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7500 Sex marker screening protocol EFGL

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Protocol status: Working
We use this protocol and it's working

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Abstract

This is a generalized protocol that Eagle Fish Genetics Lab (EFGL) uses to prepare samples for a genetic sex screening on the ABI 7500. It provides steps on normalizing samples, prepping different master mix recipes, and using the 7500 software v2.3 to complete a 7500 run.

Attachments

XLSX
7500 Quantification ...
43KB

XLSX
7500 PCR labsheets v...
17KB

Materials

- 2X TaqMan Universal PCR Master Mix **Applied Biosystems (ThermoFisher Scientific) Catalog #**4318157
- MicroAmp™ Optical 96-Well Reaction Plate **Thermo Fisher Catalog #**4316813
- MicroAmp™ Optical Adhesive Film **Thermo Fisher Catalog #**4311971
- Ultrapure Distilled, Nuclease Free Water

Equipment

Applied Biosystems 7500 and 7500 Fast Real-Time PCR Systems	NAME
Applied Biosystems	BRAND
4351107	SKU

Troubleshooting

Some guidelines before starting:

1 This is a generalized protocol for working sex assays in EFGL.

- If you're working on a newly designed assay, there could be changes to setup, reagent concentration, etc. Ultimately, it shouldn't stray too far from this.

1.1 EFGL normalizes all sample DNA to 10ng/µl before performing any 7500 runs.

1.2 For an optimal representation of results, each plate setup should ideally have a 50/50 split, or as close as possible of known males and females.

- If all samples are of unknown sex, run known sex samples as "positive controls" for comparison. Consult biologist on which samples are ideal as controls.
- Include at least 2 NTCs per assay per plate

1.3 EFGL's default 7500 experiment type is "Genotyping".

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/4387784c.pdf

Setting up project and samples

2 Obtain sample list and assay(s) needed from lead biologist/data coordinator. Samples may have been
pre-extracted (stored in -80 °C), or extract them if necessary before proceeding.

3 Use the *7500 Quantification and Input File v1.1 spreadsheet* in your project folder, to generate quantification and input files of your tray(s).

The spreadsheet template is setup so that it'll match the plate layout as you do each 7500 runs.

3.1 Enter in the project name, and tray information to keep track of samples for that tray.

3.2 Enter sample names in "Sample Information" tab starting from cell C7.

- Enter known phenotypic sex for the samples in column D. OK to leave blank if unknown.
- NTC wells have been set to wells G12-H12.

3.3 Save new file for each tray you'll run.

3.4 Repeat steps 3 to 3.3 until all samples have been mapped out.

4 To have a visual layout, head to the “7500 plate map” within spreadsheet

You can also start copying and pasting each tray map to the *7500 PCR labsheets v1.2 spreadsheet* to obtain master mix recipe. OK to do this after normalization as well.

5 Depending on sample size, determine which quantification protocol (HTX plate reader or Qubit HS) would best fit.

Note

Qubit is recommended/easier if:
- Total sample size is less than 80, or
- There’s a lot of cherry picking from several trays for each plate to run

Recommended to quantify and normalize all samples prior to starting the 7500 runs. PLAN accordingly as this section is the most time consuming

STEP CASE

Qubit HS From 25 to 41 steps

6 Prep Qubit master mix (199 µL of buffer to 1 µL of dye per sample) for 4 or 5 samples first.

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Qubit_dsDNA_HS_Assay_UG.pdf

6.1 In a Qubit assay tube, add 198 µL of Qubit master mix and 2 µL of DNA per sample. Gentle vortex, quick spin and incubate for 00:03:00 at Room temperature prior to read

6.2 If Qubit machine says, “too high”, dilute the remaining samples to 1:10 dilution. If not, proceed as normal for the remaining samples.

6.3 *(Skip if no dilution is required)* In a new unskirted PCR plate, add 1 µL of DNA to 9 µL of water. Heat seal, vortex, quick spin and use this diluted DNA for Qubit quantification.

7 Using the same *7500 quantification and input* spreadsheet, enter in the quantified DNA concentration in "Qubit + Norm" tab. It has been formulated to normalize all samples to [M] 10 µg/µL at 15 µL final volume.

