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Opentrons Dual-Index Primer Plate Workflow V.2

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ABSTRACT

This protocol is designed to utilize the Opentrons robotic automation platform to generate dual-index primer - master mix plates for PCR reactions for NGS, particularly for DNA barcoding large specimen pools with Oxford Nanopore Technologies (ONT) MinION devices. It is designed for the indexing strategy where you start with a plate of 96 different forward or reverse primers, and then use a single forward/reverse index for each PRC plate you are running for the pool. I typically use eight plates (768 reactions) of specimens for a given Flongle run. So each plate of reactions has a single unique forward tag corresponding with that plate number and a standard plate of 96 reverse tags. The protocol is broken down into three primary areas.

1. OT-2 "Working" Primer Plates - 100uM->10uM

These steps and automated protocol turns a single 100uM primer plate into four 10uM "working" primer plates.

2. OT-2 Dual-Index PCR Stock Plates

This portion of the protocol will generate four "stock" dual-index primer / master mix plates. Each of these stock plates will generate 16 PCR ready plates for a single ONT single index (Ex - ONT01). This protocol will need to be repeated twice to create stock plates for each of the eight unique indexes for a run.

3. OT-2 Dual-Index PCR Ready Plates

This protocol will create eight PCR ready plates from one PCR stock plate. Each cell has half-reactions (11.5uL; 12.5 total reaction volume with template) or quarter-reactions (5.7uL; 6.3uL total reaction volume). At the end of this step, you are left with dual indexed PCR plates that are ready for DNA template to be added and to be put in the thermocycler.

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

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PROTOCOL integer ID: 98150

MATERIALS

Equipment

Opentrons OT2 or OT2R
Plate centrifuge
200uL Pipette
1000uL pipette
or better -
Heat Sealer (optional)

Reagents

ONT-tagged Reverse Primers **eurofins genomics** 96 well plate with 96 different primers. - Eurofins or others. $396.16 - $0.172 per plate

ONT-tagged Forward Primers **eurofins genomics** 10uM Forward Primers (~1.15mL) x 8, one for each forward index $87.50 -

Molecular Water **IBI Scientific Catalog #**IB42130 (IBI Scientific): 1L is $39.23 320mL total; $0.174 per plate

PCR Master Mix (Empirical Bioscience): $770.99/100mL 100mL total; 770.99; $10.71 per plate

Consumables

Opentrons 20uL Filter Tip Racks - Opentrons $821.18 / 100 racks - $8.21/rack 11 total; $90.31; $1.254/plate

Opentrons 200uL Filter Tip Racks
or Opentrons 300uL Tip Racks - Opentrons - $660.64 / 100 racks - $6.61/rack 12 total; $79.32; $1.102/plate

1000uL Filter Tips

Bio-Rad 96 Well Plate 200 µL PCR - HSP9601: Ebay $150/50 $3/ea 13 total; $39.00; $0.541/plate

NEST 96 Well Plate 100 µL PCR Full Skirt - 402501: Direct $215/100 $2.15/ea 72 total; 2.15/plate

NEST 12 Well Reservoir 15 mL - 360102: Opentrons $250/50 $5.00/ea 1 total; $5.00; $0.069/plate

Agilent (201252-100) or NEST (360206) 1 Well Reservoir 290 mL: Opentrons $250/50 $5.00/ea 1 total; $5.00; $0.069/plate

50mL tubes: Opentrons $130/500 $0.26/ea 8 total; $2.08; /$0.028 per plate

PCR Sealing Film (Amazon): $36.74/ 100 sheets 83 total; $30.49; $0.424/plate
$1.00 per plate for foil

The totals for each item above are for a single run of 16 final PCR plates for each of the eight indexes, or 72 total plates as the output. Cost per plate comes out to around $17-$18 per plate. This equates to ~$0.17/specimen for PCR.

Initial Prep

1 Starting Point – 1 Stock Primer Plate with 96 different ONT-tagged primers. At least 90uL fluid at 100uM concentration. Spin down this plate. Transfer liquid to a BioRad 200uM plate. If you have less than 90uL in any of the cells, you will need to make modifications to the programs.

