



Red Biobancos

Institute of Health Carlos III

SOP EXTRACTION OF NUCLEIC ACIDS

Red Nacional
de Biobancos
Spanish National
Biobank Network

Working Group DNA Bank

REVISION	DONE BY	DATE	APPROVED	DATE	ENTRY INTO FORCE
00	DNA Bank	05/26/2011	Management	06/26/2015	07/19/2011
Amendments:					

Nucleic Acid Extraction Protocol

This publication is supported by the Subprogram Thematic Networks for Cooperative Research in Health of the Institute of Health Carlos III (ISCIII), within the Strategic Action on Health 2009, RD09/0076/00113

Alberto Orfao

*Coordinator of the
Working Group*

Manuel M Morente

*Coordinator of the National
Biobank Network - ISCIII*

National Biobank Network - ISCIII

www.redbiobancos.es

AUTHORS

This document was prepared with the participation of the following persons and institutions:

Coordinator:

Alberto Orfao de Matos -National DNA Bank

Assistant coordinator:

Rosa Pinto Labajo –National DNA Bank

Collaborations:

Juan Pascual Sánchez-Hospital Marqués de Valdecilla

Eugeni Aragall –Health Sciences Research Institute of the Germans Trías i Pujol Foundation

Eduarne Pedrosa Berrio –Health Sciences Research Institute of the Germans Trías i Pujol Foundation

Antía Solloso Banobre –La Coruña University Hospital

Manuel Posada de la Paz –Research Institute for Rare Diseases-Institute of Health Carlos III

Elisabet Ars Criach –Puigvert Foundation

Irene Vieitez González –Vigo University Hospital

Jacobo Martínez Santamaría –Public Health Research Center

Elena Bellmunt –University and Polytechnic Hospital La Fe

Pablo Isidro Marrón –Central University Hospital of Asturias

Raquel Coya Guerrero –Basque Biobank/Cruces University Hospital

Rocío Aguilar Quesada –Andalusian Human DNA Bank

Rosario Martinez Marín –Hospital Virgen de la Arrixaca

Teresa Escámez –Hospital Virgen de la Arrixaca

OBJECTIVE

The objective of this manual is to draw up a document in which different operating procedures (SOPs) for obtaining nucleic acids from various sample types are collected; these SOPs are used in several biobanks integrated into the National Network of the Institute of Health Carlos III (ISCIII).

The protocols have been classified according to their principle of operation, and in each of them the type of samples they are indicated for is specified, as well as the approximate yield and quality of the samples that should be obtained.

As each biobank has developed its own protocols depending on the type of samples processed and the equipment available, it is not intended to unify all procedures into a single protocol, but rather to present their characteristics, their potential suitability for different types of sample, and the possible application to different biobanks in terms of the equipment at their disposal and the level of performance required in terms of quality and quantity (maximum yield, purity and functionality) of the products to be obtained.

INDEX

1. INTRODUCTION	11
1.1. CELL LYSIS	11
1.2. PURIFICATION OF NUCLEIC ACIDS.....	11
1.2.1. Precipitation by salts and alcohols	11
1.2.2. Extraction using organic solvents.....	11
1.2.3. Adsorption on a silica column.....	11
1.2.4. Magnetic separation.....	11
2. PROTOCOLS FOR THE EXTRACTION OF GENOMIC DNA.....	12
2.1- SALT PRECIPITATION METHOD	13
2.1.1. Principle.....	13
2.1.2. Scope.....	13
2.1.3. General Protocol	13
2.1.4. Specifications for Different Types of Samples	14
2.1.5. List of Protocols based on Salt Precipitation	15
2.1.6. Yield	15
2.1.7. Quality of the Samples.....	16
2.2- DNA EXTRACTION USING ORGANIC SOLVENTS.....	17
2.2.1. Principle.....	17
2.2.2. Scope.....	17
2.2.3. General Protocol.	17
2.2.4. Specifications for Different Types of Samples	17
2.2.5. Organic Solvents Protocol that was used in the Development of this Manual	18
2.2.6. Yield.	18
2.2.7. Quality of the Samples.....	18
2.3- METHODS EMPLOYING ADSORPTION ON SILICA COLUMNS.....	18
2.3.1. Principle.....	18
2.3.2. Scope.....	18
2.3.3. General Protocol	19
2.3.4. Specifications for Different Types of Samples	19
2.3.5. List of Protocols based on Adsorption on Silica Columns	19

2.3.6. Yield	20
2.3.7. Quality of the Samples	20
2.4- PURIFICATION USING BINDING TO MAGNETIC BEADS	21
2.4.1. Principle.....	21
2.4.2. Scope.....	21
2.4.3. General Protocol	21
2.4.4. Specifications for Different Types of Samples	22
2.4.5. List of Protocols Based on Binding to Magnetic Beads.....	22
2.4.6. Yield	22
2.4.7. Quality of the Samples	22
ANNEXES.....	24

1. INTRODUCTION

The general procedure for extracting nucleic acids consists of three consecutive steps: disintegration of cells or tissues (cell lysis), inactivation of intracellular nucleases, and separation of nucleic acids from the other cell components.

Cell lysis depends largely on the type of sample to be processed. Whereas in samples such as blood, cells, certain tissues or saliva efficient cell lysis can be achieved in relatively short times, in other types of samples such as paraffin embedded tissues cell lysis requires a much more laborious pretreatment.

1.1. CELL LYSIS.

During cell lysis, structures formed by lipids and proteins are destroyed, which allows release of nucleic acids from the cell nucleus. Lysis is accomplished by a saline solution that usually contains detergents that denature proteins and/or proteases. After the nucleic acids are separated from the proteins and lipids, they are purified.

1.2. PURIFICATION OF NUCLEIC ACIDS.

Purification methods are very variable and are developed based on the physicochemical properties of DNA and RNA molecules. Some of these extraction and purification methods used in the biobanks integrated into the National Biobank Network of the Institute of Health Carlos III (ISCIII) and described in more detail in this manual are summarized below:

1.2.1. Precipitation by salts and alcohols:

After cell lysis and removal of proteins from the sample, nucleic acids are precipitated by adding isopropanol or ethanol. This method allows obtaining high purity DNA and has the advantage that the solutions used are very safe for the health of the technical staff carrying out the extraction.

