5'RACE (Rapid Amplification of cDNA ends)

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Abstract

This protocol can be used for mapping transcriptional start site(s) (TSS) of a specific gene of interest in bacteria.

By slightly modifying this protocol, whole transcriptome TSS could also potentially be mapped.

Citation: Anna Behle 5'RACE (Rapid Amplification of cDNA ends). **protocols.io**
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Guidelines

When handling RNA, samples should always be kept cold (on ice) and in an RNase-free environment. Wear gloves, wipe down surfaces with RNase-Away, use separate, RNase-free water for reactions. As a precautionary measure, samples stored long-term (> 1 month) should be kept at -80 °C.

Before starting, check the integrity of the total RNA on a formaldehyde agarose gel to check for degradation.

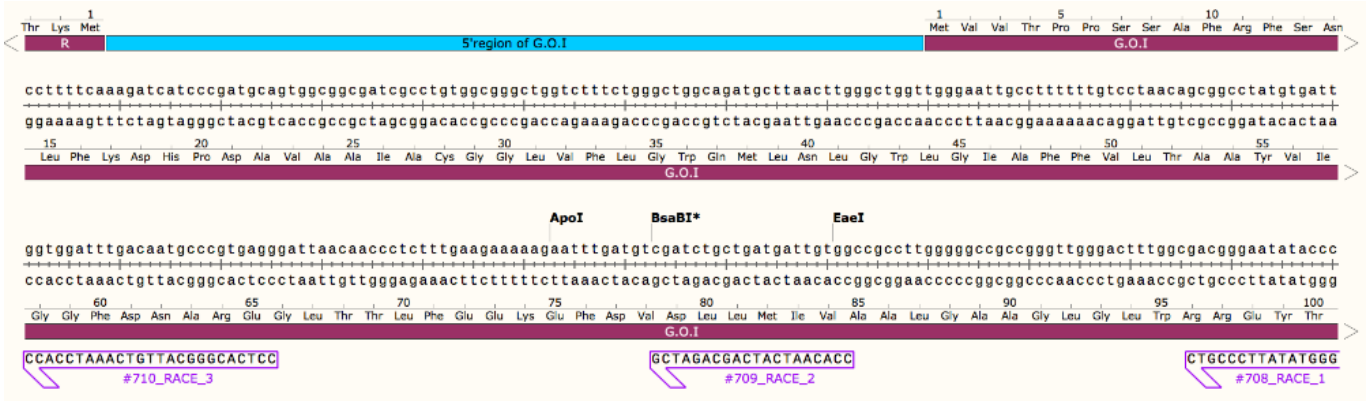
Before start

Oligos used:

5RACE_RNA	rGrUrG rArUrC rCrArA rCrCrG rArCrG rCrGrA rCrArA rGrCrU rArArU rGrCrA rArGrA rNrNrN
Adapter_Prim_1	TGATCCAACCGACGCGAC
Adapter_Prim_2 (nested)	ACCGACGCGACAAGCTAATGC

The image below shows three gene specific primers that anneal in the G.O.I for nested PCR. Blue marks the intergenic region to be mapped, most likely containing the TSS.

Gene specific primers should be carefully designed using online guidelines (e.g. here)



Materials

- DNase I (RNase-free) - 1,000 units M0303S by New England Biolabs
- RNA 5’ Pyrophosphohydrolase (RppH) - 200 units M0356S by New England Biolabs
- Q5 High-Fidelity DNA Polymerase - 500 units M0491L by New England Biolabs
- T4 RNA Ligase 1 (ssRNA Ligase) (30,000 units/ml) - 5,000 units M0437M by Contributed by users

  SuperScript® III First-Strand Synthesis System 18080-051 by Thermo Scientific
- T4 Polynucleotide Kinase - 500 units M0201S by New England Biolabs

  RiboLock RNase Inhibitor EO0381 by Thermo Scientific

  FastAP Thermosensitive Alkaline Phosphatase EF0654 by Thermo Scientific

Protocol

Growth, harvesting, RNA isolation

Step 1.

