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1 **cDNA library construction for Next Generation Sequencing (NGS) to determine the**
2 **transcriptional landscape of *Legionella pneumophila***

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19 mapping, small ncRNA
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1 **Summary**

2 The adaptation of *Legionella pneumophila* to the different conditions it encounters in the
3 environmental and in the host is governed by a complex regulatory system. Current
4 knowledge of these regulatory networks and the transcriptome responses of
5 *L. pneumophila* is mainly based on microarray analysis and limited to transcriptional
6 products of annotated protein-coding genes. The application of the Next Generation
7 Sequencing (NGS) technology allows now genome wide strand-specific sequencing and
8 accurate determination of all expressed regions of the genome to reveal the complete
9 transcriptional network and the dynamic interplay of specific regulators on a genome wide
10 level. NGS based techniques promote deeper understanding of the global transcriptional
11 organization of *L. pneumophila* by identifying transcription start sites (TSS), alternative
12 TSS and operon organization, noncoding RNAs (ncRNA), antisense RNAs and 5'-/3'-
13 untranslated regions. In this chapter we describe the construction of cDNA libraries for (i)
14 RNA deep sequencing (RNA-seq) and (ii) transcription start site (TSS) mapping using the
15 Illumina technology.

16

17 **1. Introduction**

18 In recent years, the field of microbial genomics has changed considerable due to the
19 development of new generation sequencing techniques (1). Thus, deep RNA sequencing is
20 now revolutionizing our understanding of the complexity, plasticity and regulation of
21 microbial transcriptomes (2). Recent studies using RNA deep sequencing indicate that the
22 function of untranslated transcriptional regions as posttranscriptional regulator was
23 underestimated in bacteria until now (3-6). In particular the unexpected high amount of
24 ncRNAs found so far strongly suggests that they play a more dominant and widespread
25 role in regulation of gene expression including a) transcription interference/termination b)
26 translational interference c) effects on the stability of target RNA and d) interaction with
27 RNA-binding proteins (for review see (7)). To date the *L. pneumophila* transcriptome was
28 analyzed mainly using microarrays (8-12). Recently the first study using NGS techniques
29 to analyze the intracellular transcriptome was applied to *L. pneumophila* allowing to
30 discover 70 novel small RNAs (13). However, the application of NGS for an in-depth
31 transcriptional analysis of *L. pneumophila* may revolutionize our understanding of adaptive
32 and developmental processes during infection, as it will add new levels of control to our
33 regulatory network. In the following, we present several strategies to construct strand-
34 specific cDNA libraries for Illumina sequencing. A protocol for strand-specific RNA deep

1 sequencing (RNAseq) and two different methods to enrich and define transcriptional start
2 sites (TSS mapping).

3

4 Parallel deep sequencing of the whole transcriptome (RNAseq) reveals detailed prediction
5 of gene expression by determination of length and abundance of transcripts including 5'-
6 and 3'-untranslated region or antisense transcription at different conditions. Anyhow,
7 RNAseq has its limitation as it is not possible to distinguish primary transcripts from
8 processed 5' ends, so, it cannot be used e.g. for identification of alternative transcription
9 starts or operon organization. To achieve a more precise view on the level of primary
10 transcripts it is necessary to construct specific TSS mapping libraries in which the 5'ends
11 of the RNA are highly enriched in comparison to an untreated cDNA control library. We
12 can distinguish between two different approaches to analyze the TSS region:

13 1) Terminator Exonuclease (TEX) method: differential cDNA library pairs treated (+)
14 or not treated (-) with TEX. Primary transcripts in bacteria like mRNA and ncRNA
15 (but not rRNA) are known to be protected by a triphosphate cap at the 5'end. TEX
16 digests specifically RNA having a 5'-monophosphate, but is not able to degrade
17 RNA with a 5'-PPP protection. Therefore, TEX eliminates processed transcripts
18 like partially degraded or sheared RNA leading to an enrichment of primary
19 bacterial transcripts with intact 5'-triphosphate terminus (Fig 1).

20 2) Tobacco Acid Pyrophosphatase (TAP) method: differential cDNA library pairs
21 treated (+) or untreated (-) with TAP. TAP hydrolyzes pyrophosphate of 5'-PPP
22 RNA resulting in 5' monophosphorylated end. This step is essential during the library
23 construction as the 5' RNA adapter will ligated only to a 5'-monophosphorylated
24 RNA but not to RNA with 5'-PPP terminus. For that reason, the untreated TAP (-)
25 control library include all transcripts with 5'-P terminus while the TAP (+) library
26 additionally comprise the start sites of the transcript formally protected by the 5'-
27 triphosphate cap (Fig 2).

