

Apr 12, 2023

Version 2

## PotatoMASH library construction V.2

 [G3: Genes](#) | [Genomes](#) | [Genetics](#)

DOI

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**Protocol status:** Working

**We use this protocol and it is working**

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## Abstract

We have developed PotatoMASH (Potato Multi-Allele Scanning Haplotags), a novel low cost, genome-scanning marker platform. We designed a panel of 339 multi-allelic regions placed at 1 Mb intervals throughout the euchromatic portion of the genome. These regions were assayed using a multiplex amplicon sequencing approach, which allows to genotype hundreds of plants at a cost of €5/sample.

This protocol describes the library construction part of the method. PotatoMASH libraries are made to be sequenced in Illumina sequencing platforms.

## Materials

| A   | B                           | C                 | D                 | E             | F                       |
|---|-----------------------------|-------------------|-------------------|---------------|-------------------------|
| <b>PotatoMASH step and supplies</b>           | <b>Provider / code</b>      | <b>pack price</b> | <b>pack units</b> | <b>pre ps</b> | <b>sample costs (€)</b> |
| <b>Template DNA normalization:</b>            |                             |                   |                   |               |                         |
| <i>Quant-iT™ PicoGreen® dsDNA Assay Kit</i>   | <i>Thermofisher P7589</i>   | 510               | 2000              | 1916          | 0.266                   |
| <i>Nunc™ F96 MicroWell™ Black Plates</i>      | <i>Thermofisher 236105</i>  | 112               | 50                | 4400          | 0.025                   |
| <i>10µL tips for quantitation</i>             | <i>Greiner 771290</i>       | 9                 | 1000              | 1000          | 0.009                   |
| <i>200µL tips for quantitation</i>            | <i>Sarstedt 70.760.002</i>  | 40                | 10000             | 10000         | 0.004                   |
| <i>10µL filter tips for normalization</i>     | <i>Sarstedt 70.1130.210</i> | 70                | 1920              | 1920          | 0.036                   |
| <i>96 well PCR plate</i>                      | <i>Thermo Sci. 10425733</i> | 58.27             | 25                | 2400          | 0.024                   |
| <i>plate adhesive lid</i>                     | <i>Greiner 676001</i>       | 14.3              | 100               | 9600          | 0.001                   |
| <b>PotatoMASH PCR1:</b>                       |                             |                   |                   |               |                         |
| <i>QIAGEN Multiplex PCR Plus Kit (100)</i>    | <i>Qiagen 6152</i>          | 185               | 2.55mL            | 700           | 0.264                   |
| <i>PotatoMASH Primers (n=348, 750uM each)</i> | <i>IDT</i>                  | 5133              | 20µL              | 85714         | 0.06                    |
| <i>96 well PCR plate</i>                      | <i>Sarstedt 72.1978.202</i> | 69.75             | 25                | 2400          | 0.029                   |
| <i>10µL filter tips</i>                       | <i>Sarstedt 70.1130.210</i> | 70                | 1920              | 1920          | 0.036                   |
| <i>Adhesive aluminium foil plate lid</i>      | <i>Sarstedt 95.1995</i>     | 50.5              | 100               | 9600          | 0.005                   |
| <b>PotatoMASH PCR2:</b>                       |                             |                   |                   |               |                         |
| <i>100µL filter tips to dilute PCR1</i>       | <i>Sarstedt 70.760.212</i>  | 70.8              | 1920              | 1920          | 0.037                   |
| <i>QIAGEN Multiplex PCR Plus Kit (100)</i>    | <i>Qiagen 6152</i>          | 185               | 2.55mL            | 490           | 0.378                   |



|  | A   | B                           | C      | D     | E         | F     |
|--|---|-----------------------------|--------|-------|-----------|-------|
|  | <i>i5 and i7 Primers (n=96+8) at 100μM</i>                                  | <i>IDT</i>                  | 667    | 300μL | 287<br>5  | 0.232 |
|  | <i>96 well PCR plate</i>  | <i>Sarstedt 72.1978.202</i> | 69.75  | 25    | 240<br>0  | 0.029 |
|  | <i>10μL filter tips</i>   | <i>Sarstedt 70.1130.210</i> | 70     | 1920  | 960       | 0.073 |
|  | <i>Adhesive aluminium foil plate lid</i>                                    | <i>Sarstedt 95.1995</i>     | 50.5   | 100   | 960<br>0  | 0.005 |
|  | <b>Library normalization, Pooling wells and Concentration-purification:</b> |                             |        |       |           |       |
|  | <i>SequalPrep™ Normalization Plate Kit</i>                                  | <i>Invitrogen A1051001</i>  | 1050   | 10    | 960       | 1.094 |
|  | <i>10μL tips for binding step</i>   | <i>Greiner 771290</i>       | 9      | 1000  | 500       | 0.018 |
|  | <i>200μL tips for washing step</i>  | <i>Sarstedt 70.760.002</i>  | 40     | 10000 | 100<br>00 | 0.004 |
|  | <i>200μL tips for elution step</i>  | <i>Sarstedt 70.760.002</i>  | 40     | 10000 | 100<br>00 | 0.004 |
|  | <i>plate adhesive lid</i>   | <i>Greiner 676001</i>       | 14.3   | 100   | 960<br>0  | 0.001 |
|  | <b>Size selection, Purification, Quantification and Pooling plates:</b>     |                             |        |       |           |       |
|  | <i>Buffer PB</i>  | <i>Qiagen 19066</i>         | 87     | 500mL | 640<br>0  | 0.014 |
|  | <i>15mL tubes</i>   | <i>Sarstedt 62.554.002</i>  | 55     | 500   | 480<br>00 | 0.001 |
|  | <i>QIAquick PCR purification Kit</i>  | <i>Qiagen 28704</i>         | 104.14 | 50    | 480<br>0  | 0.022 |
|  | <i>1mL tips</i>   | <i>Fisherbrand 11548442</i> | 8.92   | 1000  | 685<br>7  | 0.001 |
|  | <i>Wizard SV Gel and PCR Clean-Up System</i>                                | <i>Promega A9281</i>        | 94     | 50    | 480<br>0  | 0.02  |
|  | <i>AMPure XP magnetic beads</i>   | <i>Beckman C. A63881</i>    | 1326   | 60mL  | 576<br>00 | 0.023 |
|  | <i>100μL filter tips</i>  | <i>Sarstedt 70.760.212</i>  | 70.8   | 1920  | 307<br>20 | 0.002 |



