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(54) Title: MULTIPLEXED DETECTION OF NUCLEIC ACID TARGETS DIRECTLY FROM SAMPLES CONTAINING BLOOD

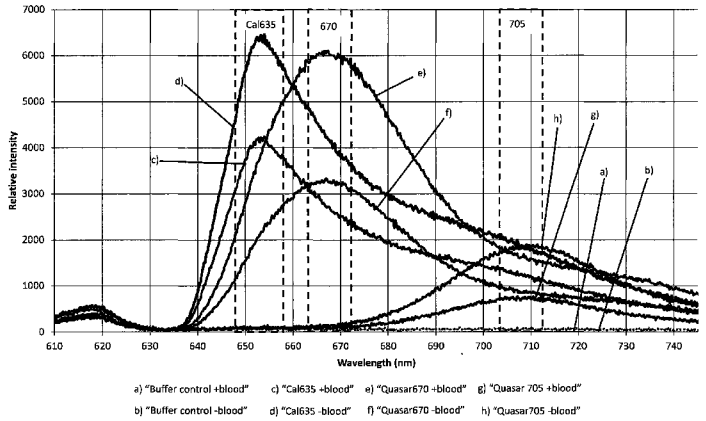

Fig. 2

Showing the spectrographic output (signal intensity against wavelength) of the fluorescence arising from 2 mW red (625nm) excitation power. Graph shows the impact of with (+) and without (-) the addition of 10% human blood. Illustrates that red centred dyes only display 30 to 50% inhibition in the presence of blood as opposed to the 85-95% observed for other visible wavelengths.

(57) Abstract: A process and apparatus for the multiplexed detection of nucleic acid targets directly from samples containing blood by quantitative real-time PCR means. The process comprises the addition of blood directly into the PCR reaction, including the nucleic acid of the target species, and an optical interrogation method centred on illumination at wavelengths exceeding 620nm. Preferably the illumination is provided by red 635nm laser diodes delivering excitation power in excess of 2mW and delivered to the sample by optic fiber, further that spectrophotometry is utilised to make possible multiplexed detection of targets in the presence of high amounts of blood. Such a process and apparatus is capable of overcoming the >90% inhibition of fluorescence that the presence of blood would normally present, rendering QPCR possible.

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— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

Multiplexed detection of nucleic acid targets directly from samples containing blood.

Field of Invention

The present invention relates to a process and apparatus for the multiplexed detection of nucleic acid targets directly from samples containing blood. A pertinent example is the concurrent detection of a target virus together with a human control gene amplified from the human DNA present in the white blood cells.

Background

The limited number of publications in this area suggest that the commonly used fluorescently labelled real-time PCR methodology cannot be utilised in the presence of any significant amounts of blood in the sample to be tested. It would clearly be advantageous for researchers and clinicians to be able to rapidly detect both pathogens and human targets directly from a blood sample, for example at the point of care, without the need to perform time consuming nucleic acid extraction.

The background to this invention lies in two distinct fields. The first is the applicant's own background knowledge in the fields of ultra-rapid PCR and the extension of this to the ability to directly amplify nucleic acids from a crude sample in a single closed tube format. The requirement for diagnostic tests that are both sensitive and take an absolute minimum time to detection has been clearly established, for example in the face of an emerging disease outbreak where in field screening would save many lives. The applicants have previously demonstrated direct rapid detection of target species from a number of matrices. One target which is both desirable and technically challenging is the direct screening of blood borne pathogens direct from a whole blood sample. The challenges comprise, firstly the amount of blood that can be accepted into the reaction and secondly the impact of the presence of this blood on the assay outcome.

The second relevant area of background information is the work of the group Barnes *et al* (Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples) (full reference of any publication is needed). There are also evolved enzymes and adjuncts designed to make direct PCR in the presence of inhibitors a possibility. This work showed that it is possible through mutation of the polymerase to make enzymes capable of amplifying DNA in the presence of whole blood. The same paper highlights the issue that thse present invention seeks to overcome,

fluorescence inhibition observed by the presence of human blood.

When real-time PCR is completed in the presence of whole human blood in a standard real-time thermal cycler there is observed a very high background and additionally an attenuation of fluorescence signals in the order of 85-95% depending on the wavelength of the dyes involved. The inventor has sought to overcome this with an intercalating dye approach but the significantly high background level from the whole human genomic DNA in the blood sample renders this approach both unsuited to clinical use but also makes multiplexing impossible. All clinical assays will have some form of internal control nucleic acid species and as such it has not been possible to perform direct diagnostic detection of target species from a whole blood sample.

The inventor has repeated earlier experiments to determine the effects of blood on fluorescence collection, however there is little background literature relating to the field of PCR since it was assumed it would not work. However, there are some papers in the field of directly fluorescently imaging blood for examining the brain and other organs. The data effectively shows why there is an 85-95% reduction in fluorescence - rendering direct multiplexed detection of nucleic acid targets direct from whole blood impossible. Firstly, there are strong absorption peaks at around 541 and 577nm and these completely absorb the fluorescence output from any common dyes in these wavelengths. Secondly, there is an observable red-shift; standard systems have narrow 10-20nm band pass filters in front of the detectors and any wavelength shift will clearly move a proportion of the light away from the wavelengths which will be collected. Thirdly, there is a strength quenching effect from the presence of the haemoglobin itself. In summary the commonly used combination of wavelength insensitive detector and the use of filters to enable the direct multiplexed detection of multiple nucleic acid targets in a single closed tube format will not work in the presence of blood.

The present invention comprises therefore an optical system for the detection of one or more nucleic acid targets from a sample containing whole blood.

Summary of the Invention

The inventors discovered that beyond the large quenching effect up to 620nm there is a much lower effect of the presence of blood, in the region of 20-30% as opposed to 85-95%

inhibition and as such the system provides a means of delivering high excitation powers of red light to the sample. The second key feature is the ability to detect the emitted fluorescence signals by the use of a spectrophotometer calibrated to collect light at 650nm and ranging as far as 750nm. By use of high excitation power red light and a spectrophotometer it becomes possible, via means of a spectral deconvolution approach, to simultaneously detect multiple target species in a single fluorescence reading. A pertinent example may be to distinguish between an infection resulting from the filovirus family while excluding infections from organisms causing similar symptoms and still have spectral space for an internal control. Standard PCR optical approaches use single point excitation sources in combination with emission filters and as such there would neither be sufficient separation between dye emission wavelengths or the ability to remove cross-talk between these channels. Systems do exist that have a red or far red excitation component, but these do not allow high level multiplexing when there is an absolute requirement for ....

According to a first aspect of the present invention therefore a method for the multiplexed detection of multiple nucleic acid targets directly amplified in a single closed tube format when the sample includes whole blood comprises the delivery to the sample of red excitation light, at greater than 620nm wavelength, at a power in the order of at least 2mW but preferably in excess of 5mW. In a preferred embodiment this excitation is delivered by a laser diode at 635nm but a range of 633-642 has proven to be applicable. To remove light emitting from the laser below the 640nm wavelength range either a band pass filter or short pass may be used. In an alternative embodiment LED excitation has been provided via coupling the LED into a large diameter core, high NA fiber and is similarly band pass filtered. Additionally a free space approach with regard to excitation has been demonstrated but the preferred embodiment is the fiber based delivery approach.

According to a second aspect of the invention there is provided apparatus for the multiplexed detection of multiple nucleic acid targets directly amplified when the sample includes whole blood, the apparatus comprising a reaction chamber arranged for effecting the amplification and means for the delivery to the sample of red excitation light, at greater than 620nm wavelength.

The excitation light is preferably delivered at a power in the order of at least 2mW but even more preferably in excess of 5mW. The excitation may be delivered by a laser diode at 635nm but a range of 633-642 has proven to be applicable. To remove light emitting from the laser below the 640nm wavelength range either a band pass filter or short pass may be

used. In an alternative embodiment LED excitation has been provided via coupling the LED into a large diameter core, high NA fiber and is similarly band pass filtered. Additionally a free space approach with regard to excitation has been demonstrated but the preferred embodiment is the fiber based delivery approach. The apparatus may incorporate a spectrophotometer for collation of the resultant emission profile.

