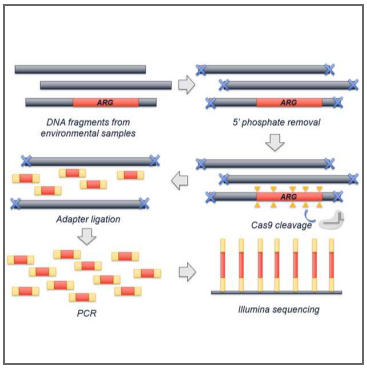

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Protocol status: In development
We are still developing and optimizing this protocol

Multiplexed CRISPR-based target-enriched next-generation sequencing for detecting antibiotic resistance genes in environmental samples

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ABSTRACT

High-throughput detection of antibiotic resistance genes (ARGs) in complex environmental samples is challenging for two reasons: 1) ARGs account for less than 0.1% of total DNA in an environmental sample, and 2) it is difficult to detect thousands of ARGs in one reaction. Conventional methods, including metagenomic sequencing and quantitative polymerase chain reaction (qPCR), have their limitations with sensitivity and target range, respectively. Here, we propose a multiplexed CRISPR-Cas9-based target-enriched next-generation sequencing (NGS) method to detect thousands of ARGs in complex environmental samples, using sewage as a testbed. This protocol includes guide RNA design, guide RNA synthesis, DNA sample preparation, CRISPR-NGS library preparation, and data processing steps. With this protocol, ARGs in low abundances can be detected with increased read depth and higher sensitivity than regular metagenomic NGS methods. This protocol is also applicable for detecting other low-abundance genetic markers, for example, bacterial virulence factors, in environmental samples.

MATERIALS

Consumables

1. Nuclease-free 1.5 mL microcentrifuge tubes
2. Nuclease-free 0.5 mL microcentrifuge tubes
3. Nuclease-free 0.2 mL PCR tubes
4. Nuclease-free 1000 µL pipette tips with filter
5. Nuclease-free 200 µL pipette tips with filter
6. Nuclease-free 20 µL pipette tips with filter
7. Nuclease-free 10 µL pipette tips with filter
8. MF-Millipore® Membrane Filter, 0.45 µm pore size (Millipore, Catalog #: HAWP04700)
9. Qubit™ Assay Tubes

Buffers and chemicals

1. Molecular biology grade water
2. Nuclease-Free Duplex Buffer (Integrated DNA Technologies, Catalog #: 11-05-01-03/11-01-03-01/11-05-01-12)
3. 100% Ethanol, molecular biology grade
4. NEBuffer™ r3.1 (New England Biolabs, Catalog #: B6003S)
5. dATP Solution (100 mM) (Thermo Scientific, Catalog #: R0141)
6. xGen™ Adapter Buffer, 300 mL (Integrated DNA Technologies, Catalog #: 10006743)
7. TE buffer
8. AMPure XP SPRI Reagent (Beckman Coulter, Catalog #: A63880/ A63881/ A63882)

Kits and master mixes

1. Phusion® High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs, Catalog #: M0531L)
2. TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific, Catalog #: K0441)
3. RNA Clean & Concentrator-5 (DNase Included) (Zymo, Catalog #: R1013/R1014)
4. Qubit™ RNA Broad Range (BR) Assay Kit (Invitrogen, Catalog #: Q10210/Q10211)
5. FastDNA™ SPIN Kit for Soil (MP Biomedicals, Catalog #: 116560200/116560300)
6. OneStep PCR Inhibitor Removal Kit (Zymo, Catalog #: D6030)
7. Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit (Invitrogen, Catalog #: Q33230/Q33231)
8. NEBNext® Ultra™ II Ligation Module (New England Biolabs, Catalog #: E7595S/E7595L)
9. NEBNext® Ultra™ II Q5® Master Mix (New England Biolabs, Catalog #: M0544S/M0544L/M0544X)