2 Calibrate the Opentrons robot. This process can take about 15 minutes. You will need a calibration block and tip racks of each size. I have designated a unit of each consumable as a "Blank" to use for calibrations.

OT-2 "Working" Reverse Primer Plates - 100uM->10uM

3 This portion of the protocol begins with a single plate of 96 different primers on a plate at 100 uM initial concentration and creates four "working" primer plates at a 10uM concentration. It adds 20 uL of each primer to four plates then 180 uL of water to each cell, bringing each cell to a 200uL total volume.

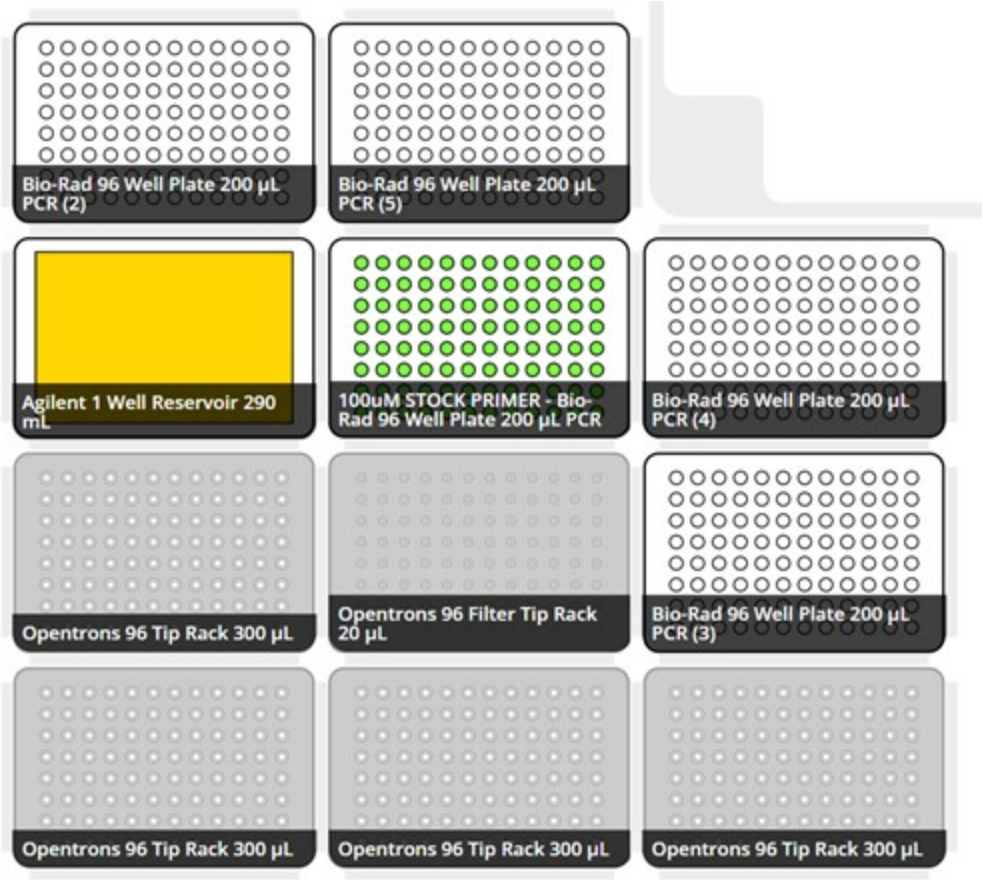
Materials:
Agilent (201252-100) or NEST (360206) 1 Well Reservoir 290 mL
96 Different Tagged 100uM Stock Primers in a BioRad 200uL 96 well Plate
(4) BioRad 200uL 96 well Plates
PCR Plate Sealing Film

Molecular Water

(1) Opentrons 20uL Filter Tip Rack
(4) Opentrons 200uL Filter Tip Racks (or 300 uL Tips)

Method:

1. Add 80mL of water to an Agilent 290ml 1 well reservoir.
2. Setup the Opentrons floor as follows:



4. Run the "**OT-2 "Working" Reverse Primer Plates - 100uM->10uM**" program. There are two versions of the program depending on whether you are working with 200uL filter tips or 300ul non-filter tips. Filter tips are suggested. If you already calibrated the device at the beginning, no need to calibrate it again. It would be good to do the labware calibration before starting the program.

