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# Improvised two-step elution of chromatographic purification of grouper's iridovirus plasmid-based vaccine by monolithic adsorbent

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Received: *28 August 2015*
Revised: *14 September 2015*
Accepted: *1 October 2015*
Online: *22 December 2015*

*Keywords:*
Grouper's iridovirus plasmid-based vaccine; two-step elution; monolithic purification; chromatography

**Abstract**

Vaccines have significantly reduced the antibiotics usage especially in the aquaculture industry. Plasmid-based vaccine offers potential effective immunity against the Grouper Iridovirus and Grouper Nervous Necrosis Virus in groupers. Chromatographic purification system is crucial for the efficient recovery of plasmid DNA vaccine construct. With monolith technology, a platform for the vaccine purification has been developed to obtain a high-throughput production of plasmid DNA vaccine in economic manner and time less. The monolith was prepared via free radical co-polymerization of glycidyl methacrylate GMA and ethylene glycol dimethacrylate EDMA in the presence of cyclohexanol as porogen at 70% porogen concentration which gave a minimum heat build-up and homogenous pore size distribution. Monolithic based purification concluded that 0.1 M NaCl as a running buffer coupled with 0.9 M of sulphate salt solution and a flow rate of 1.0 mL/min used for separation are found to be able to produce a reasonably pure DNA. First peak elution is eluting all the RNA leaving DNA alone to be eluted in the second peak. The result of the studies will advance knowledge in the control of disease in the aquaculture industry using plasmid based vaccination and high throughput plasmid purification process.

## Introduction

Orthodox vaccines limit the effectiveness in eliciting immune response as they rely on antibiotic or chemically-treated bacteria and parasites to cause cessation or attenuation in pathogenic capacity. Besides, treatment with chemicals may be detrimental to the fish through accumulation in the flesh, development of drug resistant strains, and contamination of the aquatic environment (Saglam & Yonar, 2009) while, treatment with antibiotics may not be effective and micro-organisms may develop resistance to the antibiotic. On the other hand, vaccination is an alternative way to prevent diseases in fish (Taylor *et al*., 2009; Plotkin, 2009). Application of vaccines has shown a huge reduction in antibiotics usage especially in the salmon industry (Evensen & Leong, 2013). However, vaccine development in the aquaculture field is still in the early stage and vaccines have the advantage in providing long-lasting protection without leaving any adverse effects compared to the therapeutic treatments.

There are many types of vaccines in use in the aquaculture industry and many more are under development. However, DNA vaccines offer a wide range of protective immunity (non-specific and specific) compared to inactivated vaccines induce mainly humoral responses (Anderson *et al*., 1996). The challenges of aquaculture vaccines are the bio-manufacturing and purification of vaccines (Sousa *et al*., 2008). *E. coli* lysate has high concentration of cell impurities that require a thorough removal process (Stadler *et al*., 2004; Diogo *et al*., 2005). In purification, the conventional chromatographic media has been a drawback for effective purification of plasmid DNA due to their small pore sizes (Diogo *et al*., 2005). The increasing demand on the aquaculture vaccines has triggered the studies of the development of the production of plasmid vaccines through monolithic purification. Monolith technology offers a high-throughput plasmid purification process due to its interconnected pores with large pore size.

## Methodology

### *Materials*

Ethylene glycol dimethacrylate (EDMA) (*M*w 198.22, 98%), glycidyl methacrylate (GMA) (*M*w 142.15, 97%), cyclohexanol (*M*w 100.16, 99%), AIBN (Mw 164.21, 98%), MeOH (HPLC grade, Mw 32.04, 99.93%), NaCl (Amresco, Mw 58.44, 99.5%), agarose (Promega), SDS (Amresco, Mw 288.38, 99.0%), Tris (Amresco, Mw 121.14, 99.8%), EDTA (SERVA, Mw 292.3, AG), EtBr (Sigma, Mw 394.31, 10mg/mL), 1kbp DNA marker (BioLabs, New England), Bradford reagent (Sigma B6916, 500mL).

### *Model Plasmid Vaccine*

E. *coli* plasmid iridovirus (pDREAM2.1) was provided by Dr. Kenneth F. Rodrigues of Biotechnology Research Institute, University Malaysia Sabah, Malaysia.