1.2.2. Extraction using organic solvents:

In this protocol the cell lysate is mixed with phenol, chloroform and isoamyl alcohol for the separation of nucleic acids from proteins. This method gives a high yield, but traces of organic solvents often contaminate the sample. Furthermore, the use of these substances toxic to health make it necessary to carry out the entire process in a chemical fume hood.

1.2.3. Adsorption on a silica column:

In the presence of certain salts, nucleic acids are retained by adsorption on silica columns. The columns are then washed with salt solutions to remove unbound particles, and the nucleic acids are finally eluted with water or a low-salt solution.

1.2.4. Magnetic separation:

In this method the lysed samples are mixed with magnetic beads that can bind to nucleic acids. After a series of washes for maximum purification of the genetic material, the beads are removed from the solution by a magnetic separator.

References:

- Nucleic Acid Isolation and Purification Manual*. www.roche-applied-science.com.
- DNA Isolation Methods*. World of Forensic Science, 2006. Gale Cengage.

2.- PROTOCOLS FOR THE EXTRACTION OF GENOMIC DNA.

This manual was prepared taking into account the information provided by several of the biobank members of the National Network of the ISCIII regarding the protocols used for the extraction of genomic DNA.

These protocols have been classified according to their **principle of operation** for obtaining the sample. We have developed a **general protocol** for each method, which reflects processing lines common to all protocols used in biobanks with this **principle of operation**. Given that the protocols used in biobanks may have their own particular characteristics, it is advised to follow the specifications of each protocol. For each method, the **scope**, **yield** and **quality** of the obtained DNA samples are described.

The **scope** defined in each method indicates for what type of sample this protocol is used in the biobanks that have participated in the development of this guide.

The **yield** indicates the value obtained from experimental data provided by the different biobanks that use them.

The **quality** of the obtained samples is determined by their purity, integrity and functionality.

Purity:

The purity of the sample is related to the value of maximum absorbance of the nucleic acids detected at a wavelength of 260 nm.

The absorbance ratio A260/A280 reveals whether the DNA obtained is contaminated by the presence of aromatic compounds, since they absorb at a wavelength of 280 nm. This ratio is very stable and it is generally considered that the DNA is of optimal quality when the A260/A280 ratio is more than 1.8. An A260/A 280 ratio > 2.1 is indicative of a significant amount of RNA present in the sample. Conversely, if this ratio is low (A260/A280 <1.6) the sample is contaminated with proteins or phenols. In case of contamination with proteins it will be necessary to carry out an additional treatment to remove the proteins from the DNA solution (for example, addition of proteinase K). If contamination is due to the presence of phenols, the sample needs to be cleaned with chloroform, isoamyl alcohol and ethanol.

The absorbance ratio A260/A230 is used as an additional measure to determine DNA purity, as at 230 nm the maximum absorbance of salts, carbohydrates or other contaminants present in the solution is detected. DNA is generally considered to be pure when the A260/A230 ratio is around 1.5-2.2. A ratio of less than 1.5 may be indicative of the presence of contaminants in the sample. However, it must be kept in mind that the information provided by this measure is not as accurate as the A260/A280 ratio, and that it can be distorted by a low concentration of DNA in the sample since one would be overestimating the concentration of salts in the resuspension buffer.

Integrity:

Electrophoresis in a 0.7% (w/v) agarose gel allows assessing the integrity of the DNA sample. A DNA sample of high integrity shows a single, perfectly defined band at the top of the agarose gel. A degraded DNA sample will show a smear in the gel, which will be more pronounced when the degradation of the sample is higher.

Functionality:

The fact that a DNA polymerase enzyme can use a DNA sample as a template in a PCR reaction indicates that the purity of the sample is optimal. Moreover, one can assess the degree of sample integrity based on the size of the amplified fragment.

At the end of this document two tables are included as annexes, which show the yield and quality of the samples obtained with each of the protocols used in the different centers that participated in the development of this manual.

Table 1 shows the data regarding the **yield** obtained with each protocol for each kind of sample.

Table 2 shows the data regarding the **quality** obtained with each protocol for each kind of sample. For each sample data regarding the purity (**P**), integrity (**I**) and functionality (**F**) of the purified DNA are indicated.

2.1- SALT PRECIPITATION METHOD.

2.1.1. Principle.

First cells are lysed with an anionic detergent that solubilizes the cell components and inhibits the action of intracellular nucleases. Next, proteins are denatured and eliminated by salt precipitation. The DNA in solution is precipitated with isopropanol, washed with ethanol, and finally resuspended in a DNA-stabilizing buffer.

2.1.2 Scope.

This method is used to obtain DNA from the following types of samples:

Peripheral blood: volumes from 3 ml to 20 ml (the amounts of the reagents for this method are scalable according to sample volume).

Peripheral blood mononuclear cells (PBMCs): PBMCs preparations have a volume that varies between 0.1 and 0.2 ml on average, and from 1 to 3×10^7 cells can be processed.

Cells: the number of cells that can be processed depends on the cell type and on the way the cells were obtained (e.g. cell cultures or cells purified by flow cytometry). In any case, a broad range of cells can be processed, ranging from 1×10^6 to 50×10^6 , as long as the amounts of reagent solutions are scaled up proportionally.

Saliva: 2 ml of saliva mixed with 2 ml of Oragene® preservative can be processed.

Tissues: very variable amounts of tissue, from 5 mg to 1 g of fresh frozen tissue, can be processed. This protocol can also be applied to obtain DNA from tissue fixed in formalin and embedded in paraffin (FFPE: formalin-fixed paraffin-embedded).