OT-2 Working Primer Plates - 100uM-10… 543KB

200uL Filter Tips

OT-2 Working Primer Plates - 100uM-10… 462KB

300uL Tips

5. At the end of the run you will now have four "working" primer plates. Seal three of them with a heat sealer (if available) or plastic film. These will be used at a later time. Leave one at room temperature to move on to the next steps.

Update notes: The original program used 20uL of primer + 20uL of water. This was too much liquid within a cell to seal without the liquid touching the top. The 20uL transfers also got broken down automatically by the software into a 19uL transfer + a second 1uL transfer. The new program avoids this.

OT-2 Dual-Index PCR "Stock" Plates

4 This protocol will generate four "stock" dual-index primer / master mix PCR plates with each run. A single working primer plate can do eight runs. Each of these stock plates will generate 16 PCR ready plates for a single ONT single index (Ex - ONT01). This protocol will need to be repeated twice to create stock plates for each of the eight unique indexes for a run.

Overview:

A mixture will be created by the robot for each of 96 cells that contains:

10.8uL of 10uM Reverse Primers from the primer plate
188uL of MMFW (Master Mix Forward-primer Water) if one of four forward primers for a given set.

For each of four primers, a mixture with 10,368uL of Master Mix, 1,037uL of Forward Primer, and 6,643uL of molecular water is needed. To make it even, the protocol uses 20 total mL of MMFW as a starting point for each primer. This is mixed in four 50mL tubes and transferred to the 12 well reservoir.

Materials:
NEST 15mL 12-well Reservoir (2 total)
96 Different Tagged 10uM Working Primers in a BioRad 200uL 96 well Plate 200uL
(4) BioRad 200uL 96 well Plates (8 total)
2x Master Mix (~100mL total)
Molecular Water (~240mL total)
10uM Forward Primers (~1.15mL) x 8, one for each forward index
8x 50mL tubes
PCR Sealing Film
Eliminase or similar

(1) Opentrons 20uL Filter Tip Rack (2 total)
(4) Opentrons 200uL Filter Tip Racks (or 300uL Tips) (8 total)

Method:

1. Wipe down the lab bench and Opentrons floor with Eliminase or similar.
2. Let two (2) 25mL tubes of master mix thaw at room temperature. This could take 30 minutes or more.
3. While this defrosts, pull out four (4) 50mL tubes and label them with the indexes you are about to be working with. Likely 1-4 or 5-8.
4. Create MMFW Cocktail. Each cocktail mixture would be done in a new 50mL tube and with a different primer index. In a 50mL tube add:

For a full run (makes 16 PCR ready plates for each index):

11,490 uL of master mix
1,150 uL of corresponding forward/reverse primer
7,362 uL of molecular water