7.1 If using diluted DNA, change the dilution factor number in cell D2 to obtain the actual DNA quantification.

7.2 In column D (DNA tray well), enter in the original DNA well position. Print out this sheet to begin normalization.

8 In a new unskirted PCR plate, add the calculated water + DNA sample to each well. Heat seal, vortex and quick spin .

Expected result

Your samples are now all normalized to [M] 10 µg/µL at 15 µL

8.1 Proceed with the remaining tray(s), and save all normalized trays till end of project.

9 Once all DNA samples have been normalized, in the same spreadsheet, head to the "7500 plate map" tab for each tray.

9.1 Copy each tray map and paste it to the respective tray in the *7500 PCR labsheets v1.2 spreadsheet.* Print individual sheets if necessary.

9.2 Going back to the *7500 Quantification and input* spreadsheet. Click on the "SNP Sample Sheet" tab and save it as .txt (Tab delimited) file on a flash drive.

9.3 Bring the flash drive with you to the lab, to import samples to the 7500 computer for running the experiment later.

10 EFGL uses 2 types of assays. Taqman assay, and IDT assays.

Taqman assays come ready to go with all primers and probes pre-optimized by Thermo Fisher. While IDT assays are ordered in separate components, 2 primers (forward and

reverse), and single or double probes.

Note

Master mix recipes listed are based on default concentration/volume EFGL uses for genotyping experiments along with the default cycling conditions.

STEP CASE

Taqman assays 27 steps

11 Thaw and gently vortex Taqman assay. As the tube itself doesn't fit in our vial centrifuge, gently tap tube on benchtop to bring assay down to the bottom of tube.

11.1 In a 1.5mL vial, prep the master mix. To account for pipetting error, add 2 additional samples.

Reagent	Volume per sample
TaqMan 2X Universal PCR Master Mix	5µl
Taqman assay (40X)	0.06µl
Water	3.94µl
Total	**9µl**

11.2 Gently vortex normalized DNA tray, quick spin down .

11.3 Use a 0.1 mL combitip to dispense 9 µL of master mix into the ABI plate wells.

11.4 Using a p10 multi-channel pipette, add 1 µL of normalized DNA to the respective wells

IMPORTANT! Add DNA to the side of the well to avoid creating bubbles – 7500 runs are sensitive to bubbles.

11.5 Seal the ABI plate with a MicroAmp film by using a Kimwipe to press down on seal to avoid smudges/scratches on film.

11.6 **DO NOT VORTEX**. Place a kimwipe at the bottom of the centrifuge plate holder in the centrifuge to avoid any black rubber flecks sticking to the ABI plate and do a quick spin down for about 10-15 seconds.

12 Load the ABI plate onto the 7500 machine. Orient your plate such that well A1 is at the top left corner of the loading tray, if not, your results will be flipped.

Set up 7500 Run

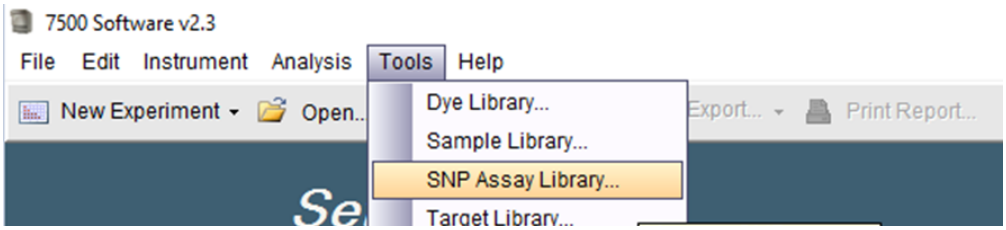
13 Turn on both 7500 machine and the computer. Plug in flash drive into computer after startup.

13.1 Launch 7500 software v2.3, and log into a profile.

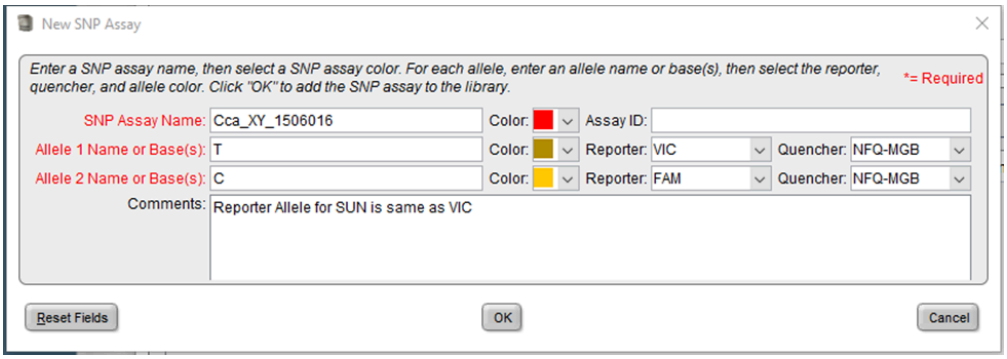
14 (Skip to step 15, if assay isn't new)

Add new SNP assay to library before proceeding with 7500 runs.

