# Protocol 1

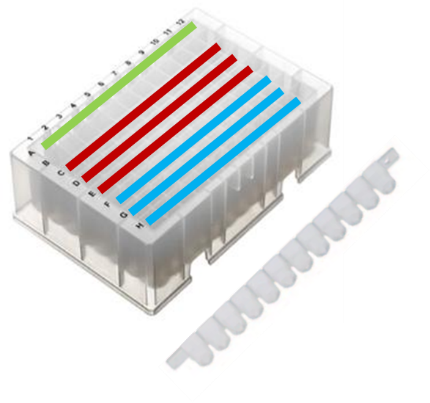
## Kingfisher plates and elution strips:

**Incubation 1:**

Row A: sample type 1 (green \* tips touching these cannot be reused\*)

C,D,E: negative beads in buffer 2 (red)

F,G,H: buffer 1 (blue)

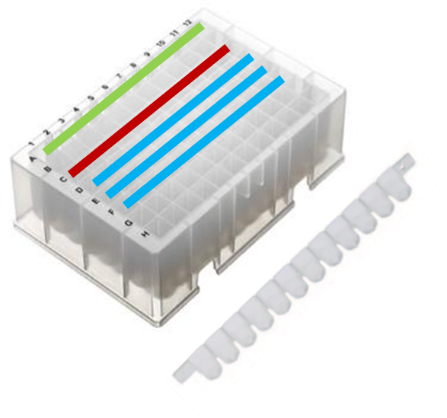


**Incubation 2:**

Row A: sample type 1 (green)

C: beads with sample type 2 in buffer 2 (red)

D,E,F: buffer 1 (blue)



## Bead preparation:

* 25 uL beads per positive sample type 1 (can be store in one tube/well per sample type 2). In the following, 3 sample type 1 and 2 sample samples type 2 is used as an example.
* 3 negative beads of 20uL per N=sample type 1 \* sample type 2, so for N=6, 18 negative beads are needed: 18 x 20uL = 360 uL

Prep of beads for incubation 1:

* Take beads, here 360 uL of homogenized bead solution
* Wash the beads 2x with 500 uL of buffer 1 & resuspend the beads in the initial volume = 360 uL of buffer 1
* Add buffer 3: 18 x 5 = 90 uL
* Incubate at RT for 30 min on shaking device (mix by pipetting?)
* Wash the beads 2x with 500 uL of buffer 1
* Finally resuspend the beads with 910uL buffer 2 and distribute 50uL to wells in row C,D,E
* Top up beads with 50uL buffer 2 (only due to robot requirements, perhaps not necessary in OT2?)

Prep of beads for incubation 2:

* Take beads, here 150 uL of homogenized bead solution
* Wash the beads 2x with 500 uL of buffer 1
* Resuspend the beads in the initial volume = 150 uL of buffer 1
* Split into n\* 50 uL (n=number of sample type 2=here 2), so 2\*50uL=100uL
* Add user specified volume of sample type 2 (should be kept cooled - need for pause and user added?)
* Incubate 30 min at 4C 500 rpm (corresponding mix by pipetting?)
* Wash the beads 1x with 500 uL of buffer 1
* Resuspend in 100 uL of buffer 1
* Add 10 uL of buffer 3
* Incubate 10 more minutes
* wash the beads 2x with 500 uL of buffer 1
* Finally resuspend the beads with 610 uL of buffer 2
* Distribute 100 uL of beads to the wells of the kingfisher plate for incubation 2 in row C.

