Primer extension assay and sequencing by dideoxynucleotide incorporation on RNA templates

Matus Valach

Abstract

The identity of a nucleotide or the presence of a bulky modification or strand break in an RNA can be determined by several approaches. When the 3′ region of the analyzed RNA is known, extending a (radio)labeled primer by reverse transcription and analyzing the reaction products using denaturing polyacrylamide gel electrophoresis and subsequent (radio)imaging allows mapping of cDNA chain-termination sites. (Based on the design of the experiment, these can be, for example, a strand break, 5′ end, pause-inducing site, or site of dideoxynucleotide incorporation.) This procedure is adapted from the previously published protocol by Walker and Lorsch (DOI: 10.1016/B978-0-12-420037-1.00020-8).

Citation: Matus Valach Primer extension assay and sequencing by dideoxynucleotide incorporation on RNA templates. **protocols.io**
dx.doi.org/10.17504/protocols.io.ejtbcnn
Published: 23 Feb 2016

Guidelines

- Estimate the concentration of the target in the sample, whether working with a purified transcript or a complex RNA mixture (e.g. total RNA, poly(A) RNA.). For annealing, RNA substrate and labeled oligo should be equimolar. If primer annealing is problematic because of prominent RNA folding (e.g. if the target is a highly structured RNA), the ratio target:primer can be varied in the range of 1:1 to 1:10 to find the optimal hybridization conditions.

Before start

- Prepare 10× annealing buffer: 0.5 M HEPES, pH 7.5 + 1 M KCl.
- Prepare 5× extension buffer: 0.5 M Tris, pH 8.0 + 50 mM MgCl2 + 50 mM DTT.
- Prepare 1 M NaOH and 1M HCl.
- Prepare 2× STOP/loading buffer: 95% deionized formamide, 0.025% SDS, 0.5 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol FF.
- Prepare 5× TBE: 49.5 mM Tris + 49.5 mM boric acid + 2 mM EDTA, pH 8.0.
- Use RNase-free water in all solutions.

Materials

Potassium chloride View by P212121

T4 Polynucleotide Kinase Reaction Buffer - 4.0 ml B0201S by New England Biolabs

AMV Reverse Transcriptase - 200 units M0277S by New England Biolabs

T4 Polynucleotide Kinase - 500 units M0201S by New England Biolabs

HEPES BP310 by Fisher Scientific

EDTA, disodium salt, dihydrate S312-500 by Fisher Scientific

Boric acid BP1681 by Fisher Scientific

Sodium Hydroxide BP359500 by Fisher Scientific

Tris(hydroxymethyl)aminomethane 327360010 by Thermo Fisher Scientific

Hydrochloric Acid by Contributed by users

NucAway™ Spin Columns AM10070 by Thermo Scientific

Magnesium Chloride AC223210010 by Fisher Scientific

Dithiothreitol BP1725 by Fisher Scientific

Formamide (deionized) BP228100 by Fisher Scientific

Sodium Dodecyl Sulfate BP166 by Fisher Scientific

Bromophenol Blue BP11525 by Fisher Scientific

Xylene Cyanol FF AC42269-0050 by Fisher Scientific

Protocol

Step 1.
To radioactively label the primer at its 5′ end, mix the following components (20 µl):

Component	Amount [µl]	Final concentration
10× polynucleotide kinase (PNK) buffer	2	1×
100 µM DNA oligonucleotide	0.5	2.5 µM
6000 Ci/mmol (10 µCi/µl) [γ-^{32}P]ATP	5	2.5 µCi/µl
10 U/µl T4 PNK	1	0.5 U/µl
Water (ddH_2O)	to 20	

Step 2.
Incubate for 45 minutes at 37 °C.

Step 3.
Inactivate the enzyme for 20 minutes at 65 °C.

Step 4.
Remove unincorporated radioactive ATP with a Sephadex column (e.g. NucAway) according to instructions.

Step 5.
To anneal the labeled primer to the RNA substrate, mix the following components (7 µl):

Component	Amount	Final concentration
10× annealing buffer	0.7 µl	1×
RNA (target)	0.1-20 pmol	

Labeled DNA oligo	0.1-20 pmol
RNase-free water (ddH_2O)	to 7 µl

Step 6.
Denature for 1 minute at 95 °C, then slowly cool the sample (at a rate of 0.1 °C/s) to *[primer T_m-5]* °C and incubate for additional 10 minutes (e.g. if the primer T_m is 55 °C, stop at 50 °C).

Step 7.
After cooling to 42 °C, immediately add the following mixture (to the final volume of 25 µl):

7.1. For regular primer extension:

Component	Amount [µl]	Final concentration
5× extension buffer	5	1×
10 mM (each) dNTPs	2.5	1 mM (each)
10 U/µl AMV reverse transcriptase	1	0.4 U/µl
RNase-free water (ddH_2O)	9.5	

7.2. For dideoxy-termination sequencing (e.g. A lane):

Component	Amount [µl]	Final concentration
5× extension buffer	5	1×
10 mM (each) dNTPs	2.5	1 mM (each)
5 mM ddTTP	1.25	0.25 mM
10 U/µl AMV reverse transcriptase	1	0.4 U/µl
RNase-free water (ddH_2O)	8.25	

Step 8.
Incubate for 45 minutes at 42 °C.

Step 9.
Add 3 µl of 1 M NaOH (final concentration 100 mM) to degrade RNA.

Step 10.
Incubate for 10 minutes at 95 °C.

Step 11.
Add 3 µl of 1 M HCl to neutralize the solution.

Step 12.
Add 31 µl of 2× STOP/loading buffer.

Step 13.
Incubate for 5-10 minutes at 95 °C.

Step 14.
Place on ice for 3 minutes.

Step 15.
Load on a pre-ran denaturing PAGE gel (1× TBE, 7M urea), run the electrophoresis, and then expose the gel to a film or phosphor-imaging screen.

Warnings

When working with radioactive material, follow the proper radiation safety procedures.