Nucleic acids

1. Double-stranded DNA template for tracrRNA (5'-AGGCGAATCAGATAATCGTTATGTCCAGACTGTATTAATACGACTCACTATAGGACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTT-3'), dissolve in molecular biology grade water to reach a final concentration of 1 ng/µL (Quan et al., 2019).
2. DNA oligo pool as the template for crRNA (5'-TAATACGACTCACTATAGXXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTATGCTGTTTTG-3') (replace the XXXXXXXXXXXXXXXXXXXX part by the designed multiplex sequences), dissolve and dilute in molecular biology grade water to reach a final concentration of 10 ng/µL (see the Equation below) (Quan et al., 2019).
3. Primers for DNA template PCR amplification: (Forward primer for both crRNA and tracrRNA: 5'-TAATACGACTCACTATAG-3'; Reverse primer for crRNA: 5'-CAAAACAGCATAGCTCTAAAAC-3'; Reverse primer for tracrRNA: 5'-AAAAGCACCGACTCGGTGCCAC-3'), dissolve in molecular biology grade water to reach a final concentration of 10 µM (Liang et al., 2015).
4. Double-stranded DNA with 5' phosphorylation "NH8B" as the external standard spike (5'-ACCCATACAAGGAACCCGGCCAGCACTACGCTCACTACGGCCGGTGGTACGGTGGGCACTCCGGTGAAATGCACGTGCTTGGCATGCCGTCAGGCCGTGAAGTCAAGCGCACCCCGGTGTTCAACATGGACAGCAACAAGATGACCATCCACATCGCCTCGCCGGCGCCGGCATACAGTCTGGGGGGAATTCAAGATGGAGAAGGGCGACGAGGTAATGGCGATCCTGACCTCGACAAGTGGAAGACCTG-3'), dissolve in molecular biology grade water to reach a final concentration of 10 ng/µL (Zhang & Ishii, 2018).
5. xGen™ UDI-UMI Adapters (Integrated DNA Technologies, Catalog #: 10006914/10005903)
6. xGen™ Library Amplification Primer Mix (Integrated DNA Technologies, Catalog

#: 1077675/1077676/1077677)

Proteins

1. TrueCut™ HiFi Cas9 Protein (5 µg/µL) (Invitrogen, Catalog #: A50576/ A50577)
2. rAPid Alkaline Phosphatase (Roche, Catalog #: 4898133001/ 4898141001)
3. RNase T1 (1000 U/µL) (Thermo Scientific, Catalog #: EN0541/ EN0542)
4. Taq DNA Polymerase with ThermoPol® Buffer (New England Biolabs, Catalog #: M0267S/ M0267L/ M0267X/ M0267E)

Equation: DNA oligo pool total mass calculation

Equation: DNA oligo pool total mass calculation

CITATION

Quan J, Langelier C, Kuchta A, Batson J, Teyssier N, Lyden A, Caldera S, McGeever A, Dimitrov B, King R, Wilheim J, Murphy M, Ares LP, Travisano KA, Sit R, Amato R, Mumbengegwi DR, Smith JL, Bennett A, Gosling R, Mourani PM, Calfee CS, Neff NF, Chow ED, Kim PS, Greenhouse B, DeRisi JL, Crawford ED (2019). FLASH: a next-generation CRISPR diagnostic for multiplexed detection of antimicrobial resistance sequences..
LINK
https://doi.org/10.1093/nar/gkz418

CITATION

Liang X, Potter J, Kumar S, Zou Y, Quintanilla R, Sridharan M, Carte J, Chen W, Roark N, Ranganathan S, Ravinder N, Chesnut JD (2015). Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection..
LINK
https://doi.org/10.1016/j.jbiotec.2015.04.024

CITATION

Zhang Q, Ishii S (2018). Improved simultaneous quantification of multiple waterborne pathogens and fecal indicator bacteria with the use of a sample process control..
LINK
https://doi.org/10.1016/j.watres.2018.03.023

BEFORE START INSTRUCTIONS

It is highly recommended to use DNA Away and RNase Away to clean all surfaces and equipment before wet lab experiments.

Multiplex crRNA design (using FLASHit as an example)

1 Create a Linux environment. It can be set up in MobaXterm (https://mobaxterm.mobatek.net/) or oth 10m
preferred terminal software. Using MobaXterm as an example, the Linux environment can be created by "Sessions" -> "New session" -> WSL. Then, select "Ubuntu" for "Distribution". Click on "OK", and the session will be created and saved.

2 Install FLASHit (https://github.com/czbiohub-sf/flash) in the Linux environment according to the instructions in "Prerequisite" on its GitHub webpage. 1h

CITATION

Quan J, Langelier C, Kuchta A, Batson J, Teyssier N, Lyden A, Caldera S, McGeever A, Dimitrov B, King R, Wilheim J, Murphy M, Ares LP, Travisano KA, Sit R, Amato R, Mumbengegwi DR, Smith JL, Bennett A, Gosling R, Mourani PM, Calfee CS, Neff NF, Chow ED, Kim PS, Greenhouse B, DeRisi JL, Crawford ED (2019). FLASH: a next-generation CRISPR diagnostic for multiplexed detection of antimicrobial resistance sequences..
LINK
https://doi.org/10.1093/nar/gkz418

3 Collect all target genes from databases. Here, as an example, all available sequences for antibiotic resistance genes (ARGs) from The Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/) were downloaded using the link: https://card.mcmaster.ca/latest/data. 10m

4 For an ARG detection project, among all downloaded ".fasta" files, "nucleotide_fasta_protein_homolog_model.fasta" was used as the input, because the ARGs that have raised high concerns such as the *CTX-M* gene families and the *mcr* gene families are included in the

protein homolog model.