|  | A                                | B                          | C   | D   | E         | F     |
|--|----------------------------------|----------------------------|-----|-----|-----------|-------|
|  | <i>Qubit™ dsDNA BR Assay Kit</i> | <i>Thermofisher Q32853</i> | 275 | 500 | 480<br>00 | 0.006 |
|  | <i>Qubit™ assay tubes</i>        | <i>Thermofisher Q32856</i> | 70  | 500 | 480<br>00 | 0.001 |

**Equipment :**

Manual pipettors, monochannel and multichannel

Electronic pipettors, monochannel and multichannel

Centrifuge for microcentrifuge tubes and plates

Vortex mixer

Plate sealer

Magnetic separation device

Gradient Thermocycler

Gel Electrophoresis Systems

Transilluminator UV

For quantification with Qubit: Qubit fluorimeter

For quantification with PicoGreen: BioTek plate reader

**Troubleshooting**

## Organize your samples in 96 well plates, quantitate template DNA, and normalize at 20ng/uL.


4h

1

2h

### Quantification with PicoGreen


In the preparation for the library construction, the first step is to quantify the template DNA. We used Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen, P7589) as detailed below:

The Quant-iT™ PicoGreen kit should be stored in  4 °C in the **dark**. An hour before take the PicoGreen kit out from the fridge and place in room temp, protect the PicoGreen reagent from light by covering the tube with aluminium foil. The Lambda DNA standard should be always kept in 4°C.

#### Notes:

*Step duration is estimated for one plate. However, the duration for each step and sub-steps of this protocol is not necessarily the result of multiplying the duration of all steps by the number of plates to process. For example, in this step 1 (Quantification with PicoGreen) the duration should be multiplied by the number of plates to process only in steps 1.3, 1.4, 1.6, 1.8 and 1.10.*

It is advised to read the manufacturer's protocol

 Quant-iT™ PicoGreen.pdf

### 1.1 Prepare the Lambda DNA standards in a serial dilution:

10m

*Note: Vortex and spin down between each dilution.*

|  | A                             | B               | C                                  | D                                       |
|--|-------------------------------|-----------------|------------------------------------|---|
|  | Concentration of standard (λ) | Volume of 1X TE | Volume and concentration of Lambda | Final DNA Concentration PicoGreen Assay |
|  | 2000 ng/ml                    | 1470 µL         | 30 µL of 100 µg/mL λ               | 1000 ng/ml                              |
|  | 200 ng/ml                     | 1350 µL         | 150 µL of 2000 ng/mL λ             | 100 ng/ml                               |
|  | 20 ng/ml                      | 1350 µL         | 150 µL of 200 ng/mL λ              | 10 ng/ml                                |



| A       | B            | C                                 | D       |
|---------|--------------|-----------------------------------|---------|
| 2 ng/ml | 1350 $\mu$ L | 150 $\mu$ L of 20 ng/mL $\lambda$ | 1 ng/ml |

- 1.2 In 50 ml tube Prepare 1X TE as following **TWICE** (the second load is for the PicoGreen working solution - in step 1.5 ):

5m

| A                   | B                        | C                         |
|---------------------|--------------------------|---------------------------|
| Reagent             | 1 $\times$ 96-well plate | 4 $\times$ 96-well plates |
| 20X TE              | 500 $\mu$ l              | 2 ml                      |
| Autoclaved dd-water | 9.5 ml                   | 38 ml                     |
| <b>Total</b>        | <b>10 ml</b>             | <b>40 ml</b>              |

Mix by vortex.

- 1.3 Dilute DNAs in black 96-well plate for quantification:

5m

**In columns 1-11:**

Pipette 98  $\mu$ l of 1X TE in each well

Add 2  $\mu$ l of each DNA sample (multichannel pipette recommended).

Mix by pipetting.

- 1.4 **In column 12 :** Fill in with 100  $\mu$ l of the standards as follows:

1m

| A         | B                         |
|-----------|---------------------------|
| Column 12 | Content                   |
| 12A       | 2000 $\mu$ g/ml $\lambda$ |
| 12B       | 200 $\mu$ g/ml $\lambda$  |
| 12C       | 20 $\mu$ g/ml $\lambda$   |
| 12D       | 2 $\mu$ g/ml $\lambda$    |
| 12E       | 1X TE                     |
| 12F       | Empty                     |
| 12G       | Empty                     |



|  |     |       |
|--|-----|-------|
|  | A   | B     |
|  | 12H | Empty |

1.5 Prepare the working solution:

5m

|  |                       |                      |                           |
|--|-----------------------|----------------------|---------------------------|
|  | A                     | B                    | C                         |
|  | <b>Reagent</b>        | <b>96-well plate</b> | <b>4 × 96-well plates</b> |
|  | 1X TE (from step 1.2) | 9.95 ml              | 39.8 ml                   |
|  | PicoGreen reagent     | 50 µl                | 200 µl                    |

Protect the working solution from light by covering the tube with aluminium foil.