Prepare bacterial culture using culture conditions of choice (e.g. high salt, oxidative stress, etc.)

Harvest cells and extract total RNA.

PROTOCOL

. Isolation of total RNA from Synechocystis (PGTX method)

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Harvest

Step 1.1.

Grow cyanobacteria to an OD_{750} of 1.

Fill a 50 mL tube with ice. Add culture until full (approx. 25 mL)

Centrifuge tube 5 min at maximum speed.

Step 1.2.

Discard supernatant. Resuspend cyanobacterial pellet in the remaining water (1mL)

Transfer to a fresh 2 mL tube. Spin down 1 min at maximum speed.

Step 1.3.

Resuspend pellet in 1 mL PGTX solution. Flash freeze and store at -80˚C for later extraction, or proceed with the next step.

REAGENTS

- PGTX by Contributed by users

SAFETY INFORMATION

PGTX contains phenol; wear safety gear and gloves

Extract

Step 1.4.

Heat samples at 95˚C in a shaking heat block. Vortex samples from time to time to ensure complete lysis.

Extract

Step 1.5.

Place samples on ice for 5 min.

Extract

Step 1.6.

Add 700 µL Chloroform/IAA. Mix well. Incubate at RT for 10 min, vortexing from time to time.

SAFETY INFORMATION

Wear safety gear

Extract

Step 1.7.

Centrifuge samples at maximum speed for 10 min to separate phases.

Transfer aqueous phase to a fresh tube.

Extract

Step 1.8.

Add 1 vol Chloroform/IAA. Mix well by vortexing. Centrifuge 10 min at maximum speed.

Transfer aqueous phase to a fresh tube.

Precipitation

Step 1.9.

Add 3 vol. of 100 % EtOH + NaOAc 10:1 to the sample. Mix well.

Precipitate 1 h at -80˚C or over night at -20˚C.

Precipitation

Step 1.10.

Centrifuge precipitated sample at 4˚C and maximum speed for at least 30 min.

Remove supernatant, making sure not to disrupt the RNA pellet.

Precipitation

Step 1.11.

Wash pellet with 70% EtOH.

Centrifuge for 15 min, 4˚C at maximum speed.

Completely remove supernatant.

Dry at RT for 5 min. Do not overdry!

Resuspend pellet in 40 µL pure, RNase-free water.

DNase I digest

Step 2.

30 µg total RNA

4 µL 10x DNase I buffer

3 µL DNase I

1 µL RNase Inhibitor (e.g. RiboLock, ThermoScientific)

H_2O ad 40 µL

Incubate 30 min at 37˚C

REAGENTS

TURBO DNA-free™ Kit AM1907 by Thermo Scientific

RiboLock RNase Inhibitor EO0381 by Thermo Scientific

DNase I digest

Step 3.

Add 1 vol. Roti Aqua P/C/I. Mix well, then centrifuge at 4˚C for 10 min at maximum speed.

Transfer to a fresh tube.

REAGENTS

Roti Aqua P/C/I X985.1 by Carl Roth

DNase I digest

Step 4.

Add 1/10 vol 3M NaOAc, pH=5.3, + 3 vol 100% EtOH.

Precipitate RNA at -20˚C over night.

Centrifuge 30 min at maximum speed and 4˚C. Discard supernatant.

Wash with 500 µL 70% EtOH. Centrifuge 15 min, 4˚C at maximum speed. Completely remove supernatant. Dry pellet for 5 min.

Resuspend RNA in 60 µL RNase-free water.

PPi-removal

Step 5.

RppH removes pyrophosphate from triphosphorylated ends of mRNA (primary transcripts in bacteria).

+RppH: Both Both primary (formerly triphosphorylated) and degraded RNA can be linked to the adapter.

-RppH: Only degraded (pyrophosphate removed) can be linked to adapter.