5 Trim the “nucleotide_fasta_protein_homolog_model.fasta” file using the Python code below to keep only the antibiotic resistance ontology (ARO) of the ARGs in the titles, because special characters cannot be processed by FLASHit. 10m

Command

```
import re

file=open(r"INPUT_FASTA_FILE_PATH_HERE")
raw_content=file.readlines()
file.close()

output_content=[]

for i in range(0,len(raw_content)):
    if i%2==0:
        aro_number=re.findall(r'ARO:[0-9]+',raw_content[i])[0]
        output_content.append('>'+aro_number.split(':')[1])
    if i%2!=0:
        output_content.append(raw_content[i].strip('\n'))

output_file=open(r" OUTPUT_TRIMMED_FASTA_FILE_PATH_HERE ",'w')

for i in range(0,len(output_content)):
    output_file.write(output_content[i]+'\n')

output_file.close()
```

Note

For DNA sequences downloaded from other databases, the above code may need to be modified. Each ARG has its own corresponding ARO in CARD.

6 In Linux terminal, activate the conda environment, then activate the environment for running FLASHit. 1m

7 *[optional] By default, FLASHit excludes the off-target sites from human genomes and the E. coli BL21 genome. If an environmental sample is expected to include undesired genomes other than these two, for example, swine genomes, users can modify the files in /flash/generated_files/ accordingly.* 5h

7.1 *Search the reference genome of the undesired off-targets from NCBI Genome database (https://www.ncbi.nlm.nih.gov/datasets/genome/) by typing the species name in the search box.*

7.2 *From the search results, click on the genome with* the *NCBI RefSeq label.*

7.3 *Download the genome sequence by choosing "RefSeq only" and "Genome sequences (FASTA)".*

7.4 *Use each ".fasta" file as an input to FLASHit following the guidance in "Creating your own library" in* the *"Workflow" section on the GitHub page of FLASHit.*

7.5 *After "Will discard xxx targets in amibiguous_targets.txt affecting xxx not necessarily unique genes." is shown on the screen, break the current FLASHit run by hitting Ctrl+C.*

7.6 *Go to the directory /flash/generated_files/target_index/, copy "all_targets.txt" to a customized directory, and rename it by the input ".fasta" file name.*

7.7 *After collecting and renaming all "all_targets.txt" files to the customized directory, split files larger than 10 Mb to separated 10 Mb files into a new directory using the command below.*

Command

```
split -b 10m INPUT_FILE_NAME OUTPUT_FILE_PATH_AND_PREFIX
```

Note

Make sure the directory only contains split files, otherwise the other files will be also renamed.

7.8 *In the new directory containing all split files, add ".txt" suffix to all files using the command below.*

Command

```
ls | while read i; do mv ${i} ${i}.txt
```

Note

Make sure the directory only contains split files. Otherwise, the other files will be also renamed.

7.9 *Copy and paste the other files smaller than 10 Mb to the directory containing all split files.*

7.10 *Run the following python code to remove replicated off-targets and organize all off-targets to the same file.*

Command

```
import argparse
import os

parser = argparse.ArgumentParser()
parser.add_argument('-i',dest='input',type=str,required=True,help='Define input txt folder')
parser.add_argument('-o',dest='output',type=str,required=True,help="Define output path for a
combined txt")
args=parser.parse_args()

full_gRNA_list=[]
dir_path=args.input.strip("'")
dir_list=os.listdir(dir_path)
for file_name in dir_list:
    print('Processing '+file_name+' ......')
    file=open(dir_path+'/'+file_name)
    raw_content=file.readlines()
    file.close()
    for i in range(0,len(raw_content)):
        raw_content[i]=raw_content[i].strip('\t\n\r')
    for i in range(0,len(raw_content),3):
        full_gRNA_list.append(raw_content[i])

full_gRNA_list=list(set(full_gRNA_list))

output_file=open(args.output.strip("'"),"w")
for i in range(0,len(full_gRNA_list)):
    output_file.write(full_gRNA_list[i]+'\n')
output_file.close()
```