28

29 **2. Materials**

30 **2.1. Strains and growth media**

31 1. *Legionella pneumophila* e.g. strains Paris, JR32 or Philadelphia-1

32

1 2. AYE medium. For 1 liter dissolve 12 g yeast extract and 10 g ACES, adjust pH to 6.9
2 with 1 M KOH. Add 10 mL of cysteine 40 g/L and 10 mL of iron pyrophosphate 30
3 g/L. Fill volume to 1 L with distilled water and filter sterilize.

4
5 3. CYE plates. For 1 liter dissolve 10 g yeast extract and 10 g ACES, adjust pH to 6.9
6 with 1M KOH, add 15 g of agar, 2 g of activated charcoal and autoclave. Add 10mL of
7 filter sterilized cysteine 40 g/L and 10 mL of filter sterilized ferric nitrate 25 g/L. Poor
8 plates.

10 **2.2. RNA extraction**

11
12 1. Resuspension buffer: ½ volume of Glucose 20% + ½ volume of Tris 25 mM pH 7.6

13
14 2. EDTA 10 mM

15
16 3. EDTA 0.5 M

17
18 4. Glass beads, Sigma (200-300 microns Sigma G1277)

19
20 5. Water treated with DEPC

21
22 6. Phenol acid pH 4.5 (Interchim)

23
24 7. Total RNA extraction, TRIzol reagent (Invitrogen,

25
26 8. Chloroforme/alcohol isoamyl 24/1 (v/v)

27
28 9. Isopropanol (RT temperature)

29
30 10. Ethanol 70% (ice cold)

31
32 11. Tris-HCl 10mM pH 7.6 EDTA 1mM

33
34 12. FastPrep Instrument to lyse bacteria

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13. 1.5 mL microcentrifuge tubes

14. Shaking platform at 37°C

15. Disposable 50 mL polypropylene tubes

2.3. cDNA library construction for Illumina sequencing

1. 10µg RNA

2. DNase I recombinant, RNase free

3. Microbe Express, Bacterial mRNA Enrichment Kit from

4. Terminator™ 5'-Phosphate-Dependent Exonuclease, TEX

5. Phenol solution, pH4.3 (Sigma, P4682-100ML); Chloroform:Isoamyl alcohol 24:1

6. RNase inhibitor (e.g. RNaseOUT™ Ribonuclease Inhibitor)

7. 3M Sodium Acetate, pH5.2

8. Ethanol p.a.; Ethanol 75%

9. Glycogen (20mg/ml)

10. Tobacco acid pyrophosphatase 10U/µl, TAP (Epicentre, T19500)

11. 5'-RNA adapter (25µM) GUUCAGAGUUCUACAGUCCGACGAU

12. T4 RNA Ligase 5U/µl

- 1 13. Superscript II Reverse Transcriptase Kit
- 2
- 3 14. RT Random primer (100 μ M) CAAGCAGAAGACGGCATAACGANNNNNN
- 4
- 5 15. RT Specific primer (100 μ M) CAAGCAGAAGACGGCATAACGA
- 6
- 7 16. RNA fragmentation kit (Ambion)
- 8
- 9 17. Alkaline Phosphatase
- 10
- 11 18. 3'-RNA adapter (100 μ M) P-UCGUAUGCCGUCUUCYGCUUGUIdT
- 12
- 13 19. T4 Polynucleotide Kinase
- 14
- 15 20. 10mM dNTP mix
- 16
- 17 21. Certified Low Range Ultra Agarose (Biorad, 161-3106)
- 18
- 19 22. Agarose gel electrophoresis equipment
- 20
- 21 23. 0.5M EDTA pH8.0
- 22
- 23 24. Distilled water DNase/RNase free
- 24
- 25 25. Thermocycler
- 26
- 27 26. Phusion HF DNA Polymerase
- 28
- 29 27. Primer 1 (25 μ M) CAAGCAGAAGACGGCATAACGA
- 30
- 31 28. Primer 2 (25 μ M) AATGATACGGCGACCACCGACAGGTTTCAGA
- 32 GTTCTACAGTCCGA
- 33
- 34 29. NucleoSpin® Gel and PCR Clean-up

