Ion AmpliSeq Library Preparation on the Janus NGS Express

Application Guide

Compatible with:

Ion AmpliSeq Library Kit 2.0

Ion AmpliSeq Panels and Primer Pools

Ion Express™ Barcode Adapters 1-96 Kits

WinPrep 4.9.0.199 or greater

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Introduction

The Janus NGS Express Ion AmpliSeq application is designed for automated preparation of amplicon libraries using Ion AmpliSeq™ Panels and Custom Primer Pools and then sequencing on the Ion Personal Genome Machine® (PGM™) System. Automating the process has the advantage of avoiding sample tracking errors and reducing sample-to-sample variability while increasing throughput. The Janus-based Ion AmpliSeq™ Application from PerkinElmer provides a pre-programmed solution for creating Amplicon Libraries on the Janus NGS Express.

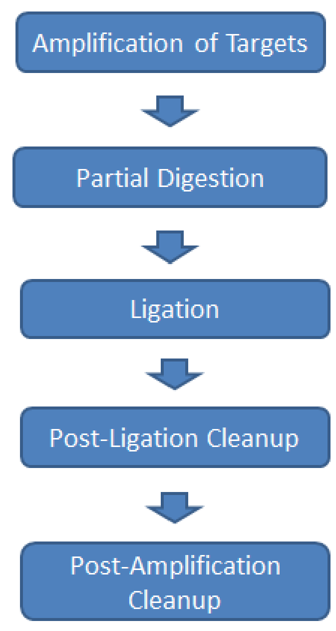


Figure 1: Overview of the steps for preparing amplicon libraries on the Janus NGS Express

Janus NGS Express Ion AmpliSeq Library Preparation DNA Workflow Overview

The NGS Express Application for creating Ion AmpliSeq Amplicon Libraries is a verified process for amplicon library preparation from genomic DNA or DNA from FFPE tissues. Samples are processed in 96-well PCR plates, and the number of samples to process is selected at the start of each run. Pre-set tip-tracking utilities written into the application guide the instrument to pick up appropriate numbers of tips and request replacement tip boxes as needed. An Inheco temperature block installed on the Janus deck allows for heated incubation for removing Ethanol in the SPRI ethanol washes. The Eppendorf IsoRack provides on-deck cooled storage of reagents. Barcoded Adapter indexing patterns may be executed during the run by arraying up to 24 different barcodes to appropriate well locations as specified by the user in an excel workbook. Easy-to-follow user interfaces guide the reagent and deck setup process and prompt the user for the off deck thermocycler steps.

Because there are several points at which the samples can be stored in the process, the overall Library Preparation was broken down into multiple smaller steps. Five independent Janus protocols are used in the Ion AmpliSeq™ Library Preparation workflow. In between each protocol an off deck step such as thermocycling is required.

	NGS Express Ion AmpliSeq Library Preparation Application	**Set-up Time**	**Run Time for 8 Samples (Does not include thermocycler time)**
1	Amplification of Targets	10 min	5 min
2	Partial Digestion	5 min	3 min
3	Ligate Adapters	10 min	9 min
4	Post-Ligation Cleanup	5 min	60 min
5	Post-Amplification Cleanup	5 min	70 min

Notes on the Automated Method

The reagent additions are very small volumes, with minimal extra volumes provided in the reagent kit. To resolve this issue, the NGS Express Ion AmpliSeq Option includes the same reagent tubes used in the Ion AmpliSeq kit. This allows users the flexibility to use the tubes directly from the kit, or to aliquot reagent into clean tubes depending on the number of samples to be run.

One challenge in automating this kit was the number of different tubes of amplicon pools provided in the different kits. Kits range from 1-4 tubes of primers, also necessitating the number of replicates of individual sample pipetted into the starting plate. To resolve this issue, the application asks the user to select the number of primer pools, and Janus will then distribute the initial sample accordingly. For example, with a primer pool of 2 tubes, 2 aliquots of each initial DNA sample will be pipetted from the DNA source into the starting sample locations for a total of 4 sample wells.

In the ligation step, the protocol allows for the use of barcoded adapters, or a standard adapter. If the user selects the barcoded adapters, they are next prompted to browse for the comma delimited sheet detailing the worklist for the barcode addition. The modifications have resulted in the development of a robust automated library preparation procedure for up to 24 samples.

In the Post Ligation Cleanup and optional Post Amplification Cleanup, adjustments may need to be made to the Ethanol wash dry time on the Inheco heater. If there is excess Ethanol present, the will caused reduced recovery. In addition, over drying in the Ethanol wash step will make it difficult to resuspend the pellet after drying, and will also result in lower recoveries. If your recoveries are low- check the Ethanol drying step carefully and adjust as necessary.

Required Materials and Reagents

Reagents

Reagent	Vendor and Part Number
Ion AmpliSeq Library Kit 2.0 (8 reactions) or equivalent	Ion Torrent 4475345
Ion AmpliSeq Cancer Panel Primer Pool (10 reactions) or equivalent	Ion Torrent 4471262 (various numbers)
Ion Xpress Barcode Adapters (optional) 1-16 Kit or equivalent*	Ion Torrent 4471250 (various numbers)
Nuclease-Free Water	Various
AMPureXP Beads	Beckman Coulter-- A63881 (60 mL)
100% EtOH	Sigma E7023 or equivalent

*up to 24 barcodes are supported

General Laboratory Equipment and Supplies

Equipment	Supplier
Microfuge	Various
Vortexer	Various
20 µL, 200 µL, and 1000 µL pipettors and appropriate barrier tips	Various
Microplate centrifuge	Various
Thermocycler**	BioRad (MJ Research) DNA Engine PTC-200, or equivalent
Plate Seals (Foil)	Various- compatible with Thermocycler and freezer storage
LabChip GX with DNA HiSens chip and reagents, or equivalent	Various
qPCR Instrument or equivalent	Various

