T4 DNA Ligase Assay Protocols

Materials

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

T4 DNA 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).

TCEP is Tris(2-carboxyethyl)phosphine hydrochloride. MWt=287. 2 mM=0.287 mg/500 mL.

Substrate:

T4 DNA ligase

12mer: 5' Phos AATTCGAGCTCG 3' (5' phosphorylated)

8mer: 5' Fluor GGCCAGTG 3' (5' fluorescein labelled)

20mer: 5' CGAGCTCGAATTCACTGGCC 3'

Annealed to give 5' ^{Fl}GGCCAGTG ^{P}AATTCGAGCTCG

3' CCGGTCACTTAAGCTCGAGC

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

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

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

Endonuclease: 40% (w/v) Glycerol, 100 mM Tris-HCl, pH8, 1 mM EDTA, 0.5 mg/mL Bromophenol Blue. (Supplied at 10X)

Also need Isoamylalcohol/Chloroform for endonuclease assay.

5X TBE Buffer: 0.445 M Tris.HCl, 0.445M Boric Acid, 10 mM EDTA.

Enzymes : T4 DNA ligase

Endonuclease Assay: Proteinase K

Exonuclease Assay: Mung Bean nuclease; ExoIII

Method

Activity Assay

a) Preparation of substrate

Mix 7.5 µL fluorescein 8mer + 30 µL phosphorylated 12mer + 30 µL complementary 20mer in a 67.5 µL volume and heat to 100˚C for 5 min. Allow samples to cool slowly (0.1˚/sec) - (anneal program on PCR machine). This gives a stock solution of 11.1 µM substrate.

DNA	Concentration (µM)	Volume (µl)
Fluorescein 8mer		7.5
Phosphorylated 12mer		30
Complementary 30mer		30

Dilute 1 in 18 with T10E1 to give a 0.62 µM substrate. This is the working solution.

Set up a MIX of ligase assay buffer (10 µL of 2X buffer per assay), oligo mix (4 µl of 0.62 µM substrate gives 0.12 µM final) and water (sterile deionised). You will need 19 µL per assay.

Reagent	**Volume (µl)**
2 x Ligase Assay Buffer	10
Oligo mix (0.62 µM)	4
water	5

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

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 (NOTE 1).

Mix the solution by gently pipetting up and down before incubating at 16˚C for 30 min.

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

Load 20 µL on a 15% urea-polyacrylamide gel (NOTE 2) run in 0.5X TBE at 300V for 50 - 55 mins (until bromophenol blue line is 2 cm from the bottom of the gel). Visualise samples using a fluoroimager (NOTE 3).

1 CELU (cohesive end unit) = dilution at which 50% of the fluorescent oligo is ligated into the larger product.

1 WEISS U = 200 CELU

To be conservative and ensure activity for customer, we use the conversion :

1 WEISS U = 600 CELU

Endonuclease Assay

Set up a MIX of ligase assay buffer (1.5 µL of 10X 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 4), and 2.5 µL of ligase enzyme (diluted appropriately) to the remaining tubes. (see NOTE 1)

Mix by gentle vortexing and incubate for 4 hours 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 (1 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 4), 2 µL of Mung Bean nuclease and ExoIII to tubes 2 and 3 respectively, and 2 µL of ligase enzyme (diluted appropriately) to the remaining tubes. (see NOTE 1)

Mix by gentle vortexing and incubate for 4 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 2) run in 0.5X TBE at 300V for 50 - 55 mins (until bromophenol blue line is 2 cm from the bottom of the gel). Visualise samples using a fluoroimager (NOTE 3).

Results

Activity Assay

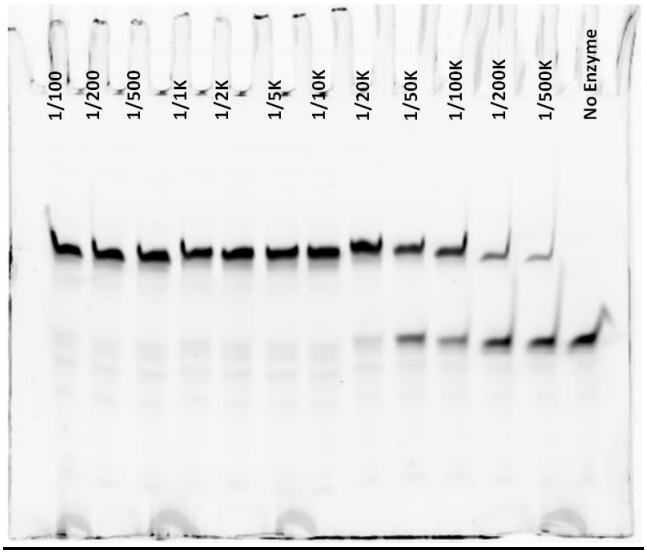

Figure 1: Example of ligase activity assay. 1 CELU is at a 1 in 50K dilution.

