Preparing ONT-tagged Primers and Master Mix for Fungal DNA Barcoding V.3

Stephen Douglas Russell[1]

[1]The Hoosier Mushroom Society

The Hoosier Mushroom Society

Stephen Douglas Russell

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We use this protocol and it's working

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ABSTRACT

ONT primer preparation has two specific aspects that are unique when comparing to Sanger sequencing protocols. The first is that each primer needs to be "tagged" - a unique ~10-15bp sequence is added to the 5' end of the forward and reverse primers. Secondarily, these tagged primers need to be "multiplexed" - meaning that each individual specimen has a unique combination of forward-tagged primer and reverse-tagged primer. These tags allow the DNA amplicons for all of the specimens to be "pooled" or mixed together for sequencing, and then to be "demultiplexed" or sorted back out, allowing the resulting sequences to be associated with the individual specimens they originated from.

It is possible to perform ONT sequencing without the tags, but you would not be able to associate the sequences with any individual specimens. Ex - If you had ten closely related Russula specimens in your sequencing run, you would be able to document sequences of all ten of the species, but you would have a difficult time associating the sequences with the individual specimens/observations they originated from. They would be sequences without faces. This may be common if you are examining the community ecology or environmental DNA of a particular location, but this result would not be ideal for most DNA barcoding goals involving specimens. Thus, if you are running 960 specimens on a Flongle flowcell, you need to have 960 unique primer combinations for the sequencing run.

The easiest way to accomplish this is to have a single unique forward primer tag for each plate you are including, combined with 96 unique reverse primers for the plate. If you are including five plates, you would have five unique forward primers (a different one for each plate) combined with a standard set of 96 reverse primers. This results in 960 unique tags for each of the 960 specimens that are being barcoded.

Note: This protocol utilized a primer plate of 96 different reverse primers. In the future I plan on ordering the primer plate of 96 unique primers for the forward ITS1F primers. This will allow ad-hoc combinations of a different ONT-tagged reverse primer to extend into the LSU region as needed.

MATERIALS

Primers

ONT-tagged Forward Primers **eurofins genomics** $87.50

ONT-tagged Reverse Primers **eurofins genomics** $396.16

Reagents and Consumables

Molecular Biology Grade Water **IBI Scientific Catalog #IB42120** (Cost in extraction step)
0.2 non-skirted 96-well PCR plates (USA Scientific/Amazon): $22.00/10 x3 = $66.00
PCR Sealing Film (Amazon): $36.74/ 100 sheets
8-strip PCR caps (USA Scientific): $11.00/125 strips x3 = $33.00
Eppendorf DNA LoBind 1.5mL tubes (USA Scientific): $31.75 per 250 tubes
PCR Master Mix (Empirical Bioscience): $206.90 shipped ($10.34 per plate; $0.108 per reaction/sample)

Equipment

Fine-tip Sharpies (Amazon): $1.84
PCR tube rack x10 (Amazon) : (Cost in extraction step)
PCR tube rack 1.5mL (Amazon): $26.42
0.5 -10uL multichannel pipette (Amazon): $155.51 (may be able to find used cheaper on Ebay)
50 - 300uL multichannel pipette (Amazon): $172.89 (may be able to find used cheaper on Ebay)
10uL filtered pipette tips (Amazon): $63.99
200uL filtered pipette tips (Amazon): $57.77
Eliminase (Ebay): $60.00
Summit Professional Freezer -20C (Facebook Marketplace / 1 / 2): $150 (gotta get lucky)
Thermocycler: (Cost in extraction step)

