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DNA extraction from clotted blood in genotyping quality

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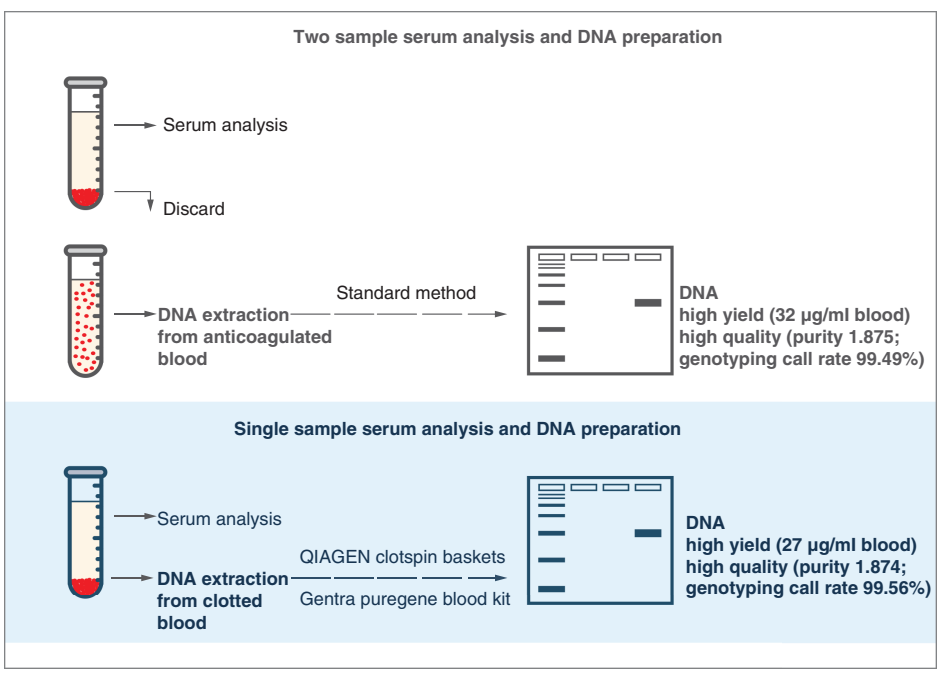
ABSTRACT

DNA extraction from frozen blood clots is challenging. Here, the authors applied QIAGEN Clotspin Baskets and the Gentra Puregene Blood Kit for DNA extraction to cellular fraction of 5.5 ml whole blood without anticoagulating additives. The amount and quality of extracted DNA were assessed via spectrophotometer and gel electrophoresis. Results from array-based genotyping were analyzed. All steps were compared with DNA isolated from anticoagulated blood samples from a separate study. The quality and concentration of DNA extracted from clotted blood were comparable to those of DNA extracted from anticoagulated blood. DNA yield was on average 27 μg per ml clotted blood, with an average purity of 1.87 (A260/A280). Genotyping quality was similar for both DNA sources (call rate: 99.56% from clotted vs 99.49% from anticoagulated blood).

METHOD SUMMARY

This study describes DNA extraction from frozen clotted blood using QIAGEN Clotspin Baskets and the Gentra Puregene Blood Kit. The concentration, yield per milliliter of blood, purity and integrity of the DNA were further confirmed via spectrophotometer and gel electrophoresis. In addition, performance for genotyping on the Illumina Global Screening Array was analyzed. Comparisons with DNA extracted from anticoagulated blood were performed.

GRAPHICAL ABSTRACT



KEYWORDS:
anticoagulated blood ● AugUR ● clotted blood ● DNA concentration ● DNA extraction ● DNA purity ● DNA yield ● frozen samples ● genotyping ● TiKoCo

Epidemiological studies at field centers are important for effective recruitment in short time. For practical constraints "in field," but also ethical concerns, the participants' burden must be reduced to a minimum, especially regarding blood collection.

For most biomarker analyses, whole blood is collected in tubes without additives to gain serum, and cellular fractions are usually discarded after centrifugation. Gold-standard DNA extraction requires buffy coat or whole anticoagulated blood [1,2], and thus blood draw with a separate tube with anticoagulant additives. The additional blood sampling can be avoided when extracting DNA from serum tubes (i.e., clotted blood), for which several methods have been described [3–11]. However, the applicability and effectiveness of these methods to obtain high-quality DNA in epidemiological-scale studies for whole-genome genotyping are unclear.

