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DE 003733986 A1 **US 6258291 B1**
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(54) Title of the Invention: **Binding surfaces**
Abstract Title: **Preparing a surface for binding to a biomolecule**

(57) A method for preparing a surface which binds or is bindable to a biomolecule through electrostatic forces, said method comprising exposing a surface to a plasma containing a monomer which is capable of forming a polymer having the appropriate electrostatic properties, under conditions in which the monomer is deposited on the surface so as to form a polymer which has said properties. The monomer may be a hydrocarbon or a halocarbon which includes a polymerisable group, such as an alkene, alkyne or acrylate group. The surface may be an assay plate, slide, culture plate, filtration medium or bonding medium. The biomolecule may be a monoclonal antibody or protein.

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Binding Surfaces

The present invention relates to the production of surfaces that allow for the immobilisation of biological molecules, in particular nucleic acid such as DNA, as well as to methods for producing these. Such surfaces may be used for example on laboratory devices and filtration media as well as on implants used in medicine.

For many biological or biochemical assays or procedures, immobilisation of biomolecules, and in particular nucleic acids such as DNA or RNA, onto surfaces is required. For example, procedures such as nucleic acid extraction, purification and/or amplification require that biomolecules such as nucleic acids in a sample are bound effectively to a surface. In further applications, immobilised nucleic acids are utilised for further investigation, such as in high-throughput screening, in amplification for example by PCR and detection, or in the arrays utilised in the so-called "lab-on-a-chip" devices.

In yet further applications, cells are cultured in vessels which are rendered permanently hydrophilic to support cell attachment and spreading.

In all cases, there is a need to modify a surface so that it interacts with a biomolecule, either a specific biomolecule or a cell, so that it becomes immobilised on the surface. This may be by means of covalent bonding, in which molecules containing reactive chemical groups are attached to the surface, and the biomolecules become attached to the reactive chemical groups, optionally in the presence of a coupling agent. For example, where the surface contains free >NH groups, such as amino or imino groups, these can be coupled to carboxy groups present in biomolecules such as nucleic acids to form amide bonds. The coupling is suitably effected in the presence of a coupling agent such as ethyldiethylaminopropyl-

carbodiimide (EDC), dimethylpimelidate and dissucinimidyl suberate, optionally in the presence of N-hydroxysuccinimide, or where the biomolecule to be bound contains amino groups of lysine, a reduction reaction using a reducing agent such as sodium cyanoborohydride to form a glutaraldehyde linkage.

However, in some cases, electrostatic charges, dipoles or temporary dipoles are sufficient to retain the biomolecule or cell in place. Such interactions tend to be of the non-specific variety and so the use of these binding mechanisms can increase the non-specific binding whilst preventing specific binding.

The nature of the surface material will depend upon the nature of the device or filtration media being prepared. Where the device is for example a microtitre or assay plate, slides or culture plates or the like, it is generally a polymeric material such as polystyrene, polyurethane, polyacrylates such as polymethacrylate, or polyalkylene such as polyethylene, polycarbonate or polypropylene. Slides and culture plates may also be glass. Filtration and binding media may take various forms such as membranes, for example cellulose or nitrocellulose membranes, or binding media such as polystyrene, silica-iron or other magnetic beads. Generally however, modification of the surface to provide effective electrostatic interactions is required for these items to bind biomolecules efficiently. The material could also be a ceramic, metal or other material for use in body implants or growing tissue or cell cultures.

The degree of binding required is very much associated to the particular assay, or process such as extraction, purification or amplification, in question and may require medium or high binding to specific biological or chemical reagents. When binding proteins and DNA for example, a hydrophobic or a

combined hydrophobic and ionic surface can change the degree of binding and so give different results.

To date, processes used in surface modification are based upon solution chemistries. However, with such processes, only a molecular layer functionalization is possible, and close control over the properties of the surface is difficult to achieve.

Plasma coating processes using monomers containing functional groups such as amines and aldehydes are described in US2006/0251693 and US2003/0054434. The applicants have found however, that plasma polymerisation of in materials such as simply hydrocarbons or halocarbons, can produce useful surfaces that effect electrostatic surfaces.

