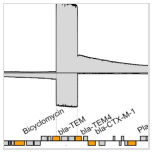

Version 2

May 16, 2021

In vitro transcription of crRNA and tracrRNA from DNA oligos for cas9 enrichment and nanopore sequencing (for Bac - PULCE) V.2

Version 1 is forked from In Vitro Transcription for dgRNA

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In Development Share dx.doi.org/10.17504/protocols.io.buk7nuzn

Olin Silander

ABSTRACT

In this protocol we describe the steps for the design and production of dual guide RNAs (dgRNAs) from DNA oligos for the enrichment of specific sequences from mixed samples. These enriched molecules can then be sequenced using long read Oxford Nanopore Sequencing. The method as outlined and applied here is termed Bac-PULCE (Bacterial strain and antimicrobial resistance Profiling Using Long reads via CRISPR Enrichment) but can be applied to enrich specific DNA molecules from any samples for downstream ONT sequencing.

For CRISPR-cas9 protocols including Bac-PULCE, we use T7 polymerase to transcribe the crRNA and tracrRNA to make dgRNA for Cas9. The two components of the dual guides are the crRNA (containing your variable 20 bp target plus a 22 bp constant region) and the tracrRNA (a 72 bp constant region).

The protocol outlined here is a combination of the two protocols below:

https://www.protocols.io/view/in-vitro-transcription-for-dgrna-3bpgimn
/dx.doi.org/10.17504/protocols.io.3bpgimn

https://international.neb.com/protocols/2013/04/02/standard-rna-synthesis-e2050

And the end product of this protocol is used for Nanopore protocol Cas9-mediated PCR-free enrichment as outlined by Oxford Nanopore - please refer to this protocol in particular for further important detail around sequencing the DNA library.

The aim of this protocol is to design and produce crRNAs and tracRNAs from DNA oligos, and combine these with Cas9 to cut specific DNA sequences. We will add a T7 RNA polymerase binding site at the 5' end of the crRNA and tracRNA to allow *in vitro* transcription, as well as a 3' tracrRNA binding site to the crRNA. To allow crRNA and tracRNA production from DNA oligos, we order our DNA oligos of the reverse complement (see below for more details).

DOI

dx.doi.org/10.17504/protocols.io.buk7nuzn

PROTOCOL CITATION

Olin Silander 2021. In vitro transcription of crRNA and tracrRNA from DNA oligos for cas9 enrichment and nanopore sequencing (for Bac - PULCE) . **protocols.io**
https://dx.doi.org/10.17504/protocols.io.buk7nuzn
Version created by Olin Silander

FORK NOTE

re-worded some steps

FORK FROM

Forked from In Vitro Transcription for dgRNA, Amy Lyden

KEYWORDS

DASH, FLASH, cas9, dgRNA, IVT, transcription, Bac PULCE

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GUIDELINES

Work in an RNAse free space! We used RNAse ZAP on all work surfaces prior to carrying out method. Use nuclease free tubes lo-bind eppendorf tubes.

MATERIALS TEXT

MATERIALS

Thermocycler **Contributed by users**

Ethanol 100% **Contributed by users** Step 11

Nuclease-free water **Ambion Catalog #AM9932** Step 2

Qubit RNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32852**

SPRI beads (homemade) or Ampure XP beads **Contributed by users** Step 9

crRNA template (60nt) **IDT** Step 2

tracrRNA template (90nt) **IDT** Step 4

10X T7 Buffer (400 mM Tris pH 7.9 - 200 mM MgCl2 - 50 mM DTT - 20 mM spermidine (Sigma 85558)) store at -80C **Contributed by users**

T7 Enzyme (10mg/mL) **Contributed by users**

NTP Set 100 mM Solution **Thermo Fisher Scientific Catalog #R0481**

Magnetic Tube Rack for 1.5mL or 15mL tubes **Contributed by users Catalog #12321D**

T7 transcription primer (18nt) **IDT** In 2 steps

STEP MATERIALS

crRNA template (60nt) **IDT** Step 2

T7 transcription primer (18nt) **IDT** In 2 steps

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NanoDrop spectrophotometer **Thermo Fisher Scientific Catalog #ND-1000**

