BCH3356 *Molecular Biology Laboratory*

Final exam: This exam is worth 30 % of the final mark for the course BCH3356

Name:_____
Student number:_____

Instructions

All questions should be answered within the space provided on THIS COPY EXAM.

You may use the endorsement of pages for your draft calculations.

Students are allowed to use a calculator.

Proctors will not answer any question during the exam.

Part I (/30 marks): Underlying principles of molecular techniques

1. BCH3356 Crossword (10%)

- ACROSS
- Before proceeding with ligation, pTrcHisB was treated with a PHOSPHATASE.
- Type of membrane used for the electrophoretic transfer: PVDF
- Restriction enzymes are also called restriction ENDONUCLEASES
- The chemical added to a liquid culture to promote the overexpression of your recombinant protein was IPTG
- A negative control is commonly used to assess the BACKGROUND signal.
- The discrimination capacity of a test in detecting a given signal: SPECIFICITY
- The transfer of a cloned fragment of DNA from one vector to another: SUBCLONING
- The ion used to recharge the affinity chromatography column used to purify your recombinant T7 RNA polymerase : NICKEL
- The ligation strategy used in BCH3356 is considered as DIRECTIONAL.
- DOWN
- Number of steps per PCR amplification cycle: THREE
- The plasmid conformation that moves the faster on a gel is the SUPERCOILED conformation.
- Process of DNA melting: DENATURATION
- The name of the destination vector used for the ligation you performed is pTRCHISB.
- pH conditions used for the miniprep: ALKALINE
- Component of the lac operon that is constitutively expressed in host cells transformed with an empty or recombinant pTrcHisB: REPRESSOR
- PCR product: AMPLICON
- Is the primer that anneals onto the antisense DNA strand: FORWARD
- Ability of a cell to take up extracellular DNA: COMPETENCY
- Reformation of complementary strands of DNA: ANNEALING

1. 0.5 was subtracted for every missing or wrong answer.

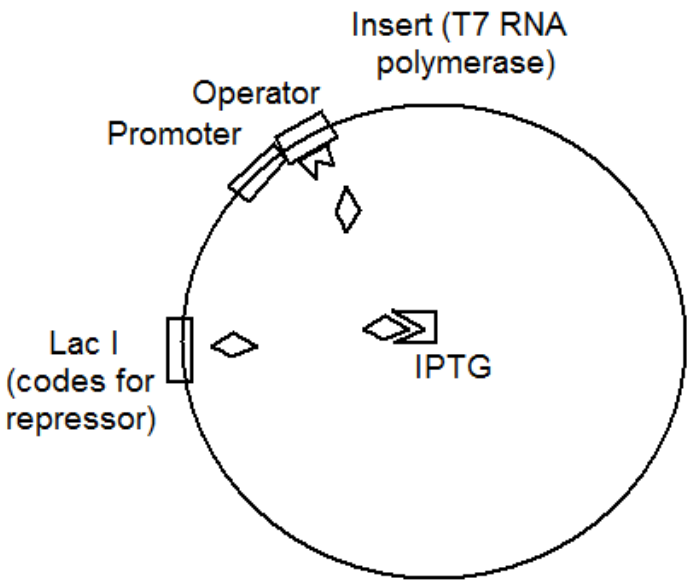
2. Identify and explain the main procedural steps for the minipreparation of plasmid DNA by alkaline lysis. Molecular details are to be emphasized. Use a separate paragraph to describe each major step. (10%)

3 MARKS: Cell lysis with NaOH for denaturation of plasmid and chromosomal DNA
3 MARKS: Neutralization with K acetate: plasmid DNA reanneals faster than chromosomal DNA because the two strands of the plasmid DNA remain entangled due to supercoiling.
4 MARKS: Centrifugation to pellet chromosomal DNA and other aggregates including denatured proteins. Plasmid DNA is found in the supernatant.

3. Explain the underlying molecular principle for immobilized metal ion affinity chromatography (IMAC) you used to purify your recombinant his-tagged T7 RNA polymerase. Molecular details should be emphasized. Make sure to explain (1) why the his-tagged T7 RNA polymerase initially binds onto the beads inside the column and then (2) why it is eluted outside the column. (10%)

2 MARKS: Resin beads are covalently coated with the NTA chelator
2 MARKS: Column is charged with nickel ions that form chelation bounds with NTA.
3 MARKS: Proteins with his-tag can be retained within the column by forming chelation bounds with nickel ions.
4 MARKS: Elution of the his-tagged protein is done by increasing the imidazole concentration. Imidazole is identical to the histidine ring side chain and it competes with his-tag for accessing nickel ions.