According to the present invention there is provided a method for preparing a surface which binds or is bindable to a biomolecule through electrostatic forces, said method comprising exposing a surface to a plasma containing a monomer which is capable of forming a polymer having the appropriate electrostatic properties, under conditions in which the monomer is deposited on the surface so as to form a polymer which has said properties.

The monomer is suitably a hydrocarbon or halocarbon monomer as described in more detail below.

By utilising plasma deposition as a means of applying the polymer to the surface, close control may be maintained of the resultant polymer structure and therefore the density and availability of the electrostatic charge. The deposition mode can be controlled and varied for instance by controlling the power of the plasma, whether or not the plasma is continuous or pulsed, the deposition time, the concentration of the monomer etc. By controlling these factors, the degree of fragmentation

and rate and nature of deposition of the monomer can be selected, and thus the structure of the polymer formed on the surface can be controlled. The structure may even be varied as the polymer is grown.

By utilization plasma polymerization either in the conventional continuous wave mode or by power modulation, and in some instances a combination of the two either as a constant build-up of polymer at a fixed density or by employing a gradient type approach to ensure good adhesion whilst retaining the desired chemical properties; very specific and desired binding characteristics can be achieved.

Depending on the degree of binding required the process gasses and vapours can be varied and fragmented to different degrees in order to give the desired surface. The surface can be assessed in numerous ways as to its fitness for purpose, however dynamic contact angle measurement is one suitable parameter which may be used to probe or test whether the desired interactions occur.

In this way, a polymeric layer carrying a predetermined electrostatic charge, for example, an appropriate level of hydrophilicity or hydrophobicity to bind a specific biomolecule may be achieved. Where hydrophilic surfaces are required, the polymer may be designed so that it has areas of high electron density, which may be achieved, for example by designing the polymer so that there are unsaturated bonds in the surface area. Hydrophobic surfaces may be produced by having for example halogenated areas, in particular perhalogenated groups, at the surface of the polymer. The use of plasma polymerisation techniques in a controlled way, can allow for such precise adaptation of the final product.

Using this method, plasmas are generated from organic molecules, which are subjected to an electrical field. When

this is done in the presence of a substrate, the radicals of the compound in the plasma polymerise on the substrate. Conventional polymer synthesis tends to produce structures containing repeat units that bear a strong resemblance to the monomer species; whereas a polymer network generated using a plasma can be extremely complex. The properties of the resultant coating can depend upon the nature of the substrate as well as the nature of the monomer used and conditions under which it is deposited.

The nature of the polymer coating applied in any particular case, will be determined by the desired end use. For instance, the nature of the monomer used will depend upon the desired properties of the final coating and the amount of such groups which are required to allow the surface to fulfil the function.

The monomers used in the process will generally comprise one or more hydrocarbon molecules but may contain halo substituents, unsaturated groups or other groups which modify the electrostatic properties and in particular the hydrophobicity or hydrophilicity. The monomer may comprise a mixture of compounds, which are the same or different. The arrangement of modifying groups within the monomer may be such that they are favourably orientated in the final polymer layer. For instance, where the monomer readily polymerises at say an unsaturated group at one end, location of say one or more hydrophobic substitutent at the other end will mean that it is likely that the hydrophobic substituents will be readily available on the surface of the final polymer layer, and thus it will have a high level of hydrophobicity. Alternatively, where hydrophilic surfaces may be required, the In this case however, since unsaturated groups may themselves be readily polymerisable, the monomer should be designed such that the group provided at the end of the molecule where polymerisation is to occur is more highly activated towards polymerisation than the unsaturated group at the other end, so that the

desired polymerisation is still preferred. In this case, an acrylate, which is a very readily polymerisable group, may be used at one end of the molecule, whilst an alkene moiety at the other may remain intact in the final polymer and provide an area of high electron density.

In addition, further modifying groups may be arranged along the polymer chain, ensuring effective capture or binding of the target biomolecule in three-dimensions.

Generally the monomers used will not contain reactive or functional groups such amino, mono-substituted amino, imino, carboxyl or carbonyl groups, which have a tendency to react to form covalent bonds with the biomolecules. (Where the monomers contain acrylate groups to facilitate polymerisation, these are suitably arranged such that they will not be at the surface of the final polymer layer, so that they do not interact with the biomolecules.)