In the preferred embodiment the system takes the form of an eight well randomly accessed system for direct in field detection (GB2015000027). This previous specification describes a system that utilises the HRM approach (US8597937) to enable direct freeze/thaw (EP2585581) mediated lysis and combined amplification in a single closed tube format. The eight well format means that all of the eight reaction vessels must transmit fluorescence signals to a single shared spectrophotometer and as such an optical fiber array forms the preferred embodiment. This consists of an octofurcated fiber array consisting of eight 200 micron core 0.22NA fibers each terminated to eight individual laser diode modules. These comprising a laser diode, control electronics, an aspheric lens for collimation and a band or short pass filter such that only light below 640nm is emitted. Each of these laser diodes is temperature controlled via means of being included within an off shoot of the HRM mechanism (US8597937) such that their temperature is held constant; laser diodes shift wavelength and excitation power with temperature shift and as such much be stabilised. The octofurcated delivery fibers are conjoined at a junction with eight collection fibers such that a double core fiber is terminated over the top of each sample reaction vessel, with one providing the excitation and a second collecting the emitted fluorescence. The eight collection fibers are brought together at the SMA connector terminus into an array of two by four as per the attached drawings and these are focused onto the spectrophotometer after passing through a long-pass filter that rejects any light below 650nm, such that only the intended emission light is imaged onto the spectrophotometer.

In use the preferred embodiment operates in a sequential fashion such that the illumination of each individual vessel in turn images one spectrum onto the detector. A means of electronic control then synchronises this illumination with both the time base and the heating system such that the contemporaneous reading from each well is taken at the correct time point.

It can clearly be envisaged that embodiments from one vessel with free space illumination to a single spectrophotometer, through different numbers of spectrophotometers and indeed differing fiber arrays can be constructed that meet the central tenet - the system must have

sufficient excitation power at a wavelength greater than 620nm and preferably utilise a spectrophotometer for collation of the resultant emission profile.

Brief Description of the Drawings

Embodiments of the invention will now be particularly described by way of example with reference to the accompanying drawings of which:

Figure 1a is a graph of relative detected intensity against excitation light wavelength, for various integration times with a no fluorophore negative control sample and no blood present;

Figure 1b is a graph as in figure 1a but with an intercalating dye and a DNA present;

Figure 1c is a graph as in figure 1a but when blood is present;

Figure 1d is a graph as in figure 1b but when blood is present;

Figure 2 is a graph showing the fluorescence arising from the use of red excitation;

Figure 3 is a perspective view of, an eight well field PCR apparatus;

Figure 4 is a plan view of the apparatus depicted in figure 3

Figure 5 is a cross section of the head array of the apparatus of figure 3;

Figure 6 is a plan view of the optical arrangement in the apparatus of figure 3;

Figure 7 depicts an optical excitation source array for the apparatus of figure 3;

Figure 8 shows a spectrophotometer;

Figure 9 shows an optical fibre array 13; and

Figure 10 shows a complete PCR apparatus exposed to show the PCR system and the optics.

Description of the Preferred Embodiment

Figures 1a-d and 2 show the effect of blood on the signal emanating from fluorescence in a sample undergoing PCR, with the vertical axes showing the relative intensity of the light and the horizontal axis showing the wavelength in nanometers (nm).

Figure 1a depicts the results from a control sample, that is one which contains no blood. The signal observed is that arising from a blue 473nm LED excitation.

Figure 1b shows the fluorescence generated by the addition of SYBR gold intercalating dye at normal concentration including the addition of 100ng (nanograms) of double stranded DNA. It shows the fluorescence saturating the detector at the higher integration times. For reference, 55,000 counts were generated over the background at 545nm.

Figure 1c shows the effect of adding 10% by volume human blood to the sample. There is an intrinsic autofluorescence with emission peaks at 560 and 610nm.

Figure 1d shows the results of setting up an identical experiment to that of figure 1b but with the addition of 10% by volume human blood to the sample. A number of absorption peaks, for example at 545nm can be observed, and also a marked increase in the autofluorescence at 610nm. Signal intensity at 545nm has dropped by over 92% compared to figure 1b.

The spectrographic output of the fluorescence from samples at 2mW red (625nm) under different parameters is shown in figure 2. The graphs show the impact of with (+) and without (-) the addition of 10% by volume human blood. It illustrates that red centred dyes Cal635, Quasar70 and Quasar 705 only display 30 to 50% inhibition in the presence of blood as opposed to the 85-95% observed for other visible wavelengths.

The figures 1a-d and 2 demonstrate in summary that the present invention overcomes the greater than 90% inhibition of fluorescence signal observed using conventional qPCR optical approaches.

As shown in figures 3 to 9 a preferred embodiment of the invention is an instrument 10 for the field detection of amplified of nucleic acid targets in the presence of whole blood. The blood may be human or that of another creature – so long as it's red!

The instrument 10 comprises eight randomly accessible reaction stations each possessing an individual optical detection head 11. There is a display monitor 12 for presenting analysis results to the end user. Each optical head 11 contains one leg of an octofurcated fiber array 13 secured in place via an SMA connector 14. Within each of these connectors is located a collimating lens 15 such that the excitation beam and the emission light emanating from the reaction vessel are collected into the fiber 13. The excitation beam is focused through a clear lid 16 to a reaction vessel 17 and to a point at the base of the reaction vessel. The distal end of the fiber array 13 is attached to eight red laser diodes 18 contained in SMA housings and each with a bandpass filter 19 to prevent unwanted wavelengths being injected into the fiber. The laser diodes 18 are driven by a PCB 2 driving the current to each device such that the excitation power can be normalised across the array 13.

Each optical detection head 11 is mounted to the apparatus via a pivot 21 enabling the head 11 to be swivelled clear of the reaction vessel 16 so that the vessel 16 can be emplaced and removed from the heating/cooling apparatus whereby PCR can be performed on the content of the vessel.

A water cooling block 22 is arranged for stabilising the temperature of the laser diode 17 assembly.

In this embodiment a laser diode 18 emitting at 635-638nm is paired with a band pass filter in the laser housing that passes 630-642 but blocks other wavelengths at OD6.to excite fluorescence in the reaction vessel.

A spectrophotometer 23 (figure 8) is arranged to receive the light emission from the reaction vessels 17 via the fibre array 13 and a paired 650 or 665nm long-pass filter 24 at the entrance to the spectrophotometer.23.

Figure 9 shows the optical fibre array 13. The fibre bundle 25 on the right comprises fibres attached to the laser diodes and contain a single core. These fibres enter a splltter 26 wherefrom emerge eight fibres now containing two cores 27, one leading back to the laser diodes 18 for excitation and the other leading to the spectrophotometer 23 for detection. The fibre 27 is the one located in the instrumenbt pivoting head.. The leg leading to the spectrophotometer 23 exits the splitter having now been combined into a single fibre 28 containing eight cores in a two by four array

Not shown in the drawings is the means by which a PCR reaction is carried out in the reaction vessels 17. The reaction vessel 17, which is of microtitre capacity,is snugly held in a reaction vessel receiving cup which is associated with the working face of a peltier cell in

such a way that a prescribed temperature can rapidly be reach in the reaction chamber of the reaction vessel. The base face of the peltier cell is associated with a heat source sink in which water is arranged to flow at a constant temperature intermediate the upper and lower temperatures of a PCR cycle.

In use whole human blood suspected of containing a potential target, such as a pathogen, is placed into the reaction vessel 17 with the correct primers and probes-labelled with for example Cy5.5, quasar 670, quasar 705 or any other multiplexed dyes known in the art. The laser is shone into the reaction vessel 17 as PCR proceeds and successful amplification generates spectra that are observed even in the presence of blood.

CLAIMS

1. A method for the multiplexed detection of nucleic acid targets directly amplified in a reaction with a sample when the sample contains blood, the method comprising excitation of the reaction at a wavelength greater than 620nm.

2. A method as claimed in claim 1 and carried out in a single closed tube format.

3. A method as claimed in claim 1 or claim 2 and wherein the excitation is effected with a power exceeding 2mW

4. A method as claimed in any one of claims 1 to 3 and wherein the excitation is effected with a power exceeding 5mW.

5. A method as claimed in any one of claims 1 to 4 and wherein the amplification reaction comprises PCR, isothermal amplification, RT-PCR.

6. A method as claimed in any one of the preceding claims and wherein subsequent processing comprises spectrally deconvoluting the multiple observed spectra arising from the plurality of signals being detected.

7. A method as claimed in any one of the preceding claims and wherein excitation is delivered at a range of 633-640nm.

8. A method as claimed in any one of the preceding claims and wherein excitation is delivered at 635nm.

9. A method as claimed in any one of the preceding claims and wherein a band pass filter or short pass is used to remove excitation below 640nm

10. A method as claimed in any one of the preceding claims and wherein the excitation is delivered by laser.

11. A method as claimed in claim 10 and wherein the excitation is delivered from a laser

diode.