***The standard sample plates used are fully skirted BioRad Hardshell 96-PCR plates. Please check if your thermocycler is compatible with this plate type. If it is not, please contact your PerkinElmer Field Application Scientist to discuss modifications to the application to support semi-skirted PCR plates.*

Materials List

Included in the Option Package:

- Software CD- Ion AmpliSeq Option for NGS Express
- Disposable Tip- 25 µL, Clear, Sterile Filter Tips
- Disposable Tip- 175 µL, Clear, Sterile Filter Tips
- Microtubes, screw cap, 0.5 mL (Sarstedt)
- Microtubes, screw cap, 2.0 mL (Sarstedt)
- Hard-Shell PCR plates, 96-well (Bio-Rad)
- PerkinElmer StorPlate 96-V, 450 µL capacity/well
- Eppendorf IsoRack (Additional to what is supplied with the NGS Express)

NGS Express Accessories & Custom Labware

Assembling the Versa Mover

The Versa Mover system can move a plate among the three front positions of the NGS Express deck. This provides automated plate movement among the magnet tile, heating/cooling tile, as well as a standard tile. The following chart provides information on the components of the Versa Mover system so you can identify the items and understand how they work. Use the Place page within the NGS Express software for step-by-step instructions on placement of each piece on the deck of the instrument.

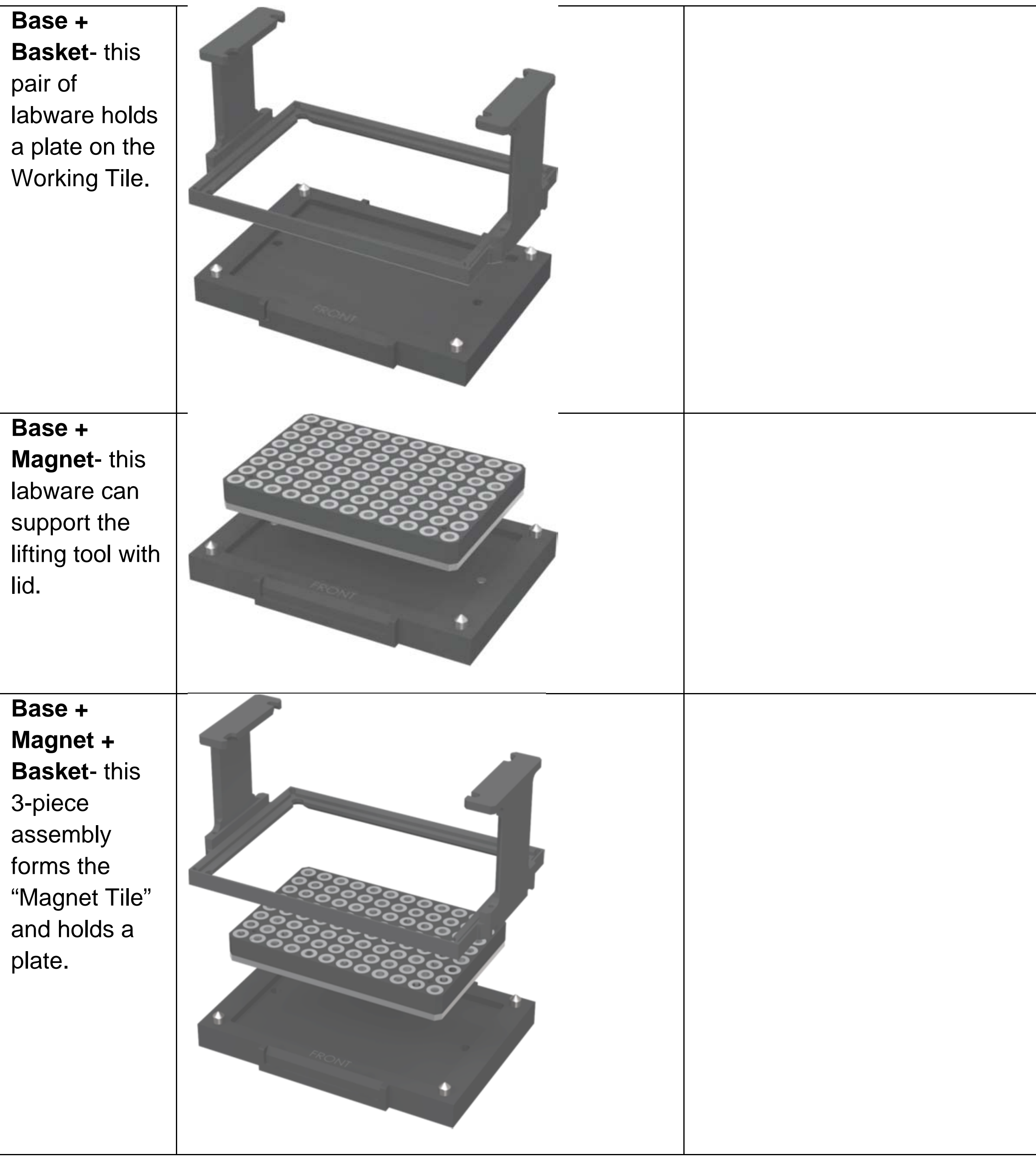
The VersaLift tool has two functions: it moves the plate to the appropriate deck location, and it can also function to lid and de-lid a plate when required.

For error-free operation, please:

- Place a universal (un-notched) disposable lid in the lifting tool. Place a clean lid in the tool at the beginning of the AmpliSeq protocol.
- Make sure the plate type you are using matches the Inheco adapter plate type. For the AmpliSeq protocol, only the PCR adapter will be used.
- Verify that the position of each part matches that shown in deck setup drawing in the software before starting each run.

