. And 5 μL standard * 4.3wells = 21.5 μL. (64.5 + 21.5 = 86)
  --if running anything in duplicate, make up a tube with 34.5 μL *master* mix and 11.5 μL DNA (this is 46μL, enough for 2 wells & some leftover: 15*2.3=34.5; 5*2.3=11.5).

6) Put the Standards and NTC in the PCR plate

This step is always done *after* the samples have been added to the PCR plate, as we use NTC to see if there was contamination. Be sure to **vortex and centrifuge**!

- From the 1.5 mL tubes that were made up (in step 5) of standard/*master* mix, add 20 μL to each of 4 correct cells of PCR plate (i.e. 100zoos goes to D1-D4, 10zoos to D5-D8, 1zoos to E1-E4, and 0.1zoos to E5-E8).
- Finally, from the 1.5mL tube that was made up (in step 5) of NTC/*master* mix, add 20 μL to each of 4 correct cells of PCR plate (i.e. D10-D11 and E10-E11.)
- **Cover PCR plate with plate sealer.**

7) Centrifuge the Plate

- Counterbalance centrifuge by adding in the dummy plate/plate holder
- Centrifuge plate at 4000 RPM for 2 minutes. Kevin does it by hitting box with PROG, then box with up arrow, which gets Program 1. Then hit start. **PCR plate is always in its holder, to keep bottom clean!!!**   *NB: Donna does 1500 RPM for 30sec. Either way is fine.*

*If using a BioRad machine to analyze samples, you may need to add fluorescein to master mix at 1.0 μl fluorescein dye per 20 μL reaction (for 20x fluorescein; precise amount can vary slightly.)

Thus, instead of using 224 μL DEPC H_2O (if making 112 wells worth!), do
112 μL Flourescein   (112 * 1.0 μL = 112 μL)
112 μL DEPC H_2O (112 * 2μL = 224μL; 224μL - 112μL = 112μL)
NB: water is added at whatever amount brings the total volume to 224 μl)

Using ABI Prism 7700 Sequence Detection System: Triplicate

If using an ABI Prism 7700 Sequence Detection System, the default amplification conditions are used: 2 min at 50°C, 15 min at 95°C; followed by 50 cycles of {15 s at 95°C, 1 min at 60°C}.   (NB: Applied Bioystems' 2x Taqman Master Mix uses only 10 min at 95°C).

- Never turn off the machine.
- Quit SDS 1.9.1 software if running.
- Open SDS 1.9.1 software, or just go to my folder, and open "Triplicate Template".
- Save As a new name (i.e. 2005Aug23triprerunsP1 if first plate of day, or 2005Aug23triprerunsP2 if second plate of day): no spaces! Use today's date.
- Verify plate setup on screen is same as your setup. If necessary:
  o Highlight appropriate cells and give samples designation: "unknown" and name: "1" for cells A1, B1, C1, etc, and "24" for cells F12, G12, H12.
  o Highlight appropriate cells and give standards info: i.e. sample name STD100; quantity 100.
  o Highlight appropriate cells and give NTC sample name: NTC.
  o If cells are not being used, put sample type as "not in use".   (i.e. if nothing is in well D12, highlight that cell, and put sample type as "not in use".)
- Can toggle between screens by hitting "Show Analysis" button, or "Show Setup" button.
- Give comments box a name and date if you want.
- *Gently* wiggle your PCR plate out of its holder, then **stick in the machine in the correct orientation**.
- Extra seal the plate using Kevin's plate sealer thing (which will simultaneously push the plate firmly into the machine). Pull big thing back, hold firmly while tightening the knob, then once it "hits", give it ¼ or so turn more.
- Where NTC vs Standard vs Unknown is, choose sample type setup; the window under Quencher should say None, not Tamra (the default).
- **ALWAYS** check default conditions from the sheet taped to the machine:
o Instruments→Diagnostics→Advanced Options
o **My exposure time for plates is 10   (if using caps it is 25!)**
o "Use spectral compensation for Real time" should be left UNCHECKED. (not too important though.)
o Check the "Use background in "spectral components" folder, and check the "Use pure spectra in "spectral components" folder (as shown on sheet taped to machine).
o **All 3 boxes in Analysis→Options should be ticked!**
- Lastly, at top of window, **verify status is idle**, then hit run.
- Wait for beep (30secs).
- Don't touch computer now.