Total Cost Outlay: $1607.74

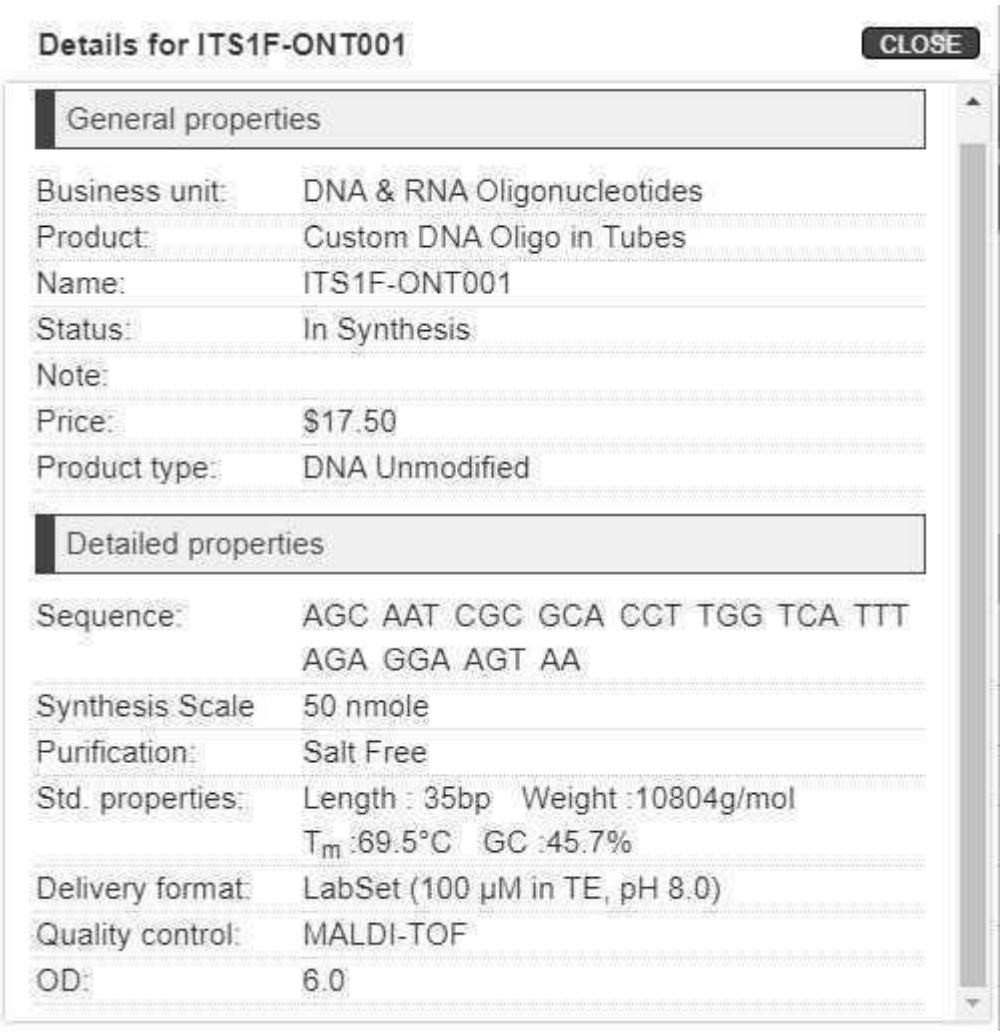
Ongoing cost per sample: minimal

Ordering ONT-tagged Primers

1 ONT-tagged Forward Primers **eurofins genomics** - Determine how many unique primers you need to order. Ex - If you are planning to utilize a Flongle cell with up to 960 specimens, you would need to order ten unique forward ONT-tagged primers. If you hope to use a 10.4.1 cell with 10,000+ specimens, you would need to order at least 105 unique forward tagged primers. ITS1F sequences and/or unique ONT primer tags can be found here:

MinION Primer Tag Sets.xlsx

$17.50 x 5



Details for ITS1F-ONT001 CLOSE

General properties

Business unit:	DNA & RNA Oligonucleotides
Product:	Custom DNA Oligo in Tubes
Name:	ITS1F-ONT001
Status:	In Synthesis
Note:	
Price:	$17.50
Product type:	DNA Unmodified

