TEACHING THE GENOME GENERATION™

PREP FOR SEQUENCING PROTOCOL

BEFORE YOU BEGIN

Several companies offer Sanger sequencing services for a fee, and may even have education discounts. Ensure you follow the protocol they recommend for sample preparation and submission.

PREREQUISITES & GOALS

STUDENT PREREQUISITES

Prior to implementing this lab, students should understand:

- The central dogma of how DNA bases code for mRNA and then for proteins
- How DNA samples were collected and prepared for PCR
- The steps that occur during the process of polymerase change reaction (PCR), as well as the reagents used
- The variants of ACTN3, CYP2C19, and/or TAS2R38
- The connection between the bands on the gel electrophoresis and the gene variants
- The purpose of using ExoSAP-IT
- The purpose of the PREP FOR SEQUENCING PROTOCOL is to prepare PCR-amplified DNA samples for Sanger sequencing

STUDENT LEARNING GOALS

1. Prepare amplified DNA products for Sanger sequencing.

NOTES

The **ExoSAP** system uses two enzymes to prepare amplified PCR products for sequencing:

Exonuclease I (Exo) digests residual single-stranded DNA primers and any extraneous single-stranded DNA fragments produced in from the PCR process

Shrimp Alkaline Phosphatase (SAP) dephosphorylates remaining dNTPs (free nucleotides) from the PCR product so they do not interfere with the sequencing reaction

CURRICULUM INTEGRATION

Use the planning notes space provided to reflect on how this protocol will be integrated into your classroom. You'll find every course is different, and you may need to make changes in your preparation or setup depending on which course you are teaching.

Course name:

1. What prior knowledge do the students need?

2. How much time will this lesson take?

3. What materials do I need to prepare in advance?

4. Will the students work independently, in pairs, or in small groups?

5. What might be challenge points for students during this lesson?

MATERIALS

REQUIRED LAB MATERIALS

Ice bath or crushed ice

Markers for labeling

Amplified DNA samples from the PCR PROTOCOL

Micropipettes & tips (size P20)

0.2 mL tubes in strips

1.5 mL tubes

Tube holders/racks

Mini-microcentrifuge

Thermal cycler

Molecular biology grade water

(F)orward sequencing primer (4 µM)

(R)everse sequencing primer (4 µM)

Invitrogen ExoSAP-IT enzyme (on ice)

WORKSTATION NEEDS

Distribute these materials to each workstation.

Micropipettes and tips

0.2 mL tubes in strips

1.5 mL tubes

Molecular biology grade water

(F)orward and (R)everse primers

Tube holders and markers for labeling

Crushed ice/ice bath

ExoSAP-IT enzyme (on ice)

Amplified DNA samples

PROTOCOL STRUCTURE

STEPS 1-5 15 minutes

Break point: samples can be stored at 4 °C for up to 48 hours

STEPS 6-8 30 minutes *(incubation period - students do not need to be present)*

Break point: samples can be stored at 4 °C for up to 48 hours

STEP 9-17 20 minutes

PROCEDURE

☐ STEP 1

Obtain 0.2 mL tubes in strips and label them with the DNA sample numbers/letters.

NOTE: Only process samples that were successfully amplified (do not process the negative control).

☐ STEP 2

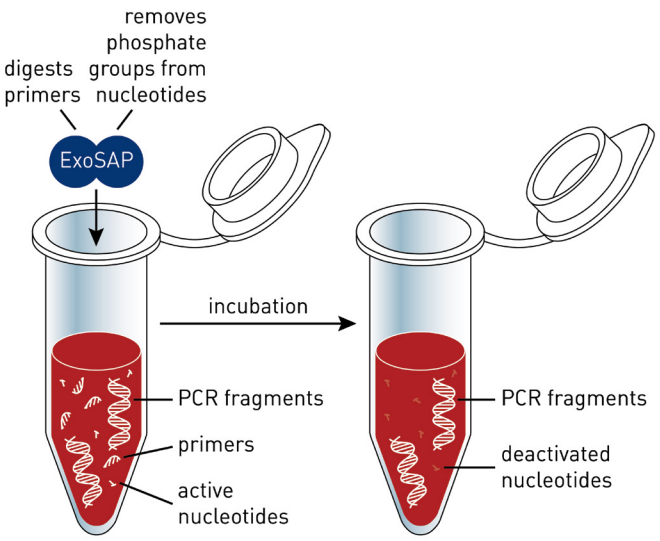
Using the P20 micropipette, transfer 5 µL of the amplified DNA samples from the PCR PROTOCOL new tubes.

☐ STEP 3

Using the P20 micropipette, add 2 µL of ExoSAP-IT to the new tubes.

WHY: There are left over PCR reagents in your PCR product tube, primers and free nucleotides in particular, which can interfere with the downstream sequencing reaction. These residual components will be eliminated and/or inactivated by ExoSAP-IT before the sample is submitted for sequencing.

NOTE: Enzymes must be kept on ice at all times.



PLANNING NOTES

☐ STEP 4

Tightly cap the tubes and flick the tube gently to mix.

