Glyoxal/borate and RNase T1-based detection of inosine residues in RNA

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Abstract

Adenosine-to-inosine (A-to-I) deamination is a functionally important modification of RNA that occurs in many metazoan nuclear transcripts, in several types tRNAs, and in mitochondrial transcripts of diplonemid protists. Several conceptually different methods exists to identify inosine residues in RNA, including the approach that exploits the cleavage after a guanosine or inosine residue by RNase T1. The digestion at Gs is blocked after an incubation in a glyoxal/borate solution and leads predominantly to cleavage 3′ to Is. This protocol blends the procedure originally conceived by Morse and Bass (DOI: 10.1021/bi9709607) and its more recent adaptation of Cattenoz *et al*. (DOI: 10.1261/rna.036202.112).

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Guidelines

- The primer extension assay to map inosine residues achieves single-nucleotide resolution, but requires more material. The RT-PCR assay is more sensitive, but for single-nucleotide resolution, it needs to be coupled to 5′ adapter-ligation and sequencing. For a practical primer extension with sufficiently intense bands for cleavage intermediate detection, the target RNA in a sample should amount to >0.1 pmol (>10 ng of a 300 nt long transcript), optimally >0.5 pmol.
- The described procedure and quantities work well for a transcript, which represents 1-2% of the RNA sample. For the primer extension assay, if more RNA sample is required to achieve >0.1 pmol of the target, scale up all components, so that guanosine residues in the sample RNA are evenly labeled with glyoxal-borate aducts.
- If a purified or synthetic RNA is being analyzed, complement the sample to the final amount of 2.5 µg RNA with non-target RNA (e.g. total yeast tRNAs) to avoid over-digestion with RNase T1. Alternatively, for an RT-PCR assay, use 1 µg of poly(A) RNA as a substrate.

Before start

- Prepare deionized glyoxal (see the protocol: Preparation of deionized glyoxal).
- Prepare 100 mM sodium phosphate buffer, pH 7.0.
- Prepare 1 M sodium borate buffer, pH 7.5.
- Prepare Tris-borate buffer (10 mM Tris-HCl, pH 7.8; 1 M sodium borate, pH 7.5).

Materials

DMSO 472301 by Sigma Aldrich

✓ Ethanol by Contributed by users

Glyoxal (40%) 50649 by Sigma Aldrich

✓ Sodium Phosphate monobasic by Contributed by users

Boric acid BP1681 by Fisher Scientific

Sodium Hydroxide BP359500 by Fisher Scientific

Tris(hydroxymethyl)aminomethane 327360010 by Thermo Fisher Scientific

RNase T1 EN0541 by Thermo Scientific

✓ Hydrochloric Acid by Contributed by users

Protocol

Step 1.
Mix the following components (100 µl):

Component	Amount [µl]	Final concentration
2.5 µg RNA (in water)	34	25 ng/µl
100 mM Sodium Phosphate, pH 7.0	10	10 mM
DMSO	50	50%
40% Deionized Glyoxal	6	2.4%

Step 2.
Incubate for 45 min at 37 °C.

Step 3.
Dilute the reaction by adding an equal volume (100 µl) of 1 M sodium borate, pH 7.5.

Step 4.
Immediately precipitate with five volumes of 100% ethanol (relative to the diluted mixture; 1000 µl).

Step 5.
Spin at >15,000 g for 30 minutes at 4 °C.

Step 6.
Discard the supernatant.

- *A white pellet of 10-20 µl is expected to have formed.*

Step 7.
Add 1 volume of 70% (aq.) ethanol to wash the pellet.

Step 8.
Directly proceed to a spin at >15,000 g for 5 minutes at 4 °C.

Step 9.

Discard the supernatant.

Step 10.
Let the pellet dry at room temperature, so that no traces of liquid remain (30-60 minutes). Alternatively, dry under vacuum for 10 minutes (without heating).

Step 11.
Solubilize the pellet in 40 µl of Tris-borate buffer (10 mM Tris-HCl, pH 7.8; 1 M sodium borate, pH 7.5). The final volume of the resuspended sample should be 47-48 µl.

- *After adding the buffer, wait for 5-10 minutes before resuspending the pellet. This makes the pellet less sticky and prevents sample loss.*

Step 12.
Add RNase T1 to a desired final concentration. If required, dilute the RNase in the Tris-borate buffer. Use the Tris-borate buffer to complete the volume to 50 µl.

- *Optimize the amount using the enzyme concentration gradient by monitoring the progress of the reaction by a Northern blot or RT-PCR. If the inosine content of the investigated site is close to 100%, 1000 U of RNase T1 will cut essentially all molecules of a transcript present at 0.5 pmol or less in 3 minutes at 37 °C.*

Step 13.
Incubate for 3 minutes at 37 °C.

Step 14.
Dilute the reaction with an equal volume of ice-cold RNase-free water and place on ice.

- ***Pause point**: Proceed directly to the next step or snap-freeze the reactions in liquid nitrogen and store at -80 °C.*

Step 15.
Extract the RNA, e.g. using the home-made Trizol substitute (see the corresponding protocol: RNA extraction using the 'home-made' Trizol substitute).

Step 16.
Solubilize the pelleted RNA in 50 µl of 100 mM sodium phosphate pH 7.0.

Step 17.
Add an equal volume (50 µl) of 100% DMSO and mix well.

Step 18.
De-glyoxalate the RNA by incubating it for 3 hours at 65 °C (with gentle mixing every 15-30 minutes).

Step 19.
Dilute the reaction with an equal volume of ice-cold RNase-free water (100 µl).

Step 20.
Extract the RNA, e.g. using the home-made Trizol substitute (see the corresponding protocol: RNA extraction using the 'home-made' Trizol substitute).

Step 21.
Resuspend the RNA in RNase-free water. Store at -80 °C or proceed directly to downstream analysis (Northern blot hybridization, primer extension, RT-PCR, RNA-Seq).

Warnings

DMSO is a very efficient solvent and easily enters through skin. Glyoxal can cause oral, dermal, and respiratory difficulties. Home-made Trizol substitute contains phenol, guanidine thiocyanate, and ammonium thiocyanate, which are all toxic. Work in gloves and use the fume hood.