1.6 Add **100 µl** of working solution in each well.  
Use the same tips to mix by pipetting.

3m

1.7 Incubate for 2 to 5 minutes at room temperature, protected from light.

5m

1.8 Measure by using a fluorescence microplate reader and standard fluorescein wavelengths (excitation ~480 nm, emission ~520 nm). The instrument's gain should be set to the well containing the highest DNA concentration (12A)

2m

1.9 Calculate template DNA concentrations:  
Subtract the fluorescence value of the reagent blank (12E) from that of each of the samples. Use corrected data to generate a standard curve of fluorescence versus DNA concentration. Determine the DNA concentration of the sample from the standard curve and multiply by the dilution (x100)

5m

1.10 Template DNA Normalization:  
Make dilutions in a new plate or add nuclease free water according to each sample concentration to bring all samples to 20 ng/µl.

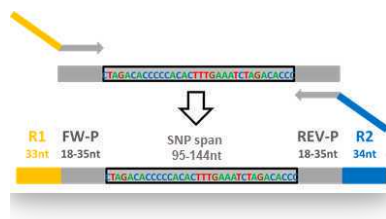
1h

## PotatoMASH PCR1 (multiplex PCR of 339 loci)

2h 15m

2 This step amplifies all PotatoMASH loci and adds Illumina sequencing primer tags R1 and R2.





**PotatoMASH primers** can be downloaded as supplemental data of the publication:  
*Leyva-Pérez, M.O.; Vexler, L.; Byrne, S.; Clot, C.; Meade, F.; Griffin, D.; Ruttink, T.; Kang, J. and Milbourne, D. PotatoMASH - a low cost, genome-scanning marker system for use in potato genomics and genetics applications.* Please cite this work if using this protocol.

If you decide to skip the library normalization (step 5) use the thermocycling conditions in **2.4 B**

*Prepare the PCR cocktail the same day for the number of plates to be processed.*

*Additional note: step duration is estimated for one plate. However, the duration for each step and sub-steps of this protocol is not necessarily the result of multiplying the duration of all steps by the number of plates to process.*


## 2.1 Prepare the **PCR1 Cocktail**: keep On ice

*Note: It is possible to prepare enough cocktail for all the plates planned to do the same day*

|  | A   | B                             | C                                 |
|--|---|-------------------------------|-----------------------------------|
|  | <b>Reagent</b>  | <b>Final content per prep</b> | <b>Cocktail for 96-well plate</b> |
|  | Nuclease free water   | 0.1 µl                        | 10 µl                             |
|  | PotatoMASH primers 125nM each primer (A pool of 678 primers=339 primer pairs) | 1.4 µl (25nM each primer)     | 140 µl                            |
|  | Qiagen Plus multiplex master mix (2x, ref:206152)                             | 3.5 µl (1x)                   | 350 µl                            |
|  | DNA (20ng/µl)   | 2 µl (40ng)                   | -                                 |
|  | <b>Total</b>  | <b>7 µl</b>                   | <b>500 µl (5 µl per sample)</b>   |

*Note: Advised order to prepare the cocktail: 1<sup>st</sup> pipette the water, 2<sup>nd</sup> add the primers, 3<sup>rd</sup> the Qiagen Master Mix.*

Gently mix by pipetting.

2.2 Dispense  5 µL of the **PCR1 Cocktail** into each well

*Note: it is advised to use 100 µl electronic pipette with dispenser function to apply the 5 µl of the PCR1 Cocktail to the plate with a 100 µl filter tip. A single tip can be used for all the plate.*

2.3 Apply  2 µL of the DNA (20ng/µl) with 10 µl filter tips,

Mix by pipetting.

Seal the plate.

Spin down in a mini plate spinner for 10 sec.

2.4 In a **gradient thermocycler** with heated lid, run PCR1 as follows:

1h 45m

**A** - Follow when performing library normalization (step 5)

|  | A                       | B             | C           | D           | E                           | F           | G                      | H           |
|--|-------------------------|---------------|-------------|-------------|-----------------------------|-------------|------------------------|-------------|
|  | <b>Step</b>             | <b>Cycles</b> | <b>Temp</b> | <b>Time</b> | <b>Annealing Temp</b>       | <b>Time</b> | <b>Elongation Temp</b> | <b>Time</b> |
|  | Initial Denaturation    | 1x            | 95 °C       | 15 min      |                             |             |                        |             |
|  | Initial amplification   | 8x            | 95 °C       | 30 sec      | 0.2°C/sec ramp down to 57°C | 30 sec      | 72 °C                  | 2 min       |
|  | Amplicon multiplication | 16x           | 95 °C       | 30 sec      | 65 °C                       | 30 sec      | 72 °C                  | 30 sec      |
|  | Hold                    | 1x            | 10 °C       | ∞           |                             |             |                        |             |

**B**- Follow when skipping library normalization (step 5):

|  | A                    | B             | C           | D           | E                     | F           | G                      | H           |
|--|----------------------|---------------|-------------|-------------|-----------------------|-------------|------------------------|-------------|
|  | <b>Step</b>          | <b>Cycles</b> | <b>Temp</b> | <b>Time</b> | <b>Annealing Temp</b> | <b>Time</b> | <b>Elongation Temp</b> | <b>Time</b> |
|  | Initial Denaturation | 1x            | 95 °C       | 15 min      |                       |             |                        |             |


|  | A                       | B   | C     | D      | E                           | F      | G     | H      |
|--|-------------------------|-----|-------|--------|-----------------------------|--------|-------|--------|
|  | Initial amplification   | 5x  | 95 °C | 30 sec | 0.2°C/sec ramp down to 57°C | 30 sec | 72 °C | 2 min  |
|  | Amplicon multiplication | 10x | 95 °C | 30 sec | 65 °C                       | 30 sec | 72 °C | 30 sec |
|  | Hold                    | 1x  | 10 °C | ∞      |                             |        |       |        |


*Note: PCR1 product can be stored at 4 °C 1-2 days or longer at -20 °C before dilution.*

## Dilute PCR1 product to be used as template for PCR2

10m

- 3 *Note that it is recommended to dilute PCR1 product the same day PCR2 will be performed.*

Centrifuge the PCR1 product plate to make sure all volume is down (e.g. for **3 min at 3000 RPM**  15 °C )

- 3.1 Add  100 µL of nuclease free water to each well to dilute 1:15.

