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Development of DNA Extraction Kit Based on Silica-Coated Magnetic Nanoparticles for Formalin-Fixed and Paraffin-Embedded Cancer Tissues

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Abstract: The aim of this study is to develop a kit for the extraction of DNA from formalin fixed paraffin embedded (FFPE) tissues using silica-coated magnetic nanoparticles $Fe_3O_4@SiO_2$ (MagSi nano) and suitable buffers. We selected the best version of synthesized MagSi nano (code M1) and optimised buffers including Lysis Buffer (code LB2) and Binding Buffer (code BB2) for extracting DNA from FFPE tissues with highest DNA recovery (84 - 103 ng/µl) and good purity (A_{260}/A_{280} around 1.8 - 2.0). Using the MagPure FFPE DNA nano kit based on the selected MagSi nano and the optimised LB2 + BB2 buffers, we successfully performed extraction of DNA from FFPE tissues of colon and nasopharyngeal carcinoma patients. The extracted DNAs from FFPE colon cancer tissues could be used as templates for downstream amplifying and sequencing *Braf* bio marker gene, and the extracted DNAs from nasopharyngeal cancer tissues could be used as templates for downstream detection of Epstein-Barr virus (EBV) using real-time Taqman PCR. In sum, the MagPure FFPE DNA nano kit is potential for extraction of DNA from FFPE tissues, and need to be further developed to improve DNA recovery yield for application in diagnostics of cancers using molecular biology.

Keywords: Silica-coated magnetic nanoparticles $Fe_3O_4@SiO_2$, DNA extraction, FFPE cancer tissue, PCR, DNA sequencing.

1. Introduction

The archives of formalin-fixed and paraffin-embedded (FFPE) tissues are extensive sources for histopathological diagnosis of diseases, especially cancers. Due to formalin-induced cross-linking of proteins, extracting DNA from FFPE tissue remains a challenge [1-3]. DNA extraction from FFPE tissues kits have been produced by well-known company in biotechnology, such as Quiagen, Promega, Thermo Scientific. These kits are developed

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using the method of silica-membrane-based nucleic acid extraction which is created by Boom and colleagues [4-6]. Mechanism of this extraction method is high affinity of the negative charged DNA backbone towards the positive charged silica particles under a condition of high concentration of chemotropic salts [7 - 9]. For robotic DNA extraction from FFPE tissues, companies such as Promega, and Thermo Scientific, have produced kits based on silica-coated magnetic micro beads. However, to the best of our knowledge, DNA extraction kits from FFPE tissues based on silica-coated nano particles are not yet commercialized or under development. Recently, our group have synthesized silica-coated magnetic nanoparticles $Fe_3O_4@SiO_2$ (magnetic nanoparticles Fe_3O_4 coated with SiO_2, named as MagSi nano) and optimised buffers to develop MagPure nano kits to extract DNA from bacteria, virus, blood cells and agarose gel [10 - 12]. In comparison to the micrometer-size silica-coated magnetic beads and silica membrane tubes, silica-coated magnetic nanoparticles have larger total surface area and superparamagnetic properties, thus they could be more functional in purification of DNA from samples [13]. Extracted DNA by MagPure kits was qualified as templates for downstream reactions such as PCR, Real time PCR, and DNA sequencing. In this study, we further developed a kit for DNA extraction from FFPE tissues based on silica-coated magnetic nanoparticles. The extracted DNA samples using the kit were tested their quality and quantity for downstream applications such as PCR combined DNA sequencing of *Braf* gene as biomarker for colon cancer tissues, and Real time PCR for detection of EBV virus from tissues of nasopharyngeal carcinoma patients.

2. Material and method

2.1. Materials

FFPE tissues samples of colon cancer were provided by Center for Gene and Protein Research, Hanoi Medical University. FFPE tissues of nasopharyngeal carcinoma patients were provided by Department of Pathophysiology, Vietnam Military Medical Institute.

MagSi nano ($Fe_3O_4@SiO_2$, magnetic nanoparticles Fe_3O_4 coated with SiO_2) with properties as listed in Table 1 was provided by a research group at Center of Nano and Energy, VNU University of Science. All other reagents were standardized for experiments in molecular biology.

