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(74) Agent: BCF LLP

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(54) Title: METHOD TO SCREEN FOR A MUTANT WITHIN A POPULATION OF ORGANISMS BY APPLYING A POOLING AND SPLITTING APPROACH

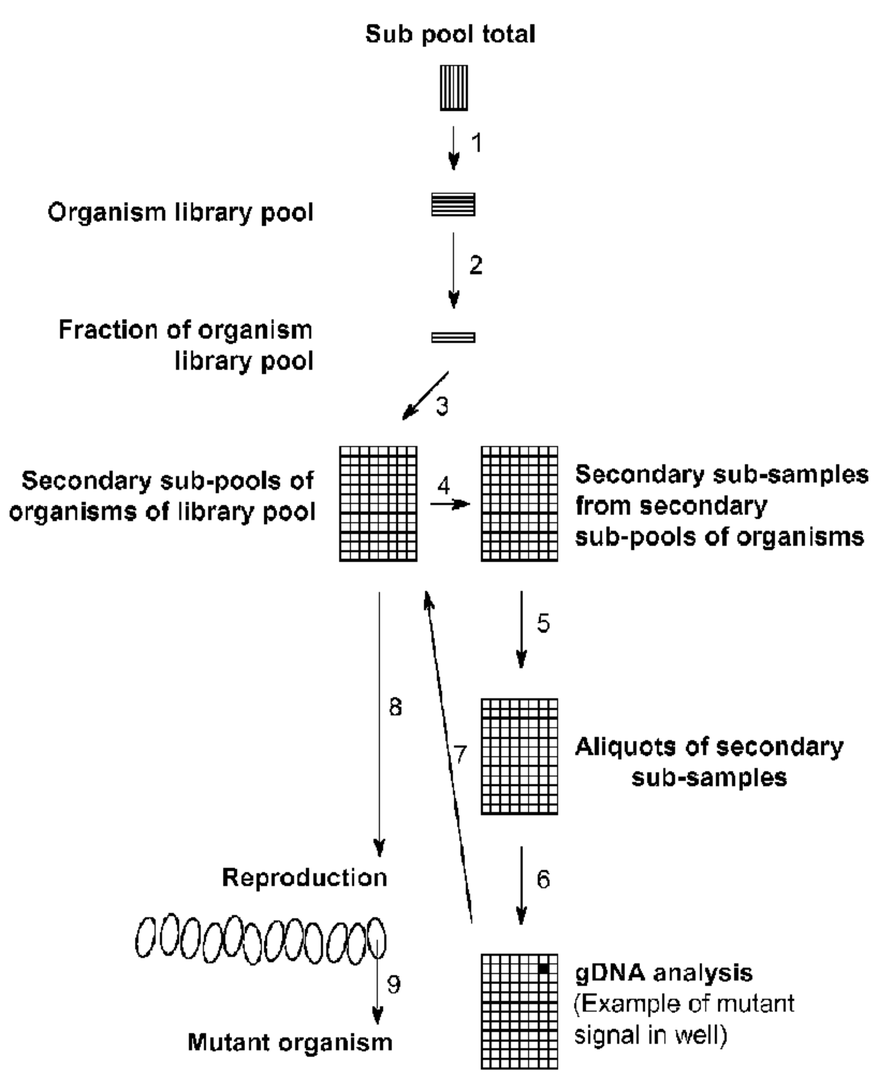

FIG. 1C

(57) Abrégé/Abstract:
In traditional plant breeding approaches, chemical mutagenesis may be utilized to introduce nucleotide substitutions at random in the genome of a plant, i.e. without possibilities to control the sites of nucleotide changes. Because of genome complexities, the statistical probability is extremely little when it comes to finding a predetermined nucleotide substitution. The present invention, however, demonstrates how a novel, alternative use of digital polymerase chain reaction (dPCR), preferably droplet dPCR (ddPCR), is developed to exploit finding of specific nucleotide substitutions in mutated genes. The entire platform comprises a screening method with a library of mutagenized organisms, digital PCR -based systems and a set-up to propagate and analyze identified, mutated organisms.

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(54) Title: METHOD TO SCREEN FOR A MUTANT WITHIN A POPULATION OF ORGANISMS BY APPLYING A POOLING AND SPLITTING APPROACH

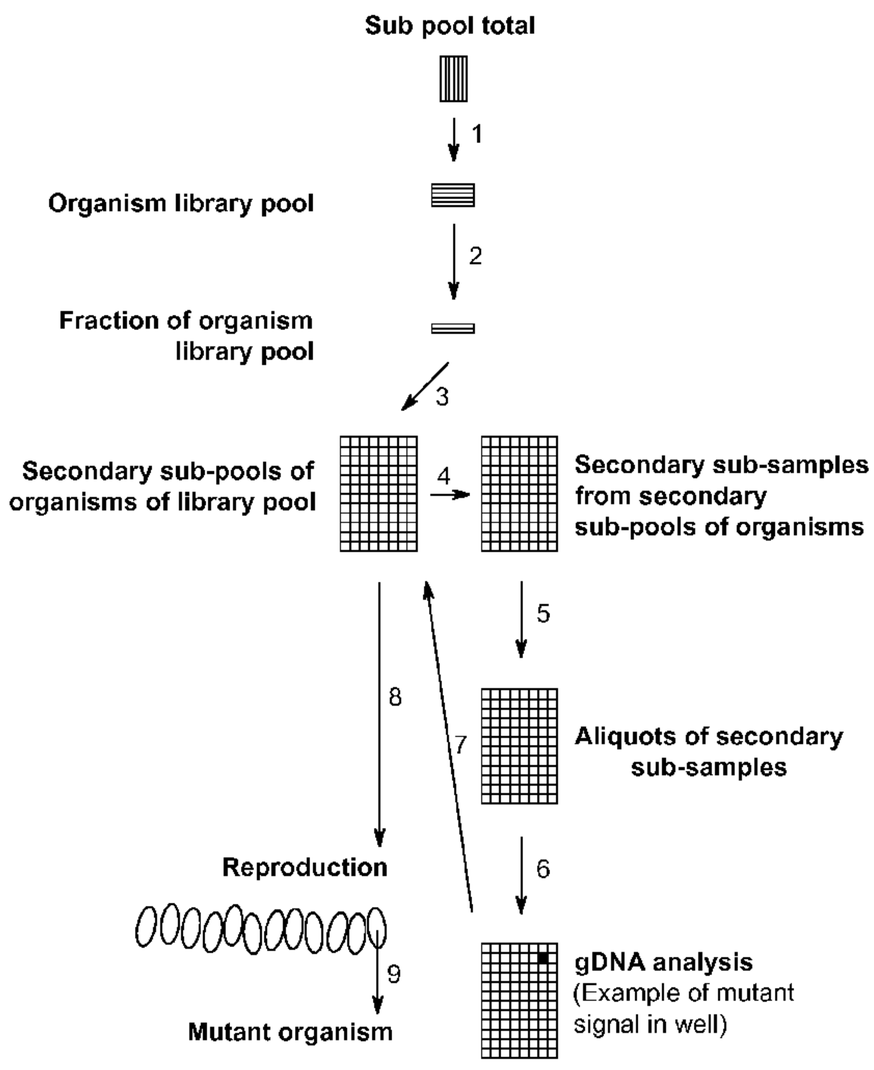

FIG. 1C

(57) Abstract: In traditional plant breeding approaches, chemical mutagenesis may be utilized to introduce nucleotide substitutions at random in the genome of a plant, i.e. without possibilities to control the sites of nucleotide changes. Because of genome complexities, the statistical probability is extremely little when it comes to finding a predetermined nucleotide substitution. The present invention, however, demonstrates how a novel, alternative use of digital polymerase chain reaction (dPCR), preferably droplet dPCR (ddPCR), is developed to exploit finding of specific nucleotide substitutions in mutated genes. The entire platform comprises a screening method with a library of mutagenized organisms, digital PCR -based systems and a set-up to propagate and analyze identified, mutated organisms.