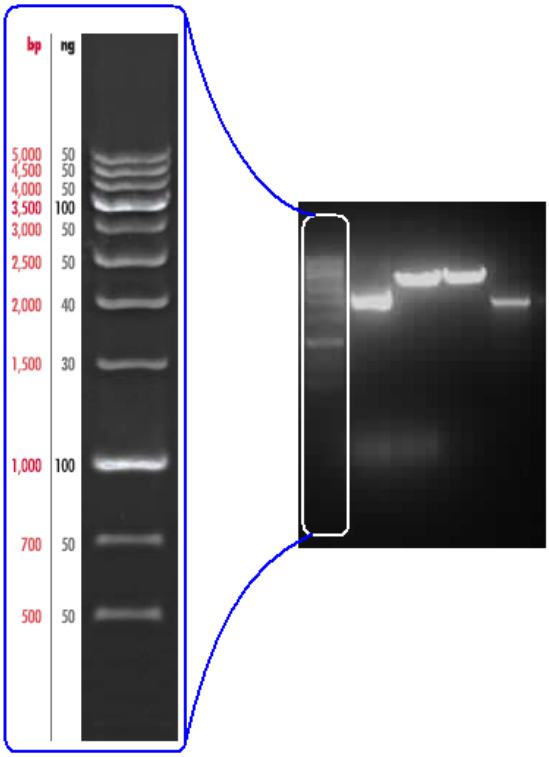
4 Explain the genetic components of the lac operon regulating the IPTG-inducible expression of T7 RNA polymerase in the TrcHisB/T7 and DH5a system you used during the semester. You should clearly explained how IPTG is able to induce protein expression. Use a diagram. (10%)



- The lac I repressor is constitutively expressed and binds onto the operator to prevent expression of the T7 RNA pol. Insert.
- IPTG binds with lac I repressor and frees the operator so transcription of T7 RNA polymerase insert can occur.
    - 2 MARKS: for lacI gene
    - 1 MARK for promoter
    - 2 MARKS for operator
    - 2 MARKS for T7 RNA pol. insert downstream the operator
    - -1 if a reference to the T7 RNA polymerase insert is missing
    - -1 for a reference to the three genes involved in galactose metabolism: lacZ, lacY and lacA (these 3 genes were missing from pTrcHisB/T7)
    - -1 if lac I was not incorporated in diagram
    - -2 if diagram was missing

Part II (/30 marks): Analytical skills

1. A group of students who completed the course last year had prepared an amplicon and a destination plasmid for a ligation procedure. Refer to their gel picture below to figure out the volumes to be used for ligation. You should assume that the amount of plasmid necessary for the ligation is 10ng with an insert:vector molar ratio of 5:1. That means that there should be 5 molecules of insert for every molecule of plasmid. Notice that the lengths for the plasmid vector and PCR amplicon used last year were 2961bp and 1851bp, respectively. Assume that the total volume of both the purified plasmid and purified amplicon was about 50ul. Explain your reasoning and calculations on next sheet then fill the summary table provided just under the gel. (15%)

