Video Article

Dried Blood and Serum Spots As A Useful Tool for Sample Storage to Evaluate Cancer Biomarkers

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Abstract

Blood sample quality is crucial to ensure accurate downstream analyses such as real-time PCR or ELISA. Correct storage of biological materials is the starting point to achieve reproducible and reliable results. All samples should be treated in the same way from blood collection to storage. Depending on the analyses to be performed, whole blood and serum samples should be stored at -20 °C or -80 °C until use. Blood/serum samples should also be aliquoted to avoid multiple freeze-thawing. Another important issue is the sample conditions during shipment from one laboratory to another. If dry ice is not available or the shipment takes longer than a few days, alternative approaches are needed. One option is to use filter paper for blood collection. Here, we propose a method for blood and serum sample collection that takes advantage of dried blood spots (DBS) and dried serum spots (DSS). We developed the procedure to extract DNA from DBS for the downstream evaluation of some single nucleotide polymorphisms (SNPs) by real time PCR. We also optimized an ELISA assay starting from proteins eluted from DSS. This method can be used with other ELISA assays or procedures evaluating proteins.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57113/>

Introduction

The main aim of the research into cancer biomarkers is the identification of new biological parameters that can be used for diagnosis, for predicting patient prognosis, and for determining whether a patient will respond to a specific treatment. This research area is fundamental for the discovery of innovative cancer treatments and plays a key role in tailored treatment.

The procedures carried out during each step of biomarker identification and validation must be reliable and reproducible. A cornerstone for the success of translational research is the correct storage of biological samples such as blood and serum. This is the first step towards obtaining high quality biological material that can be used to perform molecular biology experiments or protein assays.

Multicenter studies are often needed to recruit enough patients to obtain robust data. Not all institutes are able to store samples at -80 °C or to send samples to other international centers in dry ice. The use of filter paper for blood collection is a simple method for storing blood and serum and does not require the immediate freezing of samples^{1,2}. A drop of blood or serum can be spotted onto the paper, left to dry overnight and then stored for up to 14 days at room temperature^{1,2}. This gives researchers time to send the samples to other laboratories. The use of dried blood spots (DBS) and dried serum spots (DSS) could thus simplify the collaboration between institutes in developed and developing countries.

Given its ease of use, DBS sampling is widely used in several types of assays for serological or genetic downstream analyses. For example, in the past, DBS were frequently used for HIV screening in developing countries^{1,2,3,4,5}. Another advantage of this storage method is that blood samples can be collected from finger-pricks, thus enabling its use for newborn screening tests^{6,7,8}. Easy sample handling and transport are further advantages of DBS, especially for samples collected in remote sites where there is no laboratory equipment. In a previous publication, we used DBS and DSS to test vitamin D and vitamin D binding protein (DBP) in a series of Caucasian and African patients⁹. Our African colleagues were not able to obtain dry ice. To compare the biological markers to understand the differences in the vitamin D pathway between the two ethnic populations, we further improved the procedure using matched samples stored under standard conditions and on filter paper. After optimizing the DBS/DSS procedure, we were able to analyze DBP and vitamin D in patient serum in both cohorts. We also evaluated a number of single nucleotide polymorphisms (SNPs) after DNA extraction from whole blood for Caucasians and from DBS for Africans⁹. The present protocol permits high quality blood samples to be stored at room temperature without affecting different types of downstream analyses ranging from

molecular biology to ELISA assays. It is recommended for use to manage biological materials in multicenter studies or for centers that do not have facilities for standard storage conditions. The following protocol represents the culmination of these optimized procedures.

Protocol

Peripheral blood and serum were collected and stored from healthy donors and patients who gave written informed consent to take part in the study. The study protocol was approved by the local Ethics Committee in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

1. Blood Storage in DBS

1. Blood sample collection

1. Collect 3 mL of peripheral blood sample in a 3 mL tube with 5.4 mg of ethylenediaminetetraacetic acid (EDTA).
NOTE: Store peripheral blood as DBS as soon as possible and within 8 h.
2. Write the patient code number on the bottom right-hand corner of the saver card.
3. Carefully resuspend the blood with a 5 mL pipette.

