Techniques in Molecular Genetics

Protein and Enzyme Assays

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Day 4

- Protein&EnzymeAssays
- Start SDS Gels
- Immuno (Western) Blotting

Enzyme Assay for Acid Phosphatase

- **6.6 Enzyme assay for acid phophatase activity**
- (Modified from (Dassa et al, 1982) : http://www.jbc.org/cgi/reprint/257/12/6669)
- **Protocols**
- **Prepare sample extract**
  - Inoculate overnight cultures of WT and *rpoS* mutant in 10 ml LB and growth at 37oC
  - Collect cells by centrifugation at 4,000 rpm for 10 min
  - Discard supernatant and resuspend pellet in 10 ml LB 20mM Phosphate buffer (pH7.0) (Pi buffer), ice cold.
  - Spin down the cells at 4,000 rpm for 10 min
  - Repeat steps 3 and 4 two more times
  - Discard supernatant and resuspend pellet in 1 ml Pi buffer
  - Transfer 0.5 ml to 15 ml Falcon blue cap tube, and save the other 0.5 ml on ice
  - Sonicate until cultures turn clear (less than 5 min)
  - Transfer crude extracts to 1.5 ml Eppendorf tubes and spin down at 13,000 rpm for 10 min
  - Carefully transfer the supernatant to clean 1.5 ml tubes
  - Quantify protein concentration using Bradford assay
- **Enzyme assay**
  - Prepare reaction buffer containing : 250 mM glycine/HCl buffer with 25 mM *p*-nitrophenylphosphate (PNPP)
  - Prepare 3 tubes, labelled with (1. Control 2. WT 3. rpoS)
  - Add 0.5 ml reaction buffer into each tube
  - Incubate all three tubes in water bath at 37oC for 2 min to equilibrate
  - Start the reaction by adding 20 µl cell extract (if the protein concentration is higher than 1 mg/ml) of WT and rpoS to tube 2 and 3, respectively. Add 20 µl of Pi buffer to control tube 1
  - Incubate at 37oC for 15 min and stop the reaction by adding 1 ml of 1N NaOH
  - Transfer 1 ml reaction mixture to cuvette to measure absorbance at 410 nm
- **Calculation of activity**
  - One unit of enzyme is defined as the amount of acid phosphatase required to release 1 nmol of PNPP/min under the above defined conditions (At 25 mM, the no enzyme rate of PNPP hydrolysis was 0.49 nmol/min/ml)
  - Alternatively, one unit of enzyme was defined as 1,000 X A410 per minute per ml (Atlung et al. 1989)

Acid Phosphatase

Expression of acid phosphatase (AP), encoded by the appA gene, is RpoS dependent. You will test AP activity in cell extracts prepared from wild type and rpoS mutant strains of *Escherichia coli*.

Replication

- **Biological Replication (experiment error)**
  - Replication of the EXPERIMENTAL UNIT
  - Use at least 3 replications
  - Contributes to statistical experimental error
  - e.g. three independently prepared cell extracts
- **Technical Replication (sampling error)**
  - Repeated sampling of the same EXPERIMENTAL UNIT
  - Can use two replicates
  - Does not contribute statistical experimental error
  - e.g. two measurements of the same cell extract.
  - Technical Error (within EU error) is usually much smaller than Biological Error (between EU error)

Replication

- All assays should be replicated. All published experiments must be performed several times.
- Types of Replication
  - Biological Replication
  - Technical Replication

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(this has been done for you..)

Enzyme Assay for Acid Phosphatase

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Enzyme assay

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- Alternatively, one unit of enzyme is defined as 1,000 X A_{410} per minute per ml (Atlung et al. 1989)
- Molar extinction coefficient for p-nitrophenol = $17.700 \times 10^3\ M^{-1}\ cm^{-1}$ (this has been incorporated into the general spreadsheet I will give out

Calculations

- Prepare a table beforehand
- Label Tubes before doing experiment
- Use a spreadsheet table to record results.
- Put spreadsheet in labbook (raw and calculated data)

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Calculations-Spreadsheet

- The general spreadsheet I will post will be slightly modified from the following sheets given in class.
- Features
  - Formats numbers and decimals properly
  - Calculates data
  - Organizes information including experimental details
  - Provides some quality control indicators
  - Graphs protein standard curve

Protein..

Bradford Protein Assay (MICROASSAY)

Date 5/8/2013 20:14

-add water to standard tubes in triplicate User
-add protein standard volume to each tube, use a fesh tip for each tube (total volume)
-add Protein dye reagent (200ul) to each tube: allow 15s between additions
-mix thoroughly with a vortex mixer
-incubate at least 5 min (preferably 30min) and read at 595 in the same order as protein reagent addition

Notes: (1) the "0" protein control as it is used for both the standard curve and for background subtraction (make sure calculated and measured values are in good agreement)
(2) make sure that all unknown values fall in the linear portion of the curve--if not, dilute and reassay
(3) if possible, use Corrected A595 (Average-blank) values in the range 0.01 to 0.400
(4) if the calculated Intercept differs from the Corrected for Blank (below) by more than .005, check standard curve
(5) Use 1.0 mg/mL BSA Standard

