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Datasheet for the decision of 25 March 2010

Case Number: T 0103/07 - 3.3.08

Application Number: 00930821.4

Publication Number: 1185616

IPC: C12N 1/06

Language of the proceedings: EN

Title of invention:
Method for rapidly detecting and enumerating microorganisms in mammalian cell preparations using ATP bioluminescence

Applicant:
MILLIPORE CORPORATION

Opponent:
-

Headword:
ATP Bioluminescence/MILLIPORE

Relevant legal provisions:
-

Relevant legal provisions (EPC 1973):
EPC Art. 56

Keyword:
"Main request: inventive step (no)"
"Auxiliary request: inventive step (yes)"

Decisions cited:
G 0010/93

Catchword:
-

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Case Number: T 0103/07 **-** 3.3.08

D E C I S I O N
of the Technical Board of Appeal 3.3.08
of 25 March 2010

Appellant: MILLIPORE CORPORATION
290 Concord Road
Billerica
Massachusetts 01821 (US)

Representative: Kirkham, Nicholas Andrew
Graham Watt & Co LLP
St Botolph's House
7-9 St Botolph's Road
Sevenoaks
Kent TN13 3AJ (GB)

Decision under appeal: **Decision of the Examining Division of the European Patent Office posted 29 June 2006 refusing European patent application No. 00930821.4 pursuant to Article 97(1) EPC 1973.**

Composition of the Board:

Chairman: P. Julià
Members: T. J. H. Mennessier
T. Karamanli

Summary of Facts and Submissions

I. The applicant (appellant) lodged an appeal against the decision of the examining division dated 29 June 2006, whereby European patent application No. 00 930 821.4 with publication number 1 185 616 was refused. The application, entitled "*Method for rapidly detecting and enumerating microorganisms in mammalian cell preparations using ATP bioluminescence*", originated from an international application published as WO 00/71675.

II. The decision was based on the main and the four auxiliary requests filed on 15 February 2006 which were refused for reasons of lack of an inventive step (Article 56 EPC 1973) in view of documents D1 and D3 (see Section VIII *infra*).

III. On 2 November 2006, the appellant filed a statement setting out the grounds of appeal together with two new documents.

IV. The examining division did not rectify its decision and referred the appeal to the board of appeal (Article 109 EPC 1973).

V. On 29 October 2009, the board issued a summons to oral proceedings which was accompanied by a communication pursuant to Article 15(1) of the Rules of Procedure of the Boards of Appeal (RPBA) containing the board's provisional and non-binding opinion on substantive matters. With reference to decision G 10/93 (OJ EPO, 1995, 172), the board announced that, using its power to examine whether a requirement of the EPC which the

examining division did not take into consideration in the examining proceedings was met, it was intended to assess whether the requirements of Article 123(2) EPC and Articles 83 and 84 EPC 1973 were complied with. Furthermore, a new prior art document (see document D4 in Section VIII *infra*) was introduced by the board into the proceedings.

VI. Together with a letter dated 24 February 2010, in reply to the board's communication, the appellant filed a new main request and five new auxiliary requests to replace the requests then on file. Additional examples were annexed to the appellant's letter.

VII. Oral proceedings took place on 25 March 2010, at which the requests of 24 February 2010 were withdrawn and a new main request as a well as a new auxiliary request were filed.

(a) The main request

The main request consisted of 9 claims, of which claim 1 read as follows:

"1. A method for the detection of microorganisms in a mammalian cell preparation from a mammalian cell fermenter, the cell preparation including mammalian cells having a level of mammalian ATP, wherein the method includes the sequential steps of:

reducing the level of mammalian ATP in said cell preparation by differentially lyzing said mammalian cells with one or more detergents to extract said mammalian ATP;

treating said extracted mammalian ATP with one or more ATP hydrolysing enzymes;

incubating said cell preparation for 15 minutes at room temperature while preserving the viability of the microorganisms;

filtering said mammalian cell preparation through a micropartitioned hydrophilic/hydrophobic membrane made up of a number of hydrophilic filter sections substantially completely isolated from each other with latticed or circular hydrophobic micropartitions to immobilise said microorganisms;

washing away said detergent and said hydrolysing enzyme on said membrane;

extracting microbial ATP from the immobilised microorganisms using an extracting agent;

applying a bioluminescent reagent onto said membrane; and

detecting light emitted by said microbial ATP to indicate the presence and number of said microorganisms in said cell preparation."

