

Genome-Wide Human SNP Nsp/Sty Assay 6.0

Improvements to step 7 of the SNP 6.0 Assay, PCR cleanup, using Agencourt AMPure XP beads

This user bulletin includes updated instructions for performing step 7 of the Genome-Wide Human SNP 6.0 Nsp/Sty Assay. This document is intended for customers who are using the original SNP Assay 6.0 Kit. You may use this bulletin in conjunction with the quick reference cards (QRCs) to view relevant diagrams associated with this protocol.

About this stage

During this stage, you will:

- Pool the Sty and Nsp PCR reactions to a single deep-well pooling plate
- Add beads to each well and incubate
- Transfer the Millipore filter plate to a vacuum manifold
- Wash the PCR products with EtOH and filter
- Add EB buffer to each well and incubate at **50°C**
- Elute the PCR products using Buffer EB (Qiagen, USA)
- Vacuum and spin transfer the PCR product to a new 96-well plate

Location and duration

Location: Main lab

Duration: 3 hours, 60 minutes hands-on time

Table 1: Steps, duration, and hands-on time.

Steps in process	Step duration (minutes)	Hands-on time (minutes)
Sample/magnetic bead incubation	10	5
Initial vacuum step	40-60	5
First EtOH vacuum step (wash step)	10-15	5
Final EtOH vacuum step (dry step)	2	5
Elute DNA in Buffer EB on Jitterbug	30	<2
Re-suspend beads in Buffer EB	30	5
Elution on vacuum manifold	5	<2
Final elution, centrifuge	5	5
Total time	180	<45

Input required from previous stage

The input required is:

- Three plates Sty PCR product
- Four plates Nsp PCR product

Equipment and consumables required

Table 2: Equipment required for this stage

Qty	Item
1	Plate centrifuge equivalent to Eppendorf Centrifuge 5804 R, capable of 4°C temperature
1	Boekel Scientific Jitterbug Microplate Thermoshaker or shaker
1	ABgene 2.2 mL Deep Well Plate (ThermoFisher Scientific, cat#AB-0932)
1	Plate, elution catch, 96-well V-bottom
1	12-channel P20 pipette
1	12-channel P200 pipette
1	12-channel P1200 pipette
As needed	Pipettes and tips: 200, 20, & 10 µL
As needed	KimWipes

Table 3: Chemicals required to perform this stage.

Qty	Item	Recommended supplier
1	0.5 M EDTA, pH 8.0	Ambion, cat#9260G
1	Ammonium acetate solution for molecular biology, 7.5 M	Sigma-Aldrich, cat#A2706-1L
1	Isopropanol (2-Propanol) for molecular biology, ≥99%	Sigma-Aldrich, cat#I9516-500ML
1	Ethanol absolute, 200 proof, >99.5%	Sigma-Aldrich, cat#459844-1L (ACS grade)
1	Buffer EB	Qiagen, cat#19086
1	Water, molecular biology grade	USB, cat#71786



Important information about this stage

To help ensure the best results, carefully read the information below before you begin this stage of the protocol.

- Do not over-dry the magnetic beads during the EtOH wash step on the vacuum. Over-drying may inhibit elution of the purified DNA.
- After adding EtOH to the wells, the first vacuum step should be approximately 20 minutes. Turn off the vacuum as soon as all the wells turn to matte.
- The final EtOH vacuum step is 2 minutes only. Do not exceed 2 minutes.

- All of the liquid in each well should be pulled through the filter. Although the beads may still be moist, there should be no standing liquid on top of the beads. The wells will appear dull (matte), not shiny. If any wells are clogged, do not continue filtering. Proceed with the protocol for the samples that have been successfully purified and eluted. Repeat the experiment for the samples in the clogged wells.
- **Bring the 75% EtOH to room temperature prior to use.**
- Store and use Buffer EB at 4°C (DO NOT bring to room temperature prior to use).
- The storage temperature for the magnetic beads is 4°C (refrigerator).
- To avoid cross-contamination, pipette very carefully when pooling the PCR reactions into the deep-well plate.
- Maintain the vacuum between 20 to 24 in Hg (do not exceed 24 in Hg).
- Inspect the vacuum manifold for salt buildup after each use and clean regularly.

Procedure

1. Prepare the 75% EtOH

Dilute ACS-grade or equivalent ethanol to 75% using molecular biology grade water.

Recipe for 75% EtOH

In a 1 L measuring cylinder:

1. Pour 750 mL 100% EtOH
2. Add 250 mL molecular biology grade water.
3. Transfer to a 1 L bottle and mix well.
4. Seal tightly and store at room temperature.

Note: The 75% EtOH will be warm from mixing with water—be sure to equilibrate to room temperature before use.