Note

This code is written to be run by a command line. Users should save it as a ".py" file, and run it by typing "python3 NAME_OF_THE_PYTHON_CODE.py -i THE_DIRECTORY_PATH_CONTAINING_ALL_SPLIT_TXT_FILES -o THE_TXT_FILE_PATH_FOR_THE_OUTPUT_ORGANIZED_TARGET_LIST.txt", quotation marks not included. In addition, this code may require a large memory, especially when the input genome is large. To avoid a *potential crash, it is recommended to run this code on a server, instead of a personal computer.*

7.11 *If there are multiple off-target genomes, place all output ".txt" files generated by the above code into the same directory. The list for human genome off-targets is already provided by FLASHit with the file path "/flash/generated_files/human_guides_38.txt". Combine all off-targets into the same "all_offtargets.txt" file using the following Python code.*

Command

```
import argparse

parser = argparse.ArgumentParser()
parser.add_argument('-i',dest='input',type=str,required=True,help='Define input txt folder')
parser.add_argument('-o',dest='output',type=str,required=True,help="Define output path for a
combined txt")
args=parser.parse_args()

file=open(args.input.strip("'"))
gRNA_list=file.readlines()
file.close()

output_list=sorted(list(set(gRNA_list)))
output_file=open(args.output.strip("'"),'w')
for i in range(0,len(output_list)):
    output_file.write(output_list[i])
output_file.close()
```

Note

This code is also written to be run by a command line. Users should save it as a ".py" file and run it.

7.12 *Replace the "all_offtargets.txt" file in "/flash/generated_files/"* with *the new ".txt" file generated by the code above. Make sure to rename the newly generated ".txt" file to "all_offtargets.txt".*

7.13 *Rename "human_guides_38.txt" and "ecoli_bl21_de3_offtargets.txt" in "/flash/generated_files/" to "human_guides_38.txt1" and "ecoli_bl21_de3_offtargets.txt1" to avoid those two files to be identified by FLASHit by default.*

8 Follow the guidance in "Creating your own library" in "Workflow" section on the GitHub page of FLASH to generate a list for the multiplexed 20-nt target regions for the template of crRNA. 10m

9 Follow the guidance in "Creating a bed file of the guides" on the GitHub page of FLASHit to generate file showing the cleavage sites of the crRNA on the target genes. 10m

10 Assemble the full crRNA templates by replacing the XXXXXXXXXXXXXXXXXXXX in 5'-TAATACGACTCACTATAGXXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTATGCTGTTTTG-3' by the 20-nt sequences generated by FLASHit. 10m

11 The assembled nucleotide sequences can be used for purchasing DNA oligo pools.

Guide RNA preparation

12 Mix the DNA template for either crRNA or tracrRNA, forward and corresponding reverse primers, Phusion High-Fidelity PCR Master Mix, and molecular biology 10m

grade water in a nuclease-free PCR tube following the volumes listed in the table below. Pipette up and down 10 times or until well mixed.

A	B
Reagent	**Volume (µL)**
DNA template	4
Forward primer (10 µM)	2.5
Reverse primer (10 µM)	2.5
Phusion High-Fidelity PCR Master Mix	25
Molecular biology grade water	16
Total	50

13 Amplify the DNA templates for crRNA and tracrRNA in a thermal cycler for PCR. The steps in the ther 30m
cycle are listed below.

Step	Temperature (°C)	Time (s)	Cycles
Initial denaturation	98 °C	10	1x
Denaturation	98 °C	5	12x
Annealing	55 °C	15	
Final extension	72 °C	60	1x
Hold	4 °C	∞	1x

CITATION

Liang X, Potter J, Kumar S, Zou Y, Quintanilla R, Sridharan M, Carte J, Chen W, Roark N, Ranganathan S, Ravinder N, Chesnut JD (2015). Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection..
LINK
https://doi.org/10.1016/j.jbiotec.2015.04.024

14 Mix ATP, UTP, GTP, and CTP provided in TranscriptAid T7 High Yield Transcription Kit in 1:1:1:1 ratio 10m
nuclease-free microcentrifuge tube. Pipette up and down for 10 times or until well mixed.

15 For the transcription reaction of crRNA and tracrRNA, mix the reagents from TranscriptAid T7 High Yield Transcription Kit and the PCR-amplified DNA templates in nuclease-free PCR tubes following the volumes provided in the table below. Pipette up and down 10 times or until well mixed. A 50-µL PCR-amplified DNA template can be divided into 4 transcription reactions in this step. 10m

Reagent	Volume (µL)
Mixed NTP	16
PCR-amplified DNA template	12
5X TranscriptAid Reaction Buffer	8
TranscriptAid Enzyme Mix	4
Total	40

Note

The reagents must be added following the order from the top to the bottom in the above table.