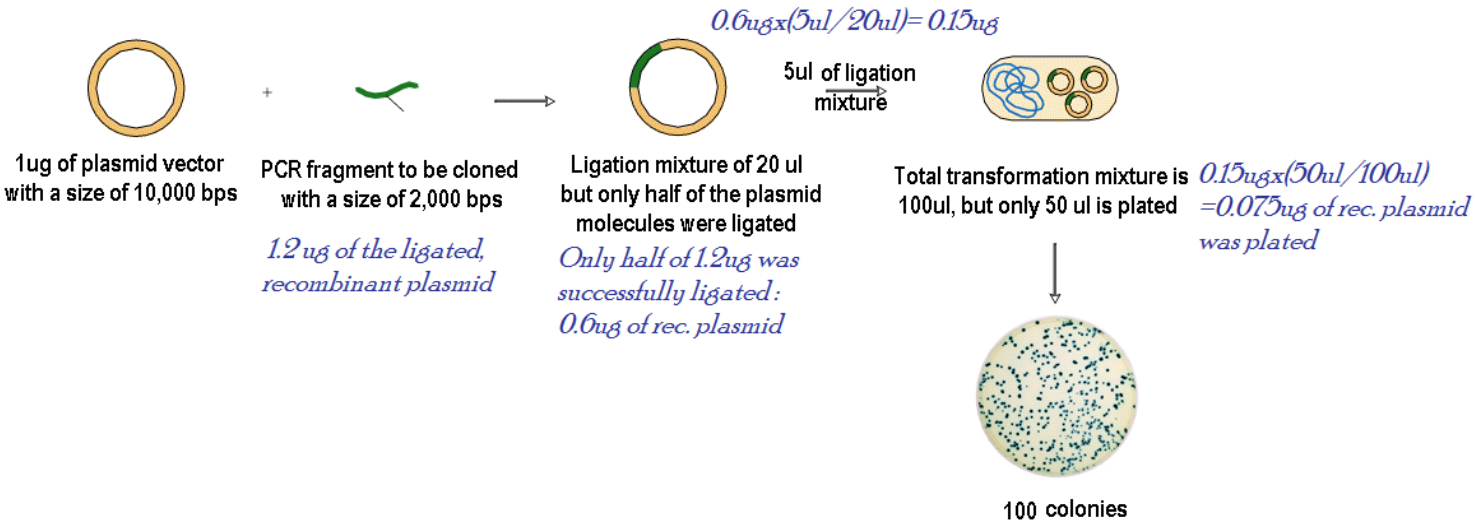


1) DNA ladder : 5ul
2) Undigested plasmid : 10 ul total (5ul + 4ul H2O +1ul loading buffer)
3) Digested, unpurified plasmid : 10ul total (5ul + 4ul H2O + 1ul loading buffer)
4) Digested, purified plasmid : 10ul total (5ul + 4ul H2O + 1ul loading buffer)
5) Digested, purified amplicon: 10ul total (5ul + 4ul H2O + 1ul loading buffer)

TREATMENT	LIGATION REACTION	H_2O (µl)	XhoI/EcoRI open plasmid 25ng (µl)	Digested and purified PCR amplicon (µl)	Ligation buffer 5X (µl)	Ligase (1U/µl)	**Total volume (µl)**
I PCR product	Purified, dephosphorylated plasmid + digested and purified PCR amplicon					2	**20**

4 MARKS for estimate of insert amount (10ng x 5 x 1851bp/2961bp = about 31.2ng of insert; or 78ng if 25ng of plasmid were used)
3MARKS for conversion of band intensities into DNA concentrations in purified insert and plasmid (lanes 4 and 5)
3MARKS for correct volume answers

Transformation yield (10%) The figure below displays an overview for a ligation and transformation procedure.

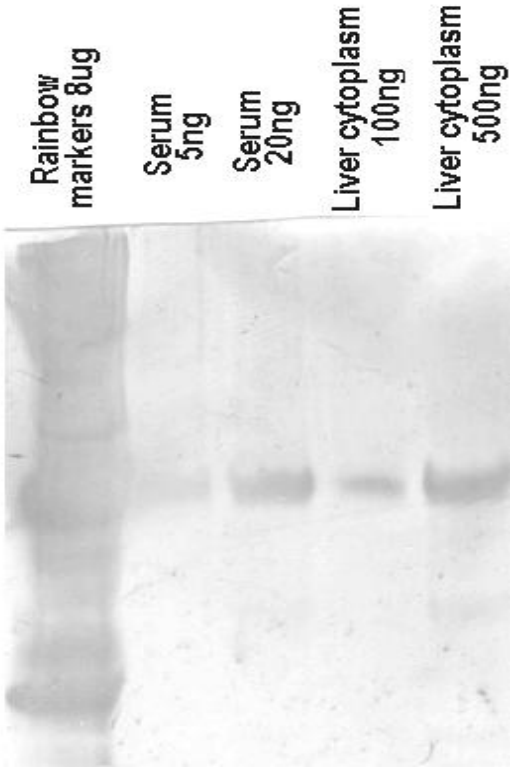


a. What's the transformation yield for that experiment? Make sure to use the appropriate units so that you can compare your experimental value with the expected yield. Show your calculations. 7 MARKS

100 colonies/0.075ug=1333cfu/ug of rec. plasmid DNA
-2 MARKS per calculation error
-2 MARKS if experimental units not provided

b. Is the experimental transformation yield reasonable? In other words, is it too low or too high relative to the expected transformation yield assuming that competent cells had been treated with calcium ions and heat-shocked? 3 MARKS
Expected transformation yield is 10^{+6}cfu/ug of plasmid DNA, but only 1333cfu/ug was obtained: this is low!
2 MARKS for mention of the expected yield
1 MARK for comparison between experimental and expected values