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[Continued on next page]

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— *with sequence listing part of description (Rule 5.2(a))*

METHOD TO SCREEN FOR A MUTANT WITHIN A POPULATION OF ORGANISMS BY APPLYING A POOLING AND SPLITTING APPROACH

FIELD OF THE INVENTION

The present invention provides highly accelerated methods and processes that make it practically possible to expand and tackle the preparation, selection and/or propagation of an organism with specific, predetermined mutation(s) in one or more nucleotides of interest (NOI). The methods of the invention are, for example, useful to advance the tempo and capacity of preparing plants with specific, predetermined mutation(s) in one or more NOI(s).

BACKGROUND OF THE INVENTION

Genetic methods to generate genetically modified (GM) organisms (GMOs) are widely available. However, for many purposes, particularly in the food and beverage industries, use of GMOs is often less desirable. Thus, there remains an enduring need to provide improved and more precise methods for traditional breeding of crops, including cereals such as barley, simply to obtain better, bespoke raw materials for application in the development and manufacture of new products. Similar shortcomings exist in relation to other organisms, including microorganisms and animals. There has, unfortunately, been a nagging lack of methods in traditional breeding approaches to address the finding of mutations at predefined nucleotides of genomes, thus limiting the progress of raw material development.

Methods are available to allow genome editing and introduction of double-stranded DNA breaks at targeted sites in an organism's genome, e.g. by CRISPR-Cas9 technology (Jiang et al., 2016). Two distinct, major complications remain associated with the CRISPR-Cas9 technology:

- Firstly, CRISPR-Cas9 technology may be regarded as GM-based. Thus, basic legislative information is missing on how authorities aim to regulate the new genetic tools on genome editing, including introduction of double-stranded DNA breaks at targeted sites in the genome;
- Secondly, there are major complications related to off-target cleavages with CRISPR-Cas9 technology.

CLAIMS

1. A method of identifying an organism of a predefined species carrying one or more predetermined mutation(s) in nucleotide(s) of interest [NOI(s)], in a target sequence, said method comprising the steps of:

a) Providing a pool of organisms of said species, or reproductive parts thereof, representing a plurality of genotypes;

b) Dividing said pool into one or more sub-pools of organisms, or reproductive parts thereof;

c) Preparing gDNA samples, each comprising genomic DNA (gDNA), from each genotype within a sub-pool, while maintaining the potential for multiplication of organisms of each genotype within said sub-pool;

d) Performing a plurality of PCR amplifications, each comprising the gDNA sample from one sub-pool, wherein each PCR amplification comprises a plurality of compartmentalised PCR amplifications, each comprising part of said gDNA sample, one or more set(s) of primers each set flanking a target sequence and PCR reagents, thereby amplifying the target sequence(s);

e) Detecting PCR amplification product(s) comprising one or more target sequence(s) comprising the mutation(s) in the NOI(s), thereby identifying sub-pool(s) comprising said mutation(s);

f) Dividing the organisms, or reproductive parts thereof, of said identified sub-pool into secondary sub-pools;

g) Preparing gDNA samples, each comprising gDNA from each genotype within a secondary sub-pool, while maintaining the potential for multiplication of organisms of each genotype within said secondary sub-pool;

h) Performing a plurality of PCR amplifications, each comprising the gDNA sample from one secondary sub-pool, one or more set(s) of primers each set flanking a target sequence and PCR reagents, thereby amplifying the target sequence(s);

i) Detecting PCR amplification products comprising one or more target sequence(s) comprising the predetermined mutation(s) in the NOI(s), thereby identifying secondary sub-pools under step i) comprising said mutation(s);

j) Identifying an organism or reproductive part thereof within said secondary sub-pool carrying said mutation(s).

2. The method according to claim 1, wherein the PCR amplification(s) of step d) is (are) performed by a method comprising the following steps:
   - Preparing one or more PCR amplifications comprising the gDNA sample, one or more set(s) of primers each set flanking a target sequence and PCR reagents;
   - Partitioning said PCR amplification(s) into a plurality of spatially separated compartments;
   - Performing PCR amplification(s);
   - Detecting PCR amplification products.

3. The method according to claim 2, wherein said spatially separated compartments are droplets, such as a water-oil emulsion droplets.

4. The method according to claim 3, wherein each droplet has an average volume in the range of 0.1 to 10 nL.

5. The method according to any one of the preceding claims, wherein each PCR is compartmentalised into in the range of 1000 to 100,000 spatially separated compartments.

6. The method according to any one of the preceding claims, wherein the PCR reagents comprises one or more mutation detection probes, wherein each mutation detection probe(s) comprise(s) an oligonucleotide optionally linked to detectable means, wherein the oligonucleotide is identical to – or complementary to – a target sequence, including a predetermined mutation of the NOI.

7. The method according to any one of the preceding claims, wherein the PCR reagents comprises one or more reference detection probe(s), wherein each reference detection probe(s) comprise(s) an oligonucleotide optionally linked to detectable means, wherein the oligonucleotide is identical to – or complementary to – a target sequence, including a reference NOI.

8. The method according to any one of claims 5 and 6, wherein the mutant detection probe(s) is (are) linked to a fluorophore and a quencher, and the reference detection probe is linked to a different fluorophore and a quencher.

9. The method according to claim 7, wherein the PCR reagents comprises an at least 2-fold excess of the mutant detection probe(s) over the reference detection probe(s).

10. The method according to any one of the preceding claims, wherein said pool of organisms is prepared by subjecting the organisms of said species, or reproductive parts thereof, to random mutagenesis.

11. The methods according to any one of the preceding claims, wherein the organism pool comprises at least 10,000, preferably at least 100,000, yet more preferably at least 500,000 organisms, or reproductive parts thereof, with different genotypes.

12. The method according to any one of the preceding claims, wherein the methods comprise a step of reproduction of the organisms, or reproductive parts thereof, within the pool, and wherein said step of reproducing may be performed simultaneously with, or subsequent to, step b) of dividing the organisms into sub-pools.

13. The method according to any one of the preceding claims, wherein the species is a plant, and steps a) and b) of said method comprise the steps of:

- Providing a plurality of seeds of a plant;
- Subjecting said seeds to random mutagenesis, thereby obtaining seeds of generation M0;
- Growing said seeds of generation M0 into mature plants, and obtaining seeds from said mature plants, wherein said seeds are seeds of generation M1;
- Optionally repeating the previous step X times to obtain plants comprising seeds of generation M(1+X);
- Obtaining seeds of either generation M1 or M(1+X) from said mature plants, thereby obtaining a pool of seeds;
- Dividing said pool of the various steps into sub-pools, wherein all seeds from a given mature plant are placed into the same sub-pool.

14. The method according to claim 13, wherein at least 100,000, such as at least 500,000 seeds, are provided.

15. The method according to any one of claims 1 to 12, wherein the species is a unicellular organism, and steps a) and b) of said method comprise the steps of:
    - Providing a plurality of unicellular organisms;
    - Subjecting said organisms to random mutagenesis;
    - Dividing the mutagenized organisms into sub-pools;
    - Subjecting each sub-pool to a step of reproduction.

16. The method according to any one of the preceding claims, wherein one or more sub-pool(s) comprising the mutation, with each divided into at least 10, preferably at least 90 secondary sub-pools.