Mix by pipetting up and down 30 times.

*Note: it is convenient to use a multichannel 100 µl electronic pipette with function 'pipette and mix' set up to dispense 100 µL and mix 40 µL volume 30x.*

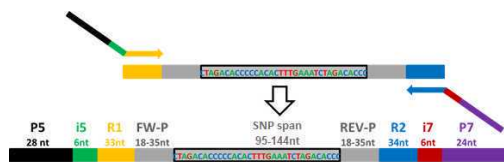
- 3.2 Spin down for 10 sec in a mini plate spinner.

*Diluted plates can be kept at 4 °C meanwhile preparing the cocktail for PCR2*

## PotatoMASH PCR2 (Adds dual barcodes)

1h

- 4 PCR2 adds Illumina capture adapter P5 and P7 and barcode indexes that identify each sample by well and by plate.




Primers i5 provide with well-specific barcodes. There is a panel of 96 primers. We use the same panel for each plate.

Primers i7 provide with plate-specific barcodes.

The 96 i5 primers and 10 i7 primers can be downloaded as supplemental data of the publication: *Leyva-Pérez, M.O.; Vexler, L.; Byrne, S.; Clot, C.; Meade, F.; Griffin, D.; Ruttink, T.; Kang, J. and Milbourne, D. PotatoMASH - a low cost, genome-scanning marker system for use in potato genomics and genetics applications.* Please cite this work if using this protocol.

Here we provide with additional i7 primers to multiplex more plates (plate 11 to plate 19)

 Additional\_i7primers\_plates11\_19.txt

By using 96 i5 barcodes in combination with 19 i7 barcodes, they can be indexed up to 1,824 samples to be joined in a single library.

#### 4.1 Prepare the **PCR2 Cocktail**: keep on ice. On ice

2m


| A                               | B                      | C                               |
|---------------------------------|------------------------|---------------------------------|
| Reagent                         | Final content per prep | Cocktail for 96-well plate      |
| Plate-specific i7 10 µM primers | 1 µl                   | 100 µl                          |
| 206152                          | 5 µl                   | 500 µl                          |
| Well-specific i5 10 µM primers  | 1 µl                   | -                               |
| PCR1 product diluted 1:15       | 3 µl                   | -                               |
| <b>Total</b>                    | <b>10 µl</b>           | <b>600 µl (6 µl per sample)</b> |

*Notes: Since i7 primers are plate-specific, you will need to prepare a different PCR cocktail for each plate. Advised order to add reagents: 1<sup>st</sup> apply the i7 primers, 2<sup>nd</sup> the Qiagen Master Mix. Mix by pipetting and spin down 10 sec.*

#### 4.2 Dispense 6 µL of the **PCR2 Cocktail** into each well

2m

*Note: it is advised to use 100 µl electronic pipette with the '(Disp.)' function to dispense 6 µl of the PCR2 Cocktail to each well in the plate with one 100 µl filter tip. A single tip can be used for all the plate.*

4.3 Add  1  $\mu\text{L}$  of the **well-specific i5 primers**.

5m

*Note: it is advised to use the manual (not electronic) 10  $\mu\text{L}$  multi-channel pipettor to add the 1  $\mu\text{L}$  of the well-specific i5 primers from the plate where the 96 primers are to your PCR2 plate. That way is easier to place each of the 96 i5 primers in each well position and to reduce the chance of mistakes. Make sure the 1  $\mu\text{L}$  is delivered by pipetting up-down a couple of times.*

4.4 Add  3  $\mu\text{L}$  of the **diluted PCR 1 product** and mix by pipetting.

5m

Seal the plate.

Spin down in a mini plate spinner for 10 sec.

4.5 Run PCR as follows:

45m

|  | A                    | B             | C           | D           | E                     | F           | G                      | H           |
|--|----------------------|---------------|-------------|-------------|-----------------------|-------------|------------------------|-------------|
|  | <b>Step</b>          | <b>Cycles</b> | <b>Temp</b> | <b>Time</b> | <b>Annealing Temp</b> | <b>Time</b> | <b>Elongation Temp</b> | <b>Time</b> |
|  | Initial Denaturation | 1x            | 95 °C       | 15 min      |                       |             |                        |             |
|  | Amplification cycles | 10x           | 98 °C       | 10 sec      | 65 °C                 | 30 sec      | 72 °C                  | 30 sec      |
|  | Final Extension      | 1x            | 72 °C       | 5 min       |                       |             |                        |             |
|  | Hold                 | 1x            | 10 °C       | $\infty$    |                       |             |                        |             |

*Note: The libraries (PCR2 product) can be stored at 4 °C 1-2 days or longer at -20 °C.*

## Library normalization (optional)

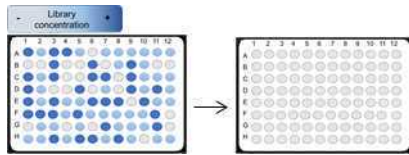
1h 30m

5 This step is optional. By having normalized the template genomic DNA prior to PCR, we minimized the differences of efficiency in each PCR. Homogeneous template DNA quality also minimizes differences in PCR yield in each well. However, if you are genotyping sets of DNA extracts within the same plate of very variable quality, we recommend to follow the complete protocol including library normalization step.

When performing this step be aware to have followed step **2.4A** and proceed at later stage with step **6.2A**.

When skipping this step be aware to have followed step **2.4B** and proceed at later stage with step **6.2B**.


In this step we normalize the PCR2 product in each well down to ~25 ng in 20  $\mu$ L (~1.25ng/ $\mu$ L).




