MED3PRC

Practical Skills in Biomedical Science

Laboratory Workbook 2019

(Part 1: Pracs 1-3)

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Total marks = ____ **/ 93.5**

Calculations

Dilutions

1. What is the dilution factor when 0.4 ml is added to 7.6 ml diluent? (1 mark)

Final volume = (0.4 + 7.6) ml = 8.0 ml
Dilution factor = (8.0/ 0.4) ml = 1:20

2. How would you prepare 20 ml of a 1:400 dilution? (1 mark)

Aliquot volume = 20 ml/400 = 0.05 ml
Add 0.05 ml of aliquot into 19.95 ml amount of diluent and mix thoroughly

3. You have to make up sufficient antibody solution to add 100 µl per well to a 96 well microtitre plate.

    a. How much antibody solution should you make (account for 5% overage)? (0.5 marks)

Total volume = (9600*5/100)+9600 = 10 080 µl

    b. How much stock antibody should you add to obtain a dilution of 1/20,000? (1 mark)

Antibody volume = 10 080 µl / 20 000 = 0.504 µl

4. You have 0.6 mL of sample, and want to dilute it all to a fiftieth of its present concentration. How much diluent will you add, and what will the final volume be? (1 mark)

c1*0.6 = (c1/50)*v2
v2 = 30 ml
Final volume = 30 ml
Diluent volume = 29.4 ml

Solutions

5. You have 250 ml of 50X TAE stock solution. What volume of the 50X TAE stock is needed to prepare 35 ml of 10X TAE? (1 mark)

50X*V1 = 10X*35 ml
V1 = 35/5 = 7 ml
Volume of the 50X TAE stock is needed = 7 ml

6. How would you <u>prepare</u> 800 ml of a 0.44 M NaCl solution (MW = 58.44 g/mole)? (1 mark)

No of NaCl moles = 0.44 M*(800/1000) L = 0.352 mol
Amount of NaCl needed = 0.352 mol*58.44 g/mol = 20.57 g

Add 20.57g of NaCl into 800 ml of diluent and mix thoroughly

7. You are asked to prepare HEPES buffered saline with the following constituents:

HEPES	10 mM
KCl	5 mM
NaCl	135 mM
$CaCl_2$	1 mM
$MgSO_4$	0.6 mM
Glucose	6 mM

You have previously made up **<u>100 mM</u>** stock solutions of HEPES, KCl, $CaCl_2$, and $MgSO_4$ and a **<u>1 M</u>** stock of NaCl. The MW of glucose is 180.16 g/mole

Calculate the volume of each solution, and the <u>weight</u> of glucose required to prepare 250 ml, 400 ml and 1 L of buffer. Fill in your answers in the table below (remember your volumes should be obtainable using standard laboratory equipment). (3.5 marks)

	[final] (mM)	[Stock] (mM)	Volume (ml)	Volume (ml)	Volume (ml)
HEPES	10	100	25	40	100
KCl	5	100	12.5	20	50
NaCl	135	1000	33.75	54	135
$CaCl_2$	1	100	2.5	4	10
$MgSO_4$	0.6	100	1.5	2.4	6
Glucose	6	MW=180.16 g/mol	g	g	g
H_2O			174.75	279.6	699
Final volume			**250**	**400**	**1000**

Protocols and Methods

8. Using the recipe-like protocol provided for you in Pracs 4 and 5, construct a scientific method for publication by filling in the gaps below. (6 marks)

ELISA

75 microliters of purified human PSA peptide (0-1000 ng/ml; Abcam AB41241) diluted in PBS (NaCl 137 mM; KCl 2.68 mM; Na_2HPO_4 8 mM; KH_2PO_4 1.5 mM; pH 7.3) was adsorbed onto microtitre plates for 1 week at 4°C. The plates were then washed 3 times with PBS and blocked with 5% (w/v) low fat milk powder in PBS-T (PBS with 0.05 Tween 20) for 45 minutes at 25 °C. Plates were washed 3 times with PBS and incubated with 75 ul polyclonal anti-PSA peptide antibody Kallikrein loop (1/500; Abcam® AB28563) for 1 hour at 25°C. Wells were washed __ with PBS and incubated with HRP-conjugated goat anti-rabbit IgG (1/15000; Sigma-Aldrich) for 1 hour at 25°C. Wells were washed 3 times with PBS (3x) and then developed using 1-step™ Ultra TMB-ELISA (Thermo Scientific). The colour reaction was stopped with 50 HCl and the final product read on a Spectrostar Nano plate reader at an optical density of 450nm.