Here the authors describe a protocol for DNA extraction from blood clots after storage at -20°C for more than 1 year using commercial products. The authors applied this protocol for DNA extraction for an epidemiological-scale field study, the Tirschenreuth SARS-CoV-2 antibody study (TiKoCo, n = 4204) [12–14]. DNA yield, quality and utility for array-based genotyping were compared with DNA extracted from anticoagulated blood from the AugUR study (n = 1282) [15]. Blood samples from this study were chosen for comparison because DNA extraction from AugUR was conducted in the same laboratory and by the same personnel. In addition, AugUR was genotyped with the very same array as TiKoCo.

Materials & methods

Sample collection & DNA extraction materials

Whole blood was collected for the baseline survey of the TiKoCo cohort, a population-based study to determine the SARS-CoV-2 serostatus in the German county of Tirschenreuth [12-14]. The TiKoCo study was approved by the Ethics Committee of the University of Regensburg, Germany (vote 12-101-0258). A single blood tube to obtain a serum sample was taken from each of the 4204 study participants in sitting position (S-Monovette® 5.5 ml, Sarstedt, Nümbrecht, Germany). The samples were processed on the same day. After centrifugation (2000× *g*, 10 min at room temperature), serum was used for anti-SARS-CoV-2 tests and clotted blood was stored at -20°C up to 415 days prior to DNA extraction.

The authors excluded 70 participants without informed consent for genetic analyses and an additional three participants without available clotted blood. For the remaining 4131 participants, blood clot preparation and DNA extraction were performed using Clotspin® Baskets and the Gentra® Puregene® Blood Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions [16] with slight modifications. The Gentra Puregene Blood Kit contains red blood cell (RBC) lysis solution, cell lysis solution, protein precipitation solution (salting-out method) and DNA hydration solution. Proteinase K, isopropanol (2-propanol) and ethanol at molecular biology grade were purchased from Thermo Fisher Scientific (MA, USA).

Protocol for DNA extraction from blood clots

Blood clot preparation & RBC lysis

In detail, frozen blood clots in 15 ml tubes were transferred from -20°C to a warming cabinet (55°C) for 10 min and thereafter immediately placed on ice. The tube was inverted to loosen the clot. The blood clot was completely poured with 5 ml RBC lysis solution into the Clotspin Basket placed on a 50 ml tube (Sarstedt). To disperse the clot, the sample was centrifuged at 2000× *g* for 5 min. The remaining clot material from the Clotspin Basket was transferred through the basket to the filtrate with 10 ml RBC lysis solution and the basket was discarded. To completely disperse the clotted material, the filtrate was vortexed vigorously for 3 s and placed for 5 min at room temperature on a circulating shaker (250 1/min). The tubes were again vortexed vigorously for 3 s and centrifuged at 2000× *g* for 5 min. The supernatant was carefully discarded, taking care that the pellet remained in the tube. If no pellet was visible, about 0.5 ml of the supernatant was kept in the tube. The tube was vortexed rigorously for 10 s and an additional 5 ml RBC lysis solution was added to the pellet, followed by vortexing for 3 s and incubation on a circulating shaker (250 1/min) for 5 min at room temperature.

White blood cell lysis

After centrifugation at 2000× *g* for 5 min to pellet the DNA-containing white blood cells, the supernatant was carefully discarded, leaving about 0.2 ml of residual liquid. The tube was vortexed rigorously for 10 s. Addition of 5 ml cell lysis solution and 25 µl of proteinase K (20 mg/ml) was followed by rigorous vortexing for 10 s. For complete lysis of DNA-containing cells, the samples were incubated overnight at 55°C.

Protein precipitation

On the next day, the samples were cooled on ice for 5 min, and 1.7 ml protein precipitation solution was added, followed by rigorous vortexing for 20 s. After centrifugation at 2000× *g* for 10 min, the samples were incubated for 2 min on ice.