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30. Bionalyzer 2100 (Agilent)

31. RNA Nano Chips (Agilent, 5067-1511)

32. DNA 1000 Chips (Agilent, 5067-1504)

3. Methods

3.1. RNA isolation

1. From a glycerol stock maintained at -80°C , streak the strain on a CYE plate and incubate it at 37°C for 72 h until you obtain colonies in stationary phase.
2. Pick colonies from the plate and grow a pre-culture in 50ml polypropylene tubes overnight, 37°C , shaking
3. Dilute the pre-culture. Grow cultures to a chosen OD and take a sample (10 ml).
4. Centrifuge in a 15ml falcon tube, 5000xg for 5 min at 4°C in a pre-cooled centrifuge.
5. Withdraw the supernatant and flash freeze the pellet on dry ice +EtOH or proceed with the next step immediately.
6. Prepare a sarstedt tube, add 500 μl phenol acid and 0.4 g glass beads.
7. Resuspend the bacterial pellet in 400 μl resuspension buffer and 60 μl EDTA 0.5 M.
8. Transfer suspension to the tubes containing phenol acid and glass beads.
9. Lyse the cells with the FastPrep apparatus with the following settings:
Speed: 6.0; Time: 30s
10. Let stand for 1 min at 4°C and repeat step 4 once more with the same settings.

- 1 11. Centrifuge for 5 minutes at 13000 rpm at 4°C. Transfer the top liquid phase to a
2 sterile eppendorf tube.
3
- 4 12. Add 1 ml room-tempered Trizol. Mix very gently with a pipette till it is 'foamy' and
5 let stand for 5 min on bench.
6
- 7 13. Add 100 µl chloroforme/IAA. Mix vigorously by shaking. Let stand for 1 minute on
8 bench.
9
- 10 15. Centrifuge 5 min at 13000 rpm at 4°C. Transfer the aqueous phase to a new
11 eppendorf tube.
12
- 13 16. Add 200 µl chloroforme/IAA. Mix vigorously and let stand for 1 minute at room
14 temperature.
15
- 16 17. Centrifuge for 5 minutes, 13000 rpm at 4°C. Transfer the aqueous phase to a sterile
17 eppendorf tube.
18
- 19 18. Add 500 µl isopropanol and agitate by inversing the tube. Let stand for 30 minutes
20 on ice.
21
- 22 19. Centrifuge 15 minutes, 13000 rpm at 4°C.
23
- 24 20. Rinse the pellet with 1 ml ice cold ethanol 70%.
25
- 26 21. Centrifuge 5 minutes, 13000 rpm at 4°C.
27
- 28 22. Withdraw the supernatant and dry the pellet (SpeedVac or airdry on bench).
29
- 30 23. Resuspend the pellet in 50 µl H₂O.
31
- 32 24. Incubate for 15 minutes at 37°C, measure the concentration aliquot and freeze to –
33 80°C or use directly for library construction

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3.2. Transcription start sites (TSS) mapping library construction

1. RNA extraction using the TRIzol reagent (see also **Note 1**)
2. Depletion of rRNA using Microbe Express (**Note 2**)
3. TEX treatment: divide the RNA in two similar aliquots and incubate one with or without TEX (TEX-/+ library, for construction of TAP-/+ library see **Note 3**):
 - Xµl depleted RNA (recovered from MicrobeExpress)
 - Xµl RNase-free water
 - 0.5µl RNase Inhibitor
 - 2µl TEX buffer A
 - 1U TEX(final volume of the reaction 20µl)
4. Incubate for 60min at 30°C
5. Terminate reaction by adding 1µl 100mM EDTA + 180ulH₂O
6. Purify RNA by organic extraction (PhenolChloroform/Isoamylalcohol, see **Note 4**).
After NaAc precipitation (Note 5), resuspend the dry pellet in 44µl distilled water
7. TAP treatment: incubate both samples (with and without TEX) independently with TAP:
8. Denature the RNA from step 3 for 10min at 65°C
9. Incubate 1min on ice
10. Add 5µl TAP buffer and 1µl TAP (10U) and incubate for 1h at 37°C
11. Extract with Phenol/Chloroform/IAA (see **Note 4**)
12. Precipitate with NaAc (see **Note 5**)
13. Resuspend dry pellet in 5.3µl distilled H₂O

