FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM

MQIP
MILK QUALITY IMPROVEMENT PROGRAM

Department of Food Science, Cornell University

Title: PCR Product Purification and Sanger Sequencing

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1. INTRODUCTION

1.1. Purpose

To outline the standard laboratory procedure for purification and subsequent sequence submission of PCR products.

1.2. Scope

This SOP applies to the Food Safety Lab, including the Laboratory for Food Microbiology and Pathogenesis of Foodborne Diseases and any experimental procedures conducted by laboratory members at other locations.

1.3. Definitions

ExoI - Exonuclease I. Exonuclease I degrades single-stranded DNA in a 3'=>5' direction, releasing deoxyribonucleotide 5'-monophosphates in a stepwise manner and leaving 5'terminal dinucleotides intact. ExoI does not cleave DNA strands with terminal 3'-OH groups blocked by phosphoryl or acetyl groups. Exonuclease I is derived from *E.coli* cells carrying a cloned *E.coli sbcB* gene (Fermentas, 2006). In the purification of PCR products, ExoI is responsible for the degradation of any excess primers in the mixture.

SAP - Shrimp Alkaline Phosphatase - Alkaline Phosphatase catalyzes the dephosphorylation of 5’ phosphates from DNA and RNA. SAP is equally efficient on either 5’-protruding, 5’- recessive and blunt ends. In the purification of PCR products, SAP is responsible for the de- phosphorylation any leftover dNTPs.

2. MATERIALS

2.1. exoSAP Purification

- Exonuclease I 10U/ul (exo)
- Shrimp Alkaline Phosphatase 1 U/ul (SAP)
- 1.5 ml microcentrifuge tubes
- Micropipette and sterile tips
- Thermocycler

2.2. Thermofisher GeneJET PCR Purification

- ThermoScientific GeneJET PCR Purification Kit #K0701, #K0702
- • Ethanol 96-100%.
- Isopropanol.
- 3 M sodium acetate, pH 5.2 (may be necessary)
- Microcentrifuge.
- 1.5 or 2 mL microcentrifuge tubes.
- Heating block or water bath (may be necessary)

3. PROCEDURES

3.1. exoSAP purification

1. Confirm good quality amplification of single product bands with agarose gel electrophoresis. exoSAP will not clean up reactions with multiple bands or bands of poor quality. Good, strong PCR amplification yields higher quality sequences.
2. Prepare a master mix containing 1 µL of Exonuclease I (10 units/µL) and 1 µL of Shrimp Alkaline Phosphatase (1 unit/µL), per 20-22 µL of PCR product. Add exoSAP master mix to sterile reservoir.
3. Pipette 2 µL of exoSAP master mix (using a multi-channel pipette) into each well of the 96 well plate containing PCR products which will undergo the sequencing reaction.
4. Heat PCR products with exoSAP in a thermocycler at 37°C for 45 minutes, and then at 80°C for 15 minutes.
5. Store plates at 4°C until proceeding to the sequencing reaction.

3.2. Thermofisher GeneJET PCR Purification

Notes: This protocol was taken from the user manual of the Thermofisher GeneJET PCR Purification Kit.

1. Prior to the initial use of the kit, dilute the Wash Buffer (concentrated) with ethanol (96-100%)
2. After the ethanol has been added, mark the check box on the bottle to indicate the completed step.
3. Examine the Binding Buffer for precipitates before each use. Re-dissolve any precipitate by warming the solution to 37 °C and cooling to 25 °C.
4. Wear gloves when handling the Binding Buffer as this solution contains irritants. All purification steps should be carried out at room temperature.
5. All centrifugations should be carried out in a table-top microcentrifuge at >12000´g (10,000-14,000 rpm, depending on the rotor type).

3.2.1. Protocol A: DNA purification using centrifuge

1. Add a 1:1 volume of Binding Buffer to completed PCR mixture (e.g. for every 100 µL of reaction mixture, add 100 µL of Binding Buffer). Mix thoroughly. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
    a. Optional (for DNA ≤500 bp): if the DNA fragment is ≤500 bp, add a 1:2 volume of 100% isopropanol (e.g., 100 µL of isopropanol should be added to 100 µL of PCR mixture combined with 100 µL of Binding Buffer). Mix thoroughly. Note. If PCR mixture contains primer-dimers, purification without

isopropanol is recommended. However, the yield of the target DNA fragment will be lower.

