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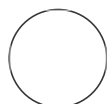
Protocol status: Working
We use this protocol and it's working

OT-2 PCR sample preparation protocol V.2

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ABSTRACT

This protocol is meant to perform samples preparation of PCR plates for 1 or more primer sets (defined number of primers per set established by the user) to the same samples, i.e, we will have for all the selected samples (we can set the initial well and number of samples) of all source plates the master mix with different primer sets (combination of primers, water and polymerase mix) in the final plates.

To run this protocol is needed a python script for an Opentrons 2 robot and an excel (.xlsx) file with several variables making the protocol modular to reactivities, volumes of transfer, type of plates, number of primers per set, etc.

In our laboratory, this protocol has been used as part of the "High-throughput workflow for the genotypic characterization of transposon library variants" also available in protocols.io to prepare PCR samples for sending to sequencing and for checking the correct introduction of the transposon in the bacterial genome.

This protocol is a set of instructions or description of the [LAP repository](#) entry **LAP-PCR-OT2-1.0.0**

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PROTOCOL integer ID: 90844

Keywords: PCR sample preparation, automation, opentrons

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GUIDELINES

This protocol was developed with python 3.7.1, OT App Software Version 6.3.1 and API level version 2.14 in a Linux 4.14.74 system (these are the OT-2 specifications). In the script several packages are used: pandas (0.25.3), openpyxl (3.1.2), math, random and numpy (1.15.1)

It has been tested with cultures from *Pseudomonas putida* KT2440 and PCR products as part of the High-throughput workflow for the genotypic characterization of transposon library variants.

The maximum number of primer sets that can be incubated with a full 96-well plate having a heater-shaker module set as True, the thermocycler set as False, the replace tip rack variable set as False **is 3**. In this scenario, **3 final plates will be created**

MATERIALS

Software

- Python 3.7.1
- opentrons software version 6.3.1
- python packages: pandas (0.25.3), openpyxl (3.1.2), numpy(1.15.1), math, random
- OT App
- Excel


OT-2 Labware

- Opentrons Tip racks


Equipment	
Opentrons 96 Tip Rack 300 µL	NAME
Tip rack	TYPE
Opentrons	BRAND
-	SKU
https://labware.opentrons.com/opentrons_96_tiprack_300ul?category=tipRack	LINK

Equipment	
Opentrons 96 Filter Tip Rack 20 µL	NAME
Tip rack	TYPE
Opentrons	BRAND
-	SKU
https://labware.opentrons.com/opentrons_96_filtertiprack_20ul?category=tipRack	LINK

- 96-well plates

Equipment	
96-well plates, flat bottom, non treated	NAME
Cell culture plates	TYPE
VWR	BRAND
734-2781	SKU
https://es.vwr.com/store/catalog/product.jsp?catalog_number=734-2781	LINK
	

- PCR plates
- Opentrons Falcon tuberack


Equipment	
Opentrons 15 Tube Rack with Falcon 15 mL Conical	NAME
OT Tube Rack	TYPE
Opentrons	BRAND
-	SKU
https://labware.opentrons.com/opentrons_15_tuberack_falcon_15ml_conical/	LINK
	

- 1.5mL eppendorfs (without the cap)
- 4°C cold-block with adaptor (file attach)

Equipment	
BRAND™ Centrifuge Tube Mini-Cooler	NAME
ColdBlock	TYPE
BRAND	BRAND
10141921	SKU
https://www.fishersci.es/shop/products/brandtech-scientific-brand-centrifuge-tube-mini-cooler-3/10141921	LINK

 adaptor_OT_coldblock.stl

Reactives:

 **Phire Green Hot Start II PCR Master Mix** Fisher Scientific Catalog #15391732

This is the reason why the transferring of the polymerase is done with a slower aspiration and dispensing rate, due to the viscosity of the reactive

Oligonucleotides:

- For spurious integration control: PS3, PS4, PS5, PS6 (Silva-Rocha et al. 2013 doi: [10.1093/nar/gks1119](https://doi.org/10.1093/nar/gks1119))
- For arbitrary PCR: ARB2, ME-O-Km-Ext-F, ME-O-Km-Int-F, ME-O-Sm-Ext-F, ME-O-Sm-Int-F, ME-O-Gm-Ext-F, ME-O-Gm-Ext-R (Martinez-Garcia et al. 2013 <https://doi.org/10.3389/fbioe.2014.00046>)
- For optional insert control: PSMCS

Equipment:

Equipment	
OT-2	NAME
Liquid handler	TYPE
Opentrons	BRAND
OT-2	SKU

Equipment	
Single Channel Electronic Pipette (GEN2) 300uL	NAME
Opentrons Pipette	TYPE
Opentrons	BRAND
-	SKU
https://shop.opentrons.com/single-channel-electronic-pipette-p20/	LINK

Equipment	
Single Channel Electronic Pipette (GEN2) 20uL	NAME
Opentrons Pipette	TYPE
Opentrons	BRAND
-	SKU
https://shop.opentrons.com/single-channel-electronic-pipette-p20/	LINK

Files Preparation

1 Preparing Customized Template

Preparing the template (a .xlsx) with the specific variables for each experiment.

Here we attach one Excel with several sheets and a PDF file explaining each variable:

1. **GeneralVariables:** variables related mainly to the labware that is going to be used
2. **SamplesPlateVariables:** variables related to the specifications of each source plate
3. **PipetteVariables:** variables related to the pipette(s) that are going to be used
4. **ReagentsPerReaction:** variables that will determine the final mix of the wells
5. **ModuleVariables:** variables related to the modules used in the protocol, the thermocycler and the heater-shaker
6. **TemperatureProfile (Optional):** PCR program that will be performed in the thermocycler if set as True in the ModuleVariables sheet
7. **Maps (Optional):** sheet(s) with the names of the samples in the source plates and will be reflected in the final plate map(s) --> *not included in the template but needed to be included if the variable Map IDs has a value.*

 Template-VariablesPCR.xlsx21KB

 PCRInstructions.pdf211KB

1.1 Fill the template with the corresponding values

1.2 Store it with the name *VariablesPCR.xlsx*

Note

The file should be spelt **exactly** *VariablesPCR.xlsx* or the Python script won't work correctly

2 Transferring file to Robot

Transfer the *VariablesPCR.xlsx* to the directory */data/user_storage* of the OT robot that we will use to perform the protocol.

Note

Before transferring any file to the OT, we need to know the IP of the robot.

This can be obtained in the Networking section of the Device that we will use.

To obtain this info go to **OT-App -> Devices -> Chosen Robot (three dots) --> Robot Settings -> Networking**

In this tab, you can see 2 types of IP; one is shown if both the robot and you are connected to the same Wifi, and the other is shown if the computer and the robot are connected via USB. Both connections can be used for this step.