Detailed properties

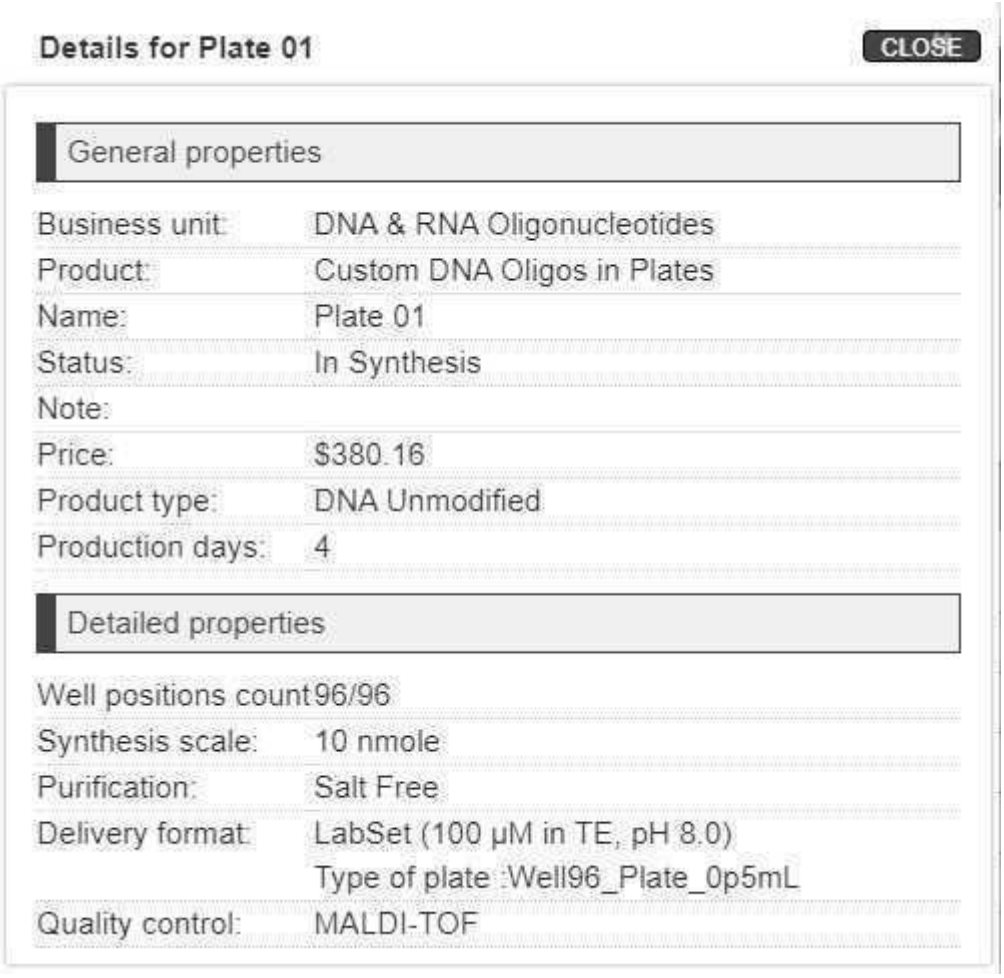
Sequence:	AGC AAT CGC GCA CCT TGG TCA TTT AGA GGA AGT AA
Synthesis Scale	50 nmole
Purification:	Salt Free
Std. properties:	Length : 35bp Weight :10804g/mol T_m :69.5°C GC :45.7%
Delivery format:	LabSet (100 µM in TE, pH 8.0)
Quality control:	MALDI-TOF
OD:	6.0

Specifications for one of the unique ONT-tagged forward primers from Eurofins being utilized in this protocol.

2 ONT-tagged Reverse Primers **eurofins genomics** - You will need to order a primer plate of 96 unique ONT-tagged reverse primers. You will only need to order a single 96 well plate. These reverse primer tags will be the same set for each plate you include in a sequencing run.

ITS4 sequences and/or unique ONT primer tags can be found here:
MinION Primer Tag Sets.xlsx
A Eurofins template ready for upload can be found here: Eurofins_PlateUpload.xlsx

$380.16 + $16 shipping



Details for Plate 01 CLOSE

General properties

Business unit:	DNA & RNA Oligonucleotides
Product:	Custom DNA Oligos in Plates
Name:	Plate 01
Status:	In Synthesis
Note:	
Price:	$380.16
Product type:	DNA Unmodified
Production days:	4

Detailed properties

Well positions count	96/96
Synthesis scale:	10 nmole
Purification:	Salt Free
Delivery format:	LabSet (100 µM in TE, pH 8.0) Type of plate :Well96_Plate_0p5mL
Quality control:	MALDI-TOF

Specifications for the "standard" 96 unique reverse ONT-tagged primers from Eurofins used in this protocol.

