**Droplet Digital PCR (ddPCR) with Bio-Rad EvaGreen**

**Materials**

Seals: Bio-Rad 1814040

Plates: 951020362 (made by Eppendorf; ask in Bailey lab for product number that’s cheaper)

DG8 Cartridges: 186-4008

Gaskets: Bio-Rad 186-3009

EvaGreen Supermix: Bio-Rad 1864033

Droplet generation oil (for EvaGreen)” Bio-Rad 1864005

**EvaGreen Sample Preparation**

The following steps can be performed in our lab before taking mixed samples and reagents to the core facility.

* According to manual, the 2x EvaGreen master mix should not be thawed and re-frozen repeatedly. Upon initial thawing, it should be allocated into sterile/clean tubes. 8-sample aliquots would be the natural unit. This would probably correspond to ~90 ul per tube (to allow for pipetting slop), but that should be determined based on expected usage.
* Mix thoroughly by vortexing the tube to ensure homogeneity because a concentration gradient may form during –20°C storage. Centrifuge briefly to collect contents at the bottom of the tube.
* Prepare reactions according to the following table



Note that 100 nM final primer concentration will correspond to 0.2 ul of 10 uM stock solution.

cDNA template volume should not exceed 6 ul in 20 ul reaction volume (because of the effect on salt concentrations).

* Mix thoroughly by vortexing the tube. Centrifuge briefly to ensure that all components are at the bottom of the reaction tube.
* For more details see EvaGreen 2x Master Mix product literature: <http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10028376.pdf>

**Droplet generation (Bio-Rad QX200 Droplet Generator)**

All of the remaining steps can be performed with equipment in Microbiology B406. You must schedule a time to use both the droplet generator/thermal cycler and the droplet reader. See instructions here:

<https://vprnet.research.colostate.edu/mqc/digital-droplet-pcr/how-can-i-schedule-time-on-the-droplet-generators-and-reader/>

* Have samples at room temp for a couple minutes before droplet generation
* First load 20 ul of each sample in the wells in the middle of the cartridges.
* Then load 70 ul of oil in each of the wells at the bottom of the cartridge (the notched corner marks the top of the cartridge). Make sure to use the correct oil. There is different oil for Evagreen vs probe-based reactions.
* In pipetting, try to ensure that samples reach the bottom of the well
* Must fill all 8 wells in the cartridge, and it must be with the actual mix and oil. Variation in viscosity could adversely affect mixing. Bio-Rad sells a “blank” solution that can replace samples at much lower cost if you plan to be doing a lot of partial cartridges.
* Load gasket into white holder (should always be in core facility with droplet generator)
* Stretch gasket over the top of the cartridge, securing with the hooks on each side. There is no top side or bottom side of the gasket. It can go on either way.
* Open Droplet Generator (QX200) by pressing button and insert cartridge. Middle light should turn on. Close droplet generator door with same button.
* Droplet generation should start automatically. 3rd green light will begin flashing and then stop flashing and go solid when the generation is complete.
* The resulting emulsions should appear milky.
* Pipet the emulsion into new PCR plate. Pipet very slowly to avoid breaking/merging droplets. Pipet the entire volume. It is not necessary that the volume is exactly the same across all wells.
* One of the users had a problem with plate sealing in the first column, so you might want to avoid the first/last columns.
* The gaskets and cartridges are disposable and can be discarded.

**Plate Sealing (Bio-Rad PX1 Plate Sealer)**

* Turn on plate sealer using the switch in the back.
* Press the open button and put in the metal block (which should next to the unit) and close the unit with the same button.
* Wait for the unit to heat up to 180 C.
* Once heated, press the Open button. Put plate into block and place foil seal on top. The foil seal should have the colored strip facing up.
* Close and press the Seal button on the screen.
* Remove block and turn sealer off when done.

**PCR (Bio-Rad C1000 Thermal Cycler)**

* Uses special deep block (and corresponding plates)
* 2 C per second ramp rate.

Load sealed plate into thermal cycler

5 min @ 95 C

40 cycles of:

30 sec @ 95 C

60 sec @ 60 C (variable, may want to modify this depending on your primers)

5 min @ 4 C

5 min @ 90 C (yes, you go back to high temp; this is part of hardening the emulsion so the droplets say in tact during storage)

Hold @ 4 C

Volume is set to 40 ul

The base program is saved under the name QX200\_DDPCR. But you might want to modify the annealing temp (don’t save over the program if you do this; just run it).

**Droplet Reading (Bio-Rad QX200 Droplet Reader)**

* Open Quantasoft software
* Evagreen is read as fam (channel 1)
* Highlight wells you want to read
* Enter settings:
  + Experiment: Usually chose ABS (for absolute). Other options include REV (rare events) and CNV (copy # variants)
  + Supermix: chose EvaGreen
  + Channel: 1
  + Target: unknown
    - \*\*Must hit apply for settings to be entered
  + Names: target (gene) and sample (template name)
  + You may need to highlight samples before pressing run (not verified)
* Open QX200 Droplet Reader
* Remove holder
* Twist up tabs to remove silver cover
* Add PCR plate, replace cover, and lock
* Close reader with same button used to open
* Click over to run tab to initiate run. When asked to save data, do not change default directory (this directory is set not to scan for viruses on external devices, which makes it possible to achieve necessary write speeds).
* Data in C:/Quantalife/Data
* When your run is completed, transfer data and template files to the Sloan lab folder within the directory. Also make copies of these data and back them up as you would with any raw data. Data files will be periodically deleted from computer in the facility.

The QuantSoft analysis software is available on our lab PC that is associated with the qPCR machine.