Chart: Images and Descriptions of VersaMover Hardware

Labware Description	Component Image(s)	Additional Info
Base- this is a custom support tile that is placed on the deck to hold a Lifting Tool w/Lid and/or a plate in the carrier Basket	FRONT	Requires the Basket in order to hold a plate. Note the 'FRONT' engraved in the base and make sure it faces the user.

Base + Basket- this pair of labware holds a plate on the Working Tile.		
Base + Magnet- this labware can support the lifting tool with lid.		
Base + Magnet + Basket- this 3-piece assembly forms the "Magnet Tile" and holds a plate.		

VersaLift- this labware is the 'handle' for the basket and also holds the disposable lid.		A lid MUST be present for the VersaMover to work correctly. The spring clip on the front of the lifting tool allows you to replace the lid as desired. The plastic pieces may be putty colored on your hardware.
Inheco + 96-PCR Adapter Tile- special geometry to match the BioRad PCR Hard Shell plate. Base color may vary.	Image of Inheco + inserted 96-BioRad PCR adapter on the deck. Base color may be black.	Image of 96-BioRad PCR adapter only

NGS Reagent Rack

Some reagent tubes will be placed in the NGS Reagent Rack which is mounted on the left side of the deck.

- AMPureXP reagent (SPRI beads) in 2.0 mL tubes
- Low TE for resuspension Buffer in 2.0 mL tube

The scalloped depressions at the outer edges hold the reagent tube caps when the tube is on the deck. Tubes are not included in the JAA image (Figure 2), so consult the Reagent Rack Maps for locations of tubes at each step in the method.

Make 80% ethanol fresh using molecular biology-grade reagents and place it in the trough. Keep the cap on the trough until you are ready to start the run.

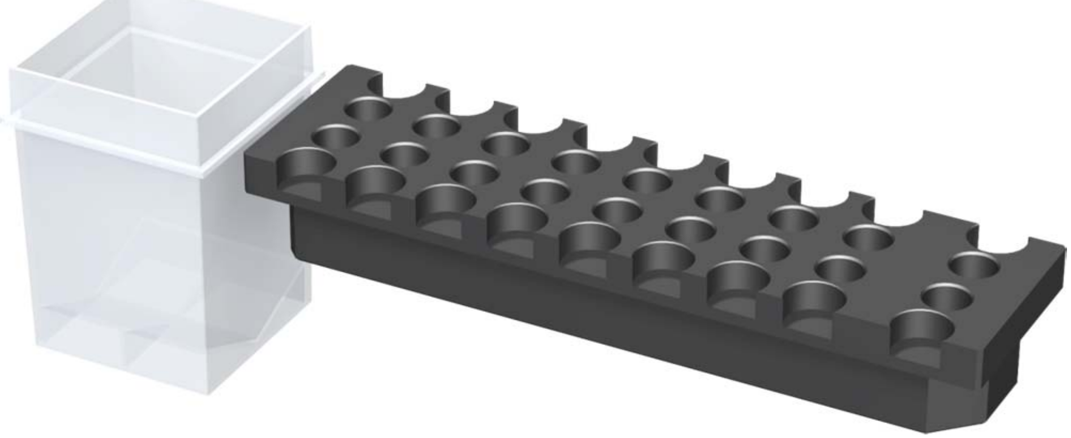


Figure 2. Left-mounted labware: reagent trough and microtube rack.

IsoRack 24-Well Cooling Block

Other tubes will be placed in the IsoRack cooling block (shown below), which is then placed on a support tile on the deck. For correct tube placement always use the cooling insert. When properly assembled, the chiller insert will be sitting about 1cm above the deck. Keep the block insert in the freezer upside down when not in use.

Some reagent tubes will be placed in the IsoRack cooling block, including:

- AmpliSeq reagents in 0.5 mL tubes
- Barcoded adapters in 0.5 mL tubes

Tubes are not shown in the JAA image, so consult the Reagent Rack Maps for locations of tubes at each step in the method.

Figure 3. Rendering of the assembled IsoRack + IsoRack cooling block.

Consumables Ordering Information

Consumable	Description	PerkinElmer Part Number	Vendor and Part Number	Number used per run of 8 samples
PCR Plates	96 Well PCR Plate, Bio-Rad Hardshell, Full Skirt	CLS 127737	BioRad HSP-9631	2
StorPlate-96V	96-well V-bottom plate, polypropylene, 450 µL capacity, 50/box	6008290		1
Boxed Tips	RoboRack 25µL Non-Conductive Filter Tips, Pre-Sterilized, 960/case	6000689		1
Boxed Tips	RoboRack 175µL Non-Conductive Filter Tips, Pre-Sterilized, 960/case	6000685		2
Lids	Lid-Universal, Robotic Friendly, Polystyrene	CLS 112785	Seahorse Bioscience 200856-100	1
Micro Tubes	0.5mL with Skirted Base		Sarstedt 72.730.711	varies
Micro Tubes	2.0mL with Skirted Base		Sarstedt 72.730.711	varies
Screw Caps	Red		Sarstedt 65.716.721	for storage only
Screw Caps	Blue		Sarstedt 65.716.723	for storage only
Trough	Perkin Elmer,4 tip, 150mL Quantity 25	6000583		1, reusable

Running the Ion AmpliSeq Library Preparation Application

Please read and familiarize yourself with all steps described in this section prior to beginning the run. For best results, the entire process should be completed in one day. If you need to stop in the process make sure to stop at a point noted in the Ion AmpliSeq™ Library Kit 2.0 Users Guide (MAN0006735) as an appropriate stopping point, and follow the directions for proper storage of the samples. Because of the break point in the method, it is common to do the application over multiple days to complete all steps including setup, sample distribution, amplification of targets, partially digesting primer sequences, ligating adapters and final clean-up and quantitation of samples.

