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(54) **METHODS AND COMPOSITIONS FOR
MULTIPLEX AMPLIFICATION OF NUCLEIC
ACIDS**

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(57) **ABSTRACT**

A method is described for predetermining ratios of primer pairs present in a single reaction vessel so as to achieve approximately equimolar yield of products. The ratios are determined as a function of the length of the amplicon and the length of other amplicons being simultaneously tested. The primers may desirably be for p53 gene sequences.

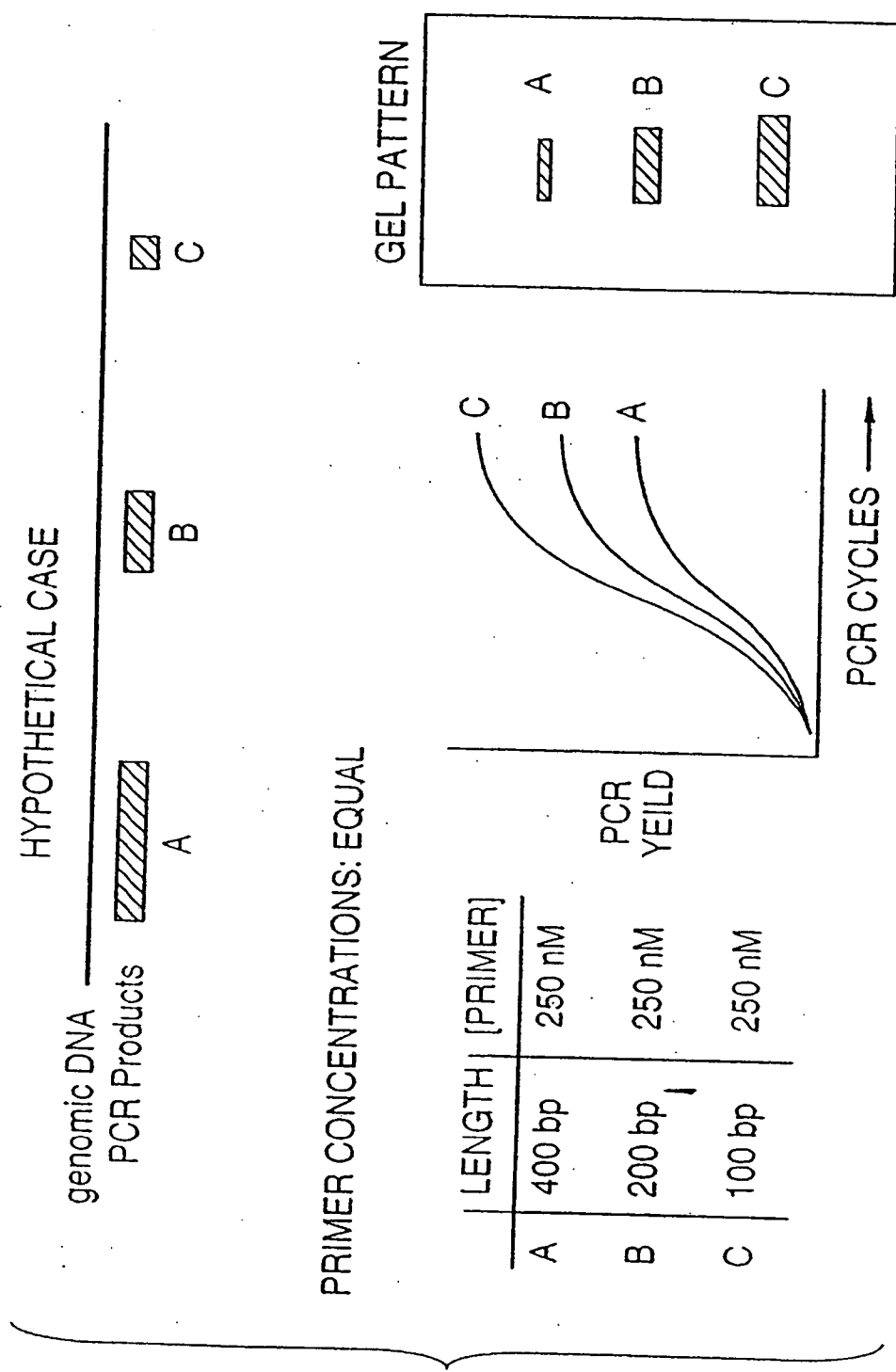


FIG. 1

FIG. 2

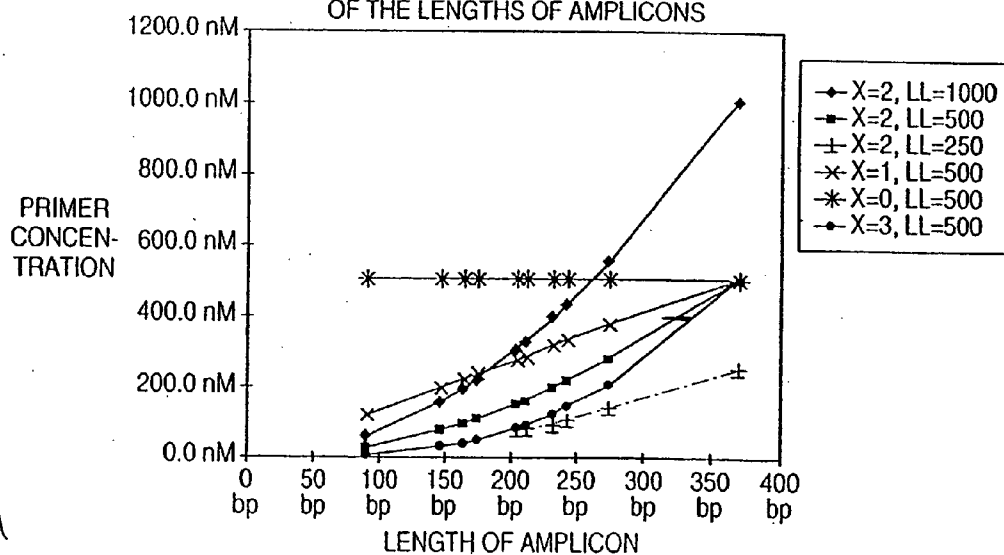
MULTIPLEX PCR PRIMER CONCENTRATIONS AS A FUNCTION OF THE LENGTHS OF AMPLICONS

| | | | | | | |
|-------------|---|---|---|---|---|---|
| VALUES OF X | 2 | 2 | 2 | 1 | 0 | 3 |
|-------------|---|---|---|---|---|---|

| | | | | | | |
|-------------------------|---------|--------|--------|--------|--------|--------|
| TYPICAL VALUES OF L_L | 1000 nM | 500 nM | 250 nM | 500 nM | 500 nM | 500 nM |
|-------------------------|---------|--------|--------|--------|--------|--------|

