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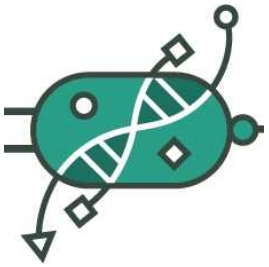
High-throughput workflow for the genotypic characterization of transposon library variants V.1



In 1 collection

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We use this protocol and it's working

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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.

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

Equipment:

Equipment	
Incubating mini-shaker	NAME
Incubating shaker	TYPE
Fisherbrand	BRAND
15554070	SKU
https://www.fishersci.es/shop/products/incubating-mini-shakers-3/15554070	LINK

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

Equipment

SPECTROstar Nano	NAME
plate reader	TYPE
BMG	BRAND
SPECTROstar Nano	SKU
https://www.bmglabtech.com/spectrostar-nano/	LINK

Equipment

Mastercycler® nexus - PCR Thermal Cycler	NAME
Thermocycler	TYPE
Eppendorf	BRAND
12304943	SKU
https://www.fishersci.es/shop/products/mastercycler-nexus-gradient-3/12304943?matchedCatNo=12304943&searchHijack=true&searchTerm=mastercycler-nexus-gradient-3&searchType=Rapid	LINK

Equipment

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

Electrophoresis machine

Wet-lab requirements:

Material

- Non-treated flat bottom sterile 96-well plates (i.e
  96-well plates flat bottom non-treated **VWR International (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
- DNA polymerase (
  Thermo Scientific™ Phire Hot Start II PCR Master Mix **Fisher 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

Note

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.

Protocol

NAME

OT-2 Media dispensing and culture inoculation protocol

CREATED BY

Biocomputation Lab

Preview

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 Grow counter-selection and glycerol stock pre-culture plates Overnight at 30 °C (*P. putida*) or 37 °C (*E. coli*) / 500 rpm 16h

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

Protocol

NAME

OT-2 Counter-Selection

CREATED BY

Biocomputation Lab

Preview

Note

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:

Protocol

NAME

OT-2 Protocol to transfer volume from several plates to a single plate

CREATED BY

Biocomputation Lab

Preview

Control PCRs

14

Safety information

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

Protocol

NAME

OT-2 PCR sample preparation protocol

CREATED BY

Biocomputation Lab

Preview

Note

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

Protocol

NAME

OT-2 PCR sample preparation protocol

CREATED BY

Biocomputation Lab

Preview

Note

The OT-2 protocol will perform the following steps:

- Prepare a PCR master mix
- Dispense 19 µL of PCR mastermix
- Transfer 2 µL of pre-culture from go to step #12

Safety information

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.

Note

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

Protocol

NAME

OT-2 PCR sample preparation protocol

CREATED BY

Biocomputation Lab

Preview

Note

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

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

	A	B	C
	98ºC	30 s	
	98ªC	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

Note

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)

Note

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

23 Annotate sequencing results by running the following protocol: 1m

Protocol

NAME

Bacterial genome annotation script using BLASTN

CREATED BY

Biocomputation Lab

Preview

Note

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

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-...

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-...

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

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)