17. The method according to any one of the preceding claims, wherein the species is a unicellular species, and step f) comprises the following steps:
    - Providing a sub-pool comprising the mutation(s) in one or several NOI(s);
    - Reproducing some, or all of the organisms, of said sub-pool in a clonal manner to obtain clonal cultures;
    - Combining a fraction of organisms from a plurality of said clonal cultures to obtain secondary sub-pools.

18. The method according to claim 17, wherein each secondary sub-pool comprises fractions of in the range of 10 to 100 clonal cultures.

19. The method according to any one of claims 1 to 14, wherein the species is a plant, and steps f) and g) comprise the following steps:
    - Providing a sub-pool comprising a plurality of seeds of a plant, wherein said sub-pool comprises one or several mutation(s) of the NOI(s);
    - Dividing the seeds of said sub-pool into secondary sub-pools, each comprising in the range of 1 to 100 seeds;
    - Obtaining a sample from each seed of the secondary sub-pools – in a manner leaving the seeds sufficiently intact to develop into a plant – and combining all samples from all seeds of each secondary sub-pool;
    - Preparing a gDNA sample from said combined samples.

20. The method according to claim 19, wherein said sample is obtained by drilling a hole into the seed followed by collecting the flour obtained.

21. The method according to any one of the preceding claims, wherein the PCR amplification of step h) comprises a plurality of compartmentalised PCR amplifications.

22. The method according to claim 20, wherein the PCR amplification(s) of step h) is (are) as defined in any one of claims 2 to 5.

23. The method according to any one of the preceding claims, wherein the species is a unicellular species, and wherein step j) of identifying the organism comprises the steps of:
    - Providing a secondary sub-pool comprising the mutation in the NOI;
    - Reproducing some or all of the organisms of said sub-pool in a clonal manner;
    - Determining which clone(s) comprise organisms comprising the mutation in the NOI.

24. The method according to any one of claims 1 to 14 and 19 to 22, wherein the species is a plant, and wherein step j) of identifying the organism comprises the steps of:
    - Providing a secondary sub-pool comprising an organism, or reproductive parts thereof, comprising the mutation in the NOI;
    - Cultivating all seed within said secondary sub-pool to allow germination, and optionally develop plants from each seed;
    - Obtaining a sample from each germinated seed;
    - Testing said sample for the presence of said mutation in the NOI, thereby identifying a plant carrying the mutation in the NOI;
    - Optionally growing said plant to maturity.

25. The method according to any one of the preceding claims, wherein the method further comprises a step of identifying a super-pool comprising one or more mutation(s) in the NOI(s), wherein the super-pool is a group of sub-pools.

26. The method according to claim 25, wherein the method further comprises the following steps performed after step c):
    - Obtaining a fraction of each gDNA sample from each sub-pool;

- Combining a plurality of fractions into super-pools, thereby obtaining gDNA super-pools comprising gDNA samples from a plurality of sub-pools, wherein gDNA from each sub-pool is only present in one super-pool;
- Performing a plurality of PCR amplifications, each comprising a gDNA sample super-pool, wherein each PCR amplification comprises a plurality of compartmentalised PCR amplifications, each comprising part of said gDNA sample, one or more set(s) of primers each set flanking a target sequence and PCR reagents, thereby amplifying the target sequence(s);
- Detecting PCR amplification product(s) comprising one or more target sequence(s) comprising the mutation(s) in the NOI(s), thereby identifying super-pool(s) comprising said mutation.

27. The method according to claim 26, wherein the method further comprises a step of enrichment of the gDNA super-pools performed before performing said compartmentalised PCR.

28. The method according to claim 27, wherein the step of enrichment comprises performing PCR amplifications on a gDNA super-pool, wherein each PCR amplification comprises a set of primers flanking the target sequence, a blocking probe and PCR reagents, wherein the blocking probe is designed to inhibit amplification of the target sequence comprising the reference NOI.

29. The method according to any one of claims 25 to 28, wherein step d) of performing PCR amplifications are performed only on samples of gDNA from sub-pool(s) comprising one of the mutation(s).

30. The method according to any one of claims 25 to 29, wherein in the range of 5 to 100 super-pools are prepared.

31. The method according to any one of claims 26 to 30, wherein the PCR amplification(s) comprising a gDNA sample super-pool is (are) performed by a method comprising the following steps:
    - Preparing a PCR amplification comprising the gDNA sample, one or more set(s) of primer(s) each set flanking a target sequence and PCR reagents;

- Partitioning said PCR amplification(s) into a plurality of spatially separated compartments, wherein each compartment has an average volume in the range of 0.1 to 10 pL;
- Performing a PCR amplification;
- Detecting PCR amplification products.

32. The method according to any one of claims 25 to 31, wherein each PCR is compartmentalised into at least 1,000,000 compartments, such as spatially separated compartments.

33. The method according to any one of claims 31 to 32, wherein said spatially separated compartments are droplets, such as a water-oil emulsion droplets.

34. The method according to any one of claims 1 to 12, 15 to 18, 21 to 34, wherein the species is a unicellular organism, for example yeast.

35. The method according to any one of claims 1 to 14, 14, 17 to 20 and 23 to 28, wherein the species is a plant, for example a flowering plant.

36. The method according to any one of claims 1 to 12, 16, 19 to 22 and 24 to 34, wherein the species is a cereal, for example barley.

37. The method according to any one of the preceding claims, wherein the mutation is a substitution of a single nucleotide.

38. The method according to any one of the preceding claims, wherein the method is a method of identifying more than one predetermined mutation.

39. The method according to claim 38, wherein the PCR reagents comprise one mutant detection probe specific for each predetermined mutation.

40. The method according to any one of claim 1 to 37, wherein the method is a method of identifying one predetermined mutation in a target sequence, and the method employs only one set of primers flanking said target sequence.

41. The method according to claim 40, wherein the PCR reagents comprise only one mutation detection probe.

5

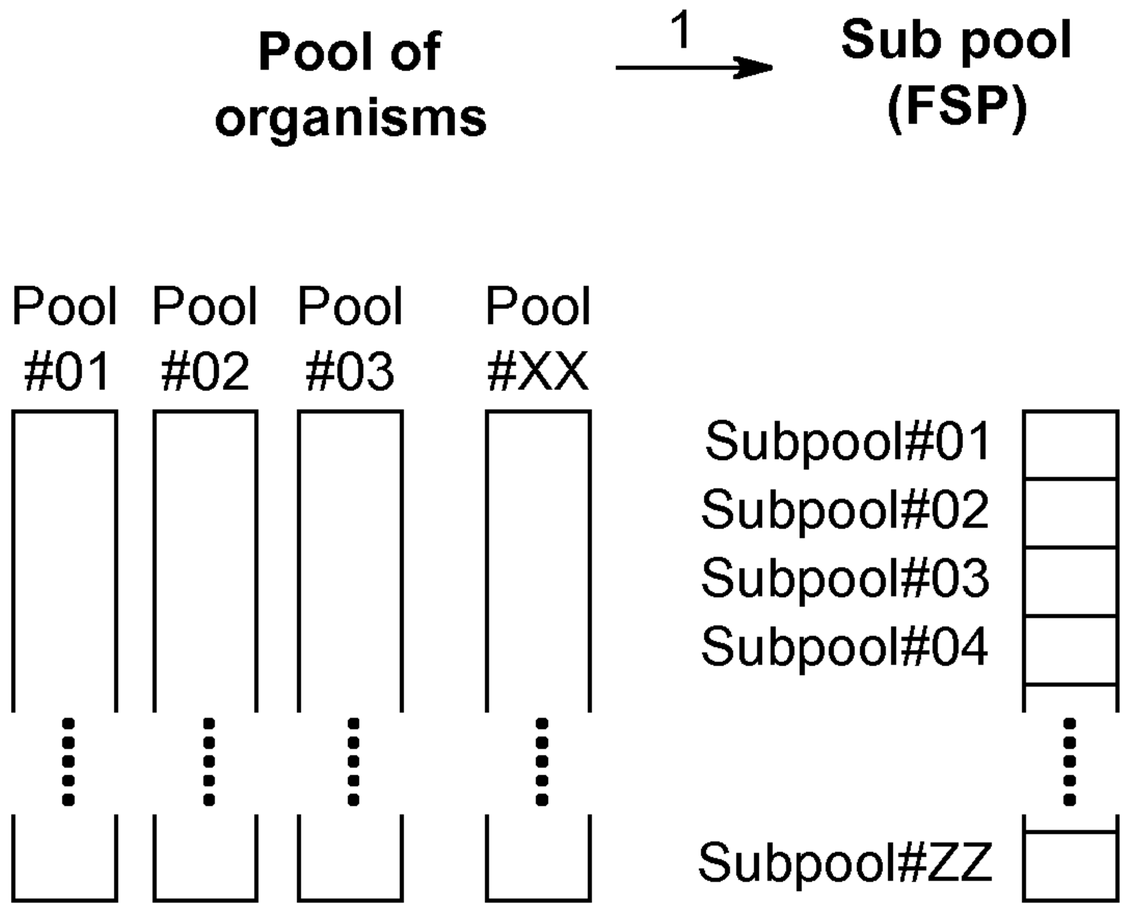
Pool of organisms
1
Sub pool (FSP)
Pool #01
Pool #02
Pool #03
Pool #XX
Subpool#01
Subpool#02
Subpool#03
Subpool#04
Subpool#ZZ

