***Bd* and Bacterial qPCR**

*By MYC*

Universal bacterial primers:

515F (5’ –GTGCCAGCMGCCGCGGTAA -3’)

806R (5’ –GGACTACHVGGGTWTCTAAT-3’)

*Bd* primers (Boyle *et al* ):

ITS1-3 (5’- CCTTGATATAATACAGTGTGCCATATGTC -3’)

5.8S Chytr (5’- AGCCAAGAGATCCGTTGTCAAA- 3’)

G-block sequence for standard curve:

CAGAAGAAGCACCGGCTAACTCC**GTGCCAGCAGCCGCGGTAA**TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGG**ATTAGATACCCTGGTAGTCC**ACCGAGTTGCCCGT**CCTTGATATAATACAGTGTGCCATATGTC**ACGAGTCGAACAAAATTTATTTATTTTTTCGACAAATTAATTGGAAATTGAATAATTTAATTGAAAAAAATTGAAAATAAATATTAAAAACAACT**TTTGACAACGGATCTCTTGGCT**CTCGCAACGATGAAGAACGCAGCGA

Blue base pairs are 515F/806R primer targets; purple base pairs are ITS1-3/5.8S Chytr primer targets. G-block sequence was created by combining the 16S rRNA V4 sequence from *E. coli* (Ehresmann *et al.* 1972) and ITS1-3/5.8S sequence for *Bd* (Schloegel *et al.* 2012) with additional random sequences to pad either end of the fragment.

All primers and G-blocks can be ordered from IDT technologies, which is accessible via CU Marketplace as a vendor. Primers are typically ordered with standard de-salting in 25nmol quantities (cost ~ $5-10 each). G-blocks are ordered in 500ng quantities ($90 for a <500bp fragment). Lead time is generally very fast—a few days.

*Diluting primers*

1. Do not open primer tube after arrival; primers may have attached to lid. Spin down primer tubes (dry) at 6000g for 1 minute.
2. Check how many nmol of DNA is in the tube (should be between 20-30nmol). Multiply this number by 10 to get how many uL of PCR-grade water to add to dilute to 100uM.
   1. For example, if you received 21.8nmol of product, add 218uL of PCR-grade water (or TE buffer) to create 100uM solution
   2. TE buffer can be used for more stability. TE buffer contains EDTA which inhibits PCRs at high concentrations, but the concentration used in primer dilutions is typically too low to matter. Since primers are inexpensive, we typically just use PCR-grade water since long-term storage is not a concern.
3. In laminar flow hood with filtered pipette tips, add the appropriate volume of water to each vial. Vortex briefly, spin down at 6000g for 1 minute, and then incubate at 50C for 3 minutes.
4. Vortex and centrifuge down at 3500g again.
5. Primers are ready to be further diluted.
   1. Tip: Make 150uL aliquots (15uL 100uM primer + 135uL PCR water), which is “just enough” for 1 full plate of PCR/qPCRs when using 1.25uL of primer per reaction
   2. Primers are stable at 4C for a couple weeks. Store at -20C for longer term storage.