(b) The auxiliary request

The auxiliary request consisted of 8 claims of which claim 1 read as follows:

"1. A method for the detection of microorganisms in a mammalian cell preparation from a mammalian cell fermenter, the cell preparation including mammalian cells having a level of mammalian ATP, wherein the method includes the sequential steps of:

reducing the level of mammalian ATP in said cell preparation by differentially lyzing said mammalian

cells with **osmotic shock and** one or more detergents to extract said mammalian ATP;

treating said extracted mammalian ATP with one or more ATP hydrolysing enzymes;

incubating said cell preparation for 15 minutes at room temperature while preserving the viability of the microorganisms;

filtering said mammalian cell preparation through a micropartitioned hydrophilic/hydrophobic membrane made up of a number of hydrophilic filter sections substantially completely isolated from each other with latticed or circular hydrophobic micropartitions to immobilise said microorganisms;

washing away said detergent and said hydrolysing enzyme on said membrane;

extracting microbial ATP from the immobilised microorganisms using an extracting agent;

applying a bioluminescent reagent onto said membrane; and

detecting light emitted by said microbial ATP to indicate the presence and number of said microorganisms in said cell preparation."

(emphasis added by the board)

Claims 2 to 8 were dependent on claim 1 and were directed to particular embodiments thereof.

VIII. The following documents are referred to in the present decision:

(D1) P. E. Stanley, Methods in Enzymology, Vol. 133, 1986, pages 14 to 22;

(D3) H. Tanaka et al., Wat. Res., Vol. 31, No. 8, 1997, pages 1913 to 1918;

(D4) E. W. Chappelle et al., Methods in Enzymology, Vol. 57, 1978, pages 65 to 72.

IX. The submissions made by the appellant, insofar as they are relevant to the present decision, may be summarised as follows:

Main request

Article 56 EPC 1973

The method according to claim 1 included a non-obvious step of opening or lysing the mammalian cell preparation by the way of a differential extraction which did not damage the contaminant microorganisms. Furthermore, use was made of a particular membrane which was found to be especially useful to immobilise the microorganisms efficiently and without damage, and which, at the same time, allowed to wash away the detergents and the freed ATP before microbial ATP was extracted and measured.

Document D1 was silent as to the type of membrane to be used and did not suggest that the microorganisms should be immobilised thereon. Nor did it indicate that the cell preparation should be incubated for 15 minutes at room temperature after treatment with the ATP hydrolysing enzyme, an incubation which had been found to be critical to allow time for the hydrolysing enzyme to break down the ATP from the mammalian cells in the sample while not affecting bacterial cell viability.

Document D1 did not disclose that the somatic cells were derived from a fermenter and therefore did not address the technical problem of the invention. It taught about extracting specifically somatic cells but did not go on to say that, having done so and having hydrolysed the mammalian ATP, the somatic cells and the mammalian ATP should be washed away.

Document D1 was a publication of 1986, pooling together a number of ideas under a common heading. It was not directed at detecting and measuring microorganisms in the presence of somatic cells.

Document D3 was not concerned with reducing the level of ATP derived from mammalian cells and removing it therefrom.

Document D4 did not disclose whether or not the filtering would damage the microorganisms.

Auxiliary request

Article 56 EPC 1973

Whereas osmotic shock was known to be efficient at opening or lysing mammalian cells, there was no suggestion in the available state of the art of using in combination an osmotic shock and one or more detergents to lyse mammalian cells. Example DD, as submitted with the letter of 24 February 2010, showed that the combined use of an osmotic shock and of one or more detergents was associated with an unexpected improved detection of microorganisms in comparison with

the use of either an osmotic shock or a detergent alone.