| | AMPLI- CON | LENGTH | X=2, $L_L=1000$ | X=2, $L_L=500$ | X=2, $L_L=250$ | X=1, $L_L=500$ | X=0, $L_L=500$ | X=3, $L_L=500$ |
|----------|---------------|--------|--------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| LONGEST | 4 | 368 bp | 1000.0 nM | 500.0 nM | 250.0 nM | 500.0 nM | 500.0 nM | 500.0 nM |
| | 5 | 272 bp | 546.3 nM | 273.2 nM | 136.6 nM | 369.6 nM | 500.0 nM | 201.9 nM |
| | 8 | 241 bp | 428.9 nM | 214.4 nM | 107.2 nM | 327.4 nM | 500.0 nM | 140.4 nM |
| | 11 | 231 bp | 394.0 nM | 197.0 nM | 98.5 nM | 313.9 nM | 500.0 nM | 123.7 nM |
| | 10 | 210 bp | 325.6 nM | 162.8 nM | 81.4 nM | 285.3 nM | 500.0 nM | 92.9 nM |
| | 6 | 204 bp | 307.3 nM | 153.7 nM | 76.8 nM | 277.2 nM | 500.0 nM | 85.2 nM |
| SHORTEST | 7 | 175 bp | 226.1 nM | 113.1 nM | 56.5 nM | 237.8 nM | 500.0 nM | 53.8 nM |
| | 2 | 164 bp | 198.6 nM | 99.3 nM | 49.7 nM | 222.8 nM | 500.0 nM | 44.3 nM |
| | 9 | 146 bp | 157.4 nM | 78.7 nM | 39.4 nM | 198.4 nM | 500.0 nM | 31.2 nM |
| | 3 | 90 bp | 59.8 nM | 29.9 nM | 15.0 nM | 122.3 nM | 500.0 nM | 7.3 nM |