DNA precipitation & washing

For the precipitation of DNA, the supernatant was carefully transferred in a 50 ml tube, containing 5 ml isopropanol. The samples were mixed by gently inverting the tube 50-times and centrifuged at 2000× *g* for 3 min. The supernatant was carefully discarded, and the tube was drained on a clean piece of absorbent paper without losing the pellet. In preliminary experiments, the purity of DNA from samples that showed red- to brown-colored pellets after DNA precipitation was below 1.6 (A260 nm/A280 nm). Therefore, the DNA pellet was inspected visually and with the two following options. (1) If the pellet was not small and white but large and red to brown, 2 ml of cell lysis solution and 10 µl proteinase K were added, followed by incubation at 55°C for 2 h in a warming cabinet or at room temperature overnight on a circulating shaker (250 1/min). For removal of residual proteins and other cellular contaminants, the samples were cooled on ice for 5 min and 1 ml protein precipitation solution was added, followed by rigorous vortexing for 20 s. After centrifugation at 2000× *g* for 10 min, the samples were incubated for 2 min on ice. DNA precipitation was done with 3 ml isopropanol. The samples were mixed by gently inverting the tube 50-times and centrifuged at 2000× *g* for 3 min. The supernatant was carefully discarded. The tube was drained on a clean piece of absorbent paper, taking care that the pellet remained in the tube. (2) For small and white pellets – or after option 1 – 5 ml of 70% ethanol was added immediately and the tubes were inverted until the pellet was detached. After centrifugation at 2000× *g* for 3 min, the supernatant was carefully discarded, and the DNA pellet was air-dried at room temperature for 10 min or until the pellet got glassy.

DNA hydration

The addition of 0.5 ml DNA hydration solution for a large pellet or 0.3 ml for a smaller pellet was followed by incubation at 65°C in a warming cabinet for an hour. To fully dissolve the DNA, the samples were put on a shaker overnight at room temperature. On the next day, the samples were centrifuged briefly and the solved DNA was transferred to a 2 ml cup and stored at -20°C. To avoid cross-contamination of samples, all pipetting steps were carried out using filter tips (different manufacturers).

The protocol is available at www.protocols.io/view/dna-extraction-5qpvobjw7l4o/v1.

DNA extraction from anticoagulated blood

For 1282 participants from the second baseline survey of the German AugUR study [15], DNA samples extracted with a very similar protocol, but from EDTA-anticoagulated blood, were available (AugUR2). Both protocols use a salting-out method to remove proteins and other cellular contaminants. In brief, the differences in the AugUR2 DNA extraction protocol from the one used for clotted blood are the following: whole blood from AugUR2 was stored at -20°C in 2.7 ml EDTA-containing monovettes (Sarstedt). No Clotspin Baskets were used. After thawing, RBC lysis was done in 9 ml lysis buffer (155 mM NH_4Cl, 20 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4). Lysis of DNA-containing cells was performed with 1.6 ml SE buffer (75 mM NaCl, 25 mM Na_2EDTA, pH 8.0), 10 µl proteinase K (20 mg/ml) and 100 µl 20% sodium dodecyl sulfate overnight. For salting-out, 1.7 ml SE buffer and 1 ml saturated NaCl were used, followed by centrifugation at 40°C. Extracted DNA was solved in 0.3 ml DNA hydration solution (10 mM Tris/HCl pH 8.0, 1 mM Na_2EDTA) overnight.

Evaluation of DNA concentration & quality

Two µl of DNA was used to determine quantity (A260 nm) and quality (A260 nm/A280 nm) on a Tecan Infinite® 200 PRO plate reader with i-control 1.10.4.0 software and a NanoQuant plate (Tecan Life Sciences, Männedorf, Switzerland). To assess potential DNA degradation or shearing, electrophoresis with 1 µl undiluted DNA was performed on 0.8% agarose gels.

Evaluation of DNA by genotyping

Genotyping of single nucleotide polymorphisms for both studies, TiKoCo and AugUR2, was conducted by Life & Brain GmbH, Bonn, Germany, using the Infinium Global Screening Array-24, GSA-MD, version 3.0 (Illumina, Inc., CA, USA) following the manufacturer's instructions, applying 200 ng of DNA. A minimum DNA concentration of 60 ng/µl for genotyping is recommended. All samples meeting this criterion were genotyped. A total of 730,059 variants were available on the genotyping array ("GSAMD-24v3-0-EA_20034606_A1" manifest file).