2. Blood spotting on DBS

1. Pipette and transfer one spot of blood (50 μ L) onto the saver card (**Table of Materials**) with a 50–200 μ L tip.
2. Repeat step 1.2.1. to have a total of 3–5 blood spots. Discard the old tip and take a new one for each blood aliquot.
3. Leave the DBS to dry overnight at room temperature in a horizontal position over an open non-absorbent surface, avoiding direct sunlight. Leave the cover of the saver card open to facilitate blood drying.
NOTE: Any particulate in air or dust during this drying step does not affect downstream applications.
4. Store the card at room temperature in a plastic bag with desiccant until use.

2. DNA Extraction Starting from DBS According to the DNA Extraction Kit Datasheet (Section for Dried Blood Spots)

1. Sample preparation

1. Use 3 dried blood spots from the card. Cut each of the dried blood spots from the card and separate them from the card with tweezers. Cut the separated dried blood spots into small pieces (diameter of about 1 mm).
2. Place the small pieces into a 1.5 mL centrifuge tube.

2. Sample digestion

1. Add 180 μ L of lysis buffer 1 provided in the kit (**Table of Materials**) to the tube. Add 20 μ L of Proteinase K. Do not mix lysis buffer 1 with Proteinase K before pipetting into the 1.5 mL tube with blood spotted card. Mix by vortexing.
2. Place the tube in a thermoincubator and incubate at 56 °C for 60 min. If the thermoincubator is not equipped with a shaker, vortex the sample every 10–15 min for at least 10 s, taking care not to let the temperature of the sample decrease. Briefly centrifuge the tubes to remove drops from the lid.

3. Wash step

1. Add 200 μ L of lysis buffer 2 (**Table of Materials**) and vortex for 10 s.
2. Place the tubes in a thermomixer or heated orbital incubator and incubate at 70 °C for 10 min. If the thermoincubator is not equipped with a shaker, vortex the samples once for at least 10 s, taking care not to let the temperature of the samples decrease.
3. Repeat step 2.3.1. Transfer the 400 μ L of lysate from the 1.5 mL tube to elution column (**Table of Materials**). Centrifuge the column at 6,000 x g for 1 min.
4. Place the elution column into a new 2 mL collection tube and discard the old one.
5. Add 500 μ L of wash buffer 1 (**Table of Materials**) and centrifuge at 6,000 x g for 1 min.
6. Place the column into a new 2 mL collection tube and discard the old one.
7. Add 500 μ L of wash buffer 2 (**Table of Materials**) and centrifuge at 6,000 x g for 1 min.
8. Place the column into a new 2 mL collection tube and discard the old one.
9. Centrifuge at 20,000 x g for 3 min to dry the membrane.

4. Elution step

1. Place the column into a new 1.5 mL centrifuge tube and discard the collection tube.
2. Place 50 μ L of distilled water onto the center of the membrane. Incubate for 10 min at room temperature.
3. Centrifuge the tube at 20,000 x g for 1 min. Collect the eluate and place it again on the center of the membrane.
4. Centrifuge the tube at 20,000 x g for 1 min. Discard the membrane.
5. Measure the DNA concentration using a spectrophotometer.
6. Store DNA at -20 °C until use.
NOTE: DNA extracted from DBS can be used to perform several analyses such as SNP evaluation by Real Time PCR or DNA Sanger sequencing.

3. Serum Storage for DSS

1. Blood sample collection

1. Collect 7 mL of peripheral blood sample in a 7 mL tube without EDTA. Leave the blood sample to coagulate for 30 min at room temperature.
2. Write the patient code number on the bottom right-hand corner of the saver card.
3. Centrifuge the blood sample at 980 x g for 15 min at room temperature.
NOTE: Store serum as DSS as soon as possible and within 8 h.
4. Carefully remove the supernatant from the tube and transfer it to a new 15 mL tube. Carefully resuspend the blood with a 5 mL pipette.

2. Serum spotting in DSS

1. Pipette and transfer one spot of serum (50 μ L) onto the saver card. Repeat this step to have a total of 3–5 blood spots.
NOTE: Be very careful to pipette exactly 50 μ L of serum into each spot. Serum should not go beyond from the dashed line of the card.
2. Leave the DSS to dry overnight at room temperature. Leave the cover of the saver card open to facilitate blood drying.
3. Store the card at room temperature in a plastic bag with desiccant until use. If the storage is longer than 14 days, store the card at -20 °C.

4. ELISA Assay Starting from DSS

1. Elute protein from DSS.

1. If necessary, thaw a DSS for each sample. Cut DSS into small pieces (about 1 mm in diameter). Put the pieces into a 1.5 mL tube with 400 μ L of PBS.
2. Shake the samples overnight at 4 °C.
NOTE: Shake them at low intensity. PBS should not wet the cover of the tube during shaking.