MULTIPLEX [PRIMERS] AS A FUNCTION OF THE LENGTHS OF AMPLICONS



METHODS AND COMPOSITIONS FOR MULTIPLEX AMPLIFICATION OF NUCLEIC ACIDS

RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Application Ser. No. 60/050,405, filed on Jun. 20, 1997, the text of which is expressly incorporated herein.

BACKGROUND OF THE INVENTION

[0002] The polymerase chain reaction (PCR) is a simple and versatile method to amplify in vitro a specific segment of DNA for subsequent study (Saiki et al., *Science* 230:1350 (1985); Saiki et al., *Science* 235:487 (1985)). The PCR method has gained widespread use in biomedical research, and has revolutionized the accurate and early diagnosis of many inherited and acquired genetic disorders (Eisenstein, *N. Engl. J. Med.* 322:178 (1990)), particularly those caused by point mutations or small insertions or deletions including sickle cell anemia (Saiki et al., *Science* 230:1350 (1985)), hemophilia A (Kogan et al., *N. Engl. J. Med.* 317:985 (1987)), Tay-Sach's disease (Myerowitz, *Proc. Natl. Acad. Sci. USA* 85:3955 (1988); Myerowitz et al., *J. Biol. Chem.* 263:18587 (1988)), cystic fibrosis (Riordan et al., *Science* 245:1066 (1989)), and many others. With PCR, it is also possible to detect heterozygotic carriers in recessive disorders.

[0003] Polymerase chain reaction (PCR) is used for a variety of purposes. PCR can be used to amplify genomic DNA or other sources of nucleic acids for analysis. It is often desirable to be able to achieve equimolar yields of different length amplicons when performing multiplex PCR or multiple PCR reactions. Having an approximately equimolar yield of amplicons is particularly useful, for example, when approximately equal representation of certain regions of genomic DNA amplified after multiplex PCR is desired. Prior to the methods of present invention, finding the appropriate experimental conditions useful to achieve this result has been difficult because PCR amplifies nucleic acids having different lengths with different efficiencies. The yield of longer amplicons is often less than the yield of shorter amplicons because of those differences in PCR amplification efficiency. **FIG. 1** shows the difference in yields that one might expect, for example, when starting with equal primer concentrations used to amplify amplicons of varying lengths: A, B, C. There is a continuing need in the art for methods which permit the amplification of different sequences with the same efficiency so that approximately equimolar products result.

[0004] It is an object of the present invention to provide a method of performing multiplex PCR which achieve approximately equimolar products.

[0005] It is another object of the invention to provide a set of primers for amplification of p53.

[0006] It is yet another object of the invention to provide a set of primers for amplification of p53 to achieve approximately equimolar products.

[0007] It is still another object of the invention to provide a mixture of primers for performing multiplex PCR.

[0008] These and other objects of the invention are provided by one or more of the embodiments provided below.

In one embodiment of the invention a method of performing multiple polymerase chain reactions in a single vessel is provided. The method comprises the steps of priming DNA synthesis on a template in a vessel with at least two sets of primers. The primers are present in the vessel at a predetermined ratio which is described by the formula:

$$C_A = C_L (L_A + L_L)^2$$

[0009] C_A is the concentration of primers for an amplicon A. C_L is the concentration of primer for the longest amplicon. L_A is the length of the amplicon A. L_L is the length of the longest amplicon.

[0010] Another embodiment provided by the invention is a method of performing multiple polymerase chain reactions in a single vessel. The method comprises priming DNA synthesis on a genomic p53 template in a vessel with ten sets of primers which amplify exons 2-11 of p53. The primers are shown in SEQ ID NO: ID NOS: 1-20. The primers are present in the vessel at the following ratio: exon 2 (89.4): exon 3 (26.9): exon 4 (450): exon 5 (245.8): exon 6 (-138.3): exon 7 (101.8): exon 8 (193.0): exon 9 (70.8): exon 10 (146.5): exon 11 (177.3).

