

Research Article

E. coli BL21 Bacterial and Newcastle Pestikal lasota Viral Ghosts Preparation, DNA/RNA Elimination/Purification/Isolation/Quantification Using Agarose Gel Entrapment Bubble Method

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Abstract

Sponge like protocol is a new protocol able to turn most microbes including viruses to empty cells or empty envelopes. The original protocol guarantees full evacuation, however, in some cases it need a validation. In this study, simple tools are described to separate the genetic constituents of both partially prepared *E. coli* BL21 and Newcastle Pestikal lasota strain ghosts. The used methods describe the preparation of entrapment bubble (in the agarose gel) as a proposed step for separating the genetic materials from the other constituents. That might solve some technical problems particularly during the virus ghost preparation. The removed DNA and/or RNA by the agarose have been collected. Different simple methods have been used in subsequent steps such as the electrophoresis using the electrical current alone or with the dialysis bag, and the double Eppendorf tube centrifugation method. The DNA and/or RNA have been measured spectrophotometrically using quartzes cuvette at 260_{nm}. This study is a new tool for separating the genetic elements from the microbial ghosts. The method could be either used for the evacuation processes, DNA RNA isolation and purification and for concentrating virus (es)/ ghost virus (es). It could be used also in other technical applications such industrial and pharmaco/medical applications etc.

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Keywords: Agarose gel; *E. coli* BL21 ghost cells; Microbial ghosts; Newcastle pestikal lasota ghost

Abbreviations

MGC: Minimum Growth Concentration
MIC: Minimum Inhibition Concentration
RBC: Red Blood Cells
SDS: Sodium Dodecyl Sulfate
SL: Sponge Like
SLP: Sponge Like Protocol

Introduction

The ancient Egyptian might be the first how succeeded to completely evacuating cells from their soluble contents, a process known as mummification. It might be that the first remarked known ghost cell (s) is existed in the first sample examined under the first microscope by Antonie van Leeuwenhoek [1]. That because microbial ghosting is a natural phenomenon [1]. In fact, we turning microbes to ghosts or lysis them daily by attacking the microbes which invade our bodies or inter to it normally, during the feeding process or by other routes. An example is the lysozyme in our mouth's saliva which is a part of our innate immune system.

Weibull (1956) is succeeded to prepare *Bacillus megaterium* as protoplast. Its protoplast has been given the name "ghosts" [2]. The phage ghosts and bacterial ghosts have been known in the middle of the 19th century because of the development of the molecular biology and the microscopes [3]. Red Blood Cells (RBCs) ghosts have been also prepared. Other forms of different cells have the ghosts feature have been described [4]. The concept of bacterial lysis by the bacteriophage *E* lysis protein (from a cloned bacteriophage Φ X174 gene) leads to establish a protocol restricted to the gram negative ghost cells [5-8]. To avoid pathogenic islands and antibiotic resistance genes in the BGs vaccine preparation, a nuclease degraded the DNA [9,10].

A new protocol proves success with all types of microbes has been introduced. It has been given the name Sponge Like (SL) protocol, which is derived from using the critical chemical concentration of some active chemical compounds or the critical activity of some proteins and enzymes. Some physical and biological parameters that could induce evacuation has been used [11]. SL protocol found acceptance and proved to be efficient when applied to different microbes [11-17]. The prepared bacterial cells using the SL protocol are maintained correct cell structures and surface antigens.

Lysozyme is an enzyme able to lysis most of the bacterial strains and it has been used in the original SL protocol [11]. Harsh exothermophilic spore former, protease and keratinase producer *Bacillus stearothermophilus* strain has been prepared as ghost cells using the minimum activity concentration of the native/purified hen white egg lysozyme [11,18,19]. Using the bio-critical concentration of a single compound such as H₂O₂ is enables the evacuation of the Newcastle

virus from their RNA constituents using the same H_2O_2 concentration which applied to *E. coli* JM109 and *E. coli* BL21 [20]. The bio-critical concentration of H_2O_2 the *E. coli* strains has been in the range and succeeded to cure the Newcastle virus from its RNA [20]. It is of great important to keep the body oxidant in certain limit, and not deteriorate them totally by over use of the antioxidant [21].

