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MITRA, ROBI D., US

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(54) Titre : AMPLIFICATION PAR REPLIQUE DE RESEAUX D'ACIDES NUCLEIQUES

(54) Title: REPLICA AMPLIFICATION OF NUCLEIC ACID ARRAYS

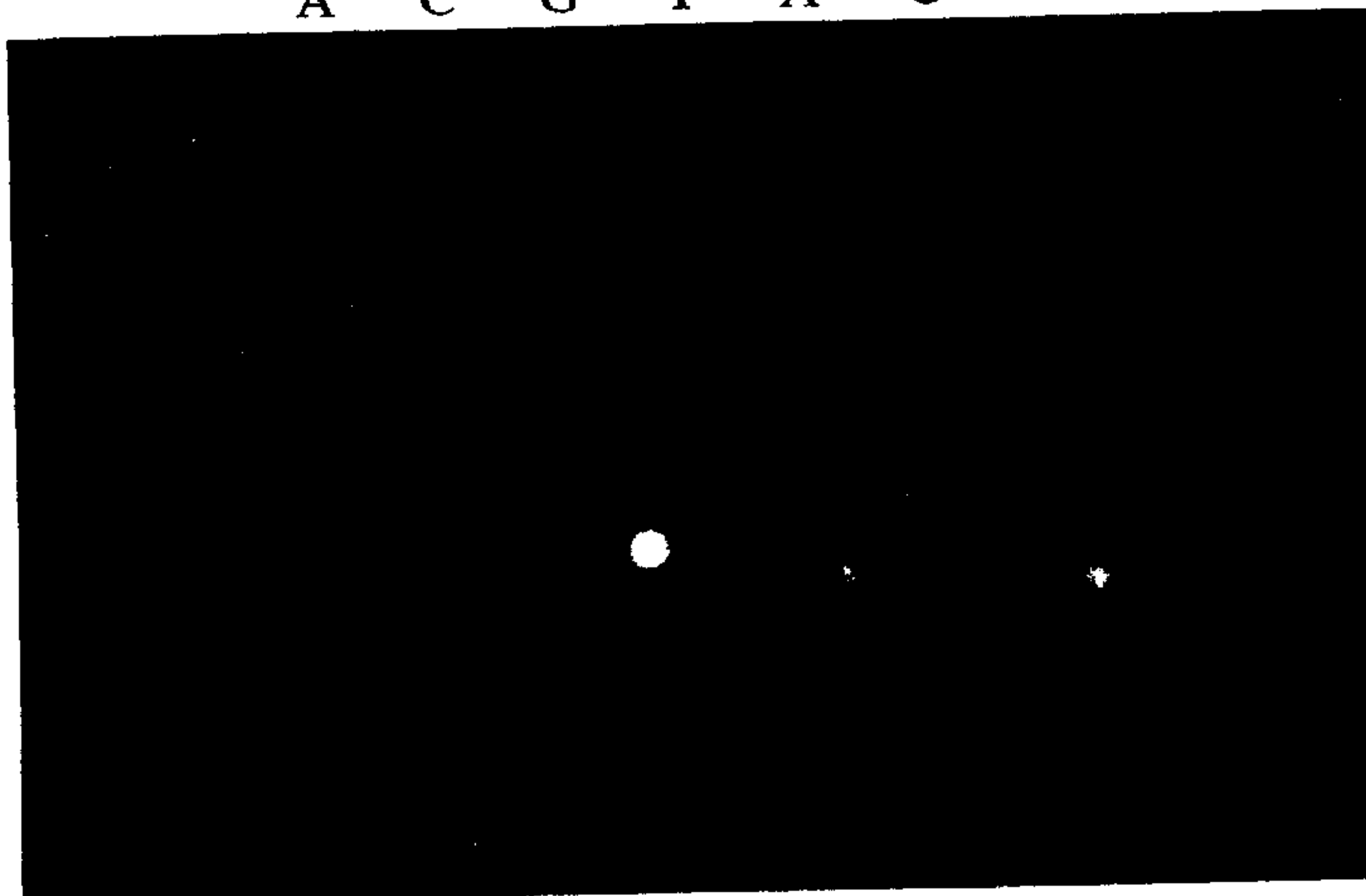
CYCLE
ADDED:

1	2	3	4	5	6
A	C	G	T	A	C

Black = Unlabeled Nucleotide
Red = Fluorescent Nucleotide

1

2



Sample 1:

3'	P'	A	G	C	T	G	T	... 5'
5'	P	T	C					

Sample 2:

3'	P'	G	T	C	T	A	T	... 5'
5'	P	C	A					

(57) Abrégé/Abstract:

Disclosed are improved methods of making and using immobilized arrays of nucleic acids, particularly methods for producing replicas of such arrays. Included are methods for producing high density arrays of nucleic acids and replicas of such arrays, as well as methods for preserving the resolution of arrays through rounds of replication. Also included are methods which take advantage of the availability of replicas of arrays for increased sensitivity in detection of sequences on arrays. Improved methods of sequencing nucleic acids immobilized on arrays utilizing single copies of arrays and methods taking further advantage of the availability of replicas of arrays are disclosed. The improvements lead to higher fidelity and longer read lengths of sequences immobilized on arrays. Methods are also disclosed which improve the efficiency of multiplex PCR using arrays of immobilized nucleic acids.



ABSTRACT

Disclosed are improved methods of making and using immobilized arrays of nucleic acids, particularly methods for producing replicas of such arrays. Included are methods for producing high density arrays of nucleic acids and replicas of such arrays, as well as methods for preserving the resolution of arrays through rounds of replication. Also included are methods which take advantage of the availability of replicas of arrays for increased sensitivity in detection of sequences on arrays. Improved methods of sequencing nucleic acids immobilized on arrays utilizing single copies of arrays and methods taking further advantage of the availability of replicas of arrays are disclosed. The improvements lead to higher fidelity and longer read lengths of sequences immobilized on arrays. Methods are also disclosed which improve the efficiency of multiplex PCR using arrays of immobilized nucleic acids.

SEQUENCE LISTING

<110> President and Fellows of Harvard College

<120> Replica Amplification of Nucleic Acid Arrays

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<140> PCT/US00/06390

<141> 2000-03-10

<150> US 09/267,496

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<170> PatentIn Ver. 2.1

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moiety at the 5' end

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75066-32E

91

CLAIMS:

1. A method for determining the nucleotide sequence of the features of an immobilized nucleic acid array, said method comprising the steps of:

5 a) ligating a first double-stranded nucleic acid probe to one end of a nucleic acid of a feature of said array, said first double stranded nucleic acid probe having a restriction endonuclease recognition site for a restriction endonuclease whose cleavage site is separate
10 from its recognition site and which generates a protruding strand upon cleavage;

b) identifying one or more nucleotides at the end of said polynucleotide by the identity of the first double stranded nucleic acid probe ligated thereto or by extending
15 a strand of the polynucleotide or probe;

c) amplifying the features of said array using a primer complementary to said first double stranded nucleic acid probe, such that only molecules which have been successfully ligated with said first double stranded nucleic
20 acid probe are amplified to yield an amplified array;

d) contacting said amplified array with support such that at least a subset of nucleic acid molecules produced by said amplifying are transferred to said support;

e) covalently attaching said subset of nucleic
25 acid molecules transferred in step (d) to said support to form a replica of said amplified array;

f) cleaving the nucleic acid features of the array with a nuclease recognizing said nuclease recognition site of said probe such that the nucleic acid of the features is
30 shortened by one or more nucleotides; and

75066-32E

92

g) repeating steps (a)-(f) until the nucleotide sequences of the features of said array are determined.

2. The method of claim 1 wherein said nucleic acid probe comprises four components, each component being
5 capable of indicating the presence of a different nucleotide in said protruding strand upon ligation.

3. The method of claim 2 wherein each of said components of said probe is labeled with a different fluorescent dye and the different fluorescent dyes are
10 spectrally resolvable.

4. The method of claim 1 wherein after said step (e) and before said step (f), the features of said array are amplified.

5. The method of claim 1 wherein amplification is
15 performed by PCR.

6. The method of claim 1 wherein:

i) after one or more cycles using said first double stranded nucleic acid probe in step (a), a distinct nucleic acid probe is used, in place of said first double
20 stranded nucleic probe in step (a), said distinct nucleic acid probe comprising a restriction endonuclease recognition site for a restriction endonuclease whose cleavage site is separated from its recognition site, said distinct nucleic acid probe also comprising sequences such that a primer
25 complementary to said distinct nucleic acid probe will not hybridize with said first double stranded nucleic acid probe; and

ii) a primer complementary to said distinct nucleic acid probe is used in place of said primer
30 complementary to said first double stranded nucleic acid

75066-32E

93

probe in step (c), so that selective amplification of those features which successfully completed the previous cycle of restriction and ligation occurs.

7. The method of claim 6 wherein a new distinct nucleic acid probe is used after each cycle of restriction and ligation, said new distinct nucleic acid probe comprising a sequence such that a primer complementary to that sequence will not hybridize to any probe used in previous cycles.