Sample & Reagent Volume Calculator

Consult the Sample & Reagent Volume Calculator worksheet **"Reagent Volume Calculator-Ion AmpliSeq Sample Prep.xlsx"** for required sample and reagent volumes. The workbook is located in the file path: C:\Packard\Janus\Bin.

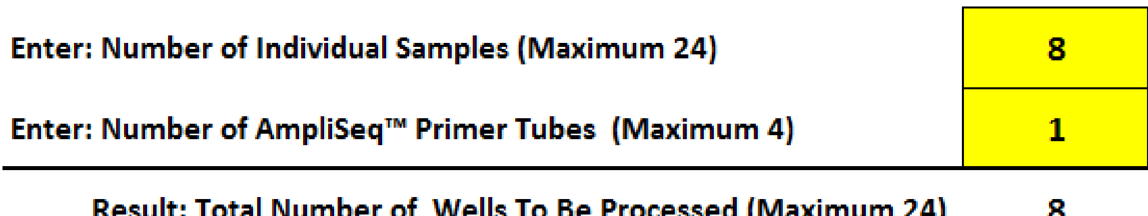


Figure 4 Input box in the Reagent Volume Calculator

To use the calculator, simple enter the number of Samples you wish to run in the input box, then enter the number of tubes of primer that will be used per sample. The worksheet will then calculate the sample and reagent volumes needed for the run.

Ion AmpliSeq Reagent Calculator for Janus NGS Express

Enter: Number of Individual Samples (Maximum 24)	8
Enter: Number of AmpliSeq™ Primer Tubes (Maximum 4)	1
Result: Total Number of Wells To Be Processed (Maximum 24)	8

	Reagent	Vol/Rxn (µl)	Tube Size	Dead Vol (µl)	No. Tubes	Total Vol/Tube*	
Step 1- Amplification of Targets	HiFi Master Mix	4	0.5 mL	3	1	35	
	Primer Pool	10	0.5 mL	3	1-4	83	
Step 2- Partially Digest Primer Sequences	FuPa Reagent	2	0.5 mL	3	1	19	
Step 3- Ligate Adapters	Switch Solution	4	0.5 mL	3	1	35	
	DNA Ligase	2	0.5 mL	3	1	19	
	Ion AmpliSeq Adapters*	2	0.5 mL	3	1	19	
	Diluted Barcodes*	2	0.5 mL	3	1-24	8	
	* Use either AmpliSeq Adapters or Diluted Barcodes						
Step 4- Purify	AMPure XP Beads	45	2.0 mL	10	4	100	
	80% Ethanol	340	Trough	3000	1	6	mL
Step 4a- Elute for Quantitation by qPCR	Low TE	50	2.0 mL	10	1	410	
Step 4b- Elute & prepare for Amplification	Pre-mix Platinum PCR MMix + Library Amplification Primer	52	2.0 mL	3	1	419	
Step 5- Post-Amplification Clean Up	AMPure XP Beads	85	2.0 mL	10	4	180	
	80% Ethanol	340	Trough	3000	1	6	mL
	Low TE	50	2.0 mL	10	1	410	

* µL unless specified

Figure 5 The Reagent Volume Calculator-Ion AmpliSeq Library Prep

Save the modified spreadsheet with its original name in its original file path. If desired, you can print the "Sample & Reagent Volume Calculator-Ion AmpliSeq Library Prep" worksheet to use as a guide while setting up reagents.

Sample Preparation

Samples should be loaded into consecutive wells starting in column 10 in a BioRad Hardshell 96-well PCR plate. The initial volume of sample is determined by the number of primer pool tubes. Refer to the Reagent Volume Calculator-Ion AmpliSeq Library Prep worksheet. Samples should be pipetted into a BioRad Hard Shell plates starting in Column 10 as indicated below.

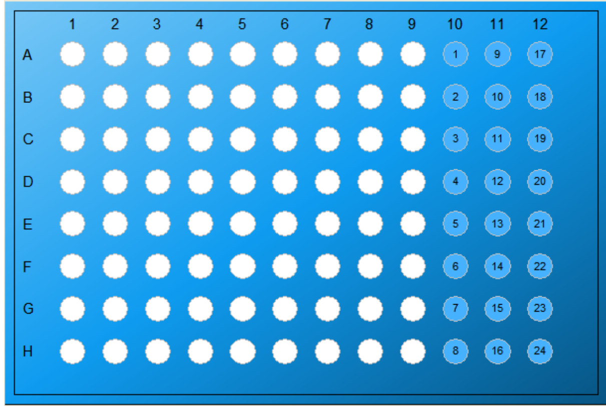


Figure 6: Location of Samples in the BioRad Hard Shell PCR plate

Before Running the Janus NGS Express

The day before your run, the following should be performed:

- Place the inserts for the Eppendorf chiller blocks into a -20C freezer. It is recommended to place the block upside down to keep the bottom foil surface as flat as possible. The inserts can be stored in the -20C freezer when not in use.
- Put a clean lid into the VersaLift to assure there is no cross-contamination between samples.
- Make sure the system liquid carboy is filled with de-ionized water (house-distilled or de-ionized, Milli-Q is also suitable). This will allow the system liquid to de-gas overnight, which will reduce the potential for air bubbles in the lies during the run.
- Check the waste container. To avoid overflow, make sure the waste container is empty or contains a volume of less than 25%.

The day of the run, if necessary, boot up the NGS Express system by first starting the Janus and the Inheco units, then starting the PC controller.