2.1.3 General Protocol.

- Step 1.** Add lysis solution to the sample. In this step it is necessary to ensure an efficient cell lysis, and therefore one can proceed to the next step of the protocol if the sample has a homogeneous aspect; conversely, if cell bodies are observed in the solution, incubation at 37°C or 65°C is recommended to achieve complete homogenization of the sample. In any case, samples are stable in lysis solution for at least 2 years at room temperature, so that the process can be stopped at this step; samples must be stored in the dark and the process can be continued at a later time.
- Step 2.** To obtain an RNA-free sample, add RNase and incubate at 37°C for 15- 45 min. This step is not necessary for peripheral blood samples, since in these samples contamination with RNA is virtually undetectable. It is advisable in other samples though, such as cells, saliva or tissues. However, the amount of RNase to add is proportional the type and amount of starting sample, and it is recommended to follow the directions of the corresponding protocol specific for each type of sample.
- Step 3.** A saline solution is added to precipitate the cytoplasmic and nuclear proteins of the sample. The sample is then vigorously vortexed for 20-30 seconds. Next, the sample is centrifuged at the speed and time needed to ensure complete precipitation of the proteins. The

precipitated proteins are seen at the bottom of the tube as a brown pellet. The supernatant must be clear and without particulate matter or brownish traces; if this is not the case, the sample must be incubated for 5 minutes on ice and centrifuged again.

- Step 4.** The supernatant containing the DNA in solution is transferred to a fresh tube with isopropanol. The sample is mixed with the isopropanol by gently inverting the tube approximately 50 times. In this step the appearance of the DNA strand is seen. However, observation of this strand will depend on the amount of DNA sample processed.
- Step 5.** Centrifuge the sample to precipitate the DNA at the bottom of the tube. The DNA is seen as a whitish precipitate.
- Step 6.** Decant the supernatant carefully and place the tube with the DNA precipitate in an inverted position on a clean piece of absorbent paper to remove as much of the remaining isopropanol as possible.
- Step 7.** Add Ethanol to 70%, cap the tube and invert gently several times to wash the DNA pellet.
- Step 8.** Centrifuge the sample and remove the ethanol by decanting or with a pipette tip (very carefully, because the DNA may come off the bottom of the tube). Make sure that the DNA precipitate remains visible at the bottom or the walls of the tube.
- Step 9.** Excess ethanol is removed by placing the tube upside down on a clean piece of absorbing paper or by leaving the tube to air dry for a few minutes until no more traces of ethanol are observed. However, it is important not to let the pellet become too dry because this makes subsequent resuspension difficult.
- Step 10.** Hydrate the DNA with an adequate buffered solution, with TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA), or with sterile water.
- Step 11.** It is recommended to incubate for about 1 hour at 65°C to facilitate resuspension of the DNA. Following this incubation, the DNA in solution is stirred at room temperature until checking with a pipette tip verifies that the DNA is fully resuspended without lumps or viscous debris in the solution.

Note: the amounts of the different solutions used in this protocol may vary depending on the type and amount of starting sample. Because different kits based on the salt precipitation method are used, it is recommended to follow the specific instructions of the kit regarding the quantities of reagents, incubation times, and centrifugation times and velocities specifically indicated for each type of sample.

2.1.4. Specifications for Different Types of Samples.

Whole blood samples: for these samples a previous selective lysis of red blood cells is required.

To do this, add three volumes of a red blood cell lysis solution to the initial volume of whole blood to be lysed. Mix the sample by inversion and leave to act for 5 minutes; invert the tube gently several times during the incubation. If the sample is completely lysed, a color change to a clearly darker shade is observed. Next, centrifuge to precipitate the leukocytes of the sample; they remain attached to the bottom of the tube while the supernatant containing the lysed red blood cells is decanted. A small residual volume of 200-400 µl of liquid should be left to resuspend the leukocytes by vigorous vortexing for about 20 seconds. Then lyse the leukocytes according to **step 1** of the general protocol described above.

This additional lysis of erythrocytes can also be done with samples of peripheral blood mononuclear cells (PBMCs) if red blood cells are observed in the sample.

Saliva samples: before processing saliva samples collected in a container with Oragene® preservative, it is recommended to incubate the sample at 50°C for 1-2 hours in order to maximize the final yield of DNA. This incubation time can be increased if it is deemed necessary to increase the final yield of the sample.

Tissue samples: tissue samples must be homogenized so that the cells are more exposed to the action of the reagents. There is a wide variety of methods for the disruption of tissue, such as sonication, mechanical grinding, using a mortar, adding glass beads, and vigorous stirring. In some DNA extraction protocols the cell lysis solution is added after homogenization of the sample, while in other protocols sample homogenization is carried out in the lysis solution itself. Because the tissue disruption process involves the release of proteases and other enzymes involved in the degradation of various cell components, enzyme inhibitors are commonly used to prevent cell degradation during processing. In addition, working quickly and in the cold with such samples minimizes the risk of enzymatic degradation.

For FFPE tissue preparations the paraffin must be removed from the sample to the greatest possible extent before DNA extraction. To this end, the sample is washed repeatedly with xylene (or some other type of dewaxing solution) and ethanol.

Note: the specifications given are common to all DNA extraction methods described in this work manual.

2.1.5. List of Protocols based on Salt Precipitation.

The following protocols are used by Biobanks who participated in the development of this manual.

-**Miller SA, Dykes DD, Polesky HF (1988).** A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* **16**:1215.

-**Gentra® Puregene® Handbook** for purification of archive-quality DNA from: human whole blood, bone marrow, buffy coat, buccal cells, body fluids, cultured cells, tissue, mouse tail, yeast, bacteria. Qiagen. (Hilden, Germany). www.qiagen.com.

-**Qiagen Genomic DNA Handbook** for blood, cultured cells, tissue, mouse tails, yeast, bacteria. Qiagen. (Hilden, Germany). www.qiagen.com.

-**Flexigene® DNA Handbook** for purification of DNA from: human whole blood, buffy coat, cultured cells. Qiagen. (Hilden, Germany). www.qiagen.com.

-**RealPure "SSS" kit.** Durviz s.l.u (Valencia, Spain). www.durviz.com.

-**DNA Isolation Kit for Mammalian Blood.** Roche. (Basel, Switzerland). www.roche.com.