12. A method as claimed in claim 11 and wherein the laser diode is kept at a constant temperature.

13. A method as claimed in claim 11 or claim 12 and wherein the laser diode is part of a module comprising a laser diode, control electronics, an aspheric lens for focusing.

14. A method as claimed in any one of claims 11 to 13 and wherein the laser diode is part of a module comprising a laser diode, control electronics, an aspheric lens for focusing.

15. A method as claimed in any one of claims 1 to 9 and wherein excitation is provided by a light emitting diode (LED)

16. A method as claimed in claim 15 and wherein excitation is provided via coupling the LED into a large diameter core, high numerical aperture (NA) fibre and is similarly band pass filtered into a large core, high NA fibre.

17. A method as claimed in any one of the preceding claims and comprising effecting and controlling the reaction employing a peltier cell having a working face arranged to heat and cool the reaction and a base face kept at a constant temperature intermediate the upper and lower temperatures of the reaction.

18. A method as claimed in claim 17 and wherein the Peltier cell base face is located on a heat exchanger through which flows liquid at the said constant temperature.

19. Apparatus for the multiplexed detection of multiple nucleic acid targets directly amplified when the sample includes blood, the apparatus comprising a reaction chamber arranged for effecting the amplification and means for the delivery to the sample of red excitation light, at greater than 620nm wavelength,

20. Apparatus as claimed in claim 19 and having fluorescence collection means comprising a spectrophotometer.

21. Apparatus as claimed in claim 19 or claim 20 and in single closed tube format.

22. Apparatus as claimed in any one of claims 19 to 21 and having excitation means arranged to operate with a power exceeding 2mW

23. Apparatus as claimed in any one of claims 19 to 22 and having excitation means arranged to operate with a power exceeding 5mW.

24. Apparatus as claimed in any one of claims 19 to 23 and wherein the amplification reaction comprises PCR, isothermal amplification, RT-PCR.

25. Apparatus as claimed in any one of claims 19 to 24 and arranged to deliver excitation at a range of 633-640nm.

26. Apparatus as claimed in any one of claims 19 to 24 and arranged to deliver excitation at 635nm.

27. Apparatus as claimed in any one of claims 19 to 25 and incorporating a band pass filter or short pass in front of the excitation source to remove excitation below 640nm

28. Apparatus as claimed in any one of claims 19 to 27 and incorporating a laser diode to deliver the excitation.

29. Apparatus as claimed in claim 28 and having means to keep the laser diode at a constant temperature.

30. Apparatus as claimed in claim 27 or claim 28 and wherein the laser diode is part of a module comprising a laser diode, control electronics, an aspheric lens for focusing.

31. Apparatus as claimed in any one of claims 19 to 27 and incorporating a light emitting diode (LED) as the excitation means.

32. Apparatus as claimed in claim 31 and wherein the LED is coupled into a large diameter core, high numerical aperture (NA) fibre and the apparatus further comprises a band pass filter feeding into a large core, high NA fibre.

33. Apparatus as claimed in any one of claims 19 to 32 and comprising a peltier cell having a working face arranged to heat and cool the reaction and a base face arranged to be kept at a constant temperature intermediate the upper and lower temperatures of the reaction.

34. Apparatus as claimed in claim 33 and wherein the Peltier cell base face is located on a heat exchanger through which is arranged to flow liquid at the said constant temperature.

35. Apparatus as claimed in any one of claims 19 to 34 and comprising a fiber array arranged to convey light between the light source and the reaction vessel and between the reaction vessel and a light detector.

36. Apparatus as claimed in claim 35 and wherein the fiber array consists of 200 micron core 0.22NA fibers

37. Apparatus as claimed in any one of claims 19 to 36 and comprising eight randomly accessed microtitre reaction wells.

38. Apparatus as claimed in claim 37 and having an octofurcated fiber array.

39. Apparatus as claimed in claim 38 and wherein the octofurcated delivery fibers are conjoined at a junction with eight collection fibers.

40. Apparatus as claimed in claim 39 and wherein a double core fiber is terminated over the top of each sample reaction vessel, with one providing the excitation and a second collecting the emitted fluorescence.

41. Apparatus as claimed in claim 39 or claim 40 and wherein the eight collection fibers are brought together at an SMA connector terminus into an array of two by four.

42. Apparatus and method as claimed in claim 40 and wherein the array of two by four collection fibers are focused onto the spectrophotometer after passing through a 665nm

long-pass filter such that only the intended emission light is imaged onto the spectrophotometer.

43. Apparatus as claimed in claim 42 and comprising a sequencer arranged for sequencing the order of excitation of the reaction vessel contents in order to present a unique signal at the spectrophotometer in respect of each reaction vessel.

44. Apparatus and method as claimed in any one of claims 19 to 43 and wherein light is conveyed between the light source, the reaction vessel and the collector by a lens and mirror arrangement.

45. A method for the multiplexed detection of nucleic acid targets directly amplified in a reaction with a sample when the sample contains blood, and substantially as hereinbefore described with reference to the accompanying drawings

46. Apparatus for the multiplexed detection of nucleic acid targets directly amplified in a reaction with a sample when the sample contains blood, and substantially as hereinbefore described with reference to the accompanying drawings

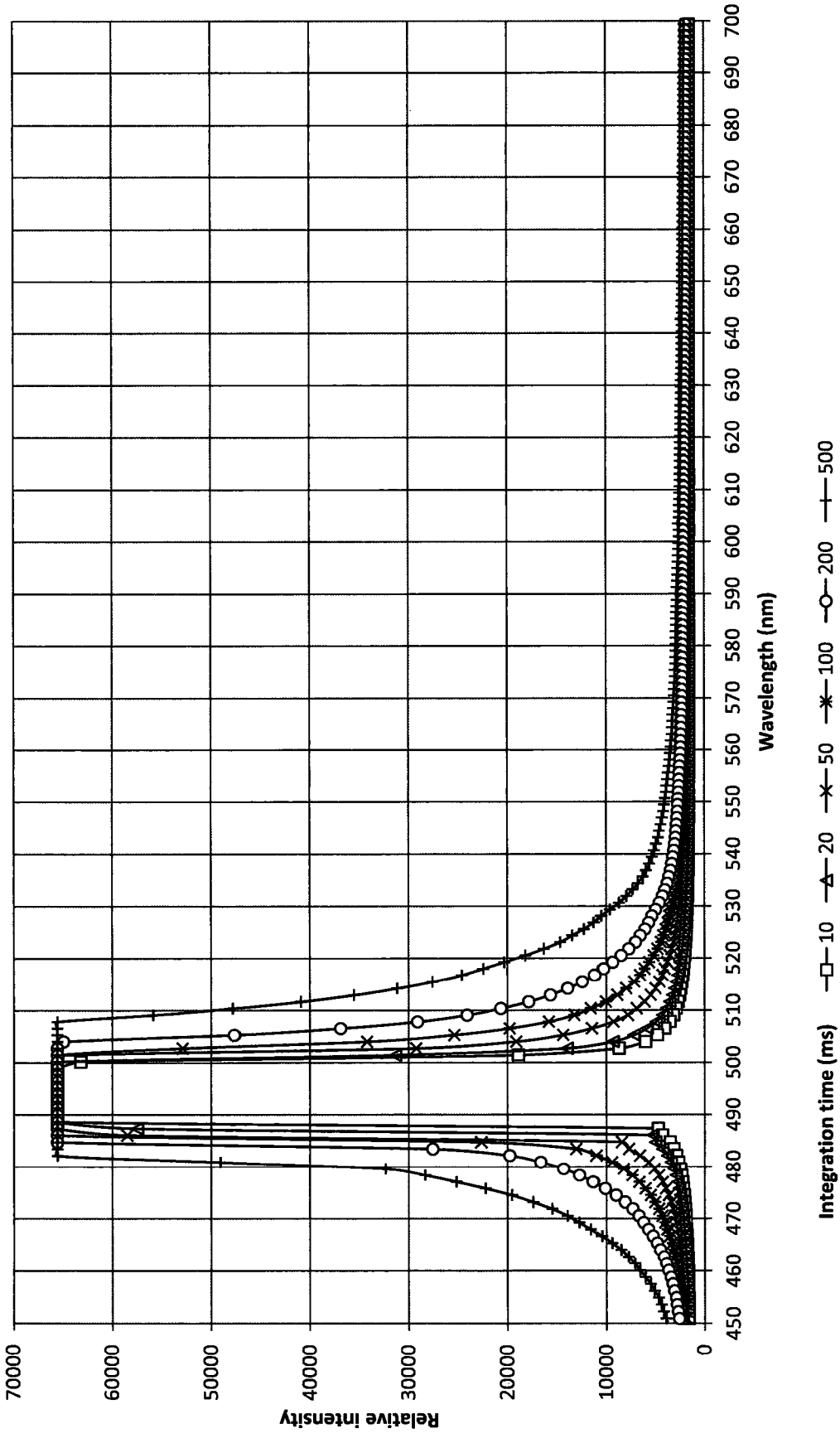


Fig. 1a

Shows a no fluorophore negative control sample with no blood added. The signal observed is that arising from the blue 473nm LED excitation.