Western analysis (10 marks) The Western analysis result obtained by one group of students from last year is displayed below. Assume that the band that was detected corresponds to rat serum albumin.



c. Refer to the results displayed on the figure above to discuss the relative abundance of rat albumin between the two fractions that were investigated, i.e., serum and liver cytoplasm. Show your calculations and justify your reasoning. 3 MARKS

Intensities in lanes 3 (20 ng of serum) and 5 (500ng of liver cytoplasm) are similar although 25 times more protein was loading in lane 5! That means that the target, rat serum albumin, is 25 times less abundant in the liver cytoplasm fraction compared to the serum fraction. Similar comparison between lanes 2 (5ng of serum) and 4 (100ng of liver cytoplasm) gives a similar relative abundance.
-1.5 MARKS if a quantitative relative estimate was missing

d. Estimate the sensitivity of the Western analysis. 3 MARKS

A band was detected with only 5ng of serum and 100 ng of liver cytoplasm. The sensitivity for the Western analysis was therefore ≤ 5ng of serum protein and ≤100ng of liver cytoplasm.
-1 MARK if the protein source (serum or liver cytoplasm) was not indicated

e. Describe two experimental steps you could modify to improve the specificity of the Western analysis. 4 MARKS
2 MARKS per correct answer (increase washing temperature or length; longer blocking; more specific antibodies, …)
Only the first two experimental steps were taken into consideration!

Part III (/30 marks): *Design of a subcloning procedure*

Parental plasmid: Presume that (1) the exact full length for the CDS of the thermotolerant DNA polymerase from *Thermus thermophilus* (Appendix 3) had already been cloned into M13mp18 (Appendix I) between the XmaI and PstI recognition sites.

Destination plasmid: It is desirable to transfer the insert coding for the complete CDS of the thermotolerant DNA polymerase from *Thermus thermophilus* from the parental plasmid into pTrcHisA (Appendix 2).

Purpose: You need to overexpress and purify the thermotolerant DNA polymerase for your own research.

a. Design two primers that you would use to amplify the complete CDS of the thermotolerant DNA polymerase assuming that the goal is to complete the subcloning procedure. Provide a diagram showing the exact alignment positions of each of your two primers along the DNA template. Don`t forget that your primers should be designed and engineered for protein expression. Justify your reasoning. 15 marks

```
For forward primer

     Sense 5'ATG GAG GCG ATG CTT CCG CTC TTT G...3'
           5'ATG GAG GCG ATG CTT CCG C3'
Antisense 3`TAC CTC CGC TAC GAA GGC GAG AAA C...5`'
```

Primer engineering with about 3 extra nucleotides and a restriction site (XhoI, SacI BglII, PstI, KpnI or EcoRI) at the 5' end. Zero, 1 or 2 extra nucleotides were necessary to maintain the insert in frame with the His-tag (XhoI: +0 nt; SacI: + 0 nt; BglII: + 2nts; PstI: +1 nt; KpnI: +2 nts; EcoRI: +2 nts).

```
For reverse primer
  Position 3071 5'GAC TGG CTT TCC GCC AAG GGT TAG GGG GGC ...3' Position 3100 Sense
                3'CTG ACC GAA AGG CGG TTC CCA ATC5'

                3'CTG ACC GAA AGG CGG TTC CCA ATC CCC CCG ...5' Antisense
```

Primer engineering with about 3 extra nucleotides and a restriction site (SacI BglII, PstI, KpnI, EcoRI OR BstBI) at the 5' end. It is not necessary to add extra nucleotides to maintain the frame beyond the STOP codon!

Let's say we fit the insert between XhoI and EcoRI sites:
Forward primer: 5' NNN(CTCGAG)ATG GAG GCG ATG CTT CCG C3'
Reverse primer: 5' NNN(GAATTC)CTA ACC CTT GGC GGA AAG CCA GTC3'

5 MARKS for designing the part of each primer annealing onto the CDS
5 MARKS for the engineering of each primer (2x5=10 MARKS)
-2.5 MARKS if a primer was not positioned properly to subclone the CDS located between positions 590 and 3094 of the taq polymerase.
-2.5 MARKS if insert was out of frame
-2.5 MARKS per error

Let's assume now that the subcloning procedure was successfully completed with the two primers indicated in your answer to question a. You then want to verify the ligation detail at both ends of your insert by DNA sequencing. Design two sequencing primers you would use to confirm that your ligation had been done as expected. Use a diagram showing the exact annealing position of each of your two primers along the recombinant vector containing the complete CDS for the thermotolerant DNA polymerase. Also indicate the first 15 nucleotides you first expect for each of your two sequencing reactions. Explain your reasoning. 10 marks

The purpose was not to sequence the insert, but to sequence the 'ligation detail' or ligation boundaries. There are several possibilities.

