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Version 2

# Multiplexed CRISPR-based target-enriched next-generation sequencing for detecting antibiotic resistance genes in environmental samples V.2

 [Water Research](#)

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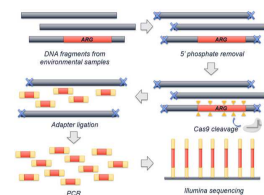
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**We use this protocol and it's working**

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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

### Equipment

1. PCR thermal cycler
2. Qubit fluorometer
3. Microcentrifuge
4. Magnetic separation rack for 0.2 mL tubes
5. Pipettes, 0.5 - 10  $\mu$ L, 2 - 20  $\mu$ L, 20 - 200  $\mu$ L, 100 - 1000  $\mu$ L, adjustable volume
6. Bead beater or vortex with bead-beating adapters

### Consumables

1. Nuclease-free 1.5 mL microcentrifuge tubes
2. Nuclease-free 0.2 mL PCR tubes
3. Nuclease-free 1000  $\mu$ L pipette tips with filter
4. Nuclease-free 200  $\mu$ L pipette tips with filter
5. Nuclease-free 20  $\mu$ L pipette tips with filter
6. Nuclease-free 10  $\mu$ L pipette tips with filter
7. MF-Millipore® Membrane Filter, 0.45  $\mu$ m pore size (Millipore, Catalog #: HAWP04700)
8. 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 I 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 XXXXXXXXXXXXXXXXXXXXXXX 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'-ACCCATACAAGGAACCCGGCCAGCACTACGCTCACTACGGCCGGTGGTACGGTGGGCACTCCGGTGAAATGCACGTGCTTGGCATGCCGTCAGGCCGTGAAGTCAAGCGCACCCCGGTGTTCAACATGGACAGCAACAAGATGACCATCCACATCGCCTCGCCGGCGCCGGCATACTGCTGGGGGAATTCAAGATGGAGAAGGGCGACGAGGTAATGGCGATCCTGACCTCGACAAGTGGAAGACCTG-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. Alt-R™ S.p. HiFi Cas9 Nuclease V3 (Integrated DNA Technologies, Catalog #: 1081060/1081061/10007803)
2. rAPid Alkaline Phosphatase (Roche, Catalog #: 4898133001/ 4898141001)
3. RNase A, DNase and protease-free (10 mg/mL) (Thermo Scientific, Catalog #: EN0531)
4. Thermolabile Proteinase K (New England Biolabs, Catalog #: P8111S)
5. Taq DNA Polymerase with Standard Taq Buffer (New England Biolabs, Catalog #: M0273S/M0273L/M0273X/M0273E)

Total mass of ssDNA in an oligo pool (g)

$$= \text{moles of ssDNA (mol)} \times \left( (\text{length of ssDNA (nt)} \times 308.97 \left( \frac{\text{g}}{\text{mol}} \right) / \text{nt}) + 18.02 \text{ g/mol} \right) \times \text{number of different ssDNA sequences in the pool}$$

**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, Wilhelm 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.

<https://doi.org/10.1093/nar/gkz418>

LINK

### 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.

<https://doi.org/10.1016/j.jbiotec.2015.04.024>

LINK

### 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.

<https://doi.org/10.1016/j.watres.2018.03.023>

LINK

## Before start

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 other 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. 10m
- 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, Wilhelm 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.

<https://doi.org/10.1093/nar/gkz418>

LINK

- 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



### Citation

Alcock BP, Huynh W, Chalil R, Smith KW, Raphenya AR, Wlodarski MA, Edalatmand A, Petkau A, Syed SA, Tsang KK, Baker SJC, Dave M, McCarthy MC, Mukiri KM, Nasir JA, Golbon B, Imtiaz H, Jiang X, Kaur K, Kwong M, Liang ZC, Niu KC, Shan P, Yang JYJ, Gray KL, Hoad GR, Jia B, Bhando T, Carfrae LA, Farha MA, French S, Gordzevich R, Rachwalski K, Tu MM, Bordeleau E, Dooley D, Griffiths E, Zubyk HL, Brown ED, Maguire F, Beiko RG, Hsiao WWL, Brinkman FSL, Van Domselaar G, McArthur AG (2023)

. CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database.