14.1 Go to Tools, and click on "SNP Assay Library..."



14.2 Select "New...", and enter the SNP assay name and details. Here's an example:

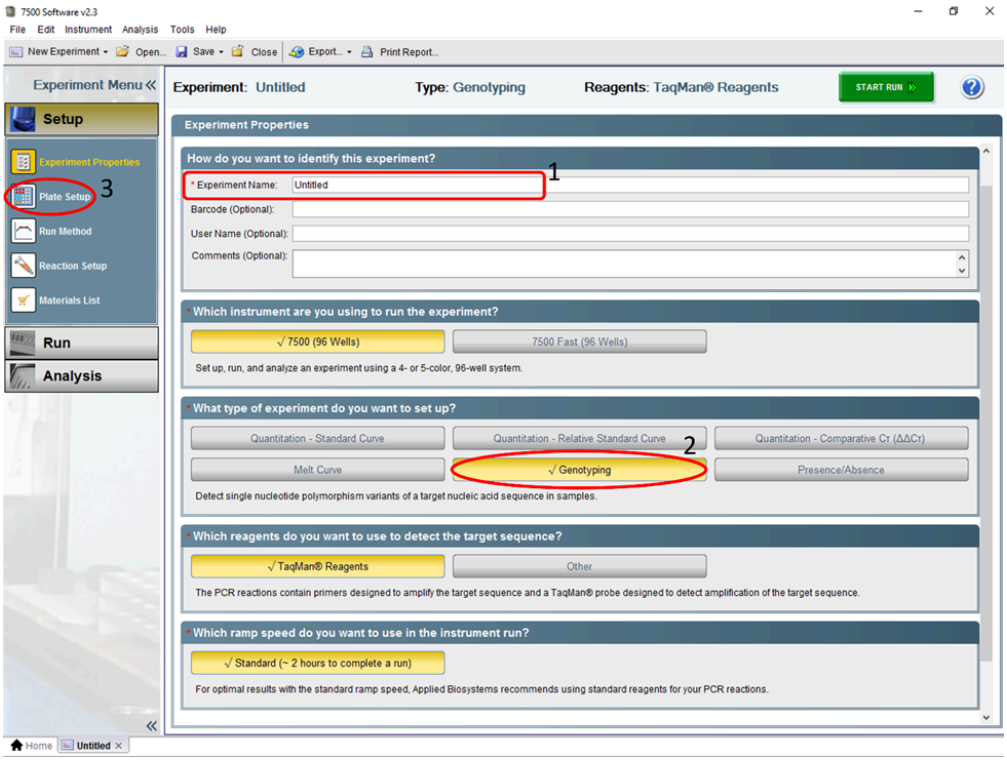


Click "OK" to save assay. Now this assay is available for future runs.

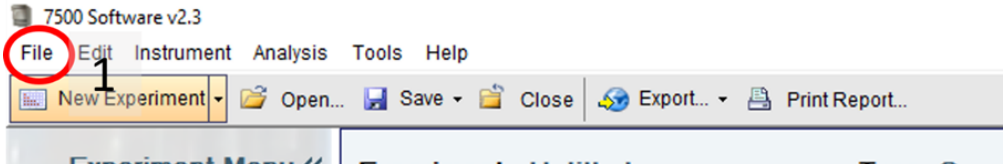
15 Click on Advance Setup in the Set Up column of the home page.

15.1 On the Experiment properties page, make the following edits:

1. Edit experiment name - following project name would be ideal
2. Select "Genotyping" for the type of experiment. Click "OK" to the pop-up. Leave everything as default. (Not shown in picture, at the very bottom of page, leave "Pre-PCR Read", "Amplification" and "Post-PCR Read" boxes checked)
3. Click on "Plate Setup" to import samples.



