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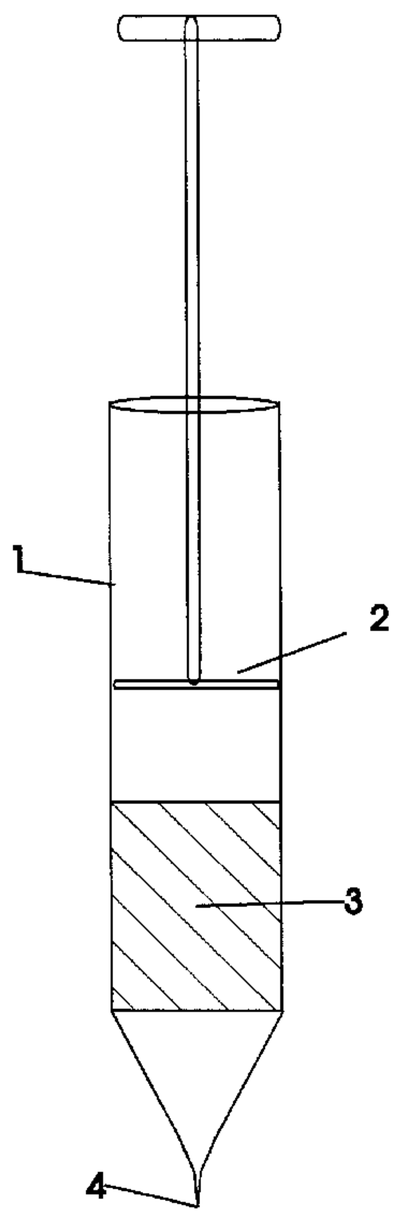
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A method for extracting biomolecules such as DNA from a liquid mixture comprises passing the liquid up and down through a solid phase incorporating an adsorbent for the biomolecule.

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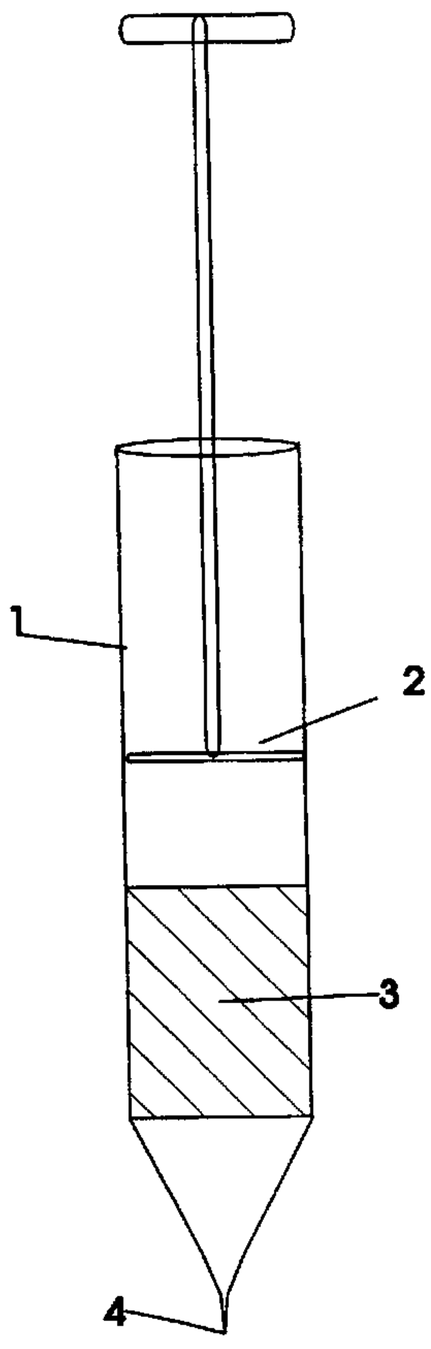
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Sample Processing Device

The present invention relates to a sample processing device and in particular to a sample processing device that can purify biomolecules from crude starting materials such as blood, tissue, plants, microbes, agricultural, food etc. The system can also be used to purify or manipulate any biomolecule or compound from aqueous or non-aqueous samples in a fully automated or manual mode.

Conventional chromatography columns are not suitable for direct extraction of biomolecules from crude starting materials containing particulate matter, viscous material, or cellular debris. This is due to the type of solid phase employed and the design of the column or cartridge that are prone to blocking or clogging. This also applies to mini-chromatography columns processed using a vacuum or centrifugation to pass the liquid over the solid supports. This clogging problem is exacerbated when attempting to extract high molecular weight DNA without shearing it into small fragments. Clogging of columns or cartridges may be due to frits (a thick, rigid, porous disc/membrane or plug) with small pore sizes or the small size of standard solid phase particles (usually less than 200 microns in diameter) and/or close packing of stationary solid phase material (dependant on size and shape and compressibility).

We have now devised equipment and a method which reduces these problems.

According to the invention there is provided equipment for extracting a material from a liquid mixture containing the material which equipment comprises a container containing a solid phase able to adsorb the material to be extracted and a reversible suction means adapted to apply suction to the solid phase to draw up the liquid mixture over the solid phase and which is able to be reversed so as to pass the liquid back over the solid phase.

