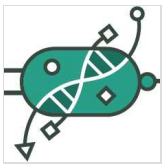

Oct 27, 2022

High-throughput workflow for the genotypic characterization of transposon library variants

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biocomp.cbgp

ABSTRACT

This is a workflow for the genotypic characterization of transposon library variants. It has been developed using an open-source Opentrons OT-2 robot, BLASTN for genomic annotations and modular sub-protocols (e.g., PCR sample preparation, OT-2 volume transfer, OT-2 counter selection, etc) that can be used for other tasks, thus providing a general-purpose pipeline.

All steps follow a 96-well plate format for high-throughput analysis. The protocol is described for the characterization of transposon library variants generated with SEVA-Sib pBAMD1-x and pBLAM1-x plasmid sets that follow Standard European Vector Architecture (SEVA, https://seva-plasmids.com) and can be amplified with the standard PS1-PS6 primers. After the description of the protocol we present the results of an example generated at our laboratory (https://biocomputationlab.com) using the soil bacterium *Pseudomonas putida* KT2440 as acceptor strain.

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PROTOCOL CITATION

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High-throughput, Transposon library, marC9, Tn5, SEVA, OT-2, Opentrons, genotypic characterization, genotyping, bacterial genome, 96-well

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PROTOCOL INTEGER ID

70220

GUIDELINES

This workflow comprises the following sections: 1) Colony picking in selective media 2) Counter-selection and glycerol stocks pre-cultures 3) Colony selection in OT-2 liquid handler robot 4) Master 96-well plate for PCR steps 5) Control PCRs (spurious plasmid integration control and cargo insertion control) 5) Arbitrary PCRs 6) Sequencing and annotation. There is an additional section with an example on how to run the script.
We recommend the use of an OT-2 protocol specially if more than 2 libraries are to be analyzed. However, we recommend to do counter-selection in the OT-2 liquid handling robot even for one plate to avoid human errors. Note that other pipettes can be used to run the workflow in the OT-2 but these were deemed the most appropriate for the overall workflow to minimize pipette changes.

MATERIALS TEXT

Equipment:

Incubating mini-shaker
Incubating shaker
Fisherbrand **15554070**

OT-2
Liquid handler
Opentrons **OT-2**

SPECTROstar Nano
plate reader
BMG **SPECTROstar Nano**

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Mastercycler® nexus - PCR Thermal Cycler
Thermocycler
Eppendorf **12304943**

Centrifuge Tube Mini-Cooler
Cold Block
BRAND **10141921**

Electrophoresis machine

Wet-lab requirements:

Material

- Non-treated flat bottom sterile 96-well plates (i.e 96-well plates flat bottom non-treated **VWR Avantor Catalog #734-2781**)
- Sterile breathable membrane for 96-well plate (i.e Greiner Bio-One Sellador BREATHseal™ **Fisher Scientific Catalog #11920667**)
- Storage membrane for 96-well plate (i.e Thermo Scientific™ Láminas de papel de aluminio adhesivas para placas de PCR **Fisher Scientific Catalog #10130853**)
- PCR plates (pink ones)
- Lids for plates

Enzymes

- DNA polymerase with green buffer

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

Thermo Scientific™ Phire Hot Start II PCR Master Mix **Fisher**

- DNA polymerase (**Scientific Catalog #15361732**)
- Agarose

Oligonucleotides:

- Spurious integration control: PS3, PS4, PS5, PS6 (
- 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 (https://doi.org/10.3389/fbioe.2014.00046)
- Optional insert control: PSMCS

Dry-lab requirements:

- Python 3
- Command-line BLASTN

Colony picking in selective media 1d

1 Dispense **100 µL** of selective media (M9-citrate for *P. putida* or Luria-Bertani plus 20 ng/µL nalidixic acid for *DH5α E. coli*) plus **transposon cassette antiobiotic** in a 96-well plate

