This document contains:

- **Question Worksheet (1):** Restriction Mapping
- **Question Worksheet (2):** qPCR
- **References to Question Worksheets**

Question Worksheet (1)

Terms to be familiar with before you start to solve the test: restriction endonucleases, DNA polymerase, exonucleases, agarose gel electrophoresis, autoradiography, DNA synthesis.

Restriction endonuclease mapping of a DNA fragment was performed in the experiment described in this test. The fragment was generated by *Eco*RI (cleavage site: 5′-G↓AATTC-3′) and *Bam*HI (cleavage site: 5′-G↓GATCC-3′) digestion. Only one strand of the recognition sites is shown; the *arrows* indicate the actual cleavage sites. The purified DNA fragment was incubated *in vitro* in the following mixture: DNA fragment, DNA polymerase lacking the 5′→3′ exonuclease activity, dATP, dTTP, dGTP, [α-^{32}P]dCTP, ions, optimal pH.

After incubation the DNA fragment was extracted with chloroform, divided into five samples, and the samples were incubated for various times (0, 2, 5, 10, 120 min) with *Alu*I restriction enzyme. The products of digestion were separated by gel electrophoresis. The autoradiogram of the gel is shown in Fig. 1.

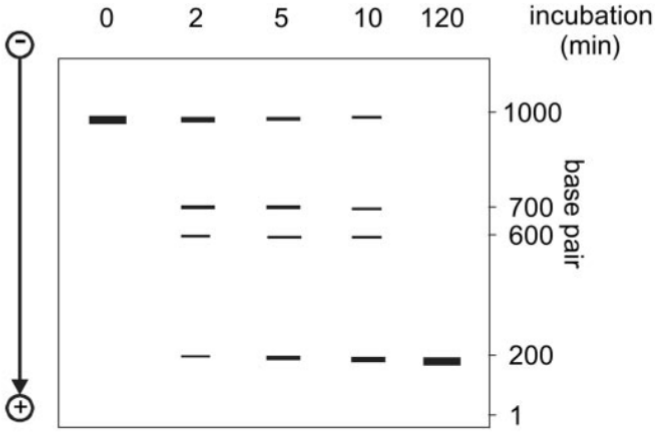

FIG. 1. **Autoradiogram of the agarose gel electrophoresis-fractionated *Alu*I digestion products.** The *arrow* indicates the direction of electrophoresis.

EXPERIMENT ANALYSIS

The following statements are related to the information presented in the description of the experiment. Based on the information given, select one of the following: A. The statement is supported by the information given; B. The statement is contradicted by the information given; C. The statement is neither supported nor contradicted by the information given.

1.____ The DNA fragment was labeled by repair synthesis.
2.____ All *Bam*HI-*Eco*RI fragments contain one labeled phosphate group.
3.____ The ^{32}P atom is located at the 3′-end of the labeled strand.
4.____ The *Eco*RI-*Bam*HI fragment is 1000 bp-long.
5.____ *Alu*I digestion generates 100-bp fragments, as well.
6.____ The *Alu*I enzyme is contaminated with 3′→5′ exonuclease.
7.____ The sample digested for 2 min contains only labeled DNA fragments (does not contain unlabeled fragments).

FIVE-CHOICE COMPLETION

Select the one best answer.

8.____ Which map (see Fig. 2) is consistent with the results of the experiment? E = *Eco*RI, A = *Alu*I, B = *Bam*HI cleavage sites.

A. A map.
B. B map.
C. C map.
D. D map.
E. None of the above.

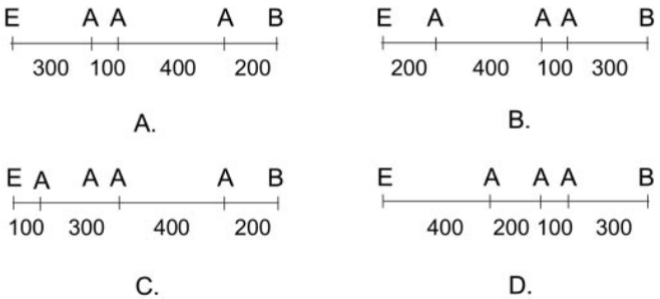

FIG. 2. **Possible restriction endonuclease maps of the DNA fragment.**

‡ To whom correspondence should be addressed. E-mail: jozsef.szeberenyi@aok.pte.hu.