-**Tissue DNA Extraction kit-AGF.** AutoGen. (Massachusetts, USA). www.autogen.com.

-**Oragene DNA Saliva Extraction-AGF.** AutoGen. (Massachusetts, USA). www.autogen.com.

2.1.6. Yield.

The expected yield for each of the above protocols is as follows:

-**Miller et al., (1988):** The expected yield is approximately 25 µg DNA/ ml whole blood.

-**Gentra® Puregene® Handbook:** the yield obtained in whole blood samples varies from 25-50 µg DNA/ ml blood, and the average yield is about 35 µg DNA/ ml blood.

In saliva samples the average yield is around 55 µg DNA /ml saliva, as long as the right amount of saliva (≈ 2 ml) was collected in the corresponding container.

In cell samples the yield is between 5 and 7 µg DNA/ 10⁶ cells, although this number is very variable depending on the cell type.

-**Qiagen Genomic DNA Handbook:** the yield is in the range of 25-35 µg DNA / ml blood.

-**Flexigene® DNA Handbook:** the average yield is about 37 µg DNA/ ml blood and around 13 µg DNA/ 10⁶ cells in samples of peripheral blood mononuclear cells.

-**RealPure “SSS” kit:** the yield varies from 15-45 µg DNA/ ml blood, and the average yield is about 35 µg DNA/ ml blood.

-**DNA Isolation Kit for Mammalian Blood:** the yield varies between 2-5 µg DNA / 10⁶ cells (it is generally estimated that 1 ml of whole blood contains an average of 7 x 10⁶ leukocytes). This kit has also been used for bone marrow samples, and a yield similar to that for blood was obtained.

-**Tissue DNA Extraction kit-AGF:** the average yield for fresh frozen tissue is about 3.5 µg DNA/ mg tissue.

In fixed and paraffin-embedded tissue the average yield is estimated to be around 30 µg DNA/ 3 tissue sections of 8 microns. These amounts can be highly variable depending on the type of tissue processed.

-**Oragene DNA Saliva 2 Extraction-AGF:** the average yield is approximately 140 µg DNA/ ml saliva.

2.1.7. Quality of the Samples

Purity:

The purity of DNA obtained with the salt precipitation method is very high and reaches in most cases values for the A260/A280 ratio of more than 1.8 for all types of samples mentioned above.

The A260/A230 ratio reaches values greater than 1.5 and is in most cases close to 2.0 (including fixed and paraffin-embedded tissue samples). However, in saliva samples in some cases a ratio of less than 1.5 was observed, but the integrity and functionality of the sample were not affected.

Integrity:

DNA samples obtained with this protocol can be considered as samples with a very high integrity, since usually a single defined band is seen in agarose gels. Exceptions are: **1)** saliva samples, since in some cases a slight smear can be seen in the lane, together with a predominant defined band at the top of the gel, and **2)** DNA samples from fixed and paraffin-embedded tissues, in which a pronounced smear is observed in the agarose gel.

Functionality:

In most of the protocols used, the samples allow amplification of a large DNA fragment (> 17 kb). In DNA samples obtained with the method described by **Miller et al., (1988)** amplification of 8.4 kb fragments has been obtained that allow good DNA sequencing. The functionality of the DNA samples obtained with the **Tissue DNA Extraction kit-AGF** protocol has been verified by amplifying fragments of 795 bp, 453 bp and 227 bp in both fresh frozen tissue and fixed and paraffin embedded-tissue.

2.2.- DNA EXTRACTION USING ORGANIC SOLVENTS.

2.2.1. Principle.

This protocol allows purification of DNA by the addition of phenol and chloroform, leading to the appearance of two phases: an upper aqueous phase containing the nucleic acids and an organic phase containing proteins solubilized in phenol and lipids dissolved in chloroform. For purifying DNA, phenol should have a pH≈7-8. Subsequently the DNA is precipitated from the aqueous phase with isopropanol or absolute ethanol and washed with 70% ethanol to remove salts and small organic molecules that may still be present in the sample. Finally the DNA is resuspended in an appropriate buffer.

2.2.2 Scope.

This method can be used to obtain DNA from different sample types, such as peripheral blood, cells or tissues. In biobanks integrated into the National Network of the ISCIII, this method is used to obtain DNA from blood and fresh frozen tissue.

2.2.3 General Protocol.

- Step 1.** The cells of the sample are lysed. Lysis is carried out depending on the type of sample to be processed, but before the addition of phenol lysis must be complete and the resulting lysate must be homogeneous.
- Step 2.** One volume of phenol is added to the volume of the lysate and the contents are mixed by inverting the tube for 20 seconds.
- Step 3.** The sample is centrifuged at 12,000xg for 3 minutes. After centrifugation two phases are observed: the upper aqueous phase and the organic phase. The interphase between the two is a whitish layer whose thickness will decrease as the sample becomes cleaner and contains less contaminating proteins.
- Step 4.** The aqueous phase containing the DNA is transferred to a clean tube taking care not to touch the interphase and the organic phase; these are discarded. An equal volume of a 1:1 phenol/CIA mixture is added to the aqueous phase. The mixture is homogenized by stirring.
- Step 5.** The sample is centrifuged again at 12,000xg for 3 minutes. The two phases are formed again but a considerable reduction of the interphase must be apparent. Steps 4 and 5 must be repeated as long as the interphase remains visible.
- Step 6.** The aqueous phase is transferred to a new tube and mixed with one volume of chloroform (chloroform removes phenol residues that may have remained in the sample). The aqueous phase and the chloroform are mixed by inversion for about 20 seconds.
- Step 7.** Centrifuge at 12,000xg for 3 minutes. Precipitate the aqueous phase in a clean tube with 2 volumes of isopropanol. Incubation of the mixture for 5-10 minutes is optional, but may improve the precipitation of DNA.
- Step 8.** Centrifuge at 12,000xg for 10 minutes to pellet the DNA. Discard the isopropanol. The DNA pellet should be visible at the bottom of the tube.
- Step 9.** Wash the precipitate with 500 µl of 70% ethanol. The sample is centrifuged at 12,000xg for 10-15 minutes. Optionally, a second wash can be done with ethanol to maximize sample purification.
- Step 10.** Remove the ethanol and leave the DNA precipitate to dry. Finally the DNA is resuspended in an appropriate volume of TE buffer or distilled water.