Prime the System

All air bubbles must be removed from the fluid path for optimal pipetting performance. To flush the system liquid path,

1) Open the NGS Express software by clicking on the icon.

2) Go to the **Maintain** tab and find the “Prime the Varispan Fluid Path” activity. Click the **Start Maintenance** button to initiate the system priming

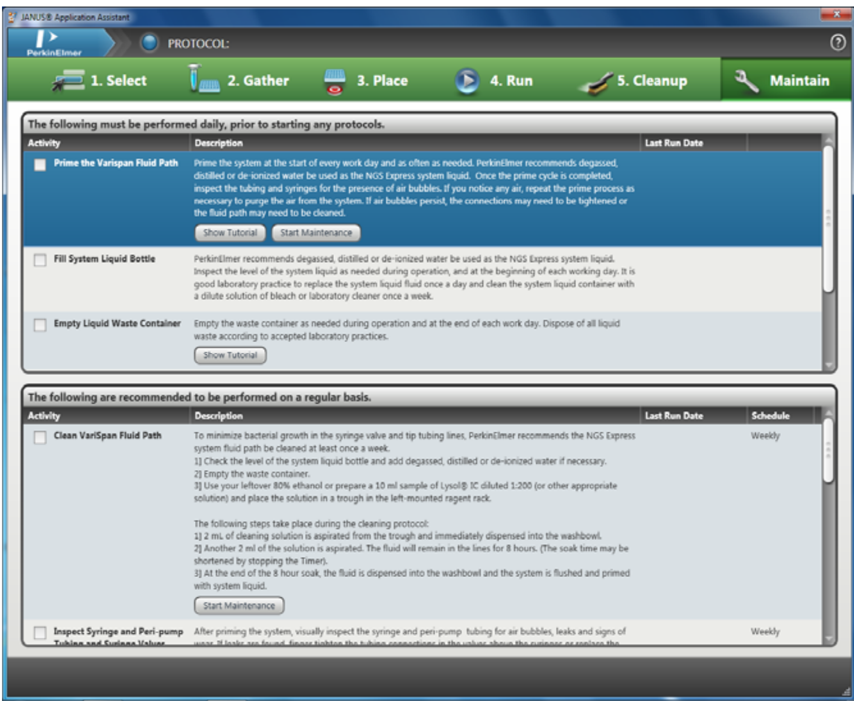


Figure 7. Maintain tab shown with Prime Varispan path highlighted.

Amplification of Targets

This protocol performs the following steps:

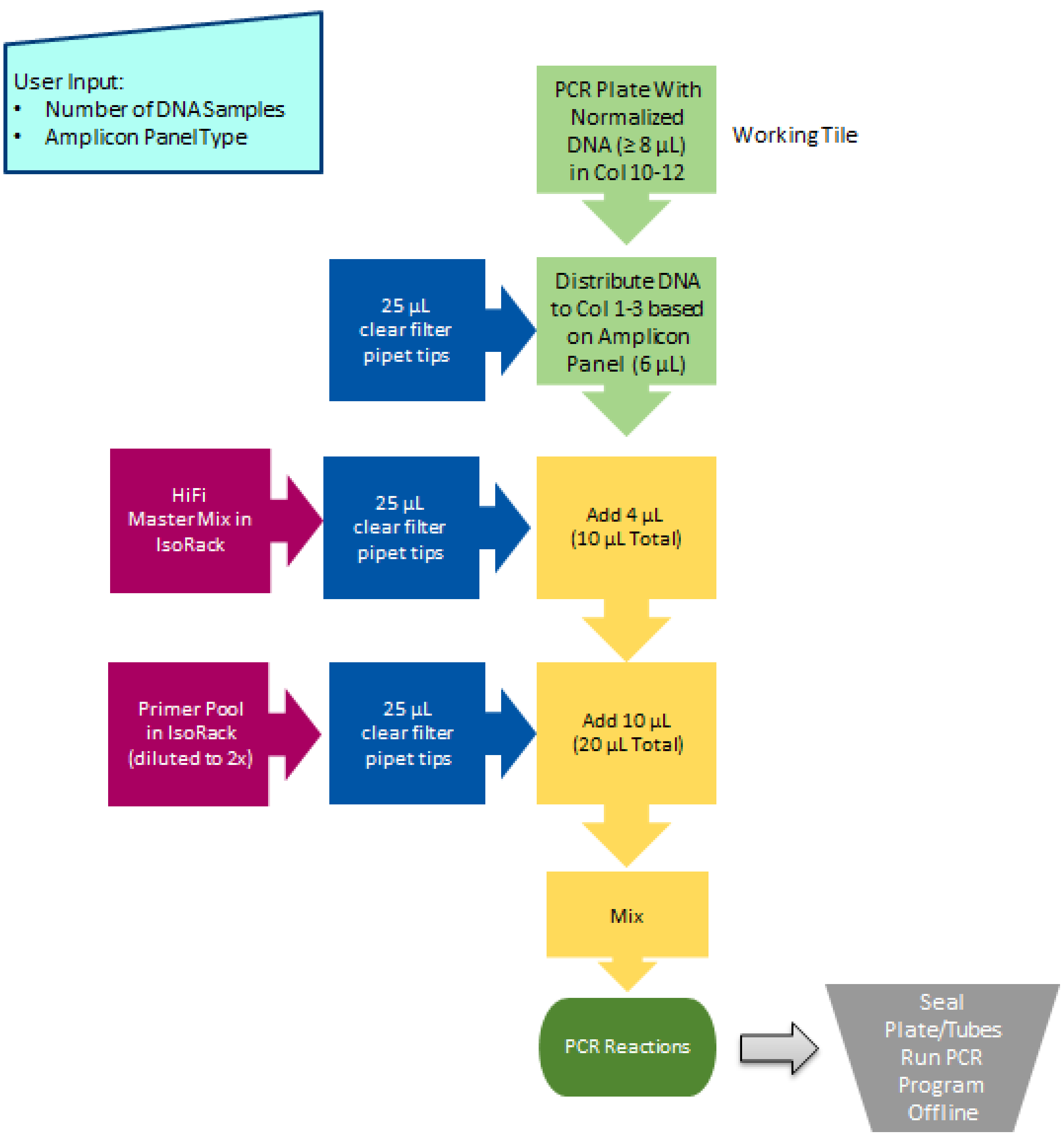


Figure 8: Flow Chart for Amplification of Targets in the Ion AmpliSeq Protocol on the NGS Express