CORRECT ANSWERS

1. B	5. A
2. A	6. B
3. A	7. B
4. A	8. A

EXPLANATIONS

Cutting DNA with both *Eco*RI and *Bam*HI generates single-stranded, 4-nucleotide-long 5′-overhangs at the ends of the cleaved DNA fragment, shown in Fig. 3.

In an *in vitro* DNA synthesizing mixture these 5′-overhangs serve as templates, whereas the shorter 3′-ends serve as primers for the DNA polymerase to make both ends double-stranded. Because the only labeled nucleotide in the mixture was dCTP, only the 3′-end of the *Bam*HI end became radioactive (multiple choice question (MCQ)[1] 1: B; MCQ 2: A; MCQ 3: A), as shown in Fig. 4.

As can be seen in the 0-min sample of Fig. 1, the undigested *Bam*HI-*Eco*RI fragment is 1000 bp-long (MCQ 4: A). Cleavages by *Alu*I produce one labeled fragment (with the radioactive "*Bam*HI-end") and one or more unlabeled fragments (MCQ 5: A; MCQ 7: B). Partial digestion (2-, 5-, 10-min samples) will produce cleavage intermediates whose sizes (700, 600, and 200 bp) will define the distance of the *Alu*I sites from the labeled end (MCQ 8: B). A 3′→5′ exonuclease contamination would have produced free, labeled dCMP, but no band shows up at the 1-nucleotide position on the autoradiogram (MCQ 6: B).

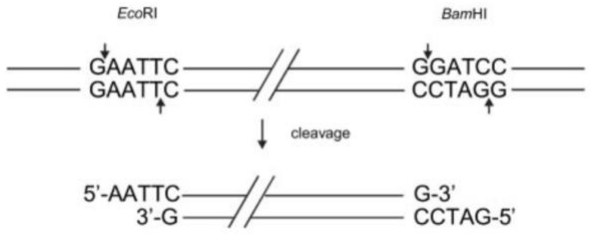

FIG. 3. **The DNA fragment used in this experiment was generated by *Eco*RI and *Bam*HI cleavages.**

[1] The abbreviation used is: MCQ, multiple choice question.

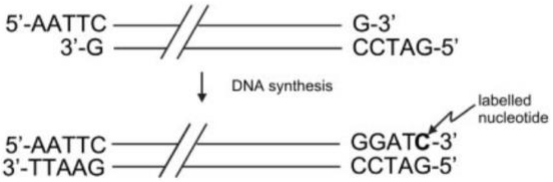

FIG. 4. **Radioactive labeling of the *Bam*HI-generated end of the DNA fragment by *in vitro* DNA synthesis.**

Question Worksheet (2)

Terms to be familiar with before you start to solve the test: polymerase chain reaction, DNA amplification, electrophoresis, breast cancer, *HER2* gene, genomic DNA, *in vitro* DNA synthesis, template, primer, Taq polymerase, 5′→3′ elongation activity, 5′→3′ exonuclease activity, deoxyribonucleoside triphosphates, DNA structure, proofreading, thermocycler, fluorescence.

THE EXPERIMENT

In traditional polymerase chain reaction (PCR), the analysis of the amplified DNA region takes place after 30–40 cycles the product is studied by electrophoresis and DNA staining. The advantage of real-time PCR is that the process can be monitored during the reaction: the extent of DNA amplification can be determined after each PCR cycle. Several different methods have been developed for this, the principle of one of the most popular techniques (TaqMan reaction)[1] is described in the following experiment.

A tumor was removed from the breast of a patient. Genomic DNA was isolated from the tumor and the surrounding normal tissue, and PCR reaction was performed using identical amounts of the two DNA samples. Reaction mixtures contained the following components.

DNA template (the genomic DNA samples); many copies of two primers specific for a region of the *HER2* gene (their binding to the template is shown in Fig. 1); many copies of a TaqMan probe, an oligonucleotide binding to one of the template strands in the region flanked by the two primers (a fluorescent reporter dye is attached to the 5′-end and a quenching molecule to the 3′-end of the probe, inhibiting the fluorescence of the reporter); Taq polymerase (heat-resistant DNA polymerase with 5′→3′ elongation and 5′→3′ exonuclease activities); the four dexoyribonucleoside triphosphates (dATP, dGTP, dCTP, dTTP).