10 8. A method of determining the nucleotide sequence of the features of an array of immobilized nucleic acids comprising the steps of:

15 a) adding a mixture comprising an oligonucleotide primer and a template-dependent polymerase to an array of immobilized nucleic acid features under conditions permitting hybridization of the primer to the immobilized nucleic acids;

20 b) adding a single, fluorescently labeled deoxynucleoside triphosphate to the mixture under conditions which permit incorporation of the labeled deoxynucleotide onto the 3' end of the primer if it is complementary to the next adjacent base in the sequence to be determined;

c) detecting incorporated label by monitoring fluorescence;

25 d) repeating steps (b)-(c) with each of the remaining three labeled deoxynucleoside triphosphates in turn; and

e) repeating steps (b)-(d) until the nucleotide sequence is determined.

75066-32E

94

9. The method of claim 8 wherein the primer, buffer and polymerase are cast into a polyacrylamide gel bearing the array of immobilized nucleic acids.

10. The method of claim 9 wherein said polyacrylamide
5 gel is macroporous.

11. The method of claim 10 wherein said polyacrylamide gel comprises up to about 25% PEG, about 3% to about 12% total acrylamide and about 1% to about 30% cross linker.

12. The method of claim 11 wherein the percentage of
10 said PEG is about 2.5%.

13. The method of claim 10 wherein said polyacrylamide gel comprises DATD.

14. The method of claim 8 wherein single-stranded binding protein is present during step (b).

15 15. The method of claim 8 wherein said single fluorescently labeled deoxynucleotide further comprises a mixture of the single deoxynucleoside triphosphate in labeled and unlabeled forms.

16. The method of claim 8 wherein after step (d) and
20 before step (e) the additional step of photobleaching said array is performed.

17. The method of claim 8 wherein said fluorescently labeled deoxynucleoside triphosphates are labeled with a cleavable linkage to the fluorophore.

25 18. The method of claim 17 wherein after step (d) and before step (e) the additional step of cleaving said linkage to the fluorophore is performed.

75066-32E

95

19. The method of claim 18 wherein said step of cleaving comprises contacting said linkage with a reducing agent.

20. The method of claim 19 wherein said reducing agent
5 is dithiothreitol.

21. The method of claim 8 wherein said oligonucleotide primer comprises sequences permitting formation of a hairpin loop.

22. The method of claim 8 wherein after a
10 predetermined number of cycles of steps (b)-(d), a defined regimen of deoxynucleotide and chain-terminating deoxynucleotide analog addition is performed, such that out-of-phase molecules are blocked from further extension cycles, said regimen followed by continued cycles of steps
15 (b)-(d) until said nucleotide sequence is determined.

23. A method of determining the nucleotide sequence of the features of an array of immobilized nucleic acids comprising the steps of:

a) adding a mixture comprising an oligonucleotide
20 primer and a template-dependent polymerase to an array of immobilized nucleic acid features under conditions permitting hybridization of the primer to the immobilized nucleic acids;

b) adding a first mixture of three unlabeled
25 deoxynucleoside triphosphates under conditions which permit incorporation of deoxynucleotides to the end of the primer if they are complementary to the next adjacent base in the sequence to be determined;

c) adding a second mixture of three unlabeled
30 deoxynucleoside triphosphates, said second mixture

75066-32E

96

comprising the deoxynucleoside triphosphate not included in the mixture of step (b), under conditions which permit incorporation of deoxynucleotides to the end of the primer if they are complementary to the next adjacent base in the
5 sequence to be determined;

d) repeating steps (b)-(c) for a predetermined number of cycles;

e) adding a single, fluorescently labeled deoxynucleoside triphosphate to the mixture under conditions
10 which permit incorporation of the labeled deoxynucleotide onto the 3' terminus of the primer if it is complementary to the next adjacent base in the sequence to be determined;

f) detecting incorporated label by monitoring fluorescence;

15 g) repeating steps (e)-(f), with each of the remaining three labeled deoxynucleoside triphosphates in turn; and

h) repeating steps (e)-(g) until the nucleotide sequence is determined.

20 24. The method of claim 23 wherein for said first or second mixtures of three unlabeled deoxynucleoside triphosphates, a mixture which comprises deoxyguanosine triphosphate further comprises deoxyadenosine triphosphate.

25 25. The method of claim 23 wherein the primer and polymerase are cast into a polyacrylamide gel bearing the array of immobilized nucleic acids.

26. The method of claim 23 wherein said single fluorescently labeled deoxynucleotide further comprises a

75066-32E

97

mixture of the single deoxynucleoside triphosphate in labeled and unlabeled forms.

27. The method of claim 23 wherein after step (g) and before step (h) the additional step of photobleaching said
5 array is performed.

28. The method of claim 23 wherein said fluorescently labeled deoxynucleoside triphosphates are labeled with a cleavable linkage to the fluorophore.

29. The method of claim 28 wherein after step (g) and
10 before step (h) the additional step of cleaving said linkage to the fluorophore is performed.

30. The method of claim 23 wherein said oligonucleotide primer comprises sequences permitting formation of a hairpin loop.

15 31. The method of claim 23 wherein after a predetermined number of cycles of steps (e)-(g), a defined regimen of deoxynucleotide and chain-terminating deoxynucleotide analog addition is performed, such that out-of-phase molecules are blocked from further extension
20 cycles, said regimen followed by continued cycles of steps (e)-(g) until said nucleotide sequence of the features of the array is determined.

32. A method of determining the nucleotide sequence of the features of a microarray of nucleic acid molecules, said
25 method comprising the following steps:

a) creating a microarray of nucleic acid features in a linear arrangement within and along one side of a polyacrylamide gel, said gel further comprising one or more oligonucleotide primers, and a template-dependent
30 polymerizing activity;

75066-32E

98

b) amplifying the microarray of step (a);

c) adding a mixture of deoxynucleoside triphosphates, said mixture comprising each of the four deoxynucleoside triphosphates dATP, dGTP, dCTP and dTTP, said mixture further comprising chain-terminating analogs of each of the deoxynucleoside triphosphates dATP, dGTP, dCTP and dTTP, and said chain-terminating analogs each distinguishably labeled with a spectrally distinguishable fluorescent moiety;

d) incubating said mixture with said microarray under conditions permitting extension of said one or more oligonucleotide primers;

e) electrophoretically separating the products of said extension within said polyacrylamide gel; and

f) determining the nucleotide sequence of the features of said microarray by detecting the fluorescence of the extended, terminated and separated reaction products within the gel.

33. The method of claim 32 wherein said amplifying is performed by PCR.

34. The method of claim 32 wherein said amplifying is performed by an isothermal method.

35. The method of claim 32 wherein said microarray of nucleic acid features in a linear arrangement is derived as a replica of features arranged on a chromosome.

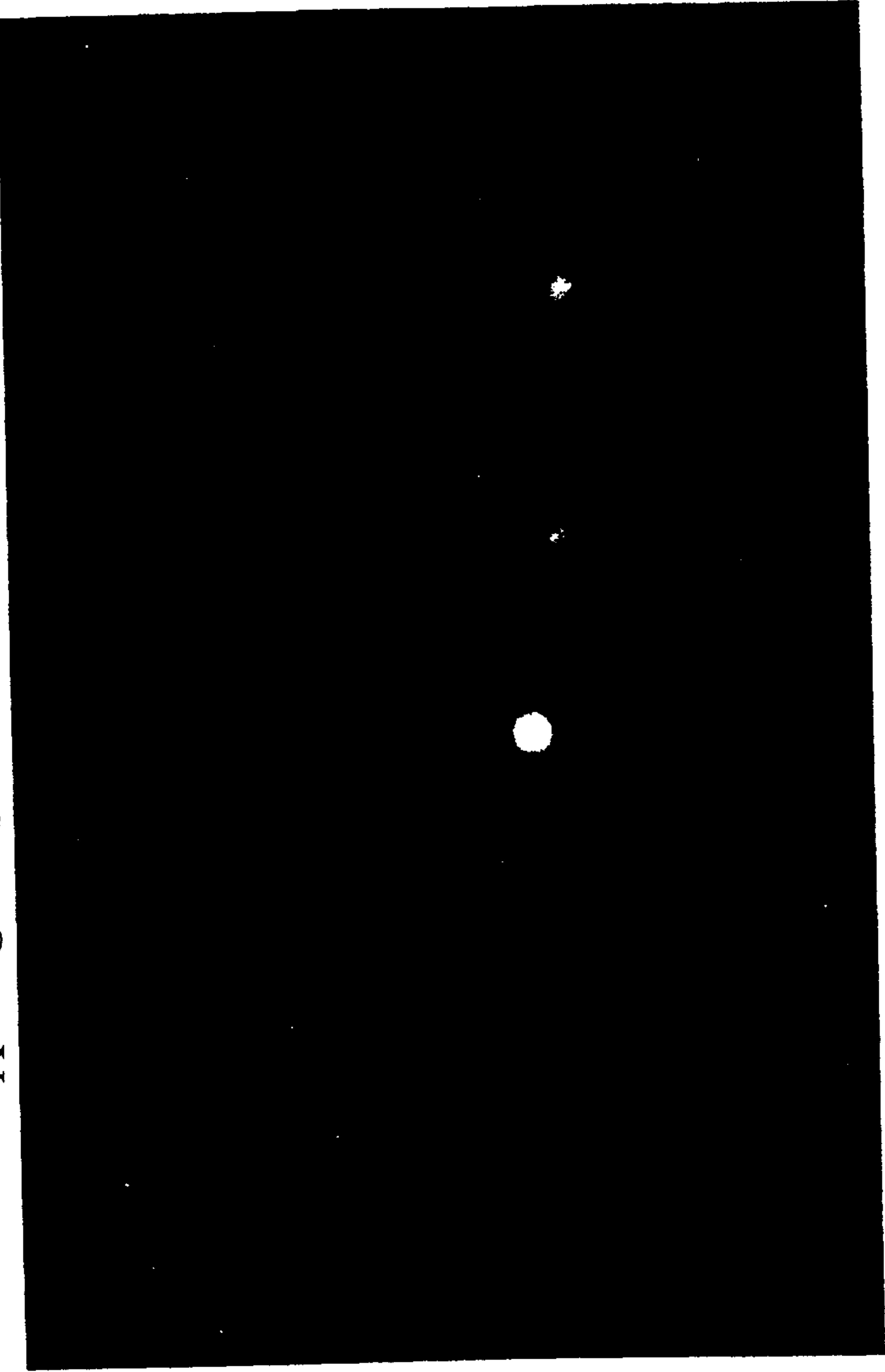
36. The method of claim 32 wherein said microarray of nucleic acid features in a linear arrangement is derived as a replica of one linear subset of features on a separate, non-linear microarray of nucleic acid features.