### *Cell Culture*

10µl of transformed cells (E. *coli* iridovirus-pdream) was cultured in 15 mL of plasmid DNA semi-defined medium (PDM) by Clarence et al., 2011 containing 50 µg/mL ampicillin at 37 $^{o}$C overnight under 180 rpm. The culture was subcultured overnight in 150 mL of PDM containing 50 µg/mL ampicillin at 37 $^{o}$C. 150 mL of culture was transferred into 1500 mL of PDM containing 50 µg/mL ampicillin at 37 $^{o}$C and fermentation was performed using bioreactor. The culture was harvested after 4 hours.

### *Preparation of E. coli Iridovirus - E. coli plasmid cleared lysate*

50 ml of *E. coli* cell culture was harvested into 50 mL of sterile falcon tube, centrifuged at 5000 g for 15 min. The cell pellet was resuspended in 3 mL of 0.025 M Tris-HCl, 0.01 M EDTA, pH 8 buffer. The resuspended cells were lysed with 3 mL of lysis solution (0.2 M NaOH, 1 % SDS) for 5 min. The lysed cells were neutralized with 4 mL of 5 M $CH_3COOK$ (subjected to pH 5.5) for 5 min. The

mixtures were separated by centrifugation at 15000 g for 10 min. A volume of 1 mL of the clarified supernatant was transferred into a fresh microtube and mixed with 1 M of sulphate salt solution. The mixture was gently inverted for 30-50 times, incubated for 30 min at 25 $^{o}$C, and centrifuged at 15000 g for 15 min.

*Synthesis of poly (GMA-EDMA) monolithic column*

The monolith was prepared via free radical co-polymerization of 21 % of GMA and 9 % of EDMA, mixed with 70 % of porogen solvents. AIBN (1 % weight with respect to monomers) was added to initiate the polymerization reaction. The mixture was sonicated for 25 minutes at room temperature. The sonicated mixture was transferred into conical polypropene column (BIORAD) sealed with parafilm on top and placed in water bath at 60 $^{o}$C for three hours. The monolith was rinsed with methanol and deionized water to remove the solvents

*Chromatographic purification of plasmid iridovirus*

2 mL of monolithic resin in a conical polypropene column was connected and configured to Next Generation Chromatography System (BIORAD). Equilibration on column was done with buffer A (deionized water) and buffer B (0.10 M NaCl) at 1.0 mL/min until a constant UV baseline was achieved. 1 mL of clarified cell lysate was injected to the sample loop. Chromatographic purification was followed by elution and washing step. 100% buffer B (1.0 M NaCl) was introduced at washing step to elute the bound DNA. The column was regenerated repeatedly at fixed output percentage of buffer B with concentration of five different sulphate salt solutions (0.6 M to 0.9 M).

*Gel electrophoresis analysis*

Analysis of plasmid iridovirus and RNA were performed via ethidium bromide gel electrophoresis using 1 kb DNA ladder (OGeneRuler 1 kb DNA Ladder, Ready-to-Use) for 45 min at 100 V. Size of the plasmid was determined under 1% gel electrophoresis using 1 kb DNA ladder. 10x TBE buffer (106 g Tris base, 27.5 g boric acid, 20 mL of 0.5 M EDTA at pH 8.0) was prepared. 1% of agarose powder was added into 1x TBE buffer and mixed to solidify forming gel. Concentration of the plasmid was determined using Image Lab$^{TM}$ Software Version 5.0 with DNA ladder as reference.

**Result and Discussion**

*Chromatography Purification*

Two peaks were obtained via monolithic purification from each run as shown in the chromatogram in Figure 1. The first and second peaks are resulted from introduced 0.1 M and 1.0 M of NaCl respectively. Five different sulphate salt solution concentrations from 0.6 M to 0.9 M showed similar trend of pH and conductivity in the chromatograms. pH started to decrease at the first peak while slowly increases approaching second peak. The fractions are collected for gel observation.

*Effect of Sulphate Salt Solution*

Two peaks were obtained via monolithic purification from each run as shown in the chromatogram in Figure 1. The first and second peaks are resulted from introduced 0.1 M and 1.0 M of NaCl respectively. Four different sulphate salt solution concentrations from 0.6 M to 0.9 M showed similar trend of pH and conductivity in the chromatograms. pH started to decrease at the first peak while slowly increases approaching second peak. The fractions are collected for gel observation.