2. Transfer up to 800 µL of the solution from step 1 (or optional step 2) to the GeneJET purification column. Centrifuge for 30-60 s. Discard the flow- through. Notes. If the total volume exceeds 800 µL, the solution can be added to the column in stages. After the addition of 800 µL of solution, centrifuge the column for 30-60 s and discard flowthrough. Repeat until the entire solution has been added to the column membrane. Close the bag with GeneJET Purification Columns tightly after each use!
3. Add 700 µL of Wash Buffer (diluted with the ethanol as described on p. 3) to the GeneJET purification column. Centrifuge for 30-60 s. Discard the flow-through and place the purification column back into the collection tube.
4. Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove any residual wash buffer. Note. This step is essential as the presence of residual ethanol in the DNA sample may inhibit subsequent reactions.
5. Transfer the GeneJET purification column to a clean 1.5 mL microcentrifuge tube (not included). Add 50 µL of Elution Buffer to the center of the GeneJET purification column membrane and centrifuge for 1 min.
    a. Note:For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50µL does not significantly reduce the DNA yield. However, elution volumes less than 10 µL are not recommended.
    b. If DNA fragment is >10 kb, prewarm Elution Buffer to 65 °C before applying to column.
    c. If the elution volume is 10 µL and DNA amount is ≥5 µg, incubate column for 1 min at room temperature before centrifugation.
6. Discard the GeneJET purification column and store the purified DNA at -20°C.

*3.2.2. **Protocol B: DNA purification using vacuum manifolds***

1. Perform DNA binding stage according to steps 1 - 2 in Protocol A above.
2. Prepare the vacuum manifold according to the supplier's instructions. Place the GeneJET purification column(s) onto the manifold. Close the bag with GeneJET Purification Columns tightly after each use!
3. Transfer up to 800 µL of the solution (from step 1 or 2 as in protocol A) to the GeneJET purification column.
    a. Note. If the total volume exceeds 800 µL, the solution can be added to the column in stages. After each application, apply the vacuum and discard the flow-through. Repeat until the entire volume has been applied to the column membrane.

4. Apply the vacuum to draw the sample through the column. Switch off the vacuum after the entire sample has passed through the column.
5. Add 700 µL of Wash Buffer (diluted with the ethanol as described on p. 3) to the GeneJET purification column.
6. Apply the vacuum to draw the solution through the column. Switch off the vacuum after the solution has passed through the column. Place the purification column back into the collection tube.
7. Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove any residual wash buffer.
    a. Note. This step is essential as the presence of residual ethanol in the DNA sample may inhibit subsequent reactions.
8. Transfer the GeneJET purification column to a clean 1.5 mL microcentrifuge tube (not included). Add 50 µL of Elution Buffer to the center of the GeneJET purification column membrane and centrifuge for 1 min.

    Note:

    - For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20- 50 µL does not significantly reduce the DNA yield. However, elution volumes less than 10 µL are not recommended.
    - If DNA fragment is >10 kb, prewarm Elution Buffer to 65 °C before applying to column.
    - If the elution volume is 10 µL and DNA amount is ≥5 µg, incubate column for 1 min at room temperature before centrifugation.

9. Discard the GeneJET purification column and store the purified DNA at - 20 °C.

3.3. Sample Preparation for Sanger Sequencing

These guidelines are based on the guidelines laid out by the Biotechnology Resource Center (BRC) and are current as of 09-30-2019. For more detailed information please refer to our handbook located here: http://www.biotech.cornell.edu/node/556

1. For each PCR product that will be sent to the BRC for Sanger sequencing, 2 samples need to be prepared, one for each primer.
2. Keep all components on ice during the procedure.
3. Place the required amount of purified PCR product and 25 pmole of primer in the specified vials (see section 3.4 for tube requirements) and bring up to 18µl with dH2O.
4. To determine the required amount of PCR product to add, use the following formula:

    - #base pairs/5.0 = amount of PCR product in ng that we need in 18ul volume.

- For example: 250bp PCR product. - 250bp ÷ 5.0 = 50ng of DNA + 25 pmole (2.5µl of a 10uM solution) primer in 18µl.
- **Note: You cannot quantify your DNA on the NanoDrop if you have already purified your PCR product with the exoSAP protocol

5. If you have not quantified your PCR product on the NanoDrop, use the following volumes in each submission tube:
   - 12.5µl of dH2O
   - 2.5µl of a 10uM primer (forward and reverse in separate submission tubes/wells)
   - 3µl of purified PCR product

3.4. Sanger Sequencing Submission

*These guidelines are based on the guidelines laid out by the Biotechnology Resource Center (BRC) and are current as of 09-30-2019. For more detailed information please refer to the handbook located here: http://www.biotech.cornell.edu/node/556