CYCLE
ADDED:

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A C G T A C

Black = Unlabeled Nucleotide
Red = Fluorescent Nucleotide

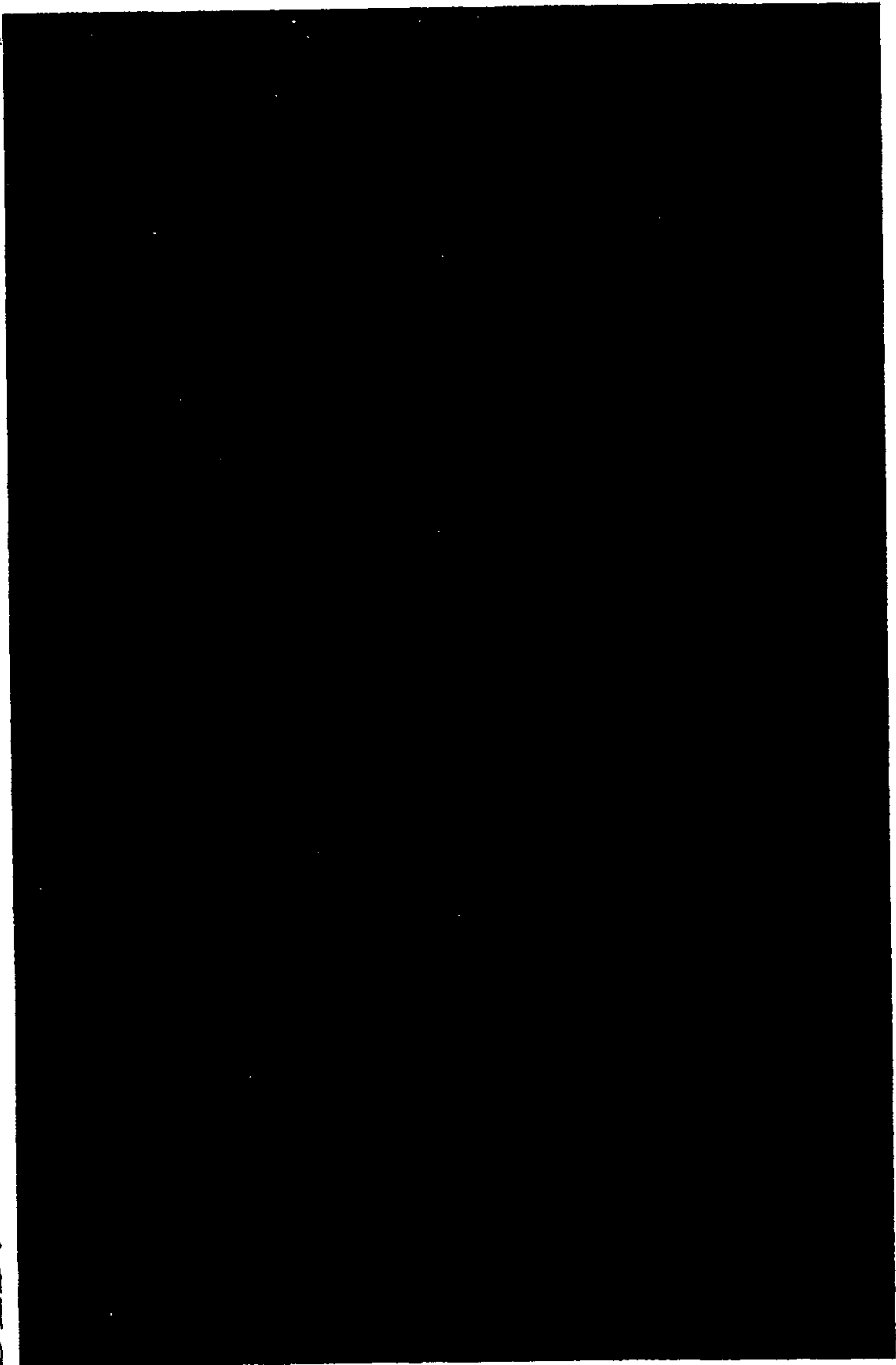


Sample 1:	3'	P'	A	G	C	T	G	T	...5'
	5'	P	T	C					
Sample 2:	3'	P'	G	T	C	T	A	T	...5'
	5'	P	C	A					

FIG. 1

CYCLE
ADDED:

1	2	3	4	5	6	7	8
A	C	G	T	A	C	G	T



1

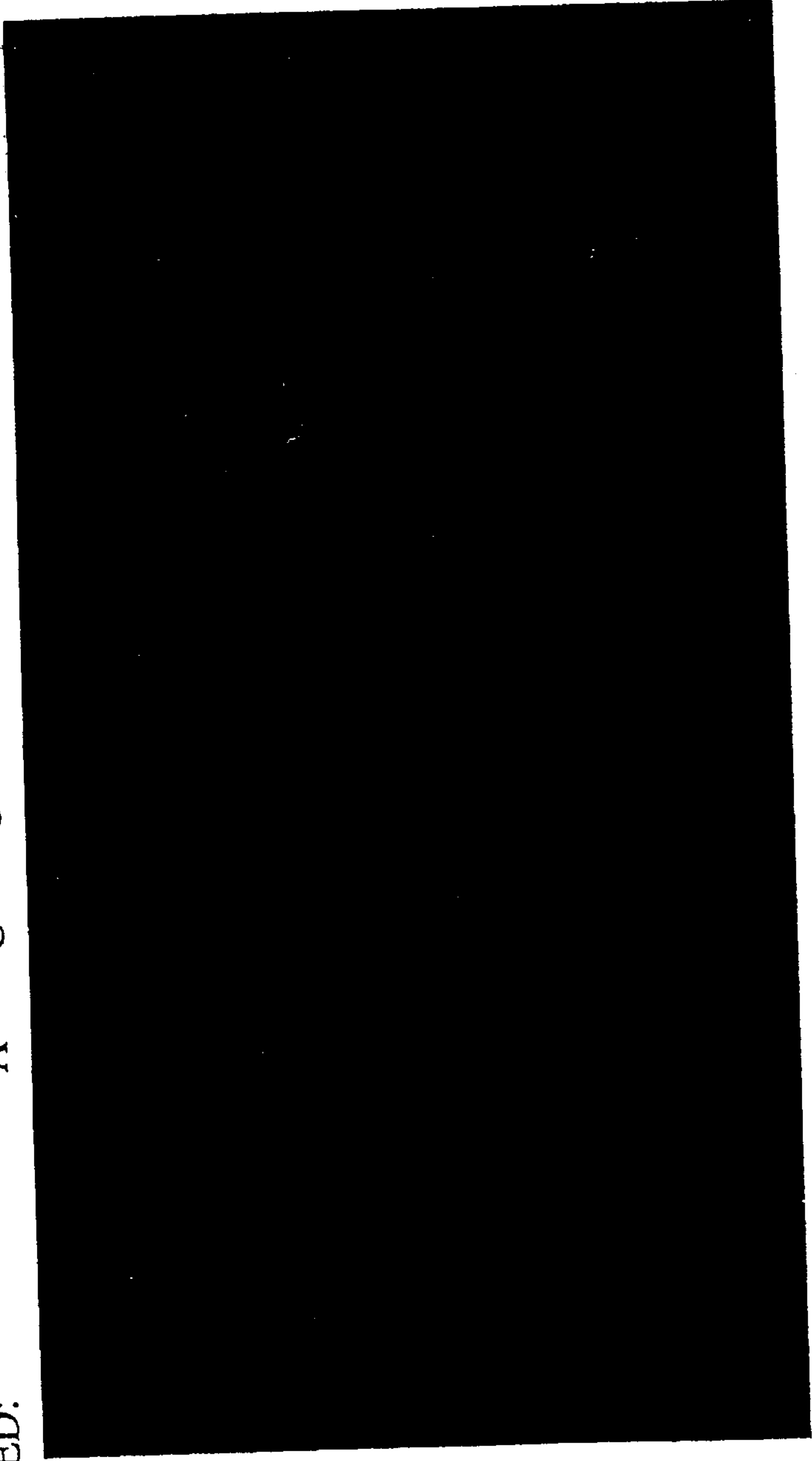
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Sample 1:	3'	P'	A	G	C	T	G	T	...5'
	5'	P	T	C	G				
Sample 2:	3'	P'	G	T	C	T	A	T	...5'
	5'	P	C	A	G				