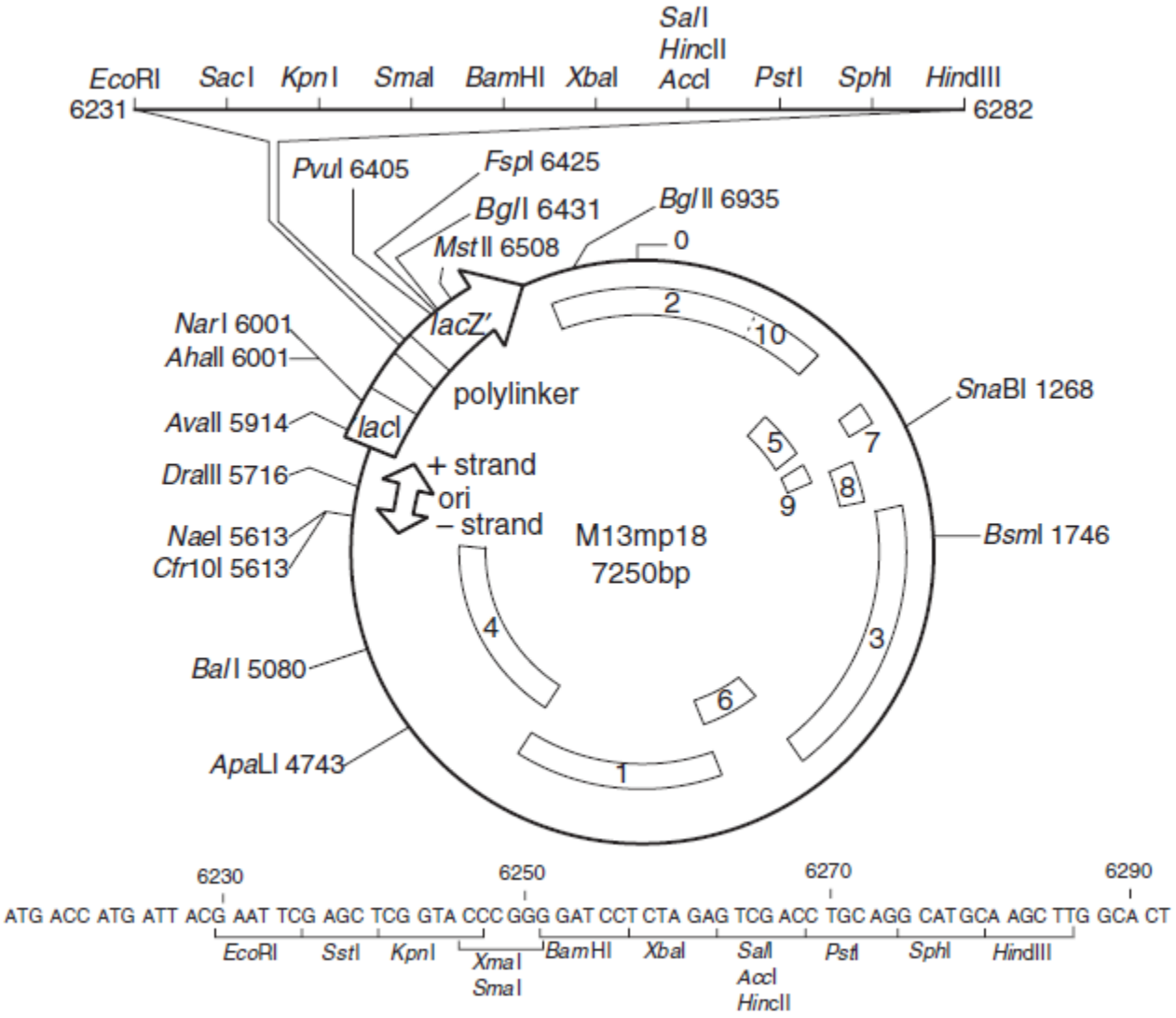
2.5 MARKS for each sequencing primer (total of 5 MARKS)