CITATION

Liang X, Potter J, Kumar S, Zou Y, Quintanilla R, Sridharan M, Carte J, Chen W, Roark N, Ranganathan S, Ravinder N, Chesnut JD (2015). Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection..
LINK
https://doi.org/10.1016/j.jbiotec.2015.04.024

16 Incubate the RNA transcription samples at 37 °C for 4 hours. 4h

Note

A white mist should be visible for a successful reaction after incubation.

17 Add 5 µL of DNase I and 5 µL of DNA Digestion Buffer provided in RNA Clean & Concentrator-5 (DNa Included) to each 40-µL RNA transcription reaction. Pipette up and down 10 times or until well mixed. 10m

18 Incubate at room temperature for 15 min. 15m

19 Follow the “Total RNA Clean-up” instructions in the user manual of RNA Clean & Concentrator-5. Use µL of DNase/RNase-Free Water to elute the purified crRNA or tracrRNA product. Incubate for 5 min before the final centrifugation to ensure maximum yield. 30m

Note

Use 1.5 volumes of ethanol to reach maximum RNA yield as suggested in the manual for 17-200 nt RNA, in the case of 40 µL transcription with 5 µL of DNase I and 5 µL of DNA Digestion Buffer added, use 225 µL of 100% ethanol. Highly recommend using low-retention pipette tips to ensure maximum RNA yield.

20 Pipette each purified crRNA or tracrRNA product up and down for 10 times or until well mixed. Take 1 µL from each purified RNA sample, dilute 100-fold in 99 µL of molecular biology grade water. Pipette up and down for 10 times or until well mixed. Quantify each 100-fold-diluted purified crRNA or tracrRNA using Qubit™ RNA Broad Range (BR) Assay Kit by adding 10 µL diluted sample to 190 µL of master mix. 20m
the

Note

This is not just a QC step. It is necessary to determine the concentration for each crRNA and tracrRNA for calculating themixing ratio before making duplexed guide RNA. Usually, the final concentrations for crRNA are >2,000 ng/µL, and the final concentrations for tracrRNA are > 4,000 ng/µL.

21 Aliquot the crRNA and tracrRNA samples and store at -80 °C before use. 10m

22 Right before making CRISPR-NGS library, mix crRNA and tracrRNA in anequi-molar ratio (see Equation 1), then add Nuclease-Free Duplex Buffer to reach a final guide RNA concentration of 1500 ng/µL (see Equation 2). Pipette 10m

up and down 10 times or until well mixed.

Equation 1: crRNA and tracrRNA equi-molar ratio mixing

Equation 2: Volume calculation for crRNA and tracrRNA mixing

Equation 2: Volume calculation for crRNA and tracrRNA mixing

23 Incubate the mixture in a thermal cycler at 94 °C for 2 min, then slowly cool down to room temperatur 7m
The guide RNA is ready to use.

DNA sample preparation (Sewage sample as an example)

24 Shake the sewage sample until well mixed, and filter 50 mL of the sewage sample through 0.45 µm pore size membrane filter. 1h

Note

The volume is determined by the turbidity of the sewage sample. For extremely turbid sewage samples, the volume can go down to 10-20 mL, and for relatively clear samples, the volume can go up to 100 or 200 mL until the filter is clogged.

25 Store the membrane filter at -80 °C until DNA extraction.

Note

The filters can be cut into two before storage. Usually, half of a filter can obtain a high enough DNA yield.

26 Extract DNA from the membrane filter using FastDNA™ SPIN Kit for Soil following the user's manual. 2h
Elute the DNA samples using 100 µL of DES provided in the kit.

27 Purify the DNA samples using OneStep PCR Inhibitor Removal Kit following the user's manual. 10m

28 Determine the concentrations of the DNA samples using Qubit™ 1X dsDNA High Sensitivity (HS) As 15m
Kit by adding 2 µL diluted sample to 198 µL of master mix.

> **Note**
>
> Usually, the DNA concentrations are higher than 10 ng/µL.