Note: CIA solution: mixture of chloroform and isoamyl alcohol in a ratio of 24: 1

TE buffer: 10 mM Tris HCl, pH 8.0, 1 mM EDTA

2.2.4. Specifications for Different Types of Samples

(See section with the same title in paragraph **2.1.4.** described on pages 14 and 15 of this manual).

2.2.5. Organic Solvents protocol that was used in the development of this Manual.

Sambrook, Fritsch, Maniatis (1989). *Molecular Cloning: A Laboratory Manual* 2nd Edition. **Vol. 3**, pages E3-E4. Cold Spring Harbor Laboratory Press.

2.2.6. Yield.

The yield obtained with this protocol in whole blood samples is around 30-70 µg DNA/ ml blood. In tissues the yield is much more variable and depends on tissue type and amount of starting sample. Nevertheless, extraction with organic solvents allows a very high yield of DNA and it is therefore a suitable method when the amount of starting sample is limited.

2.2.7. Quality of the Samples.

Purity: when this DNA extraction method used, it is crucial work very carefully to avoid contamination of the DNA solution by remnants of phenol or chloroform. The occurrence of traces of phenol or chloroform may affect the absorbance ratios that indicate the purity of the sample.

The samples obtained by this method have an A260/A280 ratio around > 1.7, although A260/A230 ratios less than 1.5 are relatively frequently observed.

Integrity: the samples show a defined band at the top of the agarose gel after electrophoresis, indicating an optimal DNA integrity.

Functionality: most samples obtained by this method are able to amplify large (> 17 kb) DNA fragments by PCR. For those samples with which bands >17 kb are not amplified, it is possible to amplify DNA fragments of approximately 7 kb long.

2.3- METHODS EMPLOYING ADSORPTION ON SILICA COLUMNS.

2.3.1. Principle.

The principle of this type of extraction method is based on the capacity of nucleic acids to be adsorbed on a silica column in the presence of high concentrations of chaotropic salts. Contaminants of the sample are removed with subsequent washes of the column, and finally the DNA is eluted with H₂O or a low ionic strength resuspension buffer at neutral or slightly alkaline pH.

2.3.2 Scope.

When a column is used for the purification of nucleic acids, it is important not to saturate the column's nucleic acid binding capacity by using an excess amount of sample. In fact, the yield and quality of the DNA (or RNA) obtained by this method depend greatly on the amount and quality of the starting sample.

This method can be used to obtain DNA from the following types of samples:

Peripheral blood: there are kits with silica columns with different retention capacities, such that blood volumes ranging from 0.02 ml to 10 ml can be processed.

Peripheral blood mononuclear cells (PBMCs): PBMC preparations may have variable volumes, since amounts from 5×10^6 leukocytes to 1×10^8 leukocytes can be processed, provided a kit is chosen with columns with the most appropriate loading capacity for the amount of starting sample.

Cells: the number of cells that can be processed depends largely on the cell type, and it is therefore recommended not to load the column with more than 1×10^7 cells when using this method for the first time.

Saliva: samples collected by swab or in a container with Oragene® preservative.

Tissues: for fresh frozen tissue it is recommended not to exceed 25 mg of sample per column, although the starting amount depends very much on the type of tissue to be processed. Fixed and paraffin-embedded tissues can also be processed by this method after removing the paraffin from the sample. When processing an FFPE tissue sample for the first time, it is advised not to use more than 3 sections with a thickness of 10 µm.

2.3.3 General Protocol.

- Step 1.** First the sample is lysed. In this lysis process proteinase K is usually added to the sample, followed by a warm incubation until lysis is complete (cell bodies should not be observed). Since not all kits employing this technique work in the same way, it is advisable to follow the specific instructions of each kit for the particular type of sample to be processed.
- Step 2.** If an RNA-free sample is required, RNase must be added to the lysate solution. In blood samples RNA contamination is negligible and therefore this step is not usually carried out. In other types of samples, which may have a high RNA content, it is recommend to add RNase following the instructions of the kit that will be used.
- Step 3.** A solution is added to the sample that promotes binding of nucleic acids to the column. This can be a solution included in the kit or absolute ethanol. The mixture is applied to the silica column fitted onto an empty tube; by centrifugation the proteins and other contaminants of the sample are eluted with the lysate solution into the tube, while the DNA is retained on the column.
- Step 4.** Transfer the column to a new tube and add a washing solution; centrifuge to elute remaining contaminants of the sample with the washing solution into the tube. This step is usually repeated twice to ensure good sample purity.
- Step 5.** The column is transferred to a new tube and is centrifuged again at maximum speed (for 1-2 minutes) to ensure complete removal of remnants of the washing solution from the column before elution of the DNA.
- Step 6.** Transfer the column to a new tube with a cap and elute the DNA from the silica column with H₂O or a low ionic strength buffer. A sufficient volume of resuspension buffer must be applied to the center of the column to ensure uniform distribution over the entire silica surface. It is advisable to incubate the column with resuspension buffer for 4-5 minutes to increase DNA yield. Centrifuge again; the DNA in solution is collected in the tube and the column is discarded.

Note: if the minimum volume of resuspension buffer is applied that ensures distribution through the entire the column, the obtained DNA solution will be more concentrated. Conversely, if a larger volume of DNA solution is desired, the sample will be more diluted. If an intermediate amount of DNA is desired in a solution that is not too dilute, the column can be eluted twice, each time with half the final resuspension volume of the sample. For example, if you want to elute with a total volume of 100 µl, a first elution is done with 50 µl and after centrifugation of the sample, keeping the column in the same tube, a second elution step is done with another 50 µl of resuspension solution.

2.3.4. Specifications for Different Types of Samples.

(See section with the same title in paragraph 2.1.4. described on pages 14 and 15 of this manual).

