StepOne Plus notes:
Before you begin, plan to compare reference samples (for consistency), extraction kits (for quantity, quality, and degradation).

- Three types of **quantitation** experiment types; Standard curve, Relative Standard curve, or the typical ΔΔCt, which is now called Comparative Ct
  - For melt curve analysis push "?" on the machine or F1 on the keyboard to access help with this.
  - Software automatically handles Taqman and SYBR Green I Dye reagents in "Design Wizard. For other reagents, you will have to go to Advanced Setup instead. Note that only SYBR Green can be used for melt curve analysis.
  - Use Primer Express 3.0 to design your own primers and probes as this software is set with StepOne optimized parameters.
  - For multiplex, SYBR cannot be used. Typically, a probe labeled with FAM is used to detect the target and a VIC labeled probe is used to detect the endogenous control. Endogenous control should be more abundant than all other targets and fairly constant across conditions and samples, and may need to be primer limited to avoid competitive PCR that may alter the Ct of less abundant template.
  - Should be validated with singleplex vs multiplex before proceeding with multiplex.
  - Primer/Probe design: Amplicons 50-150bp; G/C content 30-80% for both; avoid runs, specifically, no more than 3 Gs in a row; Tm should be 68-70°C for probes, 58-60°C for primers; NO G on 5' end of PROBE (has quenching effect) and as short as possible w/o being < 13bp; 5 nucleotides at 3' end of primer should have no more than 2 G/C bases if possible to avoid non-specific product formation.
  - Recommended cycling conditions on pg 3-28 and 29 of the Reagent Guide.
  - Optimize primer concentrations for quantitation assays – Recommended starting points 900nM for Taqman Rgts (ultimately want amount that gives the lowest Ct and highest ΔRn), 50nM for SYBR Green Rgts (Same goal but must include negative controls that don't produce product). Can validate by melt curve.
  - Optimize probe concentration – For best reproducibility, avoid probe-limiting concentrations. 250nM recommended.
- Can do **genotyping** which is an endpoint experiment to differentiate SNPs
- **Presence/absence experiments**, also endpoint experiments when you just want a yes or no without actual quantity, commonly used for parasite or pathogen detection.
  - Needs replicates, negative controls, and best tan internal positive control (IPC) to distinguish between true negatives and inhibited PCR.
  - Ideally, IPCs are run multiplexed with targets to ensure that a failed PCR is not mistaken for a negative result.
  - TAMRA not recommended for StepOne so maybe we should avoid for StepOnePlus too. Presence/absence not supported for Fast or SYBR Green master mixes and protocols.
  - Neither Fast, nor SYBR Green master mixes and protocols are supported in presence/absence experiments.
- To utilize the **VeriFlex Block** – in "Advanced Setting" under Plate Setup, you must click the box above the plate graphic that says "enable VeriFlex Block". Then in Run Module, click on the temperature you would like to vary in each zone and a set Temperature dialogue box will open allowing you to set each zone to the desired temperature.
- Can create compile various runs/experiments into **Studies** – a few conditions required – 1) Endogenous controls must be present on each reaction plate within study, 2)each experiment must have identical thermal cycling parameters.