As an additional benefit, it also provides with a first purification step: the binding buffer provides low pH condition causing the positive surface charge of coating of the library normalization plates to bind the negatively charged nucleic acid backbone. Proteins and other contaminants (such as short oligonucleotide primers) are not bound and are simply washed away.

*Tip: In this step, it is possible to use unfiltered tips, since the libraries are already prepared and barcoded, therefore, the risk of contamination is lower.*

*It is advised to read the manufacturer's protocol (Applied Biosystems, A105100)*

 [sequalprep\\_platekit\\_man.pdf](#)


## 5.1 Before starting:

- Centrifuge the PCR2 product plate to make sure all volume is down (e.g. **3 min at 3000 RPM**  15 °C )




## 5.2 Binding:

2m

- With a multichannel pipette, add  10  $\mu$ L **binding solution** (the same volume of the PCR product) into the PCR2 plate and mix completely by pipetting up and down. With the same tips, transfer 10  $\mu$ L, twice, to the normalization plate.


*Advice: Release on the upper part of the well, careful to not interrupt the coating by scratching the well sides.*

- Seal the plate and spin down in a mini plate spinner for 10 sec.
- Incubate in  Room temperature for **1 hour**
- Transfer back the amplicon/Binding Buffer mixture to the original PCR 2 plate.

*After binding is complete, the mixture can be stored at  $-20^{\circ}\text{C}$  for up to 30 days to recover more libraries later if needed. Again, be careful no to scrape the well sides with the tips during aspiration. To use 10  $\mu$ L (transfer 10  $\mu$ L twice), helps to avoid scrapings.*

## 5.3 Washing:

2m


- Add  50  $\mu$ L of **wash buffer**. Mix by pipetting up and down 35  $\mu$ L 3 times.

*Note: it is convenient to use a multichannel 100  $\mu$ L electronic pipette with function 'pipette and mix' set up to dispense 50  $\mu$ L and mix 35  $\mu$ L volume 3x. Then you can press the pipette button again to use the same tips to aspirate and discard the washing buffer.*

- Use the same tips to aspirate and discard the wash buffer.
- Invert and tap the plate on a paper towel to get rid of the remaining droplets of liquid.

## 5.4 Elution:

6m

- Elute the libraries with  20  $\mu$ L of **elution buffer** (pH 8.5–9.0). Mix by pipetting up and down 10  $\mu$ L 5 times.
- Incubate at room temperature 5 min.

*The plates with the eluted DNA can be stored at  $4^{\circ}\text{C}$  (short-term storage) or  $-20^{\circ}\text{C}$  (long-term storage).*

- 5.5 Store in   $4^{\circ}\text{C}$  until library pooling.

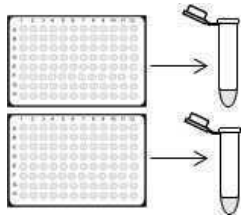
## Pooling libraries, Concentration - Purification

5h 10m

### 6 Pooling sub-libraries:

10m

At this stage, the libraries are barcoded and even normalized. We can pool a full plate (96 samples) into one 2mL tube.



We also refer to each of the pools as 'pooled sub-library'.

It is convenient to keep the plate on a cooling holder or On ice .

### 6.1 Before starting:

- Spin down the plates.
- Label one tube per plate On ice .

### 6.2 A - Follow if you have performed library normalization (step 5):

Transfer 15  $\mu$ L of each sample per 96-well plate into one 2mL tube.

**B-** Follow if you have skipped the library normalization (step 5):

Transfer 5  $\mu$ L of each sample per 96-well plate into one 1.5mL or 2mL tube.

*Note: we advise to use a 200uL electronic pipet with multi-aspirate function to take 15  $\mu$ L x 12 times (pool by rows) and deliver in the same tube. All can be done with 1 filter tip.*

*Tubes can be kept at 4 °C or On ice for short term prior to Purification steps the same day or at -20°C for longer term.*

## 7 Concentration - Purification:

5h

In this step we use the purification kit to purify, concentrate and reduce the volume of each pooled sub-library from 0.25 or 1.5 mL to 42  $\mu$ L by using the Qiaquick PCR Purification kit (Qiagen, 28104) and extra binding buffer (PB buffer, Qiagen, 19066), following manufacturer's guidelines with slight adjustments:

*Note: It is advised to read the manufacturer's protocol*

QQ\_PCR\_Gel\_Cleanup\_Kit\_0718\_W... or HB-1196-005\_HB\_QQ\_Spin\_0120\_W...

### 7.1 Before starting:

- Spin down the tubes with the pooled sub-libraries.
- Label **15 ml tube** (in case of normalized libraries, **protocol A**) or one **2mL tube** (in case of normalized libraries, **protocol B**) for each pooled sub-library for the binding




step (7.2)


- Prepare collection tubes with a QIAquick Spin column inserted for each sub-library for the binding and washing step (7.2-7.3)
- Prepare same number of 1.5 ml tubes for the elution step (7.4)

## 7.2 Binding:


**A-** Follow if you have performed library normalization (step 5):

- Prepare a **15 ml tube** for each pool with  7.5 mL of **PB**


*The ratio is 5 volumes of Buffer PB to 1 volume of the PCR product.*

- Add the  1440 µL of the **pooled sub-libraries**


**B-** Follow if you have skipped the library normalization (step 5):

- Prepare a **2 ml tube** for each pool with  1.2 mL of **PB**

**Important:** Make sure that the non-normalized pool is well mixed by vortexing a few seconds if you didn't so right after pooling the libraries. It is important because we will use only the half of the volume. The remaining volume can be stored at -20 °C in case is needed.

- Add the  240 µL of the **pooled sub-libraries**



**From now on, all steps are in common for protocols A and B:**

- **Vortex** for **5 sec** after mixing each pool with the PB and again after having processed all of the tubes.
- Pipette  600 µL of the PB-PCR product mix into the **QIAquick Spin column**.
- Centrifuge for 30 sec at 13,000 RPM and discard the flow-through.
- Repeat the last 2 steps until all the PB-PCR product mix in the tube is finished.

