Running the Quant-iT assay on the iD3 plate reader

Adapted from manufacturer's protocol.
Updated 29-Feb-2024 by Katie Murphy

HELPFUL HINTS:

- If your kit includes separate dye and buffer, allow the kit to come to room temperature before use (the dye needs to be thawed). This will take at least 30 minutes.
- Each assay plate needs to include the 8 standards. This means you can run up to 88 of your samples per plate.
- You do NOT need to run full plates. Leave any wells empty that you don't need (don't waste reagents please).
- Use solid black plates with flat-bottom wells.
- Turn on the plate reader prior to setting up your assay plate, so it'll be ready to go when needed.
- For more accurate pipetting and therefore more accurate quantification, we recommend using at least 2 µL DNA rather than just 1 µL.
- For your calculations, it is simplest if you use the same volume of DNA for all samples on a plate. But this isn't strictly necessary, as long as you record what volume of DNA you used for each sample.

MATERIALS & REAGENTS:

- DNA to be quantified
- Reservoir (for adding assay buffer to plate with a multichannel pipette)
- HS or BR Quant-iT kit
    - HS is recommended if you expect samples to be < 100 ng/ul
- Solid black 96-well plate (Corning #3915)
- Foil plate seal

PROTOCOL:

Step 1 – Setting up your assay plate

Please follow 1A if you plan to use the pre-defined software template (2A). Alternatively, you can follow 1B if you aren't using the pre-defined software protocol, which gives you flexibility in where you run your standards.

1A. Setting up your assay plate to use with our pre-defined software template

If using this protocol, you MUST load your standards in the 12th column of your assay plate.

1A.1 If not using a 1X kit: you need to make a 1X working stock. Calculate the number of reactions needed, making sure to include a few extra reactions to allow for pipetting error. For each reaction, you'll need 1 µL of dye and 200 µL of buffer.
   a. Example: for a full plate of 96 reactions, mix enough for 100 reactions – you'd need 100 µL of dye and 20mL of buffer.
   b. Once you dilute the dye, it's stable at room temp for 3 hours as long as it's protected from light.

1A.2 Add 200 µL of diluted dye to each well of your 96 well plate that you plan to use for samples or standards.

1A.3 Lightly mix your standards by inverting or vortexing, then briefly spin down. Add 10 µL of each standard to the following wells in column 12.
   a. A12 – standard 1 (least concentrated)
   b. B12 – standard 2
   c. C12 – standard 3
   d. D12 – standard 4
   e. E12 – standard 5
   f. F12 – standard 6
   g. G12 – standard 7
   h. H12 – standard 8 (most concentrated)

1A.4 Add 1-20 µL of each DNA sample to its designated well of the assay plate.
   a. We recommend 2 µL instead of 1 µL if possible, as pipetting is more accurate with 2 µL.

1A.5 Seal the assay plate thoroughly with a foil seal, then vortex to mix.

1A.6 Spin plate briefly to collect contents to the bottom of the wells.

1A.7 Incubate plate at room temperature for at least 2 minutes before reading on the iD3 plate reader.
   a. Your assay will be stable for up to two hours at room temperature, as long as you store the plate in a dark location (or keep covered with foil).

1A.8 Remove the foil seal before inserting your plate into the plate reader.

1B. Setting up your assay plate without using our pre-defined software protocol.

If using this protocol, you can load your standards anywhere on your assay plate – even in a separate plate. If you have a full plate of libraries, this would allow you to run all samples in one plate, and the standards in a second plate. Please note, you must follow Step 2B if using this protocol.

1B.1 If not using a 1X kit: you need to make a 1X working stock. Calculate the number of reactions needed, making sure to include a few extra reactions to allow for pipetting error. For each reaction, you'll need 1 µL of dye and 200 µL of buffer.
   a. Example: for a full plate of 96 reactions, mix enough for 100 reactions – you'd need 100 µL of dye and 20mL of buffer.
   b. Once you dilute the dye, it's stable at room temp for 3 hours as long as it's protected from light.