Data management, statistical analysis & literature search

Data management and statistics were done with SPSS 26.0.0.1 for Windows (IBM Corp., NY, USA). Box and whisker plots were created with GraphPad Prism, version 8.4.3, for Windows (GraphPad Software, CA, USA). PLINK 1.9 was used to analyze the genotype data [17]. For comparison of continuous parameter between two groups, the *t*-test was applied. The level of significance was set at $p < 0.05$.

A comprehensive PubMed search was conducted using the search terms "DNA extraction method human clotted blood" (access date: 1 April 2022). In addition, Labome was searched for available DNA extraction protocols (last access date: 1 April 2022) [18].

Results & discussion

DNA from clotted blood was extracted from 4131 samples, one each per participant of the TiKoCo study. Purity of the extracted DNA was high, with a mean of 1.874 (A260 nm/A280 nm; Table 1); n = 4111 (99.5%) samples yielded a purity >1.80. No difference in DNA purity between extraction from clotted blood and anticoagulated blood (n = 1282) could be observed (Table 1). Total yield of DNA per ml whole

Table 1. Comparison of concentration and quality for DNA extracted from serum clotted blood and EDTA-anticoagulated blood.

	Clotted blood	Anticoagulated blood
Study	TiKoCo	AugUR2
Blood available, n	4131	1282
Blood volume, ml	5.5	2.7
DNA purity (A260 nm/A280 nm), mean ± SD	1.874 ± 0.042	1.875 ± 0.023
DNA per 1 ml blood (µg), mean ± SD (maximum)	26.92 ± 12.56 (111.71)	32.15 ± 12.45 (103.64)
DNA concentration <60 ng/µl, n (%)	71 (1.72%)	2 (0.16%)
DNA samples used for genotyping	4060	1280
Per person genotyping call rate on Illumina Global Screening Array-24, GSA-MD, version 3.0 (%), mean ± SD (n <95%)	99.560 ± 0.003 (3)	99.493 ± 0.001 (0)
Per single nucleotide polymorphism genotyping call rate on Illumina Global Screening Array-24, GSA-MD, version 3.0 (%), mean ± SD	99.502 ± 0.050	99.427 ± 0.052

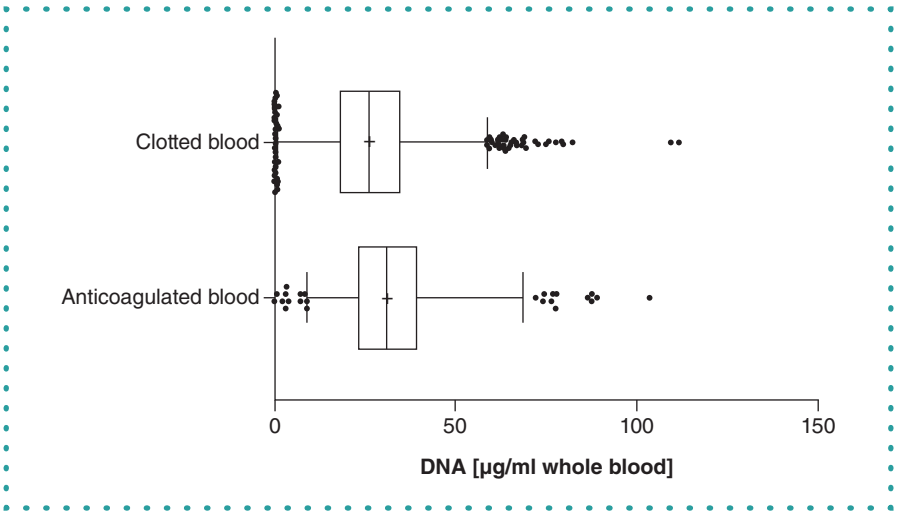


Figure 1. Distribution of total amount of DNA normalized to 1 ml of whole blood from the two DNA sources: DNA extraction from clotted blood (n=4131, TiKoCo), DNA extraction from anticoagulated blood (n=1282, AugUR2). Boxes show 25th, 50th and 75th percentiles. Whiskers range from 1st to 99th percentile with values beyond these percentiles as single points. The mean of DNA amount is shown as "+" (26.9 µg and 32.2 µg respectively; $p=2.4*10^{-38}$).