FIG. 1A

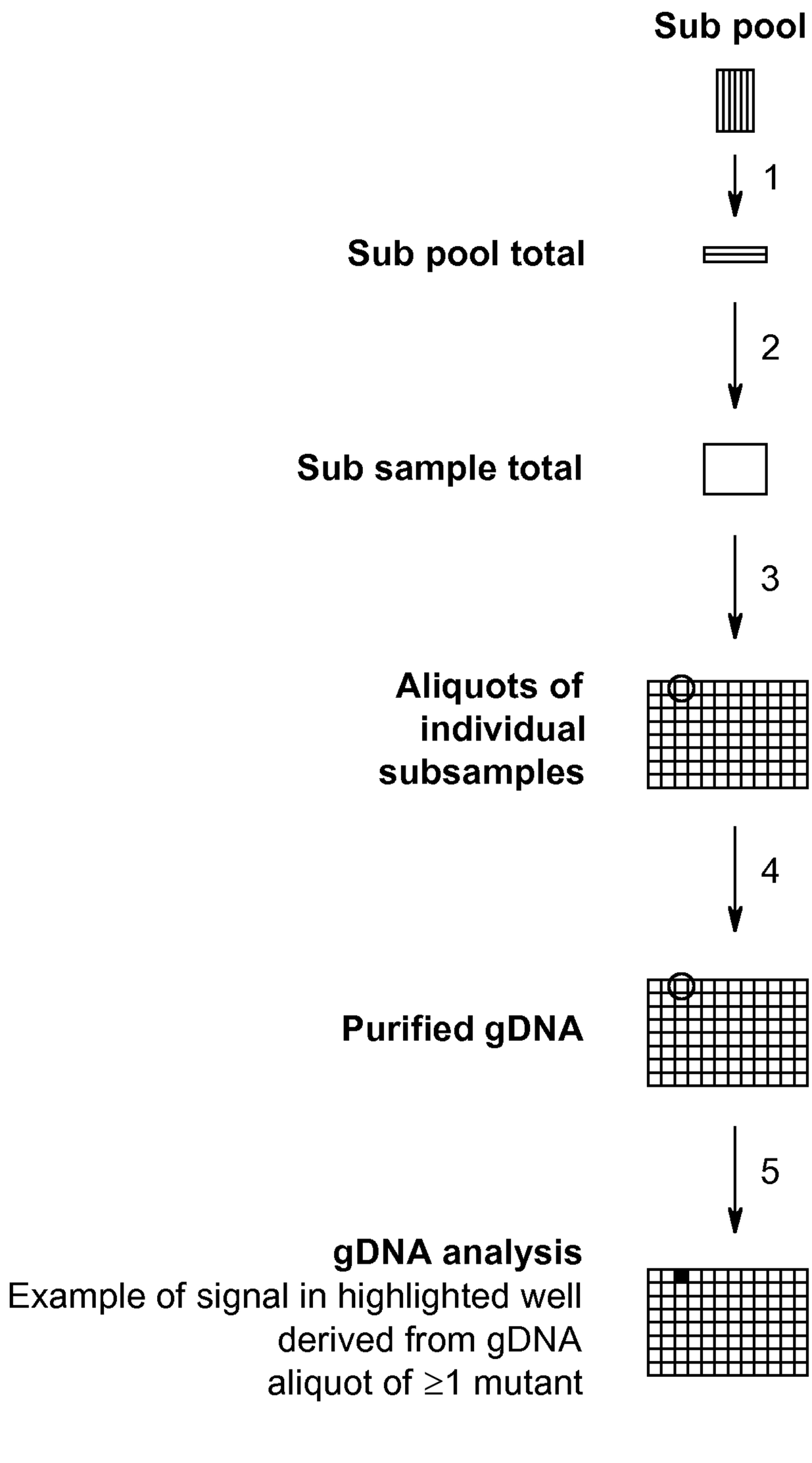
Sub pool
1
Sub pool total
2
Sub sample total
3
Aliquots of individual subsamples
4
Purified gDNA
5
gDNA analysis
Example of signal in highlighted well derived from gDNA aliquot of ≥1 mutant