<https://doi.org/10.1093/nar/gkac920>

LINK

- 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

#### new command name

```
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* 5h



genomes other than these two, for example, swine genomes, users can modify the files in `/flash/generated_files/` accordingly.

- 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 ambiguous\_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

**new command name**

```
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**

**new command name**

```
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

### new command name

```
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

#### new command name

```
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 FLASHit 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 a 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'-TAATACGACTCACTATAGXXXXXXXXXXXXXXXXXXXXXXXXGTTTTAGAGCTATGCTGTTTTG-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

6h 57m

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

| A            | B           |
|--------------|-------------|
| Reagent      | Volume (μL) |
| DNA template | 4           |



| A                                    | B   |
|--------------------------------------|-----|
| Forward primer (10 $\mu$ M)          | 2.5 |
| Reverse primer (10 $\mu$ M)          | 2.5 |
| Phusion High-Fidelity PCR Master Mix | 25  |
| Molecular biology grade water        | 16  |
| <b>Total</b>                         | 50  |

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

30m

| A                    | B                | C        | D                        |
|----------------------|------------------|----------|--------------------------|
| Step                 | Temperature (°C) | Time (s) | Cycles                   |
| Initial denaturation | 98 °C            | 10       | 1x                       |
| Denaturation         | 98 °C            | 5        | 12x for tracr; 5x for cr |
| Annealing            | 55 °C            | 15       |                          |
| Final extension      | 72 °C            | 60       | 1x                       |
| Hold                 | 4 °C             | $\infty$ | 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.

<https://doi.org/10.1016/j.jbiotec.2015.04.024>

[LINK](#)

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

10m

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- $\mu$ L PCR-amplified DNA template can be divided into 4 transcription reactions in this step.

10m

| A                                | B                 |
|----------------------------------|-------------------|
| Reagent                          | Volume ( $\mu$ L) |
| Mixed NTP                        | 16                |
| PCR-amplified DNA template       | 12                |
| 5X TranscriptAid Reaction Buffer | 8                 |
| TranscriptAid Enzyme Mix         | 4                 |
| <b>Total</b>                     | 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.

<https://doi.org/10.1016/j.jbiotec.2015.04.024>

LINK

- 16 Incubate the RNA transcription samples at 37 °C for 4-6 hours.

4h

### Note

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

- 17 Add 5 µL of DNase I (1 U/µL) and 5 µL of DNA Digestion Buffer provided in RNA Clean & Concentrator-5 (DNase I 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 30 min.

30m

- 19 Follow the "Total RNA Clean-up" instructions in the user manual of RNA Clean & Concentrator-5. Use 15 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

20m



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<sup>TM</sup>RNA Broad Range (BR) Assay Kit by adding 10 µL diluted sample to 190 µL of master mix.

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 2500 ng/µL (see Equation 2). Pipette up and down 10 times or until well mixed.

10m

$$\frac{V_{cr}(\mu L)}{V_{tracr}(\mu L)} = \frac{7}{12} \times \frac{C_{tracr}(ng/\mu L)}{C_{cr}(ng/\mu L)}$$

#### Equation 1: crRNA and tracrRNA equi-molar ratio mixing

$$\frac{V_{cr}(\mu L) \times C_{cr}(ng/\mu L) + V_{tracr}(\mu L) \times C_{tracr}(ng/\mu L)}{V_{cr}(\mu L) + V_{tracr}(\mu L) + V_{buffer}(\mu L)} = 2500(ng/\mu L)$$

#### 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 temperature. The guide RNA is ready to use.

7m