X. The appellant requests that the decision under appeal be set aside and that a patent be granted on the basis of the main request or the auxiliary request, both filed during the oral proceedings.

Reasons for the Decision

Main request

Admission into the proceedings

1. Since the main request was filed in direct reaction to objections under Article 84 EPC 1973 raised by the board at the oral proceedings against the appellant's previous main request of 24 February 2010, the board using its discretionary power under Article 13(1) RPBA decided to admit it into the proceedings.

Formal and substantive requirements of the main request

2. The subject-matter of the main request is basically derived from a combination of the claims on which the decision under appeal was based. No objections other than lack of inventive step were raised by the examining division nor does the board, after having considered the appellant's observations and arguments, see any reason to raise any of its own (see also points 18 and 19 ***infra***).

Requirements of Article 56 EPC 1973

3. Claim 1 of the main request is directed to a method for the detection of microorganisms which may contaminate mammalian cells in a fermenter. As explained on page 2, lines 1 to 3, of the application as published (see WO 00/71675), an object of the invention was to provide a highly sensitive method for the detection of low levels of contaminating microorganisms.

4. D1 is a prior art document cited in the supplementary European Search Report and correctly considered by the examining division in the decision under appeal to represent the closest state of the art. Document D1 deals with the issue of how extracting adenosine triphosphate (ATP) from microbial and somatic cells, and provides guidance to determine microbial ATP contained in mixtures of somatic and microbial cells, with one approach consisting in selectively extracting the somatic cells of the mixture using a detergent such as Triton X-100 and then hydrolysing the somatic ATP together with any free ATP with an ATPase (see on page 21 the Section entitled "***Somatic and Microbial Cells Mixed Together with Nonliving Material***"). For a more detailed description of that approach, document D1 directs the reader to its reference 43 which is document D4 in the present appeal proceedings (see the number 43 put after the term ATPase in the second paragraph of page 21).

5. Prior art document D4 investigates the determination of bacterial content in fluids using the bioluminescence-based firefly luciferase ATP assay. Factors, including interference by nonbacterial ATP in

the sample, that influence the accuracy of the measurement, and assay methods, including filtration procedures, are addressed.

5.1 On page 68, it is stated that nonbacterial ATP in the sample, including intracellular ATP from eukaryotic cells, must be removed prior to bacterial ATP extraction and assay, the removal comprising in a preferred embodiment a selective chemical lysis of nonbacterial cells by using a detergent such as Triton X-100 followed by the addition of an ATPase to hydrolyse the freed ATP.

5.2 On page 72, an assay is described which incorporates such a treatment. The mammalian cells contained in a sample fluid, which passes readily through a filter with minimal damage to the bacteria, are lysed by adding to the sample an amount of Triton X-100 (also denoted "TX" in the document, see page 71, third line) and an active ATP-hydrolysing enzyme solution (known as "apyrase"; see the paragraph beginning with the term "Apyrase" on page 70). The sample is incubated 15 minutes for ATP hydrolysis, filtered on a 0,22 µm membrane and washed. The filter is then treated with an extracting agent to remove bacterial ATP from the bacteria retained on its surface. The extract is collected as filtrate, an aliquot of which is assayed by injection of a luciferase enzyme preparation (see page 72, first three paragraphs).

6. The board observes that document D1, taken together with document D4 which is one of its own citations, describes a method for the detection of microorganisms

in a sample from which the method according to claim 1 differs only in that a particular membrane is used.

7. Thus, the technical problem faced by the skilled person may be seen as the provision of an alternative sensitive method for the detection of low levels of microorganisms contained in a mammalian cell fermenter (see page 2, lines 1 to 3 of the published application). The solution to that problem is a method according to claim 1 in which the filtration is carried out through a micropartitioned hydrophilic/hydrophobic membrane made up of a number of hydrophilic filter sections substantially completely isolated from each other with latticed or circular hydrophobic micropartitions to immobilise said microorganisms.

