Purpose: This procedure reflects the Precipio Standard Operating Procedures for Purification of PCR Products which includes the AMPure XP® PCR Purification Kit, Cycle Sequencing of AMPure XP Purified PCR Products, and Dye-Terminator Removal from Cycle Sequencing Reactions using the CleanSEQ® Dye-Terminator Removal Kit.

Protocol:

1.0 Materials and Equipment Needed:

1.1 Nuclease-free, aerosol resistant pipette tips
1.2 DistriTips Syringe, 125 µL volume
1.3 DistriTips Syringe, 1250 µL volume
1.4 0.2 mL, nuclease-free, thin-walled PCR tubes and caps
1.5 96-Well PCR Plate
1.6 Cap Strips for PCR plates
1.7 Sterile Reservoirs
1.8 Sterile 1.7 mL tube
1.9 15 mL Sterile Conical Tubes
1.10 Sequencing Plate Raised Skirt
1.11 Calibrated Pipettes, Rainin Pipettemen P-1000, P-200, P-100, P-20 P-10, and P-2 or equivalent.
1.12 Multichannel Pipettes, Rainin 1-10 µL, 5 µL – 50 µL and 50 µL – 300 µL or equivalent
1.13 Repeat Pipettor
1.14 SPRIPlate 96-well magnetic plate, Agencourt Product #: A29164 or equivalent
1.15 NIST Traceable Digital Timer, FisherBrand Model 14-649-11 or equivalent
1.16 Lab-Line Instruments, Incubator Model 203 or equivalent
1.17 Thermal Cycler, GeneAmp PCR System 9700 or equivalent

2.0 Reagents and Solutions:

2.1 AMPure XP Reagent (Beckman Coulter, Brea, CA)
2.2 ethanol, 200 Proof
2.3 molecular biology grade water
2.4 Big Dye v3.1 (Thermo Fisher Scientific, Waltham, MA)
2.5 Better Buffer (Gel Company, San Francisco, CA)
2.6 Sequencing Primer
2.7 CleanSEQ Reagent (Beckman Coulter, Brea, CA)
2.8 0.5M EDTA

3.0 Procedure-Purification of PCR Products

Note: Please use an appropriate pipetteman, multi-channel pipetteman or repetitive pipetteman for all steps below.

3.1 If not already done, prepare, in a sterile bottle, a 70% ethanol solution using 200 proof ethanol and molecular biology grade water (e.g. 700 mL 200 proof ethanol and 300 mL molecular biology grade water).
3.2 Mix the AMPure XP reagent thoroughly before use by gently shaking the bottle to re-suspend the magnetic particles. It should appear homogeneous in color.
3.3 PCR product and AMPure XP volumes:
    3.3.1 The following are guidelines for volumes used in the AMPure XP procedure.
        3.3.1.1 For ICP product, use 10 µL PCR product to 18 µL AMPure XP.
3.4 Dispense the appropriate volume of PCR Product into a 96-well PCR plate.

3.5 Dispense the appropriate volume of AMPure XP according to 3.3.1 into the 96-well PCR plate containing the PCR product and pipette mix 10 times creating a homogenous mixture. A multi-channel pipetteman and sterile reservoir for the AMPure XP may be used with large sample numbers. Centrifuge briefly as needed and let incubate for 3-5 minutes at room temperature.

3.6 Place the sample plate onto the SPRIPlate for 2 minutes to separate beads from solution.

3.6.1 Ensure the solution is clear before proceeding to the next step. If the solution is not clear, repeat step 3.6 until solution is clear.

3.7 With the sample plate situated on the SPRIPlate, aspirate the cleared solution and discard. Do not disturb the ring or pellet of separated magnetic beads. Check pipette tips before discarding solution. If magnetic beads are seen in the pipette tip dispense the solution back in appropriate well, repeat step 3.6 and attempt to extract cleared solution again. Repeat until clear solution is obtained then discard clear solution.

3.8 With the sample plate situated on the SPRIPlate dispense 200 µL of 70% ethanol to each reaction well and incubate for approximately 30 seconds at room temperature. A multi-channel pipetteman and sterile reservoir for the ethanol may be used with large sample numbers. Do not disturb the separated magnetic beads during this process.