### 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. Elute the DNA samples using 100 µL of DES provided in the kit.

2h

- 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) Assay Kit by adding 2 µL diluted sample to 198 µL of master mix.

15m

**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 Pre-mix Cas9 nuclease (25 µM) and the duplexed gRNA (2500 ng/µL) obtained from step 23 in 3:10 volume ratio. Incubate at room temperature for 2.5 hr.

- 31 Serial dilute the "NH8B" external standard 1000-fold using molecular biology grade water. Keep the 100-fold diluted standard for Qubit quantification.

5m



32 Determine the concentration of the 100-fold diluted "NH8B" external standard using Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit by adding 5 µL diluted external standard to 195 µL of master mix.

10m

33 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.

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

[LINK](#)

## Citation

Quan J, Langelier C, Kuchta A, Batson J, Teyssier N, Lyden A, Caldera S, McGeever A, Dimitrov B, King R, Wilhelm 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.

<https://doi.org/10.1093/nar/gkz418>

LINK

- 34 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                | ∞          |

- 35 Mix the Cas9-gRNA premix, blocked DNA sample, NH8B positive control, NEBuffer™ r3.1, and molecular biology grade water 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) |
| Blocked DNA sample                    | 6-8         |
| NEBuffer r3.1                         | 3           |
| NH8B positive control (1000x diluted) | 1           |
| Cas9-gRNA premix                      | 10.4        |



| A                             | B       |
|-------------------------------|---------|
| Molecular biology grade water | 7.6-9.6 |
| <b>Total</b>                  | 30      |

#### 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.

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

LINK

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

16h



37 Add 2.5 µL of RNase A to the mixture, pipette up and down for 10 times, and incubate at room temperature for 15 min to remove gRNA.

15m

38 Add 2.5 µL of Thermolabile Proteinase K to the mixture, pipette up and down for 10 times, and incubate in a PCR machine using the following thermal cycle:

| A                       | B                 |
|-------------------------|-------------------|
| <b>Temperature (°C)</b> | <b>Time (min)</b> |
| 37                      | 15                |
| 55                      | 10                |

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

10m

| A                             | B           |
|-------------------------------|-------------|
| Reagent                       | Volume (μL) |
| dATP (100 mM)                 | 2           |
| Taq DNA Polymerase            | 5           |
| Standard Taq Buffer           | 80          |
| Molecular biology grade water | 13          |
| <b>Total</b>                  | 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.

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

LINK

- 40 Add 5 μL of the dA-tailing master mix to each 35-μL mixture to reach a 40 μL total volume. Pipette up and down 10 times or until well mixed. 5m
  - 41 Incubate the mixture at 72 °C for 20 min for dA-tailing. 20m
  - 42 Dilute adapters 100-fold using molecular biology grade water or xGen™ Adapter Buffer. 10m
- Note**

Highly recommend using UDI-UMI adapters with unique molecular barcoding to reduce the biases caused by PCR.
- 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           |
| NEBNext Ligation Enhancer            | 1           |
| NEBNext Ultra II Ligation Master Mix | 30          |
| <b>Total</b>                         | 68          |

#### 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 (57 μL of SPRI beads for 68 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                       | 0.5        |



| A                                                          | B   | C   |
|------------------------------------------------------------|-----|-----|
| Discard the supernatant                                    | on  | /   |