Recently a fast plasmid slot lysis protocol has been described by Amara (2018) for DNA and plasmid isolation and ghost cells preparation [22]. Some other excellent protocols describe different type of DNA preparation [23-26]. The agarose gel electrophoresis causes a migration for the plasmids as well as their DNA contents. That enables the determination of the cells which have the right clones. In fact, the protocol has been used as an alternative solution for the white/blue selection [22,27]. Only the lysis buffer is applied for a short time. The protocol is used for microbial ghosts' preparation using the electrophoresis current to force the cells evacuation and the process, substitute the centrifugation and the washing steps in the original protocol [22,27].

Different exopolysaccharides have been used in various molecular and biotechnological applications. Agarose is a linear natural polymer with a molecular weight of about 120,000, consisting of alternating D-galactose and 3,6-anhydro-L-galactopyranose [28,29]. It is usually used for DNA and RNA isolation and sometimes used instead of the agar to measure microorganism motility and mobility.

This study aims to entrap the partially prepared bacterial/virus ghosts in a prepared agarose gel bubbles, and to eliminate/ purify/ isolate/ quantify their DNA and/or RNA contents.

Materials and Methods

Microbial strains

Bacterial strain: *E. coli* BL21 recombinant cells (Promega) has been used in this study. The *E. coli* contains a plasmid pLysS. The *E. coli* genotype is *pLysS* strain *F'*, *ompT*, *gal* [*dcm*], [*lon*], *hsdS_B*, (*r_B⁻ m_B⁻*), *gal*, *dcm*, *rne131*, (*DE3*), *pLysS* (Cam^r) [30,31].

Viral strain: Newcastle Pastikal lasota SPF virus (Hyper V-for animal health -GENER) strain obtained from local pharmacy has been used in this study. It has been preserved at -20°C at a refrigerator.

Preparation of bacterial ghost cells: *E. coli* BL21 prepared as ghost cells following Amara et al (2013) [11,15,16]. Only $NaHCO_3$ has been used instead of $CaCO_3$ as described first by Amara (2015) [17].

Preparation of viral ghost: Newcastle Pastikal lasota virus has been prepared following the methods described by El-Baky and Amara (2014) [20].

Preparation of Trapped Agarose Bubble

The trapped bubble formation

1.0% (w/v) agarose gels solution have been used where one gram of the agarose (Sigma-Aldrich, Steinheim, Germany) has been dissolved by boiling it in 100 ml TBE buffer (50 mM Tris/HCl, 50 mM Boric acid, 2.5 mM EDTA, pH 8.5) or in distilled water. The mixture has been heated till complete dissolving of the agarose and left in room temperature to reduce its temperature.

For preparing trapped bubbles, the dissolved agarose has been loaded in a plastic spectrophotometer cuvette and the agarose left till becoming viscous. Sterile syringe with a fine needle has been used. The syringe which contains air has been left 5 min in -20°C to cool the air inside. The syringe needle then has been interred in the agarose (it is recommended to use fine needle), then the cold air has been injected gently to form a single bubble. The syringe needle then removed gently to avoid the loosing of the formed bubble. The agarose left till complete dryness.

Removing of the agarose from the spectrophotometer cuvette

The down side of the cuvette is removed (using a cutter). The agarose is removed from the cuvette by pressing it gently using suitable tool. Alternatively, symmetric cuvette could be used and the bottom side only could be removed.

Sample loading in the trapped bubbles

The prepared sample, each has been injected to a single bubble till complete removal of the inside-air. The injected volume has been calculated. The needle is removed. In case of injecting the sample before removing the cuvette body, after the injection step the agarose is turned 90 degrees to keep the injected surface up. Loading dye could be added just to prove the immigration of the DNA and/or RNA, but in experiments aim to isolate native DNA and/or RNA, it is recommended to calculate the time of the DNA and/or RNA immigration or using the additional step (the dialysis page or the alternative step the double Eppendorf tubes centrifugation method) as below. The agarose containing the samples are put in a DNA electrophoresis unit (MAX FIL TUG-SYS unit Cambridge-Science Park) and covered gently with the running buffer. The gel electrophoresis electrical current then has been run at 70 V for 2-3 min (or as calculated). Both of the agarose concentration or the current electrical power could be increased or decreased to appropriate level based on the used samples. For experiments aiming to investigate only the presence of the DNA and/or RNA or plasmid, 70% glycerol can be improving the samples loading.

