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Isolation of Cell-Free DNA from Seminal Fluid

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Abstract: Cell-free DNA (cfDNA) is small short double stranded molecule that is also found in seminal fluid. It is a product of apoptotic cells in different developmental stages during spermatogenesis. To final concentration of total cfDNA in semen contributes also cfDNA secreted from living cells and cfDNA that is result of different diseases e.g. prostate cancer or infertility. Amended concentration (high or low) can be connected to prostate cancer or male infertility and can represent important non-invasive diagnostic biomarker for detection and prognosis of these pathological conditions. In this paper, I will discuss different approaches for isolation of cfDNA from seminal fluid, which includes selection of the samples, separation, isolation, extraction, purification and analysis. Today's most popular approach for isolation is the use of commercial kits based on selective binding and elution on silica-membrane technology, magnetic-bead technology or extraction with organic solvents and salting out procedure. Furthermore I will present that I tried to isolate cfDNA from semen with QIAamp DNA Mini Kit to confirm the presence of cell-free DNA in our samples. In the end I will describe problems we are facing during cfDNA measurement which are mainly associated with low concentration of cfDNA in samples.

Key words: Cell-free DNA, seminal fluid, diagnostic biomarker, prostate cancer, male infertility, cell-free DNA isolation, problems of cell-free DNA measurement.

1. cfDNA

1.1 Biological Aspects of cfDNA

The cfDNA are mostly double-stranded molecules with molecular weight significantly lower than genomic DNA. Range of wide is from 0.18 kB to 21 kB [1]. In different studies cell-free DNA was detected in all semen specimens [2]. Scientist predicts that this is a result of prominent apoptosis during spermatogenesis (e.g. spermatocytes and spermatids [2]) which results in high concentrations of cfDNA in sperm [3] with heterogenic size molecules [2]. Cell-free nucleic acids (cfDNA and RNA) are released by dying cells, actively secreted by living cells and may have biological role in the organism [3].

Data: concentration of cfDNA in seminal plasma of normozoospermia: 1.34 ± 0.65 μg mL^{-1}, with ranges from 0.51 to 2.73 μg mL^{-1} [3].

1.2 Clinical Application

Cell-free DNA can be used as important non-invasive diagnostic biomarker for detection and prognosis of prostate cancer and male infertility (sperm quality) [1]. Other applications can longitudinally monitor disease progression and response to treatment [3]. In different studies cell-free DNA was detected in all semen specimens [2]. Isolated cfDNA from semen represents easily accessible and non-invasive source of genetic material for further analysis [3].

1.3 Prostate Cancer and cfDNA

Factors that can influence cfDNA changes in cancer patients are tumor stage, tumor grade, tumor size, tumor aggressiveness and metastasis which can lead to quantitative and qualitative changes [4].

Generally higher cfDNA concentrations are found in cancer patients because of its release from tumor cells and also non-tumor cells. Due to previous researches, cfDNA in prostate cancer patients could be associated with tumor stage, Gleason grade, metastatic spread and PSA (prostate-specific antigen). Higher concentrations of cfDNA can be informative and used as independent factors for poorer outcome regarding survival or disease-free interval [4].

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Improvement of predictive value for prostate cancer can be achieved with adding cfDNA in multivariate model with age, PSA, %PSA and prostate volume [4]. Regarding this cfDNA can be suggested as independent predictor for development of prostate cancer.

Different changes may occur in cfDNA in cancer patients including changes in fragmentation (size), mutations in tumor suppressor genes and oncogenes, microsatellite alterations and hypermethylation of different genes. In different studies scientist came to different conclusions. In one study scientist did not found correlation between fragmentations of cfDNA. Conversely the other study shows that there are higher concentrations of small cfDNA fragments in prostate cancer patients. These short fragments can be useful for diagnosis and prognosis of prostate cancer [4] and are suggested as a cancer marker [2].

1.4 Men Fertility and Cell-Free DNA

One study shows that only low-molecular weight cfDNA in seminal plasma is related to specific sperm parameters important for men fertility. Due to that Low-weight cfDNA could represents marker of semen quality and fertility-related problems in the male reproductive system [2]. We could assume that low concentrations are consequence of reduce amount of cells in all or specific developmental stage during spermatogenesis which results in lower concentration of released cfDNA.