| 2nd wash with 80% ethanol                                  | on  | 0.5 |
| Discard the supernatant                                    | on  | /   |
| Air dry the beads with the lid open                        | on  | 3-5 |
| Resuspend the beads with 17 $\mu$ L of 0.1x TE buffer      | off | /   |
| Release DNA from the beads to the liquid phase             | off | 15  |
| Separate the beads from the liquid phase                   | on  | 5   |
| Transfer 15 $\mu$ L of the supernatant to a clean PCR tube | on  | /   |

- 47 Dilute the xGen<sup>TM</sup> 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<sup>®</sup> Ultra<sup>TM</sup> II Q5<sup>®</sup> Master Mix in a nuclease-free PCR tube according to the table below. Pipette up and down 10 times or until well mixed.

5m

| A                              | B                 |
|--------------------------------|-------------------|
| Reagent                        | Volume ( $\mu$ L) |
| Beads-purified DNA sample      | 15                |
| Diluted primer mix             | 10                |
| NEBNext Ultra II Q5 Master Mix | 25                |
| <b>Total</b>                   | 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    |
| Annealing            | 65 °C            | 75 s  |        |
| Final extension      | 65 °C            | 5 min | 1x     |
| Hold                 | 4 °C             | ∞     | 1x     |

- 50 0.50Purify 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                       | 0.5        |
| Discard the supernatant                           | on                       | /          |
| 2nd wash with 80% ethanol                         | on                       | 0.5        |
| Discard the supernatant                           | on                       | /          |
| Air dry the beads with the lid open               | on                       | 3-5        |
| Resuspend the beads with 33 µL of 0.1x TE buffer  | off                      | /          |
| Release DNA from the beads to the liquid phase    | off                      | 15         |
| 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 Kit by adding 2 µL diluted external standard to 198 µL of master mix.

10m

**Note**

The DNA concentration should be above 2 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 fragment analysis.

4h

**Note**

A visible peak at ~170 bp means excess adapter dimers. After pooling and before sequencing, such libraries should be size selected using SPRI beads or eGel.

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

- 54 For sequencing, we recommend doing 2×150 nt with a total number of reads of at least 25 million for each library.

## NGS read mapping

4h 41m

- 55 Download and install PRICE from <https://derisilab.ucsf.edu/software/price/index.html> to the local Linux environment.

10m

**Citation**

Ruby JG, Bellare P, Derisi JL (2013)  
. PRICE: software for the targeted assembly of components of (Meta) genomic sequence data.

<https://doi.org/10.1534/g3.113.005967>

LINK

- 56 Download and install Bowtie2 from Bioconda <https://anaconda.org/bioconda/bowtie2> to the local Linux environment.

**Citation**

Langmead B, Salzberg SL (2012). Fast gapped-read alignment with Bowtie 2.

<https://doi.org/10.1038/nmeth.1923>

LINK

- 57 Download and install UMI-tools from <https://github.com/CGATOxford/UMI-tools> to the local Linux environment.

**Citation**

Smith T, Heger A, Sudbery I (2017)  
. UMI-tools: modeling sequencing errors in Unique Molecular Identifiers to improve quantification accuracy.

<https://doi.org/10.1101/gr.209601.116>

LINK

- 58 Download and install SAMtools from <https://github.com/samtools/samtools> to the local Linux environment.

**Citation**

Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, Whitwham A, Keane T, McCarthy SA, Davies RM, Li H  
(2021). Twelve years of SAMtools and BCFtools.

<https://doi.org/10.1093/gigascience/giab008>

LINK

- 59 After sequencing, download all raw sequencing fastq.gz files.

2h



- 60 The read1, read2, and UMI should be stored in three different fastq.gz files. If only read1 and read2 files are available, use Trimmomatic (<http://www.usadellab.org/cms/index.php?page=trimmomatic>) to separate UMI into a third fastq.gz file.

**Command****new command name**