2.5 MARKS for the 15 first nts to be sequenced by each sequencing primer (total of 5 MARKS)

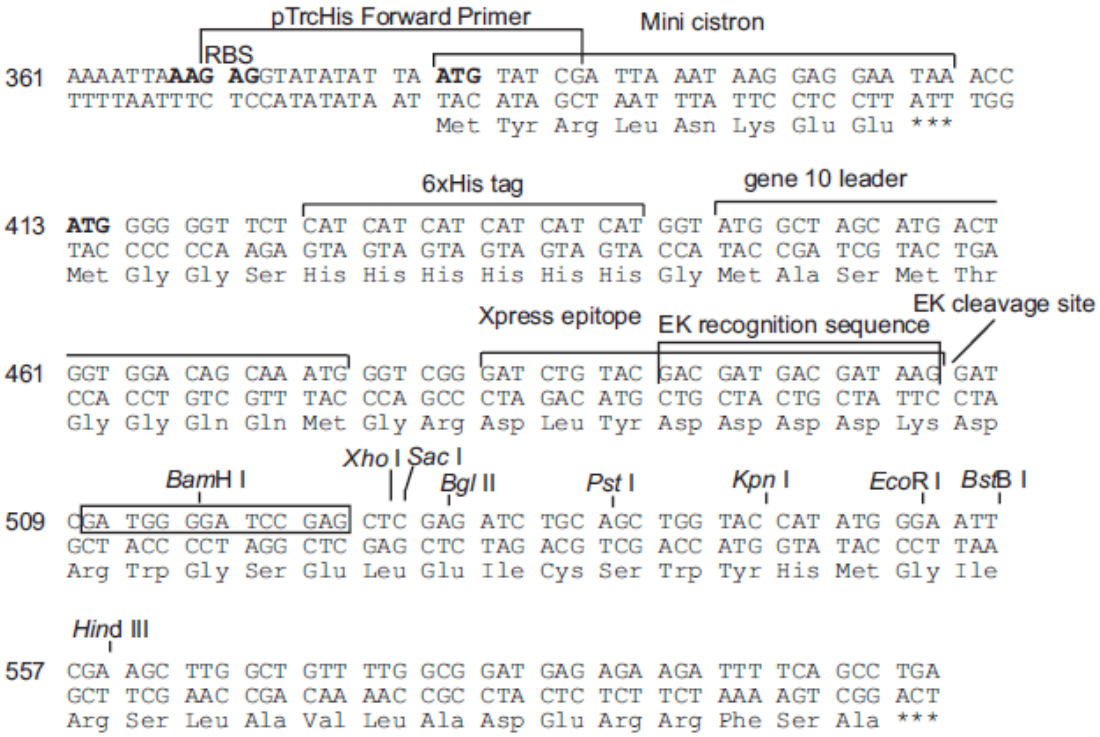
b. What would be first 20 amino acids of your recombinant protein starting from the N-terminal end? 5 marks

The recombinant protein starts with the His-tag at its N-terminal end: Met-Gly-Gly-Ser-(His)6-Gly-Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln (See pTrcHisA plasmid at positions 413-472).

Appendix I: Information relative to the vector M13mp18, a vector derived from a filamentous phage



Appendix 2: Polylinker region of the cloning vector pTrcHisA, a vector specialized for his-tagged protein expression. The full length of the vector is 4404.



Recognition sequences

- BamHI: 5'-G↑G A T C C-3'
  3'-C C T A G↓G-5'
- XhoI: 5'-C↑T C G A G-3'
  3'-G A G C T↓C-5'
- SacI: 5'-G↑A G C T C-3'
  3'-C T C G A↓G-5'
- BglII: 5'-A↑G A T C T-3'
  3'-T C T A G↓A-5'
- PstI: 5'-C T G C A↑G-3'
  3'-G↓A C G T C-5'
- KpnI: 5'-G G T A C↑C-3'
  3'-C↓C A T G G-5'
- EcoRI: 5'-G↑A A T T C-3'
  3'-C T T A A↓G-5'
- BstBI: 5'-T T↑C G A A-3'
  3'-A A G C↓T T-5'
- HindIII: 5'-A↑A G C T T-3'
  3'-T T C G A↓A-5'