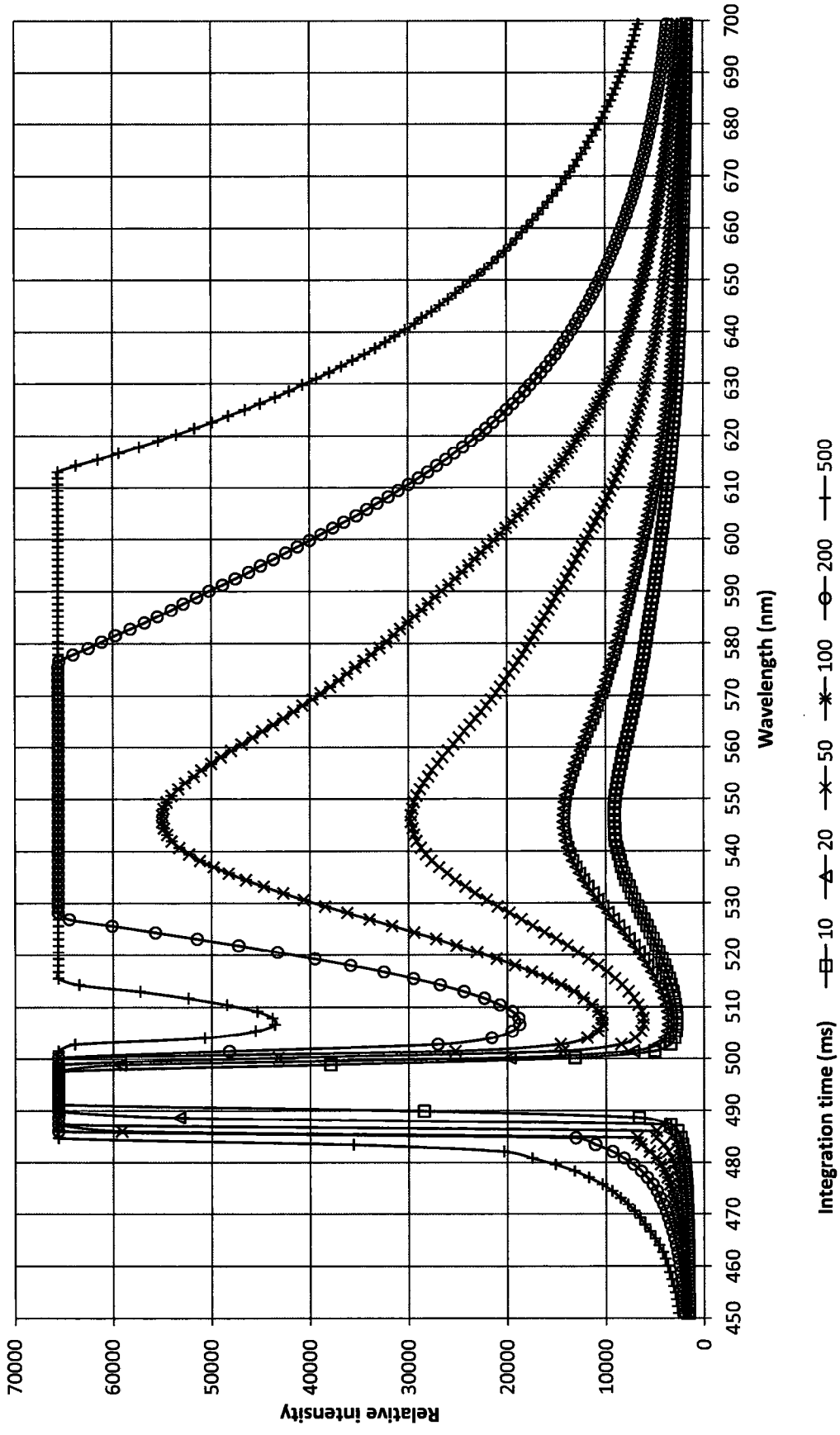


Fig. 1b

Shows the fluorescence generated by the addition of SYBR Gold intercalating dye at normal concentration plus the addition of 100ng of double stranded DNA. Fluorescence saturates detector at higher integration times and for reference generated 55,000 counts over background at 545nm.

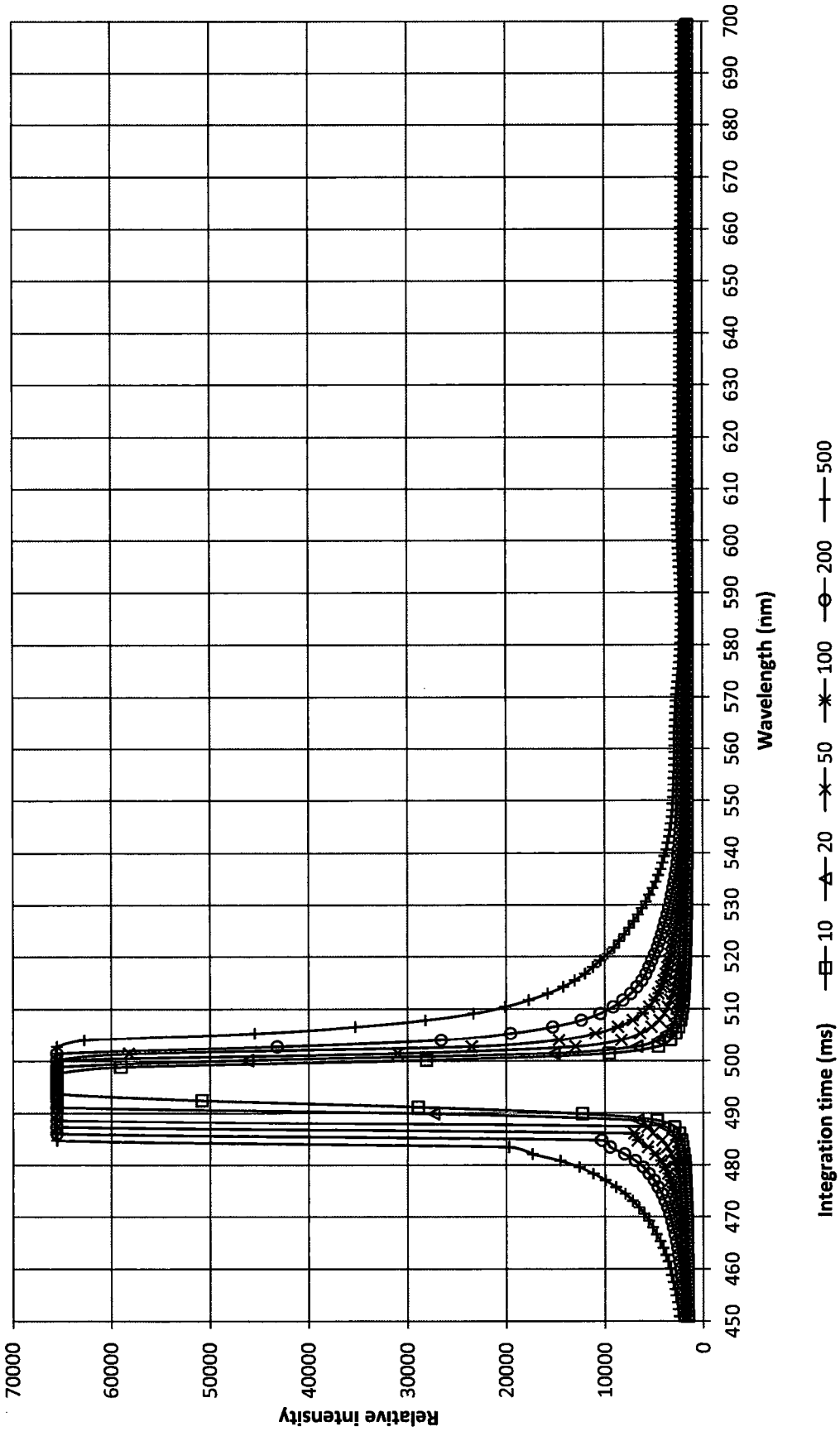


Fig. 1c

Demonstrates the intrinsic autofluorescence of the 10% human blood itself with emission peaks at 560 and 610nm