29 Aliquot and store the DNA samples at -20 °C or -80 °C until library preparation.

CRISPR-NGS library preparation

30 Right before library preparation, dilute the "NH8B" external standard 100-fold using molecular biology grade water. 5m

31 Determine the concentration of the diluted "NH8B" external standard using Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit by adding 2 µL diluted external standard to 198 µL of master mix. 10m

32 Mix the diluted Cas9 protein, the duplexed guide RNA, and NEBuffer™ r3.1 in nuclease-free PCR tubes with the volumes listed in the table below. Pipette up and down 10 times or until well mixed. 10m

	A	B
	Reagent	**Volume (µL)**
	Cas9	2
	Guide RNA	10
	NEBuffer r3.1	3

	A	B
	Molecular biology grade water	4
	Total	19

Note

It is highly recommended to use TrueCut™ HiFi Cas9 Protein (5 µg/µL) because other commercial Cas9 proteins are not able to reach the accuracy and cleavage efficiency as high as this Cas9 protein with this workflow.

CITATION

Liu Y, Tao W, Wen S, Li Z, Yang A, Deng Z, Sun Y (2015). In Vitro CRISPR/Cas9 System for Efficient Targeted DNA Editing..

LINK

https://doi.org/10.1128/mBio.01714-15

33 Incubate the above mixture at room temperature for at least 15 min to bind guide RNA to Cas9. 15m

34 Block the DNA samples by removing 5’ phosphate group using rAPid Alkaline Phosphatase with the volumes listed in the table below. 10m

	A	B
	Reagent	**Amount**
	rAPid Alkaline Phosphatase Buffer 10x concentrated	2 µL
	rAPid Alkaline Phosphatase 1 U/µl	1 µL
	DNA sample	~200 ng
	Molecular biology grade water	Fill up the volume to 20 µL

Note

You can reduce the volume to 10 µL if the DNA sample is limited. In such cases, the DNA sample input will be ~ 100 ng, and the volumes of the buffer and the phosphatase will be reduced to half of the volumes listed above.

CITATION

Gilpatrick T, Lee I, Graham JE, Raimondeau E, Bowen R, Heron A, Downs B, Sukumar S, Sedlazeck FJ, Timp W (2020). Targeted nanopore sequencing with Cas9-guided adapter ligation..
LINK
https://doi.org/10.1038/s41587-020-0407-5

CITATION

Quan J, Langelier C, Kuchta A, Batson J, Teyssier N, Lyden A, Caldera S, McGeever A, Dimitrov B, King R, Wilheim J, Murphy M, Ares LP, Travisano KA, Sit R, Amato R, Mumbengegwi DR, Smith JL, Bennett A, Gosling R, Mourani PM, Calfee CS, Neff NF, Chow ED, Kim PS, Greenhouse B, DeRisi JL, Crawford ED (2019). FLASH: a next-generation CRISPR diagnostic for multiplexed detection of antimicrobial resistance sequences..
LINK
https://doi.org/10.1093/nar/gkz418

35 Incubate the DNA-blocking reaction mixture in a thermal cycler with the following thermal conditions 15m

A	B	C
Step	**Temperature (°C)**	**Time (min)**
Incubation	37	10
Phosphatase inactivation	75	2
Hold	4	∞

36 Mix the blocked DNA samples, the mixture of Cas9 and guide RNA, and the “NH8B” external standard 10m with the volumes listed in the table below. Pipette up and down 10 times or until well mixed.

A	B
Reagent	**Volume (µL)**

	A	B
	Mixture of Cas9 and guide RNA	19
	Blocked DNA	10
	100-fold diluted “NH8B” external standard	1
	Total	30

37 Incubate the above mixture at 37 °C for 16 hours. 16h

38 Add 5 µL of RNase T1 to the mixture, pipette up and down for 10 times, and incubate at 37 °C for 15 15m to remove guide RNA.

39 Prepare the master mix for dA-tailing, using the reagents and volumes listed in the table below. Pipet 10m up and down 10 times or until well mixed.

	A	B
	Reagent	**Volume (µL)**
	dATP (100 mM)	2
	Taq DNA Polymerase	5
	ThermoPol Reaction Buffer	80
	Molecular biology grade water	13
	Total	100

CITATION

Gilpatrick T, Lee I, Graham JE, Raimondeau E, Bowen R, Heron A, Downs B, Sukumar S, Sedlazeck FJ, Timp W (2020). Targeted nanopore sequencing with Cas9-guided adapter ligation..

LINK

https://doi.org/10.1038/s41587-020-0407-5

40 Add 5 µL of the dA-tailing master mix to each 35-µL mixture after RNase T1 treatment to reach a 40 µ 5m
total volume. Pipette up and down 10 times or until well mixed.