*Diluting G-blocks*

1. Do not open g-block tube after arrival; primers may have attached to lid. Spin down primer tubes (dry) at 6000g for 1 minute.
2. Each tube should contain 500ng of G-blocks. Add 50uL of PCR-grade water (in laminar flow hood with filtered pipette tips) to get 10ng/uL concentration stock solution.
   1. To get copy number, see below
3. Vortex briefly, spin down in centrifuge at 6000g for 1 minute, then incubate at 50C for 5 minutes. Vortex again, and spin down a second time.
4. Create a standard curve by doing a serial 10x dilution from E-1 to E-9. E-1 and E-10 concentrations fail frequently. When running qPCRs, E-2 to E-9 is best to use
   1. Tip: when making serial dilutions, make a **50x** dilution of E-1 (e.g. 10uL in 490uL water) and use this to make the remaining 10x dilutions (100uL in 400uL). This way, you can add 5uL of standard instead of 1uL of standard to each reaction—it is more reliable and easier to pipette 5uL than 1uL of liquid.
   2. When transferring liquid from one standard to make another, *do not allow tip to touch liquid. “Hover” above the liquid and eject concentrated primer solution from the air. This ensures more consistent standards.*
   3. Between each dilution, vortex briefly; spin down; and incubate at 50C for 5 minutes for thermal mixing.
   4. Standard can be stored at 4C for a couple weeks. Store at -20C for longer term storage. Screw-cap tubes recommended to prevent evaporation.
5. Before every use, heat standards at 50C for 5 minutes; vortex at level ~3-4 for 5 seconds; and cenrifuge for 1 minute at 6000g. Add 5uL of E-2 to E-9 to each qPCR reaction by pipetting up and down 10x in the primer tube (filtered tip); “hovering” over the liquid with the pipette tip; and ejecting without touching the tip to the water.

*G-block copy number calculation*

Copy num (stock) = (Concentration in ng/uL)\*(Mol weight) \* (mol/fmol) \* (avogadro’s number)

= (10ng/uL)\* (fmol/ng) \* (1^-15)\* (6.022E23)

Where fmol/ng is the molecular weight of your G-block written on the tube.

More on G-blocks: <https://www.idtdna.com/pages/education/decoded/article/tips-for-working-with-gblocks-gene-fragments>

***qPCR reaction***

*(typically done in duplicate; different plates if possible, always randomize wells)*

*x1 Reaction Master Mix*

12.5uL ABsolute Master Mix with SYBR Green, no ROX (CA#: Thermo AB-1159/A)

1.25uL Primer #1, 10uM

1.25uL Primer #2, 10uM

5uL PCR H2O

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5uL template DNA\*

\*I recommend diluting DNA if 5uL/rxn is too concentrated; it is much easier to add 5uL/rxn than 1uL/rxn and you get more consistent results. If you change DNA template volume, adjust PCR H2O amount until the final reaction volume is 25uL.

Recommended plate: Agilent qPCR non-skirted plates, CA#: 410088

***qPCR* *reaction setup protocol***

1. Spray down flow hood with 70% ethanol and air-dry. Then, spray 10% bleach solution on surfaces, wipe, and allow to dry. Turn on flow hood and UV for 15 minutes. If desired, you can also put the qPCR plates and plate rack/lid in as well to UV the inside of the plate/lid for extra sterility.
2. Calculate how many reactions you need, and add 10% “ghost” reactions to account for pipetting error. Remember to include PCR blanks (No Template Controls) in your total reaction count. E.g.: for 7 rxns and 1 NTC, make at least 9 rxns. For a full 96-well plate, use 108rxns.
3. From here on out, use the flow-hood and filtered pipette tips for everything. Pipette gently, to prevent aerosols when transferring liquids.
4. Mix all reaction reagents (except template DNA) in a sterile micro-centrifuge tube (make sure total volume will fit in tube first!—see tips below for comments on this). Add water first; then primers; then ABsolute MM. (Least expensive + most volume first; most expensive reagent last). Mix by inverting tube and flicking a few times. Put on ice when not in use.
5. If using plates, put plate in a plate-rack on an ice block (Agilent qPCR non-skirted plates, CA#: 410088). Do not hover your hands or pipettes over the open plate.
6. Add 20uL of Reaction Master Mix to each well. You may use the same tip for all wells, as long as you are careful.
7. Add 5uL of DNA template into each appropriate well; fresh tip for each. I suggest having a print-out of your DNA extraction plate map printed out already (pressed against the outside of the flow-hood wall so you can see it), and to have your DNA template “arranged” in a rack in the correct order in advance. If you are doing plates, then use a “new” box of pipette tips so you can track which reactions you’ve “finished” by how many tips you’ve used in the box. Pipette up and down in DNA tube 10x (slowly), and eject contents of tip while hovering over liquid (same technique as in DNA standards).
8. Remove PCR plate from ice block and carefully seal plate with clear qPCR cover, making sure to roll/press cover on tightly over all wells.