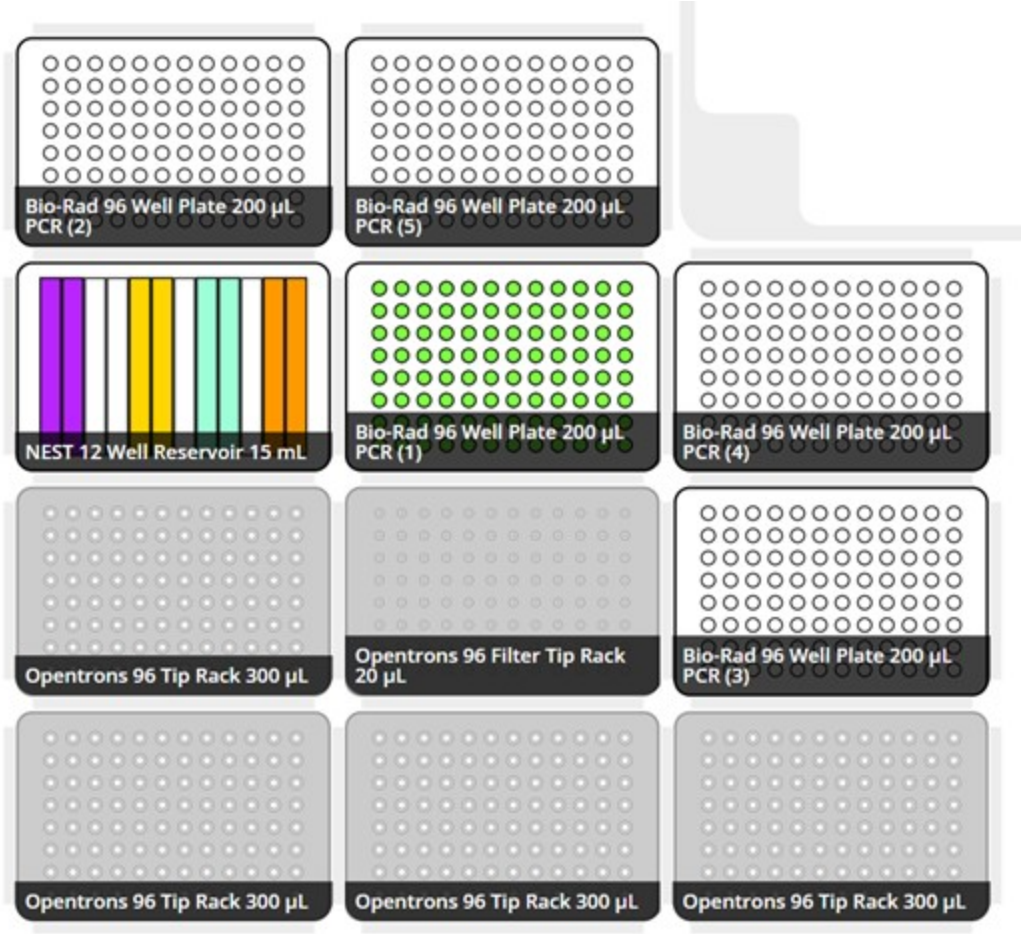
For a half run (makes 8 final PCR ready plates for each index):

5,745 uL of master mix
575 uL of corresponding forward/reverse primer
3,681 uL of molecular water

5. Turn on the Opentrons Robot. Load the appropriate program. Calibrate the Opentrons robot for the program . If you calibrated once, you will only need to do the labware calibration.

6. For a full run, add 10mL of the MMFW cocktail to each of the eight corresponding cells of a NEST 15mL 12-well reservoir as seen in the picture below. Each MMFW index mix will have 10mL split between two cells of the 12 well reservoir. Each run/reservoir will only hold four indexes. So this Opentrons program will need to be run twice to create all eight indexes. If you are doing half a run, you will only use the left cell for each index colored pictured below. The right cell will remain empty.

7. Setup the Opentrons floor as follows:



8. Run the "**OT-2 Dual-Index PCR Stock Plates**" program. This program runs with the 300uL non-filter tips. It will need to be modified if you would like to use 200uL filter tips.

OT-2 Dual-Index PCR Stock Plates Half … 382KB

Half Run (8 final PCR ready plates per index)

OT-2 Dual-Index PCR Stock Plates Full … 389KB

Full Run (17 final PCR ready plates per index)

9. Keep an eye on the tip disposal tray. You will likely need to pause the program about 25 and 40 minutes in to remove discarded pipette tips from the trash tray.

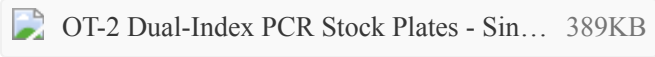
10. Upon completion, remove each of the stock primer plates from the Opentrons. If they are not going to be used immediately for the next step, seal them. If they are not going to be used immediately for the next

step, seal them with the heat sealer. If they will be used immediately, use clear plastic film to seal them temporarily.

11. Remove the remaining master mix from the 12-well reservoir into 1.5mL tubes to freeze and save for a future run. This used plastic reservoir can be discarded.

Each working primer plate should be able to run through this process nine times. That means if a single stock primer plate turns into four working primer plates, each working primer plate turns into 32 PCR Stock Plates, and each PCR Stock plate turns into 16 PCR Ready plates, then each stock primer plate will make about 2,304 dual index plates.