```
java -jar PATH_T0/trimmomatic-0.39.jar SE --threads n  
input_read1_or_2.fastq.gz output_UMI.fastq.gz HEADCROP:8
```

**Citation**

Bolger AM, Lohse M, Usadel B (2014)  
. Trimmomatic: a flexible trimmer for Illumina sequence data.

<https://doi.org/10.1093/bioinformatics/btu170>

LINK

- 61 Unzip all fastq.gz files to fastq files.
- 62 Sort all fastq files using the command below, to ensure all reads are in the same order in read1, read2, and UMI files.

**Command****new command name**

```
cat file.fastq | paste - - - - | sort -k1,1 -t " " | tr "\t" "\n" >
file_sorted.fastq
```

**Note**

Sometimes the reads in different fastq files are already in the same order. This can be checked quickly by using the "head" command for each fastq file. If the sequence identifiers that begin with the "@" character in lines 1, 5, and 9 are in the same order in read1, read2, and UMI fastq files for the same library, this sorting step can be skipped.

- 63 Extract UMI for read1 and read2 files using the following command.

**Command****new command name**

```
umi_tools extract --extract-method=string --bc-pattern=NNNNNNNNNN -I
[UMI_index_fastq_file] -S [UMI_index_fastq_file_out_nothing_left] --
read2-in=[read_fastq_file_to_add_UMI] --read2-out=
[output_read_fastq_file_with_UMI_added]
```

**Note**

Please note that the read2 in the UMI-tools command is not the read2 fastq file obtained from sequencing. This command should be run twice to add UMI into read1 and read2 files respectively. In each run, either the read1 or the read2 file name should be entered after "--read2-in="

The UMI.fastq file will no longer be used in the following steps after being added to the read1 and read2 files.

- 64 Screen the low-quality sequencing reads using PriceSeqFilter with 85% of nucleotides in a read must be 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.

1h

**Command**

new command name

```
PATH_TO_PRICE_FOLDER/Price/PriceSeqFilter -fp R1.fastq R2.fastq -rqf  
85 0.98 -rnf 90 -op R1_filtered.fastq R2_filtered.fastq
```

**Citation**

Quan J, Langelier C, Kuchta A, Batson J, Teyssier N, Lyden A, Caldera S, McGeever A, Dimitrov B, King R, Wilhelm 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.

<https://doi.org/10.1093/nar/gkz418>

[LINK](#)



- 65 Make a copy of the ".fasta" file used for generating guide RNA in Step 8. Add the sequence of the "NH8B" external standard to the end of the copied file. This ".fasta" file will be used as a reference gene list for read mapping.

#### Note

Copy and paste the entire content below to the .fasta file:

```
>NH8B
ACCCATACAAGGAACCCGGCCAGCACTACGCTCACTACGGCCGGTGGTACGGTGGGCACTC
CGGTGAAATGCACGTGCTTGGCATGCCGTCAGGCCGTGAAGTCAAGCGCACCCCGGTGTTT
AACATGGACAGCAACAAGATGACCATCCACATCGCCTCGCCGGCGCCGGCATAACAGTCTGGG
GGGAATTCAAGATGGAGAAGGGCGACGAGGTAATGGCGATCCTGACCTCGACAAGTGAAG
ACCTG
```

#### 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.

<https://doi.org/10.1016/j.watres.2018.03.023>

LINK

- 66 Index the ".fasta" file for Bowtie2 mapping.

#### Command

**new command name**

```
bowtie2-build reference.fasta reference
```

- 67 Map the sequencing reads to the reference using Bowtie2 and generate a sorted bam file using SAMtools. The unaligned reads are excluded from the output.

**Command****new command name**

```
bowtie2 -x reference -1 R1_with_UMI.fastq -2 R2_with_UMI.fastq -a --  
very-sensitive --threads n | samtools view -bSF4 - | samtools sort - -  
o sorted_output.bam -@ n
```

- 68 Index the bam file obtained from step 67 using SAMtools.

**Command****new command name**

```
samtools index sorted_output.bam -@ n
```

- 69 Deduplicate the reads in the bam file obtained in step 67 using UMI-tools.

**Command****new command name**

```
umi_tools dedup -I input.bam --output-stats=deduplicated -S  
deduplicated.bam
```

- 70 Sort and index the deduplicated bam file using SAMtools.

**Command****new command name**

```
samtools sort deduplicated.bam -o deduplicated_sorted.bam -@ n
samtools index deduplicated_sorted.bam -@ n
```

**Note**

The bam and bai files generated in this step can be viewed in Integrative Genomics Viewer (IGV).

**Citation**

Thorvaldsdóttir H, Robinson JT, Mesirov JP (2013)  
. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration.

<https://doi.org/10.1093/bib/bbs017>

LINK

- 71 Get the coverage for the ARGs detected in the library using SAMtools. Use "--ff 0" to remove the restriction of secondary alignment.

**Command****new command name**

```
samtools coverage --ff 0 -o coverage_out.tsv deduplicated_sorted.bam
```



- 72 Organize coverage files by adding ARG names and combine into one single xlsx file using the following Python code. The coverage threshold is set to 75% in this code. The reference file is ARO\_index.tsv downloaded from the Comprehensive Antibiotic Resistance Database (CARD). The xlsx file is the final output of this pipeline.

## Command

### new command name