NTP Set 100 mM Solution **Thermo Fisher Scientific Catalog #R0481**

10X T7 Buffer (400 mM Tris pH 7.9 - 200 mM MgCl2 - 50 mM DTT - 20 mM spermidine (Sigma 85558)) store at -80C **Contributed by users**

T7 Enzyme (10mg/mL) **Contributed by users**

crRNA template (60nt) **IDT** Step 2

Nuclease-free water **Ambion Catalog #AM9932** Step 2

tracrRNA template (90nt) **IDT** Step 4

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SPRI beads (homemade) or Ampure XP beads **Contributed by users** Step 9

Ethanol 100% **Contributed by users** Step 11

Qubit RNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32852**

Agilent Small RNA Bioanalyzer kit **Contributed by users Catalog #5067-1548**

BEFORE STARTING

Design crRNAs

Design the crRNAs using CHOPCHOPv3 (Labun et al 2019). Insert the region surrounding your gene target and CHOPCHOP will locate a sequence of 20 bp ending in 'NGG-3'.

5'----NNNNNNNNNNNNNNNNNNNNGG----3'

where the 20Ns are unique for your target site. Cas9 will cut between the 17th and 18th N of your target (see below), leaving a free phosphate to which the sequencing motor for Oxford Nanopore sequencing will attach. One end of the cut molecule will often remain bound by the cas9 complex, which will lower the ligation efficiency of the Oxford Nanopore motor. The end that is bound by the cas9 complex tends to be the end that does *not* contain the PAM sequence. However, the cas9 complex will not block ligation in all cases or contexts. As we employ this protocol to enrich for specific regions with a single cut, you may want to pay attention to which end will tend to have the cas9 bound, for example if you are cutting AMR genes and want to read off the gene into the genome or plasmid on which the AMR sits.

Cas9 will cut between the 17thand 18th nt of the target, yielding the following products:

5'--**NNNNNNNNNNNNNNNNN**--3' 5'-**NNN**NGG--3'

or 5'--CCN**NNN**3' 5'**NNNNNNNNNNNNNNNNN**--5'

In this protocol, we are designing DNA oligos that will be transcribed into crRNAs and the tracRNA by T7 RNA polymerase. This requires the addition of a T7 RNA polymerase binding site for each of the oligos, and requires that the crRNA and tracRNA DNA oligo sbe ordered as the reverse complement, as they will serve as the template for transcription. The tracRNA will bind at the 5' end of your *transcribed crRNA*. Together with cas9 a dgRNA complex will form.

The sequence of each DNA oligo for creation of the crRNA should be designed as follows, with the Ns replaced by your 20 bp target:

TAATACGACTCACTATAGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTATGCTGTTTTG

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The underlined sequence is the T7 RNA polymerase binding site, which will be made double stranded with the addition of the 18bp oligo below. The DNA oligo for your crRNA should be ordered **as the reverse complement** of the above sequence, as it is the **template** for the transcribed RNA.

60mer reverse complement:
5'-CAAAACAGCATAGCTCTAAAACNNNNNNNNNNNNNNNNNNNNCTATAGTGAGTCGTATTA-3'

The 18 bp oligo for annealing to the above template oligo for T7 transcription is
5'-TAATACGACTCACTATAG-3'

It should not be reverse complemented when ordering.
18mer T7:
5'-TAATACGACTCACTATAG-3'

The tracrRNA: (constant for all dgRNA)

The sequence of the tracrRNA template should be as follows:

TAATACGACTCACTATAGGACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAG TCGGTGCTTTTT

Just as with the crRNA, only the T7 binding site needs to be double stranded, with the remainder of the oligo serving as **template**, so the following oligo should be purchased from IDT:

90mer reverse complement:
AAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATGCT GTCCTATAGTGAGTCGTATTA

(the T7 oligo for double stranding is the same shown above)
18mer T7:
TAATACGACTCACTATAG

Preparation

1 RNAse Zap equipment, gloves and bench prior to starting protocol. Turn on heat block to reach a temperature of **95 °C** .