Note

To connect to the robot, an **OT-key** should have been previously generated, and it is done with the *ssh-keygen* command and **transferring the public key to the OT**.

For more information about how to generate and set the connection between your computer and the Opentrons robot, visit <https://support.opentrons.com/s/article/Setting-up-SSH-access-to-your-OT-2>

Here, we present a summary of how to transfer the files in 3 Operative Systems: *Windows*, *MacOS* and *Linux*.

MacOS/Linux

We will use the command line with scp to transfer the file *VariablesPCR.xlsx* to the OT system.

We need to perform the following command

Command

File passing from linux (our computer) to linux (OT raspberry)

```
scp -i [ot_key] [file] root@[IP_OT]:/data/user_storage
```

Note

You could face difficulties transferring files in the MacOS Ventura (13) and Sonoma (14). These problems were solved by adding the argument -O (uppercase o) to the command

Command

Transferring files to OT (MacOS 13 and 14)

```
scp -Oi [ot_keypath] [file path] root@[OP_robot]:/data/user_storage
```

Windows

There are several ways to pass files from a Windows to a Linux (for example, with a virtual machine or Windows Powershell in the latest versions of Windows).

Here, we will use **FileZilla** (<https://filezilla-project.org/download.php?type=client>).

Go to **File -> Site Manager -> New Site -> Change Protocol to SFTP**. Then, introduce in Host the OT IP, change the Logon Type to Windowskey file, change the user name to root and give the directory where

the OT key is. It should look something like this

Then press connect, and we will have a connection between our computer and the robot.

After this connection, we should be able to move out *VariablesPCR.x/sx* (in our computer) to the directory */data/user_storage* in the robot.

This method of transferring files can also be done in Linux and MacOS.

Note

Take into account that the IP of the robot could change, so it may be necessary to change the host in these connections from time to time.

3 Adding the custom labware



There is only a need to do this step when the labware you are using is not OT official or included in the OT app.

3.1 Creation of .json file

The description file can be obtained by describing the labware dimensions at <https://labware.opentrons.com/create/>

3.2 Uploading files to the OT App

In the OT app, we need to perform the following route: **Labware -> Import -> Choose File -> Select file we have created in step 3.1**

Expected result

After uploading the labware you should be able to see the new labware in the Labware tab of the OT App, all custom labware can be found more easily in the category *Custom Labware*

3.3 *Transfer labware files to the robot*



If you are using the entry **LAP-PCR-OT2-1.0.0** and **custom labware**, an additional step is needed, which is transferring a folder with the custom labware

We need to create for our custom labware a folder with the API name containing the description file (.json) called 1.json and then transfer that folder to the robot's folder `/data/labware/v2/custom_definitions/custom_beta` in a similar way as in the Step 2 but with the difference that is a directory that needs to be transferred and not a file.

Command

Transferring the custom labware to OT (Linux)

```
scp -i [ot_key] -r [directory_custom_labware] root@[IP_OT]:/data/labware/v2/custom_definitions/custom_beta
```

Note

We do not need to execute this part every time the protocol is used, only when that labware is not included in the OT official labware and these directories are not in the robot

Prepare Robot OS

4 Install needed packages



This script needs the package *openpyxl*, which is not installed by default in the OT-2 robots




Note

This step is only needed if the package is not installed in the robot, not every run of the protocol

If the package is not installed, an error when running the script in the robot will appear, while simulating the script in the app, this error will appear but you can ignore it

4.1 Connect to the robot

 [go to step #2](#) to find the IP of the robot in which you want to run the script

To connect to the robot, you can do it via ssh with the following command

Command

Connect to Linux based OT via ssh

```
ssh -i [path ot_key] root@[Robot_IP]
```

In Wsuccessfulindows, you can do this command in Windows Powershell

Expected result

If the connection has been successful you should obtain a screen similar to the following image

Note

For more information about installing packages in the opentrons robots, check the following Opentrons page: <https://support.opentrons.com/s/article/Using-Python-packages-in-Python-API-protocols>

Run protocol

5 Load script in OT-App

Now that we have transferred the variable files to the robot, we can import the script and run it in the selected robot

Note

This whole step has been developed with version 6.3.1 of the OT-App and has been tested in versions up to 7.0.2

Indications may vary from version to version of the opentrons App and the version of the script.

Software

Opentrons App

NAME

Windows >=10, Mac >=10 , Ubuntu >=12.04

OS

Opentrons

DEVELOPER

<https://opentrons.com/ot-app/>

SOURCE
LINK

5.1 *Load the script in the OT app*

Protocols -> Import -> Drag Python script

Note

The last script version can be found at <https://github.com/BiocomputationLab/LAPrepository/tree/main/LAPEntries> (the name of this file is the user's choice) in the directories with the name **LAP-PCR-OT2** followed by the version.

As well we can find the latest version of the script at <https://www.laprepo.cbgp.upm.es/repository/> with the same name as in GitHub

Software

LAP Repository

NAME

<https://biocomputationlab.com/>

DEVELOPER

www.laprepo.com

SOURCE
LINK

Note

The App with version 6.3.1 analyzes your protocol before setting a robot to run, so the labware will not be shown before assigning the protocol to a specific robot when you import it into the App.

