#### Supplemental file 2: Quantification of dsDNA from spheroids in an XFe96 spheroid microplate

The following steps describe a protocol for quantifying intra-spheroidal dsDNA using the Quant-iT PicoGreen dsDNA Assay Kit. As nuclear dsDNA in spheroids is linearly correlated with cell seeding density or spheroid volume (**Figure 9**), nuclear dsDNA content is a possible approach to normalize XF data. dsDNA is preferred over protein normalization, particularly where spheroid adhesion to assay culture plates is aided by poly-D-lysine and where pretreatments may impact protein synthesis.

This protocol assumes the final XFe assay well volume to be ~ 240 µL. Adjust the protocol as necessary for other volumes, e.g., spheroids within cell culture growth plates. Users may wish to prepare a standard curve of monolayer cells for DNA quantification to allow estimation of cell number within spheroids.

1. Carefully aspirate 190 µL of XF assay medium from each well of the XFe96 spheroid microplate, leaving ~50 µL of assay medium/well.
2. Add 50 µL/well of spheroid lysis buffer (supplemented with 20 mg/mL proteinase K), place the plate on ice, and leave to lyse for a minimum of 10–30 min.

NOTE: Shake the assay plate on a plate shaker, if necessary, to help enhance lysis. Larger spheroids may require elongated incubation, and lysis can be confirmed under the microscope. The process of freeze-thawing will also help aid spheroid lysis.

1. Add an equal volume (100 µL/well) of 1x Tris-EDTA buffer (10 mM Tris-HCl + 1 mM EDTA, pH 7.4) to reduce sample viscosity.
2. Carefully mix lysates within wells by pipette aspiration. Alternatively, use a plate shaker set at 500 rpm.
3. Generate a standard curve of lambda DNA in 100 µL of XF assay medium from 2 mg/mL–2 ng/mL DNA.
4. Add 100 µL of spheroid lysis buffer to match sample volumes and mix.

NOTE: Final standard curve will be in the range of 1 mg/mL–1 ng/mL after dilution.

1. Transfer 20 µL of the spheroid lysate and standards into separate wells of a suitable 96-well microplate for recording fluorescence (preferably black).
2. Dilute PicoGreen DMSO stock solution 200-fold to achieve final volume required to complete assay e.g., to achieve analysis of 10 samples at 100 µL final volume, dilute 4 µL of PicroGreen DMSO stock solution in 796 µL of 1x TE buffer.
3. Add 80 µL/well of PicoGreen working solution and incubate at room temperature for 2–5 min, protected from light, with gentle intermittent shaking to equilibrate.
4. Measure well fluorescence on a fluorescent-based microplate reader with an excitation wavelength of 485nm and emission wavelength of 520nm (485EX–520EM).