3.8.1 Aspirate out and discard the ethanol. If magnetic beads are seen in the pipette tip, place solution back in appropriate well, allow the solution to clear and attempt again. Repeat until clear solution is aspirated and only then discard.

3.8.2 **Repeat** wash step for a total of two washes.

3.8.3 Be sure to remove all ethanol from the bottom of the well, as it may contain residual contaminants.

3.9 Evaporate the residual ethanol in the samples by placing the sample plate uncovered in the incubator for approximately three minutes.

3.9.1 The incubator should be set to approximately 60 ºC.

3.9.2 After three minutes, inspect the sample wells to ensure all residual ethanol is removed. If ethanol is still present, repeat step 3.8 until residual ethanol has evaporated. Take care not to over dry the bead ring or pellet (bead ring or pellet appears cracked) as this will significantly decrease elution efficiency.

3.9.3 If the samples will be used immediately, proceed to Step 3.10 for elution. If the samples will not be used immediately, the dried samples may be sealed with cap strips and stored indefinitely at -20 °C.

3.10 With the sample plate **off** of the SPRIPlate, add 40 µL of molecular biology grade water to each well, pipette mix 10 times, centrifuge briefly as needed, and incubate at room temperature for two minutes.

3.11 Place the sample plate back onto the SPRIPlate for at least one minute to allow the sample to clear.

3.12 *Optional:* Once clear, pipette 30 µL of the purified DNA from the center of the wells to a clean 96-well PCR plate labeled as “AMPure” product with the appropriate sample information. Check pipette tips before dispensing solution. If magnetic beads are seen in the pipette tip dispense the solution back in appropriate well. Repeat step 3.11 and attempt to extract clear solution again. Repeat until clear solution is obtained then transfer clear solution.

4.0 Procedure-Cycle Sequencing of AMPure XP® Purified PCR Products

Note: Big Dye and any solution containing Big Dye must be kept in the dark as much as feasible. Keep reagents on ice at all times. Verify the concentration of your primers and adjust Master Mix conditions accordingly.

4.1 Determine the number of samples to undergo Cycle Sequencing. As ICE-COLD PCR is only sequenced in one direction, one Master Mix may be used for all samples. Add approximately 10 additional reactions per 96 well plate to the total to ensure sufficient volume.

4.2 Cycle Sequencing Master Mix Preparation (on ice) **Master Mix reagents are listed in bold** while individual components are in regular text. Vigorously vortex BigDye for 30 seconds before adding to the Master Mix.

4.2.1 Each reaction receives:

Reagent:	1X reaction*
Molecular Biology Grade Water	5.25
BigDye v3.1	0.25
Better Buffer	2.50
Sequencing Primer (10 µM)	1.00
AMPure Purified Product	1.00

**Multiply volumes in this table by the number of samples being tested.*

4.2.2 Cap Master Mix tube and vortex briefly and spin down.

4.3 Add 1 µL AMPure XP-purified PCR product to the designated well(s).

4.4 Add 9 µL of the Master Mix to each sample well.

4.5 If not already in the master mix, add 1 L of the designated 10 M sequencing primer.

4.6 Place cap strips on the wells, vortex, and centrifuge the samples briefly to ensure all reagents are at the bottom of the wells. Load plate on thermal cycler.

4.7 Set up the Thermal Cycling Conditions as follows:

Step	Temperature (°C)	Time (sec)	Cycle
Denature	94	30	
Primer Annealing	50	10	40
Extension	60	45	
Hold	>12	∞	

4.8 After the thermal cycling program is complete, the reactions must have the excess Dye-Terminators removed. Refer to Section 5 below to continue sample processing.

5.0 Procedure-Removal of Excess Dye-Terminators

5.1 If not already done, prepare, in a sterile bottle, an 85% ethanol solution using 200 proof ethanol and molecular biology grade water (e.g. 850 mL 200 proof ethanol and 150 mL molecular biology grade water).

5.2 If not already done, prepare, in a sterile bottle, a 0.1 mM EDTA solution from the stock EDTA and molecular biology grade water (e.g. 200 µL 0.5M EDTA and 1 L molecular biology grade water).