Table 1. Properties of MagSi nano ($Fe_3O_4@SiO_2$)

No	Properties	Values		
		M1	M2	M3
1	Concentration of MagSi nano	50 mg/ml	50 mg/ml	50 mg/ml
2	Saturation magnetisation of core Fe_3O_4 particles	64 emu/g	64 emu/g	64 emu/g
3	Average diameter of core Fe_3O_4 nanoparticles	10-20 nm	10-20 nm	10-20 nm
4	Saturation magnetisation of MagSi nano	49 emu/g	44 emu/g	35 emu/g
5	Thickness of SiO_2 layer	2-4 nm	3-5 nm	4-6 nm

2.2. Methods

2.2.1. Preparation of DNA extraction buffers

A set of nucleic acid extraction buffer was prepared as follows: (i) proteinase K 20 mg / ml (BioBasic), (ii) Lysis Buffer (LB) contained Tris-HCl and SDS at different concentrations (iii) Binding Buffer (BB) contained chaotropic salts (GuHCl, Triton X-100) and EDTA at different concentrations, (iii) Washing buffer 1 (WB1) and (iv) Washing Buffer 2 (WB2) contained Tris-HCl plus high concentration of ethanol for washing other organic compounds from DNA-MagSi nano complexes, and (v) Elution Buffer contained of Tris-HCl at basic pH to isolate nucleic acids from MagSi nano.

2.2.2. Preparation of FFPE samples

For each DNA extraction, 10 mg of FFPE tissue was cut into 8-10 thin sections of 5-10 µm thick, then added into an eppendorf tube with 500 µl mineral oil. The tube was vortexed and incubated at 60°C for 5 min to release paraffin into the mineral oil. Then, the oil was removed and the tissue was washed with ethanol 96° twice, followed by dd H_2O once. Finally, the tissue was dried at 37°C for 5 min.

2.2.3. Extraction of DNA from FFPE tissues

200 µl LB and 40 µl Proteinase K 20 mg/ml were added into an eppendorf tube then the tube was mixed thoroughly by vortexing for 10 s and incubated at initial 60°C for 60 min, then further 90°C for 60 min. After incubation, 400 µl BB, 200 µl of absolute isopropanol, and 100 µl of MagSi nano were added into the cell lysate. The suspension was mixed thoroughly, then allowed to stand at room temperature (RT) for 3 min for binding of DNA on MagSi nano. The DNA-MagSi nano complexes were collected by applying an external magnet for 10-15 s and the clear supernatant was discarded. The complexes were washed with 1 ml of WB1 and then 1 ml WB2, to remove proteins, salts and other impurities. The residual ethanol in WB2 was completely removed and evaporated by air drying at RT. Finally, 50 µl of EB was added to the complexes, and the tube was placed on a magnet in order to collect the supernatant containing genomic DNA (gDNA).

2.2.4. Measurement of concentration and purity of purified DNA

Spectrophotometer Nanodrop (ND100, Life Technology) was used to measure absorbance of purified DNA at wavelengths of 260 nm and 280 nm. A_{260} was used to calculate DNA concentrations, and ratios of A_{260}/A_{280} was used to estimate contamination levels of proteins and RNA.

2.2.5. PCR-based amplification and DNA sequencing of Braf gene using the extracted DNA as templates

The extracted DNA using optimised MagSi nano and buffers (MagPure FFPE DNA nano kit) were used as templates for PCR to amplify a specific sequence of *Braf* gene. A primer set for specific amplification of exon 15 of *Braf* gene, which generates a DNA product of 252 bps (named as Braf), contained Fw Braf 5'-TCATAATGCTTGCTCTGATAG- 3' and Rv Braf 5'- CTTTCTAGTAACTCAGCAGC-3'. 5 µl of total 50 µl purified DNA from 10 mg FFPE tissues was used for each PCR reaction with a total volume of 25 µl. PCR was performed using thermal conditions as follow: preheating at 94°C for 3 min, 35 cycles at 94°C for 30 min, 58°C for 30 s, 72°C for 30 min with a final extension at 72°C for 5 min. Amplified PCR products were run on 1,5% agarose gel followed by staining with fluorescent ethidium bromide for visualisation of DNA band under UV excitation. DNA sequencing of each PCR product was performed under service of IDT Company using either Fw Braf or Rv Braf primers and the obtained sequences were analysed using ApE software.