The DNA and/or RNA collection

After the migration of the DNA/RNA in the gel there are two simple methods have been used to collect the DNA and/or RNA. The first is the dialysis page and the second is the double Eppendorf tubes centrifugation method for collecting the DNA and/or RNA.

Isolation of DNA and/or RNA fragments using dialysis bag

Dialysis page (SERVA-VISKING[®]) with a diameter equal to 16 nm was prepared according to the manual included. The agarose gel containing the sample and the migrated DNA and/or RNA are transferred to a treated dialysis bag contains the running buffer. The bag then closed well and put in electrophoretic chamber and the electric current is allowed (70 V). The estimated running time is calculated regarding to the size of the gel. The DNA will pass through the gel to the buffer due to the effect of electric current. DNA can be attached to the surface of the dialysis bag, however by putting the bag in the opposite direction and allowing the electrophoretic current 70 V for 5 sec., that should be enough to detach the DNA and/or RNA from the surface of the dialysis bag. The DNA and/or RNA then collected by opening the bag and collecting the buffer.

Isolation of DNA and/or RNA using duple Eppendorf tubes centrifugation method

Under aseptic conditions, two Eppendorf tube are used one with small pore in its bottom covered by sterile filter paper. The migrated DNA and/or RNA agarose gel part could be cut to small slices (after full DNA and/or RNA migration) or the whole used gel which contains the sample can be used. The gel slice is putted inside the Eppendorf which contains a poor and the filter paper, then both are put in a second normal Eppendorf. The two Eppendorf are centrifuged at 4000 rpm/10 min then the speed has been elevated to 13000 rpm for 5 min (Biofuge 13, Heraeus instrument Christ, Osterode). The DNA and/or RNA will be separated from the agarose gel and will be passed through the filter paper and then through the Eppendorf poor and collected in the second Eppendorf. The centrifugation forces and time could be reduced or increased to appropriate level based on the sample type.

Visualization of the DNA and/or RNA on the gel

After electrophoresis, the gel has been stained in ethidium bromide solution for at least 10 min, followed by detecting the DNA and/or RNA under UV light at 260 nm in a UV transilluminator (SYNGENE-BIO IMAGEREADY). An image has been generated.

Determination of the DNA Concentration

The concentration of the DNA has been determined spectrophotometrically (Libra S70-Biochrom Cambridge) by measuring the absorbance at 260 nm. Quartz cuvette has been used. The absorbance has been set to 0 using the running buffer as a blank ample. An extinction $E_{260} = 1$ corresponds to $50 \mu\text{g dsDNA ml}^{-1}$ [32]. And $38.5\text{-}40 \mu\text{g ss DNA/RNA ml}^{-1}$.

Additionally, the concentration of DNA has been estimated by following the fluorescence of bands within the agarose gel that had been re-stained with ethidium bromide. The amount of DNA which has been just visible after electrophoresis, staining and photography on the gel has been set to 2 ng.

Collection of the Newcastle Pastikal lasota virus and the *E. coli* BL21 from the agarose gels

After separating the DNA and/or RNA the ghosts of the Newcastle Pastikal lasota virus and the *E. coli* BL21 were collected by opening the agarose passing inside the bubbles and its content has been separated for each. Gentle vortex at 40 rpm for the agarose sample before the cut could improve the process. The collection can be done in different point of the protocol based on the nature of the preparation.

Results and Discussion

Isolating the genetic elements from the different microbes is a basic tool in the molecular biology research. Understanding the behaviors of the different genetic elements will facilitate their isolation safely and correctly. Simplifying such tools is in demand particularly in the developmental countries where the cost of an experiment might be a critical issue [27,33-36]. Many simple enzymatic and non-enzymatic based methods have been described for either the isolation of the DNA, RNA or the plasmid DNA [27,33-36].