FIG. 1B

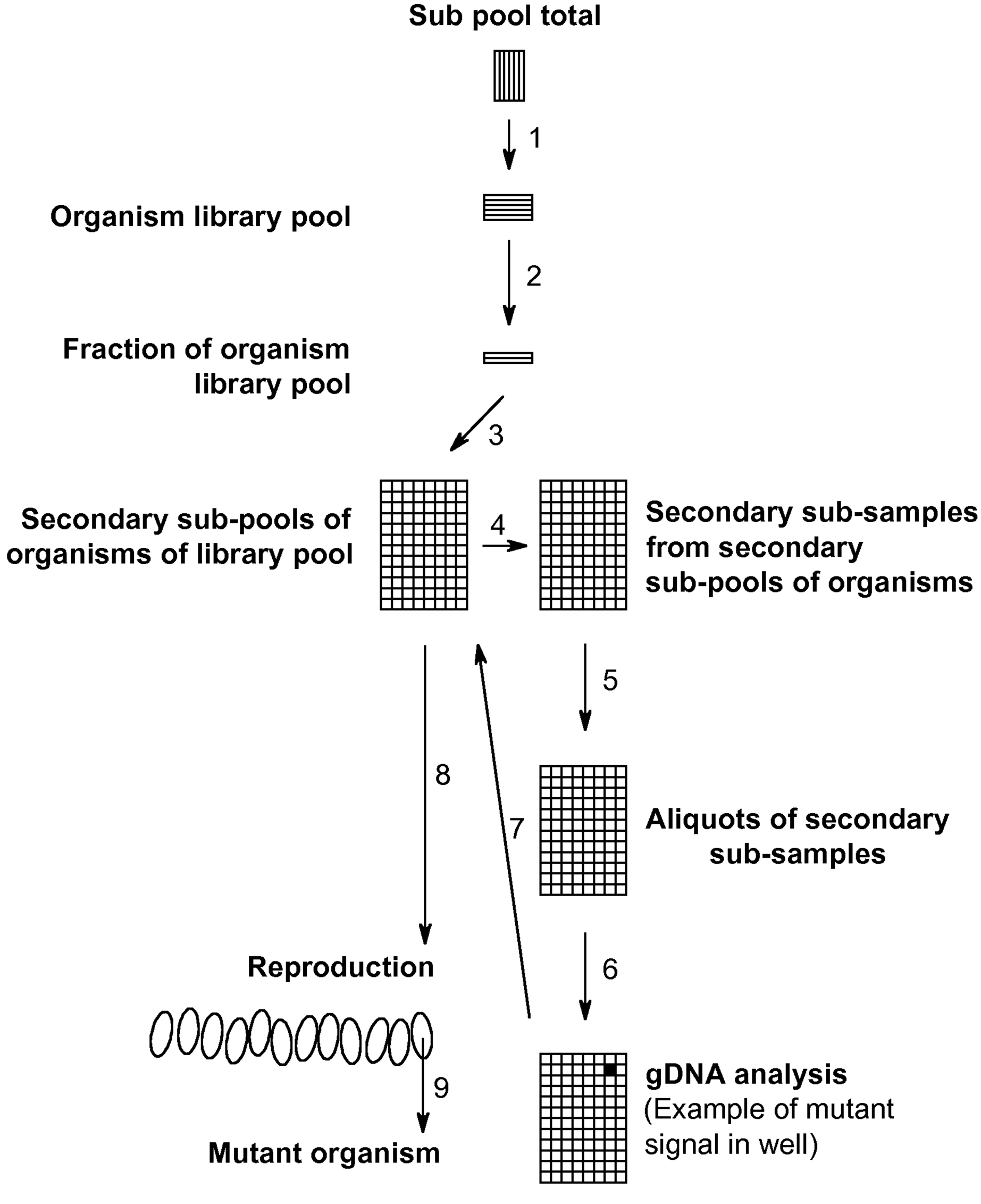
Sub pool total
1
Organism library pool
2
Fraction of organism library pool
3
Secondary sub-pools of organisms of library pool
4
Secondary sub-samples from secondary sub-pools of organisms
5
8
7
Aliquots of secondary sub-samples
6
Reproduction
9
gDNA analysis
(Example of mutant signal in well)
Mutant organism

FIG. 1C

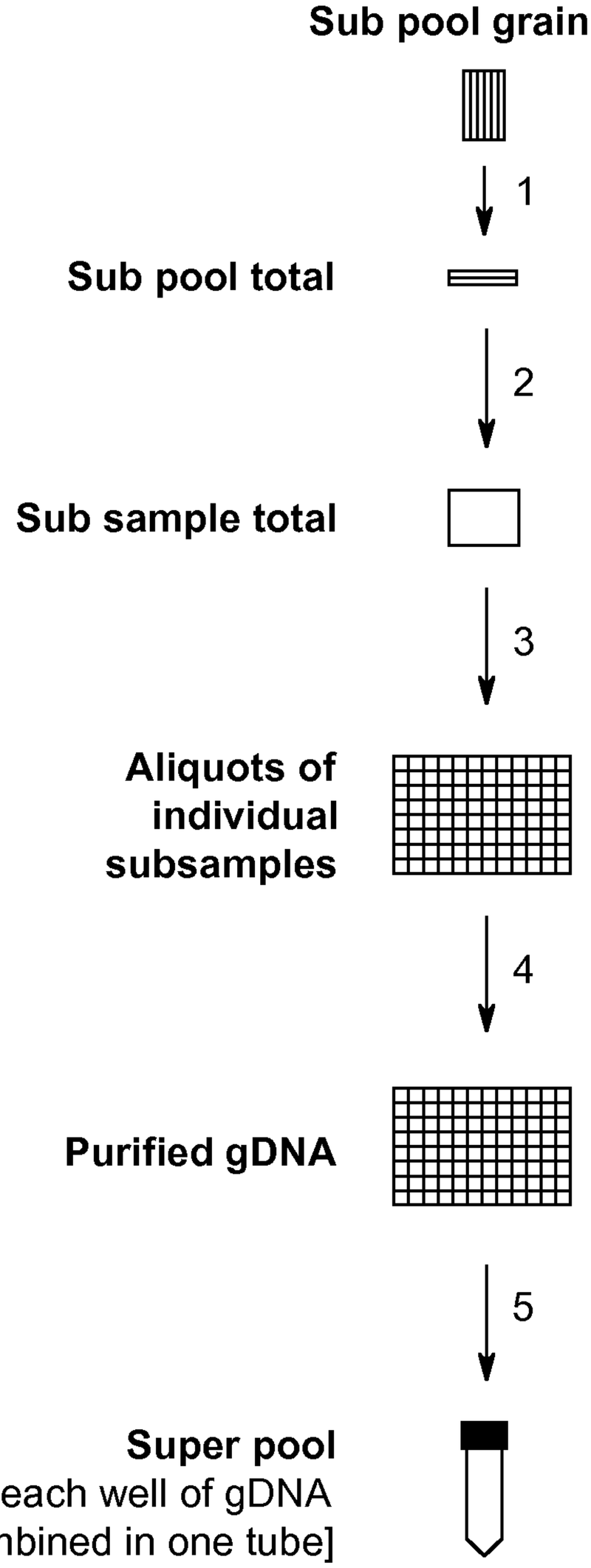
Sub pool grain total
1
Sub pool total
2
Sub sample total
3
Aliquots of individual subsamples
4
Purified gDNA
5
Super pool
[gDNA from each well of gDNA combined in one tube]

FIG. 1D

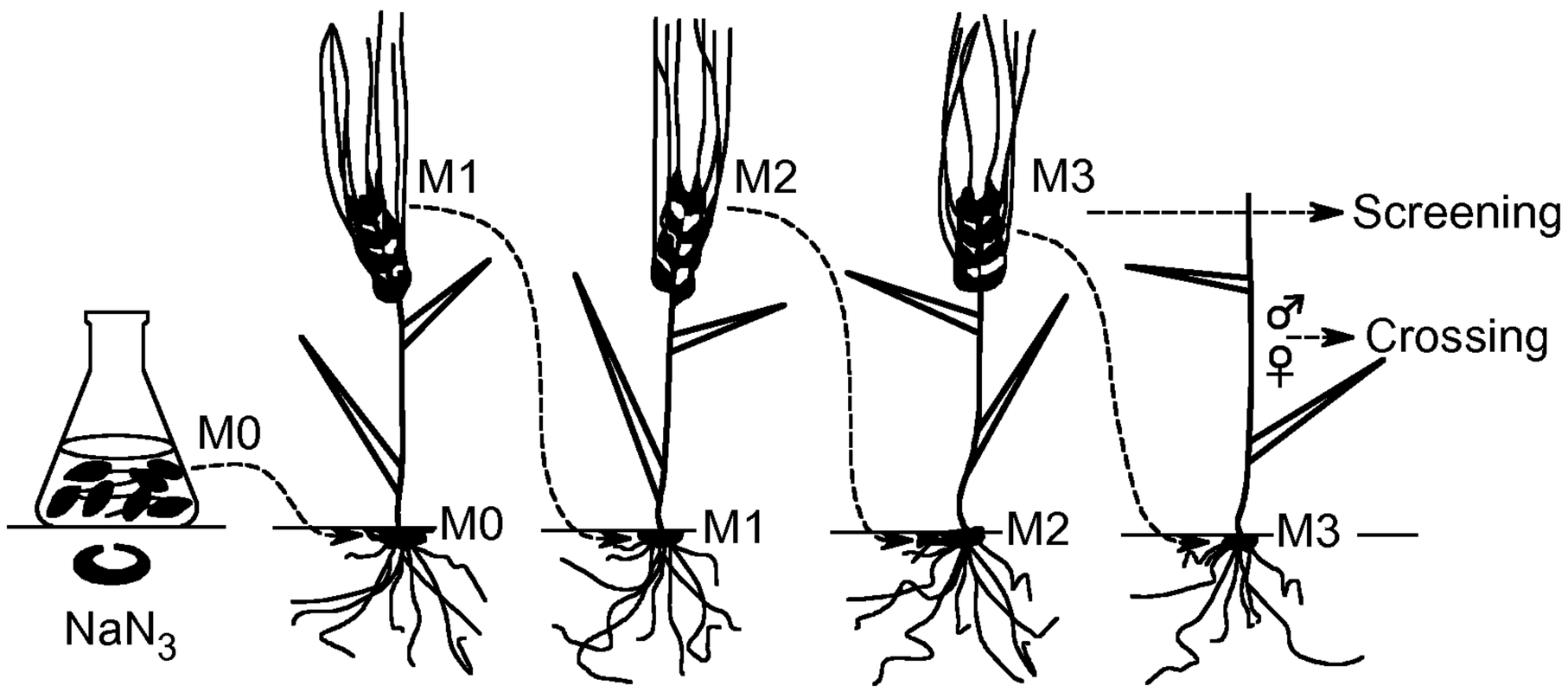
M1
M2
M3
Screening
♂
♀
Crossing
M0
M0
M1
M2
M3
NaN3