## Preparation of plates for incubation 1 and 2:

* Prepare the mastermix (**MM**). N = N(samples) \* 3 + 3 negative controls + 1 excess. Here N=22:

|  |  |  |
| --- | --- | --- |
| Reagents | Per Reaction | For 22 reactions |
| water | 16.7 | 367,4 |
| Buffer 3 | 5 | 110 |
| Buffer 4 | 2.5 | 55 |
| Primer 1 | 0.4 | 8,8 |
| Primer 4 | 0.4 | 8,8 |

* Add 25 uL of **MM** in the wells of the two elution strips (in as many wells as needed) and place them in the king fisher
* Add 200uL buffer 1 wells in row F,G,H in plate for incubation 1 and D,E,F in plate for incubation 2 as shown below:

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Neg | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A |  |  |  |  |  |  |  |  |  |  |  |  |
| B |  |  |  |  |  |  |  |  |  |  |  |  |
| C |  |  |  |  |  |  |  |  |  |  |  |  |
| D |  |  |  |  |  |  |  |  |  |  |  |  |
| E |  |  |  |  |  |  |  |  |  |  |  |  |
| F |  |  |  |  |  |  |  |  |  |  |  |  |
| G |  |  |  |  |  |  |  |  |  |  |  |  |
| H |  |  |  |  |  |  |  |  |  |  |  |  |

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| pos | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A |  |  |  |  |  |  |  |  |  |  |  |  |
| B |  |  |  |  |  |  |  |  |  |  |  |  |
| C |  |  |  |  |  |  |  |  |  |  |  |  |
| D |  |  |  |  |  |  |  |  |  |  |  |  |
| E |  |  |  |  |  |  |  |  |  |  |  |  |
| F |  |  |  |  |  |  |  |  |  |  |  |  |
| G |  |  |  |  |  |  |  |  |  |  |  |  |
| H |  |  |  |  |  |  |  |  |  |  |  |  |

* Store positive plate back in the fridge at 4 degrees Celsius
* Add the sample type 1 to row A of the negative plate and top up to 150uL with buffer 2 (due to instrument requirement)
* The incubation 1 plate should now look like this:

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | Sample type 1 + buffer 2 | | | | |  |  |  |  |  |  |  |
| B |  |  |  |  |  |  |  |  |  |  |  |  |
| C | beads | | | | |  |  |  |  |  |  |  |
| D |  |  |  |  |  |  |  |
| E |  |  |  |  |  |  |  |
| F | Buffer 1 | | | | |  |  |  |  |  |  |  |
| G |  |  |  |  |  |  |  |
| H |  |  |  |  |  |  |  |

And plate 2:

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | Free (for sample type 1 after incubation 1) | | | | |  |  |  |  |  |  |  |
| B |  |  |  |  |  |  |  |  |  |  |  |  |
| C | Beads (w/ sample type 2) | | | | |  |  |  |  |  |  |  |
| D | Washing steps | | | | |  |  |  |  |  |  |  |
| E |  |  |  |  |  |  |  |
| F |  |  |  |  |  |  |  |
| G |  |  |  |  |  |  |  |  |  |  |  |  |
| H |  |  |  |  |  |  |  |  |  |  |  |  |

## Steps in incubation 1:

1. Sample type 1 is in row A (in volume: 50-150 µL depending on instrument requirements)
2. Beads (without buffer) transferred from row C to sample in row A, incubated for 30 min at 4 or 37 °C with shaking and beads are transferred back to row C without liquid.
3. Step 2 is repeated for beads in row D
4. Step 2 is repeated for beads in row E but instead of being transferred back to row E they beads are washed by incubated for 45 sec in row F, G and H with shaking
5. Beads are collected from row H and resuspended in 30 µL **MM** in the elution strip
6. 1 µL sample type 1 in row A is diluted in 99 µL buffer 5

## Steps in incubation 2:

1. Pause for addition of new reagents (only if necessary)
2. \*\*Repeat step 1-5 from incubation 1 using sample type 1 from row A (in incubation 1) but with only 1 round of bead incubation instead of 3 (as shown in kingfisher plate for incubation 2)\*\*

* Samples for further analysis are beads in **MM** from incubation 1 and incubation 2, diluted sample type 1 (1:100) after incubation 1 and sample type 1 after incubation 2

Final bead prep:

1. Beads in **MM** after incubation 1 and incubation 2 are incubated 5 min at 95 °C
2. Immediately after, the entire 30 µL **MM** are mixed with 25 µL buffer 6 in 0,2mL PCR tubes/skirted plate.