Rather, the binding in the present case is intended to be more "loose" and so may be required in particular in situations where reversible binding of biomolecules may be required, for example in some types of immunoassay or purification device, where biomolecules such as antibodies, antigens or whole cells are required to be associated with a surface, for example on a magnetic bead or microtitre plate, and then washed off for subsequent detection or analysis. Such methods may be utilised for example in the detection of bacteria or the like for instance in food testing.

Similarly, culture plates may be prepared in which cells become "fixed" to the surface for growth purposes, but can be removed intact at a later date, for example by colony lifting techniques, for example onto membranes.

Thus monomers which may be utilised in the process include for example hydrocarbons and halocarbons, in particular fluorocarbons which are either straight chained, branched or cyclic. The molecules may be saturated, but will often include a polymerisable group such as a group with an unsaturated element, such as an alkene, alkyne or acrylate group.

As used herein, the expression "hydrocarbons" refers to organic compounds which contain hydrogen and carbon atoms. Examples of such compounds include those which comprise alkyl, alkenyl, alkynyl, aryl, cycloalkyl, alkylcycloalkyl, aryl (such as phenyl or naphthyl), alkylaryl, arylalkyl, alkylcycloalkylalkyl, alkylarylalkyl, alkenylcycloalkyl, alkenylaryl, arylalkenyl, alkenylcycloalkylalkyl, alkylarylalkenyl, alkylcycloalkylalkenyl, alkenylarylalkyl, alkynylcycloalkyl, alkynylaryl, arylalkynyl, alkynylcycloalkylalkyl, alkylarylalkynyl, alkylcycloalkylalkynyl or alkynylarylalkyl groups.

Where such groups comprise alkyl, alkenyl or alkynyl chains, these may be straight or branched. Suitably hydrocarbyl groups contain from 1 to 30, suitably from 1 to 10 and preferably from 1 to 6 carbon atoms, depending upon the desired properties of the final polymeric product.

The expression "halocarbons" refers to hydrocarbons as described above where some or all of the hydrogen atoms are substituted by a halogen atom such as fluorine, chlorine, bromine or iodine. Halogen groups will produce an area of hydrophobicity which may be desirable in particular instances such as where it is desirable to modify and particularly to reduce the surface energy or the accessible binding sites, so as to create preferred steric interactions at the surface. A particularly preferred halocarbons for hydrophobicity are fluorocarbons, but these may have further substitutions, which include chlorine, bromine or iodine groups to further increase

the range of electrostatic interactions, or be provided in mixture with chloro, bromo or iodocarbon compounds.

The number and nature of any polymerisable groups in the molecule will have an effect on the nature of the final surface as these will be more readily reacted at low power, to form a more structured polymer. Thus the relationship of these to any hydrophobic or other unsaturated groups present in terms of number and position will effect the availability and density of the hydrophobic or other unsaturated groups in the final coating.

In particular the monomer will contain from 2 to 10 carbon atoms. Preferably the monomer may contain a degree of unsaturation, and so comprise an alkene or alkyne. Alternatively, the unsaturation may be present in a polymerisable group which may be an optional substituent on or interposed within a hydrocarbon or fluorocarbon chain. A particular example of such a group is an acrylate group. Unsaturation in the molecule may assist in directly the polymerisation process as these active bonds are prone to breakage and reforming in the polymerisation.

However, even saturated compounds may be polymerised using the plasma technique, as the activation converts the monomer compounds into radicals which are able to combine together to form polymeric moieties.

Particular examples of monomers for use in the method of the invention are CH_4, C_3H_8, C_7H_{16}, C_3H_6, C_7H_{14}, cyclopentane, allyl cyclohexane, 2,2-dimethylbutane and 2-methylpropane, but these are representative molecules only and many others might be possible.

Relatively long chain monomers, for example containing from 10-30 carbon atoms, deposited under mild plasma conditions where

little fragmentation may take place will tend to give rise to well ordered polymeric layers where the arrangement of any hydrophobic or other unsaturated groups is largely retained, and may be well ordered.