[0011] According to still another embodiment of the invention a set of primers for performing multiple polymerase chain reactions in a single vessel is provided. The set comprises twenty primers having sequences as shown in SEQ ID NO: 1-20.

[0012] According to yet another embodiment of the invention a mixture of primers for performing multiplex polymerase chain reaction is provided. The primers are present in the mixture at a predetermined ratio to each other. The ratio of the concentrations of the primers is described by:

$$C_A = C_L (L_A + L_L)$$

[0013] wherein C_A is the concentration of primers for an amplicon A; wherein C_L is the concentration of primer for the longest amplicon; wherein L_A is the length of the amplicon A; and wherein L_L is the length of the longest amplicon.

[0014] The present invention thus provides the art with a method useful for performing multiplex PCR. This method is particularly useful for amplification of multiple exons of p53. Moreover, a particular primer set useful for performing such multiplex PCR is also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] **FIG. 1** shows the difference in yields that one might expect, for example, when starting with equal primer concentrations used to amplify amplicons of varying lengths: A, B, C.

[0016] **FIG. 2** illustrates the relationship for given values X and L_L , using the amplicons from different exons of the human p53 gene as an example.

DETAILED DESCRIPTION OF THE INVENTION

[0017] It is a discovery of the present invention that approximately equimolar yields of amplicons of varying lengths can be easily produced by multiplex PCR. It has been determined that varying the primer concentrations as a function of the lengths of amplicons yields approximately

equimolar amounts of amplicons of varying lengths. The relationship between primer concentration and the length of amplicons is as follows:

$$C_A = C_L (L_A / L_L)^X$$

[0018] wherein

[0019] C_A = the concentration of primers for an amplicon A;

[0020] C_L = the concentration of primer for the longest amplicon;

[0021] L_A = the length of amplicon A;

[0022] L_L = the length of the longest amplicon; and

[0023] X is usually not zero and is often between one and three.

[0024] This relationship can be placed in a computer readable medium or be used with a computer system if desired.

[0025] FIG. 2 illustrates the relationship for given values X and L_L , using the amplicons from different exons of the human p53 gene as an example. Using primer concentrations as set forth, for example in FIG. 2, one skilled in the art can determine the optimum set of primer concentrations to yield approximately equimolar yields of varying length amplicons in a multiplex or multiple PCR. Preferably, primers having both comparable base composition and comparable melting temperatures are used. Also preferably, Mg^{+2} concentration, annealing temperatures, and cycling times of the PCR are optimized prior to choosing the desired set of primer concentrations in accordance with the present invention.

[0026] PCR techniques applicable to the present invention include inter alia those discussed in PCR PRIMER: A LABORATORY Manual, Dieffenbac, C. W. and Dveksler, G. S., eds., Cold Spring Harbor Laboratory Press (1995).

[0027] The present application further provides primer sequences, primer concentrations, and experimental conditions useful in the amplification of the coding region of the human p53 gene. Particularly useful primers for amplification of exons of the p53 gene are set forth in Table 1.

Table 1

[0028] p53 Primer Set

[0029] 20 primers in 1 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, sequences:

| | |
|---------|---------------------------------------|
| Exon 2: | 5' -TCATGCTGCATCCCCACTTTCTCCTTTG-3' |
| | 5' -TGGCCTGCCCTTCCAATGGATCCACTCA-3' |
| Exon 3: | 5' -AATTCATGGGACTGACTTTCTGCTCTTGTC.3' |
| | 5' -TCCAGGTCCCAGCCCAACCCTTGTC-3' |
| Exon 4: | 5' -GTCCCTGACTGCTCTTTTACCCCATCTAC-3' |
| | 5' -GGGATACGGCCAGGCATTGAAGTCTC-3' |
| Exon 5: | 5' -CTTGTGCCCTGACTTTCAACTCTGTCTC-3' |
| | 5' -TGGGCAACCAGCCCTGTCTCTCTCCA-3' |
| Exon 6: | 5' -CCAGGCCTCTGATTCCTCACTGATTGCTC-3' |
| | 5' -GCCACTGACAACCACCCTTAACCCCTC-3' |