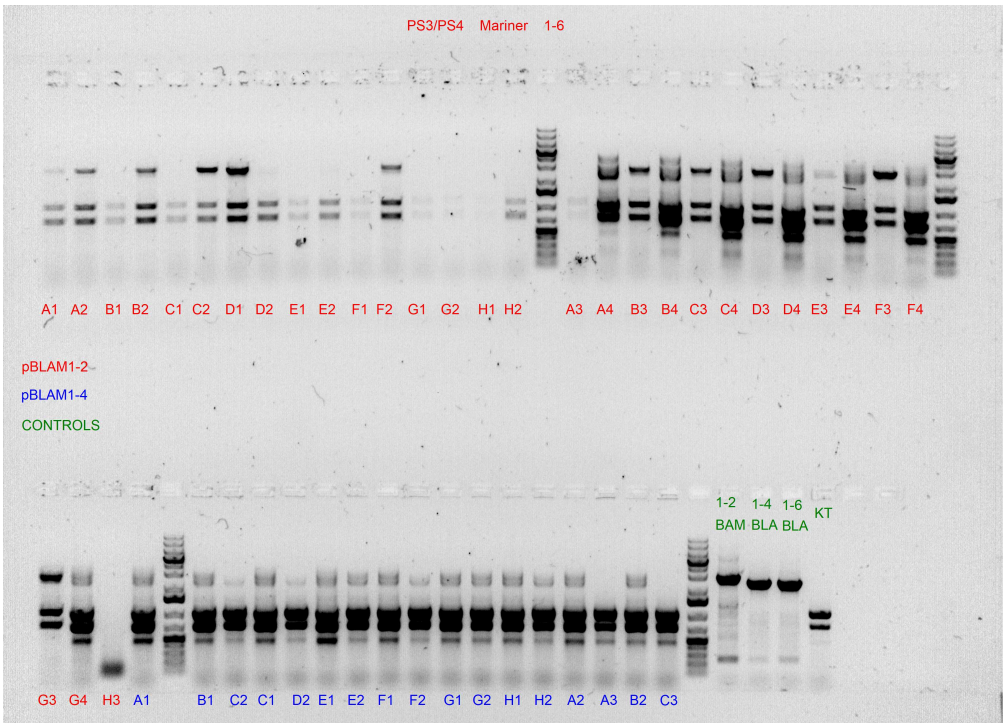
maps_master_pcr-220923_5.csv

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

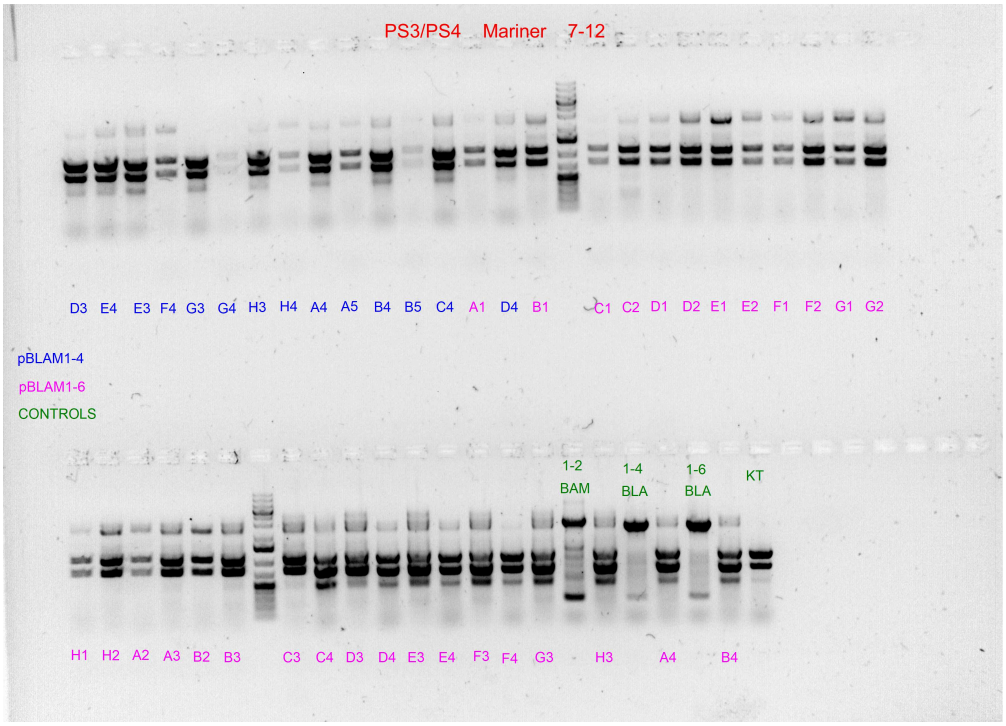
28.1 **Spurious integration control** with PS3/PS4 and PS5/PS6 SEVA oligonucleotides
The following template.csv was used for the OT-2 script in go to step #14 to prepare each mastermix with either PS3/PS4 or PS5/PS6 primer pairs and transfer cultures from Master 96-well plate