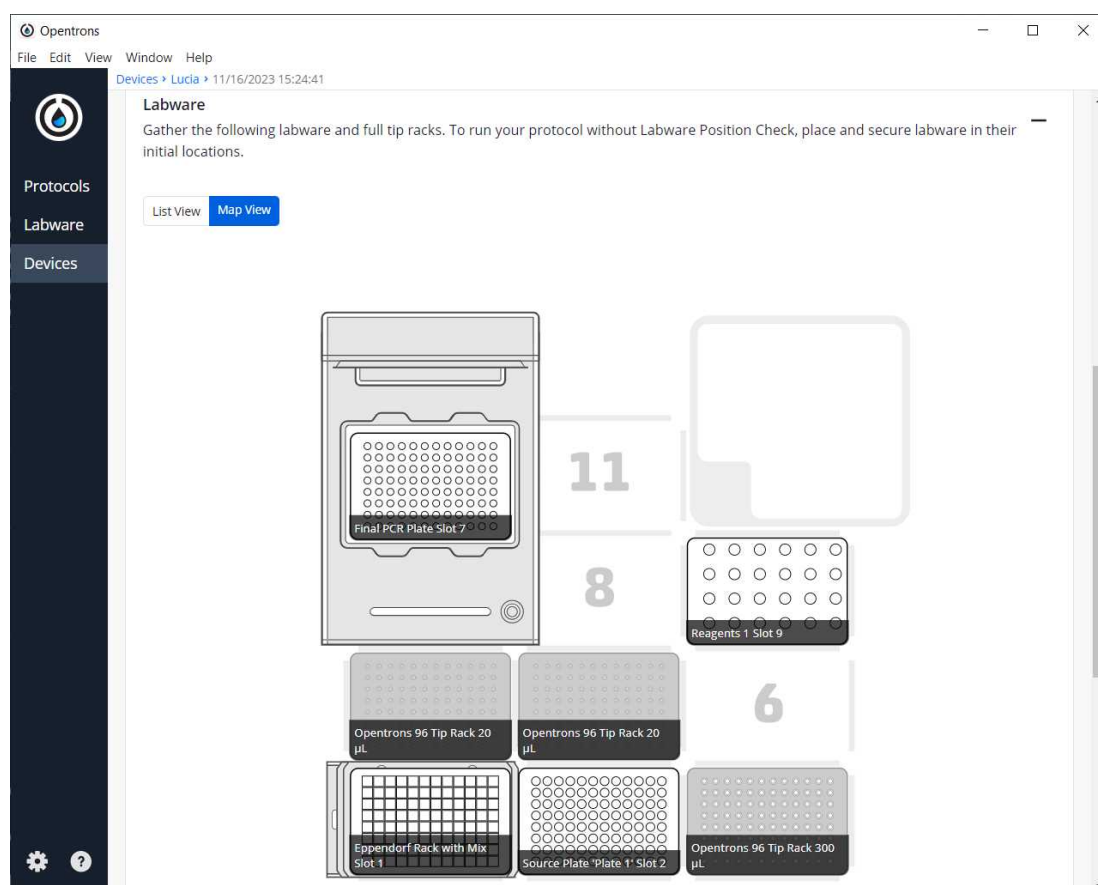
5.2 *Select Robot to Perform Script*

Click in the protocol -> Start setup -> Choose the OT where the file *VariablesPCR.xlsx* is -> Proceed To Setup

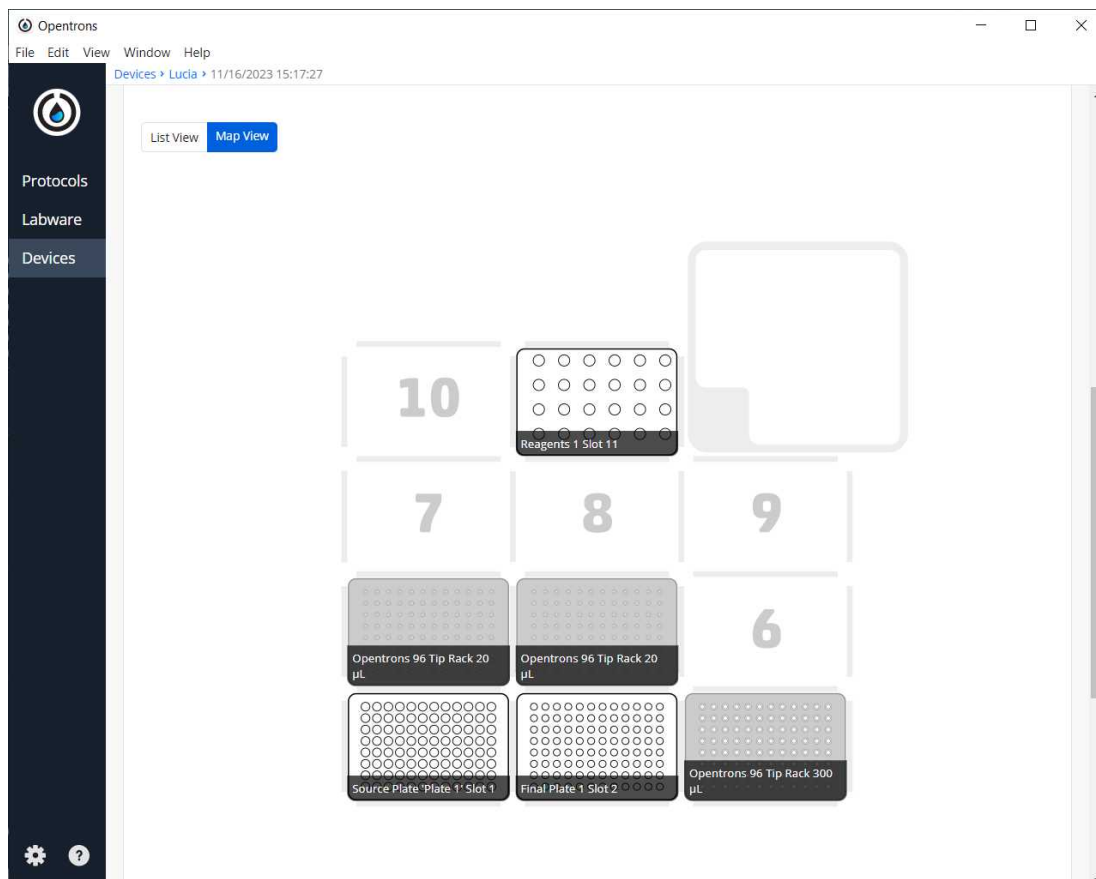
After clicking on Proceed to Setup, you should obtain the labware positions in the *Labware* tab and the reagents, with their corresponding volume, in the *Liquids* tab.

Expected result

A labware setup should look like the following image, where you can find the initial and final plates, the Eppendorf labware to store the reagents, the corresponding tips and, if included, the location of the heater-shaker (s) and thermocycler. The latter will always have the exact location in the OT-2



