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We use this protocol and it's working

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Opentrons Dual-Index Primer Plate Workflow

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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 (12.5uL). 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.

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

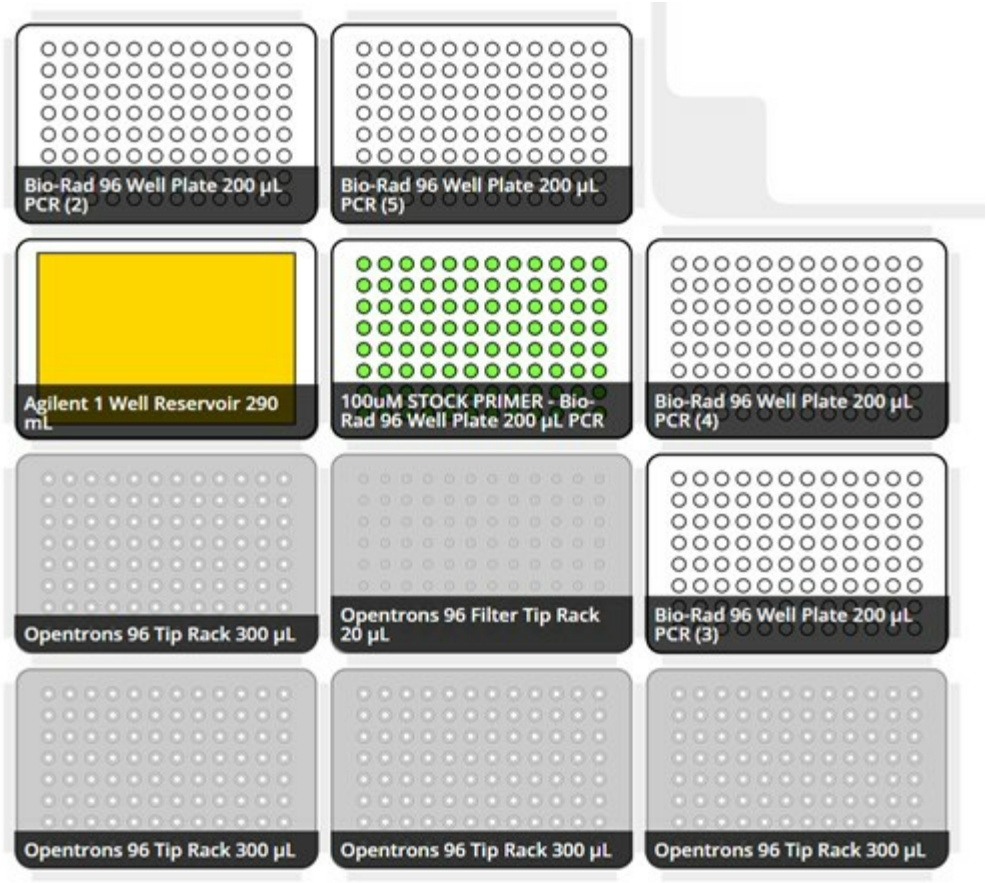
3 This portion of the protocol begins with a single plate of 96 different primers on a plate at 100 uM initial concentration and creates four "working" primer plates at a 10uM concentration. It adds 20 uL of each primer to four plates then 180 uL of water to each cell, bringing each cell to a 200uL total 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-10uM - 200uL filter tips.json543KB 200uL Filter Tips

OT-2 Working Primer Plates - 100uM-10uM - 300uL tips.json542KB 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.

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 nine 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

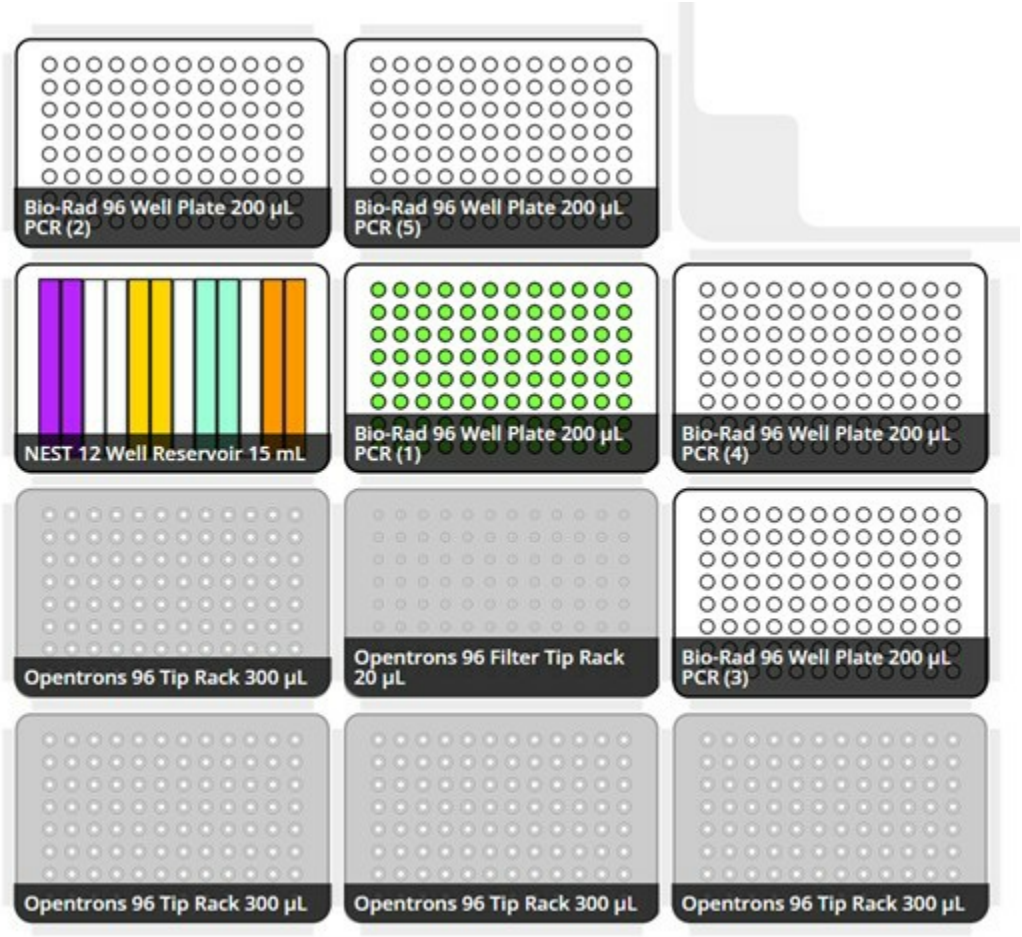
(1) Opentrons 20uL Filter Tip Rack (2 total)
(4) Opentrons 200uL Filter Tip Racks (or 300uL Tips) (8 total)