blood was 26.9 ± 12.6 µg from clotted and 32.2 ± 12.5 µg from anticoagulated samples (Table 1). The clotted blood samples that were washed twice after DNA precipitation (n = 140) showed comparable good purity (1.876 in comparison with 1.874 of samples from clotted blood without second washing step, n = 3991) and 31% lower DNA yield (18.68 µg/ml blood vs 27.21 µg/ml blood), but with sufficient mean DNA concentration (226.6 ng/µl vs 313.5 ng/µl). The minimum DNA concentration of 60 ng/µl for genotyping was achieved for 4060 (98.3%) clotted and for 1280 (99.8%) anticoagulated blood samples (Table 1); all these samples were used for genotyping. For 15 (0.4%) clotted blood samples, low amounts of DNA with concentration <5 ng/µl (too little for most high-throughput analyses) were achieved. The minimum yield for the remaining samples with a concentration between 5 and 59 ng/µl was 1.5 µg DNA in total, which is sufficient for most downstream applications after concentrating the DNA.

DNA yield per ml blood was significantly lower after extraction from clotted blood in comparison with anticoagulated blood (Figure 1). Especially, DNA concentration in samples from clotted blood was below the genotyping threshold of 60 ng/µl in 1.72% of TiKoCo samples in comparison with 0.16% from AugUR2 (Table 1). However, this is not only due to the different extraction protocols but also due to the different blood sampling processes. While AugUR2 blood collection was performed under standardized circumstances in the authors' study center, TiKoCo blood collection was performed "in the field" at three locations, including home visits for immobile individuals [12]. In AugUR2, for all samples, 2.7 ml anticoagulated blood was available. The authors observed that some TiKoCo samples did not contain the requested 5.5 ml blood. From the 71 samples resulting in DNA concentration below 60 ng/µl in TiKoCo, 63 were marked to be not fully filled blood samples. Therefore, very low DNA concentration was rather caused by the low amount of blood in the tube than in dependence of the DNA extraction method. Taking only fully filled tubes into account, the rate of samples with DNA concentration below 60 ng/µl was comparable between extraction from clotted blood (0.19%) and extraction from anticoagulated blood (0.16%).

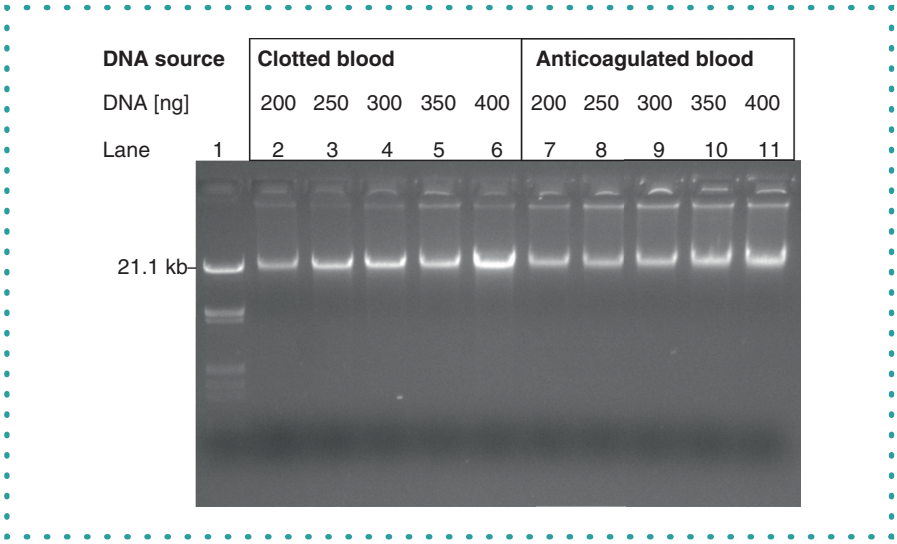


Figure 2. Gel electrophoresis of 1 μl extracted DNA on 0.8% agarose gel. Lane 1 represents λ-EcoRI-HindIII DNA marker (500 ng) with band at 21,226 bp. Lane 2 to 6: DNA samples extracted from clotted blood (TiKoCo) with 200, 250, 300, 350 and 400 ng/μl genomic DNA. Lane 7 to 11: DNA samples extracted from anticoagulated blood (AugUR2) with 200, 250, 300, 350 and 400 ng/μl genomic DNA.