2. Use the protein eluate as a serum sample for secreted protein/growth factor analysis according to ELISA kit instructions, after appropriate dilutions.

NOTE: This procedure works well for DBP detection⁹. For other ELISA assays, an optimization step may be needed.

Representative Results

We took advantage of the DBS and DSS procedures to store blood and serum at room temperature without affecting the quality of the biological material. **Figure 1** shows an example of the protein card saver without blood and after blood collection. In order to confirm that the storage on filter paper and the procedure to elute blood do not interfere with sample quality, we performed a comparison with standard storage methods. We extracted DNA from 3 matched samples of whole blood collected in a 2 mL tube or stored as DBS, as reported in the Protocol section. Five SNPs were analyzed by real time PCR. The data obtained for matched samples were 100% concordant.

With regard to ELISA analyses, we stored 8 samples of serum collected in a 2 mL tube (and stored immediately at -80 °C) and as DSS (stored at room temperature for one week and then at -20 °C). We eluted proteins from DSS, as described in the Protocol section, and then performed ELISA according to manufacturer's datasheet for DBP for the 8 samples. Results are shown in **Table 1**. The coefficient of variation (CV) between matched samples ranged from 2% to 24 %. The mean CV was 9.6%.

Figure 1: Example of DBS before and after the blood collection. (A) Protein saver card. (B) Protein saver card after blood sampling. [Please click here to view a larger version of this figure.](#)

Samples	DBP (µg/mL)		CV%
	Serum (st)	DSS	
1	66.47	65.28	2
2	213.31	216.51	1
3	164.96	145.21	14
5	109.81	120.28	9
6	162.70	130.60	24
7	124.63	117.13	6
8	149.47	132.69	12

Table 1: DPB levels in matched DSS and serum samples. DBP levels analyzed by ELISA assay. CV is the coefficient of variation calculated between the 2 obtained values. The CV percentage is calculated as follows: ((DSS value-Serum value)/DSS value) x 100%.

Discussion

This protocol investigates the potential for storing blood and serum on filter paper when the labs do not have the staff or infrastructures needed for the correct handling of blood samples. In particular, whole blood or serum collected in standard tubes or by finger-prick can be stored using this method and there is no need to freeze samples at -20 °C or -80 °C immediately after the blood collection. DBS/DSS can remain at room temperature for up to 14 days without any change in blood/serum integrity. A critical issue for the storage is the presence of moisture which affects the properties of biological material more than temperature. This problem can be avoided by using plastic bags with desiccant. Moisture becomes an issue during the storage when cards are inside the plastic bags. Problems resulting from particulate in air/droplets/dust or moisture were not observed during the overnight incubation¹⁰. We previously used filter paper to store blood and serum in a study of biological markers in Italian and African cohorts¹¹. We optimized DNA extraction from DBS and analyzed a number of SNPs in both case series. As we were also interested in evaluating protein levels, we optimized a method to elute proteins from DSS in order to perform ELISA assays. Serum storage in DSS for ELISA is more delicate than whole blood storage for qualitative analyses because laboratory researchers must be sure to pipette exactly the same amount of serum in all samples. If pipetting is not accurate, downstream analyses will be compromised. Whilst the DNA extraction procedure can be applied to other molecular analyses, the protein procedure should be optimized in matched samples for each new cytokine/secreted factor investigated. It is important to cut DSS into very small pieces and to wet the pieces of card thoroughly with PBS during the overnight incubation to ensure efficient protein elution. This is a more delicate step than that of blood elution because ELISA is a quantitative evaluation.

In recent years, this storage approach has been widely used for different applications ranging (as described in this protocol) from molecular biology to protein analysis^{12,13}. In particular, good quality mass results starting from serum spotted in saver cards have been reported¹⁴, confirming the high flexibility and reliability of the method.

Our results, in agreement with the literature, highlight the usefulness of storing samples as DBS and confirm the reliability of this procedure with respect to standard storage methods. We confirmed previous results and underlined the possibility of optimizing an ELISA assay by using it together with our storage methods. Thanks to its simplicity, developing countries can also contribute to cancer research. For example, as the majority of studies on Africans are performed on African Americans, very few data are available on native Africans⁹. The use of DBS could help to bridge this gap.

Disclosures

The authors have no conflicts of interest to disclose.

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