However, where densely packed hydrophobic or other unsaturated groups may be required, high power conditions, leading to significant fragmentation and/or the use of small chain monomers may lead to a more suitable coating.

In particular, the monomers should be sufficiently volatile to allow them to be introduced into a plasma chamber in a gas phase. Therefore, the monomer of formula (I) suitably contains less than 30 carbon atoms, for instance less than 10 and in particular less than 6 may be preferred.

In the method, in general, the substrate to be treated is placed within a plasma chamber together with one or more monomer or gas, which are able to generate the target polymeric substance, in an essentially gaseous state, a glow discharge is ignited within the chamber and a suitable voltage is applied.

As used herein, the expression "in an essentially gaseous state" refers to gases or vapours, either alone or in mixture, as well as aerosols.

The gas present within the plasma chamber may comprise a vapour of the monomeric compound alone, but it may be combined with a carrier gas, in particular, an inert gas such as helium or argon. In particular helium is a preferred carrier gas as this can minimise fragmentation of the monomer.

When used as a mixture, the relative amount of the monomer vapour to carrier gas is suitably determined in accordance with procedures that are conventional in the art. The amount of monomer added will depend to some extent on the nature of the

particular monomer being used, the nature of the substrate being treated, the size of the plasma chamber etc. Generally, in the case of conventional chambers, monomer is delivered in an amount of from 50-250mg/min, for example at a rate of from 100-150mg/min. Carrier gas such as helium is suitably administered at a constant rate for example at a rate of from 5-90sccm, for example from 15-30sccm. In some instances, the ratio of monomer to carrier gas will be in the range of from 100:0 to 1:100, for instance in the range of from 10:0 to 1:100, and in particular about 1:0 to 1:10. The precise ratio selected will be so as to ensure that the flow rate required by the process is achieved.

The plasma used may be continuous wave or pulsed depending on the degree of monomer retention required in addition to the level of durability specified. Using pulsed plasmas, in which low average powers can be achieved, a highly controllable surface covering can be obtained with minimal deterioration of the monomer, which is particularly important when retention of the monomer structure in the target polymer is required.

The applied fields are suitably of power of from 5 to 500W, suitably at about 100W peak power. When applied as a pulsed field, the pulses are suitably applied in a sequence which yields very low average powers, for example in a sequence in which the ratio of the time on : time off is in the range of from 1:3 to 1:1500, depending upon the nature of the monomer gas employed. Although for monomers which may be difficult to polymerise, time on : time off ranges may be at the lower end of this range, for example from 1:3 to 1:5, many polmerisations can take place with a time on:time off range of 1:500 to 1:1500. Particular examples of such sequence are sequences where power is on for 20-50μs, for example about 30μs, and off for from 1000μs to 30000μs, in particular about 20000μs. Typical average powers obtained in this way are 0.01W.

The fields are suitably applied from 30 seconds to 90 minutes, preferably from 5 to 60 minutes, depending upon the nature of the monomer and the substrate, and the nature of the target coating required.

Suitable plasmas for use in the method of the invention include non-equilibrium plasmas such as those generated by radiofrequencies (Rf), microwaves or direct current (DC). They may operate at atmospheric or sub-atmospheric pressures as are known in the art. In particular however, they are generated by radiofrequencies (Rf).

Various forms of equipment may be used to generate gaseous plasmas. Generally these comprise containers or plasma chambers in which plasmas may be generated. Particular examples of such equipment are described for instance in WO2005/089961 and WO02/28548, but many other conventional plasma generating apparatus are available.

In all cases, a glow discharge is suitably ignited by applying a high frequency voltage, for example at 13.56MHz. This is applied using electrodes, which may be internal or external to the chamber, but in the case of larger chambers are internal.

Suitably the gas, vapour or gas mixture is supplied at a rate of at least 1 standard cubic centimetre per minute (sccm) and preferably in the range of from 1 to 100sccm.

In the case of the monomer vapour, this is suitably supplied at a rate of from 80-300mg/minute, for example at about 120mg per minute depending upon the nature of the monomer, whilst the pulsed voltage is applied.