***Agilent machine settings***

Cycle for Universal Bacterial (515/806) primers:

95C for 15min; [94°C 45 sec; 50°C 1 min; 72°C 1:30 min] x40 cycles; 72C for 10min

Runtime is ~ 3.5h

Cycle for *Bd* primers:

95C for 15min; [95C for 15s; 60C for 1min]x50 cycles

Runtime is ~ 2h

\*\* Important to keep 15min @ 95C at beginning of qPCR cycles. This activates the ABsolute Taq.

Steps to setup machine are:

1. Turn on Agilent machine. Switch is below the power cord on the left backside.
2. Open MxPro software and create new qPCR project.
3. Set up plate map—“Unknown” are samples, “NTC” are no-template-controls, and “Standard” are you DNA standards.
   1. Choose “SYBR” for the dye for all samples and standards. No reference dye.
   2. Calculate copy number in DNA standards and input these now. The software will automatically fit a curve for you using these numbers.
4. Set up cycle conditions. See cycles above. You can “import” cycle conditions from prior projects if you are using the same one over and over again.
5. Take your reaction plate and spin down in plate-salad-spinner to ensure all liquid is at the bottom of each reaction tube. 40x “pumps” of the salad spinner is usually sufficient.
6. Put plate in machine; make sure you lift the machine lid, put in the plate, then slide and click machine lid back. Sometimes people make the mistake of putting the plate *on* the lid, which looks tempting but is incorrect. Do not be deceived.
7. Shut the sliding curved machine cover. Optional: bless the reaction in the religion of your choice, or pray to the Lab Gods for success if you are agnostic.
8. Start run. Lamp may take ~20minutes to warm-up. If leaving overnight, un-check the box that says “keep lamp on after run” to save energy/lamp life.

***General tips:***

* Annoyingly, a “full plate” of qPCR reactions (x96+12 ghosts) is too much Reaction Master Mix volume for one 2mL microcentrifuge tube (108rxns x 20uL == 2160uL). To get around this constraint, you can split plates up the following way, since you generally have to do duplicate reactions anyway:
  + For your first qPCR, do duplicates of the first two rows of your DNA plate, plus the DNA standard. Randomize order of the 2nd duplicate on the plate. This should be 5 rows of reaction total.
  + Then, for your 2nd and 3rd qPCRs, do the remaining plate (rows 3-12) with 1 row of DNA standards. This is equal to 11 rows\* 8 wells == 88rxns. Factor in 10 ghosts and you have 98rxns 🡪 1960uL of Reaction Master Mix.
    - Remember to randomize qPCR platemap for duplicate plate.
  + If you are an “advanced” user and are confident your reactions will work, you can also start with rows 3-12, and then include any failed duplicates in the same run with rows 1+2. This method maximizes efficiency.
* Before starting a series of qPCR reactions, make all stock solutions *first* to minimize contamination issues. Aliquot 500uL of PCR water into individual tubes. Aliquot ABsolute mastermix into 1400uL aliquots (108rxns). Aliquot primers into 150uL at 10uM. Create G-block standards. Gather DNA template and thaw out in fridge. All these items are stable at 4C for a couple weeks, and freeze-thaw cycles are generally more harmful for them than storing at 4C (as opposed to -20C).
* Avoid re-using stock tubes—qPCR consistency and sterility decreases dramatically if you take from the same tube of primer/ABsolute tube over and over again, for example. Try to plan out reactions in advance, so you use up exactly one aliquot each time.
* Wells 1C and 4H always evaporate in the McKenzie lab machine, not sure why. Try to make these NTC wells. When running an “incomplete” plate, try to add plain PCR water in wells 1A-H, and then use rows 2-12 for your actual reactions. This will minimize risk of evaporation and possibly avoiding having to re-do failed duplicates.