FIG. 2

CYCLE
ADDED:

1	2	3	4	5	6	7	8	9	10
A	C	G	T	A	C	G	T	A	C



1

2

Sample 1:

3'	P'	A	G	C	T	G	T	...	5'
5'	P	T	C	G	A	C			

Sample 2:

3'	P'	G	T	C	T	A	T	...	5'
5'	P	C	A	G	A				

FIG. 3

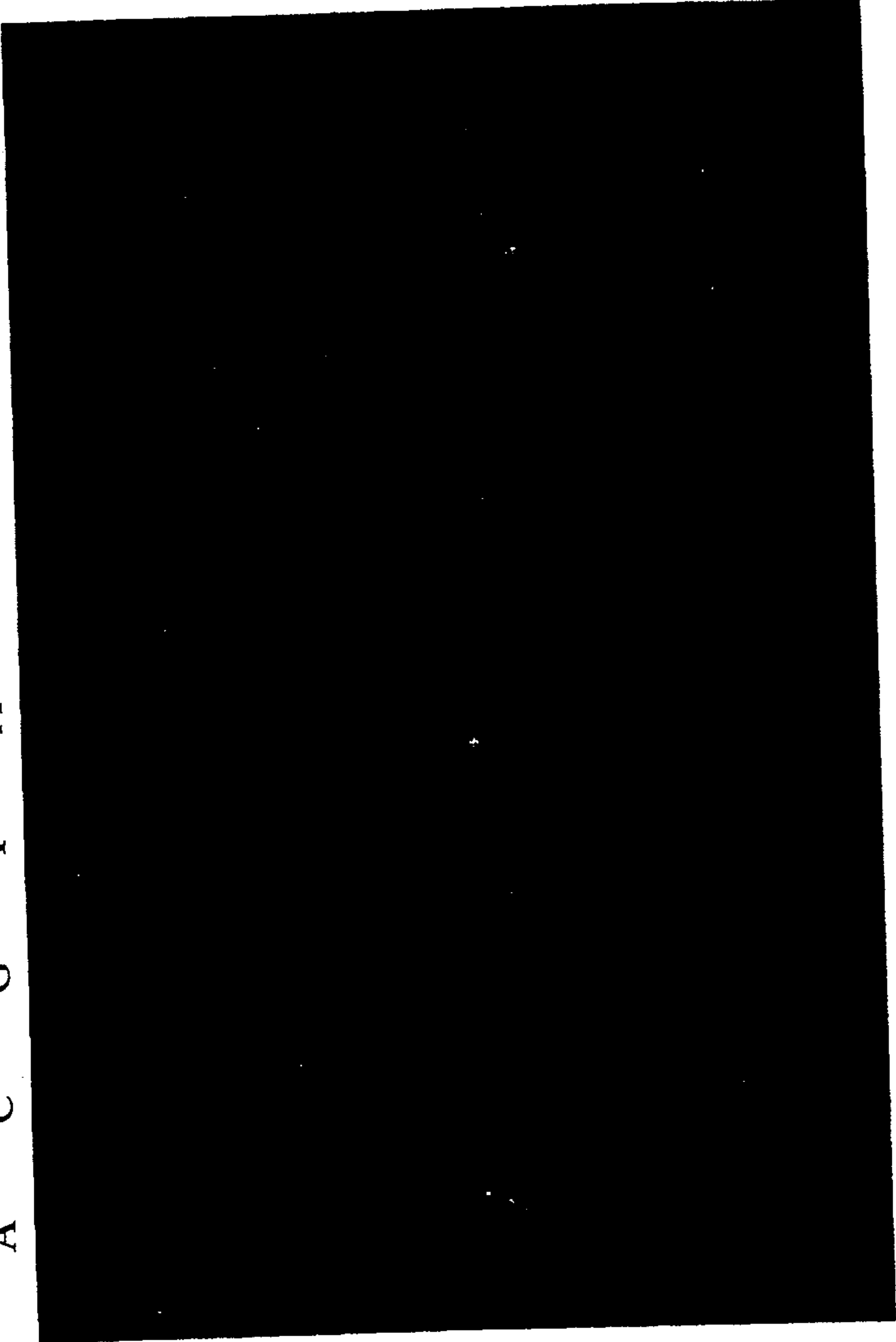
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PCT/US00/06390

4/10

CYCLE
ADDED:

1	2	3	4	5	6	7	8	9	10	11	12
A	C	G	T	A	C	G	T	A	C	G	T



1

2

Sample 1:	3'	P'	A	G	C	T	G	T	...	5'
	5'	P	T	C	G	A	C			
Sample 2:	3'	P'	G	T	C	T	A	T	...	5'
	5'	P	C	A	G	A	T			

