protocol v5.03

qPCR plate assembly with Hamilton Microlab Star

First time user? Please first read the overview of the procedure and comments at the end of this guide.

In this guide, "assay" = target gene = primer couple (+/- probe).

Procedure

1. Preparation of the lists of assays and DNA samples

1.1 Obtain the Excel model files available on GECF website to prepare the lists of assays and DNA samples.

1.2 For each DNA sample, specify the "task" and "quantity":

- **STND** task for a serial dilution of samples that will form a standard curve. Indicate the absolute or relative amount in the quantity column. *Do not indicate dilution factors, but quantities. Commas/dots are not supported, therefore multiply by 1000 if needed.*
- **NTC** for No Template Controls (or water). Indicate 0 in the quantity column.
- **UNKN** for actual samples. Indicate 0 in the quantity column.

1.3 For each assay, specify the main chemistry (SYBR, FAM, VIC…) and the quencher chemistry. *MGB quenchers are non-fluorescent. SYBR chemistry has no quencher therefore indicate "Non fluorescent".*

- All these names/values can still be changed after the qPCR run, but the dyes must be set correctly as the instrument only records corresponding wavelengths.

- Each assay and sample must have a unique name. Avoid non-standard characters, in particular ' is not supported and may cause the robot to skip samples/assays.

- The name of the sheets themselves (the tabs at the bottom of the sheet) must be "DNA" and "PRIMER" (upper cases). The files must have an .xls extension, not .xlsx.

2. Preparation of DNA sample and primers microtubes

2.1 Use the Excel calculation file ("Hamilton qPCR reagents setup guide v0.7") available on GECF website to determine how to prepare the primers/H2O and DNA sample/mastermix:

- Indicate the assay chemistry (SYBR, TaqMan or commercial premixed 20x TaqMan assays), the type of plate, the number of replicates, and define a safety margin % for the robot pipetting (20% by default, tweaking this value requires prior testing by the user).
- Indicate the number of assays and DNA samples.
- Indicate stock concentrations of your probes and primers (individual primers concentration).

- Indicate final concentrations of your probes and primers (individual primers concentrations) in the reaction. *Refer to the user manual of your mastermix for guidelines.*

2.2 Prepare the mixes according to these calculations in microtubes. *We recommend 1.7ml Axygen MCT-175-L-C or MCT-175-C. Do not use tubes whose caps tend to close again by themselves after opening.*

- Note: Mastermixes can be viscous and careful pipetting is therefore needed. Change tip between each tube. Importantly, avoid bubbles (spin briefly or remove manually)!

3. Setting up the robot software and worktable

A wizard will guide you through these steps.

3.1 Switch on the robot if needed.

3.2. Copy your DNA list and PRIMER list to the "InputAndMatrixFiles**"** folder.

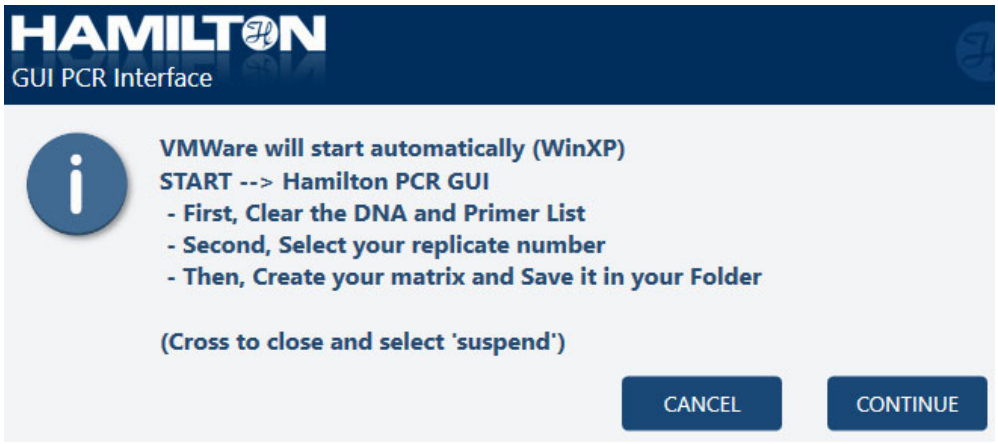
3.3 Open the Hamilton software (penguin icon). *Close and reopen it if it was already open.*