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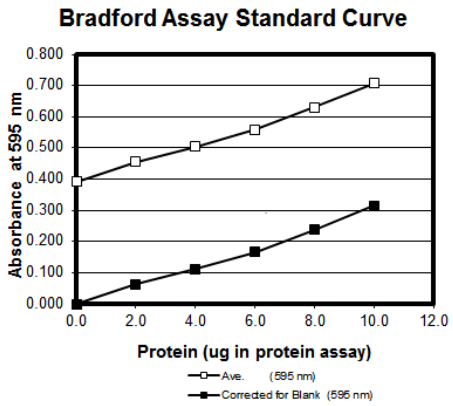
Standard Curve

BSA Protein (ug)	Volume of Standard (ul)	Volume of Water (ul)	Total Vol.	Abs At 595 nm (triplicate)			Ave. (595 nm)	Corrected for Blank
0	0.0	800.0	800.0	0.392	0.400	0.384	**0.392**	**0.000**
2	2.0	798.0	800.0	0.450	0.455	0.460	**0.455**	**0.063**
4	4.0	796.0	800.0	0.501	0.499	0.510	**0.503**	**0.111**
6	6.0	794.0	800.0	0.570	0.555	0.550	**0.558**	**0.166**
8	8.0	792.0	800.0	0.620	0.630	0.640	**0.630**	**0.238**
10	10.0	790.0	800.0	0.700	0.720	0.700	**0.707**	**0.315**



Regression

Slope	0.0307619
Intercept	-0.0049206

Unknown Samples

Sample Number	SampleID	Volume of Sample (ul)	Volume of Water (ul)	Readings Rep #1	Readings Rep#2	Average	Average-blank	Protein (mg/mL)	Error Flags 2.5%	Error Flags 5.0%
1	First sample	2.0	798.0	0.552	0.558	**0.555**	**0.163**	**2.65**		
2										
3										
4										
5										
6										
7										
8										
9										
10										
11										

- User input
- Spreadsheet calculated values
- Protein value wrong--check standard curve

Enzyme..

General Enzyme Calculation Template

Date 5/8/2013 20:16

-determine protein concentration of samples using ProteinMicroassay or ProteinMicrotiterPlateassay sheet
-determine activity of samples using standard curve calibration assay below to obtain Total Units per mL
-divide Total Units per mL by Protein (mg/ml) to obtain specific acitivity

A. Enzyme assay

1. Prepare reaction buffer containing : 250 mM glycine/HCl buffer with 25 mM *p* -nitrophenylphosphate (PNPP)
2. Prepare 3 tubes, labelled with (1. Control 2. WT 3. rpoS)
3. Add 0.5 ml reaction buffer into each tube
4. Incubate all three tubes in water bath at 37oC for 2 min to equilibrate
5. Start the reaction by adding **20 µl** cell extract (if the protein concentration is higher than 1 mg/ml) of WT and rpoS to tube 2 and 3, respectively. Add 20 µl of Pi buffer to control tube 1
6. Incubate at 37oC for **15 min** and stop the reaction by adding 1 ml of 1N NaOH **(TOTAL Volume = 1.52 mL)**
7. Transfer 1 ml reaction mixture to cuvette to measure absorbance at 410 nm

Assay Notes : **(1) make sure that you perform linearity checks**
(2) use only determine unknown values with in the standard curve (do not extrapolate..dilute and re-assay instead)
(3) biological replicates usually yields standard errors of less than 10% (5% is quite good). If yours is higher check your technique..
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ACID PHOSPHATASE ASSAY DATA

Sample	Sample ID	Protein concentration (mg/mL)				Volume of Sample (ul)	Time of Incubation(min)	OD410 READINGS						Total Activity (nmol/min/mL)				SPECIFIC ACTIVITY	Standard Error	Error Flags	
		Rep1	Rep2	Rep3	Average			Rep1		Rep2		Rep3		Rep 1	Rep 2	Rep 3	Average			10%	20%
								Sample1	Sample2	Sample1	Sample2	Sample1	Sample2								
1	Your first sample	1.30	1.50	1.45	1.417	10.0	20.0	0.600	0.600	0.610	0.605	0.650	0.650	2576.3	2597.7	2608.5	2594.2	1831.2	75.6		
2																	#DIV/0!	#DIV/0!	#DIV/0!		
3																	#DIV/0!	#DIV/0!	#DIV/0!		
4																	#DIV/0!	#DIV/0!	#DIV/0!		
5																	#DIV/0!	#DIV/0!	#DIV/0!		
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20																	#DIV/0!	#DIV/0!	#DIV/0!		

CALCULATION NOTES
Protein Concentration (from Protein Assay Table)
Rep - Biological Replicate of Experimental unit
Sample - Technical Replicate
TOTAL ACTIVITY = {(AverageOD410/1770 L mol-1cm-1) x (10E9 nmol/mol) x (1.0 l/1000mL) x 1.52mL (total assay volume) x ((1/incubation time) (min))] / sample volume (mL)
SPECIFIC ACTIVITY = TOTAL ACTIVITY/PROTEIN CONC. (e.g. S25/G25)
STANDARD ERROR = STANDARD DEVIATION of Biological Replicate Specific Activities (e.g. Standard Deviation (P25/D25, Q25/E25, R25/F25) / SQRT(3))
ERROR FLAGS - these will indicate if variation is high. If high, check to make sure numbers are typed correctly--it is easy to mistype a decimal)
-this is not operational
MOLAR EXTINCTION COEFFICIENT (ε) OF pPNP = 17,700 M-1 cm-1

=-user inputed data
= spreadsheet calculated data
= negative number (this occur if blank values are higher than measured values)
error flag - excess variation
error flag - very high varation that will likely affect ability to employ statistical tests of significance