2. Prepare the reagents

Note: Store and use the Buffer EB at 4°C (refrigerator).

3. Prepare the vacuum manifold

To prepare the manifold:

1. Connect the manifold and regulator to a suitable vacuum source able to maintain 20 to 24 in Hg.
2. Leave the vacuum turned off at this time.
3. Inspect the manifold for salt and other contaminants and clean if necessary.
4. Place the vacuum flask trap below the level of the manifold.
5. Place the standard collar on the manifold.



Note: Inspect the vacuum manifold for salt buildup before each use. If the flask trap is not placed below the level of the manifold, some solution may splash back and adhere to the bottom of the filter plate.

4. Preheat the hybridization ovens

To preheat the hybridization ovens (Affymetrix® GeneChip® Hybridization 640 or 645):

1. Turn on each oven and set the temperature to 50°C.
2. Set the RPM to 0. Turn the rotation off.
3. Allow to preheat for at least 30 minutes.

5. Pool the PCR products



CAUTION: Be very careful when pooling PCR products. Avoid cross-contaminating neighboring wells with small droplets.

To pool the PCR products:

1. If PCR products are:
 - Frozen, thaw to room temperature on the bench top in plate holders.
 - On thermal cyclers, remove them now.
2. Vortex the center of each plate at high speed for 3 seconds.
3. Spin down each plate at 2,000 RPM for 30 seconds.
4. Place each PCR plate in a plate holder on the bench top.
5. Place a deep-well pooling plate on the bench top.
6. On each PCR plate, cut the seal between each row so that it can be removed one row at a time.
7. Use a 12-channel P200 pipette set to 110 µL.
8. Remove the seal to expose row A only on each PCR plate.
9. Transfer the reactions from row A of each PCR plate to the corresponding wells of row A on the pooling plate.
10. Change your pipette tips: Change pipette tips after the PCR product from the same row of each PCR plate has been pooled on the pooling plate.
11. Remove the seal from each PCR plate to expose the next row.
12. Transfer each reaction from the same row of each PCR plate to the corresponding row and wells of the pooling plate.
13. Repeat steps 10, 11, and 12 until all of the reactions from each PCR plate are pooled.
14. When finished, examine the wells of each PCR plate to ensure that all product has been transferred and pooled.

6. Purify the pooled PCR products

In this stage you will add magnetic beads and incubate to purify the PCR product. During a 10-minute incubation, the DNA binds to the magnetic beads.

To add magnetic beads:

1. Mix the magnetic bead stock very well by vigorously shaking the bottle. Beads will settle overnight. Examine the bottom of the bottle and ensure that the solution appears homogenous.
 - a. Pour or pipette 50 mL of magnetic beads to a solution basin.
 - b. 1 mL of magnetic beads is required for each reaction. You can add in multiple batches if the solution basin is not large enough.
2. Using a manual (not electronic) 12-channel P1200 pipette:
 - a. Slowly add 1.0 mL of magnetic beads to each well of pooled PCR product.
 - b. Mix well by pipetting up and down 5 times using the following technique.
 - c. Change pipette tips for each row.

Mixing technique:

- i. Depress the plunger and place the pipette tips into the top of the solution.
- ii. Move the pipette tips down—aspirating at the same time—until the tips are near the bottom of each well.
- iii. Raise the tips out of the solution.
- iv. Place the pipette tips against the wall of each well just above each reaction and carefully dispense the solution.



*Note: The solution is viscous and sticky. Pipette carefully to ensure you have aspirated and dispensed 1 mL. **Do not use an electronic pipette.** Thorough mixing is critical to ensure that the PCR products bind to the beads.*

To incubate:

3. Cover the plate to protect the samples from environmental contaminants and allow the plate to incubate at room temperature for 10 minutes. You can use the lid from a pipette tip box to cover the wells.

7. Purify the reactions

To purify the reactions:

1. Turn on the vacuum to 20 to 24 in Hg and check the seals. Do not exceed 24 in Hg. Adjust the leak valve if necessary.

2. Ensure that the unused wells are completely sealed and cover the plate to protect it from environmental contaminants.
3. Run the vacuum until all of the liquid has been pulled through the filter (approximately 40 to 60 minutes). The wells should appear matte. You may use a flashlight to inspect the wells more closely.
4. Confirm wells are matte (dull color).
 - a. Use the palm of your hand to firmly tap the top of the plate twice to release the hanging drops.
 - b. Turn off the vacuum.
 - c. Remove the plate and firmly tap on KimWipes until no wet spots are observed.
 - d. Place the plate back on manifold and turn on the vacuum.
 - e. Vacuum for an additional 10 minutes.
 - f. Leaving the vacuum on, firmly tap the top of the plate again twice.
5. Leaving the vacuum on, examine each well. If any of the wells are still wet, put the plate back on the vacuum and continue filtering for up to 5 minutes.
6. Leaving the vacuum on:
 - a. Set a 12-channel P1200 to 900 μ L and add 900 μ L of 75% EtOH to each reaction.
 - b. Wait approximately 1–2 minutes (or until the volume in the wells begins to decrease).
 - c. Add another 900 μ L of 75% EtOH to each reaction (for a total of 1.8 mL EtOH).
 - d. Cover the plate.
 - e. Run the vacuum until all wells are matte (approximately 10 to 20 minutes).
 - f. Firmly tap the top of the plate twice to release the hanging drops.
 - g. Turn off the vacuum.
 - h. Remove the plate and firmly tap the plate on KimWipes until no wet spots are observed.
7. Return the filter plate to the manifold and turn on the vacuum for an additional 2 minutes ONLY. Do not exceed 2 minutes. Less than 2 minutes is okay if no excess ethanol is present in the wells or on the underside of the filter plate.



NOTE: The purpose of this step is to remove excess EtOH so that it is not carried over into the eluate. Two minutes is sufficient for this purpose. Leaving the vacuum on for more than 2 minutes may over-dry the beads, which may inhibit elution of the purified DNA.

8. Turn off the vacuum and blot the bottom of the plate on KimWipes to remove any remaining EtOH.

8. Elute the purified reactions

To elute the purified reactions:

1. Attach the elution catch plate to the bottom of the filter plate using two strips of lab tape. The filter and elution plate assembly is now referred to as the *plate stack*.



CAUTION: Do not completely seal with tape. Product will not elute if sealed.

1. Pour or pipette 3 mL of Buffer EB to a solution basin. Buffer EB should be used at 4°C (less than room temperature)

2. Using a 12-channel P200 pipette, add 60 μ L of Buffer EB to each well.
 - a. For accurate pipetting, pre-wet pipette tips with EB before dispensing. Dispense as close to the beads as possible without touching them. Buffer EB should be applied directly on top of the beads.
3. Tap the plate stack to move all Buffer EB onto the filter at the bottom of the wells.
4. Loosely cover the plate (do not seal) with a clean lid from a pipette tip box or a plate lid.
5. Place the covered plate stack in a 50°C hybridization oven on the right front side (away from the air vent) for 30 minutes.
6. Remove the plate stack from the oven and replace the plate cover with an adhesive film. Seal tightly and place the sealed plate stack on a Jitterbug for **30 minutes at setting 7**.
7. Inspect each well to verify that the beads are thoroughly re-suspended.

The beads must be thoroughly re-suspended in Buffer EB so that the DNA can come off the beads.

8. Remove the plate stack from the Jitterbug and remove the adhesive seal.

Continue elution on the vacuum manifold as follows:

- a. Remove the standard collar from the manifold.
- b. Place the plate stack inside the manifold.
- c. Seal the empty wells with adhesive film.
- d. Place the standard collar around the plate stack.
- e. Turn the vacuum on to 20 to 24 in Hg and ensure that a seal has been formed between the collar and the base of the manifold.
- f. Ensure that the unused wells are completely sealed.
- g. Cover the plate stack to protect it from environmental contaminants.
- h. Run the vacuum until all of the liquid has been pulled through the filter (approximately 5 to 15 minutes).
- i. Turn off the vacuum.
- j. Examine each well.



NOTE: There should be no standing liquid in any well and the wells should appear matte. Wet wells will look shiny. If any of the wells are still wet, continue filtering for up to 15 additional minutes.

- k. Seal the plate stack with an adhesive film and spin it down at room temperature for 5 minutes at 1,400 relative centrifugal force (RCF).

Formula and example

Use the following formula to convert RCF to RPM: $\text{RPM} = 1,000 \times \text{square root}(\text{RCF}/1.12r)$

The radius, r , is equal to the distance in millimeters between the axis of rotation of the centrifuge and the bottom of the plate bucket.

Example: on the Eppendorf 5804 R, spinning at 3,100 RPM = RCF of 1,400 (assuming $r = 133$ mm).

9. Remove the elution catch plate from the filter plate.

10. Seal the catch plate and place the plate on a Jitterbug for 5 minutes at **setting 7** (note: the higher shaking speed is important for effective mixing), then quickly spin the plate down.
11. Using a 12-channel P200 pipette, transfer 47 μ L of eluate to a new PCR plate for fragmentation (the new PCR plate is now referred to as the fragmentation plate)
12. Using a 12-channel P20 pipette, transfer 2 μ L of eluate from the fragmentation plate for OD measurement.

This stage is now complete. You may seal the fragmentation plate containing the eluate and store it overnight at -20°C or proceed directly to fragmentation.

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