```
import pandas as pd
import os
import argparse

parser = argparse.ArgumentParser()
parser.add_argument('-
i', dest='input', type=str, required=True, help='Define input tsv folder')
parser.add_argument('-
r', dest='reference', type=str, required=True, help='Define input
reference file')
parser.add_argument('-
o', dest='output', type=str, required=True, help="Define output path for
a combined excel with ARG names and drug classes")
args=parser.parse_args()

ARO_table=pd.read_csv(args.reference,sep='\t')

dir_path=args.input.strip("")
dir_list=os.listdir(dir_path)
dir_list=sorted(dir_list)
for file_name in dir_list:
    if file_name.endswith('.tsv'):
        print(file_name)
        prefix=file_name.split('.tsv')[0]
        mapping_table=pd.read_csv(dir_path+'/'+file_name,sep='\t')
        mapping_table['ARG_name']=''
        mapping_table['ARG_family']=''
        mapping_table['Drug_class']=''

        for i in range(0,len(mapping_table)):
            if mapping_table.loc[i,'numreads']==0:
                continue
            else:
                for j in range(0,len(ARO_table)):
                    if str(mapping_table.loc[i,'#rname']).strip('\t
') in ARO_table.loc[j,'ARO Accession']:

mapping_table.loc[i,'ARG_name']=ARO_table.loc[j,'CARD Short Name']

mapping table.loc[i.'Drug class']=ARO table.loc[i.'Drug Class']
```



```
mapping_table.loc[i,'ARG_family']=ARO_table.loc[j,'AMR Gene Family']
break

output_table=pd.DataFrame(columns=mapping_table.columns)
for i in range(0,len(mapping_table)):
    if mapping_table.loc[i,'coverage']>=75:

output_table=output_table.append(mapping_table.loc[i],ignore_index=True)