2.2.6. Real time PCR to detect EBV using the extracted DNA as templates

The extracted DNA using the optimised MagSi nano and buffers (named as MagPure FFPE DNA nano kit) were used as templates for PCR to amplify a specific 74 bp sequence of nonglycosylated membrane protein named

BNRF1 p143 of EBV. Primers included EBV-74 forward 5′-GGAACCTGGTCATCCTTGC-3, EBV-74 reverse 5′-ACGTGCATGGACCGGTTAAT-3', and the probe FAM 5′-CGCAGGCACTCG.TACTGCTCGCT-3′ TAMRA. 5 µl of total 50 µl purified DNA from 10 mg FFPE tissues was used for each real time PCR reaction with a total volume of 25 µl. The real-time PCR conditions included 42 cycles of 15 s at 95°C and 60 s at 60°C [7].

3. Results and Discussion

3.1. Optimisation of Lysis and Binding Buffers

The first step of our research is to optimise the two buffers including Lysis Buffer (LB) and Binding Buffer (BB) which play the most important roles in extracting DNA from FFPE tissues. We made 3 different recipes for each pair of buffers coded LB1+BB1, LB2+BB2, LB3+BB3 and tested these buffers on clinical samples of patient 1 and patient 2 following the DNA extraction methods as described in the Materials and Methods. In all samples, the same MagSi nano code M1 and 10 mg amounts of FFPE samples were used. Experiments for each buffer pair were repeated 3 times. As result, the electrophoresis data showed that extracted gDNA was fragmented into less than 1kb-size smear bands, in which and LB2+BB2 provided the brightest ones (Fig. 1A). The extracted DNAs were used as templates for PCR amplifying specific 252 bp sequences of *Braf* genes. As shown in Fig.1B, the LB2+BB2 provided the best recovery and quality of DNA templates as indicated by the brightest and the most evenly intensities of PCR bands.