2 Pick individual colonies into a 96-well plate with selective media
Tip: Keep tips inside of wells to keep track

3 Cover with a sterile breathable membrane

4

Grow **Overnight** at **30 °C** (*P. putida*) or **37 °C** (*E. coli*) / **500 rpm**

Counter-selection and glycerol stocks pre-cultures 1d

5

Measure OD_{600nm} of overnight culture grown in selective media from **go to step #4** plus **transposon cassette antibiotic** in a plate reader

6 Inoculation of **counter-selection plate** in selective media: 10m

- Dispense **100 µL** of selective media (M9-citrate for *P. putida* or Luria-Bertani plus 20 ng/µL nalidixic acid for DH5α *E. coli*) plus **ampicillin** (backbone antibiotic) to select against spurious integration events.
- Transfer **5 µL** of overnight culture from **go to step #4** to counter-selection (ampicillin) plate
- Cover with a sterile breathable membrane

For steps 6 and 7, if two or more 96-well plates are used as input it is advised to use the **OT-2** protocol below to minimize human error. Dispensed volume and culture volume inoculated should be that described in these steps. Note that steps 6 and 7 could be completed together in a single run depending on the number of initial plates.

OT-2 Media dispensing and culture inoculation protocol
by biocomp.cbgp
PREVIEW RUN

Citation: Lorea Alejaldre, Ana Mariya Anhel, ÃÂngel GoÃÂ±i-Moreno High-throughput workflow for the genotypic characterization of transposon library variants https://dx.doi.org/10.17504/protocols.io.kqdg394jzg25/v1

7 Inoculation of precultures for **glycerol stock** in rich media: 10m

- Dispense **100 µL** of Luria-Bertani media plus **transposon cassette antiobiotic** in a 96-well plate
- Transfer **5 µL** of overnight culture from **go to step #4** to counter-selection (ampicillin) plate
- Cover with a sterile breathable membrane

8 16h

Grow counter-selection and glycerol stock pre-culture plates **Overnight** at **30 °C** (*P. putida*) or **37 °C** (*E. coli*) / **500 rpm**

9

Measure OD_{600nm} of overnight culture grown in selective media plus **ampicillin** in a plate reader

Colony selection in OT-2 liquid handler robot

10

- Selection of colonies to store as glycerol stocks and do further PCR reactions by running the following OT-2 protocol with its corresponding template.csv

OT-2 Counter-Selection
by biocomp.cbgp

PREVIEW RUN

The OT-2 protocol will prepare three plates (2 glycerol stock plates and a "PCR plate") and perform the following:

- Dispense **75 µL** of PCR-grade water to "PCR plate"
- Dispense **25 µL** of 30% glycerol to two glycerol stock plates
- Transfer **25 µL** of grown pre culture in Luria-Bertani media plus **transposon cassette antibiotic** from **go to step #7** to "PCR plate" and glycerol stock plates

11 Cover glycerol stock plates with a storage membrane and store at -80ºC

12

If not proceeding to the next step right away: Store "PCR plate" at 4ºC for a few days or cover with an storage membrane and store at -20ºC for longer term

Master 96-well plate for PCR steps

13

Transfer **50 µL** of selected colonies from one or more libraries to a 96-well plate with the following OT-2 protocol:

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OT-2 Protocol to transfer volume from several plates to a single plate
by biocomp.cbgp

PREVIEW RUN

Control PCRs

14

Positive control (donor plasmid) and wild-type control (*P. putida or E. coli*) should be added to every reaction in this section.