Annealing T7 to crRNA and tracrRNA template

2 Pool your crRNA DNA oligo sequences in equimolar amounts. For small numbers of crRNAs we order templates from IDT with oligos diluted in nuclease free water at a concentration of 10µM. For larger numbers of crRNAs, order in 96-well plate format. Nuclease-free water crRNA template (60nt) **IDT** **Ambion Catalog #AM9932**

3 Add an equimolar amount of T7 primer to your crRNA pool. For example, reconstitute T7 primer to 10µM, and pool 125µL of your 10µM crRNA pool with 125µL of your T7 primer at 10µM.

T7 transcription primer (18nt) **IDT**

4 Add an equimolar amount of T7 primer to your tracrRNA. For example, if you have reconstituted your tracrRNA to 100µM, pool 125µL of your tracrRNA at 100µM to 125µL of your T7 primer at 100µM.

tracrRNA template (90nt) **IDT**

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T7 transcription primer (18nt) **IDT**

5 Anneal tracrRNA template + T7 primer and crRNA template + T7 primer by heating to **95 °C** on a heat block or thermocycler for **00:02:00** and allowing them to cool to room temperature slowly on the bench in a tube rack.

In Vitro Transcription

6 For IVT we used the T7 E2050S kit from NEB and followed the standard RNA synthesis method. Thaw and keep on ice the necessary components - NTP buffer mix.

HiScribe T7 Quick High Yield RNA Synthesis Kit - 50 rxns **New England Biolabs Catalog #E2050S**

7 Assemble the reactions in the following order in nuclease free 1.5ml lobbind tubes for both the crRNA & tracrRNA . total volume can be scaled accordingly. We usually had a total reaction volume of 90µL.

A	B	C
1x	CrRNA	TracrRNA
Nuclease free water	XµL	XµL
NTP buffer mix	30µ	30µ
Oligo RNA template	(1µg)	(1µg)
T7 Polymerase Mix	6µL	6µL
Total Volume Mix	90µL	90µL

* To find the volume of oligo RNA template required we used NEBio calculator ss: moles to mass (https://nebiocalculator.neb.com/#!/ssdnaamt)

8 Incubate at **37 °C** overnight. We usually incubate for 16 hours using a heat block.

RNA Purification with SPRI beads

9 Proceed to purification. We used Ampure beads to purify gRNAs after transcription.

SPRI beads (homemade) or Ampure XP beads **Contributed by users**

10 Equilibrate Ampure XP beads to room temperature.

11 For every 200 µL of the IVT reaction, add 300µL of 100% ethanol (1:1.5 ratio e.g. for 90 µl IVT reaction mixture then add 135 µl of ethanol). The solution should turn a cloudy white (precipitation of RNA) upon addition of ethanol. This step helps the short RNAs bind to the beads. This can be done in several 1.5mL tubes.

Ethanol 100% **Contributed by users**

12 For every 500µL of IVT reaction + 100% ethanol, add 500µL of Ampure XP beads to the solution of IVT reaction and ethanol and mix well by inverting or pipetting with a P1000.

13 Incubate at room temperature for **00:05:00** .

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14 Place the tubes on a 1.5 mL magnetic separation rack.

15 Wait **00:05:00** to allow the beads to separate if using a 1.5mL rack. Allow a longer time if necessary to ensure proper separation of beads liquid before proceeding. While waiting make up a batch of 80% ethanol for next step. 5m

16 Make up a batch of 80% ethanol for following wash steps. Usually 5ml is sufficient for washing both crRNA & tracrRNA tubes.

17 Remove and discard the supernatant while tube remains in magnetic rack.

18 Rinse the beads with 1mL of 80% ethanol if using a 1.5mL tube . It is not necessary to resuspend the beads.

19 Wait **00:01:00** then remove and discard the ethanol.

20 Repeat the wash step as described above. (Add the same amount of 80% ethanol, wait **00:01:00** , then discard the ethanol.)

21 Remove residual ethanol that collects at the bottom of the tube by using a P200 or P20.

22 Air dry the beads for **00:05:00** in a 1.5mL tube or until the beads lose their glossy appearance. Sufficiently dry beads will appear matte. Be careful not to let the beads get too dry (appearing cracked or dusty). 5m

23 Elute the RNA by resuspending the beads with an appropriate amount of nuclease-free H2O depending on the desired volume and concentration. For the proceeding nanopore sequencing we usually elute with 30-50µL nuclease free H20.