Total marks for Practical 1 (calculations): /16 marks

Practical 1 – Expression of Bcl-2

AIM - PCR

Briefly state the aim of the PCR experiment (1 mark)

PCR is performed to examine the presence of Bcl-2 plasmid in cell cultures (HL-60WT, HL-60Bcl-2 and plasmid Bcl-2/ positive control).

METHODS – PCR

Briefly outline the key steps in the method of the PCR experiment (see practicals 1 & 2) (3.5 marks)

1. Isolate genomic DNA all cell types (HL60-WT and HL60-Bcl-2)
2. Quantify the amount of DNA in each cell type and prepare cell cultures in PCR tubes (including Bcl-2 plasmid and the negative control).
3. Run the PCR machine for 30 cycles.
4. Prepare an agarose gel and set up the apparatus to do a gel electrophoresis for the PCR products.
5. Load each PCR sample into the gel and run the gel at 80 volts for about 60 minutes.
6. View the gel under a UV transilluminator to visualize the DNA bands.

EXPECTED RESULTS – PCR

Briefly indicate what you expect your results to be for each PCR reaction mix if the experiment was successful (2 marks)

PCR reaction	Expected result
No template control	No amplified product
Positive control	Amplified product
HL-60^{WT} DNA	No amplified product
HL-60^{Bcl-2} DNA	Amplified product

LAB MANUAL CALCULATIONS

ASSESSING BCL-2 EXPRESSION AT THE GENE LEVEL

Part B - Quantify DNA

After extracting DNA from your cell pellet you are asked to determine the concentration of DNA in your lysate using the nanodrop. Fill out the table below to obtain 100 ng of DNA from your lysate. (1 mark)

Cell culture (HL-60^{WT} or HL-60^{Bcl-2})	**DNA concentration (ng/µl)**	**Volume required for 100 ng of DNA (µl)**
HL-60Bcl-2	57.9	1.73

Part C - Prepare and run PCR reactions

Fill out the following table of reagents to make up the PCR reaction tubes. (2 marks)
(Reminder: you want to achieve a final concentration of 0.5 µM for each primer)

Reagent	**Volume (µl)**		
	Reaction 1	Reaction 2	Reaction 3
Genomic DNA sample (~100 ng) or plasmid DNA control (~5ng)	0	1.73	12.5
Sterile H_2O	15	13.27	2.5
GoTaq™ Green Mix	25	25	25
Forward primer (5 µM stock)	5	5	5
Reverse primer (5 µM stock)	5	5	5
Total reaction volume:	50	50	50

AIM – WESTERN BLOT

Briefly state the aim of the Western Blot experiment (1 mark)

Western blot is done to examine the relative levels of Bcl-2 protein in HL-60WT and the transgenic cells carrying the Bcl-2 transgene (HL60-Bcl-2).

METHODS – WESTERN BLOT

Briefly outline the key steps in the method of the Western Blot experiment (see practicals 1 & 2) (3.5 marks)

1. Prepare cell lysates for each cell type including HL60-WT, HL60Bcl-2 and plasmid Bcl-2.
2. Load each sample into an acrylamide gel after adding the SDS loading buffer into the samples and run the gel at 25mAmp and then at 50Amp for approximately 30 minutes,
3. Transfer the proteins from the gel to a PVDF membrane by creating a sandwich with the SDS-Page gel in direct contact with the membrane.
4. Block unbound sites of the membrane.
5. Probe the PVDF membrane with primary and secondary in order.
6. Rinse the membrane with distilled water and incubate in NBT/BCIP substrate mixture until the bands become visible and sufficiently intense.

EXPECTED RESULTS – WESTERN BLOT

Briefly indicate what you expect your results to be for each sample tested if the Western Blot experiment was successful (3 marks)

Primary antibody	Sample		
	HL-60^{WT} lysate	HL-60^{Bcl-2} lysate	Positive control
Anti-Bcl-2	positive(one band)	positive(two bands)	positive (one band)
Anti-FLAG	negative(no bands)	negative(no bands)	positive (one band)

PRACTICAL 1 QUESTIONS

1. What is PCR? Briefly explain how it works considering the steps of denaturation, annealing and extension. (2 marks)

PCR is a method to amplify a specific DNA region that lies between two regions of known DNA sequence. PCR results in a great quantity of that targeted DNA region.