Spurious integration control with SEVA primers pairs PS3/PS4 and PS5/PS6

OT-2 PCR sample preparation protocol
by biocomp.cbgp

PREVIEW RUN

If <48 cfu are to be analyzed both PS3/PS4 and PS5/PS6 spurious integration controls can be done in a single 96-well plate

15

Optional: Cargo integration control with primers PSMCS and either ME-O-Km-R/ME-O-Sm-R or ME-O-Gm-R (depending on **transposon cassette antibiotic**)

Arbitrary PCRs

16

Arbitrary PCR#1 using primer pairs ARB6 and ME-O-Km-Ext-F/ME-O-Sm-Ext-F or ME-O-Gm-Ext-F depending on transposon antibiotic cassette

OT-2 PCR sample preparation protocol
by biocomp.cbgp

PREVIEW RUN

The OT-2 protocol will perform the following steps:
- Prepare a PCR master mix
- Dispense 19 µL of PCR mastermix

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- Transfer 2 µL of pre-culture from **go to step #12**

If different primer pairs are added to a single 96-well plate, the OT-2 script should be run separately for each primer pair

17 Seal 96-well plate, place it in thermocycler and run the following PCR program:

A	B	C
98ºC	5 min	
98ºC	10 s	x6 cycles
30ºC	30 s	
72ºC	1 min 30 s	
98ºC	10 s	x30 cycles
45ºC	30 s	
72ºC	1 min 30 s	
72 ºC	5 min	
4ºC	hold	

18

Select 8-12 Arbitrary PCR#1 reactions from the 96-well plate and run them on a 1% agarose gel to verify amplification.

Several bands will appear and even DNA smears even when the reaction has worked perfectly.

19 **Arbitrary PCR#2** using primers pairs ARB2 and ME-O-Km-Int-F/ME-O-Sm-Int-F or ME-O-Gm-Int-F depending on transposon antibiotic cassette

OT-2 PCR sample preparation protocol
by biocomp.cbgp
PREVIEW RUN

The OT-2 protocol will perform the following steps:
- Prepare a PCR master mix
- Transfer 1 µL of PCR product from Arbitrary PCR#1
- Dispense 19 µL of PCR mastermix

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20 Seal 96-well plate, place it in thermocycler and run the following PCR program:

A	B	C
98ºC	30 s	
98ºC	10 s	x30 cycles
52ºC	30 s	
72ºC	1 min 30 s	
72ºC	5 min	
4ºC	hold	

21

Select 8-12 Arbitrary PCR#2 reactions from the 96-well plate and run them on a 1% agarose gel to verify amplification

Several bands will appear and even DNA smears even when the reaction has worked perfectly.

Sequencing and annotation 1m

22 Prepare a PCR plate to send to sequencing by mixing **10 µL** of unpurified **Arbitrary PCR#2** reaction and **10 µL** of 10 µM sequencing primer (ME-O-Km-Ext-F/ME-O-Sm-Ext-F or ME-O-Gm-Ext-F depending on the transposon antibiotic cassette)

These guidelines may vary depending on the sequencing service arranged for your laboratory.

23 1m

Annotate sequencing results by running the following protocol:

Bacterial genome annotation script using BLASTN
by biocomp.cbgp
PREVIEW RUN

The python script uses as input:
1. DNA sequencing results in .txt or .seq
2. Reference genome file in fasta format
3. Genome annotation file in fasta format

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Example

24 In this section we show a specific example on how to run the workflow with the OT-2 liquid handler using a starting point three 96-well plates with *P. putida* KT2440 colonies picked from matings using cargos with either a kanamycin (pBLAM1-2), streptomycin (pBLAM1-4) or gentamicin (pBLAM1-6) resistance gene.

25

Counter-selection and glycerol stocks pre-cultures using the following OT-2 protocol in **go to step #6** in two runs

25.1 The following template. csv will dispense M9-citrate media containing ampicillin to three plates. Subsequently, each plate will be inoculated from each of the three starting plates.

Variables-AntibioticPlatesCreation-OT.csv

25.2 The following template. csv will dispense LB media with either gentamicin, kanamycin or streptomycin to each plates. Subsequently, each plate will be inoculated from each of the three starting plates.

Variables-AntibioticPlatesCreation-OT.csv

26 **Colony selection in OT-2 liquid handler robot.**

Each LB plus cargo antibiotic plate will be run separately with the script in **go to step #10** . For each library there is an input .csv file for the absorbance at 600nm in M9-citrate with either kanamycin, gentamicin or streptomycin and another .csv file for the absorbance at 600nm in M9-citrate ampicillin. The LB plate from step 25.2 will be used to inoculate the two glycerol stock plates and PCR plate.