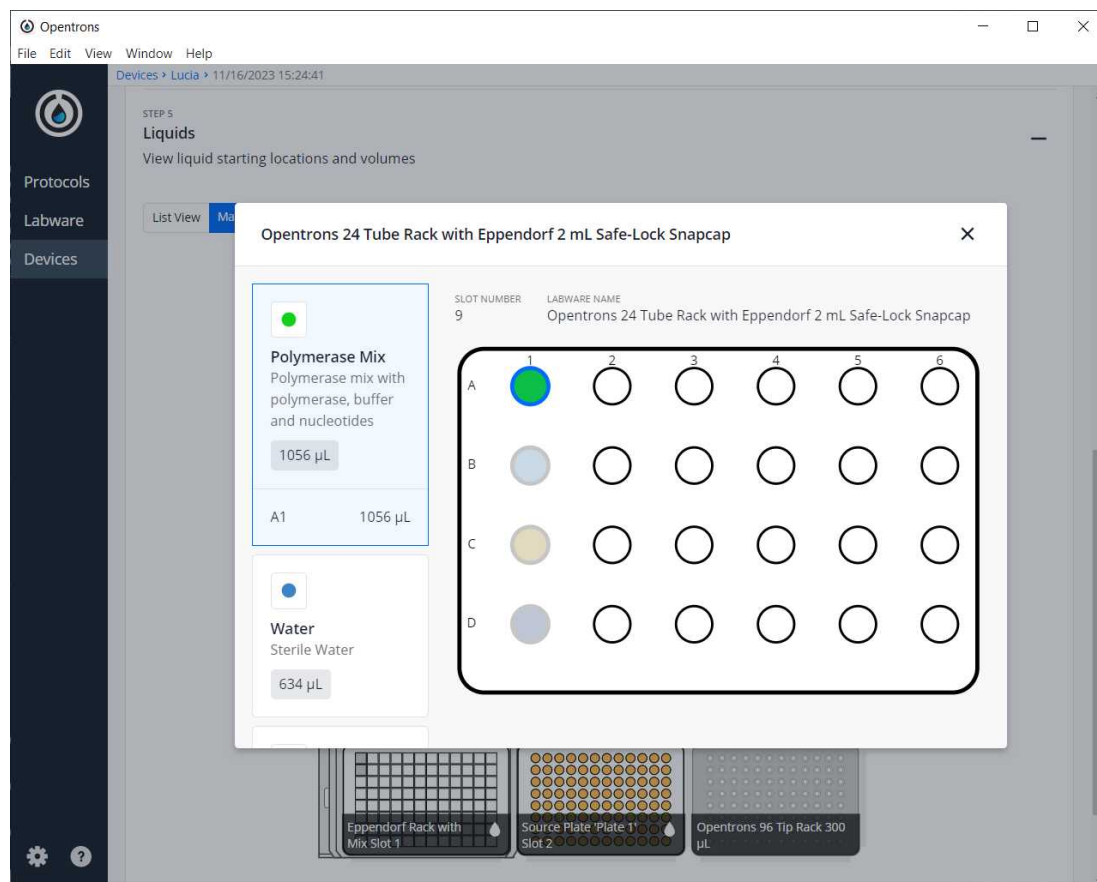
Labware set-up of an example of a PCR run with both variables 'Presence Thermocycler' and 'Presence Heater-Shaker' set as True



Labware set-up of an example of a PCR run with both variables 'Presence Thermocycler' and 'Presence Heater-Shaker' set as False

Expected result

A liquid setup should look like the following images, where you can find the samples in the initial plates and the different reagents in the Eppendorf labware with their respective volume



Liquid set up of a reagents tube rack

Note

The volume of the initial samples is established to be 90% of the max volume of the well, but it is only a recommendation, **just make sure that there is enough volume to transfer to all the final plates.**

On the other hand, the volume of the reagents is exactly what is needed, so it is **suggested to pour always more to take into account the error of pipetting**

Note

It is recommended that you perform a labware position check.

You can do it with test plates after loading the script but before cleaning the surface. That way, you reduce the probability of contamination (using the test plates and labware) and pipetting errors (position check).

6 Run Protocol in OT

6.1 *Make sure the needed calibrations are done*

Pipettes, tip racks and tip length calibrations need to be done for the items used in this run

6.2 *Labware position check is performed (if needed)*



6.3 *Clean the surface of the robot with 70% ethanol to clean and disinfect the surfaces*

Note

Check the Opentrons page <https://support.opentrons.com/s/article/Cleaning-your-OT-2?> for more information about cleaning the OT-2 robot with the proper materials.

6.4 *Set the labware and reagents as shown in the OT-App*

6.5 *Start Run*

The procedure that the robot is going to do is mainly divided into 6 parts:

1. Creation of mix(es) transferring primers, water and polymerase to new tube(s)
2. Mixing with either a pipette or heater-shaker
3. Distribute mix to all plates
4. Distribute samples to the corresponding wells (as many transferring of 1 sample as the number of primer sets)
5. Generate identity maps to be exported
6. (Optional) PCR program for thermocycler module

Expected result

One or more plates where there is a mix between the sample (DNA-template) and the set of primers, each combination of DNA template set of primer in one well

The distribution of each primer will be sequential. In other words, all the DNA templates with the first set of primers will be in the first N-wells, then all the DNA templates with the second set of primers will be in the following N-wells and so on.

A **sheet for every final plate will be created as well in an Excel file** with the given name in the shee GeneralVariables in the variable "Final Map Name" followed by the extension .xlsx in the folder */data/user_storage* of the robot where we run the script.

After-running

7 Retrieve labware from the OT

8 Importing map from robot

To retrieve the file we can  **go to step #2** and reproduce it by transferring the files to the computer.

They will be in the directory */data/user_storage*. It will be a file with an extension .xlsx and have the name provided in the input variable file

Command

Transferring files from OT to computer (Linux, macOS)

```
scp -i [path_ot_key] root@[IP_robot]:/data/user_storage/[name_map].xlsx [ultimate_path_computer]
```



Expected result

The map(s) contains a table for each final plate in the run

Each table will have the identity or name of the sample and the set of primers that are mixed in that well. If a map has been provided for the source plate, it will have that name as an identity. If not, the identity will be the well in the slot that the source plate was in the run of the script.

Example

2h 31m

- 9 We want to perform a PCR mix preparation of 94 colonies and 2 controls that are in 2 source plates(40 first colonies + 2 control samples in 1 plate and 54 colonies from the A4 well in the other plate) with 2 primer sets.

We are going to have a heater-shaker module available but not a thermocycler.

1 of the source plates will have a map of identities, but the other will not have it.