FIG. 2A

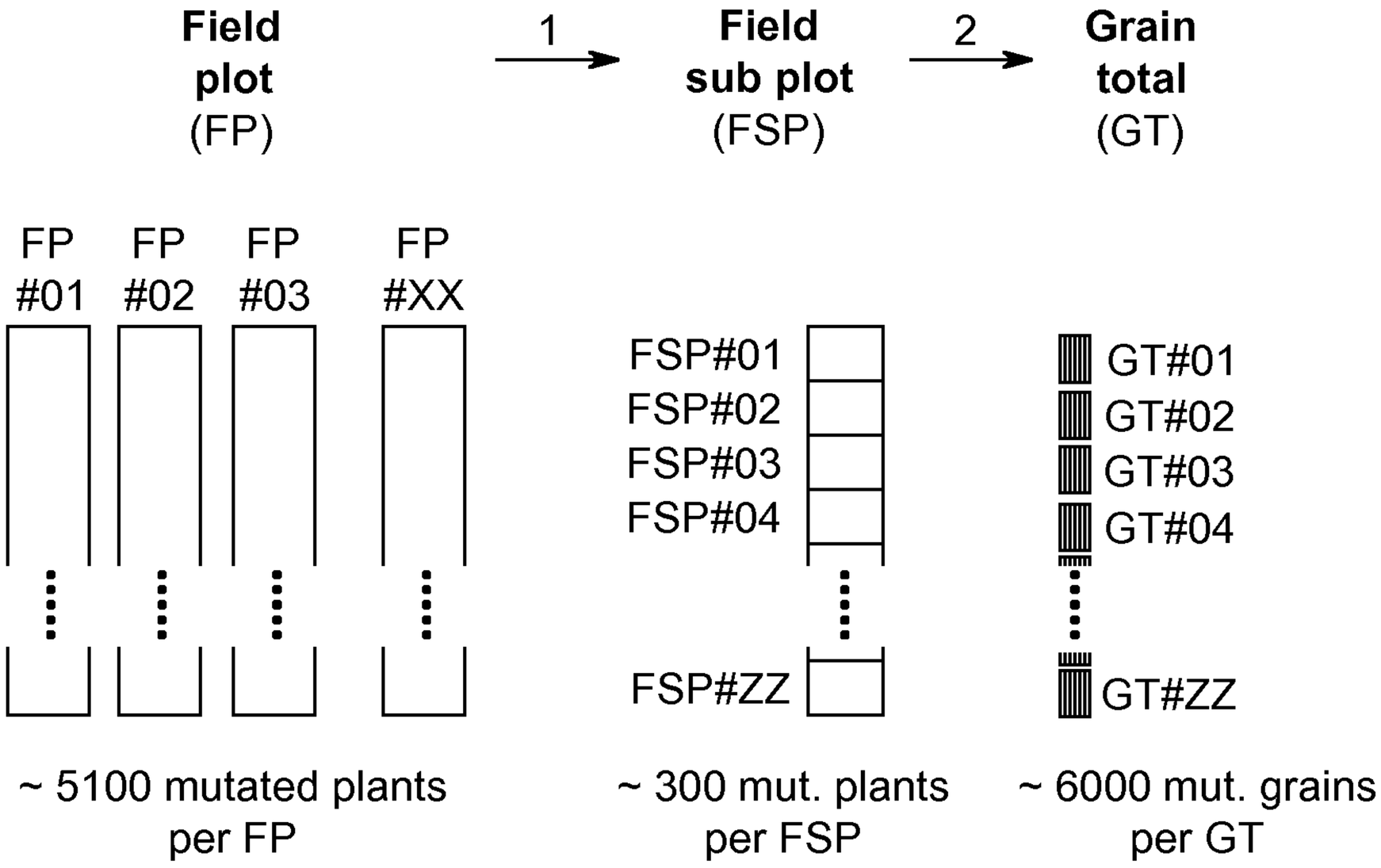
Field plot (FP)
1
Field sub plot (FSP)
2
Grain total (GT)
FP #01
FP #02
FP #03
FP #XX
FSP#01
FSP#02
FSP#03
FSP#04
FSP#ZZ
GT#01
GT#02
GT#03
GT#04
GT#ZZ
~ 5100 mutated plants per FP
~ 300 mut. plants per FSP
~ 6000 mut. grains per GT

