T4 RNA Ligase 1 Assay Protocol

Materials

T4 RNA Ligase assay buffer : 50 mM Tris.HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM TCEP, and 1 mM ATP (Supplied as 2X).

Ligase Storage Buffer : 50 mM Tris.HCl (pH 7.5), 100 mM NaCl, 2 mM TCEP, 0.1 mM EDTA, 50% (v/v) glycerol, and 0.1% Triton® X-100 (Supplied as 1X).

Stop Buffers: Activity and Exonuclease Assay: 98% (v/v) Formamide, 10 mM EDTA, 0.2% Bromophenol Blue, and 0.2% Xylene.

Endonuclease: 40% (w/v) Glycerol, 100 mM Tris-HCl, pH 8, 1 mM EDTA, 0.5 mg/mL Bromophenol Blue. (Supplied at 10X) , IsoamylChloroform.

10X TBE Buffer: 0.89 M Tris.HCl, 0.89 M Boric Acid, 20 mM EDTA.

Enzymes : T4 RNA ligase 1 & 2

Endonuclease Assay: Proteinase K

Exonuclease Assay: Mung Bean nuclease; ExoIII

Plasmid : Activity Assay Oligos:

- RNA Ligase 1: 5´ fluorescein labelled RNA 15mer (5´-GGGUCGCAGUUGACU-3´) and a 5´ phophorylated DNA 18mer (5´-TTTAATCAATTGCGACCC-3´)
- RNA ligase 2: 5´ fluorescein labelled RNA 8mer (5´-GGCCAGUG-3´); 5´ phophorylated DNA 12mer (5´-AATTCGAGCTCG-3´) and a complementary DNA 20mer (5´-CGAGCTCGAATTCACTGGCC-3´).

Endonuclease Assay: Supercoiled pBR322 (Supplied at 1 µg/µL).

Exonuclease Assay: M13 fluorescein internally labelled primer (supplied at 3.3 µM).

Method

Activity Assay

Mix a ratio of 1.6 µL fluorescein-labelled 15mer (350uM) + 22.2 µL phophorylated 18mer (100µM) (made up to 50 µL with TE buffer) for the RNA ligase 1 substrate. Dilute 1/18 and use 4 µL in 20 µL reaction. Final concs of 15-mer is 122 nM and of 18 mer is 488 nM.

So, 1 U is 50% ligation of 2.4 pmoles of substrate.

and 7.5 µL fluorescein 8mer: 30 µL phosphorylated 12mer: 30 µL complementary 20mer (in a 67.5 µL volume) for the RNA ligase 2 substrate and heat to 100˚C for 5 min. Allow samples to cool slowly (0.1˚/sec).

Set up a MIX of ligase assay buffer (10 µL of 2X buffer per assay), oligo mix (1.5 µM final for RNA ligase 1; 0.12 µM for RNA ligase 2) and water (sterile deionised). You will need 19 µL per assay.

Set up the appropriate number of tubes and aliquot 19 µL of MIX into each.

Add 1 µL of ligase storage buffer to the first tube.

Dilute the enzyme in ligase storage buffer and add 1 µL to each tube.

Mix the solution by gently pipetting up and down before incubating at 37˚C for 30 min.

Stop the reactions by adding 20 µL of formamide stop buffer.

Analyse the samples on a 15% urea-polyacrylamide gel (NOTE 1) run in 0.5X TBE at 300 V for 50 - 55 mins (until bromophenol blue line is 2 cm from the bottom of the gel). Visualise samples using a fluoroimager (NOTE 2).

Endonuclease Assay

Set up a MIX of ligase assay buffer (7.5 µL of 2X buffer per assay), supercoiled pBR322 (0.25 µL of 1 µg/µL stock) and water (sterile deionised). You will need 12.5 µL per assay.

Set up an appropriate number of tubes and aliquot 12.5 µL of MIX into each tube.

Add 2.5 µL of storage buffer to tube 1 (NOTE 3), and 2.5 µL of ligase enzyme (diluted appropriately) to the remaining tubes.

Mix by gentle vortexing and incubate for 4 hours at 37°C.

After incubation add 3 µL of 2% (w/v) SDS and 1.5 µL 10 mg/mL Proteinase K.

Mix briefly by vortexing and incubate for 30 minutes at 37 °C.

Stop reaction by adding 30 µL of 2X GSTEB and 30 µL of chloroform/isoamyl alcohol (v:v, 24:1).

Vortex vigorously ~5 secs and centrifuge for 2 minutes.

Load 20 µL of aqueous phase onto a 1% (w/v) agarose gel.

Run at 80 V for approximately 2 hours

Stain with ethidium bromide (15 mins) , destain (5 – 10 mins) in water and visualise with a transilluminator or gel documentation system.

Exonuclease Assay

Set up a MIX of ligase assay buffer (5 µL of 2X buffer per assay), M13 fluorescein internally labelled primer (2 µL of 3.3 µM stock) and water (sterile deionised). You will need 8 µL per assay.

Set up an appropriate number of tubes and aliquot 8 µL of MIX into each tube.

Add 2 µL of storage buffer to tube 1 (NOTE 3), 2 µL of Mung Bean nuclease and ExoIII to tubes 2 and 3, and 2 µL of ligase enzyme (diluted appropriately) to the remaining tubes.

Mix by gentle vortexing and incubate for 5 hours at 37°C.