The reversible suction means can be in the form of a syringe and the solid phase is contained within the syringe below the piston so that when the nozzle of the syringe is placed in the liquid mixture and the piston is withdrawn liquid is drawn up over the solid phase and when the piston is depressed the liquid is passed back over the solid phase.

Alternatively the container containing the solid phase can be attached to the nozzle of a syringe.

In another embodiment of the invention the reversible suction means comprises a pipette and there is a plug of the solid phase contained within the pipette tip.

It is a feature of the invention that the novel design allows almost any starting material to be used without clogging the automated extraction system and in a closed environment reducing the risks of contamination to the operator, instrument or to adjacent sample tubes.

The design allows the use of existing solid phase extraction methods used in chromatography as well as novel reagents and materials described below.

The invention is especially suitable for extracting large macromolecules such as nucleic acids (DNA and RNA) that tend to block or clog existing devices.

The syringe or pumping device with sucking and blowing action can be used in conjunction with a specially modified chromatography cartridge that resists clogging. For example, a biological sample e.g. animal or plant tissue, blood, cells, hair, faeces, agricultural, water, food etc. is homogenised to release the nucleic acids and then passed through a solid phase material to capture the nucleic acids, nuclei or nucleated cells. The cellular debris or contaminants pass up and down to waste leaving the nucleic acids immobilised on the solid phase support. Alternatively, the solid-phase

material can be used to remove unwanted cellular debris leaving the DNA or biomolecule in solution.

The material to be extracted can be passed up and down the solid phase using the pumping action of the syringe or peristaltic pump. Any type of solid phase can be used since the cartridges are designed to be interchangeable for a wide variety of solid phase extractions.

Additionally, the system will allow homogenisation of samples by introducing a shredding device in the primary cartridge.

This allows difficult samples, such as plant extracts, animal tissue, faeces, food or other samples requiring maceration to release cellular contents such as proteins or nucleic acids. The target molecules can then be captured on the solid-phase and the homogenisation and purification process is thus completely automated.

An enrichment step is often used to remove initial debris and this can be performed using flocculating agents such as cellulose, diatomaceous earth, silica gel, dextrans, PEG or any substance that promotes rapid flocculation and sedimentation of debris or contaminants without requiring centrifugation.

The instrument of the invention can be designed to handle single or multiple rows of standard syringes (disposable or non-disposable) of different sizes e.g up to 100ml . A single disposable unit with 4, 8, 12, 24 or 96 channels can also be used in conjunction with a pumping system. Thus it is able to dispense large or small volumes in an 8 by 12 tube array.

A feature of the instrument of the invention is that the final purified product can be presented in a microtitre plate format of 8 x 12 tubes or as single tubes regardless of the starting volumes of samples. Therefore a great many large samples of blood e.g.

5ml, can be processed for the extraction of DNA simultaneously and then concentrated down to small 1 ml tubes.

By using sucking and blowing rather than flow-through of reagents and samples, the disposable cartridge retains the sample completely, preventing contamination of any interfaces with the instrument such as tubes, valves and ports of the disposable cartridge. This also minimises the risk to the operator as the disposable item can be automatically discarded.

Alternatively, a whole microtitre plate can be processed containing small samples e.g. 1ml buccal cell scrapes, using smaller syringes or pumping devices.

A syringe or multi-channel disposable cartridge system is able to operate in X, Y and Z dimensions and can accommodate any pitch changes necessary to handle different sample volumes. The purified analyte may be transferred automatically to a UV/visible spectrophotometer or fluorescent photometer to estimate analyte concentration and purity.

Most conventional UV spectrophotometers require a relatively large sample to analyse in a silica quartz cuvette. Unfortunately, in biological samples the amount of analyte is often in tiny volumes or low concentration. This results in either sacrificing the whole sample or diluting into a bigger volumes which then may make detection very difficult. By having a disposable probe that dips into the sample the solution can be measured at full strength and without wastage.

The instrument design allows incorporation of electrodes or metal meshes or conductive plastic meshes that can be made positively charged to bind nucleic acids from crude extracts. The positive charge can then be turned off or reversed to release the purified nucleic acids. The pumping effect of the instrument allows rapid mixing increasing the contact of the target molecules. In one format there could be a mesh,

bead or tip incorporated as a plastic disposable that can carry a positive charge by applying a potential difference or induction. As the sample passes across the mesh or membrane the nuclei acids bind and can be released as the charge is reversed or switched off.

Large highly porous or non-porous solid phase beads may be used to avoid clogging and maintain high flow rates. For example; porous plastic beads with a diameter of 150microns or greater with very large pores e.g. 1 to 20 microns, made from polypropylene, polyethylene or any polymer with a natural affinity for specific biomolecules. Other materials can also be used; cellulose, agarose, glass, silica or any suitable material that may be derivatised to extract a target analyte.

The solid-phase may be derivatised with imidazole groups, amine, carboxy or any group with an affinity for nucleic acids or the target molecule/compound. The beads may be used with a frit or membrane or a single hole or multi hole mesh depending on the flow rates required.

The beads may be composed of material that has an inherent affinity of biomolecules such as poly vinyl pyridine that is positively charged at pH 4 and will bind DNA and elute it at pH 8. Any polymeric compound can be converted into beads or particles or surfaces for binding and may include groups such as pyrazole, pyrole, pyrroldine, indole, pyrimidine, nucleic acid bases, imidazole, imines, amines, lysines or any groups that have a pKa in the range of 3 to 12. Preferably a pKa of 5 to 8 is employed to maintain physiological conditions if biological samples are being processed and can thus be manipulated by pH to turn a positive charge on or off.