3.4 Click on the "Play" button.

3.5 Enter username, optional (*default User ID*). Press OK.

3.6 Reply "No" when asked whether you already have a worklist. *You will create it now.*

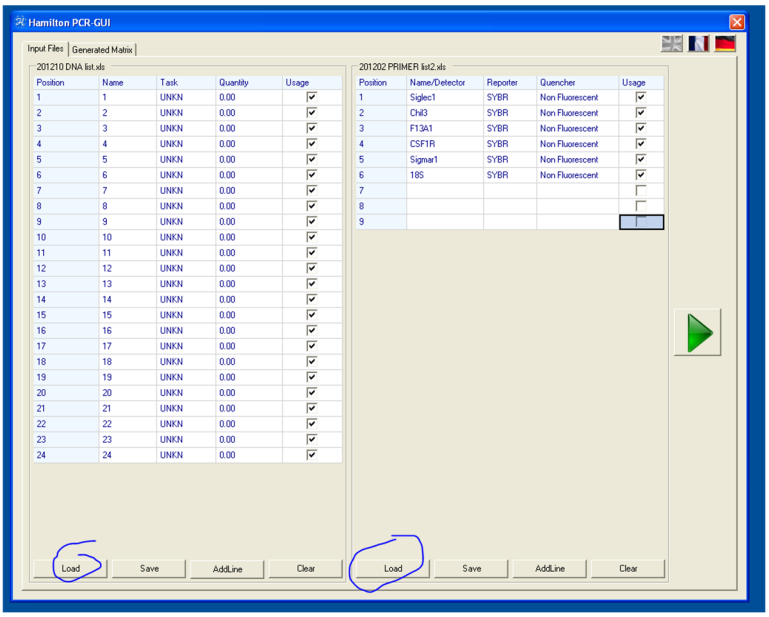
3.7 A window appears saying that you will use the Hamilton PCR Graphical User Interface (GUI) through a virtual machine (VMWare) to create the worklist. VMWare program opens automatically.



Since VMWare window may open minimized, check on the Windows taskbar at the bottom of your desktop and maximize it if needed.

3.8 Within the VMWare, open Hamilton GUI software. *It may already be open.*

3.9 In the Hamilton PCR GUI, select the "Input Files" tab first. *The Input files tab initially shows the DNA list and PRIMER list from the last user (like in the image below)*



3.10 In order to import your DNA samples and assays lists, click on “Load” button of the DNA (left side) and PRIMER (right side). *Sometimes the lists presented at this step include empty lanes (like in the image above, in the assay table), which will crash the software later… go back to your Excel lists, select a few rows just below your last sample/assay and delete these rows, then begin again the import procedure.*

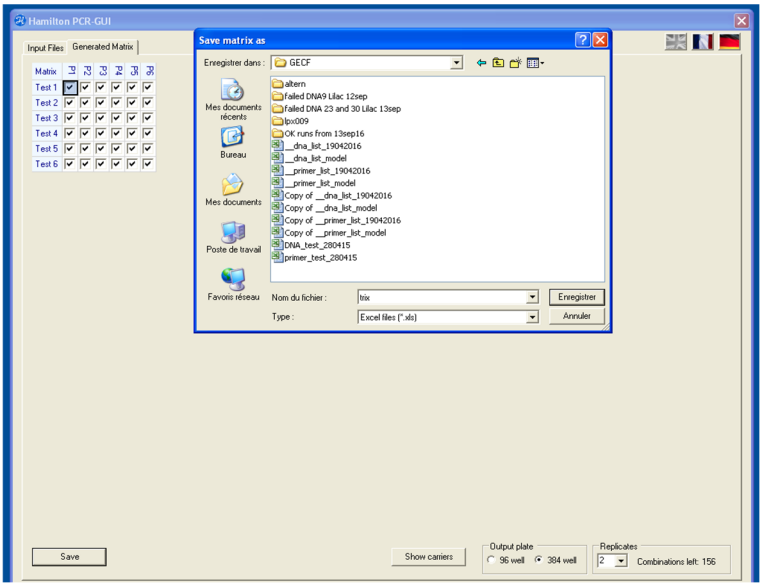
3.11 Only after loading your lists, click on the green “play” button.