26.1 pBLAM1-2: .csv files and template .csv to run script

Variables-ColonieScreening-OT.csv

220920_M9Cit_ant_LibpBLAM12.csv
220921_M9Cit_amp_LibpBLAM12.csv

Output from the run (map with identifiers)

map_220921_LibpBLAM12.csv

26.2 pBLAM1-4: .csv files and template .csv to run script

Variables-ColonieScreening-OT.csv

220920_M9Cit_ant_LibpBLAM14.csv
220921_M9Cit_amp_LibpBLAM14.csv

Output from the run (map with identifiers)

map_220921_LibpBLAM14.csv

26.3 pBLAM1-6: .csv files and template .csv to run script

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Variables-ColonieScreening-OT.csv

220920_M9Cit_ant_LibpBLAM16.csv
220921_M9Cit_amp_LibpBLAM16.csv

Output from the run (map with identifiers)

map_220921_LibpBLAM16.csv

27 **Master 96-well plate for PCR steps** to combine cultures from different transposon libraries in a single 96-well plate

The following template.csv is to be run with the OT-2 script in **go to step #13**

Variables-SamplesMerging-OT.csv

Output from the run (map with identifiers)

maps_master_pcr-220923_5.csv

28 **Control PCRs** to account for spurious integrations and the correct integration of the cargo

28.1 **Spurious integration control** with PS3/PS4 and PS5/PS6 SEVA oligonucleotides
The following template.csv was used for the OT-2 script in **go to step #14** to prepare each mastermix with either PS3/PS4 or PS5/PS6 primer pairs and transfer cultures from Master 96-well plate

Variables-PCRs-OT.csv

Gels after PCR protocol in thermocycler

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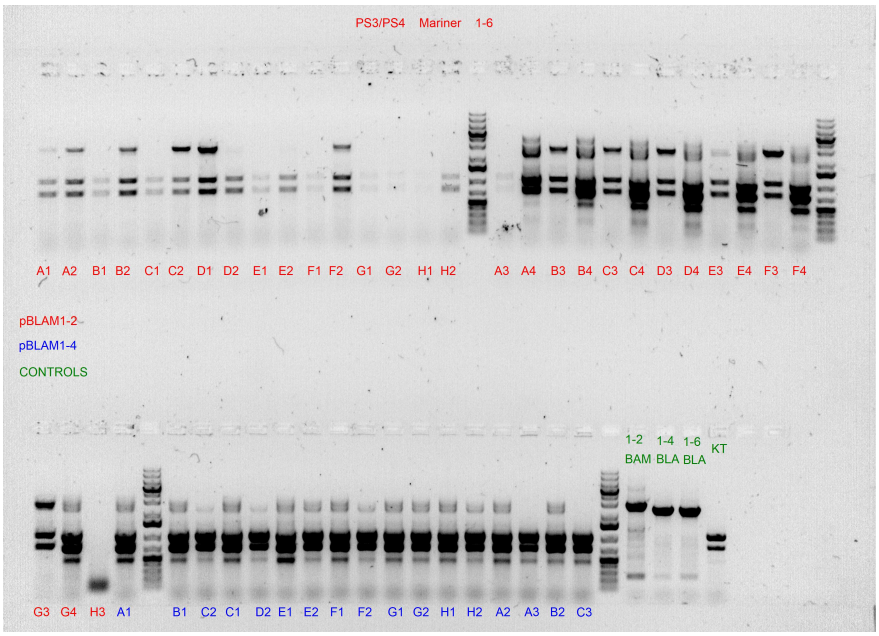
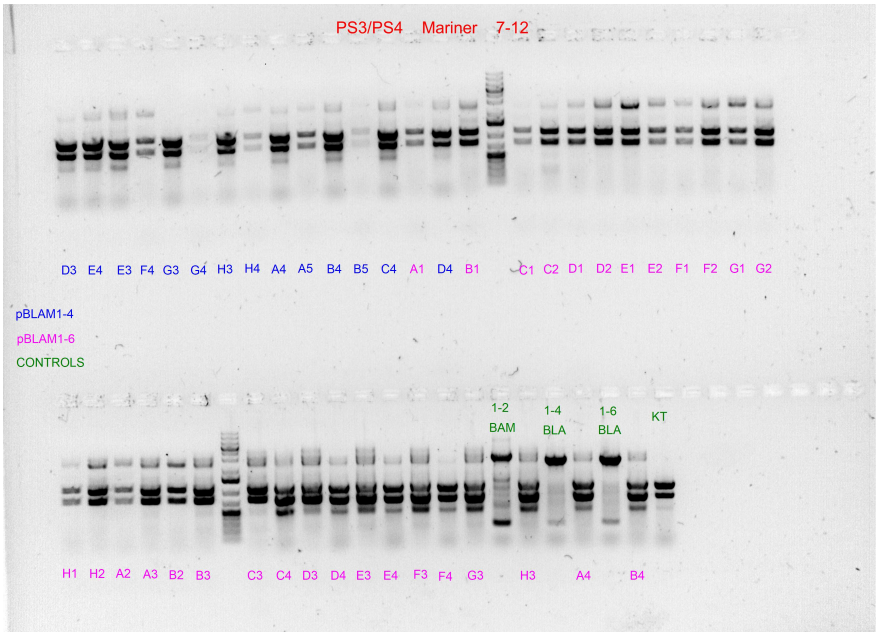