The first step of PCR, denaturation is performed to separate the two strands of the DNA helix by heating the tubes containing DNA up to about 94 degrees of Celsius.

The vial is cooled down to about 58 degrees of Celsius. Then the annealing process begins. The two primers that are complementary to the opposite strands, bind to the known regions of the DNA sequence.

Primers start replicating and making complete copies of the complementary strands with the help of Taq polymerase enzyme. Usually at this stage, the temperature of the test tubes are raised up to 72 degrees of Celsius as that is the optimum temperature for the taq polymerase function.

This cycle is normally done 30 times to harvest a great amount of the interested DNA sequence.

2. What is the purpose of the control reactions in the PCR – no template control and positive Bcl-2 control? (1 mark)

No template control is there to identify any contaminations in the samples. No template control shouldn't have any bands after being run in the gel. If it gives bands and there are corresponding bands in the other samples as well, we can say that the samples may have been contaminated.
Both HL60-Bcl-2 and positive Bcl-2 control should give one band each in the same position. Positive control is there to compare the thickness between the two bands and determine the amount of product that has been amplified.

3. Approximately what size band would you expect to see if your PCR successfully amplified full length Bcl-2 gene? Why are we not amplifying the entire gene? (1 mark)

450-550 base pairs
We are not interested in the entire gene. Only the plasmid Bcl-2 sequence that encodes for the transfected Bcl-2 protein.

4. How can we tell the difference or detect transgenic Bcl-2 compared with a wildtype Bcl-2 gene by PCR? (1 mark)

Transgenic Bcl-2 gene is bigger than the wild type Bcl-2 gene as it contains an additional DNA sequence that encodes for the FLAG region of the corresponding protein. Therefore, the band for transgenic Bcl-2 will be located a little higher in the gel than the band for wildtype Bcl-2.

Additionally as the primer we use during PCR is specific for the Bcl-2 region plus a little bit of the plasmid, there has to be a greater amplification in product of HL60-Bcl-2 than the wild type. Therefore, a thicker band should be visible for HL60-Bcl-2 in the gel than the wild type Bcl-2.

Total marks for Practical 1 (Expression of Bcl-2): /22 marks

Practical 2 – Expression of Bcl-2 (cont.)

LAB MANUAL CALCULATIONS

Part A – Prepare an agarose gel for PCR

For these calculations, refer to Prac 2 in your lab manual.

Indicate the amount of agarose you needed to weigh out to obtain a 0.8 % (w/v) solution when diluted with 0.5 X TBE. (1 mark)

Amount of agarose to obtain 0.8 % (w/v) solution: 0.8 grams

Indicate the volume of SYBRSafe stain you need to add to your agarose/TBE solution to obtain 0.01 % (v/v). (1 mark)

Volume of SYBRSafe stain to obtain 0.01 % (v/v) solution: 10 microliters

PRACTICAL 2 QUESTIONS

PCR

Refer to your DNA gel (or a gel deemed successful by the demonstrator) for the following questions.

1. Which samples(s) was there no PCR product? Why? (1 mark)

HL60-WT cell sample, no template control
Because no DNA sequences have been amplified in those samples during PCR.

2. Why did we perform this PCR? (1 mark)

PCR is performed to examine the expression of Bcl-2 in a gene level.

3. What is the difference between what we are detecting in the PCR and western blot today? Why do we need to detect the presence of both? (1 mark)

Bcl-2 gene is detected in PCR while the Bcl-2 transgenic protein is detected in western blot.
We need to detect the presence of both to make sure that the Bcl-2 transgene in HL60-Bcl-2 actually produces the transgenic Bcl-2 protein through translation.

4. If there was a PCR product for both HL-60^{WT} and HL-$60^{Bcl\text{-}2}$ samples, where would the primers most likely bind to? (1 mark)

Just the gene sequence that encodes for the Bcl-2 protein (Not the gene and a part of the plasmid).

5. What is the overall conclusion from this PCR experiment? (1 mark)

HL60-WT cells do not contain the Bcl-2 plasmid gene that encodes for transfected Bcl-2 whereas HL60-Bcl-2 cells do contain that particular gene.