Variables-PCRs-OT.csv

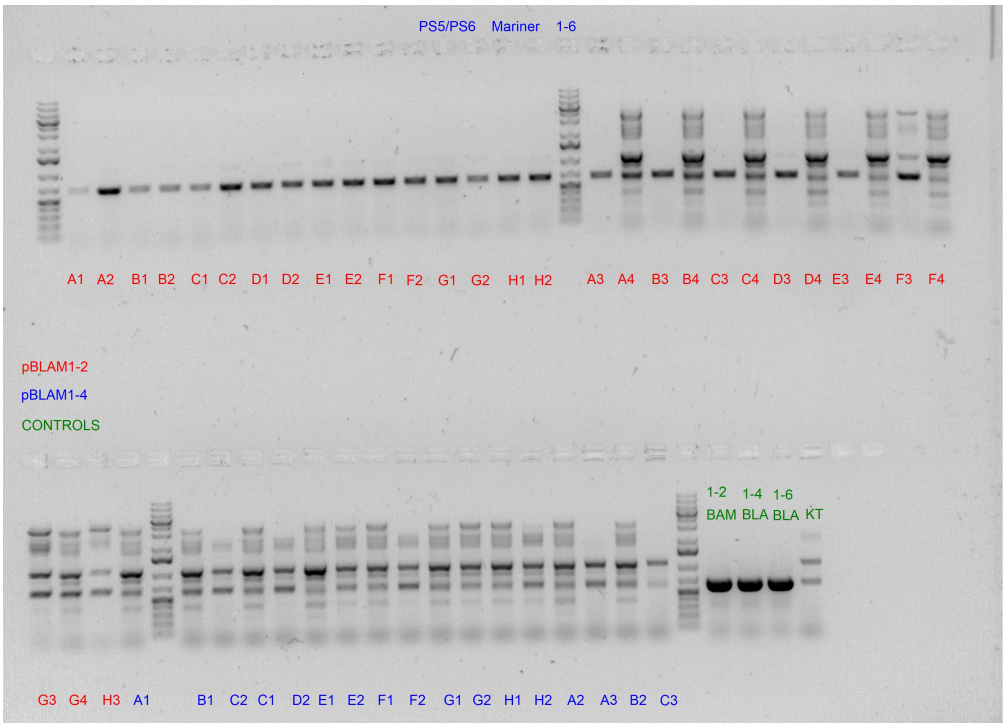
Gels after PCR protocol in thermocycler



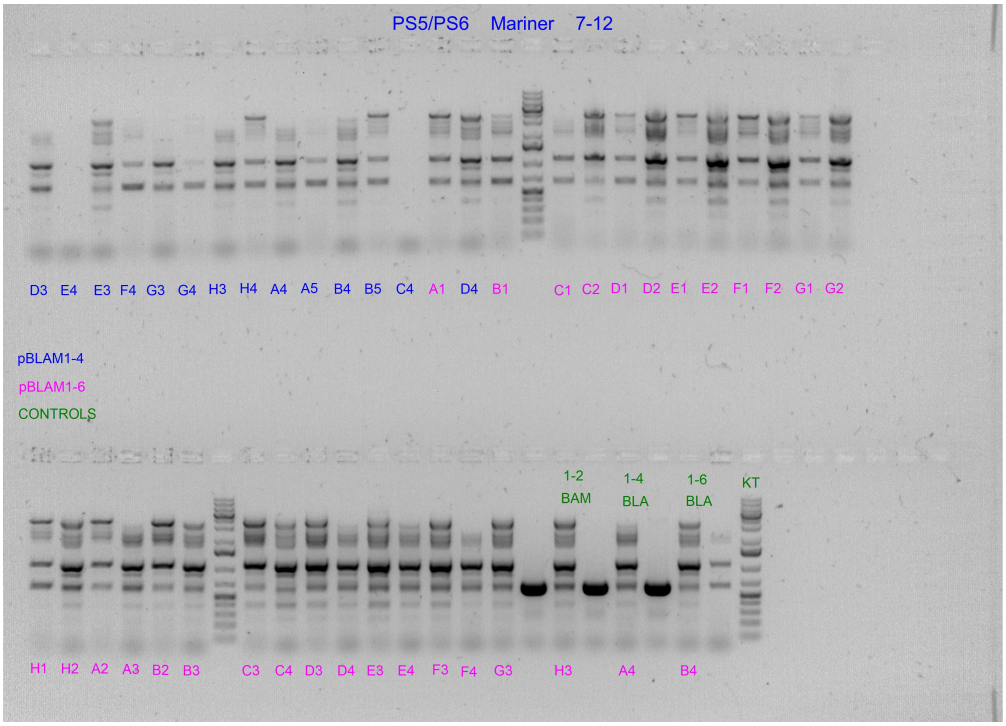
Gel of the first 7 rows with the primers ps3 and ps4



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