8. The question to be answered for the assessment of inventive step is whether the skilled person would have found an incentive in any of the available prior art documents to replace in the method of document D1, as detailed in document D4, the 0,22 µm membrane by a membrane as that disclosed in the application.

9. Prior art document D3 would not have escaped the attention of the skilled person faced with the underlying technical problem because it dealt with the problem of designing a sensitive method which can be used to detect low concentrations of microorganisms.

10. The method of document D3 relies on the use of a special membrane of the type referred to in claim 1. This membrane makes possible a low concentration of microorganisms in the samples on the surface, the dispersal of the microorganisms uniformly and the

prevention of the microbial ATP from spreading and diluting (see in the left-hand column of page 1914, the paragraph entitled "*RMD membrane filter*" and the legend of Figure 1). This method can be used to detect low concentration of microorganisms (see lines 13 to 17 in the right-hand column of page 1914).

11. It is not doubtful that the skilled person would have readily realised that the membrane described in document D3 qualifies as a filter appropriate to the detection of low concentrations of microorganisms and would have used it in place of the 0,22 µm membrane used in the method of document D4, thereby arriving at the method according to claim 1.

12. The appellant argued that document D3 was not relevant for the reason that it was not concerned with a method that included the reduction of mammalian ATP contained in a mixture of mammalian cells contaminated with microorganisms. However, this is not the point at issue. The high relevancy of document D3 in the present assessment of inventive step lies indeed in the disclosure of the remarkable ability of the particular membrane referred to therein, directly linked to its structure, of allowing a low concentration of microorganisms to be spread homogenously on its surface and, thereby, allowing also an accurate detection of the same. Thus, the appellant's argument is not tenable.

13. Equally untenable is the appellant's argument that document D1 is not relevant for the reason that the mixture of somatic cells and microbial cells as referred to on page 21 does not specifically include a mixture as contained in a mammalian cell fermenter,

i.e. mammalian cells contaminated with microorganisms. Indeed, the non-exhausted list of cell mixtures referred to at the top of page 21 includes mixtures, such as rumen contents and clinical samples, which comprise mammalian cells contaminated with microorganisms and therefore do not differ substantially from the mammalian cell preparation referred to in claim 1. No inventive contribution can be associated with the selection of a mammalian cell preparation from a mammalian cell fermenter. Nor is this selection emphasised in the application as providing any particular advantageous effect.

14. The further argument, that in the method of document D4 keeping undamaged the microorganisms was not an issue, was contradicted by the indication made in the first paragraph on page 72 of that document, wherein it is stated that the sample was passed through a filter "with minimal damage".

15. Also the appellant's argument that an incubation of 15 minutes was unexpectedly found to be advantageous to allow the ATP hydrolysing enzyme(s) to hydrolyse the mammalian ATP, is invalidated by the description in document D4 of such an incubation (see third paragraph on page 72).

16. Thus, the board concludes that the subject-matter of claim 1 of the main request does not involve an inventive step and that, therefore, it does not comply with Article 56 EPC 1973 and, as a result thereof, the main request can not form a basis for the maintenance of the patent in amended form.

Auxiliary request

Admission into the proceedings

17. Since the auxiliary request was filed in direct reaction to objections under Article 56 EPC 1973 raised by the board against the appellant's main request, the board using its discretionary power under Article 13(1) RPBA decided to admit it into the proceedings.

Requirements of Article 123(2) EPC

18. Support can be found for the method according to claim 1 on page 5, lines 3 to 16 and 20 to 30 taken together with page 1, lines 8 to 14 (for the feature mammalian cell fermenters) of the application as published (WO 00/71675, the content of which correspond to that of the application as filed), with the lysis step (involving an osmotic shock combined with the use of one or more detergents) being specifically described ***inter alia*** on page 5, at line 14. The additional features contained in the dependent claims are described ***inter alia*** on page 5, lines 16 to 19 (see claim 2); page 6, lines 6 to 7 (see claim 3); page 6, lines 13 to 16 (see claim 4); page 6, lines 3 to 5 (see claim 5); page 6, lines 17 to 18 (see claim 6); page 6, lines 7 to 13 (see claim 7) and page 5, lines 7 to 8 (see claim 8). Thus, the auxiliary request as a whole complies with the requirements of Article 123(2) EPC.