We will use a computer with a Windows 10 system

9.1 Prepare variable file

10m

Excel temple filled and saved with the name *VariablesPCR.xlsx*



9.2 Upload custom labware to app

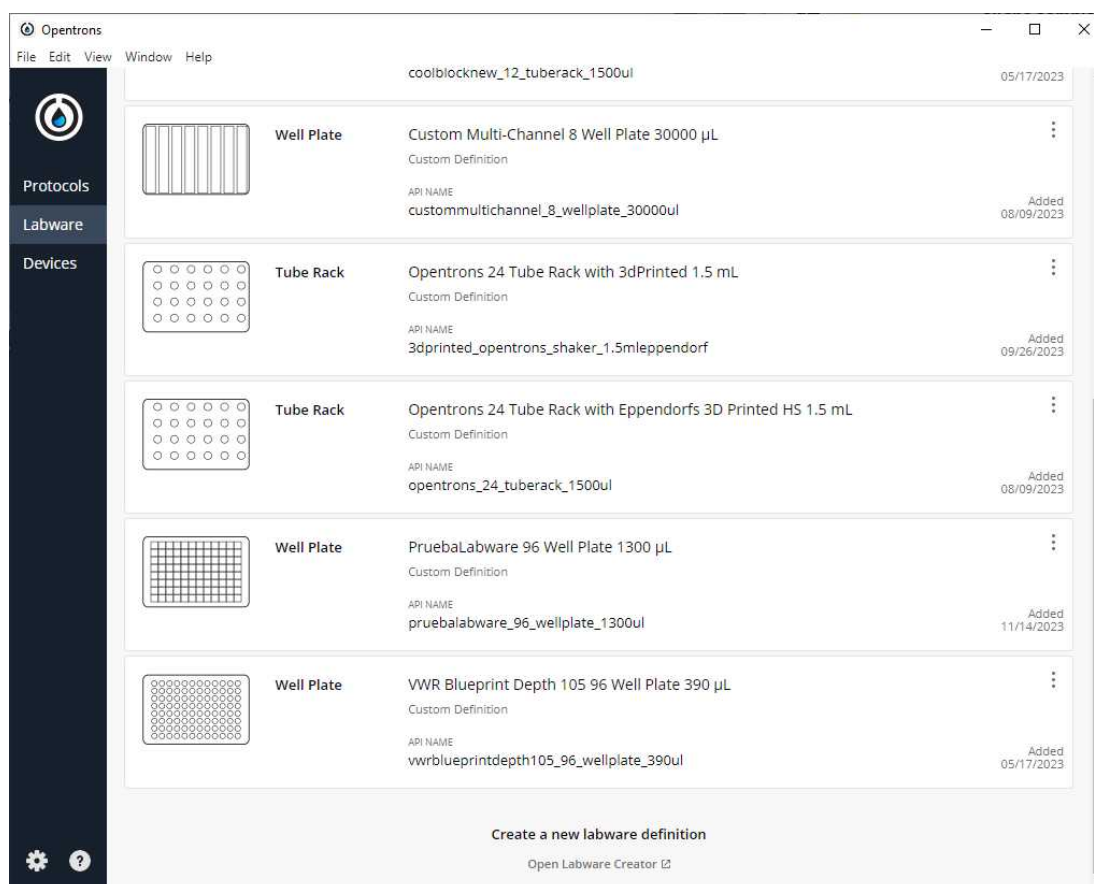
2m

We are using a custom labware called *vwrblueprintdepth105_96_wellplate_390ul* and *3dprinted_opentrons_shaker_1.5mleppendorf* that has been created with the labware creator that opentrons offer (<https://labware.opentrons.com/create/>)

`vwrblueprintdepth105_96_wellplate_390ul.json` 11KB

`3dprinted_opentrons_shaker_1.5mleppendorf.json` 3KB

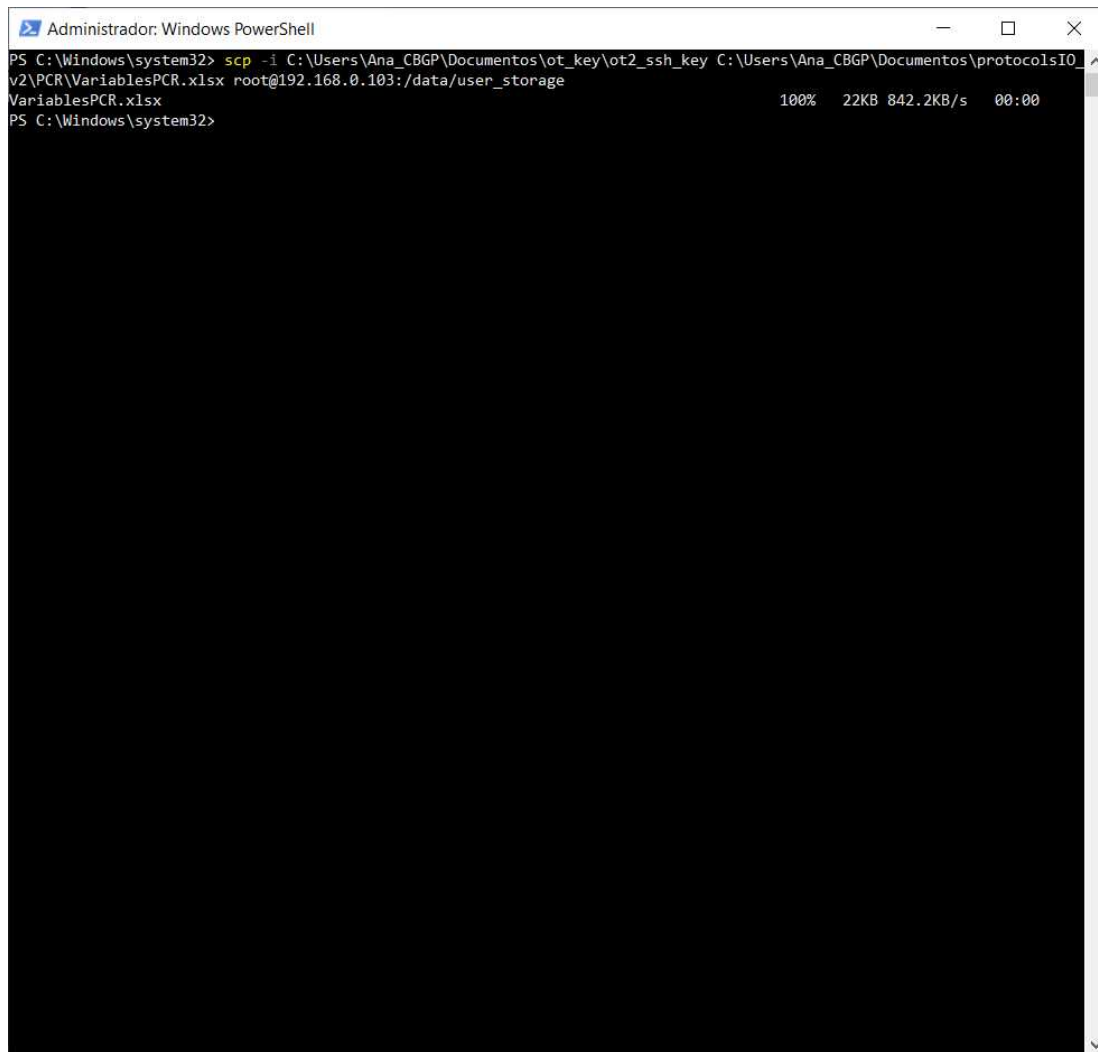
Upload it to the labware and make sure it is loaded in the app



List of custom labware recorded in the Opentrons App

9.3 Export the variable file to *the /data/user_storage* folder in the robot

2m



```
Administrador: Windows PowerShell
PS C:\Windows\system32> scp -i C:\Users\Ana_CBG\Documents\ot_key\ot2_ssh_key C:\Users\Ana_CBG\Documents\protocolsIO_v2\PCR\VariablesPCR.xlsx root@192.168.0.103:/data/user_storage
VariablesPCR.xlsx 100% 22KB 842.2KB/s 00:00
PS C:\Windows\system32>
```

command line window with scp commands to transfer the variables .xlsx from our computer to the OT-2

9.4 Because we are using version 1.0.0 of the script in this example, we will transfer the directory of the labwares as well (here we have attached a zip, but it is the folder the one that must be transferred, not the zip) 2m