-continued

| | |
|----------|---|
| Exon 7: | 5' -GCCTCATCTTGGGCCTGTGTTATCTCC-3' |
| | 5' -GGCCAGTGTGCAGGGTGGCAAGTGGCTC-3' |
| Exon 8: | 5' -GTAGGACCTGATTTCCCTACTGCCTTGC-3' |
| | 5' -ATAACTGCACCCTTGGTCTCTCCACC-3' |
| Exon 9: | 5' -CACTTTTATCACCTTCTTCCCTCTTTC-3' |
| | 5' -AACTTTCCACTTGATAAGAGGTCCCAAGAC-3' |
| Exon 10: | 5' -ACTTACTTCTCCCCTCTGTGTTGCTGC-3' |
| | 5' -ATGGAATCCTATGGCTTTTCCAACCTAGGAAG-3' |
| Exon 11: | 5' -CATCTCTCCTCCCTGCTTCTGTCTCTAC-3' |
| | 5' -CTGACGCACACCTATTGCAAGCAAGQGTTC-3' |

[0030] Table 2 shows particularly useful concentrations of the primers set forth in Table 1 for multiplex PCR amplification using the experimental conditions set forth in Table 3.

TABLE 2

| Primer Concentrations in p53 Primer Set | | | |
|---|----------|--------|--------------|
| | Amplicon | Length | Primer Concs |
| Longest | 4 | 368 bp | 450.0 nM |
| | 5 | 272 bp | 245.8 nM |
| | 8 | 241 bp | 193.0 nM |
| | 11 | 231 bp | 177.3 nM |
| | 10 | 210 bp | 146.5 nM |
| | 6 | 204 bp | 138.3 nM |
| | 7 | 175 bp | 101.8 nM |
| Shortest | 2 | 164 bp | 89.4 nM |
| | 9 | 146 bp | 70.8 nM |
| | 3 | 90 bp | 26.9 nM |

Values of X 2
Typical values of C_L 450 nM

[0031]

TABLE 3

| Multiplex PCR | | | |
|--|------------|--------------|----------------|
| Start with 250 ng of Template DNA. | | | |
| PCR Components for 100 ul PCR in 0.2 ml thin walled tubes: | | | |
| | Stock Conc | Final Conc | for 1 reaction |
| Buffer (No Mg) | 10 X | 1 X | 10.0 ul |
| MgCl ₂ | 25 mM | 2.5 mM | 10.0 ul |
| dATP | 10 mM | 200 uM | 2.0 ul |
| dCTP | 10 mM | 200 uM | 2.0 ul |
| dGTP | 10 mM | 200 uM | 2.0 ul |
| dTTP | 10 mM | 200 uM | 2.0 ul |
| Taq GOLD | 5 U/ul | 10 U | 2.0 ul |
| p53 Primer Set | 20 X | 1 X | 5.0 ul |
| Water | | | |
| Human genomic DNA | | 250 ng | |
| | | Total Volume | 100.0 ul |

Final Concentrations in Buffer (No Mg) are 10 mM Tris-HCl (pH 8.3), 50 mM KCl
Taq GOLD is AmpliTaq Gold™ from Perkin Elmer catalog # N808-0243
PCR Cycles:

| | | |
|------------|-------|--------|
| 35 Cycles: | 94 C. | 10 min |
| | 94 C. | 30 sec |
| | 60 C. | 30 sec |

TABLE 3-continued

| Multiplex PCR | |
|---------------|--------|
| 72 C. | 45 sec |
| 72 C. | 10 min |

To visualize amplicons by gel Analysis:
 Visualize PCR products on 4% NuSieve Agarose Gel
 NuSieve™ Agarose 3:1 is from FMC catalog # 50092
 Load 5 ul of PCR + loading buffer
 Use 50 bp Ladder (Gibco/BRL catalog # 10416-014) as size marker
 Run gel at 125 Volts for 30 min. to 90 min.