2. Samples

For standardization of method Semen donors must be men with normozoospermia (following WHO guidelines). Sexual abstinence of donors is required [1]. Also routine andrological analyses of semen must be performed—cells should be negligible and round. Predictive parameters of men fertility should be examined: concentration, rapid progression, strict normal morphology, heat-included hyper activation and capacity index from the sperm penetration assay. We should separate each sample in more aliquots—one or more for analysis and one which should be frozen up to at least 6 months for later investigation or repetitions if needed [2].

3. Separation

Cells from seminal plasma must be separated from sperm supernatant—we can achieve that with centrifugation: low speed centrifugation to avoid cell lyses (e.g. first 400 x g for 10 min and then seminal plasma again on 200 x g for 20 min) followed by filtration and ultracentrifugation or high speed centrifugation (16,000 x g for 5 min) [1]. Main advantage of high speed centrifugation is that seminal plasma can be used for directly cfDNA while sample after centrifugation at low speed need to be further treated [1].

3.1 cfDNA Isolation/DNA Extraction and Purification

Nowadays, there is a wide selection of known techniques with many modifications for isolation of cfDNA. Most widely used are numerous kits for extraction of cfDNA from blood (plasma and serum), urine and other body fluids among which we can also count seminal fluid. The most used are different commercial kits from different producers. Some manufacturers are QUIAGEN, Macherey-Nagel (NucleoSpin kits), EpiGentek, Thermo Fisher Scientific and many others. These kits are based on selective binding and elution on silica-membrane technology, magnetic-bead technology or extraction with organic solvents and salting out procedure. After isolation cfDNA is suitable for a broad range of downstream applications, including next-generation sequencing, real-time PCR, digital PCR and others.

Great importance is that we use isolation methods that capture all of DNA fractions [4].

3.2 Silicone Column Binding

These commercial kits are based on silica-gel membrane technology by selective binding and

stepwise elution of DNA [4]. During the procedure DNA is reversible bind onto silica membranes, particles or beads at defined pH and high concentration of chaotrophic salts. The binding is result of interaction between positive silica particles and negative charged DNA. In next step, contaminants are washed away and with the application of low salt buffer we elute and collect the DNA [5].

We can use Automated/manual kits e.g. QIAamp DNA blood mini kit or QIAamp circulating nucleic acid kit. The latter is from QUIAGEN with following capillary gel electrophoresis.

This approach is popular for isolation of high-quality DNA via adsorption chromatography [5]. In one study Modified Isocratic Capillary electrophoresis was used. Agarose gel inside the capillary tube was to enhance detection of trace amounts of cfDNA [2].

Biggest pro for this method is time—usage of commercial kits and automated techniques represents fastest way of cfDNA isolation. They are also easily available and can be ordered on internet. Manufacture's protocols guide the user throughout the whole process. Therefore the use is simple. We can isolate DNA from very small volume of sample and also obtain concentrated DNA from diluted samples [6]. Manually we can do this with binding-washing-elution procedure on DNA purification column. This kind of DNA purification column is easy to manipulate therefore can be suitable for different applications in laboratories. Isolation time of cfDNA is approximately 30 minutes [1].

The main problem when using commercial kits it`s that cfDNA yield can vary to 50%. This is because of the loss of short DNA fragments (< 100 bp) during the isolation process. Fragments do not bind to column or bind to tight. Consequently they cannot be eluted from the column [7]. This represents great problem, because this fragments are usually most informative and contain different mutations especially in cancer patients [1]. In the future scientists should try to develop columns and commercial kits that will be able to capture fragments of all sizes. Moreover when extraction is performed automatically the scientist does not have insight in procedure. Manipulation of different conditions is therefore not possible.

3.3 Extraction with Organic Solvents E.G. Phenol-Chloroform Separation and Salting-Out Procedure

There are many known protocols of phenol-chloroform extraction e.g. protocols according to Schmidt et al. [8], Yuan et al. [9], and Hufnagl et al. [10], and a THP (triton/heat/phenol) protocol according to Xue et al. [11].