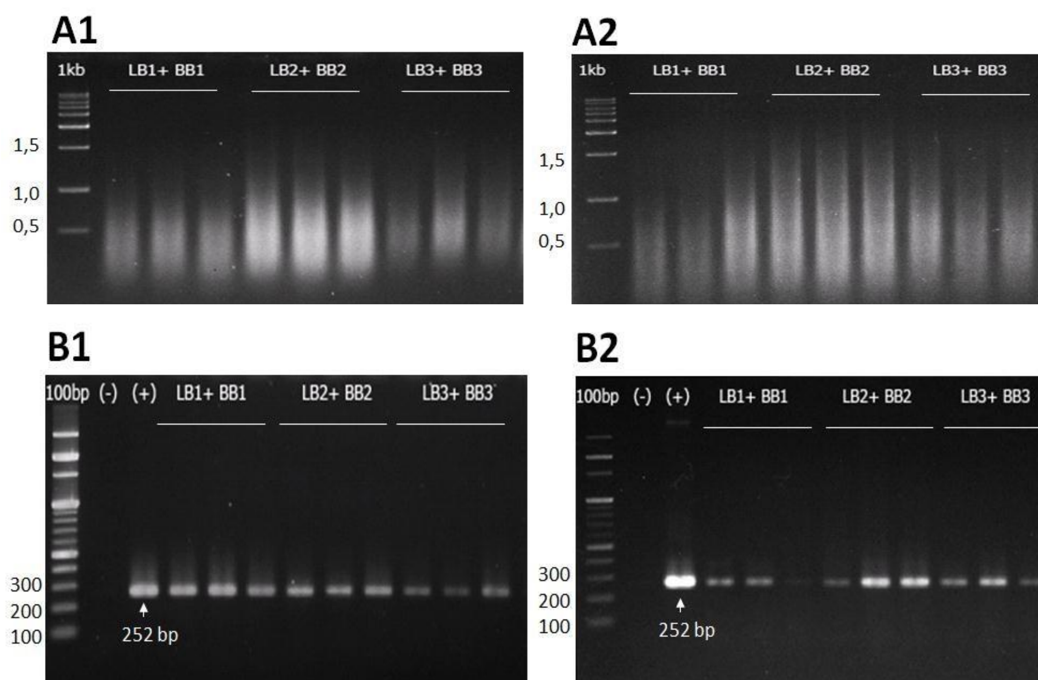


Figure 1. Agarose-gel electrophoresis of DNAs extracted from FFPE tissues and of their specific PCR products of *Braf* genes when using different pairs of Lysis and Binding buffers.

A. 1% agarose-gel electrophoresis of DNA extracted from FFPE tissues of patient 1 (A1) and patient 2 (A2) using different lysis and binding buffers (LB1+BB1, LB2+BB2, BL3+BB3). B. 1.5% agarose-gel electrophoresis for PCR products amplifying *Braf* genes of patient 1 (B1) and patient 2 (B2) using extracted DNAs by MagPure kit using 3 different pairs of buffers (LB1+BB1, LB2+BB2, LB3+BB3).

DNA extracted from 2 patient samples was evaluated concentration and purities using optical density method. The extracted DNA using LB2+BB2 buffers had highest absorbance values in samples of both patients (102.8 ± 6.94 ng/µl for the patient 1 and 84.1 ± 4.99 ng/µl for patient 2, $n = 3$) (Fig. 2). This data was consistent to the data obtained in Fig. 1, in which LB2+BB2 buffers provided the best results. All DNA samples extracted using 3 pairs of buffers had values of A_{260}/A_{280} ranging between 1.9 - 2.2 (Table 2), indicating they all had good purity. Taken together, we selected LB2+BB2 buffers for further steps in development of the kit.

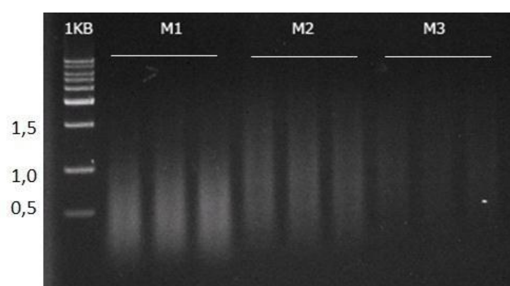
Table 2. Yield and purity of DNA extracted from FFPE tissues of colon cancer patients using different pairs of Lysis and Binding buffers

Buffer	Concentration of DNA (ng/µl)		A_{260}/A_{280}	
	patient 1	patient 2	patient 1	patient 2
LB1+BB1	53.6 ± 5.75	60.0 ± 18.22	2.00 ± 0.02	2.03 ± 0.05
LB2+BB2	**102.8 ± 6.94**	**84.1 ± 4.99**	**1.95 ± 0.02**	**1.97 ± 0.03**
LB3+BB3	44.2 ± 10.38	58.2 ± 9.99	2.21 ± 0.18	2.04 ± 0.07

3.2. Selection of the most suitable MagSi nano

Using a similar approach, we tested three types of Magsi nano particles coded M1, M2, M3 (with different saturation magnetisation of silica-coated $Fe_3O_4@SiO_2$ particles and thickness of silica layer as described in Table 1) together with the optimised LB2+BB2 buffers to extract DNA from FFPE tissues. We could not perform experiments on the same FFPE tissue of patient 1 and 2 as the amount of tissue sample was limited. Thus, we performed on FFPE tissue of patient 3 and experiments for each MagSi nano version were repeated 3 times. The results of DNA electrophoresis on 1% agarose gel showed that gDNA extracted by the three MagSi nano structures were all highly fragmented into smear bands, in which M1 provided the brightest bands (Fig. 2A).