FIG. 4

5/10

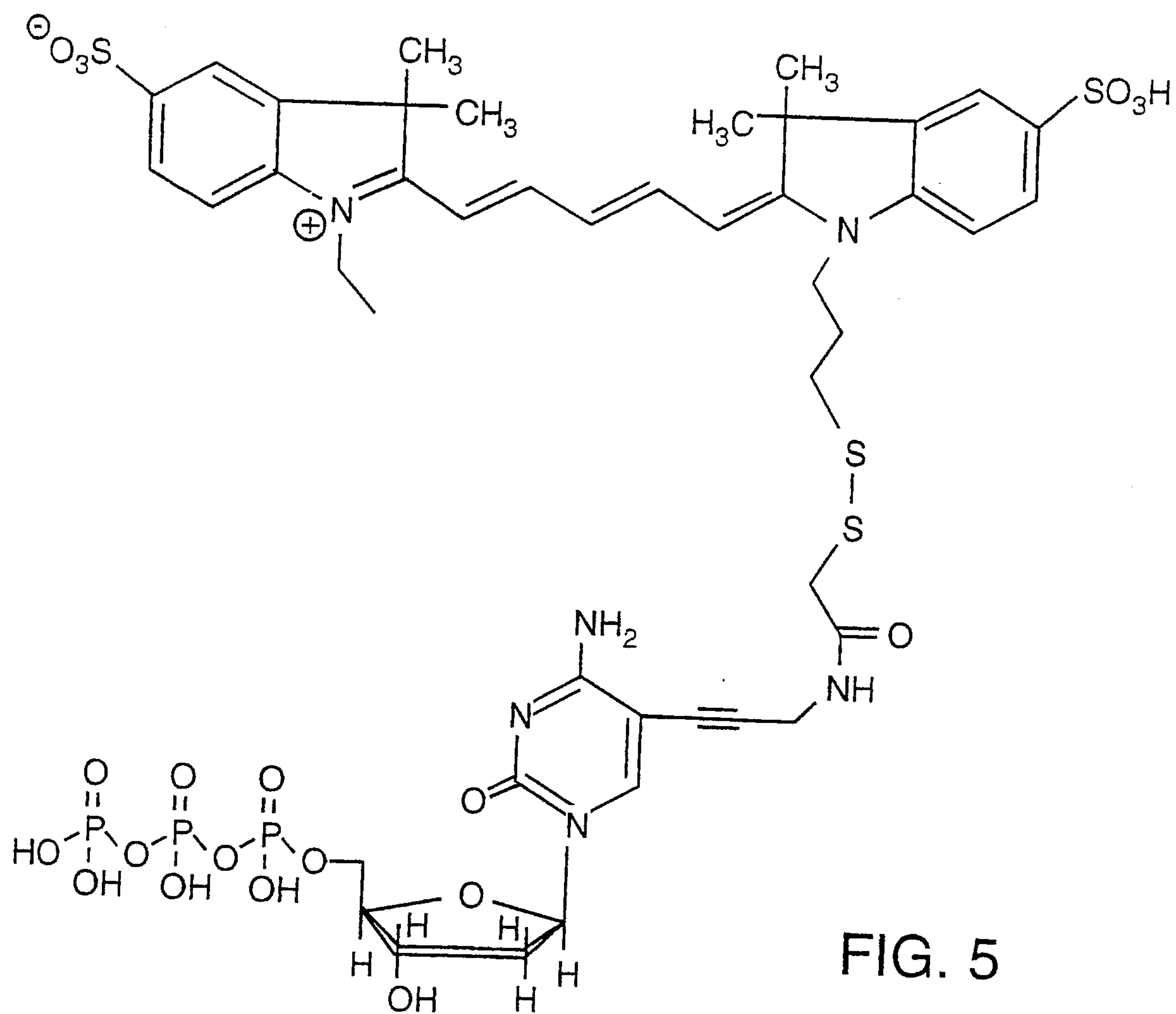
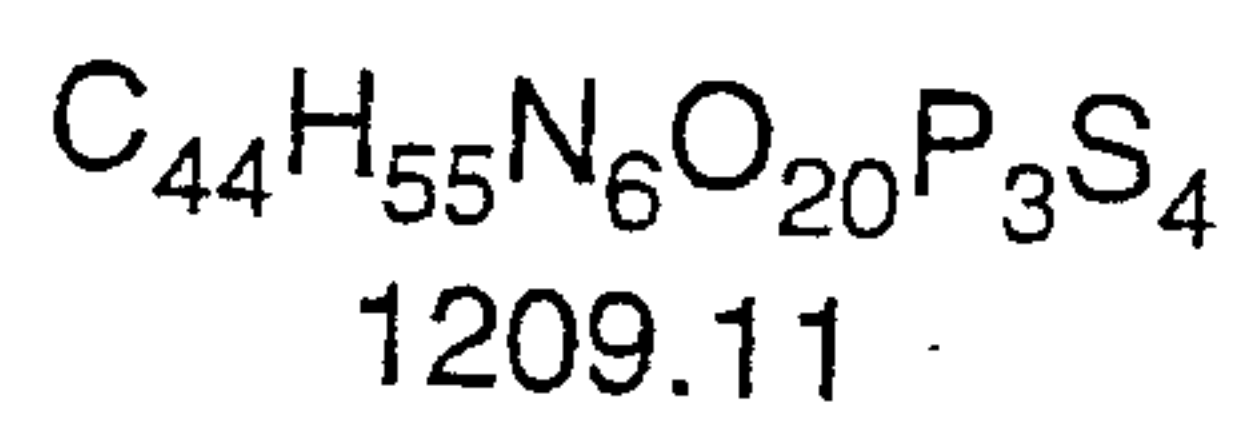
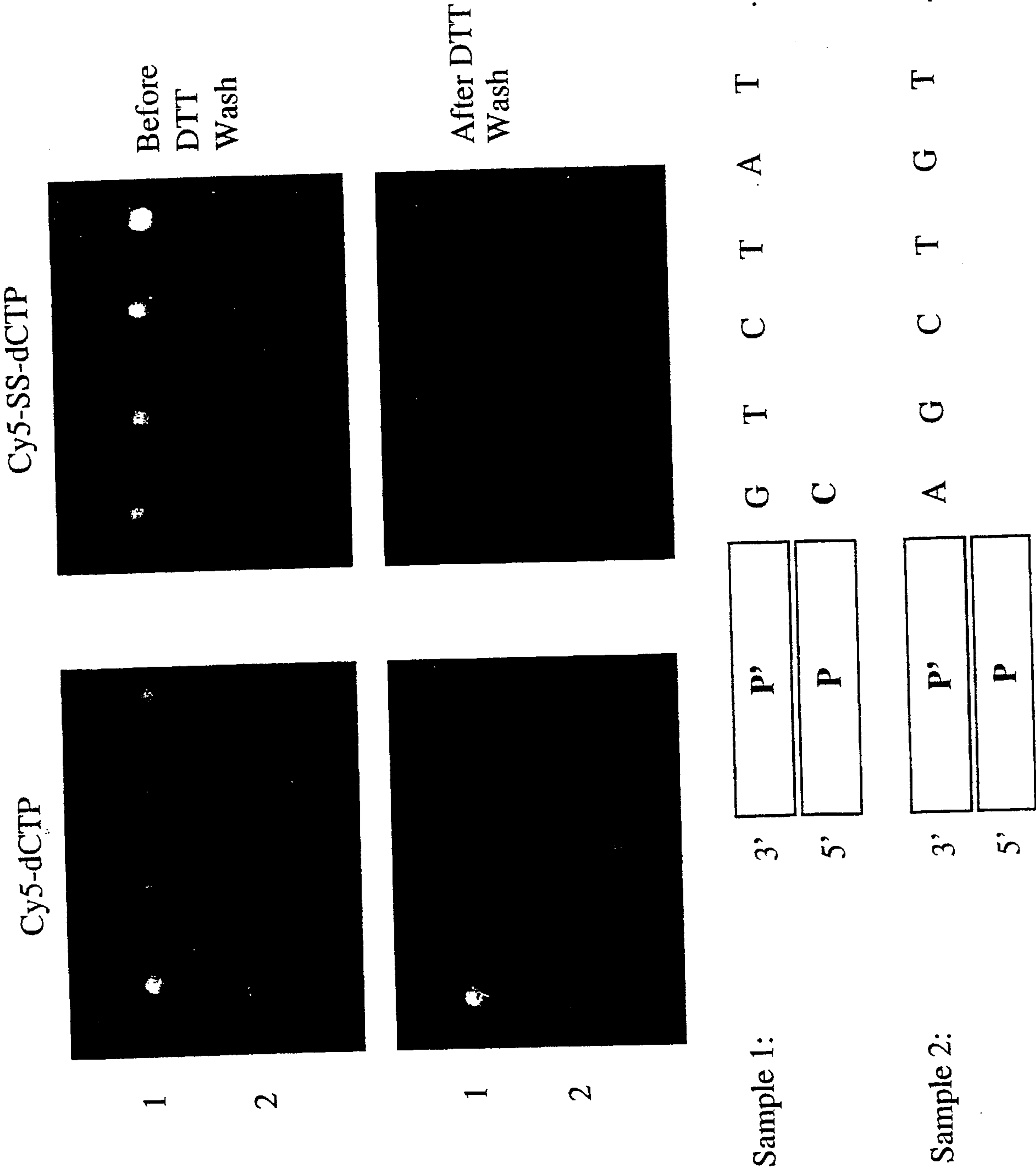