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14. 5'-RNA adapter ligation:

5.3µl RNA

1.2µl 5'-RNA adapter (25µM)

Incubate mix for 10min at 65°C, put on ice for 1min and add

1µl T4 RNA ligase buffer

1µl ATP solution

0.5µl RNase inhibitor

1µl RNA ligase

And incubate for 6h at 20°C, following 4°C over night

15. Reverse Transcription using RT Random primer

10µl RNA +1.5µl random primer (100µM)

Denature at 65°C for 10min, put on ice 1min and add

6µl first strand buffer

1.5µl DTT (100mM)

1.5µl dNTP

1µl RNase inhibitor

7µl RNase-free H₂O

1.5µl Superscript II RT

16. Incubate 10min at 25°C, following 1h at 42°C

17. Size fractionation on a low range ultra 2% agarose gel (**Note 6**)

Cut the zones between 100-170nt and 170-250nt and purify independently with columns (e.g. Nucleospin)

Combine the samples of the two “zones”, precipitate and resuspend pellet in 30µl distilled water

18. PCR amplification (**Note 7**)

30µl template cDNA

10µl Phusion HF buffer

8µl H₂O

0.5µl Primer 1 (25µM)

- 1 0.5µl Primer 2 (25µM)
- 2 0.5µl dNTP (25mM)
- 3 0.5µl Phusion DNA Polymerase
- 4
- 5 98°C 1min
- 6
- 7 98°C 10sec
- 8 60°C 30sec
- 9 72°C 30sec
- 10 15 cycles
- 11
- 12 72°C 10min
- 13 4°C
- 14
- 15 19. Purify PCR reaction with columns (e.g. Nucleospin)
- 16
- 17 20. Precipitate and resuspend in 10µl
- 18
- 19 21. Check quality and quantity with the Bioanalyzer (DNA 1000)
- 20
- 21 22. Ready for Sequencing with Illumina

23 **3.3. RNAseq library construction**

- 24
- 25 1. RNA extraction (Trizol method, see also Note 1)
- 26 2. Depletion of rRNA using Microbe Express
- 27 After NaAc precipitation, resuspend the dry pellet in 10µl distilled H₂O
- 28 3. Fragmentation: the RNA was metal-catalyzed heat fragmented to sizes of around
- 29 100-200nt using the RNA fragmentation kit
- 30
- 31 1.1µl 10x fragmentation reagent (buffered zinc solution)
- 32 10µl RNA
- 33 Incubation at 70°C for 5min
- 34 Terminate reaction by putting on ice and add 1.1µl stop solution

1 After NaAc precipitation (**Note 5**), resuspend the dry pellet in 26µl RNase-free
2 distilled H₂O

3

4 4. RNA dephosphorylation:

5 a) TAP treatment

6 Denature the RNA from step 3 for 10min at 65°C

7 After 1min on ice, add 3µl TAP buffer and 1µl TAP (10U) and incubate for 1h at
8 37°C

9 b) Alkaline Phosphatase treatment

10 Add 14µl distilled H₂O, 5µl 10x Dephosphorylation buffer and 1µl Alkaline
11 Phosphatase (10U) and incubate for 30min at 37°C

12

13 Phenol/Chloroform/IAA extraction (**Note 4**), NaAc precipitation; resuspend the dry
14 pellet in 5.9 µl distilled water

15

16 5. 3'-RNA adapter ligation

17

18 5.9µl RNA

19 0.6µl 3'adapter (100µM)