- Appendix 3: Sequence of the Thermotolerant DNA polymerase from *Thermus thermophilus*

```
      FT   5'UTR           1..589
      FT   CDS             590..3094
      FT   3'UTR           3095..3221
    1 tctagaggaa gcatgagcct caccctggca gacaaggtgg tctacgagga ggagatccag
   61 aaaagccgct tcatcgccaa ggcggccccc gtggcctcgg aggaggaggc cttggcgttt
  121 ttggccgaga accgggagcc tgaggccacc cacaacggcc acgcctacaa gatcggcctc
  181 ctctaccgct tctctgacga cggggagccc tcgggcaccg caggcaggcc catcctccac
  241 gccatagagg cccagggcct ggaccgggtg gcggtcctgg tggtgcgcta cttcggcggg
  301 gtgaagctcg gggccggggg gcttgtgcgg gcctacgggg gggtggcggc ggaggcctta
  361 aggcgggcgc ccaaggtccc cttggtggag cgggtggggc tcgccttcct cgtgcccttc
  421 gccgaggtgg gccgggtcta cgccctcctg gaggcccgcg cccctgaagg ccgaggagac
  481 ctacaccccg gaggcgtgcg cttcgccctc ctcctcccca agcccgagcg ggaaggtttc
  541 ctcagggcgc tcctggacgc cacccgggga caggtggccc tggagtagca tggaggcgat
  601 gcttccgctc tttgaaccca aaggccgggt cctcctggtg gacggccacc acctggccta
  661 ccgcaccttc ttcgccctga agggcctcac cacgagccgg ggcgaaccgg tgcaggcggt
  721 ctacggcttc gccaagagcc tcctcaaggc cctgaaggag gacgggtaca aggccgtctt
  781 cgtggtcttt gacgccaagg ccccctcctt ccgccacgag gcctacgagg cctacaaggc
  841 ggggagggcc ccgacccccg aggacttccc ccggcagctc gccctcatca aggagctggt
  901 ggacctcctg gggtttaccc gcctcgaggt ccccggctac gaggcggacg acgttctcgc
  961 caccctggcc aagaaggcgg aaaaggaggg gtacgaggtg cgcatcctca ccgccgaccg
 1021 cgacctctac caactcgtct ccgaccgcgt cgccgtcctc caccccgagg gccacctcat
 1081 caccccggag tggctttggg agaagtacgg cctcaggccg gagcagtggg tggacttccg
 1141 cgccctcgtg ggggacccct ccgacaacct ccccggggtc aagggcatcg gggagaagac
 1201 cgccctcaag ctcctcaagg agtggggaag cctggaaaac ctcctcaaga acctggaccg
 1261 ggtaaagcca gaaaacgtcc gggagaagat caaggcccac ctggaagacc tcaggctctc
 1321 cttggagctc tcccgggtgc gcaccgacct ccccctggag gtggacctcg cccaggggcg
 1381 ggagcccgac cgggaggggc ttagggcctt cctggagagg ctggagttcg gcagcctcct
 1441 ccacgagttc ggcctcctgg aggcccccgc ccccctggag gaggcccccc ggcccccgcc
 1501 ggaaggggcc ttcgtgggct tcgtcctctc ccgccccgag cccatgtggg cggagcttaa
 1561 agccctggcc gcctgcaggg acggccgggt gcaccgggca gcagacccct tggcggggct
 1621 aaaggacctc aaggaggtcc ggggcctcct cgccaaggac ctcgccgtct tggcctcgag
 1681 ggaggggcta gacctcgtgc ccggggacga ccccatgctc ctcgcctacc tcctggaccc
 1741 ctccaacacc acccccgagg gggtggcgcg gcgctacggg ggggagtgga cggaggacgc
 1801 cgcccaccgg gccctcctct cggagaggct ccatcggaac ctccttaagc gcctcgaggg
 1861 ggaggagaag ctcctttggc tctaccacga ggtggaaaag cccctctccc gggtcctggc
 1921 ccacatggag gccaccgggg tacggcggga cgtggcctac cttcaggccc tttccctgga
 1981 gcttgcggag gagatccgcc gcctcgagga ggaggtcttc cgcttggcgg gccacccctt
 2041 caacctcaac tcccgggacc agctggaaag ggtgctcttt gacgagctta ggcttcccgc