FIG. 5



6/10



7/10

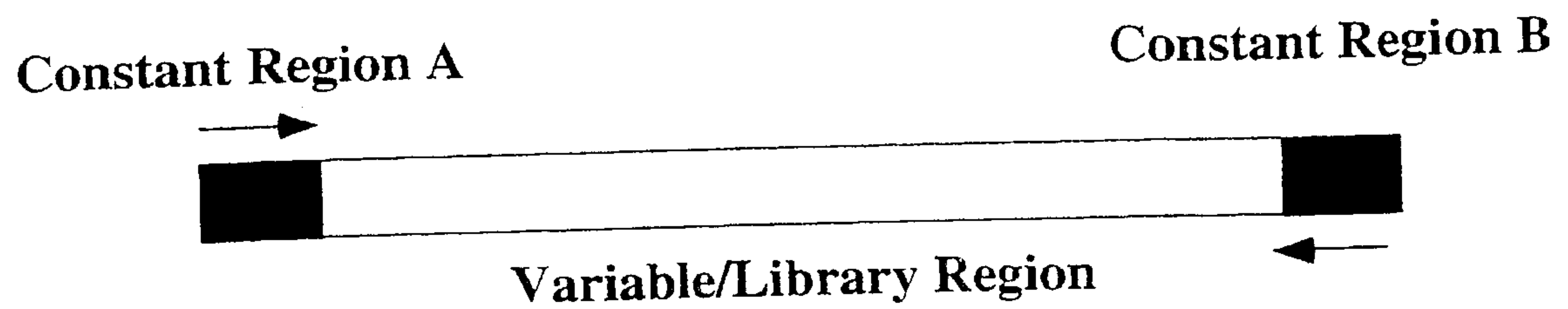


FIG. 7

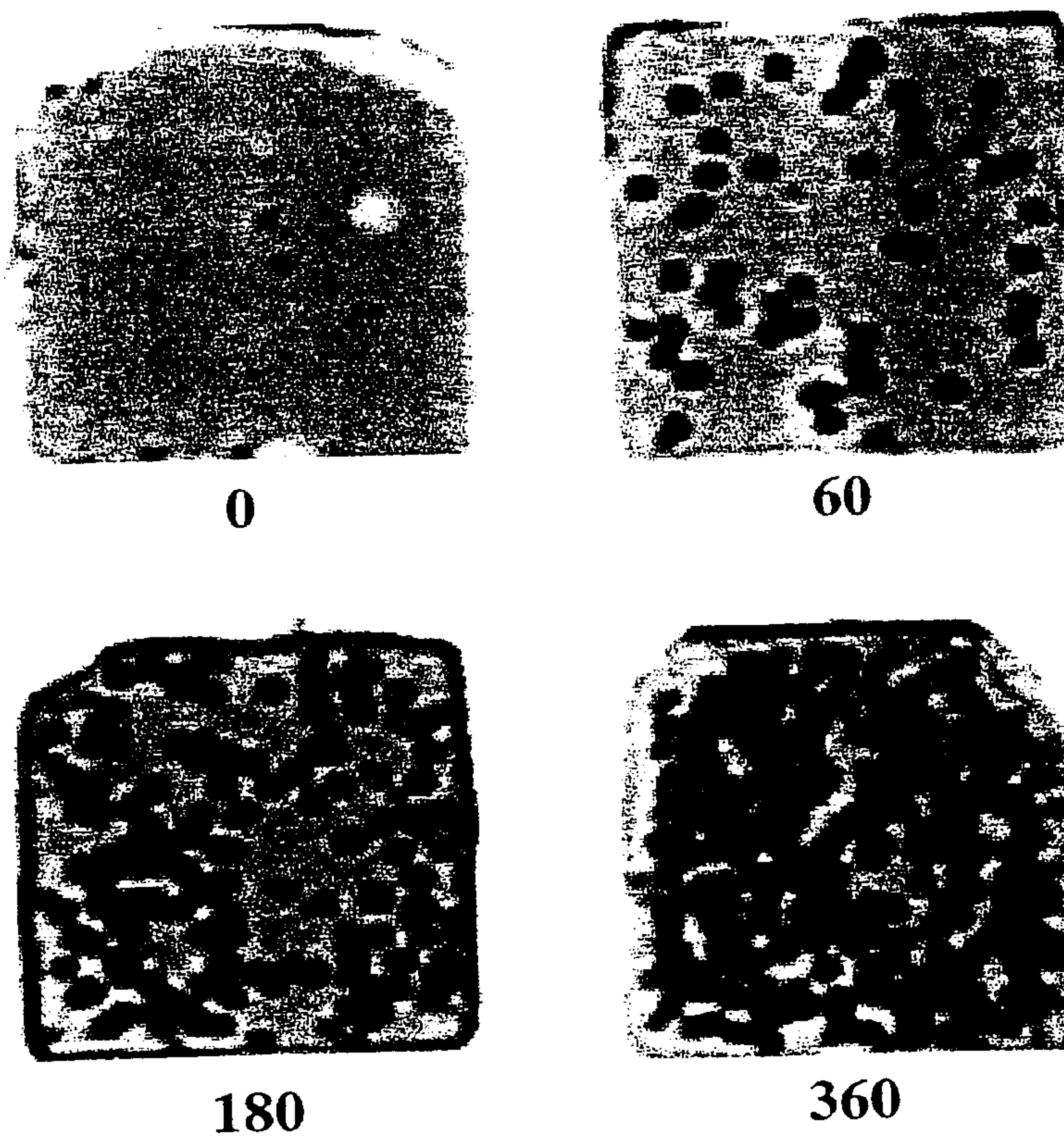


FIG. 8A

8/10

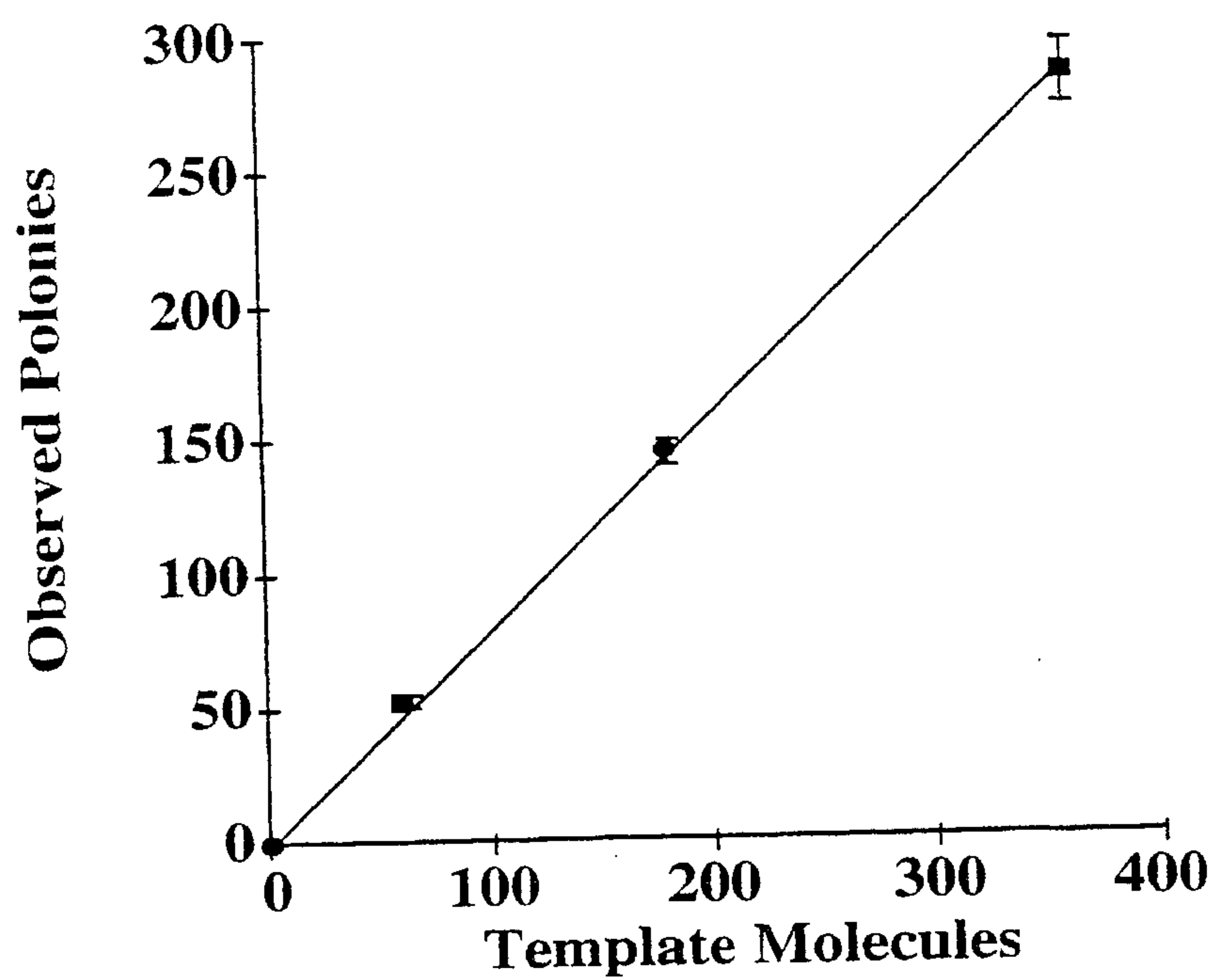


FIG. 8B

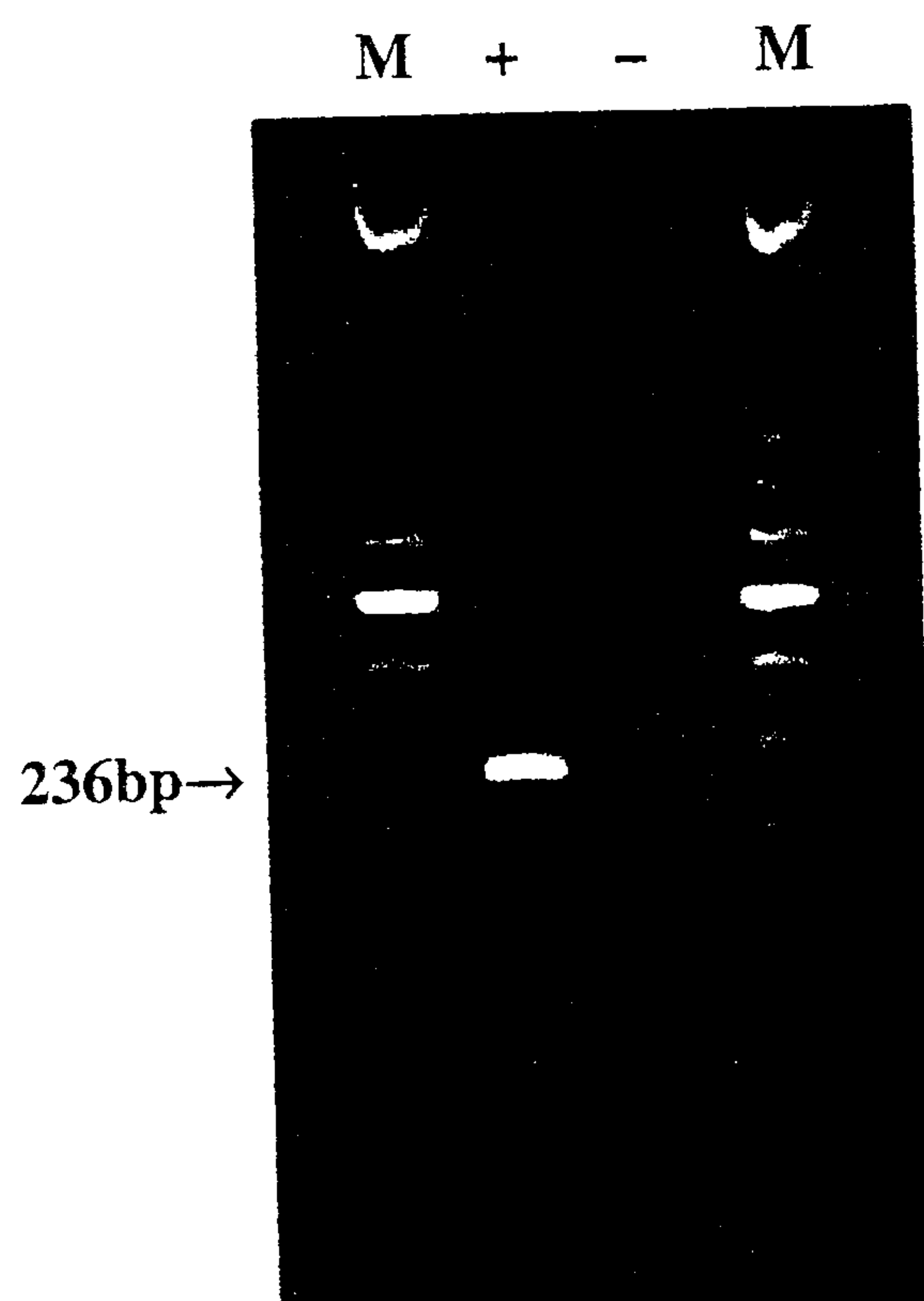


FIG. 8C

WO 00/53812