The initial step of Amplification of Targets involves taking the normalized sample DNA from Columns 10-12 and distributing it to columns 1-3 at a volume of 6µL.The sample will be distributed based on the number of primer pools to be used. The users can select 1-4 primer pools. Figure 9 shows the plate map for how the samples will be distributed in the plate based on the number of primer pools selected.

One Replicate:

1	9	17
2	10	18
3	11	19
4	12	20
5	13	21
6	14	22
7	15	23
8	16	24

Three Replicates:

1	3	6
1	4	6
1	4	7
2	4	7
2	5	7
2	5	8
3	5	8
3	6	8

Two Replicates:

1	5	9
1	5	9
2	6	10
2	6	10
3	7	11
3	7	11
4	8	12
4	8	12

Four Replicates:

1	3	5
1	3	5
1	3	5
1	3	5
2	4	6
2	4	6
2	4	6
2	4	6

Figure 9: Sample Map for Ion AmpliSeq Library prep based on the number of Primer Pools

Setting up the reagents - refer to the Sample & Reagent Volume Calculator worksheet to determine the volume of reagents needed for the number of samples to be run. The reagents should be placed in the Eppendorf IsoRack with the chiller block as shown below:

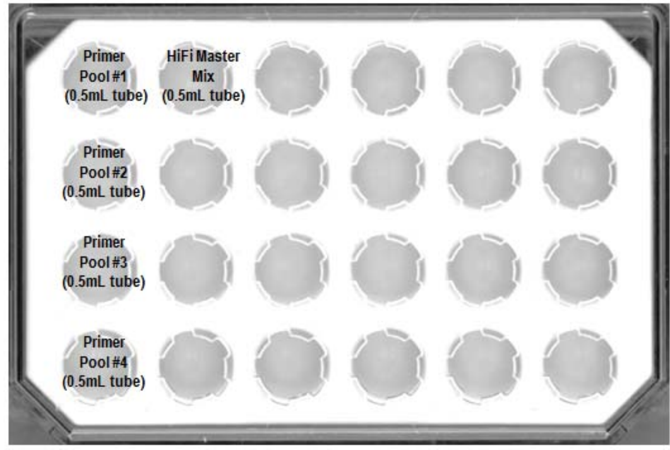


Figure 10: Well Map of the Eppendorf IsoRack with Primer Pools and HiFi Master Mix for the Amplification of Targets step.

Protocol 1- Amplify Targets

This protocol will start by distributing 6µL of the DNA sample to columns 1-3 based on the number of primer pool tubes. For each primer tube used, 1 aliquot of each DNA sample is transferred. For example, with 1 primer pool tube one DNA sample is transferred. If there are 4

primer pool tubes, the same DNA has 6µL of DNA transferred into 4 wells, one for each primer pool tube. Next 2µL of the HiFi Master mix is added to each well of DNA. Finally, 10µL of primer pool is added to each sample, and the sample is mixed.

To Start the Protocol,

1. Open the NGS Express software by clicking on the Janus Application Assistant (JAA) icon.
2. The list of available protocols will appear.
3. Select the protocol “Ion AmpliSeq 1_Amplify Targets” by clicking on it once.
4. Questions related to this protocol will appear in the lower panel.
    a. Enter the number of unique DNA samples you want to process.
    b. Enter the number of primer pool tubes to be used (1-4).
    c. If you wish to generate a Run Report, check the Run Report box. If checked, a report will be generated including the following:
        i. Name or User ID- this optional file allows you to record which operator is running the instrument.
        ii. Kit Barcode- use the handheld barcode scanner or type in the kit barcode. This information will be saved in the Run Report.