41 Incubate the mixture at 72 °C for 20 min for dA-tailing and Cas9 inactivation. 20m

42 Dilute adapters using molecular biology grade water or xGen™ Adapter Buffer based on the total DN 10m
input, according to the table below.

	A	B
	Total DNA input (ng)	**Adapter dilution ratio**
	>100	100x
	<100	200x

43 Ligate adapters to targeted DNA fragments using the reagents and volumes listed in the table below. 10m

	A	B
	Reagent	**Volume (µL)**
	dA-tailed DNA sample	35
	Diluted adapter	2.5
	NEBNext Ligation Enhancer	1
	NEBNext Ultra II Ligation Master Mix	30
	Total	68.5

Note

The reagents must be added following the order from the top to the bottom in the above table. After adding each reagent, pipette up and down 10 times or until well mixed. The ligation master mix should be well mixed before adding to the reaction.

44 Incubate the ligation mixture at room temperature for 15 min. 15m

45 Dilute 1x TE buffer 10-fold to make 0.1x TE buffer. 5m

46 Purify the adapter-ligated DNA samples using AMPure XP SPRI beads with beads:DNA ratio of 0.8:1 (5 µL of SPRI beads for 68.5 µL of adapter-ligated DNA sample). The detailed SPRI beads cleaning steps are listed in the table below. 1h

	A	B	C
	Step	**On/Off the magnetic rack**	**Time (min)**
	Bind DNA sample to the beads	off	5
	Separate the beads from the liquid phase	on	5
	Discard the supernatant	on	/
	1st wash with 80% ethanol	on	2
	Discard the supernatant	on	/
	2nd wash with 80% ethanol	on	2
	Discard the supernatant	on	/
	Air dry the beads with the lid open	on	3-5
	Resuspend the beads with 17 µL of 0.1x TE buffer	off	/
	Release DNA from the beads to the liquid phase	off	10
	Separate the beads from the liquid phase	on	5
	Transfer 15 µL of the supernatant to a clean PCR tube	on	/

47 Dilute the xGen™ Library Amplification Primer Mix 2-fold by adding an equal volume of molecular biology grade water. 5m

48 Mix the beads-purified DNA sample, diluted primer mix, and NEBNext®Ultra™ II Q5® Master Mix i nuclease-free PCR tube according to the table below. Pipette up and down 10 times or until well mixed. 5m

	A	B

	A	B
	Reagent	**Volume (µL)**
	Beads-purified DNA sample	15
	Diluted primer mix	10
	NEBNext Ultra II Q5 Master Mix	25
	Total	50

49 Incubate the mixture above in a thermal cycler using the thermal cycle listed in the table below. 1h

	A	B	C	D
	Step	**Temperature (°C)**	**Time**	**Cycles**
	Initial denaturation	98 °C	30 s	1x
	Denaturation	98 °C	10 s	22x for >100 ng DNA input; 30 x for <100 ng DNA input
	Annealing	65 °C	75 s	
	Final extension	65 °C	5 min	1x
	Hold	4 °C	∞	1x

50 Purify the PCR product using AMPure XP SPRI beads with beads:DNA ratio of 0.9:1 (45 µL of SPRI beads for 50 µL of PCR product). The detailed SPRI beads cleaning steps are listed in the table below. 1h

	A	B	C
	Step	**On/Off the magnetic rack**	**Time (min)**
	Bind the PCR product to the beads	off	5
	Separate the beads from the liquid phase	on	5
	Discard the supernatant	on	/
	1st wash with 80% ethanol	on	2
	Discard the supernatant	on	/
	2nd wash with 80% ethanol	on	2
	Discard the supernatant	on	/
	Air dry the beads with the lid open	on	3-5

	A	B	C
	Resuspend the beads with 33 µL of 0.1x TE buffer	off	/
	Release DNA from the beads to the liquid phase	off	10
	Separate the beads from the liquid phase	on	5
	Transfer 30 µL of the supernatant to a clean tube	on	/

51 Determine the DNA concentration of the library using Qubit™ 1X dsDNA High Sensitivity (HS) Assay by adding 2 µL diluted external standard to 198 µL of master mix. 10m

Note

The DNA concentration should be above 1 ng/µL for a successful library.

52 According to the DNA concentration, take 1-2 µL of the library and dilute to ~1 ng/µL for the fragment analyzer. 4h

Note

A visible peak at ~170 bp means excess adapter dimers. Such libraries should be cleaned up after pooling and before sequencing using SPRI beads or eGel.