I will describe protocol from Thermo Fisher for Phenol/Chloroform Extraction followed by Ethanol Precipitation. First we have to add one volume phenol: chloroform: isoamyl alcohol to my sample and mixed by vortex or with hand. Next step is centrifugation after which we carefully remove aqueous layer and transport it into new tube. Lastly ethanol precipitation is performed. We add Glycogen, NH_4OAc and ethanol to the aqueous phase and leave mixture on -20 °C overnight to achieve DNA precipitation. We can store sample at -80 °C or centrifuge them to get the pellets of DNA. We carefully remove the supernatant and add ethanol. We use centrifugation again and remove supernatant. In the end we have to dry and resuspend the DNA by pipetting and centrifuging briefly to collect the sample and place it on ice [12].

According to the different studies, previously mentioned procedures have higher recovery of DNA than ones using binding columns [7] e.g. salting-out DNA isolation procedure has higher recovery as commercial kits because of loss of small fragments of cfDNA during the isolation process [1]. It was confirmed that greater amount of small fragments of cfDNA was isolated with this method. Another advantage is that a lot of steps in the procedure can be adjusted. Therefore, we can gain greater recovery and higher efficiency with manipulation of specific conditions.

One of main drawbacks is that methods are time-consumable and are not so easy to perform in comparison with other methods e.g. binding kits.

3.4 Magnetic Beads

Principle of this method is that target DNA binds on magnetic beads/particles, coated with Antibodies which bind DNA or have surface that reversibly interacts only with DNA (e.g. silica). After incubation of sample with magnetic beads, we separate the beads with bound DNA in magnetic field. Other contaminants stay in solution and can be selectively removed. In the end, the beads are washed and DNA is eluted and ready for further use [13].

On the market we can found kits from different manufactures with this technology e.g. The *EpiQuik*™ Circulating Cell-Free DNA Isolation Kit (1), or MagMAX Cell-free DNA Isolation kit.

The major advantage is that there is no need for sample preparations with centrifugation or vacuum [13]. Moreover there is absence of the precipitation step (which often compromises yield and purity). Sample input volume can vary the high sample volumes and minimize sampling error [14]. Samples can be processed automatically, semi-automatically or manually and with automated techniques we can achieve high-throughput applications [5]. Technology enables reproducible recovery of high-quality DNA. Isolated DNA can be used and analyzed with different quantification and qualification methods. This approach is relatively fast and DNA can be directly used [15]. This method is known by its simplicity [5].

A con of this method is variability in the efficacy of extraction. It also required specialized instruments which can represent additional cost [5].

4. Practical Work

In our research, we used QIAamp DNA Mini Kit for isolation of cell-free DNA from sperm. Firstly, we tried to isolate whole DNA in sperm to confirm the effectiveness of used method. In the next step we proceed with cfDNA. We followed the Protocol for isolation of genomic DNA from sperm using the QIAamp DNA Mini Kit.

Before starting we had to equilibrate sample and buffer AE in room T and prepare Buffers AW1, AW2 and Buffer X2 by following recepies:

AW1: 19 mL AW1 concentrate + 25 mL Ethanol (96-100%)

AW2: 13 mL AW2 concentrate + 30 mL Ethanol (96-100%)

Buffer X2: 20 mM Tris Cl (pH 8), 20 mM EDTA, 200 mM NaCl, 80 mM DTT, 4% SDS, 250 μg/mL Proteinase K (just before use)

Then we perform isolation following these steps:

(1) Into 1.5 mL tube we add 100 μL sperm plasma and 100 μL buffer X2, mixed and incubate at 55 °C for at least 1 h with occasionally mixing.

(2) Add 200 μL Buffeer AL and 200 μL of ethanol and mix by vortexing

Then we follow regular procedure of step 5 to 8 of the tissue Protocol of the QIAamp DNA Mini Kit

(3) We apply the mixture including precipitating into spin column (in a 2 mL collection tube)—we had to pay attention that we do not wett the rim. After closing we centrifuged the column with sample at 6,000 x g for 1 min. We placed QIAamp column in 2 mL collection tube and discard the tube containing filtrate.

(4) After opening of the spin column we add 500 μL Buffer AW1, closed and centrifuge at 6,000 x g (8,000 rpm) for 1 min. We placed column in 2 mL collection tube and discarded tube with filtrate.