Nuclease-free Water **Contributed by users**

24 Flick the tubes to combine and allow the RNA to elute off the beads by incubating at room temperature for **00:10:00** . 10m

25 If necessary, pulse-spin the tubes to collect any liquid along the sides of the tubes.

26 Place the tubes on the magnetic rack and allow them to separate until water is clear. This will take 5-10 minutes for a

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1.5mL tube.

27 Collect the eluted RNA, being careful not to take up beads.

Quantify, anneal and aliquot dgRNA

28 Using the XR RNA Qubit kit, quantify 1µL of the eluted tracrRNA and 1µL of the eluted crRNA. Follow standard XR RNA Qubit protocol. if concentration is too large to read initially make a 1 in 10 dilution.

Qubit™ RNA XR Assay Kit **Thermo Fisher Scientific Catalog #Q33223**

29 Store RNA at **-80 °C** in small aliquots (we usually store 3µl-6µl aliquots in PCR tubes) in order to avoid freeze-thaws. The crRNA & tracrRNA should be frozen separately. Annealing to make the dgRNAs should be done only prior to sequencing.

Preparing input DNA *From Oxford nanopore protocol - please refer to their protocol for further detail

30 Prepare 5µg of high molecular weight genomic DNA (a range of 1-10µg can be used but we have found that higher DNA input works better).

31 Prepare DNA in nuclease free water at a 24µl total volume in a PCR tube. Flick to resuspend and spin down.

Preparing cas9 ribonucleoprotein complexes (RNPs)

32 Preheat thermal cycler to **95 °C** . Thaw an aliquot of NEB CutSmart Buffer, mix by vortexing, and place on ice.

33 In a PCR tube add in the following order (ensure crRNA & tracrRNA are equimolar - 100µM):

A	B
Duplex Buffer	8µL
crRNA pool (100µM)	1µL
tracrRNA (100µM)	1µL

* To help calculate the volume required from the amount of crRNA & tracrRNA previously made, we have used the bioline calculator. (https://www.bioline.com/media/calculator/01_07.html - *Calculation of the molar concentration*)

Pipette the mixture up and down. Place in thermal cycler for 5 mins at **95 °C**

34 Cool to room temperature and spin down to pool liquids - the crRNA : tracrRNA complexes will now be annealed.

Forming RNPs

35 In a 1.5ml Eppendorf tube

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A	B
Annealed tracrRNA : crRNA complex	10µL
10x NEB cutsmart buffer	10µL
Nuclease free H2O	79.2µL
Pyrogenes Hifi Cas9 nuclease	0.8µL
Total Volume	100µL

* further information can be found in the Oxford Nanopore protocol for scaling this reaction down accordingly.

Mix by flicking the tube, and incubate at room temperature for 30 mins. Place on ice. The RNP complexes should now be formed.

Phosphorylate genomic DNA

36 In a PCR tube add:

A	B
10x CutSmart Buffer	3µL
Your Genomic DNA	24µL
Total reaction Volume	27µL

Mix gently by flicking the tube and then spin down.

CutSmart Buffer - 5.0 ml **New England Biolabs Catalog #B7204S**

12m

37 Add 3µl of CIP to the tube

Using a Thermocycler, incubate at **37 °C** for **00:10:00** , **80 °C** for **00:02:00** then hold at **20 °C** .

Alkaline Phosphatase, Calf Intest (CIP) - 1,000 units **New England Biolabs Catalog #M0290S**

Cleave and dA-tail the target DNA

38 Thaw dATP tube, vortex to mix, and place on ice.

39 In a 1.5ml Eppendorf lobind tube make up dATP mix to 10mM. Vortex to mix and spin down (e.g. 1µl of dATP to 9µl nuclease free water for a 100mM stock), then place on ice.

Nuclease-free Water **Contributed by users**

20m

40 To the PCR tubes containing the 30µl of desphophorylated DNA add:

A	B
Desphosphorylated genomic DNA sample	30µl
Cas9 RNPs	10µl
10 mM dATP	1µl
NEB Taq polymerase	1µl
Total reaction volume	42µl

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Carefully mix the contents by inversion of tube, spin down and place in thermal cycler.