Bacterial ghosts are intact, inactive, evacuated bacterial cell with correct 3D structure and surface antigens. Ghost techniques increase the safety of the killed vaccines, while maintaining their antigenicity

due to mild preparation procedures. SL protocol is, guarantee an inactive microbial cells with correct surface antigen and 3D structure. Ghost cells may express and/or carry several expressed, surface antigens, plasmid DNA carrier, drug as delivery system, adjuvants, and dendritic cell inducer. Unlike attenuation, inactivation of microbes is based on complete killing for the microbes using chemical, biological or physical tools. However, the SL protocol is a fast, cheap and in house protocol for microbial cell evacuation [11,15-17]. It describes bacterial cell evacuation from its cytoplasmic contents. It has been optimized using Plackett-Burman design to map the best cells' evacuation experimental conditions, which give the best un-deformed 3D structure and un-deteriorated surface antigens [11,15]. Then the best experimental conditions are used to fasting the protocol time [11]. Its main concept is to determine the Minimum Inhibition Concentration (MIC) and the Minimum Growth Concentration (MGC) of chemical compounds able to induce pores in the cell walls [1,11-17,19,37-39]. The used chemical compounds until now are Sodium Dodecyl Sulfate (SDS), NaOH, NaHCO_3 , H_2O_2 , CaCO_3 , NaCl and ethanol. Each of those compounds has been selected based on their effect on the cell wall, genetic elements or the protein contents [28,40-52]. In addition, 60% ethanol has been used to improve the DNA extraction processes [11]. Apparently, the original protocol published by Amara et al. (2013), has been the first protocol describes the use of the MIC and the MGC of the used chemical compounds for ghost cells preparations. In the original protocol only one colony is survived and the whole experiment batch has been degraded by activating the lysozyme gene using chloramphenicol [11]. However, the main prepared ghost cells have been prepared from the highest quality yield experiments as reintroduced in the reduced protocol [11]. Controlling the cells 3D structure during their evacuation steps using the light microscope guarantee full evacuation with nearly no effect on the 3D or the surface antigens. For each microbe there is a need for doing some adjustments, that due to the differences in the cell wall or envelopes/cote constituents, but after adjusting the evacuation conditions, the protocol is reliable and straightforward.

The idea of the protocol has been used to evacuate the Newcastle virus [20]. Recently, the virus has been prepared as empty viral ghost using the bio-critical concentration of the H_2O_2 [21]. Newcastle virus (LaSota strain-RNA virus) has been used as a model [21]. It is a RNA virus. The images of the agarose gel as well as the spectrophotometer spectra at $260/280_{\text{nm}}$ have been proved successful release and degradation to the virus RNA [21].

Other microbes such as the eukaryotic *Saccharomyces cerevisiae*, *Aspargillus nige*, *Aspargillus flavus* have been prepared as ghosts. [53-55], additionally the oyster mushroom's spores have been turned as ghosts [56]. The first drug delivery system using the SLP with the gossypol acetic acid and *E. coli* and *Saccharomyces cerevisiae* has been published by Amara (2015) [53].

To prove that the protocol concept is universal and could exceed the chemical compounds to the biological ones, lysozyme and proteinase K have been used with the spore former extremothermophilic *B. stearothermophilus* [18]. The enzymes when used in their minimum killing activity concentrations and their minimum living activity concentrations succeeded to turn the spore former bacteria to ghost cells [18]. It enables the control of the pore formation by many controlling factors, variables and steps. One could say that this protocol and its concept enable the evacuation of the biological cells or viruses

without harming their cell wall/virus cote, or their outer surface antigen [37-39].

Many technical solutions have been discussed in the different published papers where each microbe considers as case by case study. The SL protocol not deals only with the cell deactivation but also deal with its complete evacuations. Despite using the electron microscope, the protocol considers to be in a house tool for microbial ghost formation. One technical problem has been deviated the protocol to use some sophisticated instruments, it is the virus collection with constant amount during the ghost preparation steps, particularly during the separation of their genetic elements from their cote.

This study has been modified to be able to isolate the DNA, RNA and other cell components, as well as the microelements that could pass from the agarose gel during the electrophoresis without changing the cell load concentration. Additionally, it could be used for different target experiments include ghost cells preparation, DNA/RNA/protein isolation etc.