5.3 Mix the CleanSEQ reagent thoroughly before use by gently shaking the bottle to resuspend the magnetic particles. It should appear homogeneous in color.

5.4 Add 5 µL of CleanSEQ to the completed cycle sequence reactions.

5.5 Add 60 µL of 85% ethanol to the samples and pipette mix 7 times creating a homogenous mixture. A multi-channel pipetteman and sterile reservoir may be used with large sample numbers. Centrifuge briefly as needed.

5.6 Place the sample plate onto the SPRIPlate for 3-5 minutes to separate beads from solution.

5.6.1 Ensure the solution is clear before proceeding to the next step. If the solution is not clear, repeat step 5.6 until solution is clear.

5.7 With the sample plate situated on the SPRIPlate, aspirate the clear solution and discard. Do not disturb the ring or pellet of separated magnetic beads. Check pipette tips before discarding solution. If magnetic beads are seen in the pipette tip dispense the solution back in appropriate well, repeat step 5.6 and attempt to extract clear solution again. Repeat until clear solution is obtained then discard clear solution.

5.8 With the sample plate situated on the SPRIPlate, dispense 100 µL of 85% ethanol to each reaction well and incubate for approximately 30 seconds at room temperature. Do not disturb the separated magnetic beads during this process.

5.8.1 Aspirate out and discard the ethanol. If magnetic beads are seen in the pipette tip place solution back in appropriate well, allow the solution to clear and attempt again. Repeat until clear solution if aspirated and only then discard.

5.8.2 **Repeat** wash step for a total of two washes.

5.8.3 Be sure to remove all of the ethanol from the bottom of the well, as it may contain residual contaminants.

5.9 Evaporate the residual ethanol in the samples by placing the sample plate uncovered in incubator for approximately 4-7 minutes.

5.9.1 The setting of the incubator should be at approximately 60 ºC.

5.9.2 After 4-7 minutes, inspect the sample wells to ensure all residual ethanol is removed. If ethanol is still present, repeat step 5.9 until residual ethanol is evaporated.

Note: Be careful not to over dry the sample beads. Agencourt warns that this can degrade the BigDye.

5.10 With the sample plate **off** of the SPRIPlate, add 40 µL of 0.1 mM EDTA to each well of the sample plate. Centrifuge briefly as needed and incubate at room temperature for 5 minutes.

5.11 Place the sample plate onto the SPRIPlate and allow the sample to clear for 3-5 minutes.

5.12 Once clear, pipette 30 µL of the purified DNA from the center of the wells to a clean ABI 3730 full-skirt clear plate.

5.12.1 Check pipette tips before dispensing solution. If magnetic beads are seen in the pipette tip dispense the solution back in appropriate well, repeat step 5.11 and attempt to extract clear solution again. Repeat until clear solution is obtained then transfer clear solution.
Note: DO NOT allow beads to be present in the sequencing samples as they can damage the instrument.

5.12.2 If plate is not full of samples, add at least 25 µL of 0.1 mM EDTA to the empty wells. Centrifuge plate briefly.

5.13 Obtain a cartridge and a clean 3730 rubber septa. Check for any tears in the septa. Place the septa on the plate and the plate into the cartridge.

5.14 Load the plates onto the sequencer (ABI 3730xl) with appropriate 1X CE running buffer.
5.15 Use the following parameters to run the ICE COLD-PCR sequencing reactions:

Name	Value	Range
Oven_Temperature	60	18...70 DegC
PreRun_Voltage	15.0	0...15 kV
PreRun_Time	180	1...1800 sec
Injection_Voltage	1.5	0...15 kV
Injection_Time	15	1...90 sec
First_ReadOut_Time	200	100...16000 ms
Second_ReadOut_Time	200	100...16000 ms
Run_Voltage	13.4	0...15 kV
Voltage_Number_Of_Steps	30	0...100 Steps
Voltage_Step_Interval	15	0...180 secs
Voltage_Tolerance	0.6	0...6.0 kV
Current_Stability	30.0	0...2000 uA
Ramp_Delay	450	1...1800 sec
Data_Delay	250	1...1800 sec
Run_Time	1000	300...14000 sec