To determine degradation and the molecular weight of the genomic DNA, gel electrophoresis was performed. With the DNA extraction method for clotted blood described here, no degradation of DNA could be observed. The integrity of the DNA was comparable to extraction from anticoagulated blood, showing a high molecular, compressed band (Figure 2).

Genotyping for both TiKoCo and AugUR2 was performed identically at Life & Brain GmbH, Bonn, Germany, applying the Illumina Global Screening Array, version 3. The per person call rate was very high for both DNA sources, with 99.6% for DNA from clotted and 99.5% from anticoagulated blood. The per single nucleotide polymorphism call rate for all 730,059 variants was at the same range, with 99.5% for DNA from clotted and 99.4% from anticoagulated blood (Table 1).

The authors evaluated the influence of storage time on DNA yield for TiKoCo clotted blood samples. While all 4131 samples were stored at -20°C at the same day of blood draw, DNA extraction processing stretched over a period, resulting in a range of storage time from 190 to 415 days. The authors found a small but significant decrease in DNA yield over blood storage time: the difference between the 25% shortest (190–247 days) and the 25% longest storage times (386 to 415 days) was 2.2 μg DNA per ml blood ($p = 3.6*10^{-5}$). However, the observed decrease of DNA yield after 1-year -20°C storage time was low (8%) in comparison with previous reports of 1-month storage at 4°C, with a drop from 37.1 μg to 0.439 μg per 4 ml clotted sample [5]. Interestingly, significant differences in genotyping call rate over the storage time of blood clots at -20°C was observed, but with higher call rate in samples that were stored longer (25% shortest vs 25% longest storage time = 99.50% vs 99.63%; $p = 1.6*10^{-30}$). This may have been by chance, but it shows that genotyping performance did not decrease with blood samples stored at -20°C for a longer period. Storage of blood clots at -20°C for more than 1 year or at even lower temperature warrants further investigations. Since serum tubes do not include DNA stabilization agents (e.g., EDTA), frozen storage is highly recommended in general.

Several protocols and reports on DNA extraction from human clotted blood and after different storage times exist in the literature. The authors performed a PubMed search with the terms "DNA extraction method human clotted blood," resulting in 151 hits. Manual curation of these PubMed hits pointed out several fitting protocols and studies, summarized in Table 2. Methods with manual homogenization or slicing of blood clots were not considered further, since they are impractical for high-throughput DNA extraction.

Adkins *et al.*, in their 2002 published analysis, also used Clotspin tubes and the PureGene DNA purification kit (formerly distributed by Gentra) and tested the extracted DNA for performance in single nucleotide polymorphism genotyping [10]. This was done in only 15 samples. The authors of the present study used the protocol presented in this paper on more than 4000 clotted blood samples, added an optional washing step to improve DNA purity, analyzed the effect of blood clot storage at -20°C over time and tested performance on whole-genome genotyping.

Time needed for DNA extraction is critical for planning personnel resources and feasibility, particularly for epidemiological-scale studies. Hands-on time for DNA extraction from clotted blood was moderate and comparable to our standard DNA extraction method from anticoagulated blood, both with two overnight steps that increase DNA yield. An additional 20 min should be planed for the Clotspin step needed for clotted blood, with effective time depending on the number of parallelly processed samples. The throughput for both methods is limited by the availability of equipment, such as centrifugation capacity and place on circulating shakers. Under ideal equipment conditions, a single technician could process more than 200 samples per day.

Table 2. Selected publications from PubMed search "DNA extraction method human clotted blood."