3.12 This generates a matrix that it is now shown in the “Generated Matrix” tab.

3.13 On the newly generated matrix, unselect primers/templates combination that you don’t want.

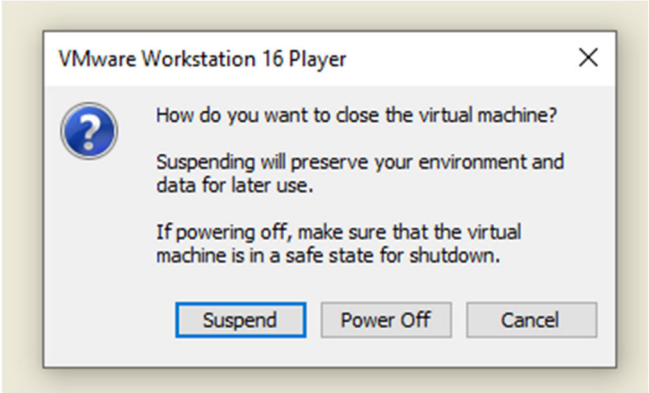
3.14 On the bottom right of the screen, check that the relevant plate format (96w or 384w) and number of replicates (3 recommended) are correctly set.

3.15 On the bottom left of the screen click on Save to save the matrix. To facilitate locating the file later, save it in a subfolder of the “InputAndMatrixFiles” folder (press “move up” button if needed)



In case you have changed the input files tab after generating a matrix, you may not be able to save. In this happens, close the Hamilton PCR GUI, reopen it and load again the input files.

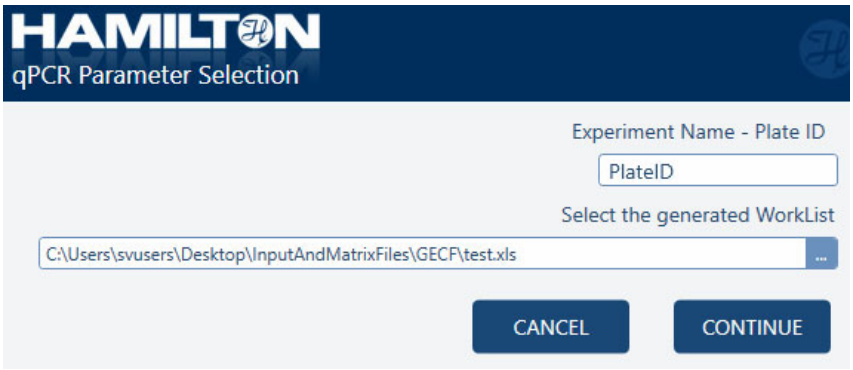
3.16 Close the VMWare software. Then click on “Suspend” in the message below.



3.17 Now you can go back to the Penguin Hamilton software in Windows 10, and click “Continue”

3.18 Enter Experiment Name, optional (default PlateID)

3.19 Select the generated Worklist that you have just saved through VMWare. Press Continue.

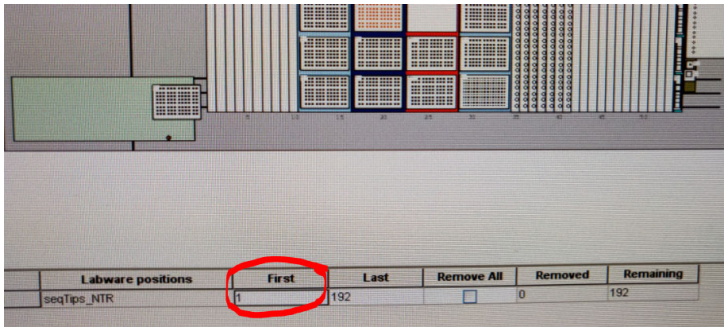


3.20 Select your mastermix type: #1 (standard) for Applied Biosystems PowerUP SYBR mastermix, or when you are not sure. Select #2 for very viscous mastermixes, such as Applied Biosystems Power SYBR mastermix or some SYBR mastermixes from Qiagen or Roche.

3.21 Select NTR Tips rack

3.22 Follow the indications of the wizard to set up the robot worktable:

- positions 11-16: 96 well block. It must be free of tips before starting the run. If there are tips left, throw them away.
- positions 17-23: 50 µl tips ("NTR", Hamilton ref # 235947). The 3 front racks must be full, the one on the back can be partially filled. Check that no tip is missing in the middle of the racks. Indicate the position of the first tip in the partially filled rack of the rear (circled field below), according to the numbering sheet displayed on the robot:



- positions 35-37: Carrier for DNA samples, loaded from rear to front at position 35. Each carrier can hold up to 32 microtubes. Subsequent ones are then loaded on additional carriers placed immediately towards the right, starting from the rear. Microtubes lids must be fully opened and pushed against the metal ridge. *Check the absence of bubbles.*
- positions 39-41: Carriers for assays microtubes. *Same remarks as for DNA samples. Check the absence of bubbles.*
- positions 23-28: 96w plates (4titude ref # 4ti-0740), which serve as intermediate mixing plates. The software indicates whether 1 or 2 plates must be placed.
- positions 29-34: The 384w (4titude # 4ti-0384/C, skirted) or 96w plate (Microamp 96w # N8010560 for standard 96w block; or MicroAmp Fast 96-Well, # 4346907, for Fast block) in which

the qPCR is performed. *Make sure you do not touch the bottom of the plate and only lay it on clean surfaces. Orientation: plate faces you.*