20 Incubate mix for 10min at 65°C, put on ice for 1min and add

21 1µl T4 RNA ligase buffer

22 1µl ATP solution

23 0.5µl RNase inhibitor

24 1µl RNA ligase

25 Incubate for 6h at 20°C, following 4°C over night, purify with

26 Phenol/Chloroform/IAA extraction and resuspend RNA after NaAc precipitation in

27 10µl distilled water

28

29 6. Re-phosphorylation of 5' end

30

31 10µl RNA

32 2µl PK buffer

33 1µl ATP solution

34 0.5µl RNase inhibitor

- 1 1µl Polynucleotide Kinase
2 5.5µl RNase free H₂O
3
4 7. Size fractionation on a low range ultra 2% agarose gel
5 Cut the zones between 100-170nt and 170-250nt and purify with columns (e.g.
6 NucleoSpin). Precipitate with NaAc and resuspend in 5.3µl distilled H₂O
7
8 8. 5'-RNA adapter ligation:
9
10 5.3µl RNA
11 1.2µl 5'-RNAadapter (25µM)
12 Incubate 10min at 65°C, on ice for 1min and add
13 1µl T4 RNA ligase buffer (Epicentre)
14 1µl ATP solution
15 0.5µl RNase inhibitor
16 1µl RNA ligase
17 And incubate for 6h at 20°C, following 4°C over night
18
19 9. Reverse Transcription using RT Specific primer
20 10µl RNA +0.5µl RT Specific primer (100µM)
21 Denature at 65°C for 10min, on ice 1min and add
22 6µl first strand buffer
23 1.5µl DTT (100mM)
24 1.5µl dNTP
25 1µl RNase inhibitor
26 8µl RNase-free H₂O
27 1.5µl Superscript II RT
28
29 Incubate 1h at 42°C
30
31 10. Size fractionation on a low range ultra 2% agarose gel (**Note 6**)
32 Cut the zones between 100-170nt and 170-250nt and purify independently with
33 columns (e.g. Nucleospin)

1 Combine the samples of the two “zones”, precipitate and resuspend pellet in 30µl
2 distilled water

3

4 11. PCR amplification (see also **Note 7**)

5

6 30µl template cDNA

7 10µl Phusion HF buffer

8 8µl H₂O

9 0.5µl Primer 1 (25µM)

10 0.5µl Primer 2 (25µM)

11 0.5µl dNTP (25mM)

12 0.5µl Phusion DNA Polymerase

13

14 98°C 1min

15

16 98°C 10sec

17 60°C 30sec

18 72°C 30sec

19 15 cycles

20

21 72°C 10min

22 4°C

23

24 Purify PCR reaction with columns (e.g. Nucleospin), precipitate and resuspend in

25 10µl

26

27 12. Check quality and quantity with the Bioanalyzer (DNA 1000)

28 13. Ready for Sequencing with Illumina

29

30 **4. Notes**

31

32 1. Extracted RNA must be treated with DNase I, purity and concentration can be
33 determined by measuring the absorbance at 260nm and 280nm. Nevertheless, to

- 1 guarantee best quality a Bioanalyzer analysis should be performed with total and rRNA-
2 depleted RNA.
- 3 2. Microbe Express is performed according to the manufacture's instruction with 2x10 μ g
4 of total RNA for each condition as starting material.
- 5 3. Alternatively to the comparison of libraries treated with and without TEX, a TAP-/+
6 library can be constructed. For this purpose no TEX treatment is necessary! Instead,
7 divide the depleted RNA after step 2 (MicrobeExpress treatment) in two similar aliquots
8 and incubate one fraction with, the other without TAP as described in step 7-10 and
9 continue with step 11.
- 10 4. Phenol/Chloroform/IAA extraction: Add 50% of Phenol to the sample and mix
11 vigorously by vortexing. Add the same amount of Chloroform/Isoamyl alcohol and mix
12 well. Centrifuge at maximal speed for 5 min, transfer the supernatant to a new tube and
13 add 1 vol of Chloroform/Isoamyl alcohol, mix vigorously, centrifuge and transfer
14 supernatant into a new tube
- 15 5. NaAc precipitation: Add 10% 3M sodium acetate (pH5.2), 2% glycogen and 2.5vol of
16 ethanol p.a. to the sample and store it min 2h (better over night) at -20°C. Centrifuge at
17 4°C for 15min at max speed and discard supernatant. Wash RNA pellet with 500 μ l ice-
18 cold 75% ethanol centrifuge again for 5min, discard supernatant and let the pellet dry
19 for 10min at RT. Resuspend the dry pellet RNase-free distilled H₂O
- 20 6. Final library with a median insert size of around 200 bp is ideal for Illumina NGS.
21 Anyhow, if you choose longer fragments you need to reduce your loading concentration
22 otherwise the clusters begin to overlap due to the length. Also a too wide range of
23 fragment size will have a negative effect on the quality and distribution of clustering.
- 24 7. Number of cycles during PCR amplification can differ between experiments depending
25 on the amount of cDNA obtained in the previous step. Typically are 12 to maximal 17
26 cycles. More cycles are not recommended as additional PCR steps may introduce a
27 significant amplification bias in cDNA representation. DNA amounts due to
28 Bioanalyzer DNA1000 analysis are typically in a range of 10-50 nM. If much less
29 material is obtained, adapt number of cycles in the PCR or increase the amount of
30 starting material. For the hybridization on the Cluster Station concentrations of 1–10
31 pM are recommended.