Gel of the first 7 rows with the primers ps3 and ps4



Gel of the last 5 rows with the primers ps3 and ps4

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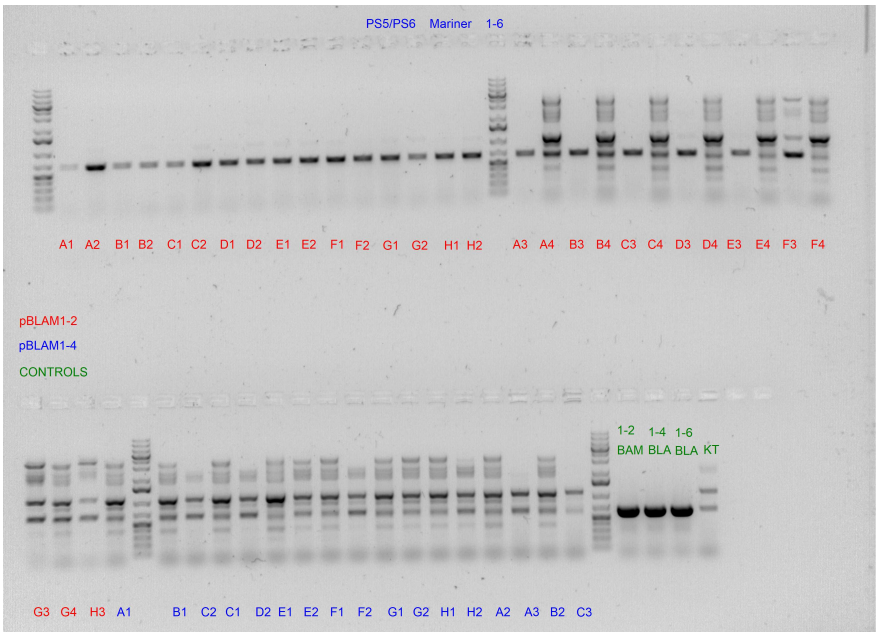
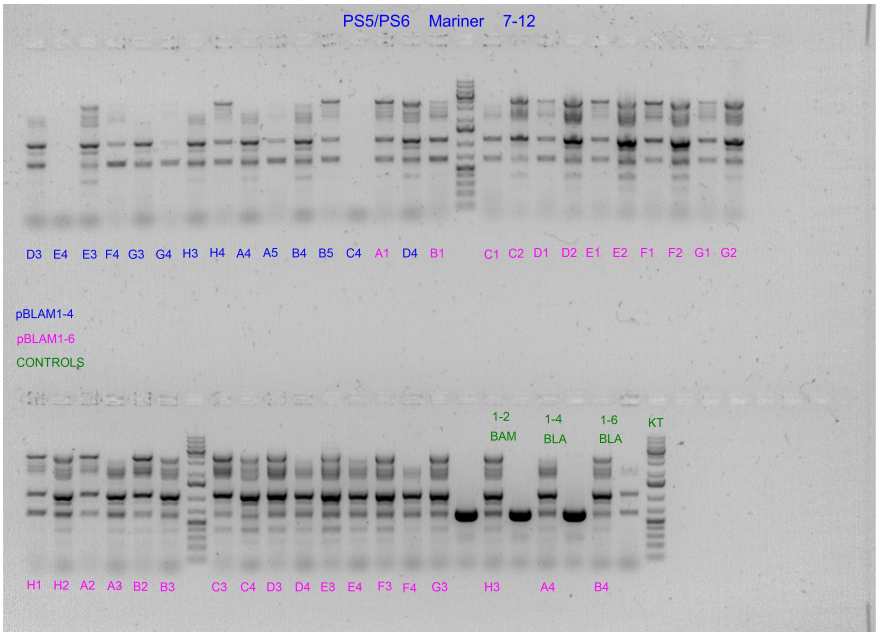