53 Store the libraries at -20 °C or -80 °C until the sequencing run.

NGS read mapping 4h 41m

54 After sequencing, download all raw sequencing data files. 2h

55 Unzip the files to get ".fastq" files for each library. 1h

56 Download PRICE from https://derisilab.ucsf.edu/software/price/index.html to the local Linux environment. Install by navigating to the PRICE directory and typing "make" in the command line tool. 10m

Note

PriceSeqFilter in the PRICE package is used for screening out low-quality reads.

CITATION

Ruby JG, Bellare P, Derisi JL (2013). PRICE: software for the targeted assembly of components of (Meta) genomic sequence data..
LINK
https://doi.org/10.1534/g3.113.005967

57 Clone the KMA repository from https://bitbucket.org/genomicepidemiology/kma/src/master/ using command below to the local Linux environment. Install by navigating to the KMA directory and typing "make" in the command line tool. 10m

Command

```
git clone https://bitbucket.org/genomicepidemiology/kma.git
```

Note

KMA is used for mapping the reads to the list of reference genes to analyze the presence or absence of the target genes.

CITATION

Clausen PTLC, Aarestrup FM, Lund O (2018). Rapid and precise alignment of raw reads against redundant databases with KMA..
LINK
https://doi.org/10.1186/s12859-018-2336-6

58 Screen the low-quality sequencing reads using PriceSeqFilter with 85% of nucleotides in a read must b 1h
in high quality, the minimum allowed probability of a nucleotide being correct is 98%, and 90% of nucleotides in a read that must be called. An example of the command for paired sequences is shown below.

Command

```
PATH_TO_PRICE_FOLDER/Price/PriceSeqFilter -fp R1.fastq R2.fastq -rqf 85 0.98 -rnf 90 -op R1_filtere
d.fastq R2_filtered.fastq
```

CITATION

Ruby JG, Bellare P, Derisi JL (2013). PRICE: software for the targeted assembly of components of (Meta) genomic sequence data..
LINK
https://doi.org/10.1534/g3.113.005967

CITATION

Quan J, Langelier C, Kuchta A, Batson J, Teyssier N, Lyden A, Caldera S, McGeever A, Dimitrov B, King R, Wilheim J, Murphy M, Ares LP, Travisano KA, Sit R, Amato R, Mumbengegwi DR, Smith JL, Bennett A, Gosling R, Mourani PM, Calfee CS, Neff NF, Chow ED, Kim PS, Greenhouse B, DeRisi JL, Crawford ED (2019). FLASH: a next-generation CRISPR diagnostic for multiplexed detection of antimicrobial resistance sequences..
LINK
https://doi.org/10.1093/nar/gkz418

59 Make a copy of the ".fasta" file used for generating guide RNA in Step 8. Add the sequence of the "NH 1m
external standard to the end of the copied file. This ".fasta" file will be used as a reference gene list.

Note

Copy and paste the entire thing below:

>NH8B
ACCCATACAAGGAACCCGGCCAGCACTACGCTCACTACGGCCGGTGGTACGGTGGGCACTCCGGTGAA
ATGCACGTGCTTGGCATGCCGTCAGGCCGTGAAGTCAAGCGCACCCCGGTGTTCAACATGGACAGCAA
CAAGATGACCATCCACATCGCCTCGCCGGCGCCGGCATACAGTCTGGGGGGAATTCAAGATGGAGAAG
GGCGACGAGGTAATGGCGATCCTGACCTCGACAAGTGGAAGACCTG

CITATION

Zhang Q, Ishii S (2018). Improved simultaneous quantification of multiple waterborne pathogens and fecal indicator bacteria with the use of a sample process control..
LINK
https://doi.org/10.1016/j.watres.2018.03.023

60 Index the ".fasta" reference gene list file with KMA using the command below. 5m

Command

kma index -i REFERENCE_GENE_LIST.fasta -o INDEX_FILE_PREFIX

CITATION

Clausen PTLC, Aarestrup FM, Lund O (2018). Rapid and precise alignment of raw reads against redundant databases with KMA..
LINK
https://doi.org/10.1186/s12859-018-2336-6

61 Map the filtered ".fastq" files to the indexed reference gene list with KMA using the command below. 15m

Command

```
kma -ipe R1_filtered.fastq R2_filtered.fastq -a -t_db INDEX_FILE_PREFIX -o OUTPUT_FILE_PREFIX
```

CITATION

Clausen PTLC, Aarestrup FM, Lund O (2018). Rapid and precise alignment of raw reads against redundant databases with KMA..

LINK

https://doi.org/10.1186/s12859-018-2336-6

62 The read mapping results for the target genes are available in the ".res" file generated by KMA.