2.3.5 List of Protocols based on Adsorption on Silica Columns.

The following protocols are used by Biobanks that participated in the development of this manual.

-**Manual PerfectPure™ DNA Blood Kit** for genomic DNA purification from whole blood and buffy coat samples. 5Prime. (Hamburg, Germany & Gaithersburg, USA). www.5Prime.com.

-**Genomic DNA from Blood**. Macherey-Nagel. (Düren, Germany). www.mn-net.com.

-**QiAamp®DNA Mini and Blood Mini Handbook** for DNA purification from whole blood, plasma, serum, buffy coat, lymphocytes, dried blood spots, body fluids, cultured cells, swabs, and tissue. Qiagen. (Hilden, Germany). www.qiagen.com.

-**QIAamp® DNA FFPE Tissue Handbook** for purification of genomic DNA from formalin-fixed, paraffin-embedded tissues. Qiagen. (Hilden, Germany). www.qiagen.com.

2.3.6. Yield.

The expected yield for each of the protocols used is as follows:

-**Manual PerfectPure™ DNA Blood Kit:** the yield ranges from about 25-40 µg DNA/ ml blood.

-**Genomic DNA from Blood:** this protocol gives an average yield of approximately 30 µg DNA/ ml blood.

-**QIAamp®DNA Mini and Blood Mini Handbook:** for blood samples this method allows to obtain a DNA yield of 25-35 µg/ ml blood.

For saliva samples extracted with Oragene® preservative, a yield of 15-25 µg DNA/ ml saliva is obtained. For saliva samples obtained by swab, about 15-25 µg DNA /swab are obtained.

In cell samples values have been obtained of around 1.5-5 µg DNA/ 10⁶ cells; DNA extraction from cells fixed in Carnoy's solution (absolute ethanol, chloroform, glacial acetic acid in a ratio of 6: 3: 1) yields amounts of 1-4 µg DNA/ 10⁶ cells.

For tissue samples the expected yield is about 0.2-1.2 µg DNA/ mg tissue, although this yield depends very much on the type and amount of tissue sample being processed.

This method has also been used with a small amount of coagulum (≈ 200 µl) as starting sample, and a yield of about 20 µg per extraction was obtained.

-**QIAamp® DNA FFPE Tissue Handbook:** values have been obtained of 37 µg DNA/ mg tissue and 25-35 µg DNA/ 10 µm thick block but, as is customary in these tissues, these amounts depend greatly on the type of sample and the quantity and quality of the starting sample.

2.3.7. Quality of the Samples.

Purity: this method allows obtaining very pure samples, but the composition of the various solutions used leads to considerable variability of the A260/A 230 ratio.

The samples obtained by this method have an A260/A280 ratio of >1.7, except when DNA is obtained from cells fixed in Carnoy's solution; in this case, an average A260/A280 ratio of around 1.65 is observed. The A260/A 230 ratio is > 1 in most cases.

Integrity: samples obtained from blood, cells and fresh frozen tissue have a very high integrity, showing a defined DNA band at the top of the agarose gel. DNA samples from saliva and cells fixed with Carnoy's solution often show a smear of the sample, although the band at the top of the gel remains predominant. In samples obtained from FFPE tissues a pronounced smear is observed along the agarose gel, reflecting increased DNA degradation.

Functionality: samples having a defined band at the top of the agarose gel are capable of PCR-amplifying a fragment > 17.5 kb. In DNA samples from saliva or cells fixed in Carnoy's medium, amplification of fragments > 17.5 kb is not always achieved. Nevertheless, in most cases amplification of DNA fragments of approximately 5 to 7 kb is achieved. The functionality of fresh frozen tissue samples has not been shown by PCR, but since both the integrity and the purity obtained with these samples are high, it is likely that large DNA fragments can be amplified by PCR.

Samples from FFPE tissues often amplify fragments of approximately 300 bp, and they have been shown to be functional in a qPCR reaction that amplifies fragments of 100-150 bp.

2.4- PURIFICATION USING BINDING TO MAGNETIC BEADS.

2.4.1. Principle.

This method utilizes magnetic beads whose surface is coated with natural or synthetic polymers that have a high affinity for nucleic acids. The magnetic beads are added to the lysed sample, which allows them to bind to DNA molecules. When a magnet is placed against the wall of the tube the beads with bound DNA cluster at the wall, while dissolved contaminants of the sample are removed by pipetting or decanting. After several resuspension/washing cycles in which the beads are retained by the magnet, the DNA is eluted in a suitable buffer.

2.4.2 Scope.

This method is used to obtain DNA from the following types of samples:

Peripheral blood: volumes between 5 and 10 ml of peripheral blood can be processed.

Peripheral blood mononuclear cells (PBMCs): PBMC preparations have a volume that varies between 0.1-2 ml on the average.

Cell cultures: up to 1.2×10^7 cells can be processed.

Amniotic fluid: volumes of 1 to 3 ml can be processed.

Saliva: 2 ml of saliva mixed with 2 ml of Oragene® preservative can be processed.

Fresh frozen tissue: it is advised not to exceed 10 mg of sample per extraction.

2.4.3 General Protocol.

- Step 1.** Lysis buffer is added to the sample together with the reconstituted protease, and incubation is done for 5 minutes to ensure that lysis is complete. When handling tissues, proteinase K is added at a final concentration of 250 µg/ ml lysis buffer and incubation is done at 56°C while stirring at 600 rpm until cell lysis is complete. In saliva samples with Oragene® preservative, no lysis buffer is added but the sample with the preservative is incubated overnight at 55° C. Unlysed material can be removed by brief centrifugation.
- Step 2.** Magnetic beads are added to the lysed sample together with a buffer that promotes binding of the magnetic beads to the DNA. Mix well and allow to incubate for 5-10 minutes.
- Step 3.** A magnetic separator is applied to the tube containing the mixture of lysate and beads for 2-4 minutes so that the magnetic beads with the bound DNA stay attached to the part of the tube in contact with the magnetic separator, and the remaining solution is eliminated.
- Step 4.** The tube is removed from the magnetic separator and a wash buffer is added. The mixture is stirred so that all beads are accessible to the wash buffer.
- Step 5.** The magnetic separator is applied again for 1-3 minutes and the wash buffer is removed.
- Paso 6.** Two new washes are done following the procedure described in step 5. When eliminating the wash buffer in the last step the tube is not removed from the magnetic separator, so that the beads stay grouped together in the part of the tube that is in contact with the separator.

