Method for Washing a Column And Method for Extracting Membrane-Bound Target Molecules

Background of the Invention

1. Field of the Invention

The present invention relates to a method for washing a column and a method extracting membrane-bound target molecules such as silica membrane-bound nucleic acids, DNA, or RNA.

2. Description of the Related Art

A variety of commercial products adopt silica chaotropic technology-based membrane columns (or generally called spin columns) to isolate and purify nucleic acids (DNA/RNA). There are one or several membranes disposed at the bottom of a column, and the space within the column can be filled with liquid. When the liquid is loaded onto the membrane column, appropriate force is applied to draw the liquid through the bottom membrane, and subsequently the drawn liquid is discarded or collected. Generally, the so-called appropriate force used to draw the liquid through membranes may be centrifugal force, when the process is manipulated on a centrifuge, or atmospheric pressure, when vacuum manifold is adopted.

Generally, the purification procedure using silica membrane chaotropic technology comprises three steps, i.e., binding, washing, and eluting. The handbook for these commercial products usually provides two procedures for the operators' choice. One is centrifuge protocol, and the other is vacuum protocol. Compared to vacuum protocol, the centrifuge protocol comprises more steps and takes longer time, and therefore it is suitable for handling less number of samples. However, the centrifuge protocol is more stable because the liquid in the column passes through the membrane(s) more completely after each centrifugation procedure, and the residue of liquid scarcely remains in the column. On the other hand, the vacuum protocol is easier to operate and takes less time compared to the centrifuge protocol, and therefore

it is suitable for handling a larger number of samples. However, the vacuum protocol is unstable because usually, the liquid in the column is unlikely to pass through the membrane(s) completely after each vacuum procedure, and there appears to be more liquid remaining in the column. In particular, at the end of the washing step, it is extremely likely that the residual washing liquid may contaminate the next eluting step and causes the eluted nucleic acid solution to contain the residual washing liquid. Consequently, such contaminant might affect the subsequent applications of the nucleic acids. Therefore, it is preferable to apply an additional centrifugation step after the washing step to eliminate the residual washing liquid. However, since the vacuum protocol can be easily manipulated at an automatic workstation, the additional centrifugation step will cause the whole procedure even more awkward.

Further, the washing liquid used in the silica chaotropic technology usually contain organic solvents such as alcohols. The main purpose of using washing liquid is to remove the undesired impurities without affecting the binding state of the nucleic acids and the membrane, while that of eluting buffers, usually water or low salt solution, is to elute the nucleic acids bound on the membrane so that they can be collected and used for the subsequent applications. Some of the applications of nucleic acids are sensitive to a small amount of organic solvents. For example, if the reagent used in the polymerase chain reaction (PCR) contains traces of alcohol, it will have noticeable inhibitory effects on the PCR. Alternatively, to eliminate the residual organic washing liquid in the membrane column, the membrane column can also be heated instead of centrifugation. But the heating condition must be adjusted to an optimal state. If the silica membrane is over-heated, it might become too dry, resulting in the bound nucleic acids' inability to be dissolved in the eluting buffer. Accordingly, the yield of nucleic acids might be reduced as a result. If the membrane is not heated enough, the residue of organic solvents cannot be eliminated.

Therefore, it is valuable to devise a method for washing a column and a method for extracting membrane-bound target molecules, such as silica membrane-bound nucleic acids, DNA, or RNA, for the application of vacuum protocol, especially for automation, to eliminate the residue of organic washing liquid, while no additional centrifugal step or heating step is necessary.

Summary of the Invention

It is an object of the present invention to provide a method for washing a column for the application of vacuum protocol, especially for automation, to eliminate the residue of organic washing liquid and/or impurities.

It is also the other object of the present invention to provide a method for extracting membrane-bound target molecules containing the above washing method.

According to the present invention, the method of washing a column is characterised in that the silica membrane column contains membrane-bound target molecules, such as nucleic acid, DNA or RNA, and impurities, and subsequently, the column is filled with a washing liquid such as eluting buffer or water having a capability of releasing the membrane-bound target molecules from the membrane to a liquid-free form, and finally, at least a portion of the washing liquid left in the column that does not pass through the membrane is subsequently removed from the column so as to leave a clean membrane column for next elution step.

According to the other aspect of the present invention, a method for extracting membrane-bound target molecules containing the above washing method comprises the steps of:

a) preparing a column containing at least one membrane at its bottom end;
b) filling the column with a solution containing the membrane-bound target molecules and impurities;
c) drawing the solution through the membrane such that the membrane-bound target molecules are bound to the membrane and the solution is drained out of the column
d) filling the column with a first washing liquid to remove the impurities while still leaving the membrane-bound target molecules bound on the membrane;
e) drawing the first washing liquid through the membrane and out of the

column;

f) filling the column with a second washing liquid having a capability of releasing the membrane-bound target molecules from the membrane to a liquid-free form;

g) removing the second washing liquid from the top of the column;

h) filling the column with an elution liquid so as to release the membrane-bound target molecules to a liquid-free form from the membrane; and

i) drawing the elution liquid through the membrane and collecting the elution liquid for the recovery of the membrane-bound target molecules.