- ☐ vwrblueprintdepth105_96_wellplate_390ul.zip1KB
- ☐ 3dprinted_opentrons_shaker_1.5mleppendorf.zip1KB

Command

Transferring the used custom labware to OT (Linux)

```
scp -i [ot_key] -r vwrblueprintdepth105_96_wellplate_390ul root@[IP_OT]:/data/labware/v2/custom_definitions/custom_beta
```

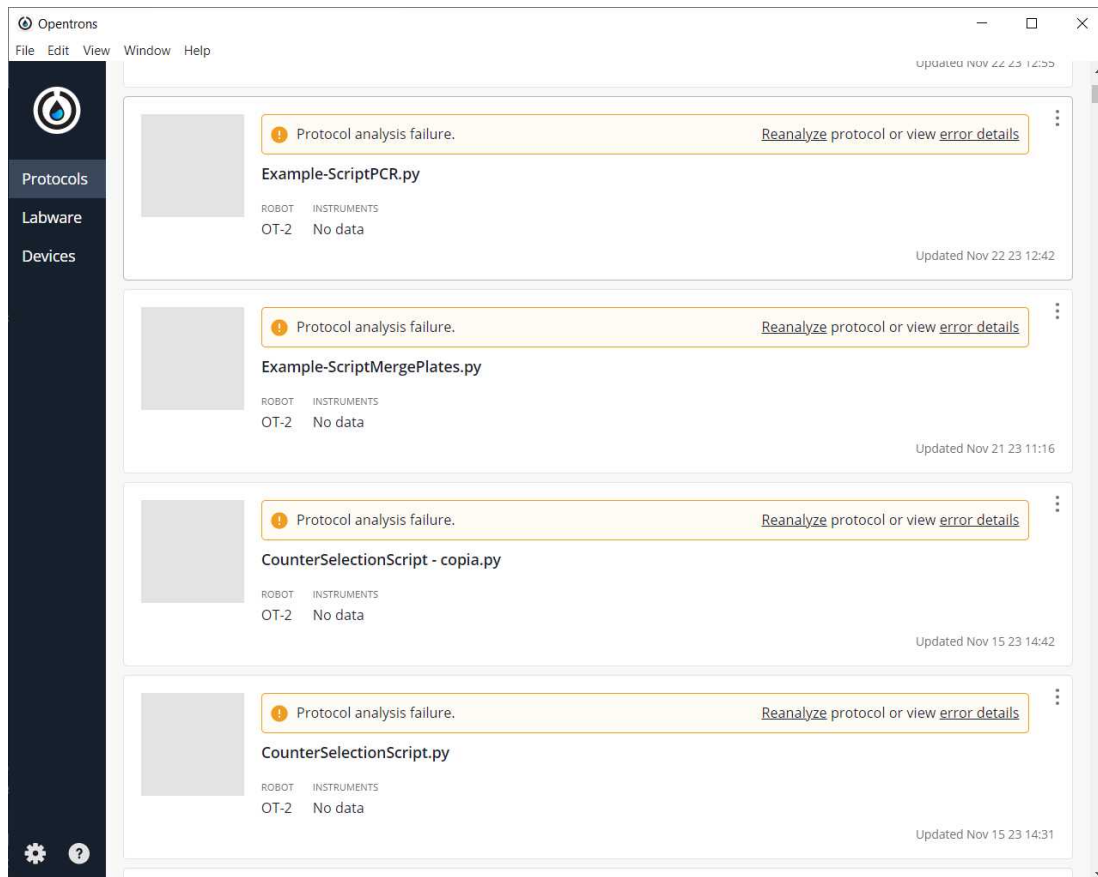
Command

Transferring the used custom labware to OT (Linux)

```
scp -i [ot_key] -r 3dprinted_opentrons_shaker_1.5mleppendorf root@[IP_OT]:/data/labware/v2/custom_definitions/custom_beta
```

- 9.5 Import the script that we have downloaded from the step [go to step #5.1](#) (I named it *ExampleScriptPCR.py*) to the OT-App 30s

 Example-ScriptPCR.py 81KB



The result of importing the Python script into the OT-App

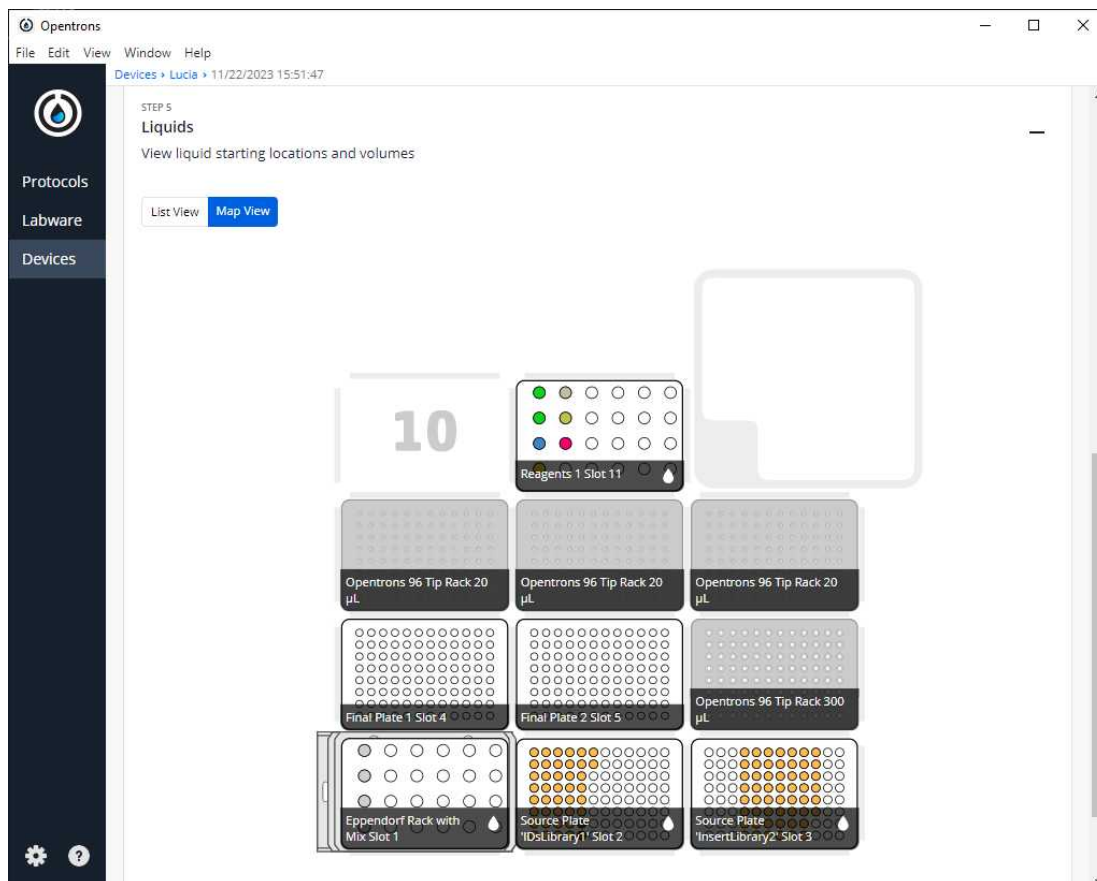
As we can see, we have an error, but that is programmed because the script is meant to work in the robot but not in your computer