Method:
1. Calibrate the Opentrons robot. If you calibrated once, you will only need to do the labware calibration.
2. 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:

    11,490 uL of master mix
    1,150 ul of corresponding forward/reverse primer
    6,643 uL of molecular water

3. Add 10mL of the MMFW cocktail to each of the eight corresponding cells of a NEST 15mL 12-well reservoir. 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.

4. Setup the Opentrons floor as follows:



5. 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 (8 indexes; four per Run; 300uL Tips).json 371KB

300uL Tips

6. 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.

7. Remove the remaining master mix from the 12-well reservoir into 1.5mL tubes. This 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.

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 (12.5uL).

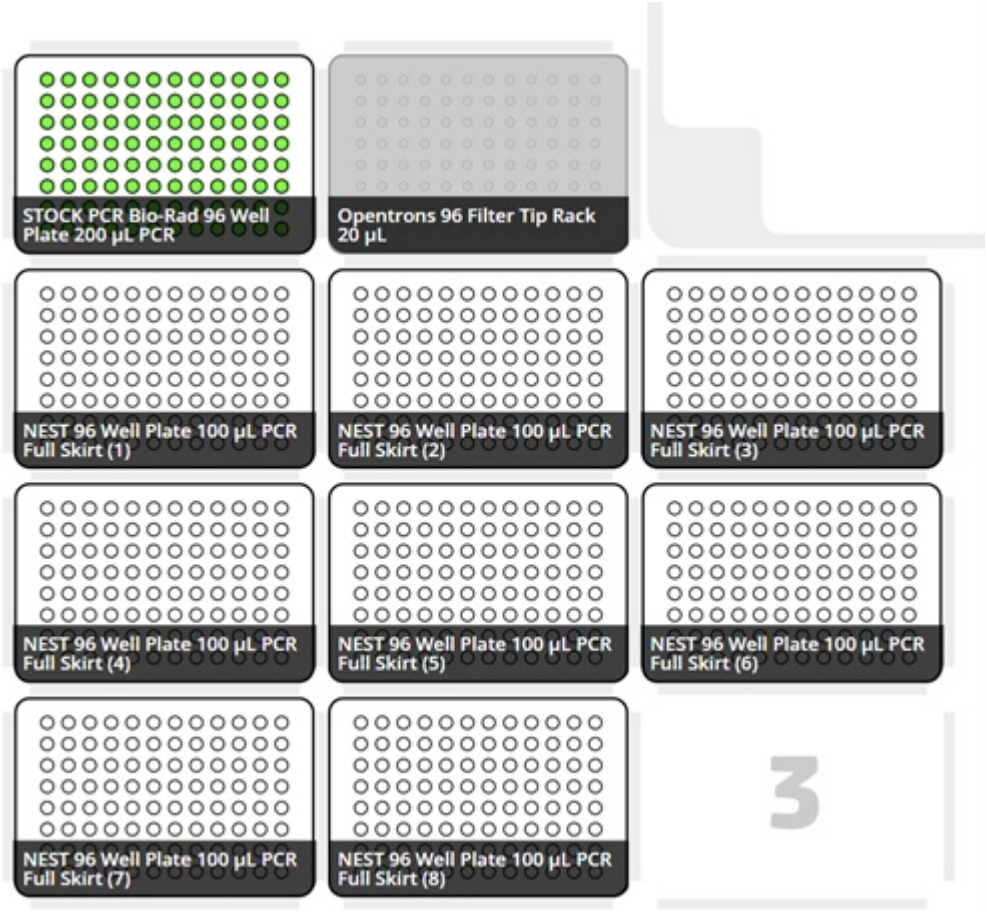
Materials:

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.json 329KB 12.5ul Reactions

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.