# Protocol 2

## NGS prep (sample and buffer annotation does not apply here)

Preparation for PCR1 (DNA samples in 96-well skirted plate) currently done row-wise:

1. Dilute 1 µL of each sample into 9 µL of water in a new 96-well plate, currently done row-wise (blue=sample liquid). :

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | n1 | n2 | n3 | n4 | n5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Sample 1 |  |  |  |  |  |  |  |  |  |  |  |  |
| Sample 2 |  |  |  |  |  |  |  |  |  |  |  |  |
| Sample 3 |  |  |  |  |  |  |  |  |  |  |  |  |
| Sample 4 |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |

\*n may vary from 3 to 6\*

1. PCR1 mix: all samples have the same reverse primer, unique forward primers for each column (n1, n2 etc.) and the number of reactions per column is R=samples per row \* 2 + 2 + 1 (for duplicates, dead volume and no-template control). Here, R=4\*2+2+1=11:

|  |  |  |
| --- | --- | --- |
|  | **1x (V in µL)** | **11x** |
| **PCR MM** | 10 | 110 |
| **Forward primer** | 0,1 | 1,1 (dilution needed for accurate pipetting?) |
| **Reverse primer** | 0,1 | 1,1 |
| **Template DNA** | 1 | - |
| **Water** | 8,8 | 96,8 |

1. PCR 1 plate - 19 µL PCR1 mix added to new 0,2mL PCR skirted plate for PCR in duplicates (see attached excel sheet for example primer combinations for PCR1 and PCR2 \* it would be great if this sheet can be provided be the user to the script\*):

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | n1 | n1 | n2 | n2 | n3 | n3 | n4 | n4 | n5 | n5 |  |  |
| Sample 1 |  |  |  |  |  |  |  |  |  |  |  |  |
| Sample 2 |  |  |  |  |  |  |  |  |  |  |  |  |
| Sample 3 |  |  |  |  |  |  |  |  |  |  |  |  |
| Sample 4 |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | control |  | control |  | control |  | control |  | control |  |  |  |

1. 1 µL DNA sample from 1:10 dilution (step 1) added to corresponding wells in PCR 1 plate (n1, n2 etc.)
2. Pause for qPCR on different machine (option to run PCR in OT2 with user specified cycle number and melting, annealing and elongation temperatures added to script)

\*Pause for normalization\*

Preparation for PCR2 – for each pool PCR2 is performed in replicates:

1. The number of replicates per pool is: 4, 3 and 2 replicates are made for <6, 6-7 and 8 sample, resp.
2. Forward and reverse primers are contained in separate tubes and each sample needs individual primer combinations (see attached excel sheet for example primer combinations)
3. PCR2 mix – primer pairs are unique for each pool:

|  |  |  |
| --- | --- | --- |
|  | **1x (V in µL)** | **Mastermix of 6x per sample (1 added for dead volume)** |
| **PCR MM** | 10 | 60 |
| **Forward primer** | 0,1 | 0,6 |
| **Reverse primer** | 0,1 | 0,6 |
| **Template DNA** | 5 | - |
| **Water** | 4,8 | 28,8 |

1. PCR2 mix is added to skirted 96-well PCR plate, in this current example like so: mastermix is distributed to each row and template DNA is added to replicates

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Replicate 1** | **Replicate 2** | **Replicate 3** | **Replicate 4** |  |  |  |  |  |  |  |  |
| **Sample 1** |  |  |  |  |  |  |  |  |  | Control |  |  |
| **Sample 2** |  |  |  |  |  |  |  |  |  | Control |  |  |
| **Sample 3** |  |  |  |  |  |  |  |  |  | Control |  |  |
| **Sample 4** |  |  |  |  |  |  |  |  |  | Control |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |

1. Program is stopped before PCR2 is run in thermocycler (unless qPCR can be incorporated)