Western Blot

6. What is the purpose of blocking? (1 mark)

The membrane used in western blot has a high affinity for proteins. Therefore, to avoid nonspecific binding of the antibodies used for detecting purposes, blocking the remaining surfaces of the membrane after transferring the proteins from the gel to the PVDF membrane, is important.

7. Why is the secondary antibody anti-mouse? If a third antibody was needed to detect the secondary, what species would it be derived from? (1 mark)

The primary antibody (Anti-Bcl-2) is raised in a mouse. Therefore, to selectively bind to it, the secondary antibody should be anti-mouse.
The third antibody would be derived from a rabbit(any animal other than a goat, since the secondary antibody is raised in a goat).

8. How large is the Bcl-2 protein? What size band (in both HL-60^{WT} and HL-60^{Bcl-2} cells) are we looking for on the blot if probed with; anti-Bcl-2 or anti-FLAG antibodies? (2 marks)

	HL60 WT	HL60 Bcl-2
Anti-Bcl-2	100-200 amino acids	100-200 amino acids
Anti-FLAG	no bands	100-200 amino acids

Refer to your blot (or a blot deemed successful by the demonstrator) for the following questions. You should consider both anti-Bcl-2 and anti-FLAG probed blots.

9. Which sample(s) bound the anti-Bcl-2 antibodies? (1 mark)

HL60-Bcl-2, HL60-WT and Plasmid Bcl-2(positive control)

10. Did the antibodies detect a polypeptide with the expected size for Bcl-2? How do you know this? (1 mark)

Yes,
There are two bands (one very faint band as the same size as the band present for the positive control and another clear band)
But the expected band has less fluorescence since the anti-Bcl-2 antibody has mainly bound to the endogenous Bcl-2 and not that effectively to the transfected Bcl-2.

11. What is it about probing with either anti-Bcl-2 or anti-FLAG antibodies that helps distinguish between wildtype and transgenic Bcl-2? (1 mark)

Anti-Bcl-2 antibody binds to both the transgenic and endogenous Bcl-2 whereas Anti-FLAG anitbody only binds to the transgenic Bcl-2 as it has a FLAG region combined with the protein.
This helps to distinguish between wildtype Bcl-2(endogenous) and transgenic-Bcl-2.

12. Did we use an appropriate control for the anti-FLAG blot? If not, what should we use next time? (1 mark)

No, a plasmid Bcl-2 that has undergone epitope tagging with FLAG.

13. What is the overall conclusion from this Western Blot experiment? (1 mark)

HL60-WT cells do not contain transfected Bcl-2 protein whereas HL60-Bcl-2 cells do.

Total marks for Practical 2 (Expression of Bcl-2 cont.): /16 marks

Practical 3 – Caspase activity assay

AIM – CASPASE ACTIVITY ASSAY

Briefly state the aim of conducting the caspase activity assay in this experiment (1 mark)

To check if caspase is active or not in HL60-WT and HL60-Bcl-2 upon adding doxorubucin and therefore, to determine if apoptosis takes place or not in those cells.

METHODS – CASPASE ACTIVITY ASSAY

Briefly outline the key steps in the method of the caspase activity assay (3 marks)

1. Prepare a master-mix and keep it on ice (add AcDEVD-AFC at the end)
2. Make aliquots
3. Lyse cells
4. Add lysate to mastermix and aliquot to 96-well in duplicate
5. Read florescence at start and 30 minutes later

EXPECTED RESULTS – CASPASE ACTIVITY ASSAY

Briefly indicate what you expect your results to be for each sample tested if the caspase activity assay experiment was successful (3 marks)

Condition	Sample		
	HL-60^{WT} lysate	HL-60^{Bcl-2} lysate	Negative control
Untreated	very low caspase activity	No caspase activity	No caspase activity
Treated (with doxorubicin)	High caspase activity	No (/low) caspase activity	No caspase activity

AIM – TRYPAN BLUE EXCLUSION

Briefly state the aim of conducting the caspase activity assay in this experiment (1 mark)

Trypan Blue exclusion test is done to determine the number of viable cells in different cell suspensions.