1B.2 Add 200 µL of diluted dye to each well of your 96 well plate(s) that you plan to use for samples or standards.

1B.3 Lightly mix your standards by inverting or vortexing, then briefly spin down. Add 10 µL of each standard to the wells of your choice. Make sure to record which wells you use.

1B.4 Add 1-20 µL of each DNA sample to its designated well of the assay plate(s).

a. We recommend 2 µL instead of 1 µL if possible, as pipetting is more accurate with 2 µL.

1B.5 Seal the assay plate(s) thoroughly with a foil seal, then vortex to mix.

1B.6 Spin plate(s) briefly to collect contents to the bottom of the wells.

1B.7 Incubate plate(s) at room temperature for at least 2 minutes before reading on the iD3 plate reader.

a. Your assay will be stable for up to two hours at room temperature, as long as you store the plate in a dark location (or keep covered with foil).

1B.8 Remove the foil seal before inserting your plate into the plate reader.

Step 2 – Running your quantification assay using the id3 plate reader

You can either use a pre-defined protocol (2A), or you can set up your own run from scratch (2B).

2A. Using LAB's pre-defined plate reader protocol & analyzing within SoftMax Pro

If using this protocol, you MUST have set up your standards in column 12 of each assay plate.

2A.1 If not already on, turn on both the plate reader and the computer.

2A.2 Log onto the computer using our standard lab login info (it's printed on the side of the computer if you forget).

2A.3 Click on the corresponding desktop icon for your assay.

a. If you're running the High Sensitivity kit, click on "HS Quant-iT assay" icon.

b. If you're using the Broad Range kit, click on "BR Quant-iT kit" icon.

c. Make sure you open the correct protocol for your assay type. If you open the wrong one, your standard curve will be incorrect, and therefore your concentrations will be incorrect.

2A.4 Verify that the software is connected to the plate reader. On the upper left of the window, you should see a green checkmark on the SpectraMax device icon. If instead there is a red circle with a slash through it, do the following:

a. Double-click the SpectraMax icon.

b. After the window pops up and populates with a list of instruments, click on the SpectraMax iD3 and connect to it. It should be the only instrument listed.

c. Close this pop-up window if it doesn't automatically do so.

2A.5 Open the plate reader drawer by clicking on the "Open/Close" button in the menu bar of the software, or by clicking the eject button on the iD3 touchscreen.

2A.6 Insert your plate, with A1 on the upper left and H12 on the bottom right.

2A.7 Close the drawer by clicking on the "Open/Close" button in the menu bar of the software, or by clicking the eject button on the iD3 touchscreen.

*a. DO **NOT** MANUALLY PUSH IN THE DRAWER BY HAND!!!*

2A.8 Click the "Read" button (green arrow).

2A.9 Click "skip/okay" if there is a popup about optimization (you don't need to check any boxes).

2A.10 The read will take approximately 1 min 30 seconds per plate.

2A.11 Once the read is complete, verify that your standard curve looks correct. Remove/mask any incorrect standard value(s).

2A.12 We highly recommend forcing the y-intercept to be the fluorescent value obtained for your first standard (0 ng). You can do this in the SoftMax Pro software by following these steps:

a. Scroll until you are viewing the standard curve.

b. From the buttons that are now along the top of the software screen, select "Curve Fit Settings".
c. In the pop-up that appears, set value "A (the y-intercept) to the fluorescent value obtained for standard 1.
d. Save/close the pop-up menu.
e. Your standard curve should now show an adjusted linear equation.
f. You can verify you've done this correctly by scrolling to the table with your standard values. The back-calculated value for the first standard should now be exactly 0 ng.

2A.13 Record the total ng ("Result" column) reported for your samples.
   a. You can copy/paste this column into a spreadsheet, or you can simply export the entire output to excel.
   b. To export your data in Excel (.xls) format:
      i. Click on the Plate icon in the top left of the screen.
      ii. Select Export.
      iii. Select your entire experiment (or plates of interest) & export your raw data.
      iv. Select "column" or "plate" format, depending on your preference.
      v. Change file type to .xls.