The beads may also be converted for chelation of biomolecules such as iminodiacetic acid beads bound with ferric ions at pH 3 to bind nucleic acids. Raising the pH removes the purified nucleic acids. Calcium, Magnesium or Ammonium ions can also be used to chelate biomolecules.

The beads can be held stationary using frits or membranes as long as the pore diameters are large enough to allow easy passage of crude matter. In most cases the frits or membranes require relatively large holes (pin holes) not found in conventional materials. Alternatively the beads may be held in place by narrowing the inlet and outlet of the cartridge removing the need for frits or they may be held in place by a mesh incorporated into the design of the cartridge.

The larger beads can be allowed to move inside the column by having space inside the cartridge. This helps with mixing and reduces clogging. Alternatively, the beads can be added to the crude mixture as a suspension and then trapped in a cartridge when the target molecules are bound thus achieving separation.

Large diameter beads are also useful for preparing magnetic solid phases which are not prone to aggregation during isolation of large macromolecules such as nucleic acids. Most non-porous magnetic (paramagnetic) beads are less than 50microns but are difficult to handle when extracting genomic or microbial DNA. Large porous magnetic beads may lead to internal entrapment of contaminants as the macromolecule such as DNA bind to the outer surface of the beads, these contaminants are only released when the DNA is eluted contaminating the final preparation.

Porous membranes or frits can be modified by adding larger holes and used in spaced stacks or individually to bind biomolecules from crude extracts, e.g. blood. Existing membranes or frits block instantly when encountering crude extracts or high molecular weight DNA. The invention describes the use of a unique pore size modification that allows the treatment of crude materials in large or small volumes that is also amenable to automation.

For DNA extraction from blood, a small or large device can be constructed based on the same design. The small device relies on a standard plastic pipette tip that

incorporates a single porous plug, wadding, or frit with a pore size that prevents blocking. This can be used with a conventional pipette or syringe in a manual mode or in a fully automated pipetting station. Alternatively, they can be incorporated into Deep Well plates, Microtitre plates or PCR tubes and used with centrifugation or vacuum manifolds. The frits may also be incorporated directly inside a syringe 1ml to 60ml instead of an extra cartridge.

The plug or frit may be derivatised to bind DNA or any biomolecule. For larger extractions additional plugs or frits can be added in stacks separated by a small air barrier to avoid blocking and maintain exposed surfaces for binding the target compound.

The material for the membrane or frit may be porous polyethylene with a primary pore size of 1 to 200 microns or preferably 20 microns , or any porous plastic, porous glass, cellulose with pores large enough to allow passage of crude matter. E.g. 20 microns or larger. The material may have small pore sizes that will enable binding of target molecule, but they must also possess larger holes e.g. 0.1 mm or greater to avoid blocking. This larger hole may be at the edges or in the middle or part of a cut away section.

A variety of frits can be incorporated in a single cartridge to perform sequential or discrete separations, e.g. one frit separates the nuclei and another derivatised with silica or imidazole groups purifies the DNA further up the cartridge or in a separate device. Alternatively, the initial capture of DNA, RNA, or other biomolecules can be performed, then the analyte is washed off and carried up the syringe and precipitated with a compound such as PEG, alcohol, ammonium or sodium sulphate. The precipitated compound may then be re-captured allowing the soluble contaminants to pass to waste.

A syringe based system can be used to filter a crude sample by incorporating a filter membrane or plug at the tip of the cartridge or pipette tip. The debris is left behind as the liquid is sucked up through the plug. After rinsing the plug free of debris the clarified solution is dispensed into a new tube.

This technique can be assisted by using a filter-aid such as silica gel, titanium oxide, fibrous cellulose etc.

Following homogenisation, a filter-aid is added to flocculate and compact the debris at the bottom of the tube leaving the target molecule in the supernatant ready for processing or purification.

The filter-aid may be soluble, possess temperature dependant solubility or be insoluble.

If smaller solid-phase beads are employed for conventional extractions. For example, less than 100 micron glass, then a by-pass channel can be introduced that allows larger particles or debris to pass up and down without clogging the cartridge. A by-pass channel may be created as small tube that by passes the solid phase or a porous material with large pores e.g. 20 microns or greater, that surrounds the solid phase.

For very pure DNA further purifications can be performed with another cartridge in-line to allow the use of the salting out technique. This salting out technique can be used in conjunction with alcoholic precipitation and capture of the insoluble nucleic acids.

If smaller solid-phase beads are employed for conventional extractions. For example, less than 100 micron glass, then the solid -phase can be allowed to move internally so clogging is avoided. The introduction of ridges, spirals or obstructions inside the cartridge helps prevent the solid-phase moving in bulk maintaining good mixing and

separation of solid-phase. If the solid-phase particles are large enough of sufficiently dense, e.g. 200 micron glass, a mini-fluidised bed can be generated.