Using your knowledge of bacterial DNA replication solve the following multiple-choice questions (MCQs).[2]

‡To whom correspondence should be addressed. E-mail: jozsef.szeberenyi@aok.pte.hu.

[1]This test is based on a product description of Applied Biosystems (Foster City, CA; www.appliedbiosystems.com).

[2]The abbreviations used are: MCQ, multiple-choice question; PCR, polymerase chain reaction.

FOUR-CHOICE ASSOCIATION

(In this type of question, a set of lettered headings is followed by a list of numbered words or phrases. Select

A. if the word or phrase is associated with A only;
B. if the word or phrase is associated with B only;
C. if the word or phrase is associated with A and B;
D. if the word or phrase is associated with neither A nor B.)

A. 5′→3′ elongation activity of Taq polymerase
B. 5′→3′ exonuclease activity of Taq polymerase
C. Both of them
D. Neither of them

1. ____ Generates phosphodiester bonds.
2. ____ Cleaves.
3. ____ Cleaves hydrogen bonds.
4. ____ Is primer-dependent.
5. ____ Degrades the primers into mononucleotides in the mixture described above.
6. ____ Degrades the TaqMan probe into mononucleotides in the mixture described above.
7. ____ Has a proofreading function.

Reaction mixtures were incubated in a thermocycler capable of monitoring fluorescence. Figure 2 shows relative fluorescence values measured after each cycle using the breast tumor (A) and normal (B) DNA sample.

Study the figure and solve the following MCQs.

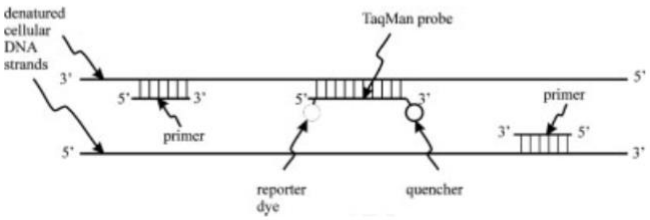

FIG. 1. The principle of TaqMan method (details in the text).

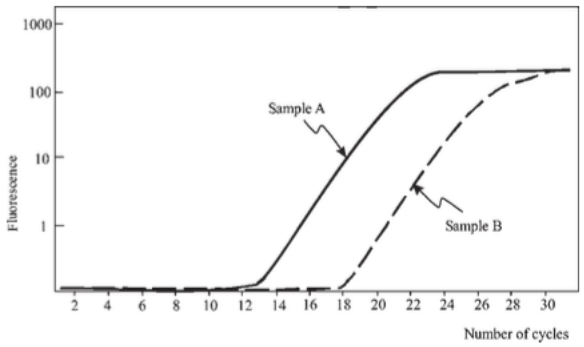

FIG. 2. Real-time PCR performed with a breast cancer (A) and a normal (B) genomic DNA sample from the same patient (details in the text).

FIVE-CHOICE COMPLETION

(This type of question consists of a question or incomplete statement followed by five suggested answers or completions. Select the one best answer.)

8. ____ As PCR reactions proceed, at one point fluorescence increases in both mixtures. What process can explain this?
   A. TaqMan probe molecules are degraded into nucleotides
   B. TaqMan probe molecules are degraded into smaller oligonucleotides
   C. TaqMan probe molecules are released from the template as intact oligonucleotides
   D. TaqMan probe molecules serve as primers for Taq polymerase
   E. TaqMan probe molecules are incorporated into the newly synthesized DNA strands.

QUANTITATIVE COMPARISON

(In this type of question paired statements describe two entities that are to be compared in a quantitative sense. Select

   A. if A is greater than B.
   B. if B is greater than A.
   C. if the two are equal or very nearly equal.)

9. ____
   A. The number of free primer molecules in Sample **A** after cycle 4.
   B. The number of free primer molecules in Sample **A** after cycle 10.

10. ____
   A. The number of free primer molecules in Sample **A** after cycle 10.
   B. The number of free primer molecules in Sample **B** after cycle 10.

11. ____
   A. The number of free TaqMan probe molecules in Sample **A** after cycle 10.
   B. The number of free TaqMan probe molecules in Sample **B** after cycle 10.

