**Table of contents**

1. **DNA Plate Preparation**
2. **Making 72x Marker Mix**
3. **Making Master-mix**
4. **Plate preparation**
5. **Thermocycler and end reads**

**384 well plate quadrants**

|  |  |
| --- | --- |
| **Washington (WA)** | **New York (NY)** |
| **Texas (TX)** | **Florida (FL)** |

1. **DNA Plate Preparation**
2. Remove 96 well plate(s) from 4°C refrigerator, briefly vortex and centrifuge.
3. Dispense 3 µl of sterile deionized water into each well (important for consistency).
4. Briefly centrifuge plates to move liquid to bottom of well.
5. Dispense 2 µl of roughly 5-15 ng/µl – DNA does not have to be normalized. Usually dispense one 96 well plate at a time in consistent WA -> TX -> NY -> FL order.
6. Briefly centrifuge plates.
7. Incubate at 65°C for at least 1:30 hours to overnight to dry overnight (plates can be saved for at least a few months at room temperature).
8. **Making 72x Marker Mix:**
9. Thaw, then vortex and centrifuge 10 ng/µl primers.
10. Combine and dilute primers:

A1 Primer 12 µl

A2 Primer 12 µl

C Primer 30 µl

H2O 46 µl

Total 100 µl

1. **Making Master-mix:**

Required materials:

* Deionized water
* PACE 2x mix
* KASP 72x marker mix.
* Centrifuge tubes (1.7mL, 2mL, 5mL, 10mL, refer to total volumes in Table 1)

**Less than 384-well plate ratios include additional padding for Meridian – can use less if pipetting manually.**

Master-mix for a full 384-well plate,

2X reaction mix 740 µl

72X assay mix 20 µl

H2O 720 µl

Total 1480 µl

Master-mix for 2/3 of a 384-well plate (288 well),

2X reaction mix 583 µl

72X assay mix 16 µl

H2O 567 µl

Total 1166 µl

Master-mix for 1/2 of a 384-well plate (192 well),

2X reaction mix 426 µl

72X assay mix 12 µl

H2O 414 µl

Total 852 µl

Master-mix for 1/4 of a full 384-well plate (96 well),

2X reaction mix 300 µl

72X assay mix 8 µl

H2O 292 µl

Total 600 µl

**Table 1**

**\*For volumes greater than one plate add the amount of plates you will be running (EX. If you want to run one and a half plates then add the 384well volumes to the 192well volumes)**

1. Remove the 72x master mix from the tube rack and one 1mL aliquot of 2x mix for every plate to be run.
2. Get out micro-centrifuge tubes for every marker that will be run and choose the appropriate volume according to the amount used (refer to **Table 1** total volumes).
3. Fill each of the tubes with sterile deionized water.
4. Fill each of the tubes with 2x mix.
5. Add the 72x mix, close the top of the tube(s).
6. Briefly vortex and centrifuge the tube(s).
7. **Plate Preparation**

Required Materials

* KASP master-mix
* 384 well plate with dried DNA
* BIO-RAD Microseal ‘B’ seal
* Roller
* 384 well steel plate anchor

1. Pipette 3 µl of KASP master mix into each well and briefly centrifuge. If running more than one marker, pipette in quadrant order WA -> TX -> NY -> FL.
2. Remove the plate(s) from the centrifuge and place one on the 384 well steel plate anchor, ensure it fits snugly and is on properly.
3. Cover each plate in microseal and press down with roller, putting pressure on first the top then each edge individually.
4. Briefly place plate(s) on vortexer/shaker and centrifuge.
5. **Thermocycler and End Reads**
6. Place plate(s) into thermocycler and run appropriate KASP protocol (see below). With new markers, start off with KASP\_31 (thirty cycles). With previously tested markers, select KASP\_34 or KASP\_37 as appropriate.
7. After program finishes, briefly centrifuge.
8. Take plate read (see section PHERAstar PLATE READER) with format PLATENAME+XX, where XX is the number of cycles above 31(so at the end of KASP\_31 will be PLATENAME+00, after another 3 cycles or after running KASP\_34 +03).
9. Re-seal edge of plate with roller.
10. Place plate(s) back into thermocycler and select program KASP\_+03. Repeat steps 2-4 until taking plate read PLATENAME+12.