Clogging of cartridges may also be avoided by the pre-addition of a mobile solid-phase to capture the target molecule. The loose solid-phase or paramagnetic beads are added as a suspension and the contaminants are washed away leaving the immobilised target compound ready for further purification in a cartridge or analysis. Any type of solid phase can be added with a preference for material that will sediment quickly or be flocculated by filter aids to avoid centrifugation or are paramagnetic.

The instrument and disposable cartridge or tip system has a variety of applications including molecular biology such as affinity purification of cell antibodies, enzymes and other proteins, purging of mixtures to remove unwanted compounds, combinatorial chemistry, ion exchange purification, hydrophobic chromatography, enzyme assays using immobilised antibodies, nucleic acids or antigens, enzyme catalysis on solid phase supports, food screening for pathogens, genomic DNA, toxins, allergens, etc., clinical sample processing for pathogenic organisms, mixing adjuvants for immunisation and making stable emulsions, pipetting larger volumes, removal of lipoproteins for cholesterol assays, detecting or concentrating pathogens in milk, food or water.

A device of the invention is described in the accompanying drawings in which:-

Fig. 1 illustrates with the solid phase inside a syringe

Fig.2 shows the use of a cartridge

Fig. 3 shows a pipette

Figs. 4, 5 and 6 show alternative cartridges and

Figs. 7, 8 and 9 show different disc arrangements.

Referring to fig. 1 a syringe (1) having a moveable piston (2) has an adsorbent solid phase (3) held within it. In use the nozzle (4) is placed within the liquid from which material is to be separated and the piston withdrawn to suck up the liquid through (3). When the piston is depressed the liquid is forced back over (3) and this process can be repeated if desired so that there is better adsorption of material from the liquid.

Referring to fig. 2 the syringe (5) with a piston (6) has nozzle (7) placed in cartridge (8) containing a solid adsorbent and the cartridge (8) has its inlet (9) placed in the liquid from which material is to be separated. When the piston (6) is withdrawn the liquid is drawn up through the cartridge and material is adsorbed, when the piston is depressed the liquid is forced back over the adsorbent in the cartridge so that there is better adsorption of material from the liquid.

Referring to fig. 4 the adsorbent material (10) can be in the form of frits or beads and can fill the cartridge.

Referring to fig. 5 there can be a by-pass channel (11) round the outside of the solid adsorbent so that larger particles can pass up and down without clogging.

Referring to fig. 6 there are discs (12) positioned within the cartridge and each disc consists of an adsorbent membrane, the discs can have large pores as illustrated in fig. 7 and can have cut away sections as shown in fig. 8 to prevent blocking. The discs can be stacked on top the other and can have a raised lip (14) as shown in fig. 9 so that the discs are only in contact through this lip.

Referring to fig. 3 a pipette (15) has an aerosol plug (16) to prevent contamination and contains a plug (16) of adsorbent material such as a porous plastic material as shown. In us the tip of the pipette (17) is placed in the liquid and liquid is sucked up over the plug (16), by blowing down the pipette the liquid is forced back over the plug (16) so that there is better adsorption of material from the liquid.

The adsorbed material can be removed from the solid phase by conventional elution methods.

The invention is described in the following examples in which the isolated or eluted products were identified using conventional laboratory analysis methods.

Example 1

Using the equipment of fig. 2 polystyrene porous carboxylated beads (200 – 500 microns or 16 - 50 mesh size) were loaded into a chromatography cartridge and held in place with plastic mesh with pore sizes of about 100 microns.

Whole blood was diluted 10 times with 10mM Ammonium Bicarbonate, 10mM Ammonium Carbonate and 0.1 % Tween 20 pH9 and sucked up and down the cartridge with a syringe and passed back through the cartridge. The dilution buffer can be any hypotonic solution that causes lysis of the red blood cell fraction, but maintains the integrity of nuclei, white blood cells or chromatin. The nuclei became immobilised on the beads and the lysed blood was taken to waste. Direct elution of the nuclear DNA was achieved using hot water. To obtain greater purity DNA, the eluate from the first cartridge was then further processed using another cartridge containing a solid-phase with poly imidazole groups.

To collect the white blood cell fraction, the same solution is made isotonic with saline and the cells were captured in a similar manner.

Example 2

Using the equipment of fig. 3 polystyrene porous carboxylated beads (200 – 500 microns or 16 - 50 mesh size) were loaded into a 1 ml pipette tip.

Whole blood was diluted 10 times with 10mM Ammonium Bicarbonate and 0.01% Tween 20 pH9 and sucked up and down the tip of the pipette. The nuclei became immobilised on the beads and the lysed blood was removed to waste. Direct elution of the nuclear DNA was achieved using alkaline detergent solutions and by boiling water.

Example 3

Using the equipment of fig. 2 agarose was treated with carbonyldiimidazole in anhydrous organic solvent and then left in water at pH 3 to maintain the imidazole groups. The derivatised agarose was placed in a cartridge and the supernatant from a plasmid alkaline lysis preparation was sucked up and down immobilising the plasmid DNA on the beads at pH 5. After washing, the plasmid DNA was eluted with 10mM Tris HCl, pH 9.

The above was repeated with carboxylated polystyrene and dextrans of various sizes and DNA obtained by elution as above.