METHODS – TRYPAN BLUE EXCLUSION

Briefly outline the key steps in the method of the caspase activity assay (1.5 marks)

1. Resuspend the cell cultures and make aliquots
2. Add the trypan blue dye and mix
3. Add the cell culture: trypan blue mix into the haemocytometer square
4. Look under the microscope
5. Calculate the cell viability

EXPECTED RESULTS – TRYPAN BLUE EXCLUSION

Briefly indicate what you expect your results to be (in terms of cell viability) for each sample tested if the trypan blue exclusion assay experiment was successful (2 marks)

Condition	Sample	
	HL-60^{WT} lysate	HL-60^{Bcl-2} lysate
Untreated	Good cell viability	Great cell viability
Treated (with doxorubicin)	Low cell viability	Great cell viability

LAB MANUAL CALCULATIONS

Part A – preparing a mastermix

Complete the protocol table to prepare 500 µl of mastermix. (2 marks)

Component	Stock concentration	Final conc in mix	Vol (µl) to add to final vol of 500 µl
HEPES buffer pH 7	1M	0.1M	50
PEG3350	50%	10%	100
CHAPS	1%	0.1%	50
Dithiothreitol (DTT)	1 M	10 mM	5
AcDEVD-AFC*	2 mM	50 µM	**Do not add this but allow for its addition in your 500µL**
Distilled H_2O			

DATA – CASPASE ACTIVITY ASSAY

3 marks

Refer to your data (or data deemed successful by the demonstrator) for the following questions.

Calculate the activity of caspase-3 as a change in fluorescence units/hr/1 x 10^6 cells by following these steps.

a. Calculate the change in fluorescence over the incubation period by subtracting the initial reading from the final reading (0.5 marks)
b. Subtract the negative control value from sample values (0.5 marks)
c. Calculate the average caspase-3 activity from your duplicate (1 mark)
d. Repeat for the other cell type using another pair's initial and final readings. (1 mark)

Cell type	Treatment	Initial Reading	Final Reading	Final - Initial	Final- initial-control	Duplicate average
HL-60^{WT}	No treatment	184.513	241.144	56.631	56.631	65.427
		202.702	276.924	74.222	74.222	
	+ doxorubicin	230.282	231.877	1.595	1.595	54.792
		193.226	301.214	107.988	107.988	
Negative control		180.941	172.546	-8.395(~0)		
HL-60^{Bcl-2}	No treatment	181.423	287.66	106.237	106.237	127.388
		211.692	329.962	148.539	148.539	
	+ doxorubicin	195.928	310.704	114.776	114.776	108.412
		188.249	290.297	102.048	102.048	
Negative control		187.168	169.473	-17.695 (~0)		

DATA – TRYPAN BLUE EXCLUSION

4 marks

Cell line =		Cell number					%viability
	Quadrant	Top Left	Bottom Left	Top Right	Bottom Right	Average	
Untreated	Trypan blue positive	3	8	7	13	7.75	95.13
	Trypan blue negative	131	262	96	116	151.25	
Treated 24h	Trypan blue positive	2	9	14	9	8.5	86.92
	Trypan blue negative	47	90	38	51	56.5	
Treated 48h	Trypan blue positive	22	21	8	30	20.25	70
	Trypan blue negative	61	38	25	65	47.25	