| Expected PCR Products: | | Order in Gel: | |
|------------------------|--------|---------------|--------|
| Amplicon | Length | Amplicon | Length |
| Exon 2 | 164 bp | Exon 4 | 368 bp |
| Exon 3 | 90 bp | Exon 5 | 272 bp |
| Exon 4 | 368 bp | Exon 8 | 241 bp |
| Exon 5 | 272 bp | Exon 11 | 225 bp |
| Exon 6 | 204 bp | Exon 10 | 210 bp |
| Exon 7 | 175 bp | Exon 6 | 204 bp |
| Exon 8 | 241 bp | Exon 7 | 175 bp |
| Exon 9 | 146 bp | Exon 2 | 164 bp |
| Exon 10 | 210 bp | Exon 9 | 146 bp |
| Exon 11 | 225 bp | Exon 3 | 90 bp |

[0032] Using the methods and reagents provided herein, we achieved multiplex PCR amplification of coding regions shown of the human p53 gene in approximately equimolar amounts. That desirable result was achieved in a single-tube reaction. The achievement of such desirable results with the

remarkable convenience of a single tube reaction further illustrates the contribution to the art made by the present invention.

[0033] The methods and compositions of the present invention are useful in virtually any context in which equimolar yields of various PCR products are desired. Such contexts include without limitation paternity testing, forensic analysis, genetic screening, polymorphism detection, and mutation analyses. The present invention can be used to amplify nucleic acids for all forms of sequence analysis known to those skilled in the art. Sequence analysis techniques includes, for example, dideoxy-sequencing and sequence analysis using high-density nucleic acid arrays: the GeneChip® probe arrays or VLSIPS™ technology of Affymetrix, Inc. High density nucleic acid arrays are discussed for example in Chee, M., Yang, R., Hubbell, E., Berno, A., Huang, X. C., Stern, D., Winkler, J., Lockhart, D. J., Morris, M. S., & Fodor, S. P., Science 5287, 610-614 (1996), U.S. Pat. No. 5,445,934, and International Publication No. WO 95/11995 corresponding to PCT Application No. PCT/US94/12305.

[0034] The p53 gene and its protein product are discussed in *Molecular Biology of the Cell*, 3rd Edition, Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D., Garland Publishing (1994) at pages 889 and 1284-1289.

[0035] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of any appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 20

<210> SEQ ID NO 1

<211> LENGTH: 28

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 1

tcattgctgga tccccacttt tcctcttg

28

<210> SEQ ID NO 2

<211> LENGTH: 28

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 2

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28

<210> SEQ ID NO 3

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: homo sapiens

<400> SEQUENCE: 3

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30

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<210> SEQ ID NO 4
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<400> SEQUENCE: 4

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<400> SEQUENCE: 13
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<400> SEQUENCE: 14
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<400> SEQUENCE: 15
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<210> SEQ ID NO 17
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<400> SEQUENCE: 18
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<212> TYPE: DNA
<213> ORGANISM: homo sapiens
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29

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```

30

1. A method of performing multiple polymerase chain reactions in a single vessel, comprising:

priming DNA synthesis on a template in a vessel with at least two sets of primers, wherein each of the sets of primers in the vessel is at a predetermined ratio, wherein the ratio is described by:

$$C_A = C_L(L_A + L_L)^2$$

wherein C_A is the concentration of primers for an amplicon A; wherein C_L is the concentration of primer for the longest amplicon; wherein L_A is the length of the amplicon A; and wherein L_L is the length of the longest amplicon.

2. The method of claim 1 wherein the template is genomic DNA encoding p53.

3. The method of claim 1 wherein the template is a cDNA encoding p53.

4. The method of claim 1 wherein the primers amplify at least 2 exons of p53 selected from the group consisting of exons 2-11.

5. The method of claim 1 wherein the primers amplify at least 4 exons of p53 selected from the group consisting of exons 2-11.

6. The method of claim 1 wherein the primers amplify exons 2-11 of p53.

7. The method of claim 4 wherein the primers are selected from those shown in SEQ ID NO: 1-20.

8. The method of claim 5 wherein the primers are selected from those shown in SEQ ID NO: 1-20.

9. The method of claim 6 wherein the primers are shown in SEQ ID NOS: 1-20.

10. The method of claim 9 wherein the primers are present in the following ratios: exon 2 (89.4): exon 3 (26.9): exon 4 (450): exon 5 (245.8): exon 6 (138.3): exon 7 (101.8): exon 8 (193.0): exon 9 (70.8): exon 10 (146.5): exon 11 (177.3).

11-24. (canceled)

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