15.2 On the Plate Setup page, click on "File" on the top left corner, and select "Import.." from dropdown.

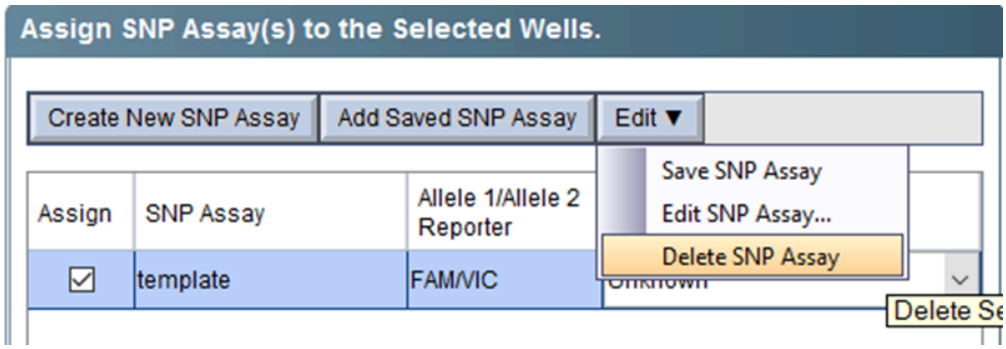


15.3 Import the saved .txt file from your flash drive. Your samples should auto-populate in the tray map on screen.

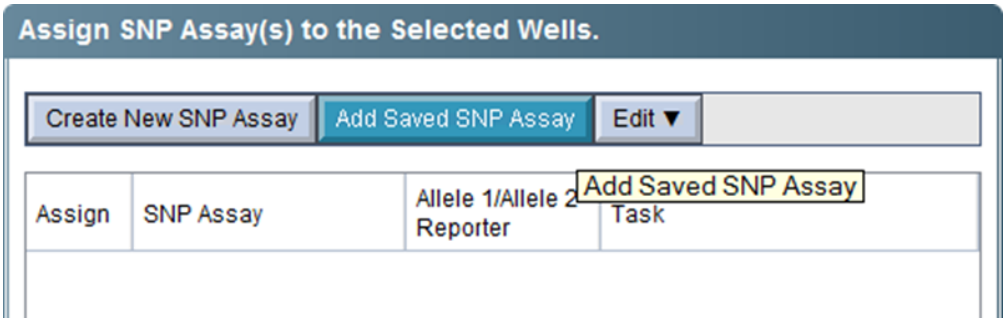
If there's an error on import, likely the .txt file was not saved correctly. Save/overwrite .txt file and try again.

15.4 In the "Assign SNP Assay(s) to the Selected Wells." box, select the assay that you'll be running with.

1. Click on the SNP assay name "template", it'll turn purple/blue upon clicking (EFGL's input file template has defaulted the SNP assay as "template", thus it needs to be switched to the correct assay)
2. Click on "Edit ", select "Delete SNP Assay"

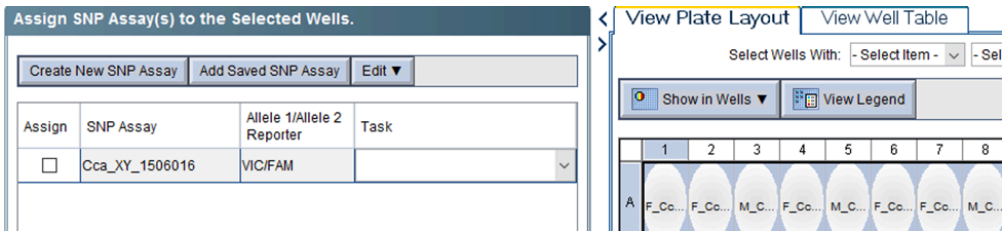


3. Once deleted, click on “Add Saved SNP Assay” and select the appropriate SNP assay(s) in pop up window. Then, click “Add selected SNP assay(s)”



15.5 Once you have the right SNP assay(s) show, select all samples to run on that assay.

- To select all samples, click on the small white square on top left corner of tray map, or select the samples individually for separate assays.
- Check the "Assign" box to assign that assay to the selected samples