FIG. 2B

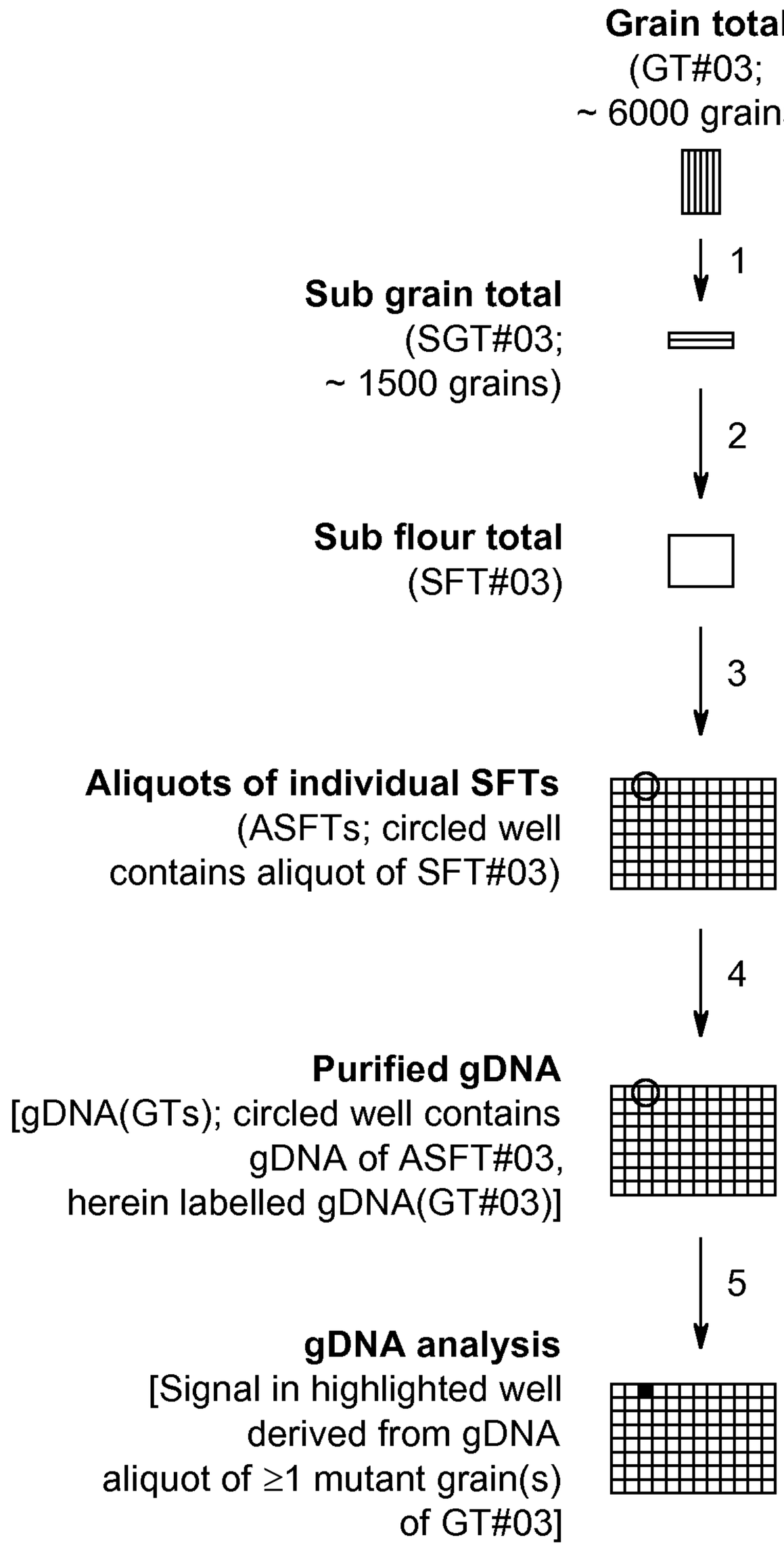
Grain total
(GT#03;
~ 6000 grains)
1
Sub grain total
(SGT#03;
~ 1500 grains)
2
Sub flour total
(SFT#03)
3
Aliquots of individual SFTs
(ASFTs; circled well
contains aliquot of SFT#03)
4
Purified gDNA
[gDNA(GTs); circled well contains
gDNA of ASFT#03,
herein labelled gDNA(GT#03)]
5
gDNA analysis
[Signal in highlighted well
derived from gDNA
aliquot of ≥1 mutant grain(s)
of GT#03]

FIG. 2C

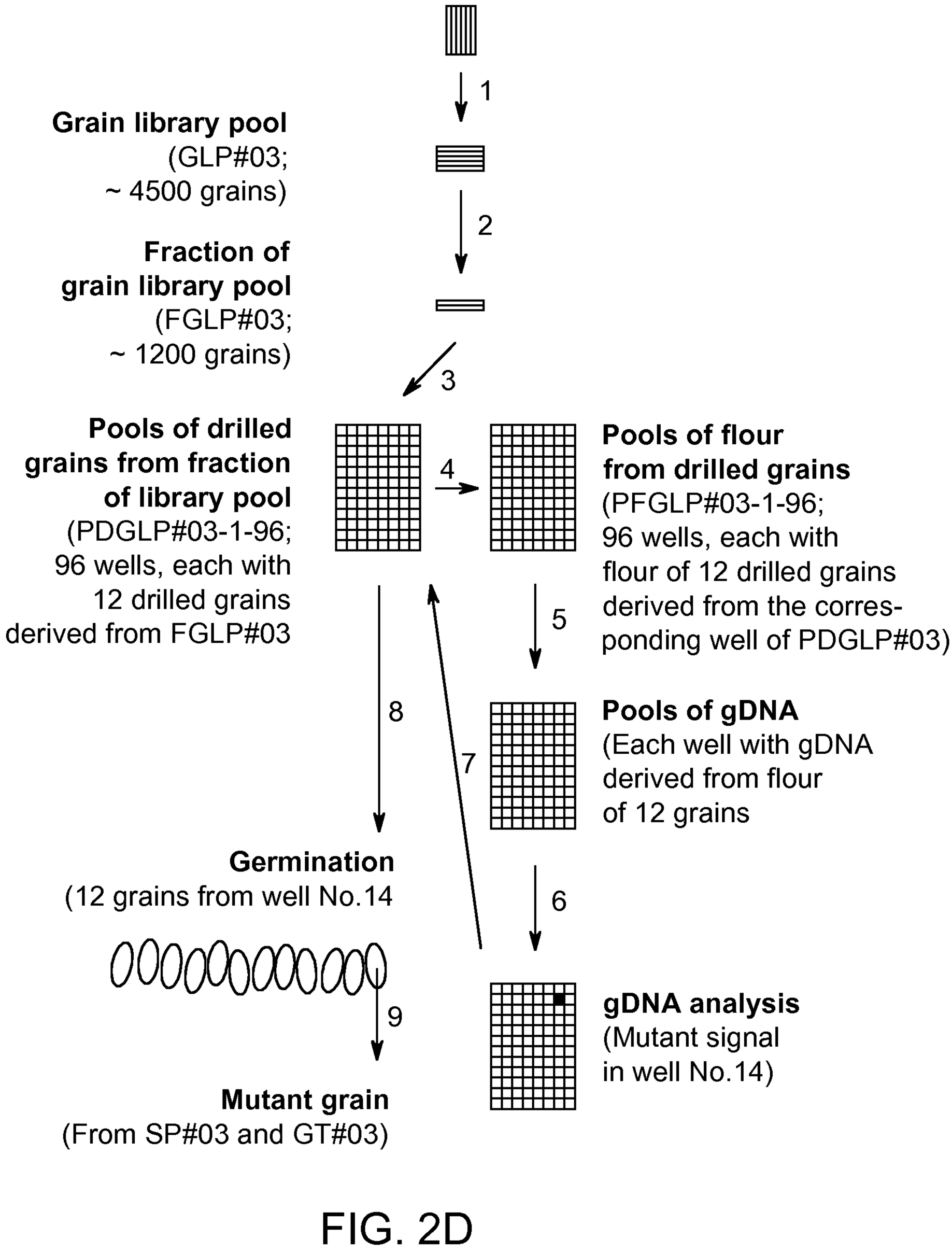
Grain total
(GT#03;
~ 6000 grains)
1
Grain library pool
(GLP#03;
~ 4500 grains)
2
Fraction of
grain library pool
(FGLP#03;
~ 1200 grains)
3
Pools of drilled
grains from fraction
of library pool
(PDGLP#03-1-96;
96 wells, each with
12 drilled grains
derived from FGLP#03
4
Pools of flour
from drilled grains
(PFGLP#03-1-96;
96 wells, each with
flour of 12 drilled grains
derived from the corres-
ponding well of PDGLP#03)
5
8
7
Pools of gDNA
(Each well with gDNA
derived from flour
of 12 grains
6
Germination
(12 grains from well No.14
9
gDNA analysis
(Mutant signal
in well No.14)
Mutant grain
(From SP#03 and GT#03)