A



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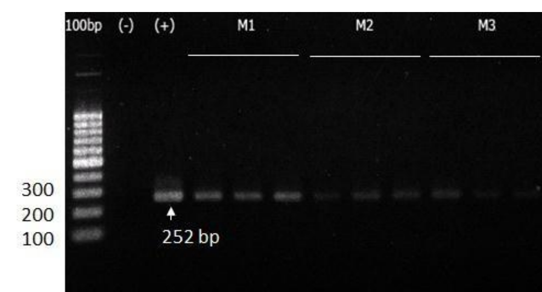

Figure 2. Agarose-gel electrophoresis of DNAs extracted from FFPE tissues and of their specific PCR products of Braf genes when using different MagSi nano versions.

A. 1% agarose-gel electrophoresis of DNA extracted from FFPE tissues of patient 3 using different MagSi nano versions (M1, M2, M3). B. 1,5% agarose-gel electrophoresis for PCR products amplifying *Braf* gene using extracted DNAs by MagPure kit using different MagSi nano versions (M1, M2, M3).

The extracted DNA was used as template for PCR amplifying specific 252 bp sequence of *Braf* gene (Fig. 2B). The M1 provided PCR bands having the brightest and the most evenly intensity (Fig. 1B). We then measured concentration and purity of DNA and found that DNA extracted by M1 particle had the highest concentration (34.47 ± 3.2 ng/µl), which was 5-fold higher than that by M2 (5.67 ± 0.8 ng/µl) and twice as much as that by M3 (16.6 ± 1.5 ng/µl) (Table 3). The data of absorbance values were consistent to the electrophoresis data obtained in Fig. 3. The purity of DNA was good with the A_{260}/A_{280} between 1.8 and 2.2 (Table 3), indicating that contamination of protein and ARN was low. Taken this data and the above data, we selected the MagSi nano M1 and LB2+BB2 buffers as major components of MagPure FFPE DNA nano kit (Fig. 3).

Table 3. Yields and purities of DNAs extracted from FFPE tissues of colon cancer patients using different MagSi nano versions.

Mag Si nano version	Concentration of DNA (ng/µl)	A_{260}/A_{280}
M1	34.47 ± 3.2	1.84 ± 0.03
M2	5.67 ± 0.8	1.83 ± 0.22
M3	16.6 ± 1.5	1.82 ± 0.06

Figure 3. MagPure FFPE DNA nano kit (100 reactions). The kit contains LB2 (200 ml), BB2 (50 ml), WB1 (50 ml concentrated), WB2 (30 ml concentrated), EB (20 ml), Proteinase K (2 ml 20mg/ml) and MagSi nano M1 (2.5 ml/tube x 2 tubes).

3.3. Downstream application of extracted DNA from FFPE tissues

DNA sequencing of Braf biomarker gene from colon cancer tissues

The PCR products of *Braf* genes from the above experiments were used as templates for DNA sequencing to check whether their sequence are readable in order to detect any mutations. As representative data obtained in Fig. 4A, we could observe sharp and clear peaks of nucleotides sequence of *Braf* gene of patient 1. The sharp peaks without any noises indicates that extracted DNA is completely free of cross-linkages, and qualified for DNA sequencing analysis. The sequence of patient 1 was analysed to be 100% identical to a sequence (start: 176309; end: 176560) of *Braf* gene posted in NCBI databases (Sequence ID: ref|NG_007873.3| *Homo sapiens Bfaf* proto-oncogene, serine/threonine kinase) (Fig. 4B). Similar data of DNA sequencing was obtained with *Braf* genes from patient 2 and 3 (data not shown).

Figure 4. DNA sequencing of biomarker *Braf* gene of patient 1 using a DNA template purified by MagPure kit.
Sequential peaks of nucleic acids of *Braf* gene of patient 1 (A) and homology analysis of the *Braf* gene of patient 1 (Query 1) to the sequence NG_007873.3 Homo sapiens *Braf* proto-oncogene from NCBI database (Sbjct) (B).