Gases or vapours may be drawn or pumped into the plasma region. In particular, where a plasma chamber is used, gases or vapours may be drawn into the chamber as a result of a reduction in the

pressure within the chamber, caused by use of an evacuating pump, or they may be pumped, sprayed, dripped, electrostatically ionised or injected into the chamber as is common in liquid handling.

Polymerisation is suitably effected using vapours of monomers which are maintained at pressures of from 0.1 to 400mtorr, suitably at about 10-100mtorr.

A particularly suitable apparatus and method for producing laboratory devices or binding or filtration media in accordance with the invention is described in WO2005/089961, the content of which is hereby incorporated by reference.

Precise conditions under which the plasma polymerization takes place in an effective manner will vary depending upon factors such as the nature of the polymer being deposited, as well as the nature of the substrate and will be determined using routine methods and/or other techniques.

The precise monomer selection and conditions used in each case will vary depending upon the exact nature of the polymeric coating required. Monomers can be designed using the criteria described above, and the conditions also selected upon the basis of whether significant monomer disruption is required or not. Once a test product has been produced, it may be probed to see whether it has the desired properties, and then the conditions modified in a routine manner, as would be clear to a skilled person, in order to provide the optimum surface.

The dimensions of the chamber will be selected so as to accommodate the particular substrate or device being treated. The chamber may be a sealable container, to allow for batch processes, or it may comprise inlets and outlets for the substrates, to allow it to be utilised in a continuous process as an in-line system. In particular in the latter case, the

pressure conditions necessary for creating a plasma discharge within the chamber are maintained using high volume pumps, as is conventional for example in a device with a "whistling leak". However it will also be possible to process drug delivery systems at atmospheric pressure, or close to, negating the need for "whistling leaks".

The thickness of the deposited polymer coating which is applied using the method of the invention will depend upon the nature of the product. Generally, the thickness of the coating may be uniform, so as to ensure that binding of biomolecule takes place evenly all over the surface of the.

Factors which may be used to control thickness include the length of exposure to the plasma and the pattern of the pulsing, as well as the pressure, flow rate and nature of the monomer.

Generally, a coating of a biocompatible polymer which is up to 5000Å thick, for example from 1-2000Å is applied for most assay and cell culture purposes.

Using the method of the invention, a variety of laboratory equipment, including assay plates and slides and culture plates, as well as filtration or binding media, such as membranes, for example cellulose, polypropylene, nylon, polytetrafluoroethylene or nitrocellulose membranes, or binding media such as polystyrene, silica or magnetic beads, with electrostatically binding surfaces may be prepared. In addition, biological implants for introduction into the body, such as replacement joints, or supports may be required to have a biologically compatible surface, on which tissue growth is to be encouraged, and these may also be treated in accordance with the method of the invention in order to achieve this.

Thus in particular, the invention provides a laboratory device, filtration or binding medium or biological implant having a polymeric layer thereon, wherein the polymeric layer comprises a functional group, able to bind or become bound to a biomolecule.

In general, when preparing a laboratory device or biological implant in accordance with the present invention, the optimum process types including the optimum monomer, deposition conditions etc., will be determined by a routine optimisation procedure. However, by utilising a plasma, it is possible to 'tailor' the surface by preparing a surface, assessing the liquid interactions for example using dynamic contact angle analysis etc. and then modifying these accordingly. For the first time the required surface chemistry and therefore the surface energy can be finely controlled tweaked to give the ideal results. Using the method of the invention, the chemistries or resulting packing densities are not restricted by the use of solution based approaches.

The process will typically take place in one step, although there may be a requirement for more than one stage, in some cases, in particular where different monomers are to be applied in sequence. Because the discharge produces activated species instantaneously, the process can be as quick as only a few seconds or minutes depending on the exact processing conditions uses.

Example 1 - Preparation of Immunoassay Plate

A polystyrene Microtitre plate for binding a monoclonal antibody or a protein with a high hydrophobic region is placed inside the appropriate sized vacuum chamber and evacuated to low pressure ~ 5 mtorr. On reaching base pressure and ensuring the out-gassing rate is satisfactory, a chemical monomer selected depending on the size and degree of interaction with the incoming biomolecule required, such as are CH_4, C_3H_8, C_7H_{16},

C_3H_6, C_7H_{14}, cyclopentane, allyl cyclohexane, 2,2 - dimethylbutane or 2- methylpropane,and gas mix are introduced to a pressure of 80 mtorr. On reaching the required operating pressure a pulsed plasma is struck using a radio frequency source at 60W peak power at a pulse on-time of 10 ms and an off time of 250 ms and the deposition of a well adhered, thin plasma polymer ensues. The deposition process runs for 10 mins after which the RF, monomer and gases are turned off and the system is evacuated to base pressure. The resulting microtitre plate with a layer is then removed and it ready for use.