(5) We opened and added 500 μL Buffer AW2, closed and centrifuge by full speed (20,000 x g; 14,000 rpm) for 3 min. In this step we could use new collection 2 mL tube and centrifuge at 20,000 x g gor 1 min.

(6) We placed spin column in 1.5 mL micro centrifuge tube and discarded tube with filtrate. After adding of 200 μL Buffer AE or distilled water we incubated mixture for 1 min at room Temperature and centrifugated at 6,000 x g (8,000 rpm) for 1 min.

(7) Lastly we eluted the DNA in 50-100 μ Buffer AE or distilled water.

5. Analysis

For quantification in different studies we can use different approaches. I will mention only some of them:

(1) Real-time qPCR—e.g. real-time PCR targeting hGDF gene [2] or human genomic targets telomerase reverse transcriptase (TERT) or other genes. It would be advisable to measure concentration of multiple reference genes for more reliable results of total cfDNA quantity [4]. For visualizing we can use different dyes e.g. SYBER GREEN I and use its fluorescence for detection of amplified products, acquiring melting curve for uniformity and calibration curve. One possibility for getting calibration curve is the use of concentrated cfDNA in serial dilutions. With this technique, we can also estimate the size distribution/fragmentation of cfDNA [2].

(2) Digital PCR

(3) Droplet digital PCR

It is recommended that for all of PCR assays targeted sequences are as short as possible [1]. We can compare quantitative PCR measurements of different reference gene assays and validation with digital PCR.

(4) NGS (Next-generation sequencing) [3]

One important aspect of isolated cfDNA is size distribution/fragmentation of it. It can be accessed with electrophoresis. In that manner, agarose gel can be used for determination of size distribution after use of gel dyes e.g. staining-SYBR-Gold fluorescent stain and following ultraviolet transillumination for visualization [1]. Fluorometric assay is recommended because is simple, robust and enough sensitive. Detection limit for this technique is around 1 ng/mL. We can also use DNA marker to track cfDNA fragment sizes [2].

We can also investigate the linearity of extraction efficiency—with applying different input volumes of seminal fluid (e.g. 1, 2, 3 and 5 mL; 3 independent sets).

For measurement of extraction efficiency, fragment size bias and yield for validation of cfDNA methods, we need quality controls. In one study they used means of improved reporting of cfDNA yield by comparing quantitative measurements of different reference gene assays in plasma samples and validating this with digital PCR [4].

6. Preanalytical and Analytical Problems of cfDNA Measurements

While working with cell-free DNA, we face with different problems. One of the main reasons for that is low concentration of cfDNA in samples. Rapid decrease in its concentration after release and sampling is also a problem. In the following, I will highlight some preanalytical and analytical problems scientists are facing when working with cfDNA:

- Sampling and processing (time interval between collection and centrifugation, storage (temperature, time), centrifugation time and forces…);
- Different alternative protocols for isolating cfDNA—high variability between studies;
- Different measurement principles—simple spectrophotometric method or sensitive fluorometric approach using different dyes (PicoGreen, SYBER Green I), different PCR assays with different targets. (e.g. telomerase, beta-actin, beta-globin) [1];
- Lack of standardization, appropriate controls, and reference materials [4];

7. Conclusions

The potential of cfDNA in seminal fluid as a prostate cancer biomarker as well as a biomarker for diagnose, prognosis and monitoring in conditions related to mane infertility is becoming increasingly apparent. Different studies show that cfDNA present in semen and its fragmentation is correlated to important sperm parameters linked to normal sperm function and presence of prostate cancer.

I tried to develop a method for extraction of cfDNA from semen. In article I described use of QIAamp

DNA Mini Kit for isolation of cell-free DNA from sperm, this approach is based on commonly used commercial kits based on selective binding and elution. Other popular methods for cfDNA isolation are magnetic-bead technology or extraction with organic solvents and salting out procedure. I suggested approaches for quantification and analysis and point out main problems of cfDNA measurement.

With this paper, I try to raise awareness of the need for development of methods for isolation, standardization of the extraction and quantification of cfDNA in seminal fluid.

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