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



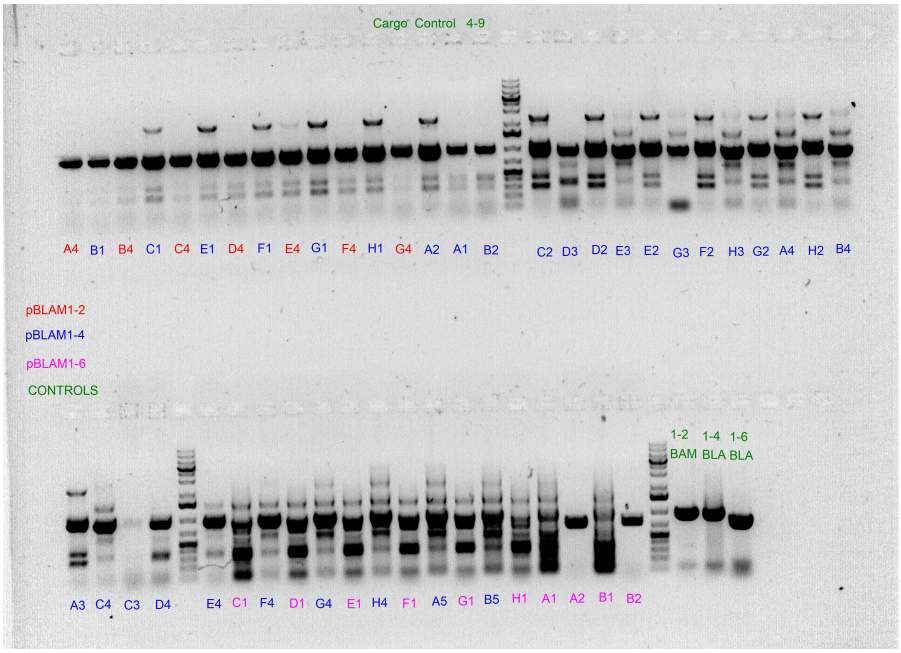
Gel of the last 5 columns with the primers ps5 and ps6

28.2 **Cargo integration control** with PSMCS and either ME-O-Km-R/ME-O-Sm-R or ME-O-Gm-R