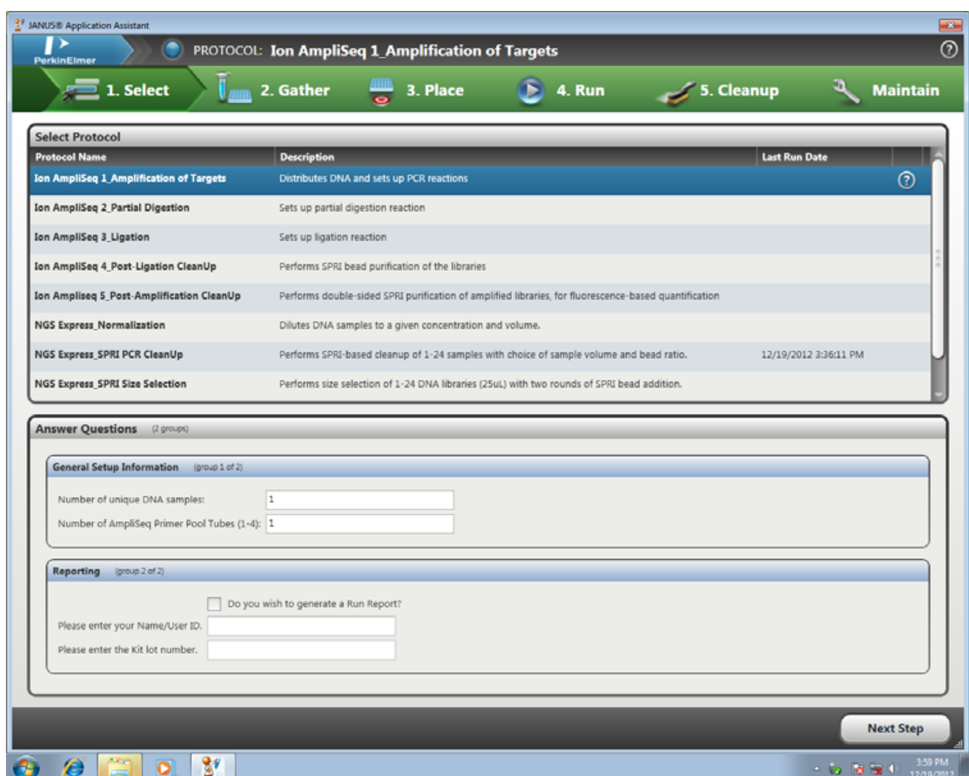


Figure 11. Select Protocol Screen with AmpliSeq 1 Amplification of Targets selected and related questions shown.

5. Place the labware and reagents on the deck.

a. Click on the Place icon in the top green bar.
b. Follow the instructions in the Place tab of the software for placing support tiles, tips, and plates on the deck. Note that your sample plate must be in the basket in the front left position on the deck, as shown in Figure 12.

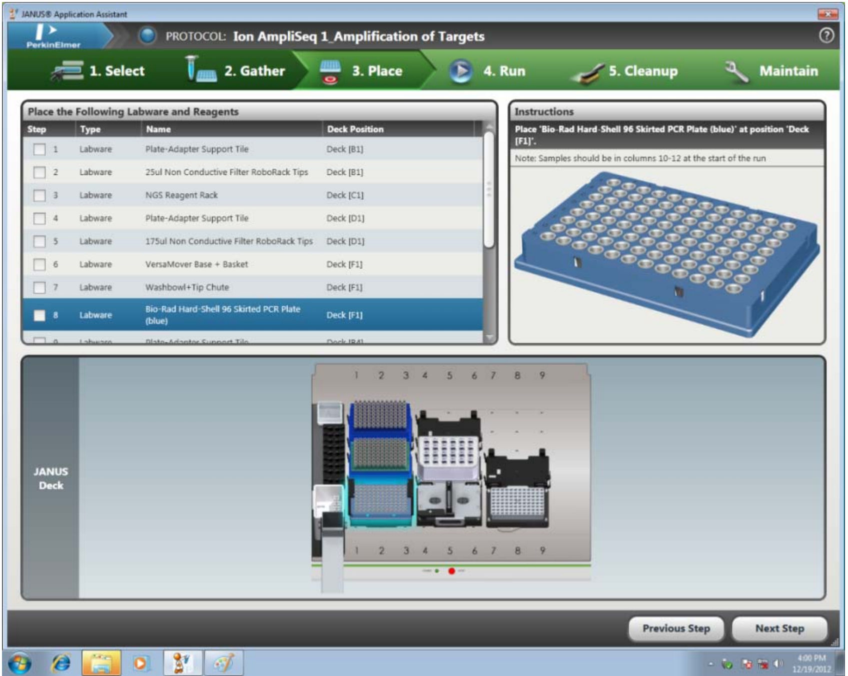


Figure 12. Place page of NGS Express software showing the location of the sample plate on the deck for Ion AmpliSeq Amplification of Targets protocol.

c. Make sure the HiFi Master Mix and primer pool(s) are in the IsoRack chilling block according to the diagram in Figure 10.

Run the Protocol

d. Click on the Run icon in the top green bar.
e. Click the Start button.
f. You will be asked to verify/change tip counts. If you are placing full tip boxes on the deck, make sure that each box shows 96/0 tips in the software. If you have full tip boxes on the deck but not in the software, click the Fill button for each tip box that is full on the deck.

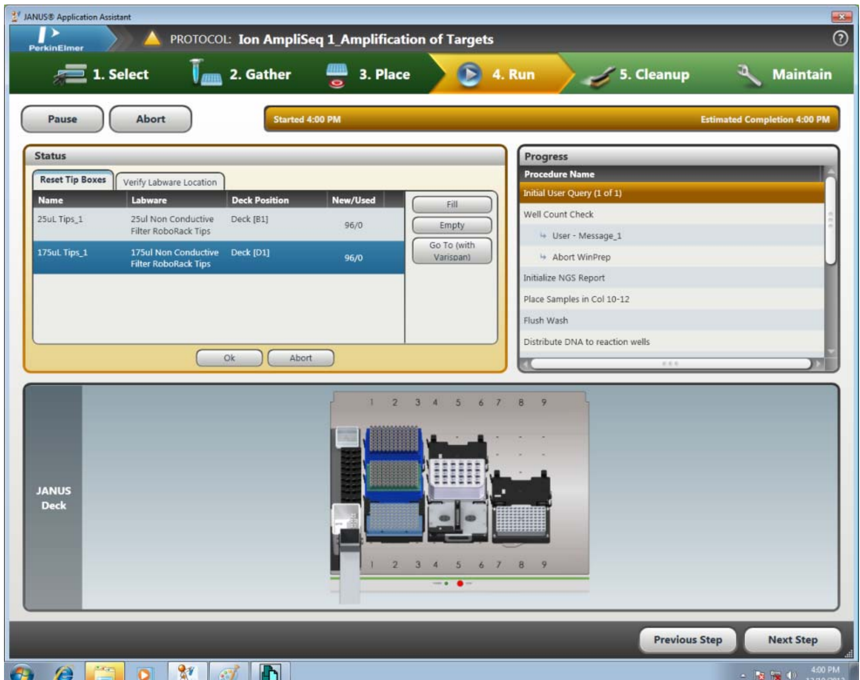


Figure 13. Interface for refilling tip boxes in JAA.

g. Click OK to commence the run.