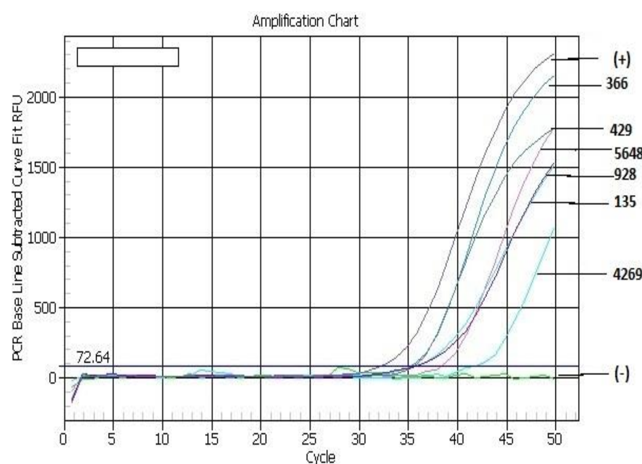

Figure 5. Amplification chart of FAM signals representing EBV during cycles of real-time PCR.
The curves of EBV-positive patients were no. 135, 366, 429, 928, 5648.
Positive control (+) and non-detectable signal of negative control (-) were run in parallel experiments.

Real time PCR detection of EBV in throat cancer tissues

In another application, we used Magpure FFPE DNA nano kit for extracting DNA from six FFPE tissue samples of throat cancer, and used the extracted DNA as templates for real-time PCR to detect Epstein Barr Virus (EBV). As shown in Figure 5, the FAM signals representing were detected in all six samples (no. 135, 366, 429, 928, 5648). Confirmation was made by no signal of FAM in a negative control and a clear FAM signal detected in a positive control. Our data indicates that the Magpure FFPE DNA nano kit could extract DNA of the EBV present in the tissue samples, and that the extracted DNA was qualified for further real time PCR detection of specific 74-bp sequence of nonglycosylated membrane protein named BNRF1 p143 of EBV.

4. Conclusion

In summary, we developed Magpure FFPE DNA kit based on optimization of the MagSi nano M1 and a pair of LB2 + BB2 buffers. The yield of DNA was about 84-103 ng/µl with low contamination of proteins and RNAs as indicated by the ratio of A_{260}/A_{280} around 1.8 - 2.0. The extracted DNAs were qualified for downstream application such as PCR, DNA sequencing and real time PCR. In addition, the extraction procedure of MagPure FFPE DNA nano kit was not required either centrifugation or vacuum filtration. Thus, the kit is potential for application in diagnostics of cancers and need to be further optimised to obtain a higher DNA concentration.

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Phát triển kit tách chiết DNA sử dụng hạt nano từ bọc silica để tách DNA từ mô ung thư được cố định bằng formalin và vùi paraffin

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Tóm tắt: Mục đích của nghiên cứu là phát triển bộ kit tinh sạch DNA từ mô ung thư cố định formalin trong thể vùi paraffin (FFPE) sử dụng hạt nano từ bọc silica (MagSi nano) và các đệm phù hợp. Chúng tôi đã lựa chọn loại hạt tổng hợp MagSi nano M1 và tối ưu hóa đệm gồm đệm ly giải LB2 và đệm gắn kết BB2 để tách chiết DNA từ các mô ung thư FFPE với lượng DNA thu hồi cao nhất (84-103 ng/µl) và độ tinh sạch tốt (A_{260}/A_{280} around 1.8-2.0). Sử dụng bộ kit MagPure FFPE DNA nano gồm hạt MagSi nano M1 và đệm LB2+BB2 đã tối ưu, chúng tôi đã tách chiết thành công DNA từ mô FFPE của bệnh nhân ung thư đại trực tràng và ung thư vòm họng. DNA tách chiết từ mô ung thư đại trực tràng có thể sử dụng làm khuôn cho phản ứng nhân gen PCR và giải trình tự gen chỉ thị khối u *Braf*, và DNA tách chiết từ mô ung thư vòm họng có thể sử dụng làm khuôn để phát hiện Epstein-Barr virus (EBV) sử dụng real-time Taqman PCR. Tóm lại, bộ kit MagPure FFPE DNA nano có tiềm năng trong tách chiết DNA từ mô ung thư FFPE, và cần được tiếp tục tối ưu để tăng lượng DNA thu hồi nhằm ứng dụng trong chẩn đoán ung thư bằng các kỹ thuật sinh học phân tử.

Từ khóa: Hạt nano từ bọc silica, tinh sạch DNA, mô ung thư FFPE, PCR, giải trình tự gen.