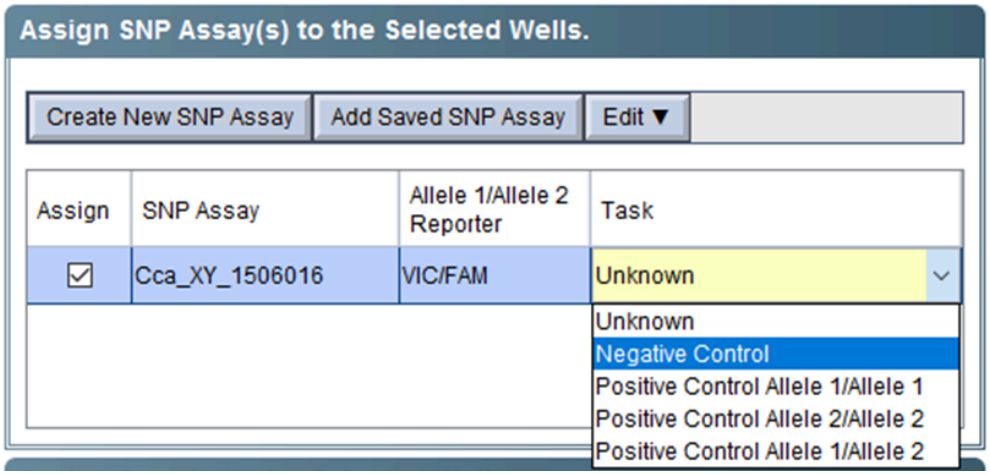


If you run a partial plate, clear all empty wells.

- Select all empty wells, right click on mouse, and select “Clear”.
- This prevents flags/errors on empty wells after the run has been completed.

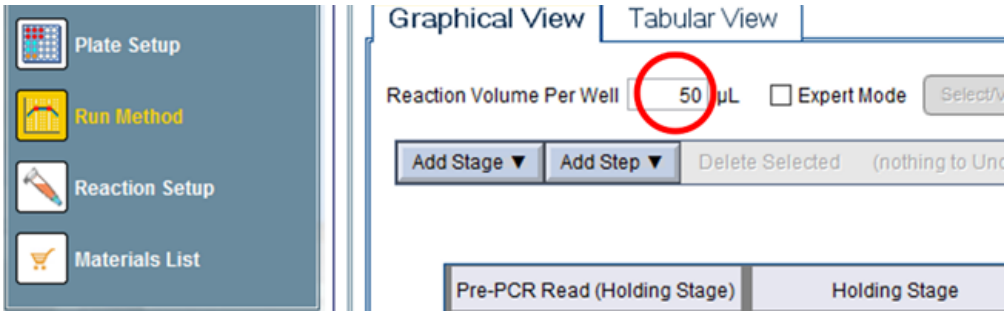
15.6 Select both NTC wells from plate layout, and click on dropdown box under “Task”

- Select "Negative Control"

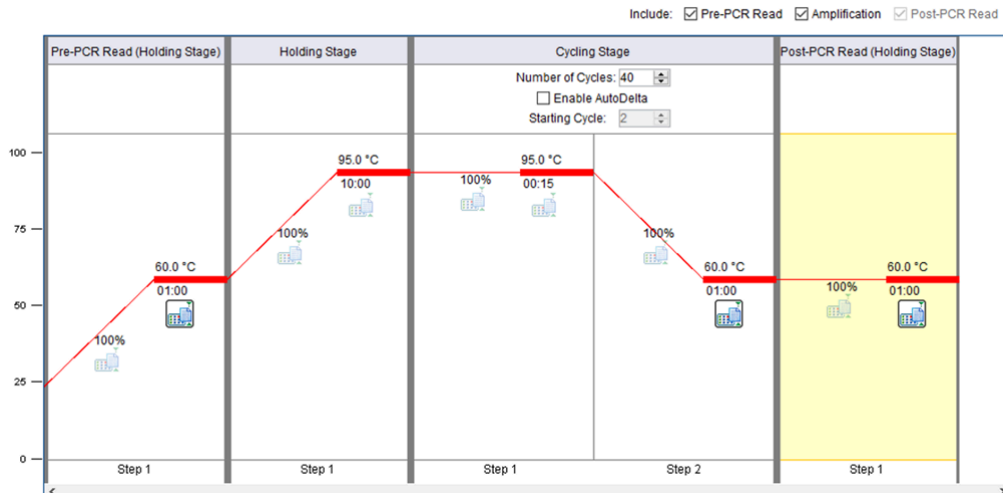


16 Next, on the Experiment Menu. Click on "Run Method".

16.1 Edit reaction volume from 50 µL to 10 µL



16.2 Make any changes to the cycle conditions, if necessary. EFGL have used the default cycling conditions on most of our assays.



16.3 Once all changes are made, click "Save".

16.4 Click on the green "START RUN" button at the top right corner to begin run. (there can be a bit of a lag, so don't click it more than once)

Default run time is approximately 1.5-2 hours.

Collect results

17 Once the run has completed, the screen will be on the Allelic Discrimination plot page.

A successful run would likely show distinct and tight clusters. In some instances, the plot will require manual calling of each sample to reflect the right calls.

17.1 Take a screenshot of plot, and export all data to flash drive. Upload plot image and raw data files to your project folder.