FIG. 2D

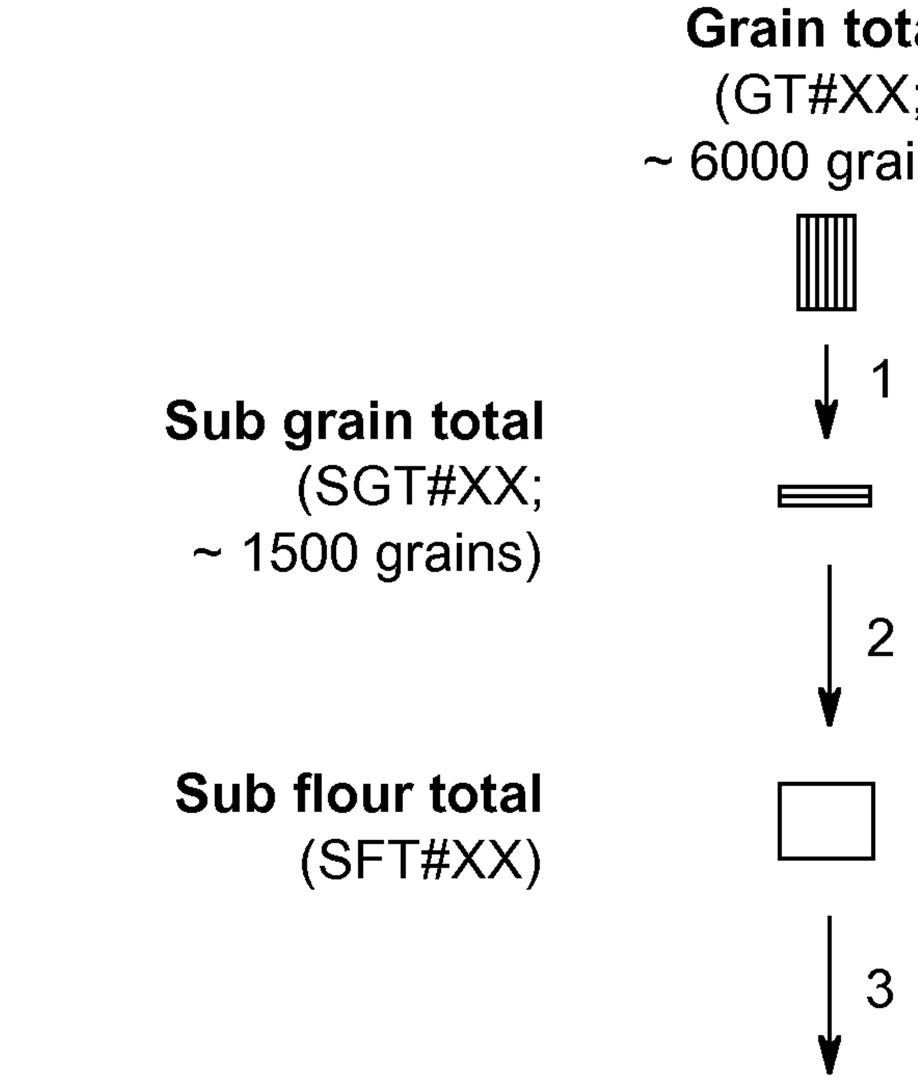
Grain total
(GT#XX;
~ 6000 grains)
1
Sub grain total
(SGT#XX;
~ 1500 grains)
2
Sub flour total
(SFT#XX)
3
Aliquots of
individual SFTs
(ASFTs#XX-1-96)
4
Purified gDNA
[gDNA(GT#XX-1-96)]
5
Super pool
[SP-gDNA#XX;
50 µl gDNA from each well of
gDNA(GT#XX-1-96)
combined in one tube]

FIG. 2E

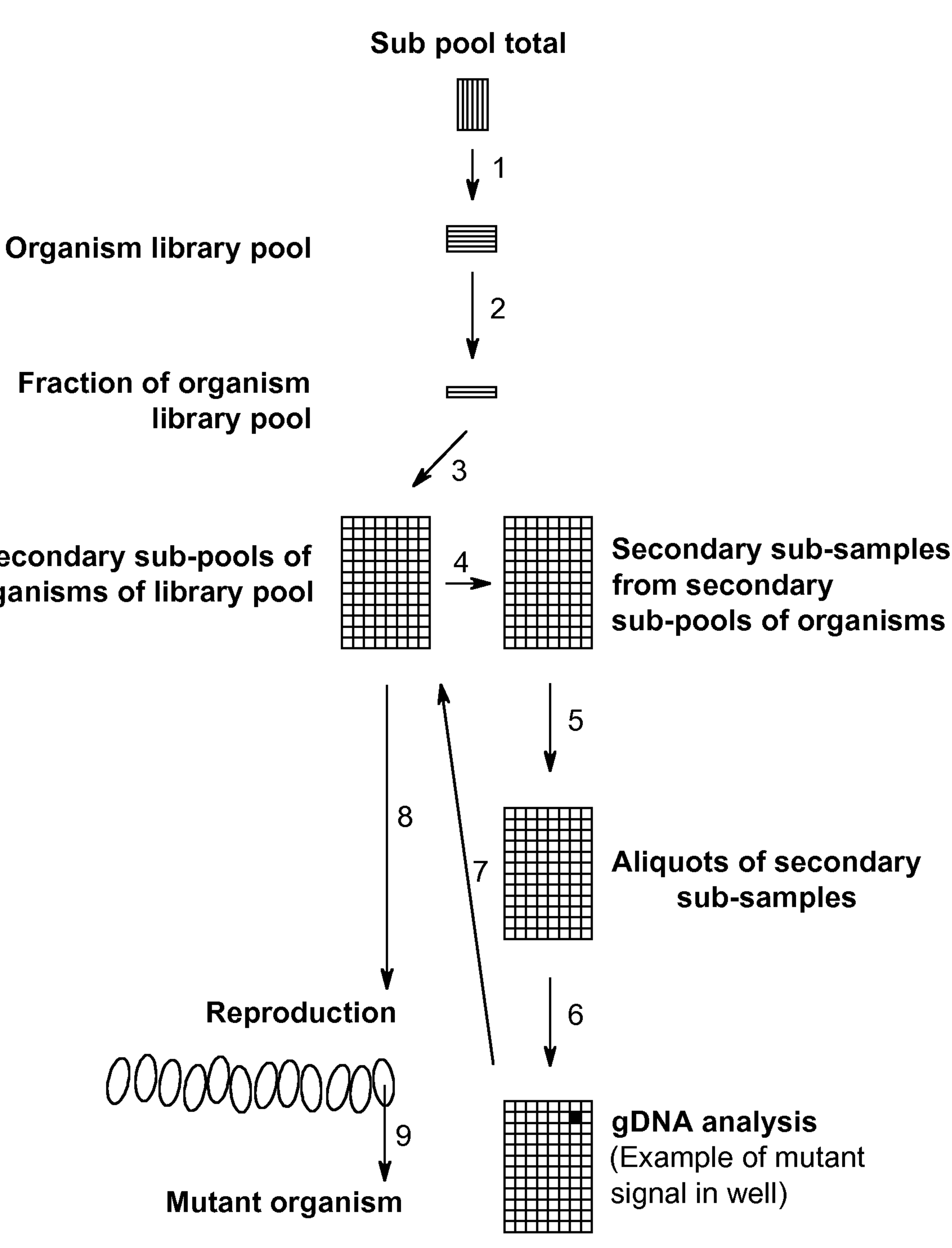
Sub pool total
1
Organism library pool
2
Fraction of organism library pool
3
Secondary sub-pools of organisms of library pool
4
Secondary sub-samples from secondary sub-pools of organisms
5
8
7
Aliquots of secondary sub-samples
6
Reproduction
9
Mutant organism
gDNA analysis
(Example of mutant signal in well)

FIG. 1C