Gel of the first 7 columns with the primers ps5 and ps6



Gel of the last 5 columns with the primers ps5 and ps6

28.2 **Cargo integration control** with PSMCS and either ME-O-Km-R/ME-O-Sm-R or ME-O-Gm-R
The following template.csv was used for the OT-2 script in **go to step #14** to prepare the mastermix and transfer cultures from Master 96-well plate

We have discarded the first 3 columns because they came as negative (without integration) in the spurious PCRs.

Gels after PCR protocol in thermocycler

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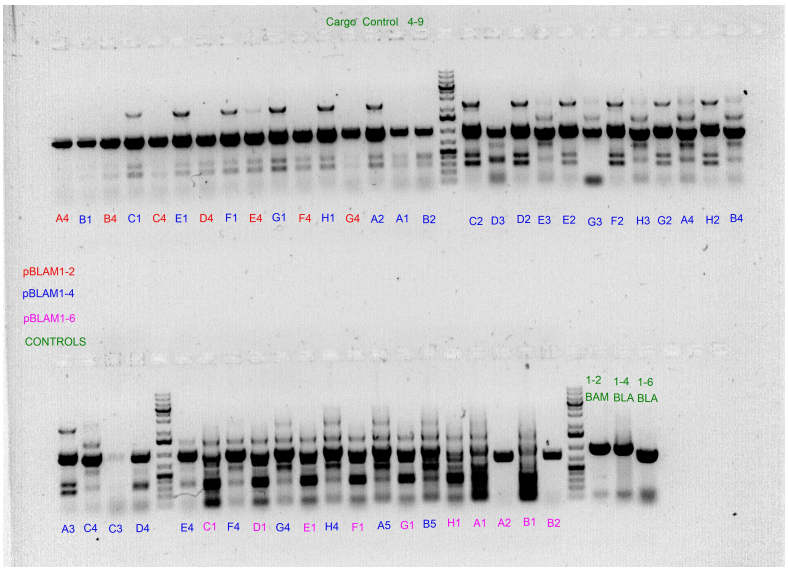
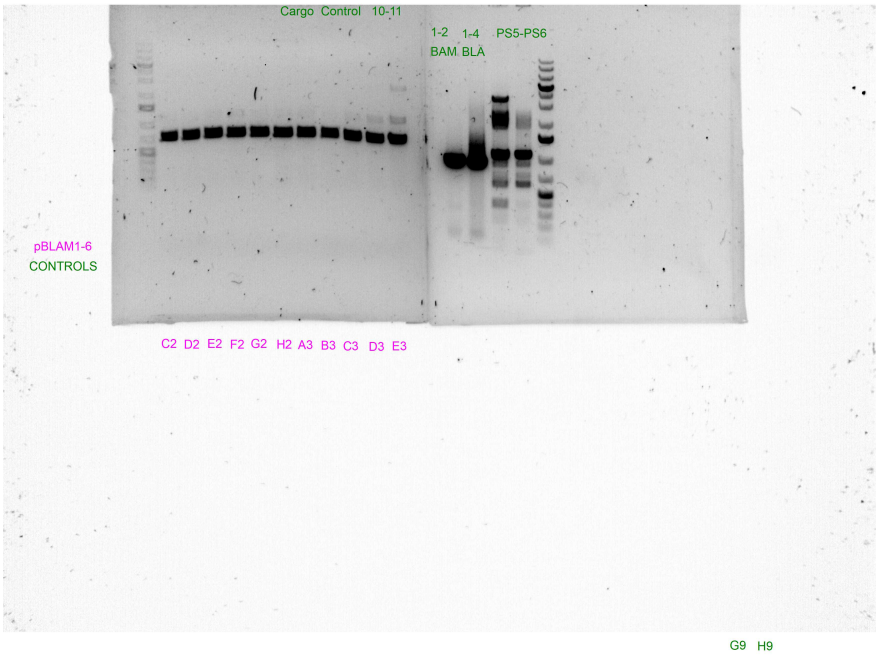