**PACE PCR Programs**

**KASP\_31** (for KASP\_34 and higher, add 3 cycles to x21):

* 94°C -- 15:00

Repeat 10x

* 94°C -- 00:20
* 65°C -- 01:00 (decreasing by -.08°C per cycle)

Repeat 21x

* 94°C -- 00:20
* 57°C -- 01:00
* 4°C -- Hold

**KASP\_+03**

Repeat 3x

* 94°C -- 00:20
* 57°C -- 01:00
* 4°C -- Hold

**PHERAstar Plate Reader**

Open the PHERAstar program, select **Run**, **Plate out**. Put the plate, which has been spun down, into the Plate Reader, then click **Plate-In**, **KASP read button**, name the plate, **Start measurement**. **Do not include 1A, 1B, 2A, or 2B in the plate name because it will interfere with the measurement.**

After the first measurement, put the plate back on the thermocycler and select KASP+ 3 Cycles. Continue taking readings and adding cycles until the clusters are well-separated.

**KlusterCaller**

In KlusterCaller, click either **Open existing project** to select the project or **Create new project**. **Click Import plate reader files** and select the plate from the list. Import the plate reader files and examine the results in KlusterCaller.

**Create and apply master plate:**

The master plate is used to indicate the location of samples and the associated marker on the plate. First, create a template of the KASP plate by concatenating the number, sample names, and marker name, if necessary, using the numbering template, then copy the information to the 3730\_384 macro file.

In KlusterCaller, right click on master plate in the project folder, select **New master plate**, name the file, click on the name of the master plate. Under **Well density**, select **384 wells**. Click **Subject IDs**, copy the cells in columns AB to AY from the 3730\_384 macro file, click cell A1, click **Paste**, **OK**. Select the location of the samples and the associated marker by holding down the control key and clicking individual columns or highlight wells by clicking and dragging. After the location of the samples has been selected, click the **DNA button.** The selected wells turn pink, click **Save**.

**Analysis:**

Click the plate reader file to be analyzed. Click the compensation F2 icon to analyze un-normalized and normalized data. The factor X and Y, and the X and Y background can be changed, after the data is normalized, to define the clusters. Make sure the data make sense whether it is normalized or un-normalized.

Left click and circle samples to change the call, if necessary. Negative samples are indicated in pink and show up as a question mark in the allele report. NTC, non-template control or blank, can also be used to identify blanks.

The value for HEX is much higher than FAM in the Y:Y cluster, HEX:FAM is about 3:1 in the X:Y cluster. In the X:X cluster, the value for FAM may be much higher than HEX, HEX may be higher or the values may be similar.

Controls can be placed in one quadrant. When viewing the data in different quadrants, mouse over the well with the control and the position of the control will appear.

Wells that contain master mix only cluster near the origin. Empty wells cluster near the origin when the data is un-normalized, but appear in a separate cluster near the allele Y cluster when normalized. Exclude empty wells when making the master plate.

For questionable samples, check the values and ratios of HEX and FAM to determine the allele call. Samples that look far from a cluster may belong to the cluster due to the auto scale.

Save changes made to the project by choosing file, select save current window.

Delete plate reader files not used for the allele report.

If 1A, 1B, 2A, or 2B is in the plate name, the results will not be visible in Klustercaller because of interference with information in the plate reader file. For example, use 1RS instead of 1A or 1B. This can be corrected by going to the end point reads, open the file of interest, change the ID, and save the file with the new name. Import the new file into the project folder. Delete the old file.

**Export allele report:**

Select the plate reader file to be used for the allele report. Right click on **Linked-master**, select **Choose master plate** from list, click on the name of the master plate, click **OK**. If there is more than one marker on the plate, the plate reader file of interest must be imported again to apply a different master plate.

After the data has been analyzed for a plate reader file, click **Submit**, which creates an allele report. Data from all plate reader files that have been submitted in a project folder will be saved in one worksheet in an Excel file. Choose **File**, **Export results**, select **SNP viewer CSV file with subject IDs**, **OK**. Select **View file in Excel**, save the Excel file in the appropriate folder on the shared drive or on a flash drive. In the Excel file, sort the subject ID in ascending order, if necessary. Copy and paste the sample names, well position, and allele call into the DNA plate record to create the allele report.

Data can also be exported using the SNP x well table. This is useful for projects like nurseries or plates that are tested for many traits. An SNP and master plate need to be selected for each plate. Choose **File**, **Export results**, select **SNP x well table**. The file will be saved as a text tab delimited file. Open Excel, select the text file, select **Delimited**, select **Tab and comma for the delimiters**, **Text qualifier =none**, **Column data format=general**. Save as an Excel file. Insert a column on the far left of the file, copy the sample ID from the 384 template used to make the master plate, and paste into the file which will match the sample ID with the master well. Sort by the number concatenated to the sample name. Copy and paste the sample names, and allele call into the DNA plate record to create the allele report.