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1

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3

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- 3 during infection and identifies seventy novel small non-coding RNAs, *PLoS One* 6,
- 4 e17570.
- 5
- 6
- 7

1 Fig 1 Example for TEX- (black) and TEX + (green) library comparison: Artemis software
2 image of the *lpp0001-0003* region. Peaks are representing the relative coverage of strand-
3 specific reads obtained from sequencing cDNA libraries with Illumina HiSeq and mapped
4 to the *L. pneumophila* Paris genome.

5

6 Fig 2 Comparison of TAP- (black) and TAP+ (green) library: Artemis software image of
7 *lpp0001-0003* region. Peaks are representing the relative coverage of strand-specific reads
8 obtained from sequencing cDNA libraries with Illumina HiSeq and mapped to the *L.*
9 *pneumophila* Paris genome.

10

Figure 1.

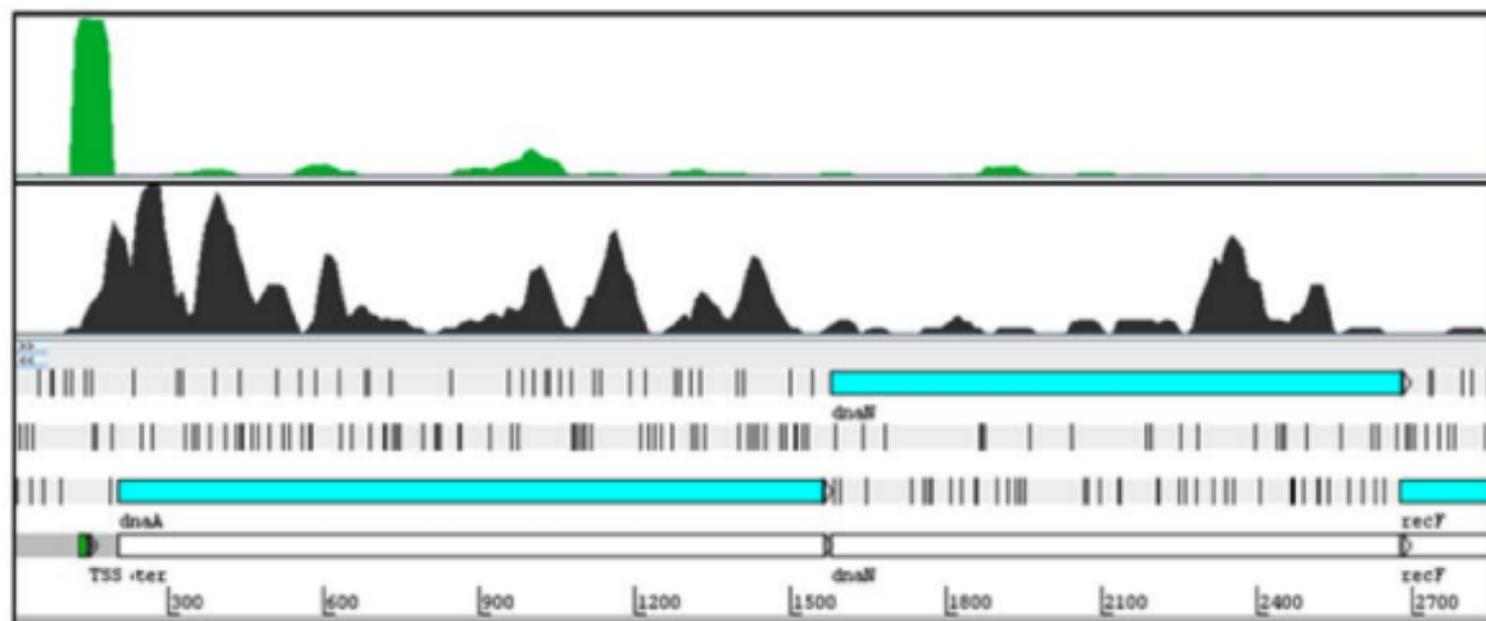


Figure 2.