Example 4

Extraction of nuclei or DNA from whole blood

Using the equipment of fig. 2 with the packing of fig. 6 whole blood was lysed with 5 volumes of 10mM Ammonium Bicarbonate containing 0.1 %Tween 20 pH9. The lysed blood was passed through several 20micron porous polyethylene frits modified with larger pores of 1 mm in diameter, housed in a plastic cartridge attached to a 2ml syringe and plunger. Each frit was spaced 3mm apart to allow free flow of liquid.

The nuclei or white blood cell fraction bound to the frit allowing all the contaminating proteins and lipids to pass through to waste in a single pass or several strokes of the plunger. The frit and nuclei was then washed to remove residual proteins using deionised water or chaotropes or alcohols or detergents such SDS or Tween 20 or combinations or lactic and salicylic acids or their salts, or poly phosphates or per chlorates and either eluted off using hot water or alkaline solutions of detergents or further purified inside the cartridge using chaotropic agents or proteases.

Example 5

Purification of buccal cell DNA

Using the equipment of fig. 3 a plug of porous polyethylene was derivatised with imidazole groups and inserted into the tip of a standard 1ml pipette tip. A further non derivatised plug was inserted at the top to act as an aerosol and liquid barrier to prevent contamination of the pipette.

A buccal scrape was taken and mixed with 0.2M guanidine isothiocyanate, 3% Tween 20, Proteinase K and 50mM MES pH5 at 30°C for 15 minutes. The mixture was then sucked up and down the tip several times allowing the DNA to bind to the derivatised plug. The plug was washed with 1mM MES pH5 and then the DNA eluted with 10mM Tris. HCI pH9. The same protocol was repeated using 0.01% to 10% SDS with or without salts and buffers. Fast degradation of the buccal cells can also be achieved using salicylic acid, lactic acid, or $MgCl_2$ at concentrations of 0.05 to 5M. Combinations of the above salts and reagents can also be used.

Example 6

1 gram of carboxylated polystyrene beads with a diameter of about 60 microns or 200 to 400 mesh was suspended in a hypotonic solution of ammonium bicarbonate 10mM with 0.1 % Tween 20 pH9. A five fold excess of this suspension was added to a 5ml blood sample and mixed once. The beads captured the nuclei and sedimented. After several washes with water, the DNA was eluted with hot water. To concentrate the DNA the equipment of fig. 2 was used with the packing of fig. 6 and the DNA was captured on a porous disc in the cartridge and subsequently eluted off in a small volume and analysed using PCR or Restriction Digestion

Example 7 Removal and purification of human IgG from serum

An agarose gel coupled to Protein A was placed in the cartridge of fig. 2 and washed with phosphate buffered saline. A solution containing human IgG in serum was sucked up and down the solid phase until all the IgG was bound. After washing the solid phase with PBS, the IgG was eluted with 0.1 M glycine, 0.1 5M NaCl, pH2.8 and immediately neutralised with Tris. HCl.

Example 8 Purification and isolation of specific white blood cell types from whole blood

Non-porous glass particles of 175 microns were coupled to CD4 monoclonal antibodies and the solid phase placed in a cartridge as in Example 4 with 80micron frits. A diluted solution of Buffy Coat was sucked slowly up and down through the glass beads immobilising the T cell sub-population which could be released.

Example 9 Recombinant protein purification

An agarose gel containing Iminodiacetic acid-Nickel ion groups was packed into a cartridge as in Example 4.

A bacterial lysate containing a recombinant protein possessing a 6 histidine tail was sucked up and down the cartridge and the protein was bound to the co-ordinated nickel. Release of the protein was effected by eluting with 0.5M Imidazole pH 6.

Example 10 Extraction of HIV RNA from serum

A cartridge as in fig. 2 was packed with 60 micron silica and a sample of serum diluted 5 times with 6M guanidine isothiocyanate, 0.1 % Tween 20, 20mM EDTA, 100mM Tris. HCl pH6 was sucked up and down through the solid phase. After washing the solid phase with isopropanol and drying the RNA was eluted using water at 60C.

Example 11 Purification of PCR reactions

A cartridge as in fig. 2 was packed with 60 micron silica and a sample of a PCR reaction diluted 5 times with 6M guanidine isothiocyanate, 0. 1 % Tween 20, 20mM EDTA, 100mM Tris-HCl pH6 was sucked up through the solid phase. After washing the solid phase with isopropanol and drying the DNA was eluted using water.

Example 12 Extraction of RNA from Liver

Fresh liver was homogenised in a mixture of 50% Phenol containing 6M Guanidine isothiocyanate, 10mM DTT, 0.1 M Sodium Acetate pH 4. Chloroform was added to

separate the phases and the top layer containing the RNA was sucked up and down through a cartridge of fig. 2 containing 60micron silica. The silica was washed with alcohol, air dried and the RNA eluted with hot water ready for processing.

Example 13 Isolation of mRNA

A cartridge was packed with COOH polystyrene beads coupled to oligo dT 30 5' NH2.

A sample of white blood cells prepared earlier in a cartridge were treated with an excess of 1% SDS, 0.5M LiCl, 10mM DTT, 10mM Tris. HCl pH 7.5 and sucked up and down through the affinity resin several times to shear the DNA and bind the mRNA. The resin was then washed in 0.1 M LiCl and air dried. Elution of mRNA was performed by hot water.

The above experiment was repeated with a carboxylated plastic porous frit as in Example 4 that was coupled to oligo dT30 and used for binding less than 5 micrograms of mRNA.