## 7.3 Washing:

- Add **750 µl Buffer PE** to the QIAquick Spin column,
- Centrifuge for 60 sec at 13,000 RPM and discard the flow-through.
- Centrifuge for 60 sec at 13,000 RPM to dry.

## 7.4 Elution:

- Transfer the QIAquick Spin column to a new 1.5 ml tube.
- Elute with  44 µL **Elution Buffer**
- Incubate in  Room temperature for **1-5 min**.
- Centrifuge for 1 min at 13,000 RPM.

## 7.5 Quality check (optional):

- Use  2 µL of the eluted pooled libraries to check quality with **nanodrop**.

Expected concentration values: 30 – 80 ng/µl.

Expected ratio  $A_{260nm}/A_{280nm}$ : 1.8 – 2.1



Expected ratio  $A_{260\text{nm}}/A_{230\text{nm}}$ : 2-2.5

## Size selection

3h

- 8 This step is meant to remove PCR1 primer-dimer amplified products in PCR2 and other unwanted secondary products from the PotatoMASH libraries. All PotatoMASH core primers are designed to produce libraries of around 300bp. Therefore, electrophoresis in agarose gel is a low-cost and easy way to separate them.


*Note that we usually perform this step the same day we perform step 7, but probably the concentrated libraries can be stored at 4°C for a day or at -20°C for longer term.*

- 8.1 Prepare **1.2 %** standard low melting point **agarose gel** in 0.5x TBE (TAE buffer can be also used).


For DNA visualization, we use GelRed dye applied to the agarose gel before polymerizing.

Make sure the wells of the gel are wide-deep enough to hold 48  $\mu\text{L}$  of sample. That can be achieved, for example, by covering three 7 mm. wide x 1mm thick comb teeth (3 wells) with scotch tape to make a wider well of 2.5 cm x 1mm thick. We recommend to leave one normal size well empty between different pools.

*Tip: Around 200mL of agarose are used for a gel 14cm long and 23 cm wide 7mm thick to get the wells deep enough to hold 48  $\mu\text{L}$ . Try to get the gel only thick enough to hold the sample but not more. The more thick is the gel, the more agarose you will have to clean up in the next step.*

- 8.2 Add  8  $\mu\text{L}$  of **loading buffer 6X** to the 40  $\mu\text{L}$  of each of the tubes with the concentrated libraries. Mix by pipetting.

- 8.3 Load the sub-libraries onto the big wells.

Load  6  $\mu\text{L}$  of p100 **DNA ladder** (e.g. NE Biolabs N3231) into at least one of middle wells in between. We do this to confirm that our main product is of the expected size and help us to spot it.

- 8.4 Run in **50 V** for **1 hour** and then run for **90 min** with **100 V**.

2h 30m

*Note: Settings used for a gel 14 cm long and 23 cm wide 0.7 cm thick, 200-230mL of agarose.*

- 8.5 Use a transilluminator UV (recommended to use low intensity like 70%) to visualise and excise the band around **300bp**. Use a clean scalpel and place it in a 2 mL tube.

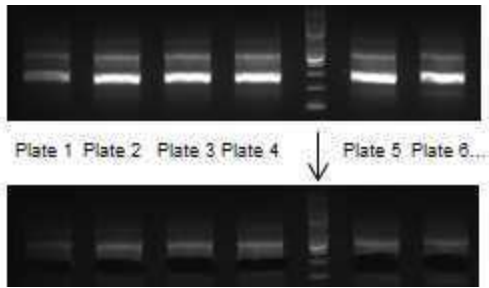


Image of six pooled sub-libraries before and after excising the band at 300bp

#### Safety information

Protect yourself from UV light by covering all exposed skin, wearing appropriate gloves and UV absorbing full face shield.

8.6 Weight the gel slices and record the weights (the weight will be important for next step, gel purification)

At that point, the gel slice can be stored in a closed tube at 4 °C for a couple of days if necessary.

## Gel purification

3h 30m

9 Purify the slice using the Wizard® SV Gel and PCR Clean-Up System Kit (Promega A9281) following manufacturer's guidelines:

*Note: It is advised to read the manufacturer's protocol*



Wizard SV Gel and PCR Clean-Up ...

### 9.1 Before starting:

- Heat a thermoblock to 65 °C
- Prepare collection tubes with SV mini-column for each sub-library for the extraction step (9.3)
- Prepare same amount of 1.5 mL tubes for the elution step (9.5)





6m


## 9.2 Dissolving the gel

- Add 10  $\mu\text{L}$  of membrane binding solution per 10mg of gel slice.
- Warm up to **50-65 °C** vortexing from time to time until the agarose completely dissolves.
- Vortex or mix by inversion.




## 9.3 Extraction of PCR product (libraries) from the dissolved gel:

- Transfer  650  $\mu\text{L}$  of the dissolved gel mixture to the SV mini-column and a collection tube
- Incubate for **1 min** in  Room temperature .
- Centrifuge for **1 min at 14,000 RPM** (16,000 x g) and discard flow-through.
- Repeat until all the dissolved gel mixture have passed through the SV mini-column.

## 9.4 Washing:

- Wash with  700  $\mu\text{L}$  of **washing solution**
- Centrifuge **1 min at 14,000 RPM** (16,000 x g). Discard flow-through
- repeat with **500  $\mu\text{L}$  of washing solution**. Discard flow-through
- Centrifuge **5 min at 14,000 RPM** (16,000 x g) to dry the column.

## 9.5 Elution:

- Transfer the mini-column to a 1.5 ml tube.
- Elute with  50  $\mu\text{L}$  **Nuclease-free water** (pre warm  65 °C )
- Incubate for 1-5 min in  Room temperature .
- Centrifuge **1 min at 14,000 RPM**

## 9.6 Quality check:

- Use  2  $\mu\text{L}$  of the eluted pooled libraries to check quality with **nanodrop**.