Preparing Forward Primer Stock

3 When your forward primers arrive in individual tubes, they will be at a 100uM concentration. You will need to make new working tubes at a 10uM concentration.

In a new 1.5uL tube, using filter tips:
900uL molecular water
100uL of 100uM forward primer

Label each tube such as "ONT001 10uM."

Preparing Reverse Primer Stock-Working-PCR Ready Plates

10m

4 Materials Required:

Molecular Biology Grade Water **IBI Scientific Catalog #IB42120**

ONT-tagged Reverse Primers **eurofins genomics**

96 well plates

Multichannel Pipettes (90uL, 10uL, 6.2uL, 4.6uL)

Your ONT-tagged reverse primers arrived in a 100uM stock solution. I typically work with 10uM primer solution for most protocols. I also typically do not like to thaw out my stock plate with any regularity, so it would be best to make multiple working plates of your reverse primers.

Summary:

100 uM Reverse Primer Stock Solution
|
"Working" Reverse Primer Plates
180uL H2O
20ul Primer
|
"PCR Ready" Reverse Primer Plates
160uL H2O
25ul "Working" primer
|
PCR Reaction
4.6uL "PCR Ready" reverse primer into each cell of PCR reaction

4.1 Wipe down your working area with Eliminase or similar.

4.2 Place 90uL of molecular water into each cell of new 96 well plates. A multichannel pipette is most efficient for this job. It is also best to use filter tips.

4.3 Transfer 10uL of reverse primer from your stock plate into each of the 96 wells, making sure each primer stays in the correct cell. Make sure to use new filter tips for each transfer.

4.4 Label each plate you create "ONT ITS4 10uM Working"

5 It is possible to use the 10uM working plates directly into your PCR reactions. However, it would require that very small amounts be added to each cell (0.625uL). These small amounts are often difficult to pipette accurately, especially with a multichannel pipette. In order to increase the volume being utilized, we can create "PCR Ready" plates that also include molecular water that we would need to be including in our PCR reaction anyway.

Each working plate should make "15-16 PCR Ready" plates.
Each "PCR Ready" primer plate should make 9-10 PCR plates.

5.1 Place 40uL of molecular water into each cell of new 96 well plates. A multichannel pipette is most efficient for this job. It is also best to use filter tips.

5.2 Transfer 6.25uL of reverse primer from your working (10uM) plate into each of the 96 wells, making sure each primer stays in the correct cell. Make sure to use new filter tips for each transfer.