Example 14 Streptavidin immobilised on solid-phase

Streptavidin was immobilised onto porous frits by mixing the protein in 0.1 M sodium phosphate buffer with 0.01% glutaldehyde pH7 as in example 4. Biotinylated primers used to generate a PCR product were then isolated on the immobilised streptavidin. The PCR product was then made single stranded using heat or 0.1 M NaOH and used for sequencing or probe analysis.

Example 15

Use of electrodes, static charge, induction, electrophoresis to isolate DNA or RNA

Whole blood was diluted down 10 times in 10mM ammonium carbonate/bicarbonate, 50mM Tris. HCl with 1 % Tween 20, 100lg/ml proteinase K pH9. Electrodes were surrounded by dialysis tubing containing the same buffer and dipped into the solution. A 12 volt direct current from a battery was connected and the nuclei or

DNA was captured on the outside of the dialysis tubing at the positive electrode after a 1 hour incubation. The DNA could be removed by elution with water.

mixture containing nucleic acid, the method employing (a) a container comprising an electrode capable of binding nucleic acid and (b) reversible suction means for drawing the liquid mixture over the solid phase, the method comprising reversibly drawing the liquid mixture over the electrode so that nucleic acid in the sample binds to the electrode surface.

26. An extraction device for extracting nucleic acid from a liquid mixture containing nucleic acid, the device comprising (a) a container containing a solid phase capable of binding nucleic acid and (b) reversible suction means for drawing the liquid mixture over the solid phase.

27. The extraction device of claim 26, wherein the container has a volume less than or equal to 100 ml and the solid phase is located within the barrel of the syringe.

28. The extraction device of claim 27, wherein the reversible suction means is a syringe and the solid phase is located in a cartridge releasably connected to the nozzle of the syringe.

29. The extraction device of claim 27, wherein the container is a pipette and the solid phase is located within the tip of the pipette.

30. The extraction device of claim 29, wherein an aerosol plug is located in the body of the pipette.

31. An extraction device for simultaneously extracting nucleic acids from two or more liquid mixtures containing

8. A method as claimed in claim 7 in which the solid phase is contained within a syringe below the piston and the nozzle of the syringe is placed within the liquid and the piston withdrawn to draw the liquid over the solid phase and then the piston is depressed to pass the liquid back over the solid phase.

9. A method as claimed in claim 7 in which the nozzle of the syringe is placed in the liquid mixture and the piston is withdrawn and liquid is drawn up over the solid phase and the piston is then depressed and the liquid is passed back over the solid phase.

10. A method as claimed in claim 7 in which the reversible means comprises a pipette and there is provided a plug of the solid phase contained within the pipette.

11. A method as claimed in any one of claims 7 to 10 in which the solid phase comprises beads adapted to adsorb the material to be extracted.

12. A method as claimed in any one of claims 7 to 10 in which the solid phase comprises a plurality of spaced apart discs.