9/10

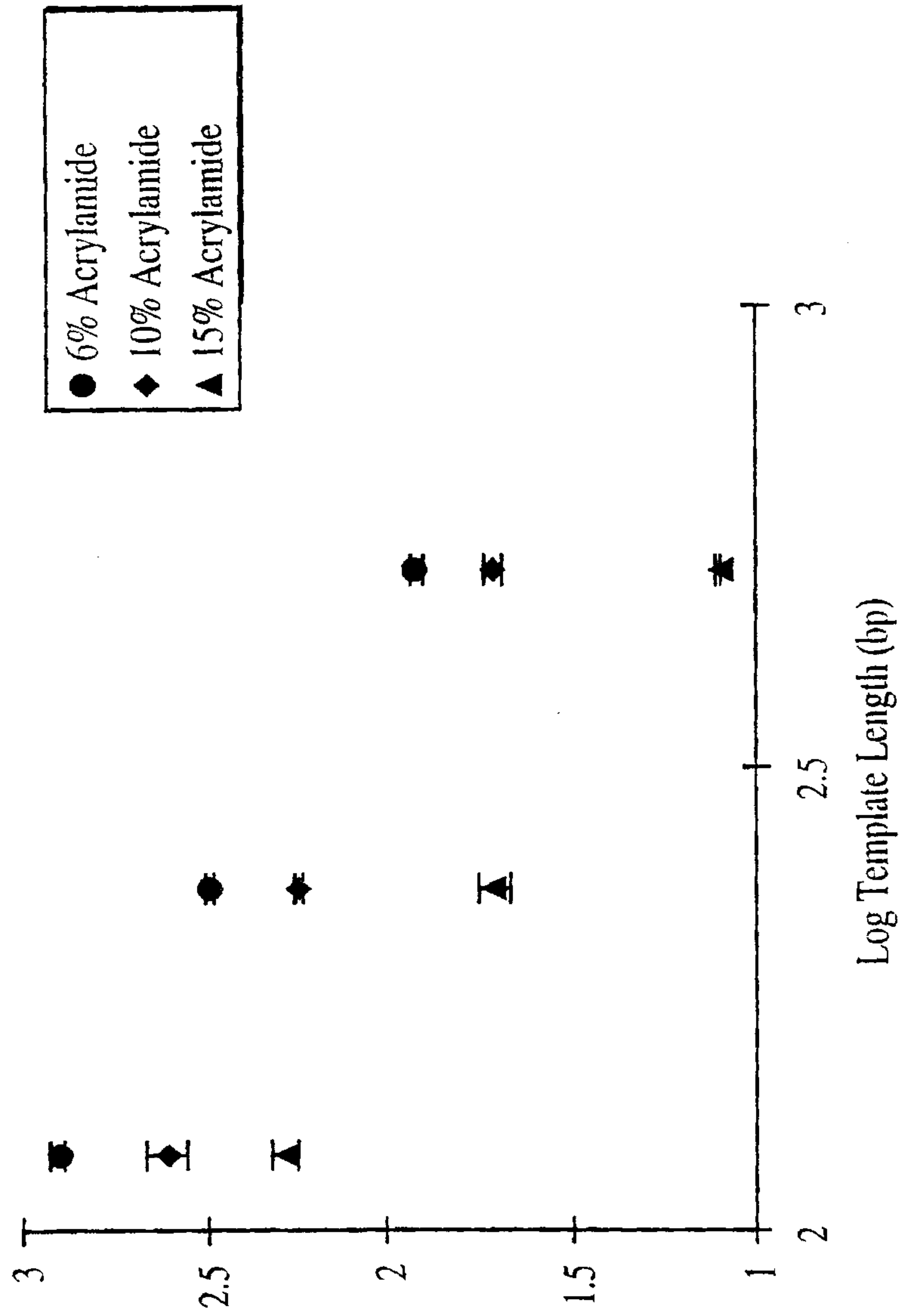


FIG. 9A

10/10

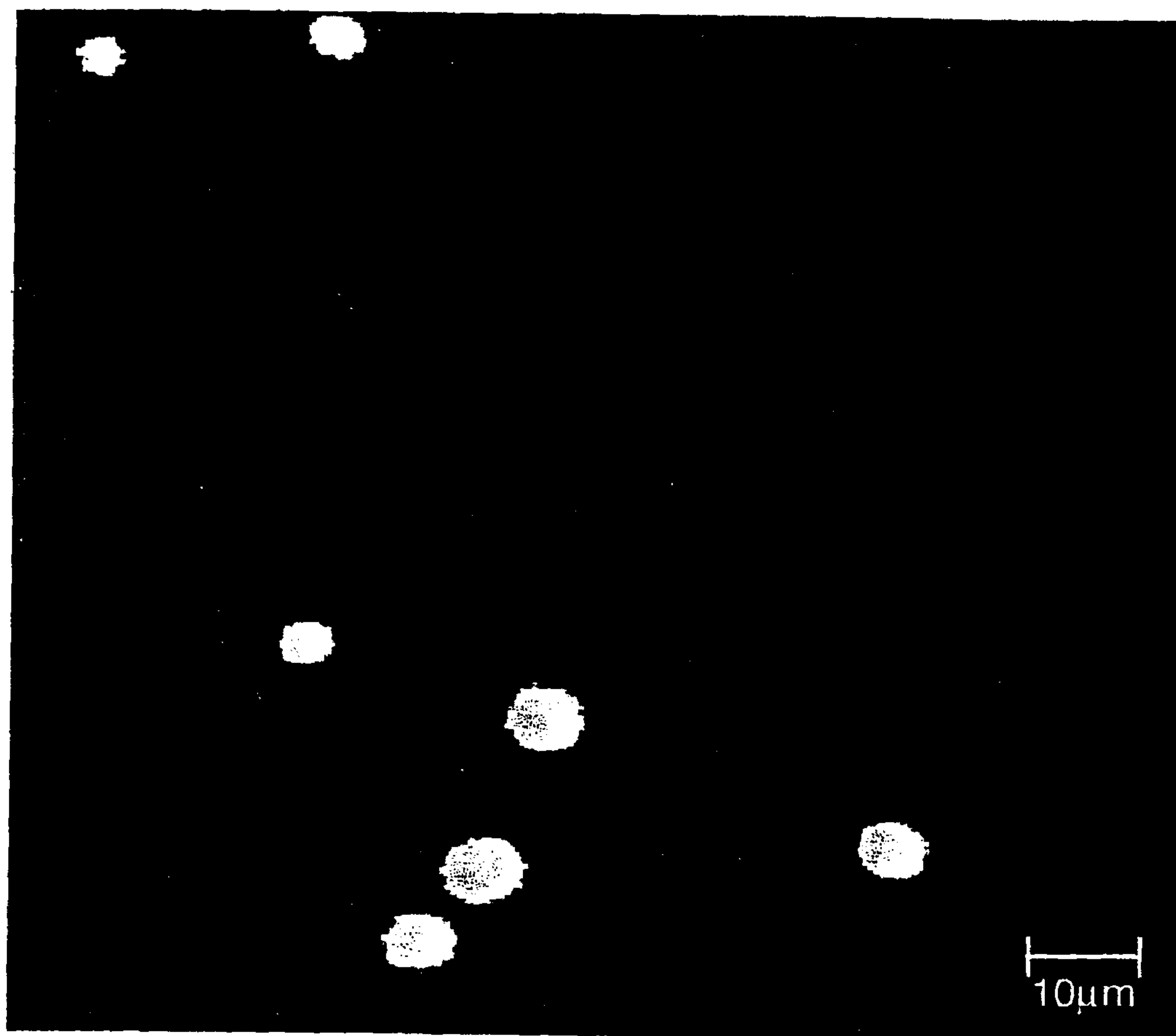


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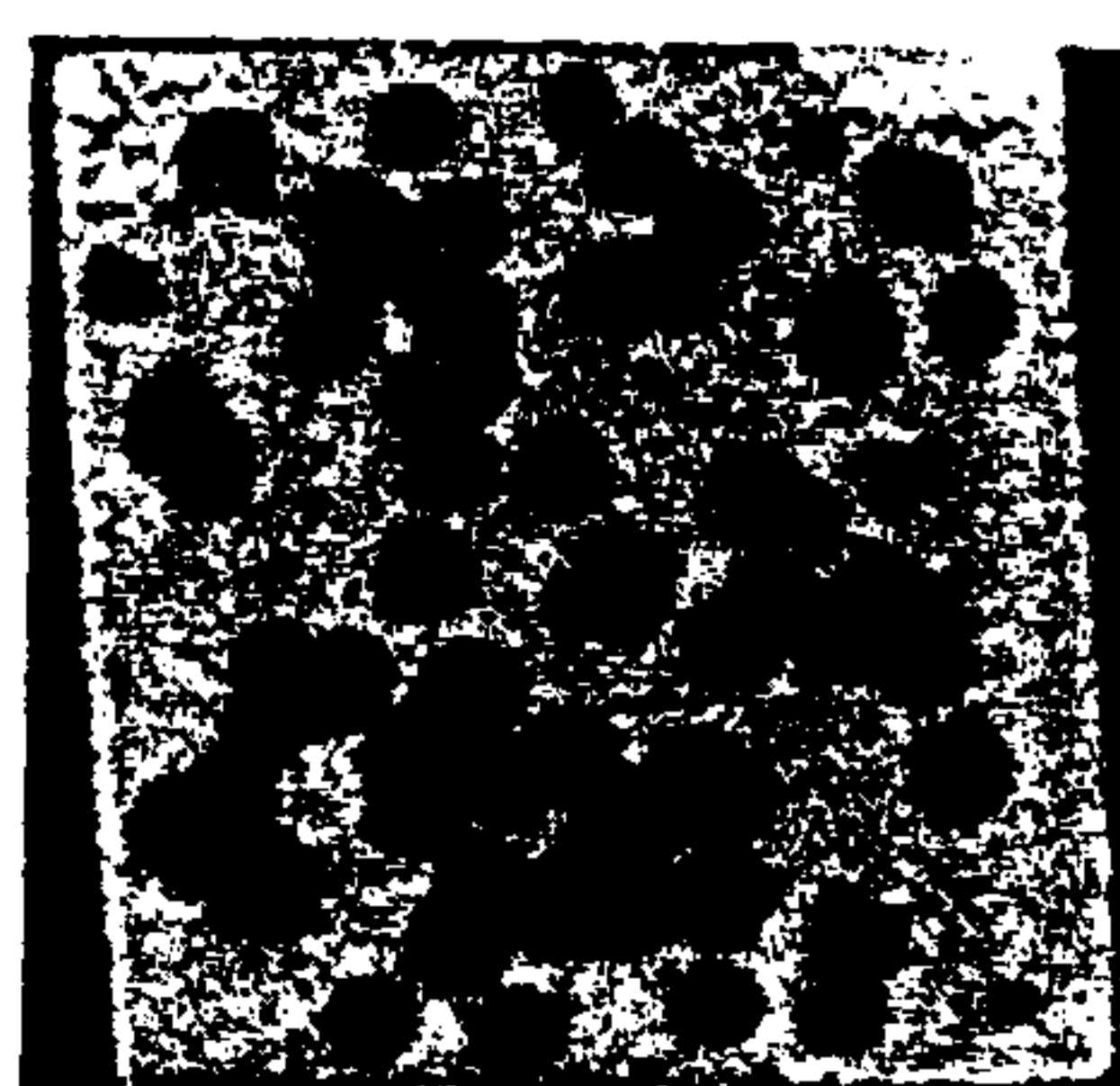


FIG. 10A



FIG. 10B

CYCLE
ADDED:

1

2