5.3 Label each plate you create "ONT ITS4 PCR Ready."

Create Master Mix / Forward Primer (MMF) Stock

6 In 1.5mL tubes, add:

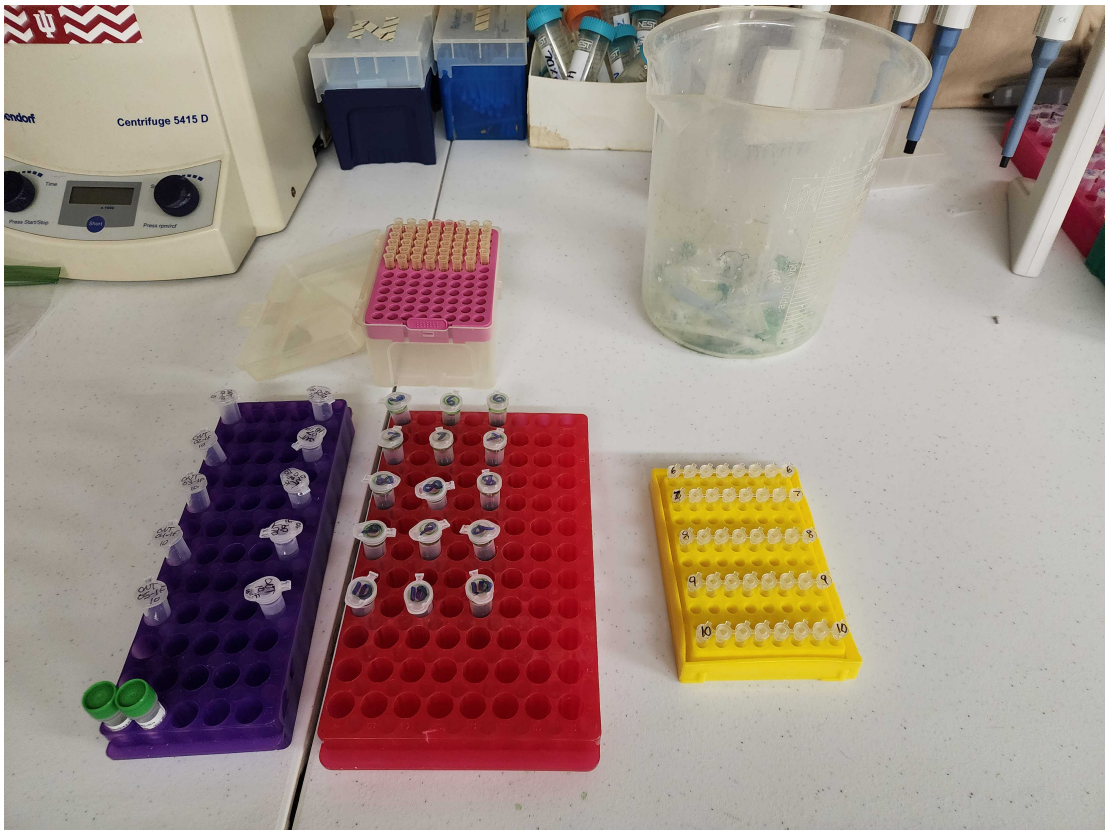
625 uL Master Mix
62.5 uL of 10uM ONT-tagged Forward Primer

Label tube with ONT name "MMF ONT001"

Each tube with this mixture will be the stock for 1 plate (100rxns). If you are running a Flongle with 960 specimens, you will need to make ten tubes, each with a different ONT-tagged forward primer.

Note: It is possible to make larger MMF batches for each forward primer and to store them in the freezer until ready for use with future runs. I will typically create three tubes of the mixture above for each forward primer when I am replenishing the stock.

I will then spec out 8-strips of each primer for easy transition to the final PCR plates.



Primers and master mix on blue. Get transferred to 1.5 MMF tubes on red. Finally into 8-stips for each primer on yellow.