12. ____
   A. The number of free TaqMan probe molecules in Sample **A** after cycle 18.
   B. The r number of free TaqMan probe molecules in Sample **B** after cycle 18.

13. ____
   A. The number of reporter dye/mononucleotide complexes in Sample **A** after cycle 18.
   B. The number of reporter dye/mononucleotide complexes in Sample **B** after cycle 18.

14. ____
   A. The number of amplified *HER2* fragments in Sample **A** after cycle 20.
   B. The number of amplified *HER2* fragments in Sample **B** after cycle 20.

15. ____
   A. The number of amplified *HER2* fragments in Sample **A** after cycle 30.
   B. The number of amplified *HER2* fragments in Sample **B** after cycle 30.

FIVE-CHOICE COMPLETION

(This type of question consists of a question or incomplete statement followed by five suggested answers or completions. Select the one best answer.)

16. ____ Why there is no detectable fluorescence in the samples after the first few PCR cycles?
   A. Because Taq polymerase degrades the TaqMan probe molecules.
   B. Because Taq polymerase degrades the primers.
   C. Because there is no DNA synthesis.
   D. Because the quenchers block the fluorescence of all reporter dye molecules.
   E. Because the fluorescence detector is not sensitive enough.

17. ____ Why fluorescence does not increase after cycle 30 in either samples?
   A. Because all primer molecules have been used
   B. Because all TaqMan probe molecules have been used.
   C. Because all template molecules have been used.
   D. A and B.
   E. A, B and C.

18. ____ What happened to the *HER2* gene in the breast tumor cells?
   A. Its copy number increased approximately 30-fold.
   B. Its copy number increased approximately 5-fold,
   C. Its copy number decreased approximately 30-fold,
   D. Its copy number decreased approximately 5-fold,
   E. Its expression decreased approximately 5-fold.

CORRECT ANSWERS

1. A
2. B
3. D
4. A
5. D
6. B
7. D
8. A
9. A
10. B
11. B
12. B
13. A
14. A
15. C
16. E
17. D
18. A

EXPLANATIONS

Taq polymerase is a heat-resistant DNA polymerase widely used in PCR protocols. Using its elongation activity it adds nucleotides to the 3′-end of template-bound primer molecules (**MCQ 1: A; MCQ 4: A**). Its 5′→3′ exonuclease activity enables it to hydrolyze the TaqMan probe into mononucleotides (**MCQ 2: B; MCQ 6: B**), liberating the reporter dye from the inhibitory effect of the quencher molecule (**MCQ 8: A**). (Taq polymerase does not have helicase/**MCQ 3: D**/ or 3′→5′ exonuclease /**MCQ 7: D**/ activity. Under the conditions used, the primers cannot be degraded by Taq polymerase /**MCQ 5: D**/.)

As DNA synthesis starts, more and more primer molecules are incorporated into the newly synthesized PCR products (**MCQ 9: A**). Since the number of template molecules increases, more TaqMan probe molecules bind to template strands and become degraded during DNA synthesis. Once the increasing fluorescence reaches the sensitivity threshold of the fluorescence detector (**MCQ 16: E**), fluorescence curves start to go up. The fact that this happens earlier in Sample A indicates that the target DNA sequences are present in larger quantity in the tumor DNA than in the genome of normal cells (**MCQ 10: B**; **MCQ 11: B**; **MCQ 12: B**; **MCQ 13: A**; **MCQ 14: A**). When the curves reach a plateau, amplification stops due to the consumption of all primer or TaqMan probe molecules (**MCQ 17: D**); at this point, the copy number of PCR products is identical in the two samples (**MCQ 15: C**). Increase in detectable fluorescence starts about five cycles earlier in Sample A than in Sample B (cycle 13 vs. cycle 18). This indicates that the *HER2* gene had been amplified 2^5-fold in the breast cancer (**MCQ 18: A**).

References

Question Worksheet 1:

Szeberényi, J. (2002). Restriction mapping. Biochemistry and Molecular Biology Education, 30(4), 258–259. https://doi.org/10.1002/bmb.2002.494030040039

Question Worksheet 2:

Szeberényi, J. (2009). Problem-solving test: Real-time polymerase chain reaction. Biochemistry and Molecular Biology Education, 37(4), 250–252. https://doi.org/10.1002/bmb.20313