Different methods have been identified the isolation of the DNA and the RNA from the different microbial strains. However, in most cases such as the alkaline lysis protocol for the plasmid preparation or the phenol protocol for the DNA preparation the protocols usually did not care with the other components of the microbial cells. Recently, Amara describe a protocol for fast slot lysis for DNA and plasmid in agarose gel separation with the recommendation to calculate the used amount in the lysis buffer to produce bacterial ghost instead of lysis the cells [22,27]. In addition, the study highlighted the use of dialysis page to separate the DNA from the bacterial cell either directly or after being loaded in the agarose.

However, in fine microbes like viruses the loading of the virus in the normal slab agarose gel electrophoresis did not granite the retrieve of the virus from the agarose. It is of great important and a technical demand to keep the virus load constant during the molecular biology protocols and experiments. One major technical limiting factor is the virus load. This study succeeded to retrieve both of the bacterial and viral ghosts without changing their load because they are entrapped in the agarose bubbles (data not shown). More studies are in need to prove that using quantitative experiments.

Even Amara highlighted the important of using DNases and RNases in the ghost cell preparation but those enzymes will need additional steps for their removing. The methods have been described in this study did not describe any use of additional proteins or enzymes, it is an additional step toward better bacterial and viral ghost coats preparation, DNA/RNA/Plasmid isolations, and the various described techniques. The data obtained from the different concentration of the isolated DNA and/or RNA of *E. coli* BL21 ghosts (DNA and/or RNA) and Newcastle Pestikal lasota viral ghosts (RNA) using the Dialysis pages and the Double centrifugation methods for DNA and/or RNA isolation as in Table 1 show various DNA and/or RNA isolated quantities. By analysis the data it is showing that the major quantities of the DNA and/or RNA are isolated during the Dialysis page step (Table 1) and (Figure 1) The quantity of the isolated RNA from the virus is less than the DNA and/or RNA isolated from the *E. coli* BL21 (Table 1) and (Figure 1) (step 7 and 8). That could be either explained that the used amount of the originally used virus load contains RNA less than that used for bacterial load. Or, the virus is very sensitive to the treatment with H₂O₂. Additionally, it might be that the first

migration step need more adjustment while the stained agarose gels show less viral RNA migrated to the bottom of the gel (Figure 1) (step 6). It is a fact that the RNA is migrated faster than the DNA. Agarose in % more than 1% will give better results. The experimental conditions did not enable the migration of the protein might have existed in the sample through the agarose, however more validation studies are needed in the future work. Meanwhile, increasing the agarose concentration will fervor better pure DNA/RNA. In contrast, using this protocol in a lower agarose concentration enable protein purification [Data not shown] [57]. Or the DNA and the RNA samples should be running separately. However, the protocol shows efficient removal for the DNA and/or RNA. This study recommended the following points:

- Specifying the target of the protocol, either being for the DNA and/or RNA isolation/purification/ quantification, ghosts validation, ghosts preparation etc. and based on the target the protocol must be directed and some of its steps could be removed or changed.
- Running each of the bacterial ghosts and the virus ghosts separately.
- Using agarose higher than 1% in case of the RNA samples.
- Eliminating the first DNA and/or RNA migration step for better DNA and/or RNA isolation.
- Using standard DNA as a control.
- Using equal side's plastic cuvettes for the entrapped bubbles preparation.
- Using voltage equal to 70V or less. For better sample collection lesser volt should be used.
- Detaching the DNA and/or RNA from the dialysis page after their separation using the electrical current.
- The method could be used for concentrating the ghosts and reduce their suspended volume by repeating the process or by using a bigger entrapped bubble, or pre-prepared unit.

Sample name	Calculated amount of DNA/RNA		
	µg ml ⁻¹		Total µg ml ⁻¹
	Dialysis page	Double centrifugation	
Running buffer	Set to 0	Set to 0	0
DNA marker	365.5	151.04	516.54
<i>E. coli</i> BL21 ghosts DNA/RNA	328.7	143.84	472.54
Newcastle Pestikal lasota viral ghosts RNA	123.36	63.2	186.56

Table 1: Different concentration of the isolated DNA/RNA of *E. coli* BL21 ghosts (DNA/RNA) and Newcastle Pestikal lasota viral ghosts (RNA) using the Dialysis pages and the Double centrifugation methods for DNA/RNA isolation.