if os.path.exists(args.output):
    with pd.ExcelWriter(args.output, mode='a') as writer:
        output_table.to_excel(writer, sheet_name=prefix)
else:
    output_table.to_excel(args.output,sheet_name=prefix)
```

### Citation

Alcock BP, Huynh W, Chalil R, Smith KW, Raphenya AR, Wlodarski MA, Edalatmand A, Petkau A, Syed SA, Tsang KK, Baker SJC, Dave M, McCarthy MC, Mukiri KM, Nasir JA, Golbon B, Imtiaz H, Jiang X, Kaur K, Kwong M, Liang ZC, Niu KC, Shan P, Yang JYJ, Gray KL, Hoad GR, Jia B, Bhando T, Carfrae LA, Farha MA, French S, Gordzevich R, Rachwalski K, Tu MM, Bordeleau E, Dooley D, Griffiths E, Zubyk HL, Brown ED, Maguire F, Beiko RG, Hsiao WWL, Brinkman FSL, Van Domselaar G, McArthur AG (2023) . CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database.

<https://doi.org/10.1093/nar/gkac920>

[LINK](#)

## Trouble shooting

73 The DNA fragments in the final libraries don't have a peak at ~370-420 bp.

**Note**

We highly recommend saving the 5  $\mu$ L dA-tailed leftover sample after taking 35  $\mu$ L out for adapter ligation in step 43. The 5  $\mu$ L leftover can be used for gel electrophoresis to check the Cas9 cleavage outcome. We recommend using 3% agarose gel for size check. If the peak of the DNA fragments in the leftover samples is at ~100-150 bp, it usually suggests a DNase residue in crRNA or tracrRNA. Kits like DNase Alert can be used to check this DNase residue. To avoid this residue, the concentration of DNase I used in step 17 should be strictly controlled at 1 U/ $\mu$ L.

- 74 DNA concentration of the final library is lower than 2 ng/ $\mu$ L.

**Note**

Make sure to dilute the adapters right before ligation. Storing adapters at -20 °C with a low concentration may cause degradation.

- 75 The yield of crRNA/tracrRNA is low.

**Note**

Elongate the transcription time by 30 min and check the formation of white mist, but don't incubate for longer than 8 hours. Make sure to use 1.5 volumes of 100% ethanol when binding the RNA to the spin column. Check the DNA template concentration after PCR in step 13.

## Citations

Quan J, Langelier C, Kuchta A, Batson J, Teyssier N, Lyden A, Caldera S, McGeever A, Dimitrov B, King R, Wilhelm 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. FLASH: a next-generation CRISPR diagnostic for multiplexed detection of antimicrobial resistance sequences.

<https://doi.org/10.1093/nar/gkz418>

Liang X, Potter J, Kumar S, Zou Y, Quintanilla R, Sridharan M, Carte J, Chen W, Roark N, Ranganathan S, Ravinder N, Chesnut JD. Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection.

<https://doi.org/10.1016/j.jbiotec.2015.04.024>

Zhang Q, Ishii S. Improved simultaneous quantification of multiple waterborne pathogens and fecal indicator bacteria with the use of a sample process control.

<https://doi.org/10.1016/j.watres.2018.03.023>

### Step 13

Liang X, Potter J, Kumar S, Zou Y, Quintanilla R, Sridharan M, Carte J, Chen W, Roark N, Ranganathan S, Ravinder N, Chesnut JD. Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection.

<https://doi.org/10.1016/j.jbiotec.2015.04.024>

### Step 15

Liang X, Potter J, Kumar S, Zou Y, Quintanilla R, Sridharan M, Carte J, Chen W, Roark N, Ranganathan S, Ravinder N, Chesnut JD. Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection.

<https://doi.org/10.1016/j.jbiotec.2015.04.024>

### Step 2

Quan J, Langelier C, Kuchta A, Batson J, Teyssier N, Lyden A, Caldera S, McGeever A, Dimitrov B, King R, Wilhelm 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. FLASH: a next-generation CRISPR diagnostic for multiplexed detection of antimicrobial resistance sequences.

<https://doi.org/10.1093/nar/gkz418>

### Step 3

Alcock BP, Huynh W, Chalil R, Smith KW, Raphenya AR, Wlodarski MA, Edalatmand A, Petkau A, Syed SA, Tsang KK, Baker SJC, Dave M, McCarthy MC, Mukiri KM, Nasir JA, Golbon B, Imtiaz H, Jiang X, Kaur K, Kwong M, Liang ZC, Niu KC, Shan P, Yang JYJ, Gray KL, Hoad GR, Jia B, Bhandu T, Carfrae LA, Farha MA, French S, Gordzevich R, Rachwalski K, Tu MM, Bordeleau E, Dooley D, Griffiths E, Zubyk HL, Brown ED, Maguire F, Beiko RG, Hsiao WWL, Brinkman FSL, Van Domselaar G, McArthur AG. CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database.

<https://doi.org/10.1093/nar/gkac920>

**Step 33**

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

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

**Step 33**

Quan J, Langelier C, Kuchta A, Batson J, Teyssier N, Lyden A, Caldera S, McGeever A, Dimitrov B, King R, Wilhelm 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. FLASH: a next-generation CRISPR diagnostic for multiplexed detection of antimicrobial resistance sequences.

<https://doi.org/10.1093/nar/gkz418>

**Step 35**

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

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

**Step 39**

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

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

**Step 55**

Ruby JG, Bellare P, Derisi JL. PRICE: software for the targeted assembly of components of (Meta) genomic sequence data.

<https://doi.org/10.1534/g3.113.005967>

**Step 56**

Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2.

<https://doi.org/10.1038/nmeth.1923>

**Step 57**

Smith T, Heger A, Sudbery I. UMI-tools: modeling sequencing errors in Unique Molecular Identifiers to improve quantification accuracy.

<https://doi.org/10.1101/gr.209601.116>

**Step 58**

Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, Whitwham A, Keane T, McCarthy SA, Davies RM, Li H. Twelve years of SAMtools and BCFtools.

<https://doi.org/10.1093/gigascience/giab008>

**Step 60**

Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data.

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**Step 64**

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