Gel of the columns 4 to 9 of the control of integration primers



Gel of the columns 10 and half of the 11of the control of integration primers

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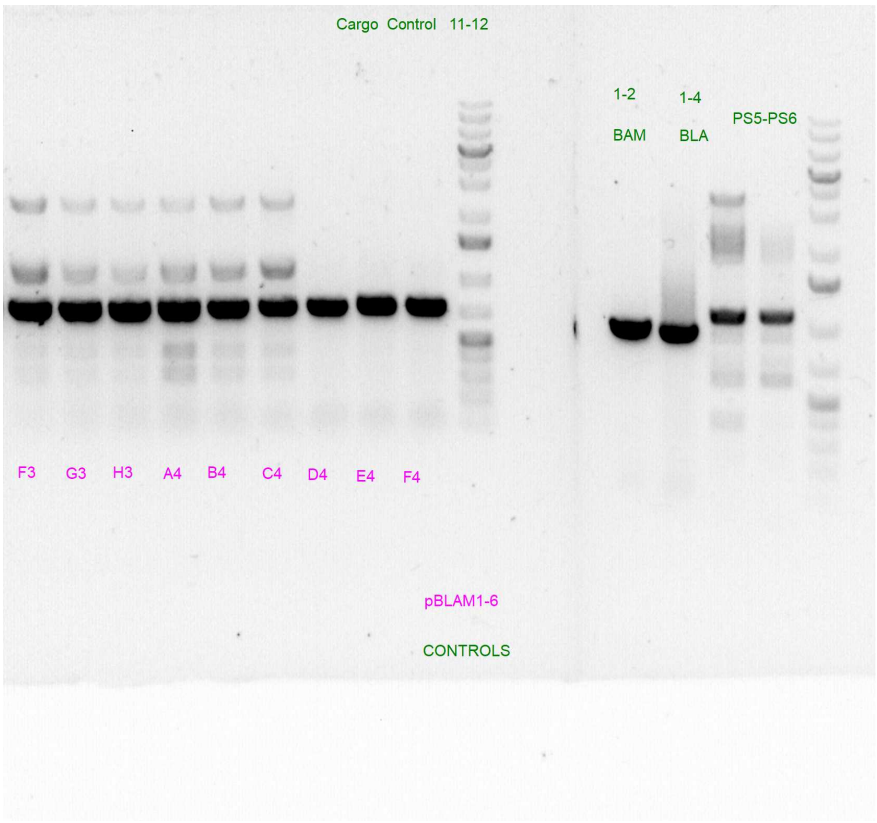