- Step 7.** A new washing solution is added on the grouped magnetic beads and incubation is done for 1 minute. Longer incubation times or dispersion of the magnetic beads during incubation can result in a lower yield, i.e. a smaller amount of DNA is obtained.
- Step 8.** The washing solution is removed and the appropriate volume of resuspension buffer is added (TE buffer pH 8.0 can also be used, or H₂O at pH 8.0). The separator is removed to mix the beads well with the DNA resuspension buffer. The solution is incubated for 10 minutes with stirring at room temperature to elute the DNA from the beads. This incubation can be done at 55°C to optimize elution of the DNA.
- Step 9.** The magnetic separator is applied to the tube for about 3 minutes to separate the magnetic beads from the DNA suspension. The DNA solution is transferred to a clean tube taking care not to carry over any magnetic beads. Repeat this last step if magnetic beads are still observed in the DNA solution.

2.4.4. Specifications for Different Types of Samples.

(See section with the same title in paragraph 2.1.4. described on pages 14 and 15 of this manual).

2.4.5. List of Protocols Based on Binding to Magnetic Beads

The following protocols are used by Biobanks who participated in the development of this manual.

- Chemagic DNA Blood Kit.** Chemagen. (Baesweiler, Germany). www.chemagen.com.
- Chemagic DNA Tissue Kit.** Chemagen. (Baesweiler, Germany). www.chemagen.com.
- Chemagic DNA Buffy Coat Kit special.** Chemagen. (Baesweiler, Germany). www.chemagen.com.
- Genomic DNA Isolation from 4 ml Saliva.** Chemagen. (Baesweiler, Germany). www.chemagen.com.
- Chemagic Amniotic Fluid Kit special.** Chemagen. (Baesweiler, Germany). www.chemagen.com.
- Chemagic DNA Cell Kit special.** Chemagen. (Baesweiler, Germany). www.chemagen.com.

2.4.6. Yield.

The yield obtained for whole blood samples is in the range of 25 to 33 µg DNA/ ml blood. For peripheral blood mononuclear cells yields of approximately 25 µg DNA/ ml blood are obtained.

With saliva samples a yield of about 25 µg DNA/ ml saliva is obtained.

For samples of cell cultures the yield is around 8-16 µg DNA/ 10⁶ cells. These numbers may vary depending on the cell type processed.

In amniotic fluid samples a yield of about 0.5-3 µg DNA/ ml sample is reached.

For fresh tissues a yield of about 2-6 µg DNA/ mg tissue has been observed. Result may vary depending on the nature of the tissue being processed.

2.4.7. Quality of the Samples.

Purity: if magnetic beads are detected in the sample the absorbance ratios can be affected; we therefore recommend reapplying the magnetic separator to the DNA solution for about 3 minutes to remove the beads that may have remained in the DNA sample.

In general, this method gives A₂₆₀/A₂₈₀ absorbance ratios between >1.7 and 2.1. In most cases the A₂₆₀/A₂₃₀ ratio is >1.5.

Integrity: after agarose gel electrophoresis of the DNA a defined band is observed at the top of the gel, indicating DNA of optimum integrity.

Functionality: most samples obtained by this method allow the amplification of large (>17.5 kb) DNA fragments by PCR.

ANNEXES

Tables YIELD obtained:

1A. Yield obtained for each type of sample using DNA extraction methods based on salt precipitation and extraction with organic solvents.

GENOMIC DNA EXTRACTION METHODS										
SALT PRECIPITATION									ORGANIC SOLVENTS	
TYPE OF SAMPLE	Miller <i>et al.</i> (1988)	Gentra® Puregene®	Qiagen Genomic DNA	Flexigene® DNA	RealPure "SSS"	DNA Isolation Kit for Mammalian Blood	Tissue DNA Extraction kit-AGF	Oragene DNA Saliva Extraction-AGF	Phenol/ Chloroform	
	peripheral blood	≈ 25 µg DNA /ml blood	≈ 35 µg DNA /ml blood	25-35 µg DNA /ml blood	≈ 37 µg DNA /ml blood	≈ 35 µg DNA /ml blood	2-5 µg DNA/ 10 ⁶ cells			30-70 µg DNA /ml blood
	PBMCs				≈ 13 µg DNA / 10 ⁶ cells					
	cells		5-7 µg DNA/ 10 ⁶ cells							
	bone marrow						2-5 µg DNA/ 10 ⁶ cells			
	cells fixed in Carnoy									
	saliva		≈ 55 µg DNA /ml saliva						≈ 140 µg DNA /ml saliva	
	fresh frozen tissue							≈ 3.5 µg DNA /mg tissue		very variable
	FFPE tissues							≈ 30 µg DNA /3 sections of 8 µm		
	amniotic fluid									
coagulum										

1B. Yield obtained for each type of sample using DNA extraction methods based on adsorption on silica columns and binding to magnetic beads.