☐ STEP 5

Spin the tubes briefly in the mini-microcentrifuge to collect the solution at the bottom of the tubes.

BREAK POINT IF NEEDED.

The DNA sample can be stored for up to 48 hours at 4 °C (refrigerator).

Expected result is to have one tube per DNA sample with 7 µL of reaction solution.

☐ STEP 6

The thermal cycler should be programmed with the digestion protocol.

EXOSAP: Digests free nucleotides and primers

Cycling conditions

1.	Digestion	37° C	15 min.
2.	Protein degradation	85° C	15 min.
3.	Final hold	4° C	forever

PLANNING NOTES

☐ STEP 7 using PTC 1000

1. Turn on the thermal cycler using the switch in back and wait for the machine to run a self-test.
2. Check that tubes are tightly capped to avoid evaporation, place the tubes in the thermal cycler and close the lid.
3. With the cursor blinking on RUN, hit PROCEED.
4. Select the appropriate protocol and hit PROCEED.
5. Prompt will ask if you want to enable the heated lid, hit PROCEED.

☐ STEP 7 using T-100

1. Turn on the thermal cycler using the switch in back.
2. Check that tubes are tightly capped to avoid evaporation, place the tubes in the thermal cycler and close the lid.
3. On the touch screen select SAVED PROTOCOLS.
4. Select the appropriate protocol and press RUN.

☐ STEP 7 using miniPCR

1. Plug the miniPCR block into both the computer and power outlet, and turn on the thermal cycler using the switch in back.
2. Check that tubes are tightly capped to avoid evaporation, place the tubes in the thermal cycler and close the lid.
3. Open the miniPCR software.
4. If the appropriate protocol does not exist, create a new protocol using the PCR template. Input the name of the protocol, times and temperatures indicated above for each step. Save the new protocol.
5. Double click the appropriate protocol. Select the miniPCR block to run the program on and click OK.
6. After two minutes of the program running, you can unplug the miniPCR block from the computer (keeping it plugged into the power outlet) and it will still run the desired program. Plug into the computer at any point to watch the temperature cycling on the software.
7. Repeat with each miniPCR block to run each.

PLANNING NOTES

BREAK POINT

The reaction will proceed for 30 minutes.

Once the protocol has completed, it will hold a constant temperature of 4 °C until samples are removed (except the miniPCR platform). It is best to remove the samples and turn off the machine within 24 hours of completing the run to avoid excessive condensation accumulation on the machine. However, samples can be left over the weekend, if necessary.

☐ STEP 8

Remove the samples after the protocol is complete, stop the program and turn the machine off.

BREAK POINT IF NEEDED

The DNA sample can be stored for up to 48 hours at 4° C (refrigerator) or up to 5 years at -20 °C (freezer).

Expected result is to have one tube per DNA sample with 7 µL of reaction solution. Nothing should look different about the solution after the reaction.

PLANNING NOTES

☐ STEP 9 **CRITICAL STEP**

Obtain two new 0.2 mL strip tubes per DNA sample to be sequenced.

☐ STEP 10 **CRITICAL STEP**

Label one tube as the forward sequencing reaction and one as the reverse sequencing reaction for each sample.

EXAMPLE: 4-F would be the label for DNA sample #4 to be sequenced with the Forward primer.

WHY: A reverse reaction is always included as a data back up in case the (F)orward reaction does not work or does not extend to the end of the sequence. If your PCR fragment is longer than ~500 bp, a (R)everse reaction will capture the sequence at the end.

☐ STEP 11

Using the P20 micropipette, add the following to the FORWARD reaction tube:

- 10 µL of molecular biology grade water
- 3 µL of ExoSAP-IT/DNA mix that has been incubated
- 2 µL of (F)orward sequencing primer

☐ STEP 12

Using the P20 micropipette, add the following to the REVERSE reaction tube:

- 10 µL of molecular biology grade water
- 3 µL of ExoSAP-IT/DNA mix that has been incubated
- 2 µL of (R)everse sequencing primer

WHY: The capillary gel electrophoresis machine can only interpret one single strand at a time, so the (F)orward and (R)everse reactions must be separated.

☐ STEP 13

Tightly cap the tubes and flick the tube gently to mix.

PLANNING NOTES

☐ STEP 14

Spin the tubes briefly in the mini-microcentrifuge to collect the solution at the bottom of the tubes.

The DNA sample can be stored for up to 48 hours at 4 °C (refrigerator) or up to 5 years at -20 °C (freezer).

Expected result is to have two tubes per DNA sample with 15 µL of reaction solution.

The DNA sample is now ready for sequencing.

☐ STEP 15

Follow the final sample labeling, shipping and packaging directions dictated by the sequencing company.

Sources of Potential Error

The most common errors for the PREP FOR SEQUENCING PROTOCOL include:

- incorrect micropipetting
- not keeping the enzymes on ice, which reduces digestion activity

Clean up

Discard all used tips and tubes in the trash except the amplified samples prepped for sequencing.

PLANNING NOTES

NEED HELP?
Email the experts: *ttgg@jax.org*