4. Running the method

4.1 Push well the DNA/primer carriers and the plate and tips holders fully towards the rear.

4.2 Check the waste bins are not full.

4.3 Make sure that there is no obstacle to the movement of the robotized arm on the worktable.

4.4 Close the front door.

4.5 Press Start in the software.

4.6 If needed already start the S200 sealer for preheating.

- Note: A full 384w assembly takes approximately 30 minutes (some combinations of numbers of assays/sample may take longer).

- Note: The reaction volume is 10ul for 384w, and 15ul for 96w.

5. Termination of the method

5.1 Close the Hamilton software.

5.3 Switch off the robot if you are likely the last user (not critical).

5.4 Get the .txt file that was generated at the end of the assembly in the folder named “OutputFiles”. *This file contains the layout of your plate that will be uploaded into the qPCR instrument.*

5.5 Inspect the remaining volumes in the microtubes to detect putative inaccurate pipetting. Throw away the intermediate plates.

5.6 Remove the assembled qPCR plate and inspect it visually to check that no obvious problem occurred (avoid touching the bottom of the plate).

5.7 Fill in the logbook with date, name and any relevant comment. Also e-mail us in case of issues.

5.8 Seal the 384w qPCR plate with the S200 sealer with an optical heat-sealing film by following the printed instructions. Or manually seal 96w plates with the available sticky films (Applied Biosystems # 4311971).

5.9 Spin the plate for 2 minutes at 2’000rpm (935g) on a large tabletop centrifuge.

General notes

- Make sure your mastermix is compatible with the qPCR instrument you plan to use (all our QuantStudio instruments behave the same in that respect).

- We strongly recommend mastermixes containing rox passive reference dye to correct for small pipetting inaccuracies.

- The assembly is performed at room temperature, since most if not all qPCR mixes contain a Hot-Start mechanism. Make sure your mix does incorporate such a mechanism.

- Maximum number of primers couples is 64. Maximum number of DNA samples is 192. The upper limit is given by the maximal number of combinations (nb primer couples x nb DNA samples x replicates) that fits into a 96 or 384 well plate. User can force the Hamilton software to omit specific combinations.

- All the plastic consumables are provided by the GECF unless microtubes. Reagents (primers, mastermixes, UNG) are to be provided by the user.

- Booking is done through the BBS GECF portal. Pricing is available on GECF web site.

- When your experimental design requires multiple qPCR plates, keep all the samples on the same plate and dispatch assays on different plates (=aka “sample maximization”).

Overview of the procedure

A list of primers and DNA samples is prepared by the user based on the GECF Excel templates. → The user manually prepares one tube for each assay mixed with water, and one tube for each DNA sample mixed with 2x mastermix. This is performed according to a calculation file provided by the GECF. → The primer and DNA lists are loaded into the Hamilton software on the PC of the robot, which creates a matrix with all assay/samples combinations, and based on that a “worklist” (= pipetting instructions for the robot). A wizard guides you during the preparation of the robot worktable. → The primers and DNA/2x mastermix are positioned in microtubes on the robot, which distributes them in an “intermediate 96w plate” for mixing. The robot then distributes these reactions in the final 384w plate with the desired number of technical replicates. → The robot outputs a .txt file. This file can be loaded in the qPCR instrument, filling in automatically samples positions, detectors, dyes and standard curve quantities when relevant.

Troubleshooting

- Refer to the sheet next to the robot, or contact us.

Changelog

- v4.00-v4.04: Added recommendations for mastermixes choice. Added comments on special characters. Many little modifications relative to historical v3 version. Clarified that cycling parameters should be modified according to mastermix user manual. Indicated not to perform an analysis in SDS while a run is ongoing. Mentioned the mastermix viscosity step at the beginning of the method. Clarified how to indicate position of the first tip to be used. Indicated to check that the new racks of tips you load do not have empty tips positions.

- v5.01 New temporary version after update to Windows 10 and the introduction of VMWare to use Windows XP.

- v5.02: Modified catalogue number of Microamp 96w plates. Several edits related to the new GUI.

- v5.03: Clarified to check that the 96 block in position 11-16 is free of tips.