2A.14 Divide your result by the volume (µL) of DNA you used for each sample to get your original ng/µL concentration.
   a. For example, if you only used 1 µL of DNA for your samples, then the outputted result is your ng/µL.
   b. If you used 5 µL of DNA, then divide the values by 5 to get your original ng/µL concentration.

2B. Setting up the plate reader from scratch & analyzing in Excel.

Use this protocol if you set up your standards in wells other than column 12, or if you prefer to analyze your data in excel yourself.

2B.1 If not already on, turn on both the plate reader and the computer.

2B.2 Log on to the computer using our standard lab login info (it's printed on the side of the computer if you forget).

2B.3 Launch the SoftMax Pro software by double-clicking the desktop icon.

2B.4 Verify that the software is connected to the plate reader. On the upper left of the window, you should see a green checkmark on the SpectraMax device icon. If instead there is a red circle with a slash through it, do the following:
   a. Double-click the SpectraMax icon.
   b. After the window pops up and populates with a list of instruments, click on the SpectraMax iD3 and connect to it. It should be the only instrument listed.
   c. Close this pop-up window if it doesn't automatically do so.

2B.5 Click on the orange gear icon to go to the Acquisition Setting menu.

2B.6 Choose/set the following:
   a. Choose Fluorescence (not Abs or Lum).
   b. Wavelengths: set to 480 and 530.
   c. Plate type: 96 well, opaque.
   d. Select all wells to read the entire plate, or you can omit empty cells/columns.
   e. You can ignore all other settings (leave them as defaults).

2B.7 Open the plate reader drawer by clicking on the "Open/Close" button in the menu bar of the software, or by clicking the eject button on the iD3 touchscreen.

2B.8 Insert your plate, with A1 on the upper left and H12 on the bottom right.

2B.9 Close the drawer by clicking on the "Open/Close" button in the menu bar of the software, or by clicking the eject button on the iD3 touchscreen.
    a. DO NOT MANUALLY PUSH IN THE DRAWER BY HAND!!!
2B.10 Click the "Read" button (green arrow).
2B.11 Click "skip/okay" on the popup about optimization (you don't need to check any boxes).
2B.12 The read will take ~1 minute 30 seconds.
2B.13 Once the run finishes, click "save" and save your experiment in your documents folder as a .sda file.
    a. This will allow you to reopen your data in SoftMax Pro if needed.
2B.14 Export your data in Excel (.xls) format:
    a. Click on the Plate icon in the top left of the screen.
    b. Select Export.
    c. Select your entire experiment (or plates of interest) & export your raw data.
    d. Select "column" or "plate" format, depending on your preference.
    e. Change file type to .xls.
2B.15 Open your file in Excel.
2B.16 Create a standard curve by creating a scatter plot of your standard data. The x-axis should be total ng of DNA from your standards, and the y-axis is their fluorescent readings.
    a. Check your assay type to determine total ng. For the HS kit, your standards will range from 0 to 100 total ng. The broad range kit will range from 0 to 1000 ng. **Note, this is TOTAL NG, not ng/µL!**
2B.17 Add a linear trendline to your standard curve, and display the equation and R^2 value.
2B.18 Remove any erroneous standards from your standard curve.
2B.19 Set the y-intercept to be the fluorescent value of your 0 ng standard (standard 1).
2B.20 Use the equation to calculate the total ng in each of your sample wells. You will be solving for x.
    a. Your y-value is the fluorescent reading from the assay.
2B.21 Divide these values by the number of µL of DNA used for each sample to determine your original ng/µL concentration.
    a. For example, if you only used 1 µL of DNA for your samples, then the outputted value IS your ng/µL.
    b. If you used 5 µL of DNA, then divide the values by 5 to get your original ng/µL concentration.