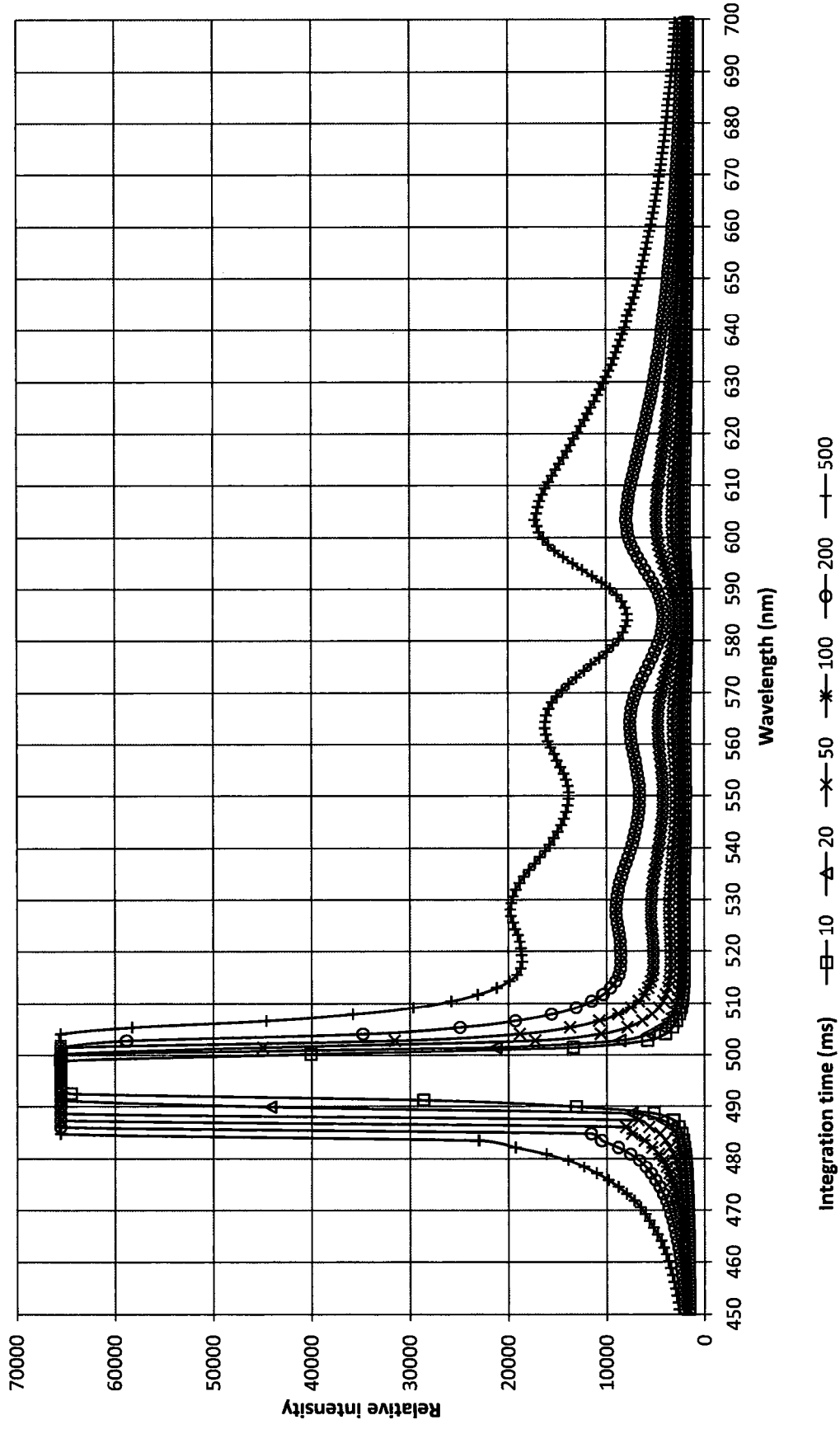
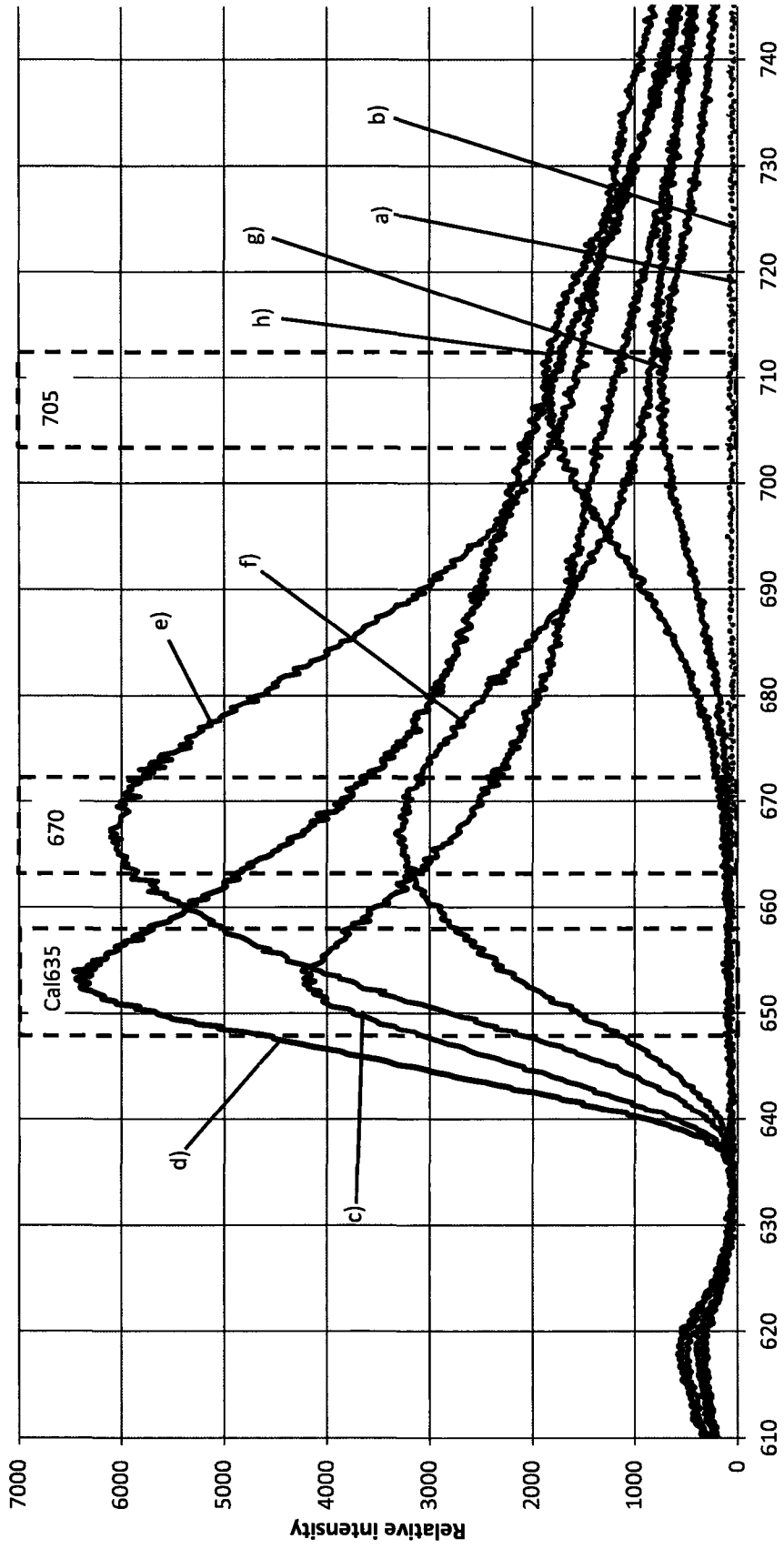


Fig. 1d

Identical experimental set up to Fig 1B but with the addition of 10% by volume human blood. A number of absorption peaks, for example at 545nm can be observed and also a marked increase in the autofluorescence at 610nm. Signal intensity at 545nm has dropped by over 92% compared to figure 1b.



a) "Buffer control +blood" c) "Cal635 +blood" e) "Quasar670 +blood" g) "Quasar 705 +blood"

b) "Buffer control -blood" d) "Cal635 -blood" f) "Quasar670 -blood" h) "Quasar705 -blood"

Fig. 2

Showing the spectrographic output (signal intensity against wavelength) of the fluorescence arising from 2 mW red (625nm) excitation power. Graph shows the impact of with (+) and without (-) the addition of 10% human blood. Illustrates that red centred dyes only display 30 to 50% inhibition in the presence of blood as opposed to the 85-95% observed for other visible wavelengths.