Stop the reactions by adding 5 µL of formamide stop buffer.

Analyse the samples on a 15% urea-polyacrylamide gel (NOTE 1) run in 0.5X TBE at 300 V for 50 - 55 mins (until bromophenol blue line is 2 cm from the bottom of the gel). Visualise samples using a fluoroimager (NOTE 2).

Results

Endonuclease Assay

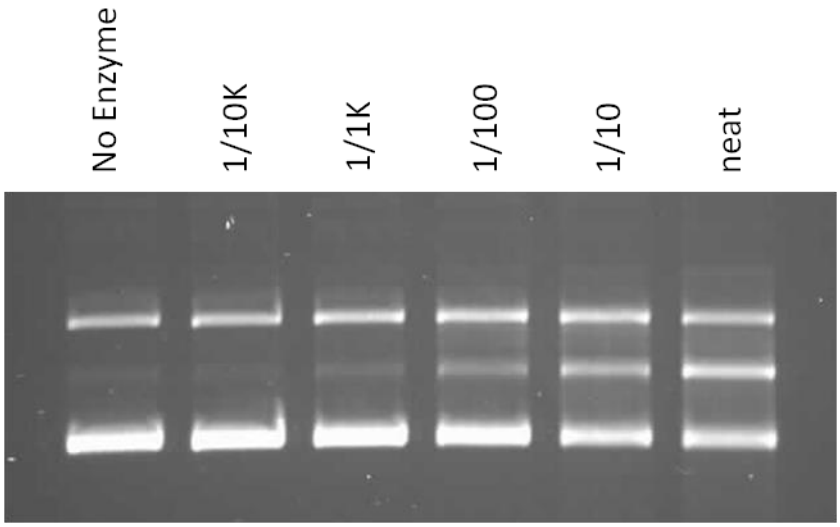

Figure 1: Example of an endonuclease assay. Nuclease activity evident in lanes 3 – 6.

Exonuclease activity

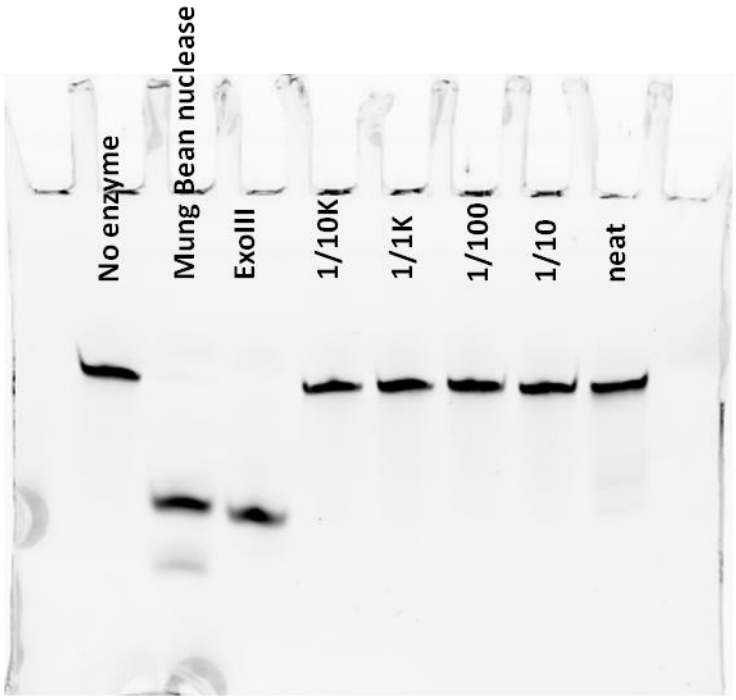

Figure 2: Example of an exonuclease assay. Mung Bean nuclease and ExoIII are positive controls.

Notes

1) 15% denaturing gel: 4.8 g Urea, 1 mL 10X TBE, 1 mL MQ dH_2O, 5.4 mL 40% Acrylamide (19:1), 100 µL AmPS (ammonium persulphate), 7.5 µL TEMED. Makes 2 gels. Heat the urea, acrylamide, TBE and water gently with stirring until the urea is dissolved. Quickly add the TEMED and AmPS and poor the gels immediately. It is recommended that the gels are run at 300 V for 30 min before the samples are loaded. When loading samples do not load in the first or last wells.

2) The fluoroimager (Fujifilm FLA-7001) is located on the second floor of the Chatt building room 208. Login to the computer adjacent to the Fujifilm FLA-7001 (on the left). Open the lid and place the glass stage into the machine lining up the white arrows. Place the gel on the glass stage noting its position on the grid. Close the lid firmly. Open the FLA-7001 software and select Flourescence. For the Activity Assay select FITC under the method dropdown. For the Exonuclease Assay select M13. At the top of the window select the folder in which you wish to save the image and label it. Make sure the red box (sample area) matches where you placed the gel on the stage. Select Start Scan. Once it has completed the scan open the Multiguage software and open the image. Select File – Export and save image as an 'Original image by Gray.TIFF' and an 'Original image by Color.TIFF'. Close both programs (select return and then close the FLA-7001 program). Remove gel and clean the stage with water and ethanol wiping with KIMCARE wipes ONLY. Return the stage to its cover and logout of the computer.

3) The storage buffer contains a high concentration of glycerol therefore the total storage buffer added should not exceed 10% of the final volume.