9.6 Run the protocol in the robot that we have transferred the Excel file

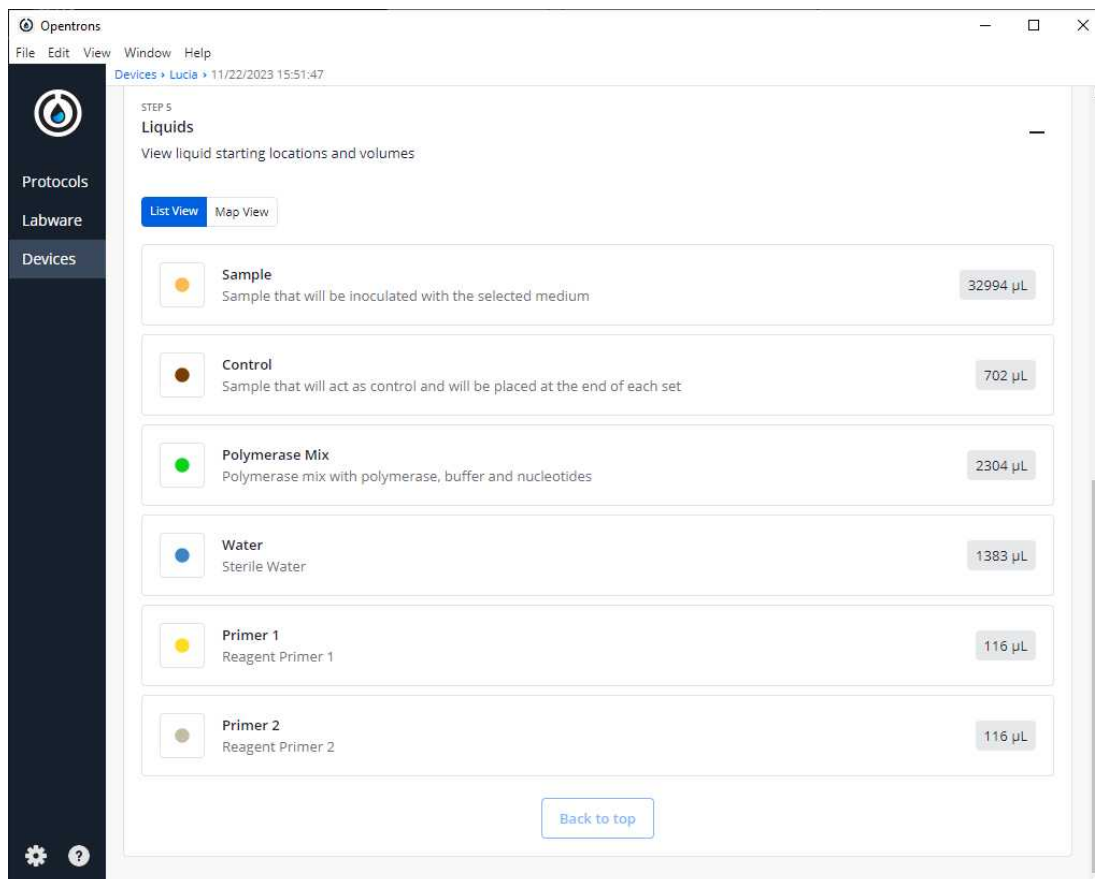
1m

Example-ScriptPCR.py -> Start setup -> Select robot in which we are going to run the protocol

If we do not have any errors, the output should look similar to the following pictures



Labware and liquid set-up layout



Volumes of the different reagents needed to perform the protocol


9.7 Turn the HEPA filter module

30s



9.8 Clean the platform of the robot that we are going to perform the protocol

2m

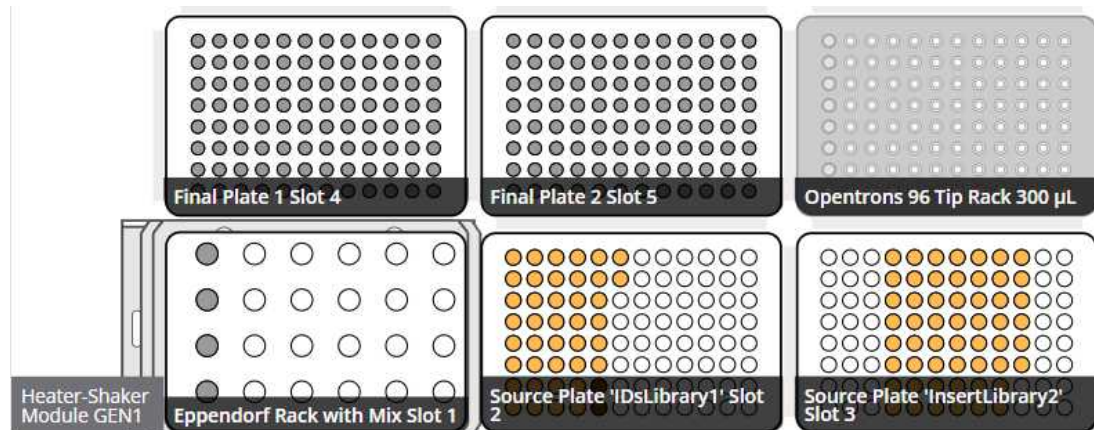
9.9 Prepare all reagents and labware in the places the App is showing and take into account the notes
step  go to step #5.2 Notes