Other objects, advantages and novel features of the present invention will be drawn from the following detailed description of preferred embodiment of the present invention with the accompanying drawings, in which:

Descriptions of the Drawings

Fig. 1A illustrates a step of the method according to a preferred embodiment of the present invention;

Fig. 1B illustrates a step of the method according to a preferred embodiment of the present invention;

Fig. 1C illustrates a step of the method according to a preferred embodiment of the present invention;

Fig. 1D illustrates a step of the method according to a preferred embodiment of the present invention;

Fig. 1E illustrates a step of the method according to a preferred embodiment of the present invention;

Fig. 1F illustrates a step of the method according to a preferred embodiment of the present invention;

Fig. 1G illustrates a step of the method according to a preferred embodiment of the present invention; and

Fig. 1H illustrates a step of the method according to a preferred embodiment of the present invention.

Detailed Description of the Invention

A membrane in a column (or a spin column) generally is semi-permeable for liquid and a column has at least one membrane at its bottom end. When the column is filled with a liquid, the liquid can be blocked and usually retained for about one or several minutes. However, due to the influence of osmosis, the liquid can still go downwards and pass through the membrane slowly. Because of this phenomenon, when a liquid having a capability of releasing the membrane-bound target molecules from the membrane to a liquid-free form, is loaded into the column, the bound target molecules will be further brought into the membrane with the liquid pulled downwards by osmosis as soon as the liquid dissolves the bound target molecules to be a free form. This almost leaves no chance for the free target molecules to move upwards back to the liquid which is still retained in the column. Consequently, the target molecules and the liquid remained in the column are separated by the membrane and it can be assumed that the liquid in the column hardly contains the target molecules.

Accordingly, after a membrane column, such as a silica or glass membrane spin column, has been bound with target molecules, such as nucleic acid, DNA, or RNA and contains impurities, such as non-target molecules, for example, residuals of organic washing buffer or chaotropic salts after a washing step, a washing liquid having a capability of releasing the membrane-bound target molecules from the membrane to a liquid-free form, such as water or an elution buffer compatible with the subsequent applications of the target molecules, is loaded into the column, the target molecules will be further brought into the membrane with the washing liquid pulled downwards by osmosis as soon as the washing liquid dissolves the bound target molecules to a free form. Consequently, the target molecules and the liquid remained in the column are separated by the membrane. Accordingly, almost all of the target molecules are in or have passed through the membrane, while most of the impurities are still contained in the washing liquid and remained in the column. While at least a portion of the washing liquid in the column that does not pass through the membrane is removed, the impurities will be also removed together and almost none or a tiny amount of the target molecules are lost, which can subsequently effectively reduce the contaminations from the previous washing step (if any) for the subsequent eluting step.

Accordingly, a method for extracting membrane-bound target molecules containing the above washing method may comprise the steps of:

a) preparing a column 10 containing at least one membrane 11;
b) filling the column 10 with a solution 12 containing membrane-bound target molecules and impurities (referring to Fig. 1A);
c) drawing the solution 12 through the membrane 11 such that the membrane-bound target molecules are bound to the membrane 11 and the solution 12 is drained out of the column 10 (referring to Fig. 1B);
d) filling the column 10 with a first washing liquid 13, which is often organic, so as to remove the impurities while still leaving the target molecules bound on the membrane (referring to Fig. 1C);
e) drawing the first washing liquid 13 through the membrane 11 and out of the column 10 (referring to Fig. 1D);
f) filling the column 10 with a second washing liquid 14, such as an eluting buffer or water, having the capability of releasing the membrane-bound target molecules from the membranes 11 to a liquid-free form (referring to Fig. 1E);
g) removing the second washing liquid 14 from the top of the column 10 (referring to Fig. 1F);
h) filling the column 10 with an elution liquid 15 so as to release the target molecules to a liquid-free form from the membrane11 (referring to Fig. 1G);
i) drawing the elution liquid 15 through the membrane 11 and collecting the elution liquid for the recovery of the membrane-bound target molecules (referring to Fig. 1H).

Experiment:

Genomic DNA is purified using the QIAGEN QIAamp DNA Blood Mini kit according to the vacuum protocol. Briefly, a 1.7 ml blood sample in a 5 ml tube is mixed with 170 μl QIAGEN protease and 1.7 ml buffer AL, then, incubated at 56°C for 16 min, adding 1.7 ml ethanol to the sample, and mixed again by pulse-vortexing for 20 sec, then, aliquot 630 μl into 8 QIAamp spin columns, continuing the wash steps. At the end of the washing step, each spin column is weighted to approximately evaluate the amount of the residual washing liquid in the column; the average weight

of spin column is increased to 25 mg +/- 2 mg as compared to their original weight. Then, the 8 columns of samples are divided into 4 groups:

1. In Group I, 200 μl of eluting buffer (AE) is applied to the spin columns without further removing the residual washing buffer in the column, followed by a centrifugation step to elute the genomic DNA.