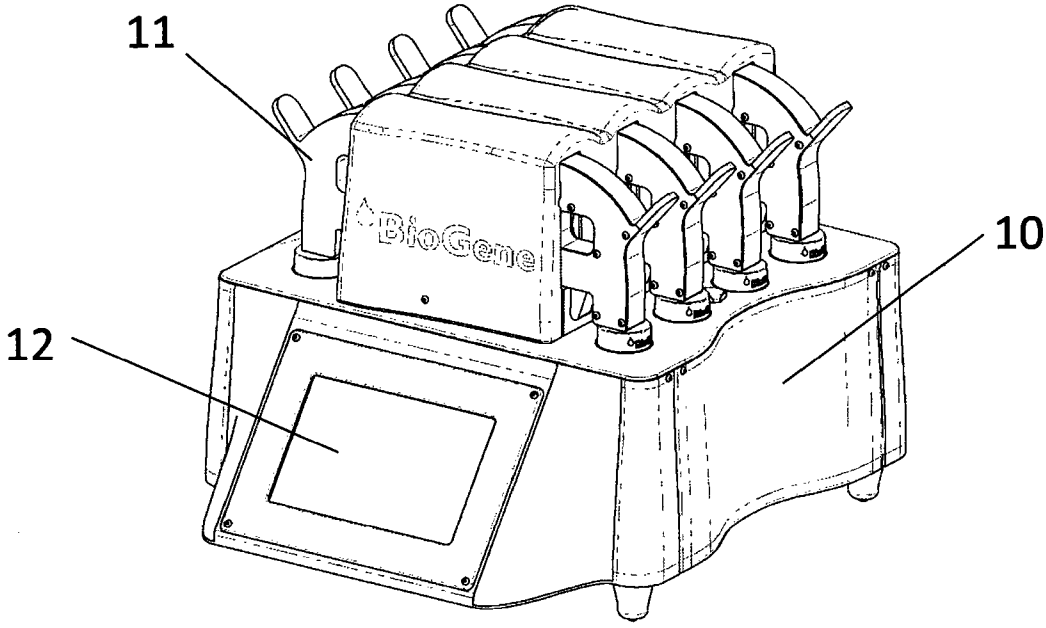


Fig 3.

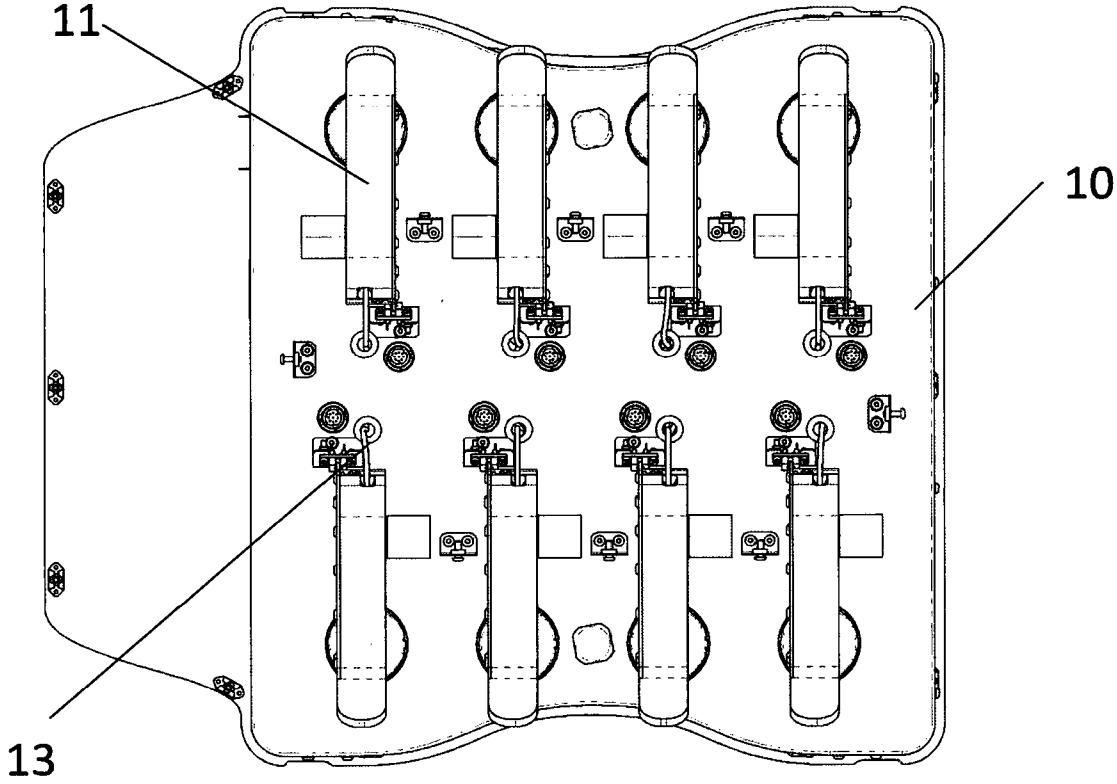


Fig 4.

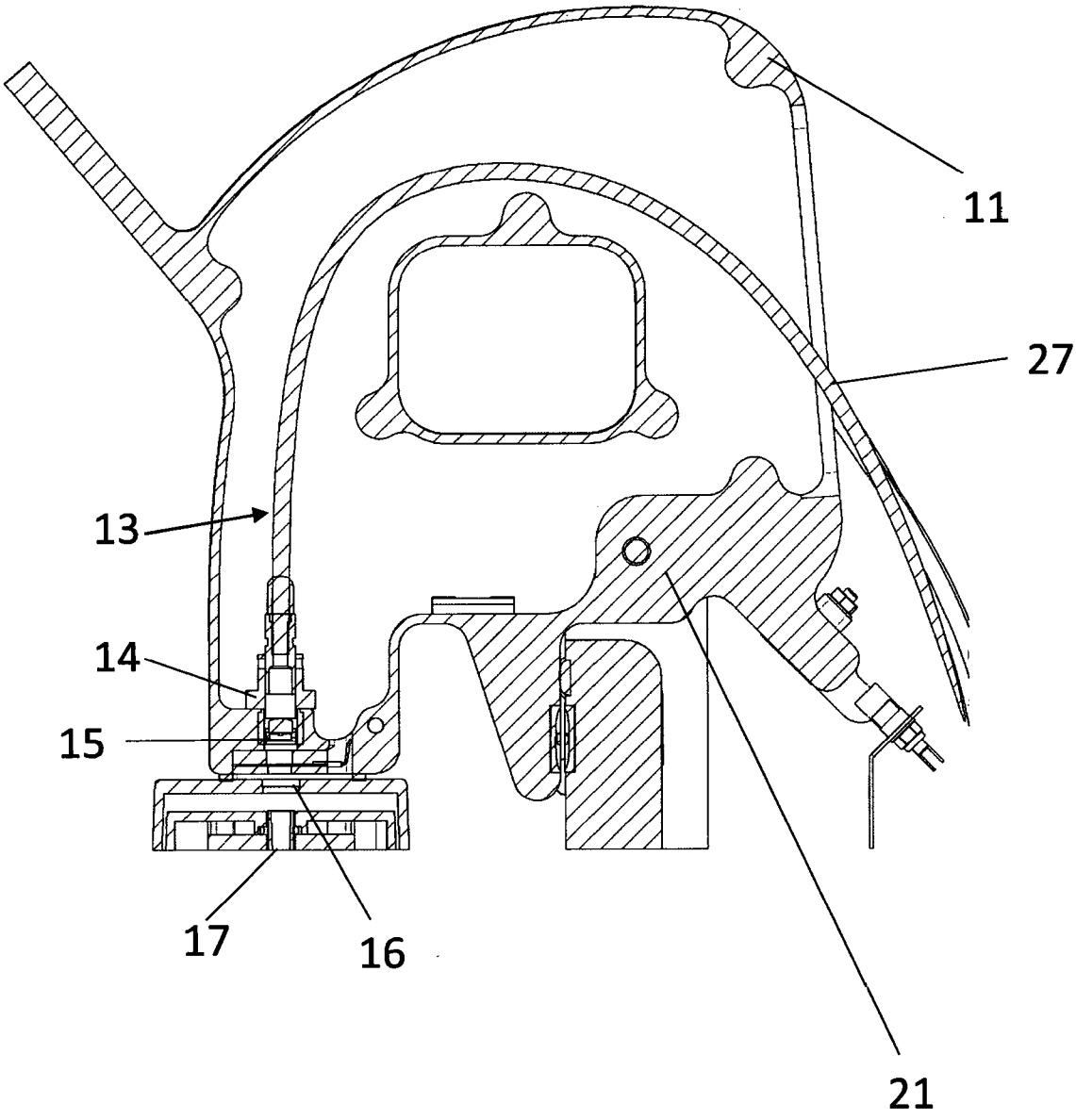


Fig 5.