1. Place an order online:
2. Samples will be accepted only **after** an order has been placed using our online ordering system which can be reached from our website: http://www.biotech.cornell.edu/brc/genomics-facility For **Sanger Sequencing, template +/- primer submission, Single Tube(s) or 96-well plate**. Batch upload is used for both tube and plate submissions, where you may upload a text file instead of typing in your sample names.
3. Sample vial requirements: We accept samples in two formats:
4. Tubes: 500ul standalone screw top vials from the following companies. USA Scientific (ordered thru Fisher): 1405-9799; VWR: 16466-052; VWR: 16466- 036 (Tubes only, 16466-084 Caps ; Fisher: 50-476-676 (Our “purple cap” tubes are an acceptable tube to use)
5. ***DO NOT USE*** the following tubes, they cause problems due to having a “lower bottom” than the tubes we recommend, so the liquid level sits lower in the tube, and the robot can miss pipetting the sample. Fisher:02681-333, Fisher: 02-682-559 and Fisher: 14-755-274. If you have tubes that you think are compatable please email us a picture. We will get back to you if they will work with the robotics we use or not.
6. **Plates**: Any PCR style plate can be used for full services Sanger Sequencing (template and primer submissions). Fill your samples down the columns as followed. Sample 1-A1, Sample 2-B1, Sample 3-C1 and so one. Fill the following column Sample 9-A2, Sample 10-B2, Sample 11-C2 and so on throughout the

entire plate. The last 2 wells, H12 and G12 on the 96 well plate must be left empty for controls.

7. **Shipping:** We recommend ***shipping*** your plates at room temperature and to use strip caps on the top of your plate as seen here. The uses of adhesive seals is not recommend and can cause contamination due to sample leakage. Adhesive seals are acceptable for dropping of plates in person. (Above example for shipping)
8. **Large batches of samples** (71 or more) can be submitted in a plate and run at a reduced cost ("our Plate Pricing"). The Plate Pricing discount is only available on samples submitted in a 96-well PCR style plate. However, please note that failure reruns are not available with Plate Pricing. ***We prefer to receive large numbers of samples in plates (16 or more), rather than in individual tubes, as it allows us to process the samples more efficiently*.** Feel free to cut the plate and use the rest later. Always fill from the upper left hand side down. When less than 71 samples are submitted in a 96-well plate, tube price will be charged. Please contact us before submitting full plates of samples so that we may go over the submission details with you.
9. **Sample** Drop off and Mailing:
   Samples may be dropped off in room 147 in Biotechnology Building from 8:00 AM to 4:30 PM Monday through Thursday and 8:00 AM to 4:00 PM on Fridays and may also be sent via FedEx or US Mail. Our mailing address is **526 Campus Road, 147 Biotechnology Building, Cornell University, Ithaca NY 14853**. Please note that there is no delivery on Saturdays or holidays and we have received numerus broken tubes and plates, so please cushion and seal your plates before mailing.

4. TROUBLESHOOTING

4.1. For the Thermofisher GENEjet kit:

4.1.1. Low DNA yield

1. Inefficient DNA binding
   - Verify that a 1:1 volume of Binding Buffer is added to the reaction mixture. Ensure the solutions are mixed well.
   - Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the solution color is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
2. Inefficient membrane wash
   - Ensure that the recommended volume of ethanol has been added to the Wash Buffer (concentrated) prior first use.
3. Inefficient DNA elution
   - Add Elution Buffer directly to the center of the membrane and not to the side of the GeneJET purification column.
   - Use 20-50 µL of Elution Buffer and ensure that the volume completely covers the surface of the membrane.
   - Increase the Elution Buffer volume twice or perform two elution cycles when purifying larger amounts of DNA. (e.g., >15 µg).
   - In step 4 of Protocol A (step 7 of Protocol B), ensure all residual wash buffer is removed from the membrane. Longer centrifugation time (extra minute) can aid in removal of wash buffer.

4.1.2. PCR reaction mixture does not contain DNA

- Check for the presence and yield of the PCR product by running an aliquot of the reaction on an agarose gel.

4.2. Downstream reactions are unsuccessful

1. Presence of residual ethanol
   - In step 4 of Protocol A (step 7 of Protocol B), ensure all residual wash buffer is removed from the membrane. Longer centrifugation time can aid in removal of wash buffer.
2. Inefficient membrane wash
   - Ensure that the collection tube is not overfilled during the wash step and that any of the wash buffer has remained in the bottom of the GeneJET purification column. Always discard the flow-through after centrifugation.
3. Eluate contains excess salt

- Ensure that the wash step 3 of Protocol A is effective. Incubate the GeneJET purification column with the Wash Buffer for several minutes before proceeding to centrifugation.

4. DNA does not remain in an agarose gel well

- In step 4 of Protocol A (step 7 of Protocol B), ensure all residual wash buffer is removed from the membrane. Longer centrifugation time can aid in removal of wash buffer.

5. REFERENCES

- "Sanger Sequencing Handbook"
- "User Guide: GeneJET PCR Purification Kit" Thermofisher Scientific, 2019