Requirements of Articles 84 and 83 EPC 1973

19. The board is satisfied that the claims of the auxiliary request complies with the requirements of Article 84

EPC 1973. Furthermore, it considers that the description as a whole including Examples A to D on pages 6 to 7 would provide the skilled person with all the necessary guidance to carry out the method according to claim 1 or of any one of dependent claims 2 to 8. It is noted that Example D on page 7 describes a differential lysis of mammalian cells with osmotic shock (with MilliQ Water) and two detergents (Triton X-100 and SDS). Thus, the requirements of Article 83 EPC 1973 are also met.

Requirements of Article 56 EPC 1973

20. Claim 1 of the auxiliary request differs from claim 1 of the main request in that the lyse of the mammalian cells is not obtained by the only effect of one or more detergents but by the same combined with an osmotic shock (see Section VII, *supra*).

21. None of the available prior art describes the use of an osmotic shock for lysing mammalian cells, let alone in combination with one or more detergents. Furthermore, the results of Example DD, as submitted with the letter of 24 February 2010 (see pages 16 and 17 thereof), show an unexpected higher detection of the contaminating microorganisms when one or two detergents are used in combination with an osmotic shock compared to the detection of the same when the lysis is obtained upon either an osmotic shock or the action of one or more detergents alone.

22. Taking document D1 as the closest state of the art (see points 4 to 6 *supra*), the technical problem to be solved may be seen as the provision of an alternative

sensitive method for the detection of low levels of microorganisms contained in a mammalian cell fermenter (see page 2, lines 1 to 3). The solution to that problem is a method according to claim 1 in which (i) the differential lyse of the mammalian cells is obtained by the combined effect of one or more detergents and of an osmotic shock, and (ii) the filtration is carried out through a micropartitioned hydrophilic/hydrophobic membrane made up of a number of hydrophilic filter sections substantially completely isolated from each other with latticed or circular hydrophobic micropartitions to immobilise said microorganisms.

23. The question to be answered for the assessment of inventive step is whether the skilled person would have found an incentive in any of the available prior art documents to replace in the method of document D1, as detailed in document D4, the 0,22 µm membrane by a membrane as that disclosed in the application **and** to carry out the differential lysis by adding an osmotic shock to the detergents used.

24. It is not doubtful that, for the reasons explained in point 9 ***supra***, the skilled person would have readily realised, as already acknowledged in point 10 ***supra***, that the membrane described in document D3 qualifies as a filter appropriate to the detection of low concentrations of microorganisms, such as those that may be contained as contaminants in a mammalian cell fermenter.

25. However, as indicated in point 21 *supra*, the skilled person would have found no guidance at all in the available state of the art to combine one or more detergents with an osmotic shock and would not have been aware that such a combination is synergistic. Therefore, in the light of the prior art on file, the board considers that the skilled person would not have arrived at the method of claim 1 in an obvious manner.

26. Thus, the board concludes that the subject-matter of claim 1 of the auxiliary request involves an inventive step. The same conclusion applies to the subject-matter of dependent claims 2 to 8. Therefore, the auxiliary request complies with Article 56 EPC 1973.

Concluding remark

27. Since the auxiliary request meets the requirements of the EPC, it forms the basis for the grant of a patent.

Adaptation of the description

28. At the oral proceedings, the appellant adapted the description to the auxiliary request. The board is satisfied that the description was satisfactorily amended in accordance with the EPC.

Order

For these reasons it is decided that:

1. The decision under appeal is set aside.

2. The case is remitted to the examining division with the order to grant a patent in the following version:

   Claims Nos.: 1 to 8 according to the auxiliary request, filed during the oral proceedings
   Description pages: 1 to 7, filed during the oral proceedings.

The Registrar: The Chairman:

A. Wolinski P. Julià