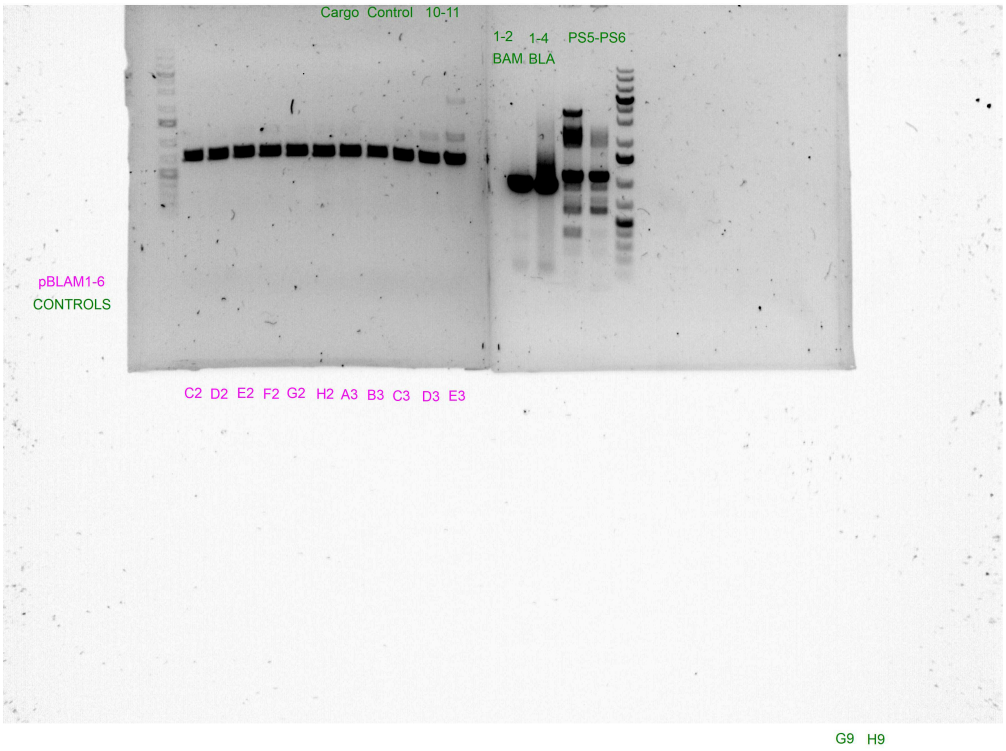
The following template.csv was used for the OT-2 script in go to step #14 to prepare the mastermix and transfer cultures from Master 96-well plate