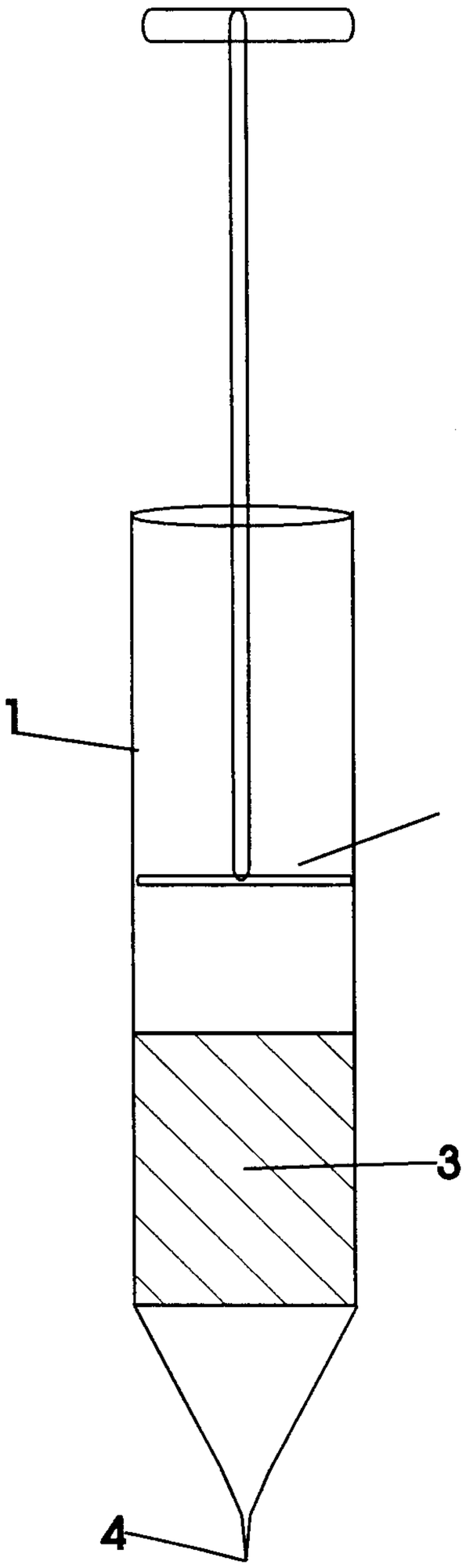


Fig. 1

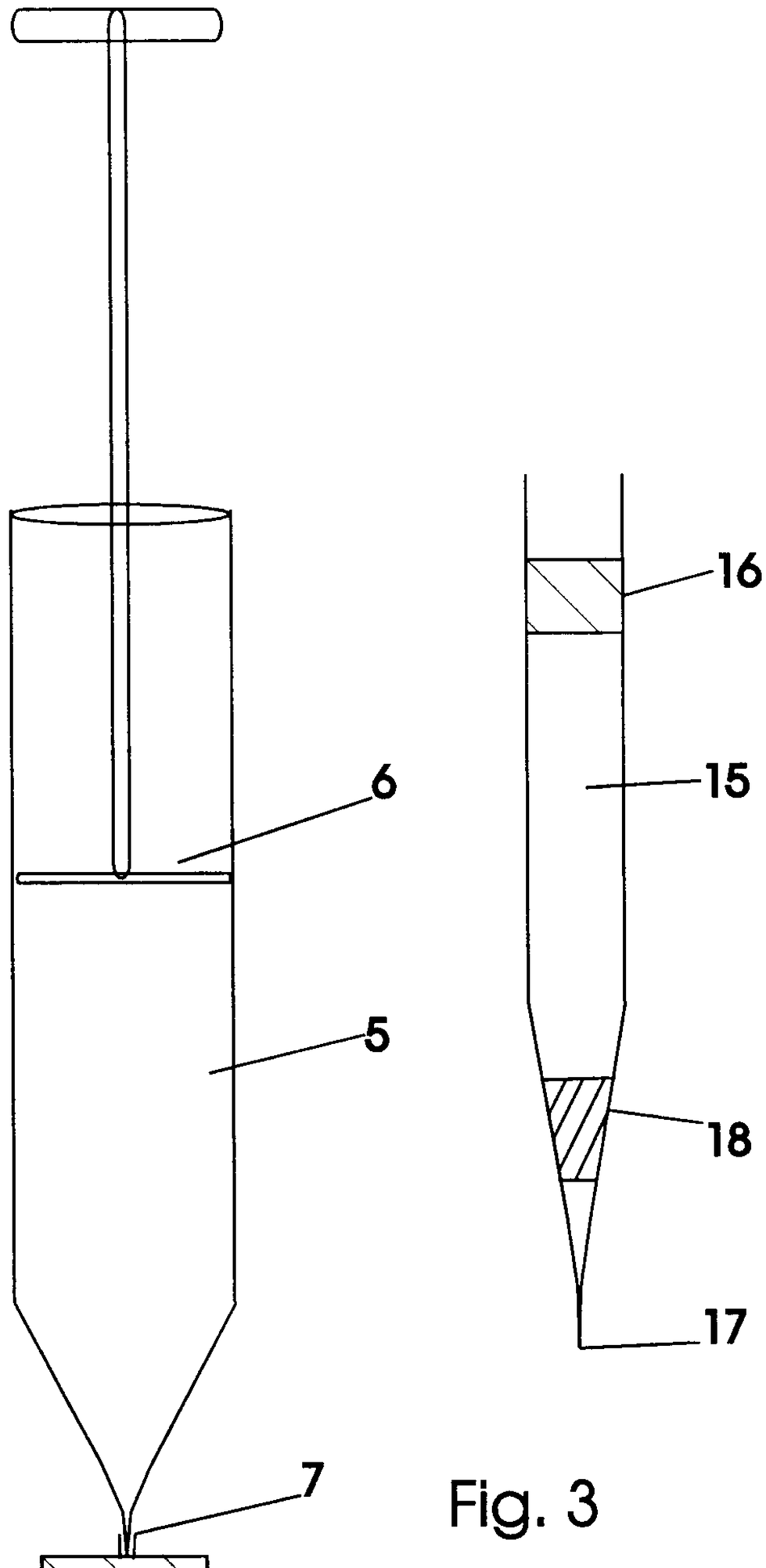


Fig. 3

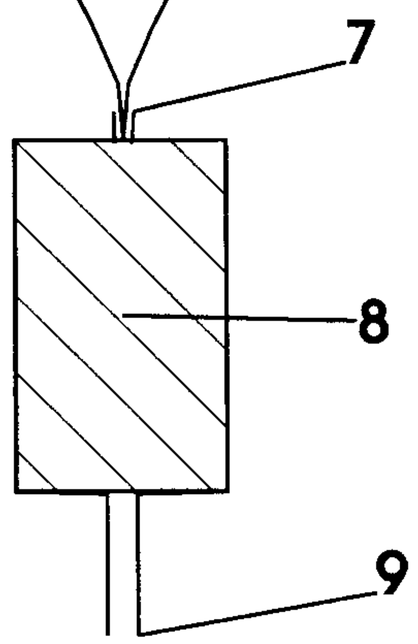


Fig. 2

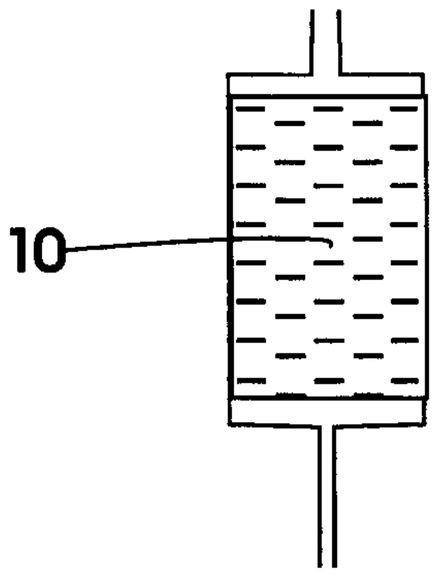


Fig. 4

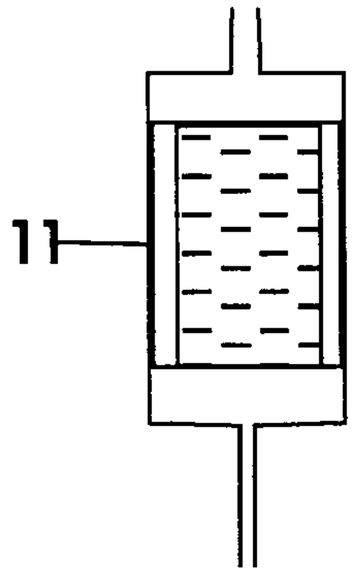


Fig. 5

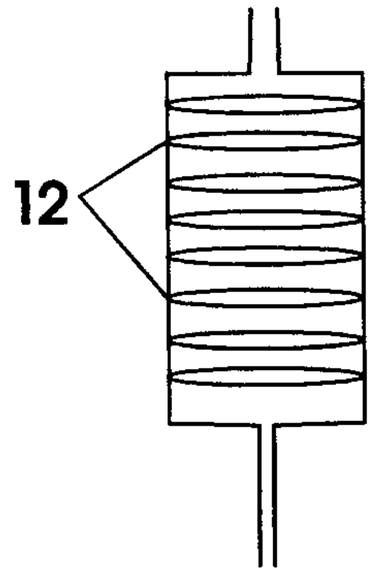