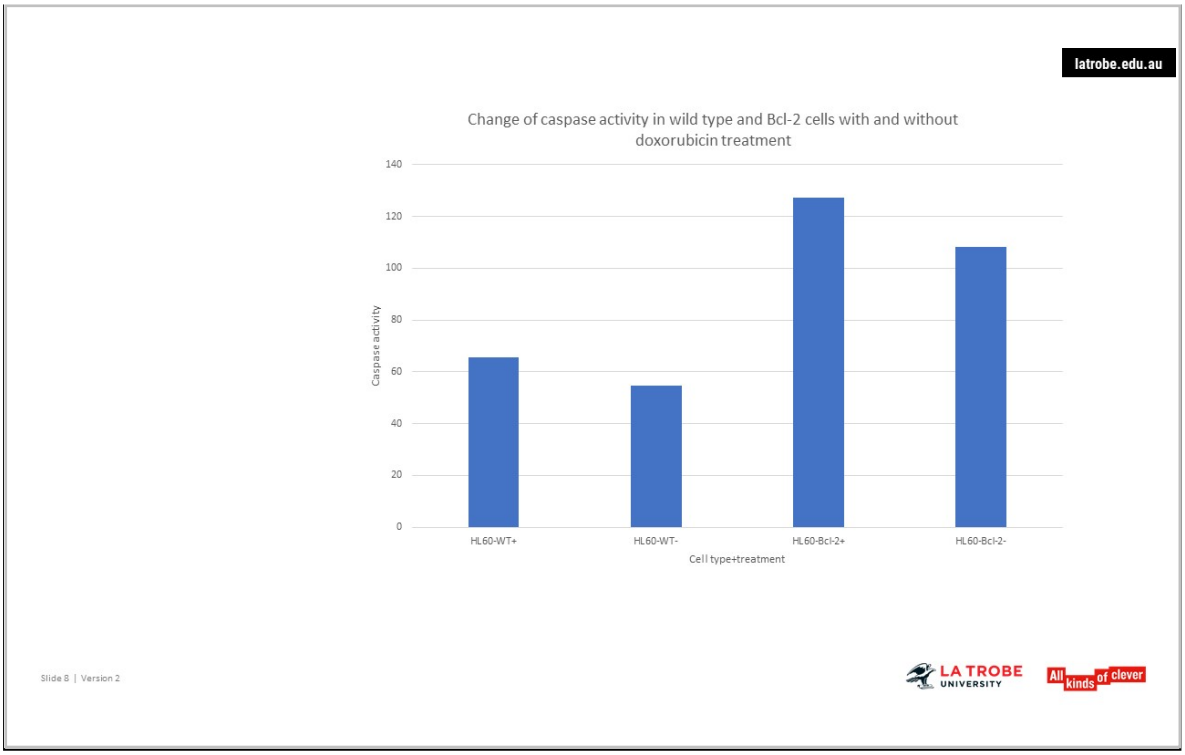
DATA COMPARISON

Obtain class data for trypan blue exclusion assay and determine for each condition (4 marks):

1. Overall average. In excel use the formula: =average(values)
2. Standard deviation. In excel use the formula: =stdev(values)

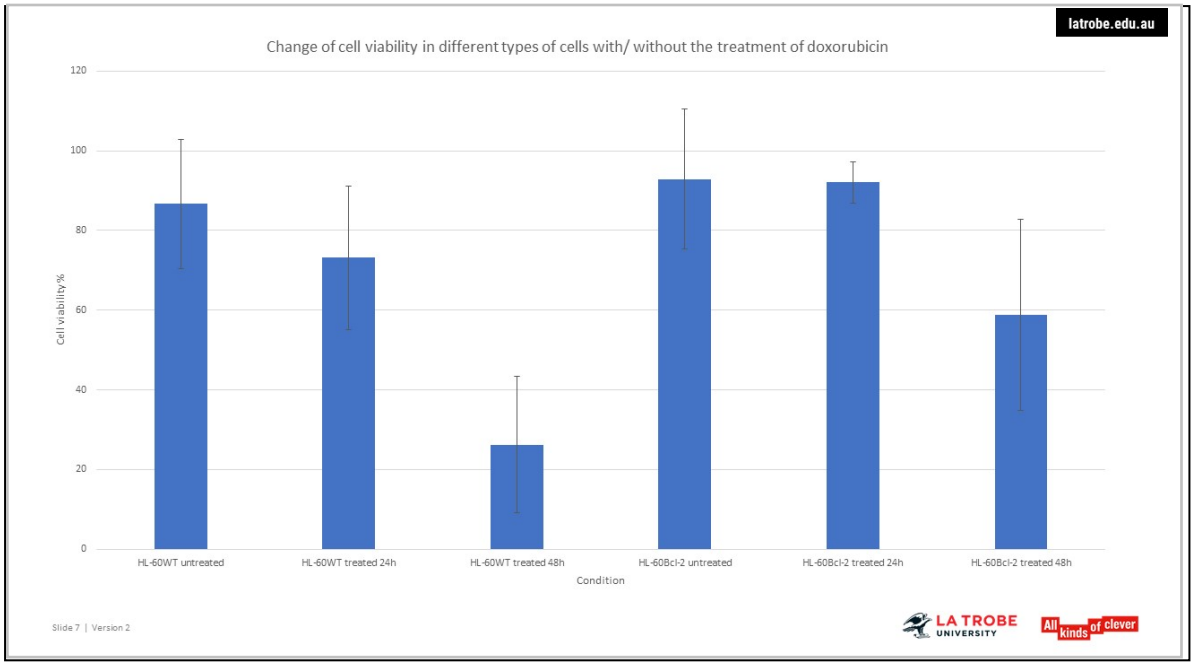
		HL-60^{WT} cells		HL-60^{Bcl-2} cells	
		Class average	%viability +/- SD	Class average	%viability +/- SD
Untreated	Trypan blue positive	8.30	86.67% +/- 16.19	4.29	91.88%+/- 17.55
	Trypan blue negative	81.78		109.38	
Treated 24h	Trypan blue positive	23.97	73.11%+/- 18.03	5.78	92.04%+/- 5.20
	Trypan blue negative	60.17		68.96	
Treated 48h	Trypan blue positive	27.65	26.21%+/- 17.08	30.54	58.78%+/- 23.94
	Trypan blue negative	13.20		61.25	