Incubate sample for the following cycle: **37 °C** for **00:15:00** **72 °C** for **00:05:00** and hold at **4 °C**

.

Adaptor Ligation

41 Thaw Ligation Buffer (LNB) at room temperature, mix via pipetting due to viscosity. Place on ice.

Thaw the adaptor mix (AMX), mix by flicking tube and pulse spin. Return to ice.

Place Ampure XP beads at room temperature

42 Using a wide-bore tip transfer contents from the previous step from the PCR tube into a 1.5ml lobind eppendorf tube.

43 In a seperate 1.5mL Eppendorf Tube assemble:

A	B
Ligation Buffer (LNB)	20µl
Nuclease free water	3µl
NEBNext Quick T4 DNA ligase	10µl
Adapter Mix (AMX)	5µl
Total reaction volume	38µl

Mix by pipetting up and down to ensure the viscous LNB is incorporated

44 Add 20µl of this adapter ligation mix to the cleaved and dA-tailed sample in the other 1.5ml eppendorf tube. Mix gently by flicking up and down. immediately after mixing, add the remainder of the adapter ligation mixture to the cleaved and dA-tailed sample, which will yield an 80µl ligation mix. 10m

Mix by flicking the tube and pulse spin to collect droplets.

Incubate mixture for **00:10:00** at room temperature.

Purify adapter ligated DNA - AMpure XP bead purification

45 Resuspend the AMpure XP beads by vortexing.

Thaw the short fragment buffer (SFB) and EB from SQK-LSK109.

46 Add 1x volume (80µl) of TE pH8.0 to the ligation mix and mix by gentle flicking of the tube. Then add 0.3x volume (48uL) of AMPure XP beads to the ligation sample. 10m

Gently invert the tube and then spin down.

Incubate sample at room temperature for **00:10:00** - do not pipette or agitate sample.

47 Spin down the sample and pellet on a magnetic rack, then pipette off the supernatant.

48 Using 250µl of Short Fragment Buffer (SFB), resuspend the beads and flick to incorporate. Return tube to magnetic rack. Allow beads to pellet.

Remove supernatant via pipette and discard.

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Repeat this SFB wash step.

49 After removal of the supernatant, let the sample dry for **00:00:15** . Add 13uL of Elution Buffer (EB). Incubate for 10m 15s **00:10:00** at room temperature.

Pellet beads using the magnetic rack and remove 12uL of eluate to a clean 1.5mL Eppendorf DNA LoBind Tube.

This prepared library will be used for loading into the flow cell. Store on ice until ready to load.

Priming and loading the flow cell 5m

50 Thaw sequencing buffer (SQB), loading beads (LB), Flush tether (FLT) and one tube of flush buffer (FB). After thawing place on ice.

51 Mix sequencing buffer (SQB) and flush buffer (FB) tubes by vortexing, spin down and return to ice.

Mix the flush tether (FLT) by pipetting, then return to ice.

52 Open the lid of the MinION sequencing device and slide the flow cell's priming port cover clockwise so that the priming port is visible.

With a P1000µl pipette set to 200µl insert tip into the priming port and turn the wheel until the dial shows 220-230µl whereby a small volume of buffer will be seen in the tip.

53 Prepare the flow cell priming mix: add 30µl of flush tether (FLT) directly to the tube of flush buffer (FB) and mix by pipetting up and down.

54 Load 800µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. 5m

Wait **00:05:00**

55 Thoroughly mix the contents of the Loading beads (LB) via pipetting.

56 In a new 1.5ml lobind eppendorf tube prepare the library for loading:

A	B
Sequencing Buffer (SQB)	25µl
Loading Beads (LB)	13µl
DNA library	12µl
Total volume	50µl

57 Gently lift the sample port cover to make the sample port accessible.

Load 200µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles.

58 Mix the prepared library by gently pipetting up and down immediately prior to loading.

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59 Add 50µl of sample to the flow cell via the sample port in a slow drop wise fashion. Ensure each drop flows into the port before adding the next.

60 Gently replace the sample port cover, close the priming port and replace the MinION lid.

Starting the sequencing run 5m

61 Follow the steps as outlined by Oxford Nanopore to start the sequencing run.

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