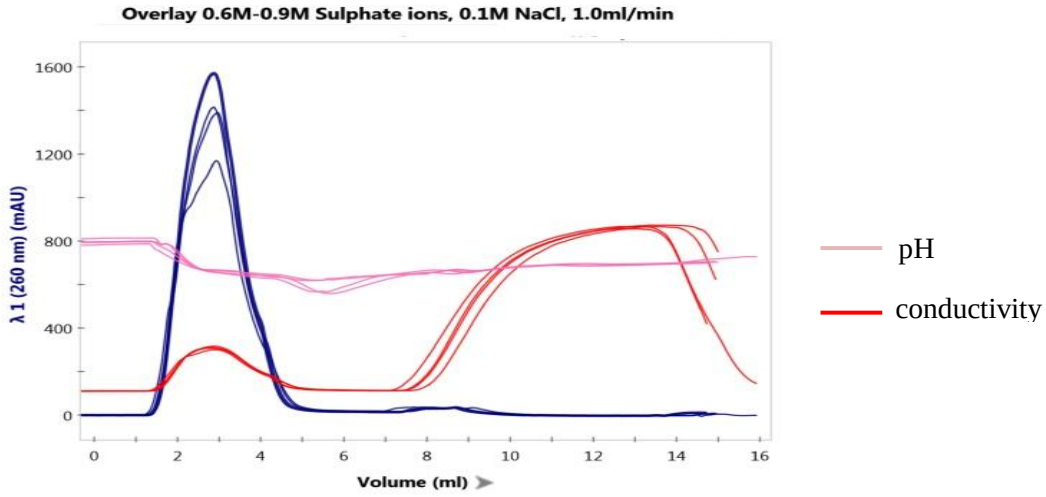


**Figure 1**: Stacking of 0.6 M - 0.9 M chromatogram of E. *coli* plasmid DNA extraction using Next Generation Chromatography System (BIORAD) via alkaline lysis. Different sulphate salt solution concentration of **0.6 M - 0.9 M** are tested. 0.1 M NaCl and 1.0 M NaCl are fixed for the first peak elution and second peak elution respectively. Flow rate of 1.0 mL/min.

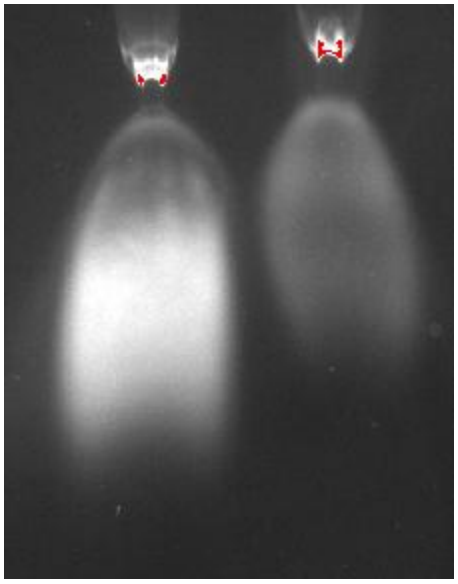

**Figure 2**: E. *coli* plasmid DNA extraction via alkaline lysis with chemical modification of sulphate salt solution. Analysis was performed 1% agarose in 60 mL TBE x 1 buffer at 100 V for 0.7 h.

*Gel Electrophoresis Analysis*

Figure 3 showed successful removal of RNA contaminant at 0.9 M and 1.0 M sulphate salt solution while there is still RNA contaminant at 0.6 M to 0.8 M sulphate salt solution. However, decreasing trend of RNA concentration was observed from 0.6 M to 0.8 M sulphate salt solution in the second

elution. Plasmid DNA is present in the first elution and second elution for five different concentration tested. At the first elution of 0.9 M sulphate salt solution, small amount of plasmid DNA wasted and all RNA is eluted leaving a reasonable pure plasmid DNA in the second elution.

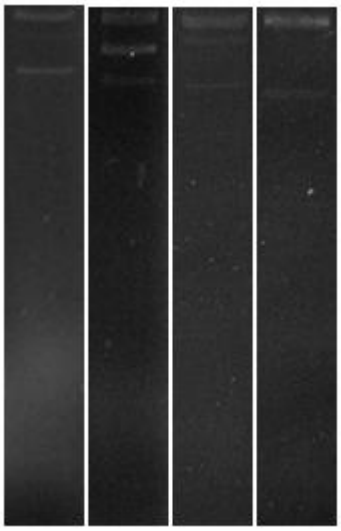

**Figure 3**: Monolithic chromatography of E. *coli* plasmid DNA extraction using Next Generation Chromatography System (BIORAD) via alkaline lysis of second peak elution with 0.6 M to 0.9 M sulphate salt solution concentration. A fixed NaCl concentration of 0.1 M is applied during the first peak elution and NaCl concentration of 1.0 M is fixed for the second peak elution. Flow rate of 1.0 mL/min. Analysis was performed 1% agarose in 60 mL TBE x 1 buffer at 100 V for 0.7 h.