Expected concentration values: 5 – 25 ng/ $\mu\text{L}$ . (*The higher yields are expected for non-normalized libraries. Note that nanodrop measurements are not accurate for very small concentrations*)

Expected ratio  $A_{260\text{nm}}/A_{280\text{nm}}$ : 1.8 – 2,5

Expected ratio  $A_{260\text{nm}}/A_{230\text{nm}}$ : 0 – 1

Store in  4 °C until next step.

## Purification with Magnetic AMPure XP beads

2h 30m

- 10 The last purification step is performed with Magnetic AMPure XP beads (Beckman C. A63881) on each of the pooled sub-libraries and by using a magnetic stand for 1.5mL microcentrifuge tubes.

This step is necessary to achieve the level of library purity needed for posterior Illumina sequencing. Note that the Expected ratio  $A_{260\text{nm}}/A_{230\text{nm}}$  of the pooled sub-libraries after gel purification are not good.

As an additional benefit, the method utilizes a double-size selection steps that will further purify remaining traces of PCR products larger or smaller than 300bp that weren't properly separated during the size selection by agarose gel.

In the first selection, beads will bind in higher proportion to fragments larger than 400 bp. Then we recover the clear supernatant with fragments smaller than 400 bp. In the second selection, beads will bind in higher proportion to fragments larger than 200 bp and by discarding the clear supernatant we will discard fragments smaller than 200 bp.



*Note: It is advised to read the manufacturer's protocol*



SPRIselect User Guide.pdf

## 10.1 Before starting:

1m

- Prepare  5 mL **85 % Ethanol**.
- Magnetic Ampure XP beads stock to be stored  4 °C
- Resuspend the Ampure XP beads stock Suspension (usually in a bottle) carefully until complete homogenization.

*Tip: It is convenient to separate from the bottle the volume of ampure beads you'd need for all tubes into a small tube, which is easier to vortex and get homogeneous every time is needed.*

- Spin down the sub-libraries (if they were in the fridge or freezer) and measure the volume of library sample (~ 46 µl).

## 10.2 First purification:

- Vortex again the AMPure beads.
- Add 0.5 x volumes of AMPure beads (~ 23 µl) of the sample volume (~ 46 µL) and mix by pipetting 10 times.
- Vortex for **30 sec**.
- Incubate in room temperature for **1 min**.
- Place the reaction tube on a magnetic stand for 1-5 min to allow the AMPure beads to settle to the magnet until the supernatant is clear.
- Recover the clear supernatant, measure and record the volume. Place in a new tube.



*Note: it is advised to use an electronic pipet with function 'manual (man.)' to pipette very slowly and measure the recovered volume.*

## 10.3 Second selection:



- Vortex the AMPure beads again.
- Add 0.6x volumes of AMPure beads (~ 42 µL) to the recovered supernatant (~ 70 µL). Mix by pipetting 30 times.
- Vortex again for 1 min. incubate at room temperature for 1 min.
- Place the reaction tube on the magnetic stand for and allow the AMPure beads to settle to the magnet.
- Remove and discard the clear supernatant

*Notes: It is ok to leave a droplet at the bottom, it will wash in the next step with the ethanol. Careful not to aspirate any AMPure beads.*


#### 10.4 Wash with 85 % ethanol

- While the reaction tube still on the magnet, add  180 µL of **85% ethanol**
- incubate at  Room temperature for **30-60 seconds**.
- Carefully Remove and discard the ethanol supernatant by pipetting.
- Leave the tube **open** for 30 sec to dry.

#### 10.5 Elution:

- Remove the reaction tube from the magnet
- Add  30 µL of **Nuclease-free water**.
- Mix the total elution volume by pipetting 30 times to resuspend the beads
- Incubate at  Room temperature for **1 minute**.
- Place the reaction tube on the magnetic stand for 1 min or more and allow the Ampure beads to settle to the magnet.
- Carefully transfer by pipetting the eluted pooled sub-library (~ 27 µl) into new tubes.

#### 10.6 Quality check:

- Use  2 µL of the eluted pooled sub-libraries to check quality with **nanodrop**.

Expected concentration values: 7 – 35 ng/µl. (*The higher yields are expected for non-normalized libraries. Note that nanodrop measurements are not accurate for very small concentrations*)

Expected  $A_{260\text{nm}}/A_{280\text{nm}}$  ratio: 1.8 – 2.2

Expected  $A_{260\text{nm}}/A_{230\text{nm}}$  ratio: 1.5 – 2

### Sub-libraries quantitation and final pooling into a single library

**30m**

#### 11 Quantitation:

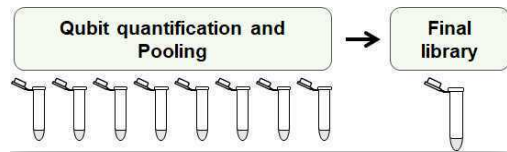
Sub-libraries quantitation using Qubit™ dsDNA BR Assay Kit (Thermofisher Q32853)

following manufacturer's guidelines  Qubit\_dsDNA\_BR\_Assay\_UG.pdf

Given the expected yield, we recommend to use 3-5 µL of each sub-library to quantify them with the Broad spectrum assay kit. Alternatively, The HS (high sensitivity) assay kit can be used.


### Pooling sub-libraries into a single library:

Calculate the volume from each sub-library to be pooled so there is the same amount in ng of each sub-library in the volume of the final library. Pool the sub-libraries and mix by pipetting.




Make sure that your final PotatoMASH library meets the sample requirements for Illumina sequencing of pre-made libraries (PCR free library), which usually are >0.5-1ng/  $\mu$ L (quantified by Qubit), >20  $\mu$ L or more volume depending of the amount of sequencing data required.