		GENOMIC DNA EXTRACTION METHODS									
		ADSORPTION ON SILICA COLUMNS				BINDING TO MAGNETIC BEADS					
		Manual PerfectPure™ DNA Blood Kit	Genomic DNA from Blood	QiAamp®DNA Mini and Blood Mini	QiAamp® DNA FFPE Tissue	Chemagic DNA Blood Kit	Chemagic DNA Tissue Kit	Buffy Coat Kit special	Genomic DNA Isolation from Saliva	Chemagic Amniotic Fluid Kit special	Chemagic DNA Cell Kit special
TYPE OF SAMPLE	peripheral blood	25-40 µg DNA /ml blood	≈ 30 µg DNA /ml blood	25-35 µg DNA /ml blood		25-33 µg DNA /ml blood					
	PBMCs							≈ 25 µg DNA /ml blood			
	cells			1.5-5 µg DNA/ 10 ⁶ cells							8-16 µg DNA/ 10 ⁶ cells
	bone marrow										
	cells fixed in Carnoy			1-4 µg DNA/ 10 ⁶ cells							
	saliva			15-25 µg DNA /ml or swab					≈ 25 µg DNA /ml saliva		
	fresh frozen tissue			0.2-1.2 µg DNA /mg tissue			2-6 µg DNA /mg tissue				
	FFPE tissues				≈ 37 µg DNA/ mg 25-35 µg DNA/ 10 µg						
	amniotic fluid									0.5-3 µg DNA /ml sample	
	coagulum			≈ 20 µg /200 µl							

Tables observed **QUALITY** in the samples:

2A. Quality observed for each type of sample using DNA extraction methods based on salt precipitation and extraction with organic solvents.

		GENOMIC DNA EXTRACTION METHODS								
		SALT PRECIPITATION							ORGANIC SOLVENTS	
		Miller et al. (1988)	Genra® Puregene®	Quiagen Genomic DNA	Flexigene® DNA	RealPure "SSS"	DNA Isolation Kit for Mammalian Blood	Tissue DNA Extraction kit-AGF	Oragene DNA Saliva Extraction-AGF	Phenol/ Cloroform
TYPE OF SAMPLE	peripheral blood	P: A260/280 : 1.8-2 I: defined band at top of gel F: PCR amplification > 8.4kb, high quality sequencing	P: 260/280 >1.7-2.1; A260/230: >1.5 I: defined band at top of gel F: PCR amplification >17 kb	P: 260/280: >1.7-2.1; A260/230: >1.5 I: defined band at top of gel F: PCR amplification >17 kb	P: 260/280: >1.8; A260/230: > 2 I: defined band at top of gel	P: 260/280: >1.7-2.1; A260/230: >1.5 I: defined band at top of gel F: PCR amplification >17 kb	P: 260/280: >1.8; A260/230: >1.5 I: defined band at top of gel F: PCR amplification >17 kb			P: 260/280 >1.7; A260/230: <1.5 I: defined band at top of gel F: PCR amplification >17 kb in most samples. Always amplification of 7 kb.
	PBMCs				P: 260/280 >1.8; A260/230: >1.9 I: defined band at top of gel					
	cells		P: 260/280 >1.7-2.1; A260/230: >1.5 I: defined band at top of gel F: PCR amplification >17 kb							
	bone marrow									
	cells fixed in Carnoy									
	saliva		P: 260/280 >1.7-2.1; A260/230: <1.5 I: defined band with slight smear F: PCR amplification >17 kb in most samples. Always amplification of 7 kb.							P: 260/280: >1.7; A260/230: >1.4
	fresh frozen tissue								P: 260/280: >1.9; A260/230: >1.8 I: defined band at top of gel F: PCR amplification bands: 795, 453 and 227 bp	P: 260/280 >1.7; A260/230: <1.5 I: defined band at top of gel F: PCR amplification >17 kb in most samples. Always amplification of 7 kb.
	FFPE tissues								P: 260/280: >1.9; A260/230: >1.8 I: pronounced smear F: PCR amplification bands: 795, 453 and 227 bp	
	amniotic fluid									
coagulum										

P: purity
I: integrity
F: functionality

2B. Quality observed for each type of sample using DNA extraction methods based on adsorption on silica columns and binding to magnetic beads.

		GENOMIC DNA EXTRACTION METHODS									
		ADSORPTION ON SILICA COLUMNS				BINDING TO MAGNETIC BEADS					
		Manual PerfectPure™ DNA Blood Kit	Genomic DNA from Blood	QiAamp®DNA Mini and Blood Mini	QiAamp® DNA FFPE Tissue	Chemagic DNA Blood Kit	Chemagic DNA Tissue Kit	Chemagic DNA Buffy Coat Kit special	Genomic DNA Isolation from Saliva	Chemagic Amniotic Fluid Kit special	Fluid DNA Kit special
TYPE OF SAMPLE	peripheral blood	P: A260/280 > 1.9; A260/230: >2.1	P: A260/280 >1.7; A260/230: 1-1.5 I: defined band at top of gel F: PCR amplification >17 kb	P: A260/280 >1.7; A260/230: >1.5 I: defined band at top of gel F: PCR amplification >17 kb		P: A260/280 >1.7-2.1; A260/230: >1.5 I: defined band at top of gel F: PCR amplification >17 kb					
	PBMCs							P: A260/280 >1.7-2.1; A260/230: >1.5 I: defined band at top of gel F: PCR amplification >17 kb			
	cells			P: A260/280 >1.7; A260/230: >1 I: defined band at top of gel F: PCR amplification >17 kb							P: A260/280 >1.7-2.1; A260/230: >1.5 I: defined band at top of gel F: PCR amplification >17 kb
	bone marrow										
	cells fixed in Carnoy			P: A260/280 >1.65; A260/230: >1 I: band at top of gel with smear F: PCR amplification >7 kb							
	saliva			P: A260/280 >1.7-2; A260/230: >1 I: defined band with smear F: PCR amplification >7 kb					P: A260/280 >1.7-2.1; A260/230: >1.5 I: defined band at top of gel F: PCR amplification >17 kb		
	fresh frozen tissue			P: A260/280 >1.7-2 I: defined band at top of gel			P: A260/280 >1.7-2.1; A260/230: >1.5 I: defined band at top of gel F: PCR amplification >17 kb				
	FFPE tissues				P: A260/280 >1.7-2; A260/230: >2 I: pronounced smear F: PCR amplification = 300 bp qPCR amplification = 100-150 bp						
	amniotic fluid									P: A260/280 >1.7-2.1; A260/230: >1.5 I: defined band at top of gel F: PCR amplification >17 kb	
	coagulum			P: 260/280 >1.7; A260/230: >1.5							

P: purity
I: integrity
F: functionality

Red Nacional
de Biobancos
Spanish National
Biobank Network



Red Biobancos

Institute of Health Carlos III