Exam 3 Solution

METHODS IN CELL BIOLOGY

1. SHORT ANSWER (30 points) Briefly define or answer the following.

   A. RFLP

   Restriction Fragment Length Polymorphism, method used to genotype samples based on differences in restriction enzyme sites

   B. Dideoxynucleotides

   Nucleotides with a hydrogen at the 3' position instead of the hydroxyl. Use as a chain terminator in sequencing reactions

   C. compatible ends

   Overhangs produced by restriction enzymes which are complementary to each other

   D. A DNA solutions with a A_{260}/A_{280} ration < 1.6 is likely to be overly contaminated with _____.

   Protein

   E. What are the two components of the gap penalty?

   Open and extend

   F. BLAST

   A computer program used to search for similar DNA or protein sequences in a database

   Basic Local Alignment Search Tool

   G. Why is sometimes advisable to treat vectors with phosphatase before ligating with foreign DNA?

   Phosphatase treatment minimizes self-ligation of the vector

   H. What are the 3 different types of ends produced by restriction enzymes?

   Blunt ends, 3'-overhangs, 5'-overhangs

   I. E-value

   A statistical score used in blast searches to evaluate the significance of matches

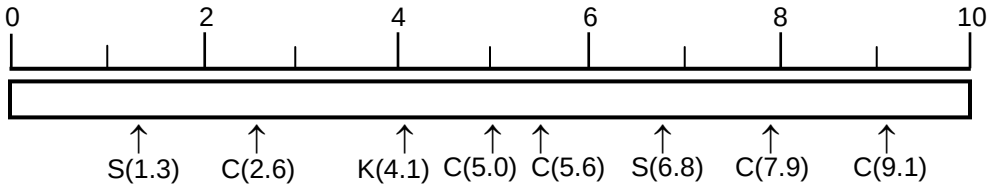
J. His_6 tag

Six consecutive histidines that are used in expression vectors for the affinity purification of recombinant proteins

2. (20 Points) What is cDNA? Describe how cDNA is prepared in regards to preparing recombinant DNA libraries. Discuss things that one needs to consider in choosing between cDNA and genomic DNA in regards to preparing a recombinant DNA library.

- cDNA refers to copy or complementary DNA. This is a DNA copy of RNA molecules
- factors to be considered in choosing between cDNA and gDNA:
  - level of expression of gene of interest in available material
  - intron/exon structure of the gene
  - goals of preparing recombinant clone (eg., expression of recombinant protein)
  - ease of preparation
- preparation
  - isolate RNA
  - use reverse transcriptase to make a RNA/DNA hybrid molecule
  - remove RNA strand and make second DNA strand
  - attach restriction sites to ends for cloning

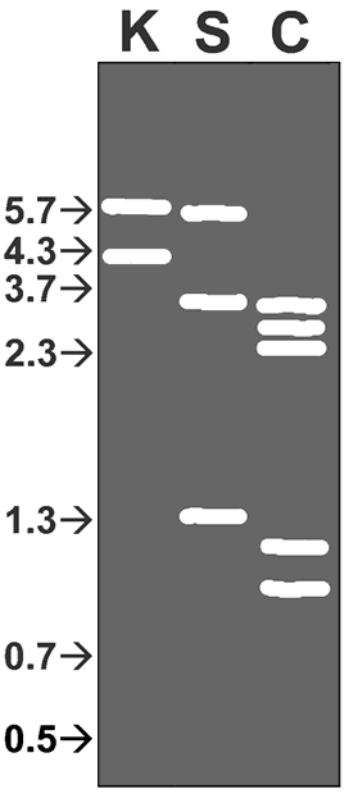
(25 points) You have cloned a 10 kb DNA fragment and sequenced it in its entirety. Analysis of the sequence for restriction enzyme sites generates the following map:



where the letters S, C, and K represent the restriction enzymes *Spe*I, *Cla*I, and *Kpn*I, respectively, and the numbers in parentheses represent the map position in kb from the left-hand side.

A. To confirm this computer generated restriction map you carry out the restriction enzyme digestions on the isolated 10 kb fragment. The purified DNA fragment had an A_{260} = 0.59. How many μl of this DNA do you need to add in order to have 1 μg of DNA in the reaction?

(0.59)(50 μg/ml) = 29.5 μg/ml, therefore 1 μg/29.5 μg/ml = 0.34 ml = 34 μl



B. The resulting ethidium bromide stained gel (shown at the right) indicates that the computer-generated map is in error. In particular, one of the predicted *Cla*I sites is not present in the DNA fragment and represents a sequencing error. Why do you know this?

- The 5 *Cla*1 sites should have produced 6 fragments. Therefore, one of the predicted *Cla*1 sites is probably missing.

C. Is it possible to unambiguously identify the restriction site which is in error based upon the gel? If so, which site is it (give the number in the parenthesis following the enzyme or circle the site). If not, indicate which two (or more) sites might be in error.
    - The lack of the 0.6 kb fragment indicates that either the Cla-5.0 or the Cla-5.6 is missing. It is probably not possible to definitively determine which of the two sites is missing, but arguments to this affect were accepted.