Analyzing Triplicate Data

- When run is done, hit File→Save.
- Pry used PCR plate out of machine with the tool there.
- If you ever clear any cell after this point, you **MUST** then SAVE AS a new name, or else original data will be lost!!! Use the original name plus "-ol" after it to signify outliers removed.
- Analysis→Analyze.
- If you need to see this amplification plot in the future, use Analysis→Amplification Plot, or Apple + G shortcut with the cells you want highlighted.
- Set the Threshold: use the log scale (double-click the y-axis then choose "log scale" in display box), and move the horizontal bar about halfway up the linear portion of lines; just make sure it is BELOW high curves, and ABOVE the NTC lines.
    - NB: *Donna uses a delta Rn of 0.10 to standardize all assays.*
- Verify the NTC's came out negative (Ct=50).
- In linear scale (double-click the y-axis then choose "linear scale" in display box), look for bad lines that drop way below the NTC. Figure out which cell(s) caused it, and Edit→Clear that cell(s).
- NB: Any time you Edit→Clear a cell, you will need to re-analyze!
- Set Baseline: the defaults of "Start: 3 and Stop: 15" are fine, but better is a Stop of 2 less than the earliest rise point (e.g. Stop: 22 perhaps). Use linear scale (double-click the y-axis then choose "linear scale" in display box) to visualize determine this.
- Hit "Update Calculations". Hit OK.
- NB: Anytime you make changes and re-analyze, you will have to reset threshold and baseline and Update Calculations again.
- Analysis→Standard Curve. Double click the x-axis and change minimum value to 0.01, and max to 10,000. Look at the standard curve: correlation coefficient better be above 0.980, hopefully above 0.990! Verify that all samples fall between the standard curves.
- Check standards by highlighting them (always including an NTC cell), then Analysis→Amplification Plot (or shortcut Apple+G). Quadruplicates should be close values, that are 3.3 cycles apart from other other standards.
- If any of your standards are outliers, Edit→Clear their cells.
- After re-analyzing, and fixing baseline and threshold, see if your R^2 increased. If it increased, File→Save. If it decreased, quit without saving, then re-open file and try something else.
- To look at each sample, highlight its triplicate, then Analysis→Amplification Plot.
- More troubleshooting can be done at Analysis→Multicomponents
- File→Export→Results Name it: e.g. "2005Aug23P1 results". Hit "Export".
- In Excel, Save As a Microsoft Excel file, **NOT** as Tab Delimited.
- Sort by IPC, then Sample Name in Excel.
- Final result must be multiplied by 100, as samples has been diluted 1/100.
- Add three columns, one with =(cell)*100 and one with =AVG(3 cells), and one with the corresponding swab number.
- If data is lost, you can always retrieve it: Open your template, and then do File→import→labview→mac HD→runs f→choose your run by date and time.

Preparing Primers/Probes/Standards (that have just arrived from company)

Keep in ice bucket while preparing. Wear gloves. Have access to vortexer (to mix) and centrifuge (to bring liquid to bottom). Vortex and centrifuge after every mixing step.

Once the primers/probe/standards are prepared, all are stored frozen!!! (regardless of what temp they were stored at upon arrival from company). But remember **Taqman 2x master is always refrigerated.**

PRIMERS:

[(Micrograms/tube) / (Molecular weight/10)] x 1000 = volume in μL of the TE 1x buffer you must add to give 100 μM (using DEPC water is fine if you don't plan to keep vials over 6 months; for long-term storage though, TE 1x buffer is better).

For "Scale = 40" (the smallest size you can order):
ITS1-3 arrives at 225microgram per tube. MW = 8867
5.8S arrives at 184microgram/tube. MW = 6752
--Thus:
ITS: (225/886.7) x 1000 = 254 μL ← so add 254 μL TE to give 100 μM solution.
5.8S: (184/675.2) x 1000 = 273 μL ← so add 273 μL TE to give 100 μM solution.

For "Scale = 200" (the next size up you can order):
ITS1-3 arrives at 1687microgram per tube. MW = 8867
5.8S arrives at 763microgram/tube. MW = 6752
--Thus:
ITS: (1687/886.7) x 1000 = 1903 μL ← so add 1903 μL TE to give 100 μM solution.
5.8S: (763/675.2) x 1000 = 1130 μL ← so add 1130 μL TE to give 100 μM solution.

Now these need to be made into 18 μM. To make 1mL of 18 μM, use $C_1 V_1 = C_2 V_2$:
100μM x V_1 = 18μM x 1,000μL. which means V_1 = 180 μL. So in a 1.5mL or 2mL microtube, add 820 μL DEPC H_2O, (**NOT** the TE!!!) then 180 μL primer (which adds up to 1mL.)
…Do this for each primer, and you'll have about 8 trays worth of primers prepared.

Probe:

MGB comes already at 100 μM.
We need it at 5 μM, and we'll make it into that 900μL at a time. This is about 8 plates worth.
Thus, 100μM x V_1 = 5μM x 900μL means V_1 = 45 μL. So in a 1.5mL microtube, add 855 μL DEPC H_2O, (**NOT** the TE!!!) then 45 μL MGB probe (which adds up to 900 μL).

Standards:

Donna's tube came as 500 μL of 200,000 zoospores per mL solution.
(That equals 1000zoospores per 5 μL).

Vortex Donna's 1000 tube beforehand!
- Add 72 μL DEPC H$_2$0) to each of 4 tubes (labeled 100, 10, 1 and 0.1). *Note: do NOT label the 1 zoospore tube as 1.0 as it will get confused with the 0.1 when viewed upside down).*
- Then take 8 μL from Donna's tube, and add it to the 100 epptube,
- Then take 8 μL from the 100 zoospore tube and add it to the 10 zoospore tube.
- Then take 8 μL from the 10 zoospore tube and add it to the 1 zoospore tube.
- Then take 8 μL from the 1 zoospore tube and add it to the 0.1 zoospore tube.

NB: Donna did 45 μL DEPC H$_2$0 with 5 μL standard.

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NB:
If you want to TEST your standards: you'll need to make a master mix that is enough to test your standards/NTC:
Make 26 reactions worth (NB: we NEED 22 reactions, so we make a bit extra; thus 26), so add these in this order to a 1.5mL microtube labeled "MM":   (4 quadruplicate standards plus sextuplicate NTC)
- 260 μL 2x Taqman Master Mix   (26*10 μL = 260 μL)
- 26 μL ITS1 primer   (26*1 μL = 26 μL)
- 26 μL 5.8S primer   (26*1 μL = 26 μL)
- 26 μL MGB2 probe   (26*1 μL = 26 μL)
- 52 μL DEPC H$_2$O   (26*2 μL = 52 μL)
                                                      (Adds up to 390 μL)