 2101 cttggggaag acgcaaaaga caggcaagcg ctccaccagc gccgcggtgc tggaggccct
 2161 acgggaggcc cacccccatcg tggagaagat cctccagcac cgggagctca ccaagctcaa
 2221 gaacacctac gtggaccccc tcccaagcct cgtccacccg aggacgggcc gcctccacac
 2281 ccgcttcaac cagacggcca cggccacggg gaggcttagt agctccgacc ccaacctgca
 2341 gaacatcccc gtccgcaccc ccttgggcca gaggatccgc cgggccttcg tggccgaggc
 2401 gggttgggcg ttggtggccc tggactatag ccagatagag ctccgcgtcc tcgcccacct
 2461 ctccggggac gaaaacctga tcagggtctt ccaggagggg aaggacatcc acacccagac
 2521 cgcaagctgg atgttcggcg tccccccgga ggccgtggac cccctgatgc gccgggcggc
 2581 caagacggtg aacttcggcg tcctctacgg catgtccgcc cataggctct cccaggagct
 2641 tgccatcccc tacgaggagg cggtggcctt tatagagcgc tacttccaaa gcttccccaa
 2701 ggtgcgggcc tggatagaaa agaccctgga ggaggggagg aagcggggct acgtggaaac
 2761 cctcttcgga agaaggcgct acgtgcccga cctcaacgcc cgggtgaaga gcgtcaggga
 2821 ggccgcggag cgcatggcct tcaacatgcc cgtccagggc accgccgccg acctcatgaa
 2881 gctcgccatg gtgaagctct tcccccgcct ccgggagatg ggggcccgca tgctcctcca
 2941 ggtccacgac gagctcctcc tggaggcccc ccaagcgcgg gccgaggagg tggcggcttt
 3001 ggccaaggag gccatggaga aggcctatcc cctcgccgtg cccctggagg tggaggtggg
 3061 gatgggggag gactggcttt ccgccaaggg ttaggggggc cctgccgttt agaggaagtt
 3121 caaggggttg tccctcagaa acgcctccag gggaacgccc tctgggccta ccaggaggcc
 3181 tttagcccca aaggtgcggg tgaaggcttc caggccctgg //
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Appendix 4: Restriction Sites within the DNA sequence provided in appendix 3

#	Enzyme	Specificity	Sites & flanks	Cut positions (blunt - 5' ext. - 3' ext.)
1	AgeI	A^CCGG_T	list	*706/710
2	AleI	CACNN^NNGTG	list	2228
3	ApaLI	G^TGCA_C	list	1589/1593
4	BamHI	G^GATC_C	list	2373/2377
5	BcgI	_NN^(N)$_{10}$CGA(N)$_6$TGC(N)$_{10}$_NN^	list	*652/650+686/684
6	BclI	T^GATC_A	list	#2478/2482
7	BfuAI	ACCTGCNNNN^NNNN_	list	2343/2347
8	BmgBI	CAC^GTC	list	*1951
9	BsaI	GGTCTCN^NNNN_	list	470/474
10	BsaWI	W^CCGG_W	list	706/710
11	BsgI	GTGCAG(N)$_{14}$_NN^	list	731/729
12	BsmBI	CGTCTCN^NNNN_	list	*1042/1046
13	BspHI	T^CATG_A	list	2874/2878
14	BspMI	ACCTGCNNNN^NNNN_	list	2343/2347
15	BsrFI	R^CCGG_Y	list	*706/710
16	BsrI	ACTG_GN^	list	3077/3075
17	BtsI	GCAGTG_NN^	list	1130/1128
18	DraIII	CAC_NNN^GTG	list	*1891/1888
19	EagI	C^GGCC_G	list	*1582/1586
20	EcoP15I	CAGCAG(N)$_{25}$^NN_	list	1629/1631
21	HindIII	A^AGCT_T	list	2689/2693
22	HpyCH4III	AC_N^GT	list	2587/2586
23	KasI	G^GCGC_C	list	*366/370
24	MslI	CAYNN^NNRTG	list	2228
25	NarI	GG^CG_CC	list	*367/369
26	PflFI	GACN^N_NGTC	list	1046/1047
27	PvuII	CAG^CTG	list	2062
28	SbfI	CC_TGCA^GG	list	1577/1573
29	SfoI	GGC^GCC	list	*368
30	SphI	G_CATG^C	list	2932/2928
31	TspRI	_NNCASTGNN^	list	1130/1121
32	Tth111I	GACN^N_NGTC	list	1046/1047
33	XbaI	T^CTAG_A	list	1/5
34	XmnI	GAANN^NNTTC	list	2897