Fig. 6

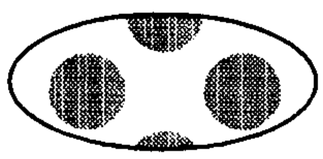


Fig. 7

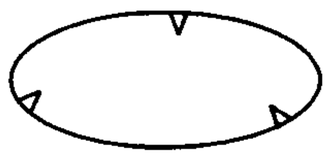


Fig. 8

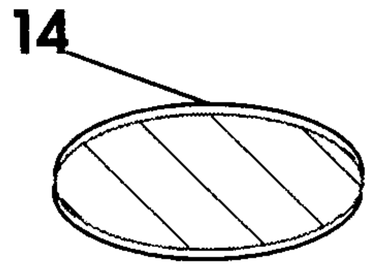
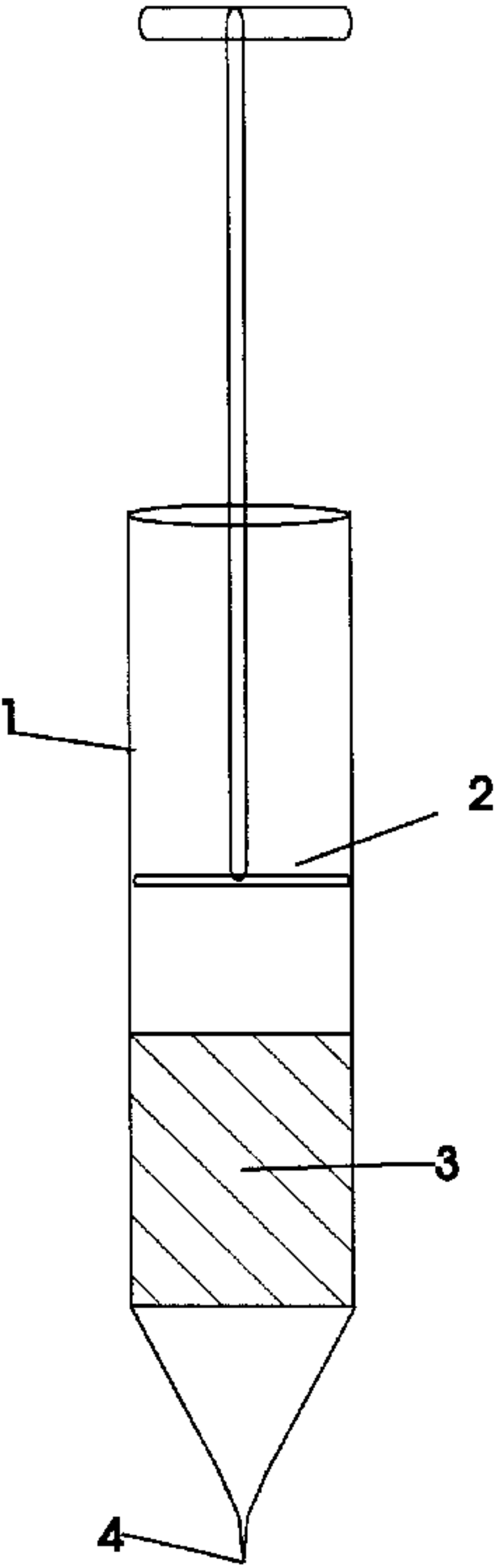


Fig. 9


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