3

4

5

6

A

C

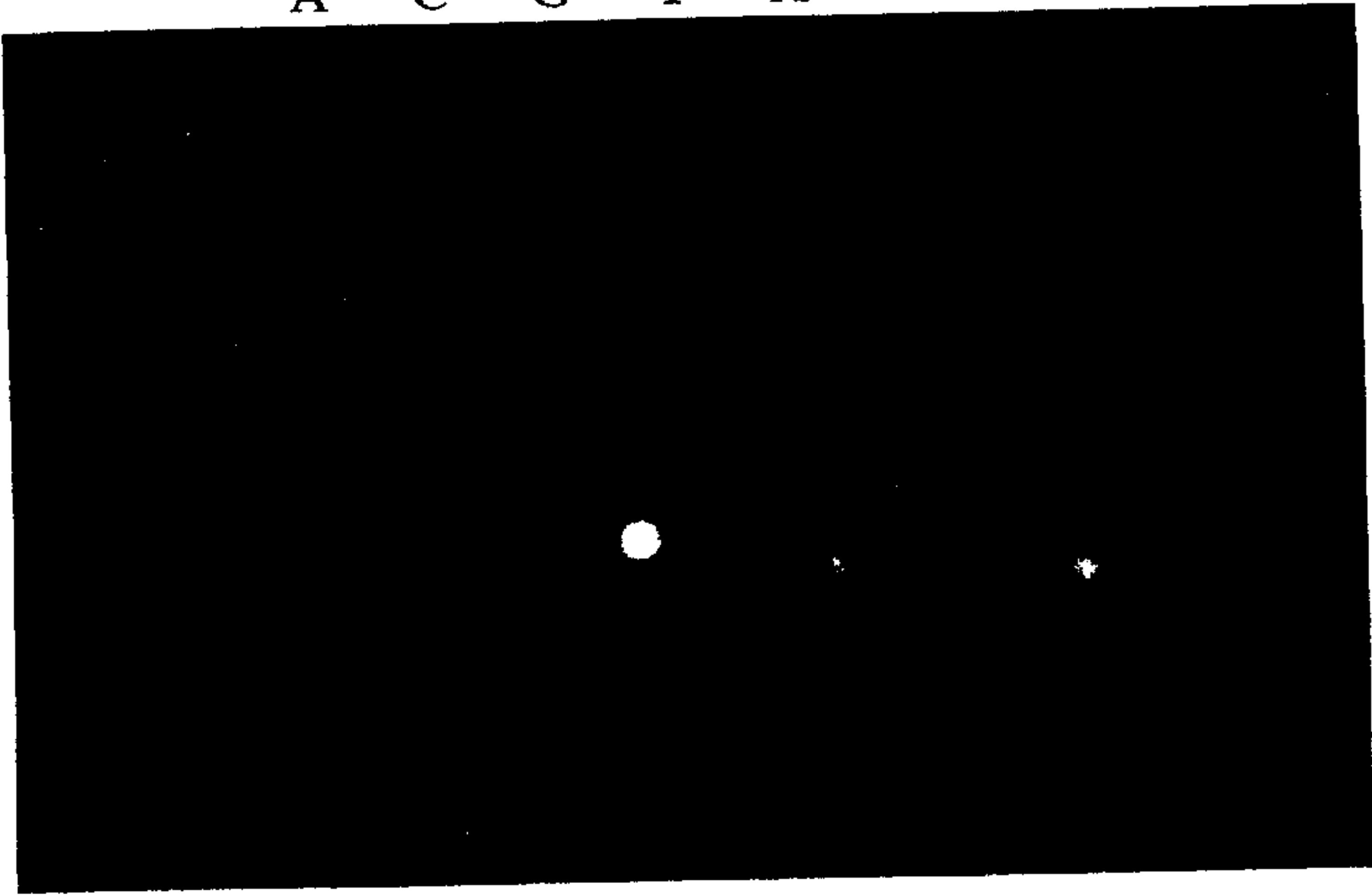
G

T

A

C

Black = Unlabeled Nucleotide
Red = Fluorescent Nucleotide



Sample 1:

3'

5'

P'

P

A

G

C

T

G

T

...

5'

T

C

Sample 2:

3'

5'

P'

P

G

T

C

T

A

T

...

5'

C

A