2. In Group II, an additional centrifugation step is included to remove the residual washing liquid in the column, then, the spin column is weighted again, about 20 mg of the residual washing liquid in the column were removed by centrifugation. Then, adding 200 μl of eluting buffer (AE), and the genomic DNA is eluted by the other centrifugation step.

3. In Group III, 850 μl of water is added into the spin column, and then is removed immediately. Finally, adding 200 μl of eluting buffer (AE) and eluting genomic DNA by centrifugation.

4. In Group IV, 850 μl of buffer (AE) is added into the spin column, and then is removed immediately. Finally, adding 200 μl of elute buffer (AE) and eluting genomic DNA by centrifugation.

The DNA quantity (O.D.260) of each sample is determined by the photo-spectrometer so as to evaluate the yield of nucleic acids. In addition, quantitative PCR (QPCR) using QuantiTect SYBR Green PCR kit (QIAGEN) is employed to evaluate the influence of different washing methods on QPCR by amplification and detection of GAPDH; 15 μl of eluate containing genomic DNA from each sample is mixed with 35 μl of QPCR master reagent containing primers. The results are listed in Table 1 below.

Table 1

Group	Sample	Yield (OD260)	Ct of QPCR
I	1	6.5 μg	27.0

	2	6.8 μg	27.5
II	3	6.6 μg	22.8
	4	6.7 μg	23.0
III	5	7.0 μg	22.8
	6	6.4 μg	23.0
IV	7	6.7 μg	22.9
	8	6.6 μg	22.9

From Table 1, it can be seen that the yields of four Groups are all close to each other, therefore, the method of the present invention does not reduce the yield of nucleic acid preparation, while adding and removing the additional washing liquid, such as eluting buffer AE or water, has the capability of releasing the nucleic acid from silica membrane. Further, analysis of the inhibition effect of the residual washing liquid in the columns from the previous washing step using QPCR shows that, Group I, without removing the residual washing liquid in the columns, has Ct values of about 27, whereas Group II has Ct values of about 23 using centrifugation to remove the residual washing liquid in the columns. The result obviously shows the inhibition effect of QPCR due to the co-elution of genomic DNA with the residual washing liquid in the columns. The Ct values of QPCR in Groups III and IV are all about 23 and close to the Ct values of Group II, showing that the method of the present invention can effectively remove the residual washing liquid in the columns from the previous washing step.

In conclusion, the present invention offers a method for washing a column and a method for extracting membrane-bound target molecules which are characterised by using an eluting buffer, water, or even any expected buffer as an additional washing liquid to effectively remove the impurities or residual washing liquid in the column from the previous washing step. The present invention can replace the

aforementioned additional centrifugation or heating step between the traditional washing step and the eluting step without reducing the yield of target molecules (nucleic acids) for silica membrane chaotropic technology using a membrane column or a spin column, while it is so easy for operation, especially for automation.

While the invention has been described in terms of a preferred embodiment, those skilled in the art will recognise that the invention can still be practiced with modifications, within the spirit and scope of the appended claims.

What is claimed is:

1. A method for washing a column containing at least one membrane, impurities and membrane-bound target molecules, characterised in that:

   a washing liquid having a capability of releasing the membrane-bound target molecules from the membrane to a liquid-free form is loaded to the column and at least a portion of the washing liquid left in the column that does not pass through the membrane is subsequently removed from the column as to effectively remove the impurities in the column.

2. The method of Claim 1, wherein the membrane is a silica or glass membrane.

3. The method of Claim 1, wherein the target molecules are nucleic acids, DNA, or RNA.

4. The method of Claim 1, wherein the washing liquid is an elute buffer.

5. The method of Claim 1, wherein the washing liquid is water.

6. A method for extracting membrane-bound target molecules comprising the steps of:
   a) preparing a column containing at least one membrane at its bottom end;
   b) filling the column with a solution containing the membrane-bound target molecules and impurities;
   c) drawing the solution through the membrane such that the membrane-bound target molecules are bound to the membrane and the solution is drained out of the column
   d) filling the column with a first washing liquid to remove the impurities while still leaving the membrane-bound target molecules bound on the membrane;
   e) drawing the first washing liquid through the membrane and out of the column;
   f) filling the column with a second washing liquid having a capability of releasing the membrane-bound target molecules from the membrane to a liquid-free form;

g) removing the second washing liquid from the top of the column;

filling the column with an elution liquid so as to release the membrane-bound target molecules to a liquid-free form from the membrane; and

h) drawing the elution liquid through the membrane and collecting the elution liquid for the recovery of the membrane-bound target molecules.

7. The method of Claim 6, wherein the membrane is silica or glass membrane.

8. The method of Claim 6, wherein the target molecules are nucleic acids, DNA, or RNA.

9. The method of Claim 6, wherein the first washing liquid is organic.

10. The method of Claim 6, wherein the second washing liquid is an elute buffer.

11. The method of Claim 6, wherein the second washing liquid is water.