In either event, discuss how a double-digest with *Cla*I and one of the other two enzymes will confirm your conclusion, or unambiguously resolve which restriction site is in error. In other words, explain your choice of enzyme and describe which fragments will change in size and how these changes will resolve the ambiguity or support your conclusion. It is not necessary to describe how all of the fragments will change in size. You only need to discuss the fragment(s) which are central to your argument. Feel free to letter or otherwise label the fragments to make the discussion easier.

- A double digest with Cla1 and either of the other 2 enzymes could be used to resolve the ambiguity. However, the Cla1 + Kpn1 double digest is probably the easier to explain and interpret.

Cla1 + Kpn1

- The 4.1 kb Kpn1 fragment would be convert into fragments of 2.6 and 1.5 kb irrespective of which site was missing.

- The 5.9 kb Kpn fragment would be cleaved into fragments whose sizes depended on which Cla1 site was missing:
    - If Cla-5.0 is missing, then the 5.9 kb fragment would be converted into fragments of 1.5, 2.3, 1.2 and 0.9 kb.
    - If the Cla-5.6 is missing, then these fragments would be 0.9, 2.9, 1.2 and 0.9.
- There may still some ambiguity because some of the fragments produced by the double digest are the same size and cannot be resolved. But those bands should have an increase in intensity.
- Furthermore, the Cla1 pattern will be altered according to which site is missing:
    - If Cla-5.0 is missing, then the top band of the three bands close together will disappear
    - If Cla-5.6 is missing, then the bottom band of the three bands close together will disappear.

Cla1 + Spe1

- The 1.3 kb Spe band will not change in the double digest
- The 3.2 kb Spe band will be converted into fragments of 1.1, 1.2 and 0.9 kb irrespective of which Cla site is in error.
- The 5.5 Spe fragment will be cleaved into fragments whose sizes depend on which Cla1 site is missing:
    - If the Cla-5.0 is missing, then the fragments will be 1.3, 3.0, and 1.2.
    - If The Cla-5.6 is missing, then the fragments will be 1.3, 2.4, and 2.8
- As discussed above, the change in the Cla pattern is probably more definitive:
    - If Cla-5.0 is missing, then the middle and bottom bands of the 3 bands close together will disappear
    - If Cla-5.6 is missing, then only the middle band of the 3 bands close together will disappear. (And there will be a small shift in the sizes of the top and bottom bands.)

4) (20 points) Answer one of the three following essay questions.

a) Briefly describe how mass spectrophotometry is used in the analysis and identification of proteins. What are the advantages of using the mass spectrophometer as compared to the more conventional N-terminal sequencing of proteins or peptides? How is the mass spectrophotometer used in high throughput protein analysis?

Key points:

- A peptide fingerprint can be produce for a protein and this fingerprint compared to databases for protein identification
- Tandem MS/MS can sequence peptides. Advantages:
    - Increase sensitivity (need less protein)
    - Overcome problems of blocked N-terminus

- High throughput methods involve:
  - digesting complex mixtures of the proteins with proteases
  - separating peptides and sequencing
  - procedures can be automated
  - provides proteomic information

b) What is stringency and how is it related to the melting temperature of DNA? Discuss how each of the following affects the stringency of hybridization: temperature, ionic strength (ie, salt concentration), the %GC composition, the length of the probe, the specific activity of the probe (ie, the amount of radioactivity incorporated into the probe). What type of information is obtain from comparing hybridizations carried out at different stringencies?

- Stringency refers to the conditions under which single-stranded nucleic acids will hybridize
- It is relative term usually expressed in terms of high, moderate, or low stringency in relation to the melting temperature
- The stringency is reflective of the degree of homology between the two nucleic acids. At high stringency only highly homologous DNA will hybridize. Therefore comparisons at different stringencies will provide information about the relative degree of homology between the probe and target. Also provide information about optimal hybridization conditions.
- Increasing the temperature raises the stringency
- Increasing the salt concentration lowers the stringency
- The % GC and probe length do not really affect the stringency (conditions) but a higher GC content or longer probe leads to a stronger hybridization, so in that respect, increasing the %GC or probe length would lower the stringency
- The specific activity of the probe has no affect on stringency or hybridization. It will only affect the amount of time for the autoradiography or the strength of the signal.

c) Discuss the basic method and things that are needed to carry out PCR. What is the variation of PCR that allows for quantification of the amount of template present in the sample? Describe the differences between conventional PCR and this technique in terms of the procedure and things needed.

Key points:

- PCR amplifies a region of DNA between 2 primers
- Things needed:
  - Thermocycler
  - Target DNA

    - Pair of primers hybridizing on opposite strands and generally < 1 kb apart
    - Taq polymerase (heat stable)
    - Nucleotides and other buffer components
- Basic method
    - Mix target DNA, primers, polymerase, nucleotides
    - Set thermocycler for appropriate times and temperatures for denaturation, annealing, extension
    - Carry amplification and analyze products by gel electrophoresis
- Real time PCR used to quantify samples
- Differences from conventional pcr:
    - Need light cycler which can measure fluorescence while reaction is progressing
    - Need to add fluorescent probe to quantify dsDNA
    - Determine the threshold cycle and this is used to quantify template