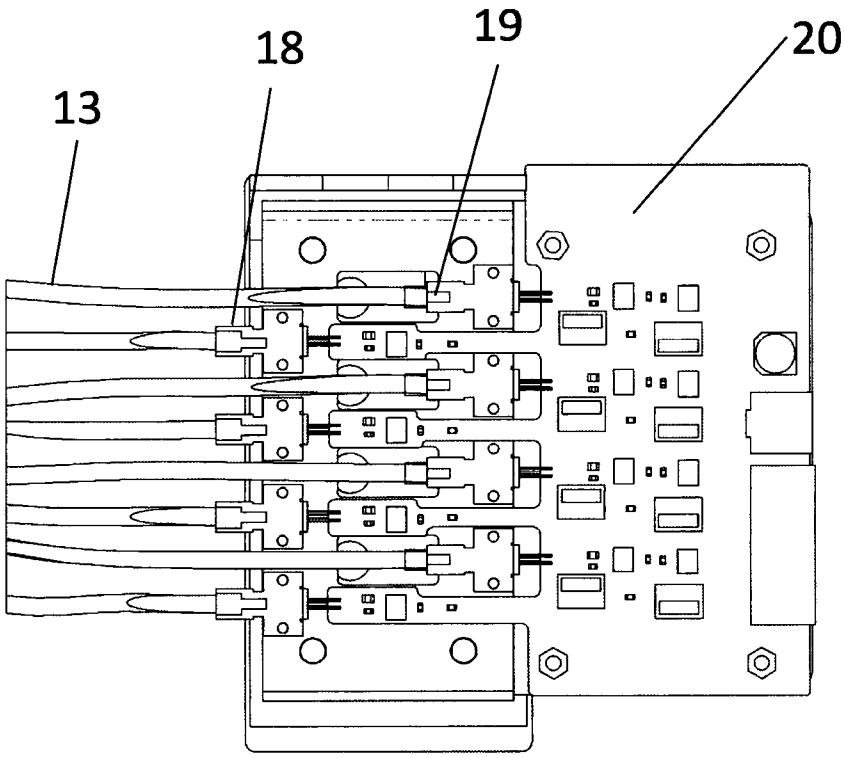


Fig 6.

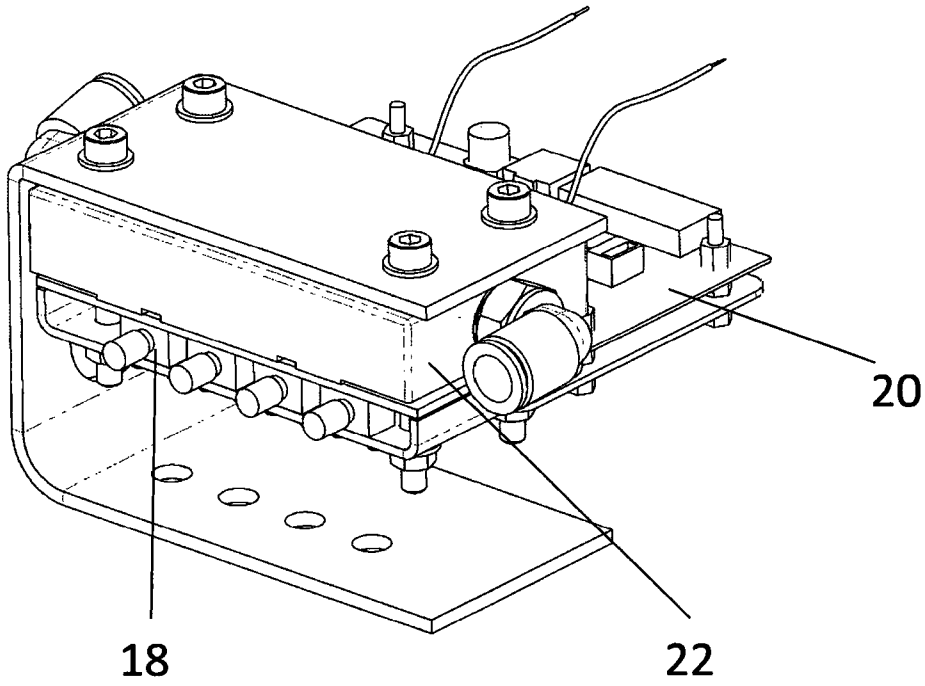


Fig 7.

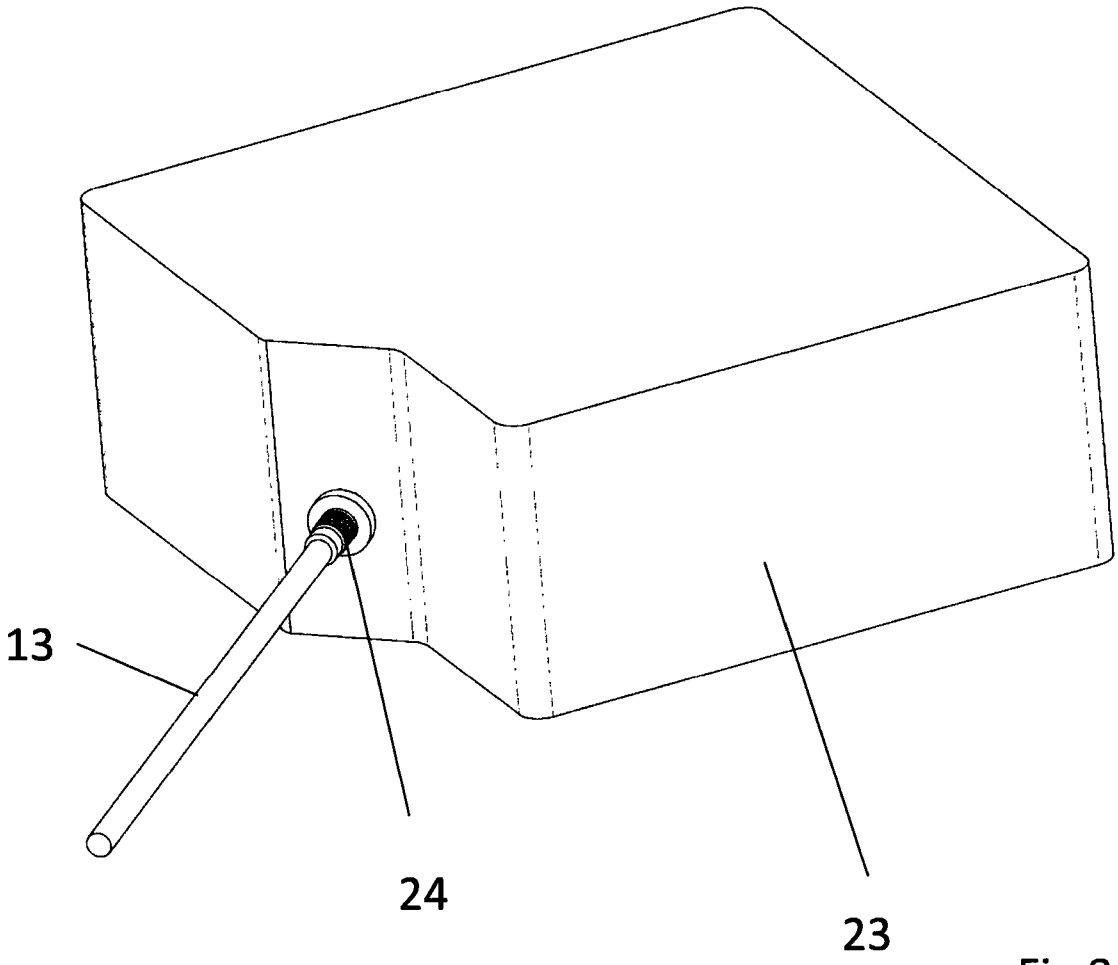


Fig 8.