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

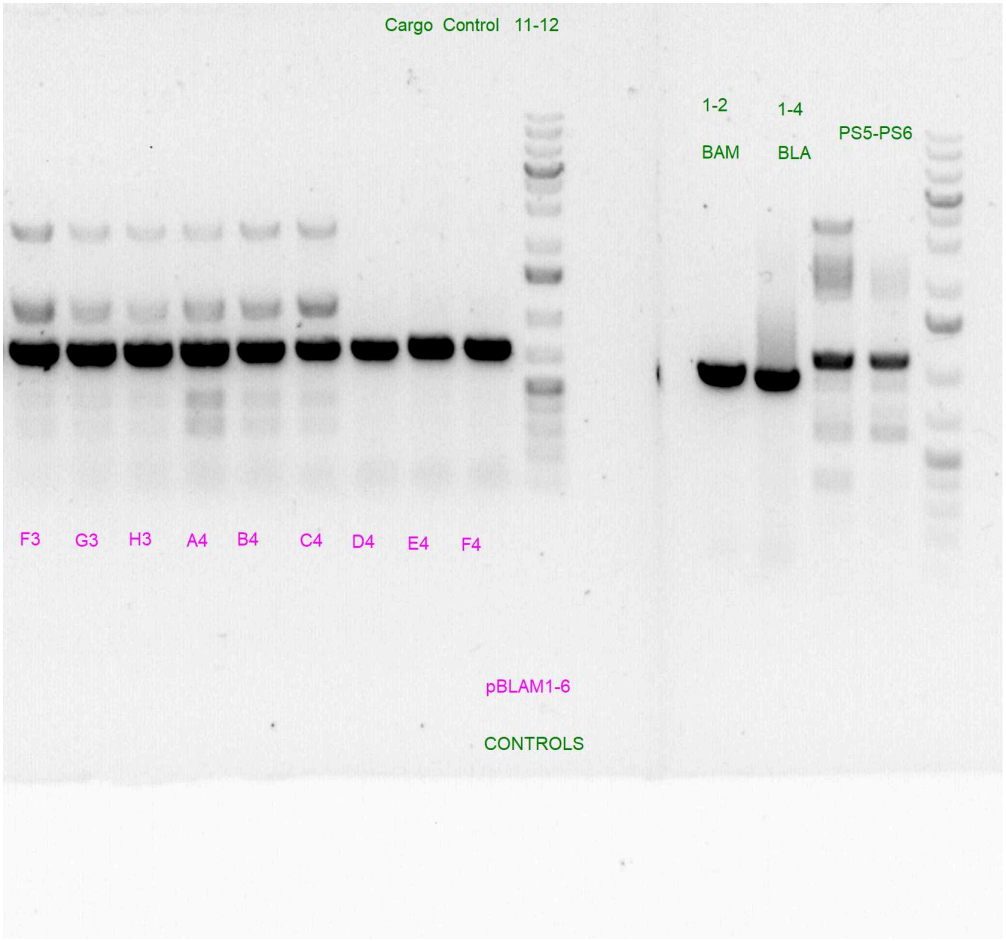
Gels after PCR protocol in thermocycler



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



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**

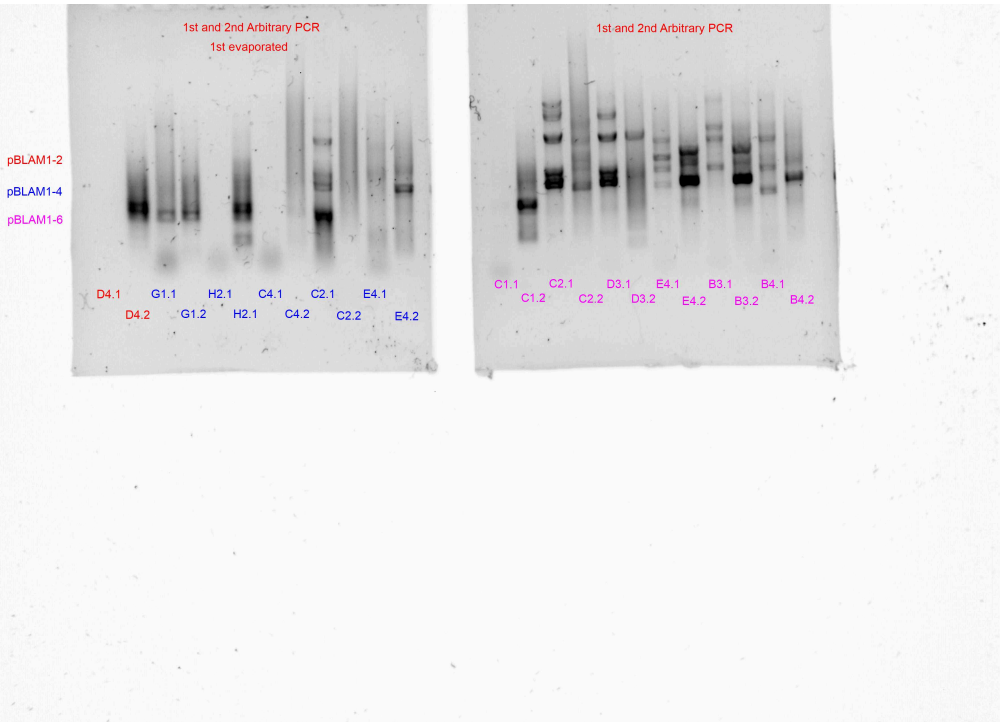
The following three different template.csv were used (one for each primer pair) in three independent runs with the OT-2 script in go to step #19 using the Arbitrary PCR#1 plate as input and the same output plate to amplify the genomic context around each transposon cassette

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



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....

Pseudomonas_putida_KT2440_110....

sequencing_results.zip

Note

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