Publication	Key message	Ref.
Simple and rapid method for extraction of DNA from fresh and cryopreserved clotted human blood. Garg UC *et al. Clin. Chem.* 1996.	Nylon mesh and Qiagen (Gentra) PureGene. Reduced DNA yield compared with extraction from anticoagulated blood. No reduction of DNA yield from frozen clots (at least 1 week).	[3]
Utilizing genomic DNA purified from clotted blood samples for single nucleotide polymorphism genotyping. Adkins KK *et al. Arch. Pathol. Lab. Med.* 2002.	Comparable to the protocol presented in this paper. Extracted DNA tested for single nucleotide polymorphism genotyping. No report on the influence of long-term frozen storage of blood clots.	[4]
A simple method for DNA isolation from clotted blood extricated rapidly from serum separator tubes. Se Fum Wong S *et al. Clin. Chem.* 2007.	Good yield with fresh samples, massive reduction for stored blood clots.	[5]
An alternate method for DNA and RNA extraction from clotted blood. Zakaria Z *et al. Genet. Mol. Res.* 2013.	Extraction of DNA and RNA from clotted blood, sonication for 3 h.	[6]
High-quality and -quantity DNA extraction from frozen archival blood clots for genotyping of single-nucleotide polymorphisms. Bank S *et al. Genet. Test. Mol. Biomarkers* 2013.	Testing different commercial kits. Promega Maxwell 16 Blood Purification Kit and Qiagen Puregene performed best.	[7]
Higher DNA yield for epidemiological studies: a better method for DNA extraction from blood clot. Zhou G *et al. Genet. Test. Mol. Biomarkers* 2019.	High performance of extracted DNA in next-generation sequencing; needs homogenizer.	[8]
A method for improving the efficiency of DNA extraction from clotted blood samples. Mardan-Nik M *et al. J. Clin. Lab. Anal.* 2019.	Ball bearing metal shots in blood clot-containing tubes to break down the blood clot in combination with modified salting-out DNA extraction method. "The main challenge in this method is pipetting in each step to dissolve the pellet."	[9]

Conclusion

This protocol for DNA extraction from clotted blood resulted in a high yield and high quality of DNA, comparable to a similar protocol using anticoagulated blood. We also established the utility of clotted blood-derived DNA for genotyping arrays and documented comparability across the two DNA sources indicated by similar genotype call rates.

DNA extraction from frozen clotted blood using QIAGEN Clotspin Baskets and the Gentra Puregene Blood Kit can be recommended as an efficient way to get high-quality material for genetic analyses from the same blood tube used for serum biomarker analytics, which can be very interesting for epidemiological field studies. Importantly, the DNA yield from blood clots with a mean of 27 μg/ml blood is sufficient for genotyping, whole-genome sequencing and methylation analysis.

Future perspective

Field work in epidemiological studies is a fast way to recruit participants and to obtain health-related data. Genetic analyses are becoming increasingly important to understand molecular mechanisms of diseases. An effective workflow at the point of recruitment is required, especially in collecting biomaterials. Many human biomarkers and antibodies can be measured in serum. To avoid separate blood draws, DNA extraction from remaining blood cells is recommended and feasible with the protocol reported here, even after storage at -20°C for 1 year.

Executive summary

- Our report "DNA extraction from clotted blood in genotyping quality" aims to establish a feasible method to isolate high quality DNA from frozen blood clots and to compare performance for large scale genotyping with DNA extracted from anticoagulated blood.
- The main objective is to reuse stored blood clots from serum samples to avoid additional blood draw with separate tubes. Quantity and quality of DNA isolated from clotted blood should be the same as for gold-standard DNA extraction.
- Our extraction protocol results in DNA with no degradation and high purity. Measures for DNA quantity and quality are presented. Genome-wide genotyping for DNA extracted with our protocol performs at the same high-quality level as with gold-standard extracted DNA.
- In conclusion, DNA extraction from clotted blood applying our protocol is an effective way to avoid additional blood draw in epidemiological studies without any limitations in quality for downstream applications.

Author contributions

KJ Stanzick: methodology and experimentation, validation, data analysis, manuscript writing; J Simon: methodology and experimentation, validation, reviewing and editing of the manuscript; ME Zimmermann: methodology, validation, reviewing and editing of the manuscript; R Wagner, K Überla: reviewing and editing of the manuscript, study PIs of TiKoCo; D Peterhoff, M Schachtner, H-H Niller: sample preparation, reviewing and editing of the manuscript; IM Heid: reviewing and editing of the manuscript, study PI of AugUR; KJ Stark: supervision, methodology, validation, data analysis, manuscript writing

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The TiKoCo study protocol was approved by the Ethics Committee of the University of Regensburg (vote 20-1867-101) and adopted by the Ethics Committee of the University of Erlangen (vote 248_20 Bc). The AugUR study protocol, study procedures and data protection strategy were all approved by the Ethics Committee of the University of Regensburg, Germany (vote 12-101-0258). The studies comply with the 1964 Declaration of Helsinki and its later amendments. All participants provided written informed consent.

Data sharing statement

The datasets generated and analyzed during the current study are not publicly available due to data privacy of study participants. Data on DNA quality are available on request.

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