Further scaling this protocol If you would like to scale this protocol further for even larger batches, a protocol can be found below that would 4x the total number of plates that are made. Instead of creating stock plates for four different indexes during this run, you would make four stock plates for a single index. You would then run this protocol 8 times (if you are using 8 indexes; 8 plates on a nanopore run) rather than twice. That would end with 64 plates per index, rather than 16.



Single Index (60+ final PCR ready plates of a single index)

OT-2 Dual-Index PCR Ready Plates

5 This protocol will create eight PCR ready plates from one PCR stock plate. Each cell has half-reactions (11.5uL) or quarter-reactions (5.7uL).

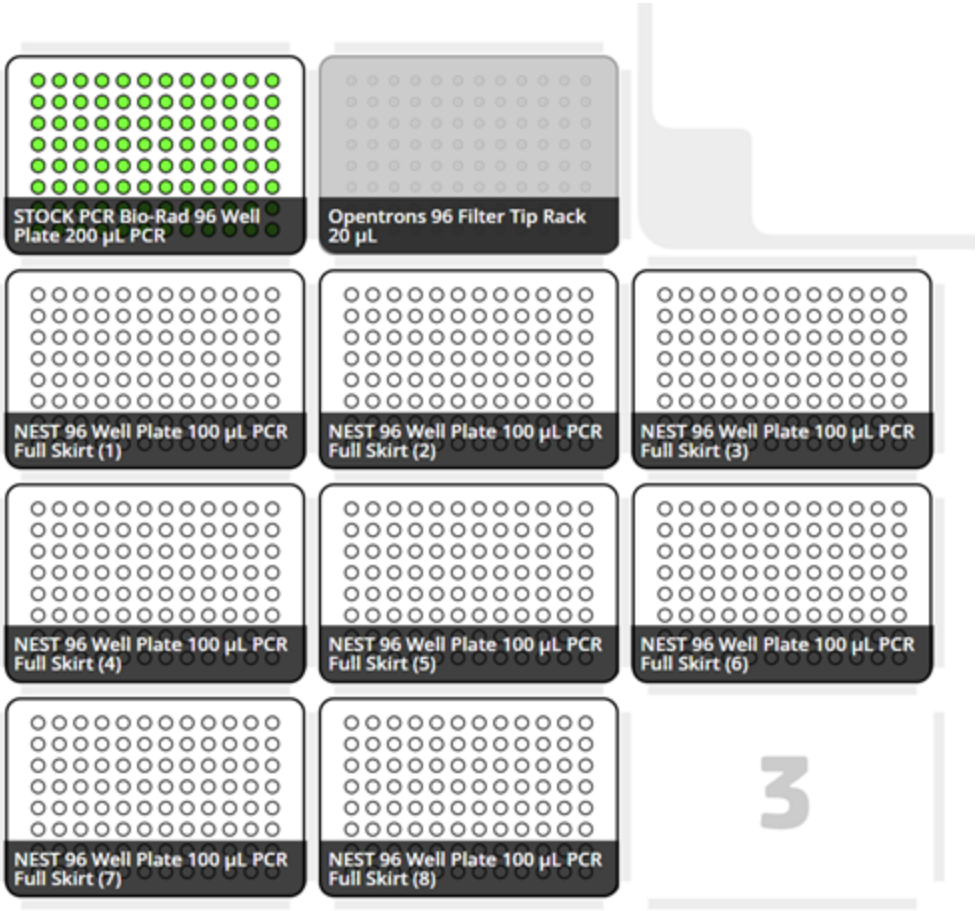
Materials:

96-well Stock PCR Plates (from previous step)
(8) NEST 100uL 96 well PCR Plates (72 total)
(1) Opentrons 20uL Filter Tip Rack (8 total)

Method:

1. Run the labware calibration for this new setup.

2. Setup the Opentrons floor as follows:



3. Run the "**OT-2 Dual-Index PCR Ready Plates**" program:

OT-2 Dual-Index PCR Ready Plates - 1_… 444KB Half-reactions (11.5uL per cell)

OT-2 Dual-Index PCR Ready Plates - 1_… 444KB Quarter-reactions (5.7uL per cell)

4. Upon completion, remove each of the PCR Ready plates from the Opentrons. Seal them with the heat sealer if you are shipping them, or plastic press-on film if you will be using them in-house.