Gel of the columns 11 and half of the 12 of the control of integration primers

29 Arbitrary PCRs

29.1 Arbitrary PCR#1

The following three different template.csv were used (one for each primer pair) in three independent runs with the OT-2 script in **go to step #16** using the same input and output plate to amplify the genomic context around each transposon cassette

Variables for source plate 1 arbitrary PCR 1: **Variables-PCRs-OT.csv**

Variables for source plate 2 arbitrary PCR 1: **Variables-PCRs-OT.csv**

Variables for source plate 3 arbitrary PCR 1: **Variables-PCRs-OT.csv**

29.2 Arbitrary PCR#2

The following three different template.csv were used (one for each primer pair) in three independent runs with the OT-2 script in **go to step #19** using the Arbitrary PCR#1 plate as input and the same output plate to amplify the genomic context around each transposon cassette

Variables for source plate 1 arbitrary PCR 2: **Variables-PCRs-OT.csv**

Variables for source plate 2 arbitrary PCR 2: **Variables-PCRs-OT.csv**

Variables for source plate 3 arbitrary PCR 2: **Variables-PCRs-OT.csv**

Gel results for arbitrary PCRs

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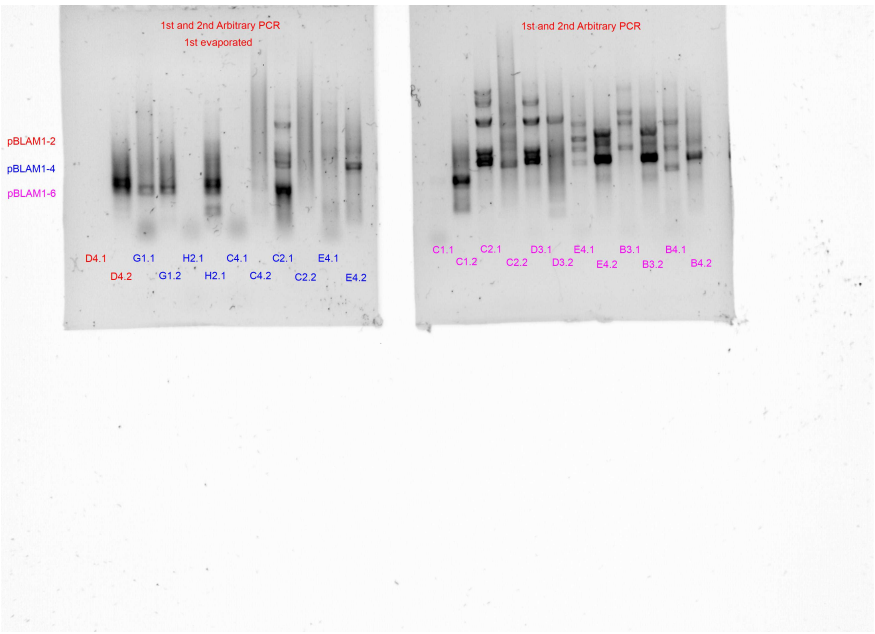

Gel for several wells in the final plate of the arbitrary PCRs. .1 is result of the first PCR and .2 is the second PCR

30 **Sequencing and annotation** of arbitrary PCR#2

The following files were used:

alignment_and_annotation_blastn.py | Pseudomonas_putida_KT2440_110.fna | Pseudomonas_putida_KT2440_110.csv | sequencing_results.zip

We only sent 61 samples, that is why sequencing_results.zip has 61 files

Citation: Lorea Alejaldre, Ana Mariya Anhel, ÃÂngel GoÃÂ±i-Moreno High-throughput workflow for the genotypic characterization of transposon library variants https://dx.doi.org/10.17504/protocols.io.kqdg394jzg25/v1