Conclusion

The SL protocol guarantees full microbial evacuation. This study is an additional step for the original protocol evaluation, DNA.RNA separation, examining the ghost cells quality, concentrating the ghost cells and validates ghost cells free DNA and/or RNA. Successfully, this preliminary study introduces for the first time, a method where the microbes did not being lost or their load is diluted during the agarose electrophoresis with subsequent safe steps for isolation of their DNA and/or RNA contents.

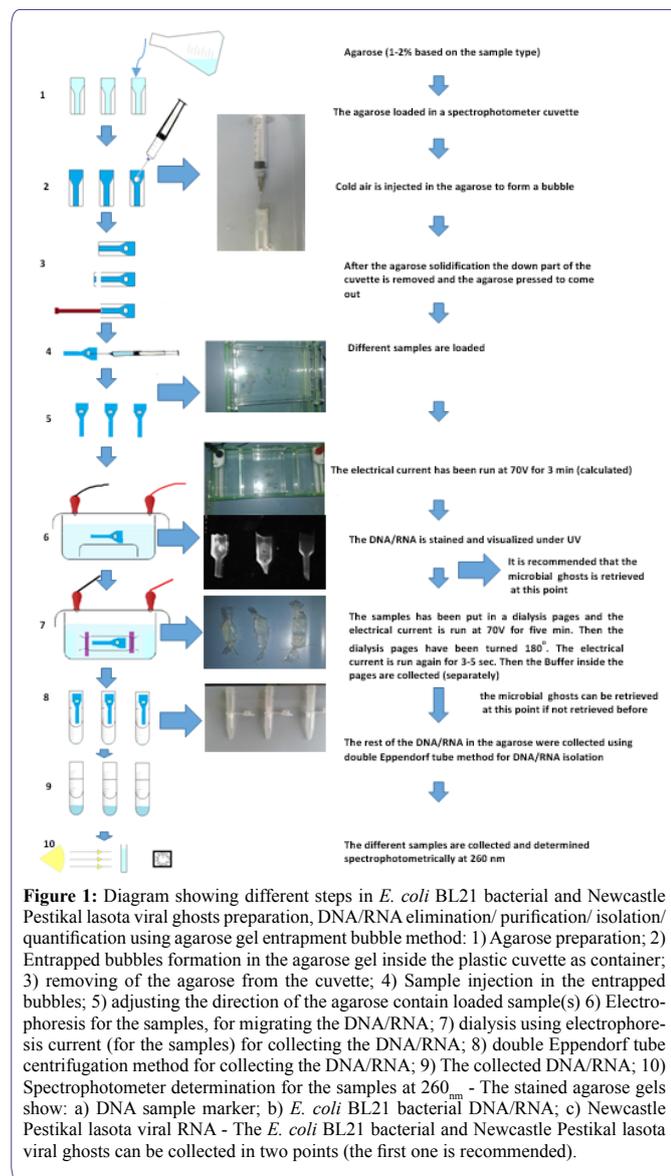


Figure 1: Diagram showing different steps in *E. coli* BL21 bacterial and Newcastle Pestikal lasota viral ghosts preparation, DNA/RNA elimination/ purification/ isolation/ quantification using agarose gel entrapment bubble method: 1) Agarose preparation; 2) Entrapped bubbles formation in the agarose gel inside the plastic cuvette as container; 3) removing of the agarose from the cuvette; 4) Sample injection in the entrapped bubbles; 5) adjusting the direction of the agarose contain loaded sample(s) 6) Electrophoresis for the samples, for migrating the DNA/RNA; 7) dialysis using electrophoresis current (for the samples) for collecting the DNA/RNA; 8) double Eppendorf tube centrifugation method for collecting the DNA/RNA; 9) The collected DNA/RNA; 10) Spectrophotometer determination for the samples at 260_{nm}. - The stained agarose gels show: a) DNA sample marker; b) *E. coli* BL21 bacterial DNA/RNA; c) Newcastle Pestikal lasota viral RNA - The *E. coli* BL21 bacterial and Newcastle Pestikal lasota viral ghosts can be collected in two points (the first one is recommended).

Conflict of Interest

The author declares that there is no conflict of interest of any kind.

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