Endonuclease Assay

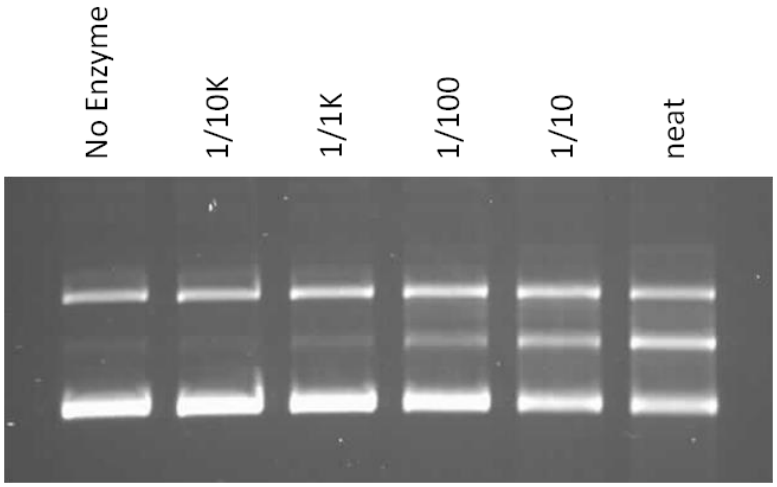

Figure 2: Example of an endonuclease assay. Nuclease activity evident in lanes 3 – 6.

Exonuclease activity

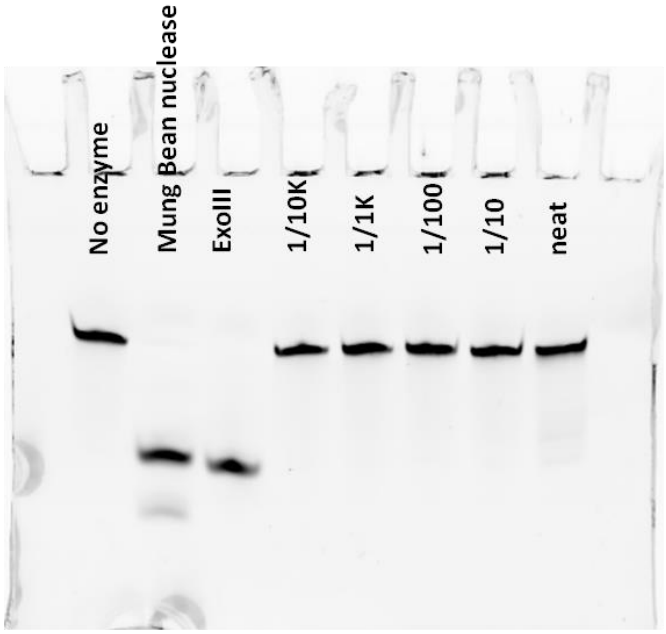

Figure 3: Example of an exonuclease assay. Mung Bean nuclease and ExoIII are positive controls.

Notes

1) Activity assays here are used to work out how active the enzyme is in CELU. Thus it is recommended that serial dilutions between 1/100 to 1/100 000 are made. 4000 U/µL CELU = 20 U/µL WEISS. For conservative estimate use 12000 U/µL CELU = 20 U/µL WEISS. So, for example, if there are 50,000 CELUs/µL then this equates to ~83Weiss U/µL.

2) 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 APS (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 APS and pour the gels immediately. It is recommended that the gels are run at 300V for 30 min before the samples are loaded. When loading samples do not load in the first or last wells.

Reagent	**Amount**
Urea (solid)	4.8 g
5 x TBE	2 mL
40 % Acrylamide (19:1)	3.75 mL
Water	2 mL
10% APS	100 µL
TEMED	7.5 µL

3) 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.

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

For 500 ml 5X TBE buffer	
0.445 M Tris.HCl	26.92 g
0.445 M Boric acid	13.76 g
10 mM EDTA	10 mL of 0.5M EDTA

	Assay buffer 10X	Final 1X Conc	Amount/50 mL	10 mL
1M Tris pH 7.5	500 mM	50 mM	25 mL	5 mL
1M MgCl2	100 mM	10 mM	5 mL	1 mL
TCEP powder	20 mM	2 mM	0.287 g	0.057 g
ATP powder	10 mM	1 mM	0.28 g	0.056 g
pH then add ATP. Need to pH to pH 7.5 at 25 °C and 10X conc for final pH 7.5 at 1X conc at 16°C				
	Dilution buffer 1X			
1M Tris pH 7.5	50 mM		2.5 mL	
5M NaCl	100 mM		1 mL	
TCEP powder	2 mM		0.028 g	
0.25M EDTA	0.1 mM		20 uL	
Triton X-100	0.1% (v/v)		50 uL	
Glycerol	50%		25 mL	