Example 2 - Magnetic binding media

Magnetic iron-silica beads for use in the purification of DNA are placed inside the appropriate sized vacuum chamber and evacuated to low pressure ~ 5 x mtorr. On reaching base pressure and ensuring the out-gassing rate is satisfactory, a chemical monomer which includes only hydrocarbon components such as allyl cyclohexane is introduced at a pressure of 80 mtorr. On reaching the required operating pressure a pulsed plasma is struck using a radio frequency source at 60W peak power at a pulse on-time of 10 ms and an off time of 250 ms and the deposition of a well adhered, thin plasma polymer ensues. The deposition process runs for 10 mins after which the RF, monomer and gases are turned off and the system is evacuated to base pressure. The resulting beads can be used to reversibly capture cells in for example, food samples and for the purification of DNA samples.

Claims

1. A method for preparing a surface which binds or is bindable to a biomolecule through electrostatic forces, said method comprising exposing a surface to a plasma containing a hydrocarbon or halocarbon monomer optionally containing an acrylate group, which is capable of forming a polymer having the appropriate electrostatic properties, under conditions in which the monomer is deposited on the surface so as to form a polymer which has said properties.

2. A method according to claim 1 wherein the monomer is a hydrocarbon or halocarbon which includes a polymerisable group.

3. A method according of claim 2 wherein the polymerisable group is an alkene, alkyne or acrylate group.

4. A method according to any one of the preceding claims wherein the plasma is applied as a pulsed field.

5. A method according to claim 4 wherein the plasma is applied in a sequence in which the ratio of the time on : time off is in the range of from 1:3 to 1:1500.

6. A method according to any one of claims 1 to 3 wherein the plasma is applied as a continuous wave.

7. A method according to any one of the preceding claims wherein the surface is of an assay plate, slide, culture plate, filtration medium or binding medium.

8. A laboratory device, filtration or binding medium or biological implant having a polymeric layer thereon, wherein the polymeric layer is able to bind or become bound to a

biomolecule using electrostatic forces only, obtainable by a method according to any one of the preceding claims.

Intellectual Property Office

Application No:	GB1413567.7	**Examiner:**	Mr Martin Price
Claims searched:	1-8	**Date of search:**	23 January 2015

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	1-8	US 5229172 A Cahalan - see e.g. the abstract
X	1-8	US 2003/0198968 A1 Matson - see e.g. claims 1, 15, 35, 36
X	1-8	EP 1435262 A1 Max-Planck - see e.g. claims 1, 4, 10
X	1-8	US 6258291 B1 Chow - see e.g. claim 1 and the abstract
X	1-8	WO 2004/040308 A1 Plasso Technology - see e.g. claim 1 and page 11 lines 1-23
X	1-8	WO96/36877 A1 Neomecs - see e.g. claims 18, 21, 24
X	1-8	US 2009/0111713 A1 Tsao - see e.g. the figures and paragraph 0024
X	1-8	DE 3733986 A1 Mueller-Schulte - see e.g. see WPI abstract number 1989-123229 and EPODOC abstract
X	1-8	WO 01/31339 A1 Univ Sheffield - see e.g. the claims

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Intellectual Property Office

Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC[X] :

Worldwide search of patent documents classified in the following areas of the IPC

A61L; C08F; C08J; C12M; C12Q; G01N

The following online and other databases have been used in the preparation of this search report

EPODOC, WPI, TXTE

International Classification:

Subclass	Subgroup	Valid From
C08F	0002/52	01/01/2006
A61L	0033/00	01/01/2006
C08J	0007/18	01/01/2006
C12Q	0001/68	01/01/2006
G01N	0033/545	01/01/2006