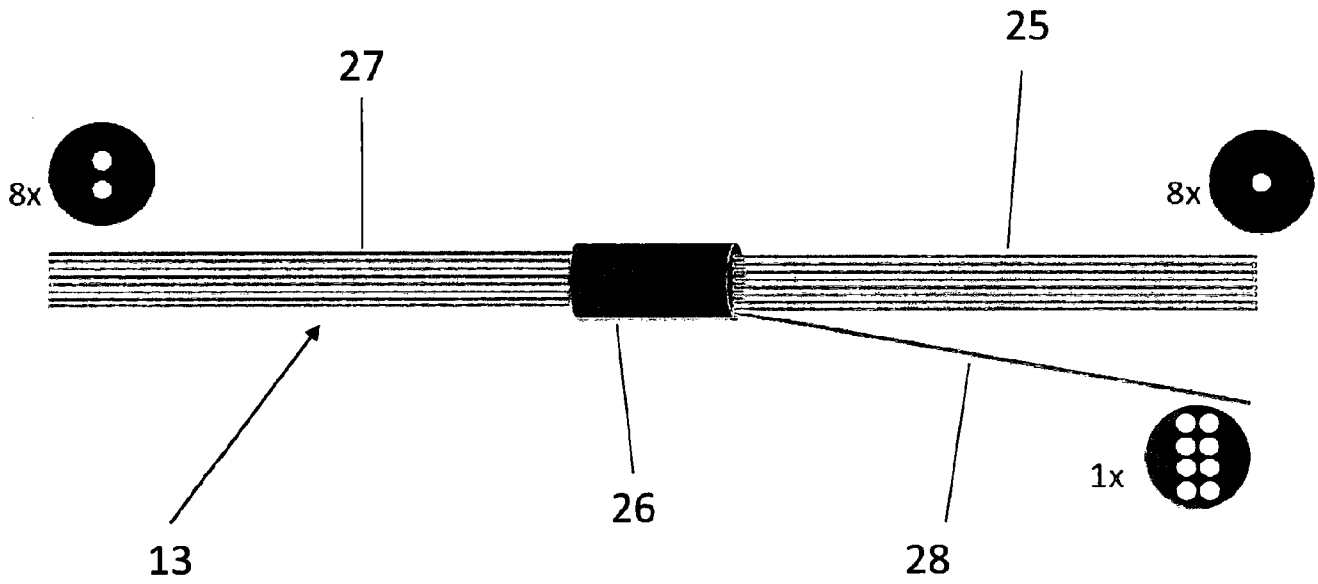


Fig 9.

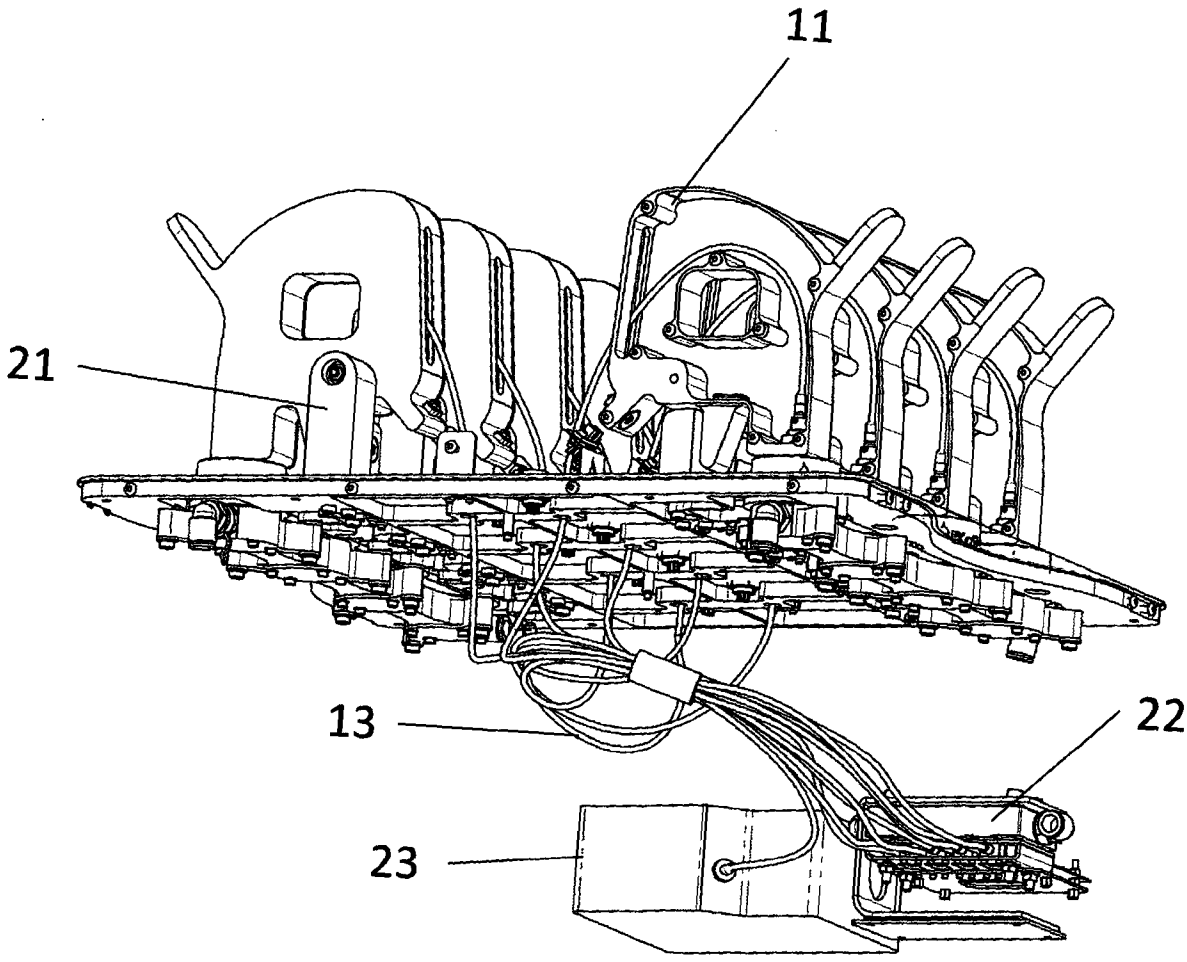


Fig 10.

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/000046

A. CLASSIFICATION OF SUBJECT MATTER
INV. B01L7/00 C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
B01L C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	"MxPro QPCR Software for Mx3000P and Mx3005P QPCR Systems", , 1 March 2009 (2009-03-01), pages 1-433, XP55276973, Retrieved from the Internet: URL:http://www.agilent.com/cs/library/user manuals/public/MxPro_Manual.pdf [retrieved on 2016-06-01] page 13, last par.; page 198, last par.; screenshots on pages 47, 51 and 52 ----- -/--	19,46

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 June 2016	29/06/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Leber, Thomas

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZHIAN ZHANG ET AL: "Direct DNA Amplification from Crude Clinical Samples Using a PCR Enhancer Cocktail and Novel Mutants of Taq", THE JOURNAL OF MOLECULAR DIAGNOSTICS, vol. 12, no. 2, 1 March 2010 (2010-03-01), pages 152-161, XP055001258, ISSN: 1525-1578, DOI: 10.2353/jmoldx.2010.090070 page 156, right col. and Fig. 5	1-18, 20-46
Y	A. CASTLEY: "Clinical Applications of Whole-Blood PCR with Real-Time Instrumentation", CLINICAL CHEMISTRY, vol. 51, no. 11, 1 November 2005 (2005-11-01), pages 2025-2030, XP055167243, ISSN: 0009-9147, DOI: 10.1373/clinchem.2005.055327 page 2015, right col.; page 2026, left col.; Fig. 1;	1-18, 20-46
Y	BRIAN J TAYLOR ET AL: "Real-time PCR detection of Plasmodium directly from whole blood and filter paper samples", MALARIA JOURNAL, BIOMED CENTRAL, LONDON, GB, vol. 10, no. 1, 19 August 2011 (2011-08-19), page 244, XP021092124, ISSN: 1475-2875, DOI: 10.1186/1475-2875-10-244 page 2 right col.; Fig. 1	1-18, 20-46
Y	LUTZ ERIC LEHMANN ET AL: "A multiplex real-time PCR assay for rapid detection and differentiation of 25 bacterial and fungal pathogens from whole blood samples", MEDICAL MICROBIOLOGY AND IMMUNOLOGY, SPRINGER, BERLIN, DE, vol. 197, no. 3, 16 November 2007 (2007-11-16), pages 313-324, XP019630543, ISSN: 1432-1831 title; abstract	1-18, 20-46

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