Polymethacrylate monolith is well known of trapping target biomolecules via functionalization with various ligands such as amino-based functional groups (Ongkudon & Danquah, 2011). The significance of this study is to improvise the DNA yield via two-step elution using non-functionalized polymethacrylate monolithic column without addition of enzyme. The whole purification required two steps elution with peak 1 generally focusing on eluting all the RNA to free up plasmid DNA in second peak by using 1 M NaCl.

We describe an improvised two-step elution that could relies on the balance in between NaCl concentration and sulphate salt solution concentration through the interaction between the negative charge of RNA with NaCl molecules at optimum concentration for an easy and cost effective production of plasmid DNA yield. This could be achieved when optimum NaCl concentration is eluting all the RNA in the first peak as a result of shielding effect thus retaining only plasmid DNA to be eluted in the second peak at higher NaCl concentrations. And to do that, we have been trying to optimize the concentration of NaCl. However, the yield in second peak is jeopardized as a result as the plasmid DNA is also reduce simultaneously with RNA during the elution of 1$^{st}$ peak albeit in much lower quantity. Finding the balance in between the concentration of sulphate salt solution and NaCl will be crucial in limiting the quantity of plasmid DNA compromised for the sake of RNA elimination (Eon-Duval & Burke, 2004).

It was evident that the optimum NaCl concentration of 0.27 M NaCl coupled with 0.8 M sulphate salt solution in the loading buffer gives a reasonable pure plasmid DNA. However, plasmid DNA

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showed an increasing trend of intensity when improvised with increasing sulphate salt solution concentration from 0.6 M to 0.9 M (as seen in Figure 3). Theoretically, an increasing trend of sulphate salt solution concentration should decrease the intensity of plasmid DNA and interestingly, the intensity of plasmid DNA could be seen improvised at lower NaCl concentration of 0.1 M when coupled with 0.9 M sulphate salt solution thus increasing the recovery percentage of plasmid DNA from 15.68 to 70.97 as shown in Table 1. This indicates NaCl has the tendency of compromising more pDNA in exchange for RNA removal as compared to sulphate salt solution. The studies showed that the optimum NaCl concentration in the loading buffer is around 0.1 M NaCl coupled with 0.9 M sulphate salt solution which compromised lesser plasmid DNA while removing RNA.

**Table 1**: pDNA pDream2.1 recovery at coupling concentration of 0.27 M NaCl and 0.8 M sulphate salt solution Vs 0.1 M NaCl and 0.9 M sulphate salt solution

| **[NaCl] [Sulphate salt solution]** | **0.27M NaCl, 0.8M Sulphate salt solution** | **0.1M NaCl, 0.9M Sulphate salt solution** |
|---|---|---|
| **Total Yield of pDNA Recovery (ng)** | 10.76 | 53.46 |
| **% of pDNA Recovery** | 15.68 | 70.97 |

We decided to fix the concentration of NaCl during first peak elution to 0.10M NaCl while manipulating the concentration of solution X used in lysis phase as it seems that sulphate salt solution is more bias in resolving RNA and DNA compared to NaCl, hence the objective of limiting the amount of plasmid DNA compromised during first peak can be achieved. It was suggested that 1 M NaCl is sufficient to overcome the binding interaction that might have occurred during sample loading and elution while 0.1 M NaCl introduced in the first elution was evident to be able to isolate plasmid DNA using 0.9 M sulphate salt solution as shown in Figure 3.

## Conclusion

This research focuses on the improvisation of the plasmid yield through purification using non-functionalized polymethacrylate monolith with chemical modification during lysis at optimum sulphate salt solution coupled with optimum concentration of sodium chloride used in the first peak elution to remove all RNA. Reasonable pure plasmid DNA can be improved at optimum concentration of 0.1 M NaCl and 0.9 M sulphate salt solution. These findings will lead to the possibility of producing alternative methods in controlling the disease in the aquaculture.

## Acknowledgement

The authors would like to thank University Malaysia Sabah for the support in carrying the project. This project was funded by Research Acculturation Grant Scheme (RAG0002-SG-2012).

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