+**Alkaline phosphatase:**Negative control; no ligation possible because all 5'phosphates are removed.

1	2	3
+RppH	**-RppH**	**+FastAP**
5µg DNase-treated RNA	5µg DNase-treated RNA	5µg DNase-treated RNA
10 µL 10x NEB 2 buffer	10 µL 10x NEB 2 buffer	10 µL 10x FastAP buffer
5 µL RppH	5 µL H_2O	5 µL FastAP

1 µL RNase Inhibitor	1 µL RNase Inhibitor	1 µL RNase Inhibitor
H_2O ad 100 µL	H_2O ad 100 µL	H_2O ad 100 µL

Incubate for 1 h at 37˚C.

Possible positive control:

+PNK: labels all dephosphorylated mRNA with a 5' phosphate.

REAGENTS

RNA 5’ Pyrophosphohydrolase (RppH) - 200 units M0356S by New England Biolabs

NOTES

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When working with eukaryotic RNA, RppH can also be used for decapping, but NEB 10x Thermopol buffer needs to be purchased instead of NEBuffer 2.

PPi-removal

Step 6.

Inactivation of enzymes:

Add 1 vol. Roti Aqua P/C/I. Mix well, then centrifuge at 4˚C for 10 min at maximum speed.

Transfer to a fresh tube.

Add 1/10 vol 3M NaOAc, pH=5.3, + 3 vol 100% EtOH + 1 µL glycogen (ThermoScientific, RNA-grade)

Precipitate RNA at -20˚C over night.

Centrifuge 30 min at maximum speed and 4˚C. Discard supernatant.

Wash with 500 µL 70% EtOH. Centrifuge 15 min, 4˚C at maximum speed. Completely remove supernatant. Dry pellet for 5 min.

Resuspend RNA in 67 µL (**1, 3**) / 134 µL (**2**) RNase-free water.

RNA-adapter ligation to 5'-end

Step 7.

5´-RNA-oligo linker:

GUGAUCCAACCGACGCGACAAGCUAAUGCAAGANNN 5´

T4 RNA Ligase 1 catalyzes the ligation of 5' phosphorylated ssRNA to 3' OH.

5'-end of RNA oligo should not be phosphorylated. This will prevent undesired ligation to mRNA 3' end.

	1 (+RppH)	2a (-RppH)	2b (-RppH)	3 (+FastAP)
treated RNA	67 µL	67 µL	67 µL	67 µL
RNase Inh.	2 µL	2 µL	2 µL	2 µL
10x Ligase buffer	10 µL	10 µL	10 µL	10 µL
RNA Oligo linker	1 µL	1 µL	/	1 µL
ATP (10 mM)	10 µL	10 µL	10 µL	10 µL
T4 RNA Ligase 1	10 µL	10 µL	10 µL	10 µL

Incubate 1 hour at 37˚C (or, alternatively, 12 h at 17˚C)

REAGENTS

T4 RNA Ligase 1 (ssRNA Ligase) - 1,000 units M0204S by New England Biolabs

RNA-adapter ligation to 5'-end

Step 8.

Inactivation of enzymes:

Add 1 vol. Roti Aqua P/C/I. Mix well, then centrifuge at 4˚C for 10 min at maximum speed.

Transfer to a fresh tube.

Add 1/10 vol 3M NaOAc, pH=5.3, + 3 vol 100% EtOH + 1 µL glycogen (ThermoScientific, RNA-grade)

Precipitate RNA at -20˚C over night, or at least 1 h at -80˚C

Centrifuge 30 min at maximum speed and 4˚C. Discard supernatant.

Wash with 500 µL 70% EtOH. Centrifuge 15 min, 4˚C at maximum speed. Completely remove supernatant. Dry pellet for 5 min.

Resuspend in 20 µL RNase-free water.

cDNA synthesis

Step 9.