Insert an appropriately labelled graph of YOUR caspase activity assay results (3 marks)



*To correctly insert your graph, copy your completed graph from Excel into Powerpoint then right click on graph and "Save Picture As" as JPEG file, then upload the JPEG file. It is your responsibility that the graph is correctly uploaded

Insert an appropriately labelled graph of the CLASS trypan blue exclusion assay results (with SD) (3 marks)



PRACTICAL 3 QUESTIONS

1. Why does measurement of caspase-3 activity give you an indication of apoptotic cell death? (1 mark)

Caspase-3 is pro-apototic. Therefore, it's active when a cell undergoes apoptosis. The amount of caspase-3 activity indicates upto which extent, apoptosis takes place in a cell suspension.

2. Why were cells treated with doxorubicin? (1 mark)

Doxorubicin is a chemotherapy drug. It induces DNA damage. DNA damage and cell stresses stimulate apoptosis. Therefore, cells are treated with doxorubicin to activate the cell death pathway (and consequently to activate the downstream caspases if the cells are susceptible to apoptosis).

3. Compare fluorescence of lysates from treated and untreated cells for each cell type. What does this assay tell you about doxorubicin treatment? (2 mark)

This assay should've suggested that the highest level of caspase activity is in HL60-WT cells that are treated with doxorubicin as it has no expression of anti-apoptotic Bcl-2 present to resist the affect of doxorubicin.

HL60-Bcl-2 cells should've demonstrated a low level of caspase activity when treated/ untreated with doxorubicin as the over expressed Bcl-2 blocks the affect of doxorubicin.

However, we assume that an error has occurred in our practical as our data suggests otherwise.

4. How does cell viability change with a longer incubation in doxorubicin (24h vs 48h)? What effect does Bcl-2 over expression have on this? (1 mark)

Cell viability decreases with a longer incubation in doxorubicin. Over expression of Bcl-2 (as Bcl-2 is anti-apoptotic) resists the effect of doxorubicin. However, as the incubation period increases Bcl-2 over expressed cells too become a little susceptible to apoptosis.

5. Compare your observations for the caspase activity assay and the trypan blue exclusion assay. Do both assays support the same trend in regards to Bcl-2 over expression? (1 mark)

Both assays should support the same trend in regards to Bcl-2 over expression.

However, as our caspase activity assay has not worked properly it does not agree with what the trypan blue exclusion assay suggests.

6. Can we make conclusions on the effect of Bcl-2 over expression on apoptosis by only considering the trypan blue exclusion data? Why? (2 marks)

No, trypan blue treatment is not specific to apoptotic cells. It has an effect on all dead cells including apototic as well as necrotic cells.
(Trypan blue cannot differentiate between necrotic and apoptotic cells)

7. What is the overall conclusion from this caspase activity assay? (1 mark)

Caspase is not activated in HL60-Bcl-2 as it is in HL60-WT after being treated with doxorubicin. This suggests that HL60-Bcl-2 is resistant to doxorubicin and therefore doesn't undergo apoptosis as HL60-WT.

8. What is the overall conclusion from this trypan blue exclusion assay? (1 mark)

Wild type cells successfully undergo apoptosis and therefore the number of dead cells in a cell suspension increases after the cell death pathway is triggered.
The number of dead cells does not increase as much in Hl60-Bcl2 cell suspension suggesting that these cells are resistant to apoptosis.

Total marks for Practical 3 (Caspase activity assay): /39.5 marks