5m

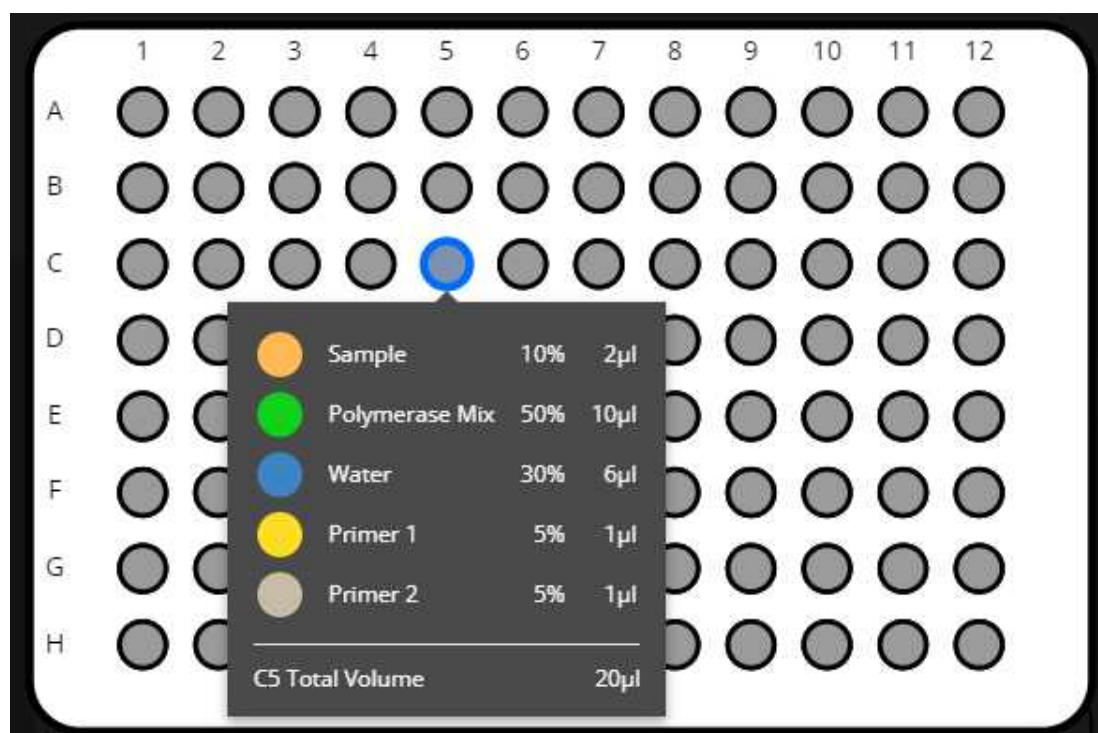
9.10 Start run

2h

Expected result



Final layout of source and final plates in the run



Example of the content of C5 in the labware *Final Plate 1 Slot 4*

Here, we will obtain the mix between the PCR mixes and the samples selected by the values in the variable file. These positions are seen in the image by the grey wells, and we can see the info of the plate and the media in the plate on slot 4 in this case

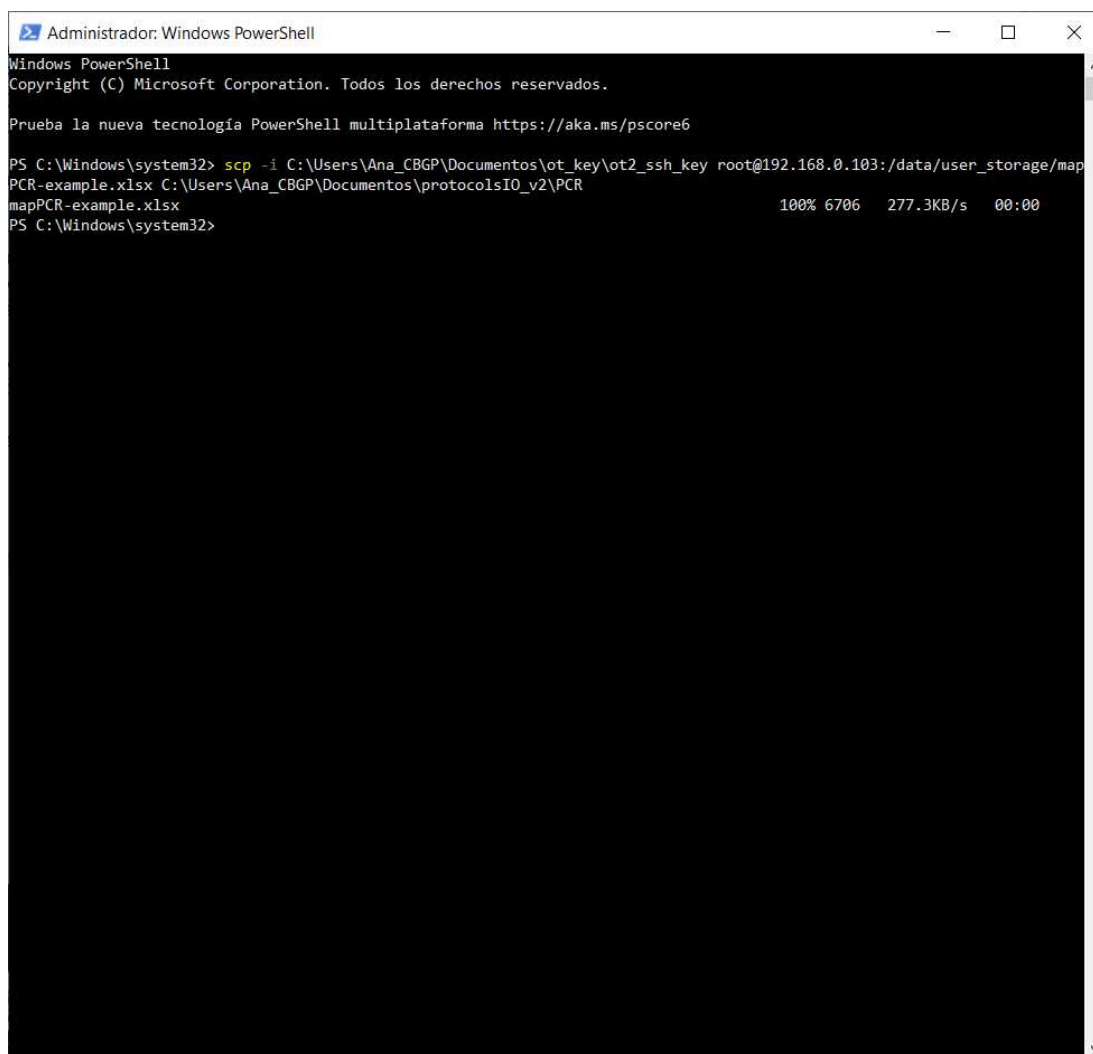
9.11 Retrieve labwares from the OT

5m

9.12

Retrieve the final maps that are going to be in the file. In this case, they will be called *example-mapPCR-example.xlsx* (name that is stated in the variable file in the variable *Final Map Name*)

1m




```
Administrador: Windows PowerShell
Windows PowerShell
Copyright (C) Microsoft Corporation. Todos los derechos reservados.

Prueba la nueva tecnología PowerShell multiplataforma https://aka.ms/pscore6

PS C:\Windows\system32> scp -i C:\Users\Ana_CBG\Documents\ot_key\ot2_ssh_key root@192.168.0.103:/data/user_storage/map
PCR-example.xlsx C:\Users\Ana_CBG\Documents\protocolsIO_v2\PCR
mapPCR-example.xlsx                               100% 6706   277.3KB/s   00:00
PS C:\Windows\system32>
```

command line windows with the transfer command of the final file with the map(s) from the OT to our computer

 mapPCR-example.xlsx10KB