5 µL linked RNA (add H_2O up to 32.5 µL)

2 pmol gene specific primer 1 (0.2 µL of 10µM stock)

Combine RNA and gene-specific primer; denature at 95˚C for 5 min, then chill on ice.

10 µL 5x First strand buffer

1 µL 10 µM dNTPs

1 µL RNase Inhibitor (e.g. RiboLock, ThermoScientific)

2.5 µL 0.1 M DTT

2 µL Superscript III

Incubate 1 hour at 55 ˚C.

REAGENTS

SuperScript® III First-Strand Synthesis System 18080-051 by Thermo Scientific

cDNA synthesis

Step 10.

Add 1 µL RNase H; incubate at 37˚C for 20 min.

REAGENTS

RNase H - 250 units M0297S by New England Biolabs

NOTES

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RNase H specifically removes RNA from DNA:RNA hybrid molecules, but not ss- or dsDNA.

cDNA synthesis

Step 11.

Add 1 vol. Roti Aqua P/C/I. Mix well, then centrifuge at 4˚C for 10 min at maximum speed.

Transfer to a fresh tube.

Add 1/10 vol 3M NaOAc, pH=5.3, + 3 vol 100% EtOH + 1 µL glycogen (ThermoScientific, RNA-grade)

Precipitate RNA at -20˚C over night, or at least 1 h at -80˚C

Centrifuge 30 min at maximum speed and 4˚C. Discard supernatant.

Wash with 500 µL 70% EtOH. Centrifuge 15 min, 4˚C at maximum speed. Completely remove supernatant. Dry pellet for 5 min.

Resuspend cDNA in 21 µL RNase-free water.

RACE-PCR

Step 12.

Perform a PCR with Adapter-specific primer and one nested gene specific primer.

5 µL 5x Q5 rxn buffer

5 µL 5x High GC buffer

0.5 µL Adapter-Primer 1

0.5 µL Gene specific primer (nested)

0.5 µL dNTPs

4 µL cDNA

0.5 µL Q5 Polymerase

H_2O ad 25 µL

Cycling conditions:

98˚C 2 min

98˚C 10 sec |

xx˚C 5 sec | repeat 30-35x

72˚C 10 sec |

72˚C 5 min

Make sure to include a control PCR on DNase-treated RNA to ensure there is no genomic contamination.

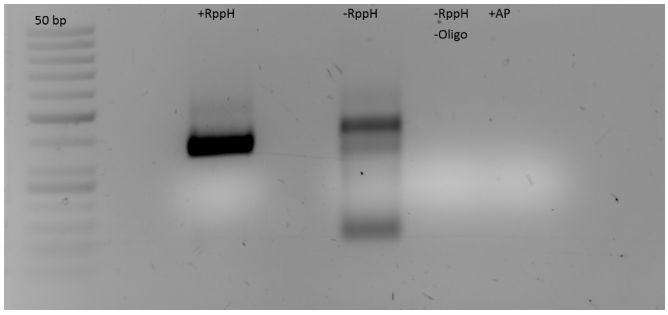
Sequencing

Step 13.

Run PCR products on a 1.8% agarose gel for approx. 1.5 h, or, in case of very small fragments, a 3% NuSieve Agarose gel.

If an RppH+-specific band is observed:

Excise and gel-extract band with the highest molecular weight.



To increase specificity, an additional PCR with nested primers may need to be performed.

NOTES

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It is possible that a gene contains multiple TSS. In case of multiple +RppH specific bands, all of them should be analyzed.

Sequencing

Step 14.

Subclone RACE-PCR fragment into cloning vector of choice, e.g. pJET or TOPO.

Transform DH5α with ligation mix.

Sequencing

Step 15.

Perform colony PCR on colonies from transformation to ensure successful ligation.

In the case of positive clones, purify the plasmids and sequence them.

At least five, possibly more, should be analyzed.

Warnings

PC/I is toxic, handle with care (wear gloves, protective goggles and lab coat).