Create Master Mix Plates

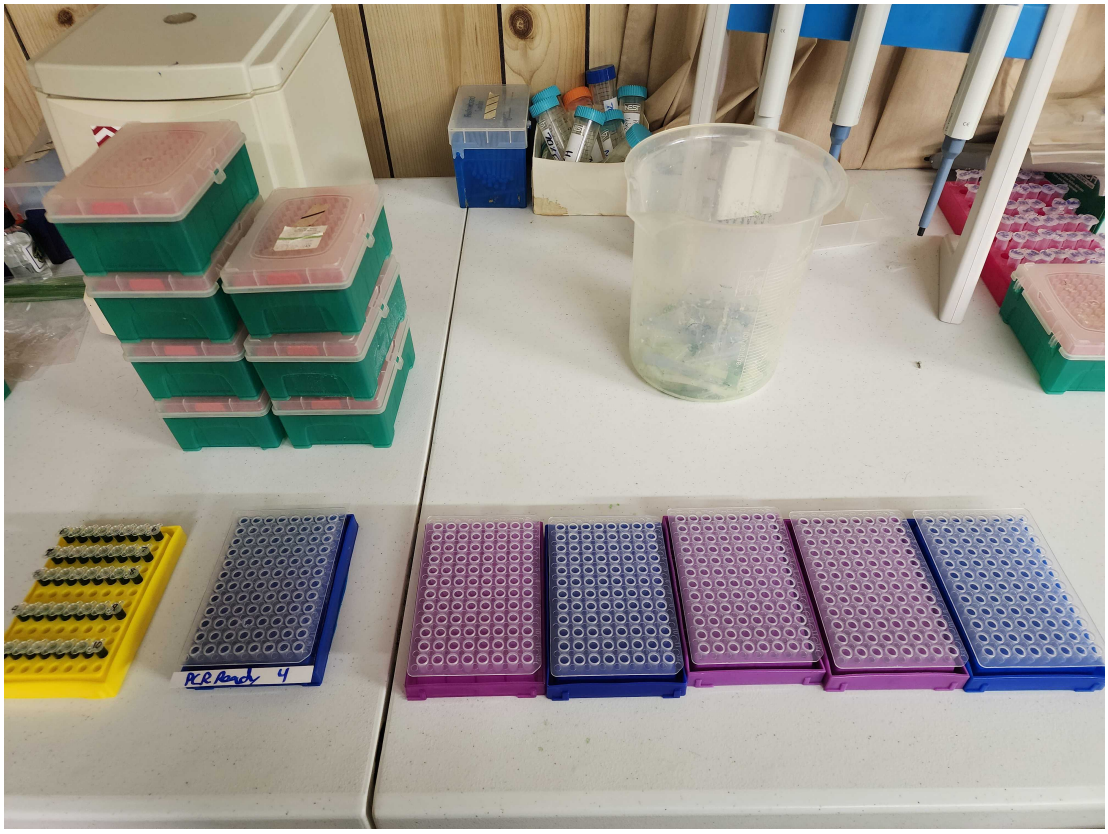
7 Make a determination of how many plates you will need for this sequencing run. For a Flongle, this will likely be 5-10. For example, if you are running 960 specimens, you will need to set out 10 new 96 well plates. However, this is often too much concentration time for a single sitting. I typically create primer plates in batches of five.

We will be performing 1/2 reactions (12.5uL total volume).

Quick Summary:
Each cell of each plate will have a different ONT-tagged primer combination and a total of:

4.6 "PCR Ready" reverse primer mix
    4uL Molecular water
    0.625 ONT-tagged reverse primer
6.9 MMF
    6.25uL of Master Mix
    0.625 ONT-tagged forward primer
1.1ul DNA template

12.5uL total reaction volume



MMF stock for each primer on yellow. PCR Ready reverse primer plate in the middle on blue. Five plates ready to accept the mixture of the two on the right.

7.1 Add 4.6uL from a "PCR Ready" reverse primer plate to each cell.

Note: It will likely be easiest to use a multichannel pipette for this step. Row 1 to five plates using a single set of tips, row 2 to five plates using a new set of tips, etc.

*Be sure to use new tips for each row of cells. You will use 96 different tips for this step. I use non-filtered tips here in order to save money.

It is imperative that you do not dip tips back into the wrong row of cells in-between plates or after they have been used. If you dip into the wrong row of the PCR Ready plate, you will need to dispose of the entire remainder of the PCR Ready plate. This step and the next one will require at least an hour of intense concentration and counting. Make sure you will have no distractions during this time.

7.2 Add 6.9uL of the MMF stock into each cell of the new 96 well plates. REMINDER: Each new plate needs different MMF stock from a different ONT-tagged primer.

I keep 8-strip tubes filled with premade MMF for each ONT forward primer. This way I can quickly thaw the strip and lay out plates for each primer as needed. I use non-filtered tips for this step in order to save money.

You will need to use a whole box of tips for each plate you are working with for this step. It is imperative that you do not dip tips back into another cell after they have been used. If you do, you will need to dispose of the remainder of the MMF mix.

7.3 Place the completed plates in the freezer until they are ready to be utilized.

7.4 Once you are ready to add the template, utilize 1.1ul of DNA template for each cell.

NOTE: Check each tip of the pipette to ensure the requisite amount is contained in the tip. It will likely be easiest to use a multichannel pipette for this step.

7.5 Cap off the plate with PCR Sealing Film.

7.6 Label each plate with the forward primer ONT name. "ONT001 P1 ITS1F-4"

Run Thermocycler Program

8 Standard ITS program:

94C for 1 min

Repeat 30X:
94C for 1 min
51C for 1 min
72C for 1 min
72C for 8 min
10C to stop