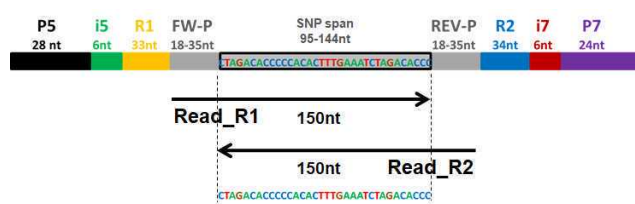
Sequencing service companies usually offer demultiplexing of libraries and for that the ask the barcode of each sublibrary. Here is an example of how to code the sublibraries:

 Example of barcoding.xlsx

You can also demultiplex the data yourself and you will need the i5 and i7 barcodes of each sample. The tags are written in the name of each primer, but here you have them in a table:

 i5\_i7\_barcodes.xlsx

This method has successfully genotyped 765 samples in a whole lane of Illumina HiseqX platform using 50% PhiX, obtaining paired-end 150nt reads (56Gb of data). The libraries have been also proven to be successful for sequencing in NovaSeq 6000 platform.



PotatoMASH library structure in relation to paired-end 150nt Illumina reads forward Read\_R1 and reverse Read\_R2.



## Library quality check (optional)

1h 30m

- 12 Library quality check (optional) by 'Illumina PCR' (PCR with known primers which anneal to Illumina capture adapters P5 and P7).

This is an alternative method to check the quality of your libraries without the need to use bioanalyzer or fragment analyzer.

It can be performed only on the Final library or to the sub-libraries before pooling them, in case you would prefer to further purify one of them before pooling.

The primers will anneal to any PCR product containing P5 and P7, so you can detect if you still have too many small fragments in your sub-libraries or final library.

### Before starting:

Order the primers:

P5 primer: 5'-AAT GAT ACG GCG ACC ACC GA-3'

P7 primer: 5'-CAA GCA GAA GAC GGC ATA CGA-3'

- 12.1 Dilute the library/libraries to test to 0.1 ng/μL. You will only need 1μL of this dilution.

- 12.2 Prepare the Illumina PCR cocktail:

|  | A  | B        | C                       |
|--|--|----------|-------------------------|
|  | Reagent  | 1 sample | 7 samples               |
|  | Nuclease-free water                            | 3 μl     | 21 μl                   |
|  | Qiagen Master Mix (2x)                         | 5 μl     | 35 μl                   |
|  | Illumina primers 10 μM (mix of both, 5μM each) | 1 μl     | 7 μl                    |
|  | library (0.1 ng/ul)                            | 1 μl     | -                       |
|  | Total  | 10 μl    | 63 μl (9 μl per sample) |

Distribute 9 μL of the cocktail per 0.2mL PCR tube and add 1μL of your library at 0.1 ng/μL. Mix by pipetting and /or spin down the tubes.

- 12.3 Run PCR as follows:

45m

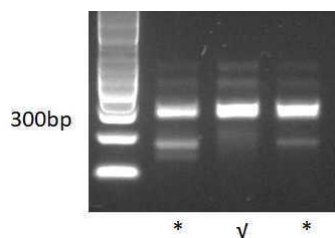
|  | A    | B      | C                  | D    | E             | F    |
|--|------|--------|--------------------|------|---------------|------|
|  | Step | Cycles | Denaturati on Temp | Time | Annealing and | Time |



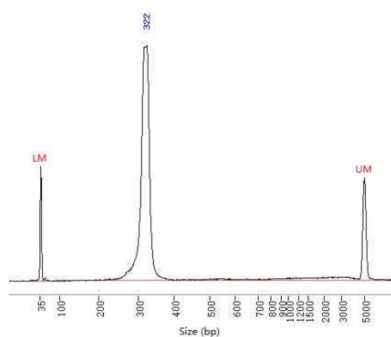
|  | A                      | B   | C     | D      | E                      | F      |
|--|------------------------|-----|-------|--------|------------------------|--------|
|  |                        |     |       |        | <b>Elongation Temp</b> |        |
|  | Initial Denaturation   | 1x  | 90 °C | 1 min  |                        |        |
|  | Annealing & Elongation | 16x | 95 °C | 15 sec | 63 °C                  | 45 sec |
|  | Hold                   | 1x  | 10 °C | ∞      |                        |        |

*Be aware that the optimal elongation temperature for the polymerase in the Qiagen multiplex master mix is 72°C and not 63°C. However, it works nicely anyway, and the annealing and elongation are done at the same temperature making the program shorter. Proper optimization is always welcome though.*

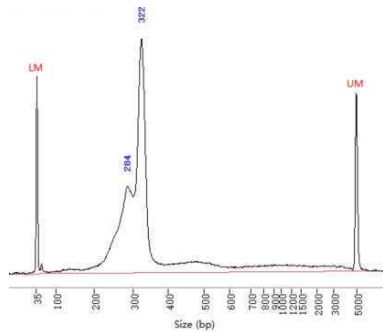
- 12.4 Run the PCR product (10 µL) mixed with 2 µL of loading buffer (6x) on 1.2 % agarose gel. Use ladder p100.



Illumina PCR product on agarose gel



✓ (sample 2)  
Fragment analyzer electropherogram



\*(sample 3)

### Fragment analyzer electropherogram

3 examples of libraries tested. Notice that brightness/contrast have been increased to highlight the unspecific bands

✓: the library passed quality test made in fragment analyzer by the sequencing service. This library was made skipping the library normalization (protocol B).

\*The libraries were set *on hold* by the service because they contained small fragments. However, the customer can ask these *on hold* libraries to be sequenced anyway and they were successful.

We don't have examples of libraries failing quality control to be rejected for sequencing. However, we advise to keep in mind that the risk of having too much unspecific libraries is to lose part of the sequencing data in non-informative data.