Consult the Workflow Chart for details on the steps being performed by the NGS Express.

Your samples will be in the plate on the magnet tile, in the wells shown below:

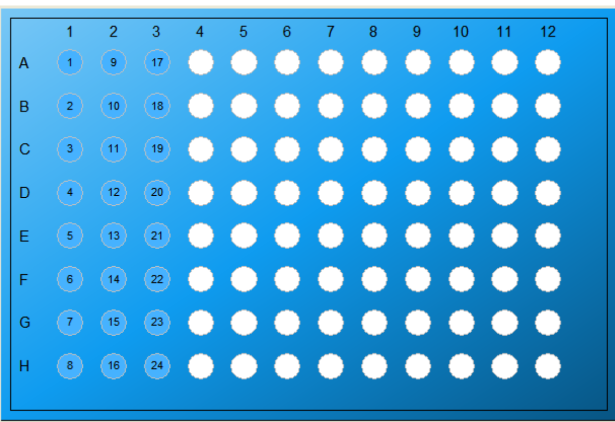


Figure 14. Sample locations at the completion of protocol 1.

The plate should be sealed and moved to the thermal cycler to run the temperature protocol as indicated in the in the Ion AmpliSeq™ Library Kit 2.0 Users Guide (MAN0006735).

Partial Digestion

This protocol performs the following steps:

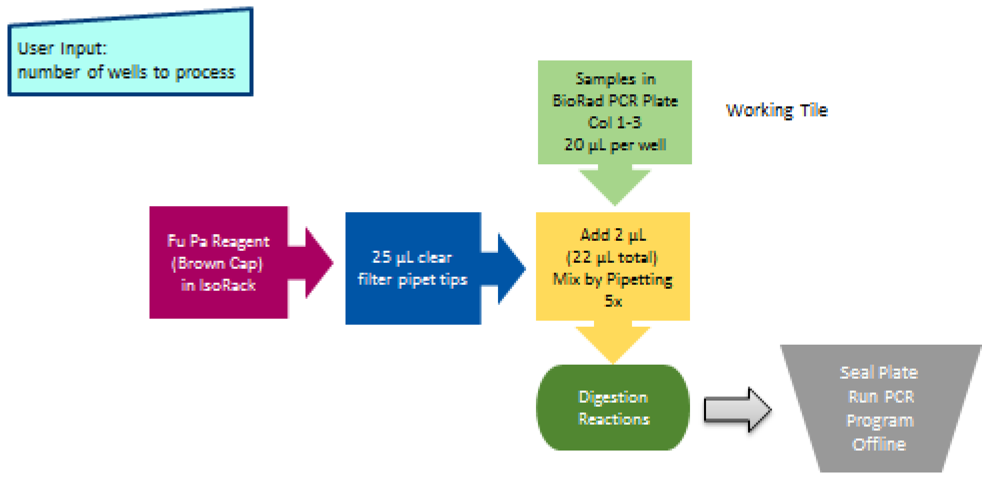


Figure 15: Flow Chart for Partial Digestion in the Ion AmpliSeq protocol on the NGS Express

Setting up the reagents- refer to the Sample & Reagent Volume Calculator worksheet to determine the volume of reagents needed for the number of samples to be run. The reagents should be placed in the Eppendorf IsoRack with the chiller block as shown below:

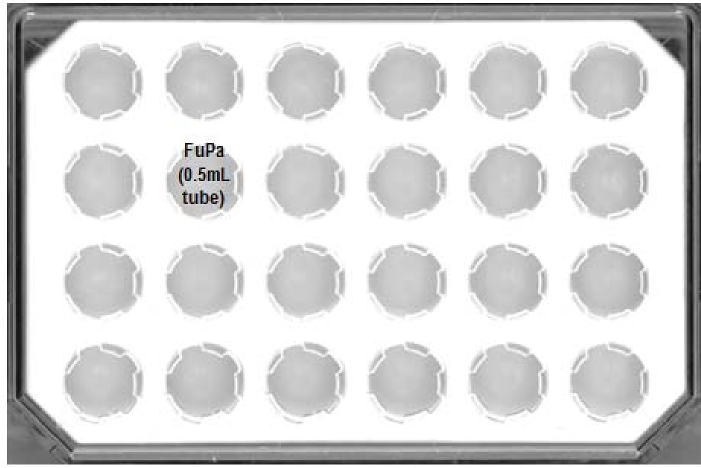


Figure 16: Well Map of the Eppendorf IsoRack, with FuPa reagent for the Partial Digestion step.

Protocol 2- Partial Digestion

This protocol will add 2µL of FuPa reagent to each sample and mix.

To Start the Protocol,

1. Open the NGS Express software by clicking on the Janus Application
2. Assistant (JAA) icon.
3. The list of available protocols will appear.
4. Select the protocol “Ion AmpliSeq 2_Partial Digestion” by clicking on it once.
5. Questions related to this protocol will appear in the lower panel.
    a. Enter the number of samples you want to process.
    b. If you wish to generate a Run Report, check the Run Report box. If checked, a report will be generated including the following:
        i. Name or User ID- this optional file allows you to record which operator is running the instrument.
        ii. Kit Barcode- use the handheld barcode scanner or type in the kit barcode. This information will be saved in the Run Report.

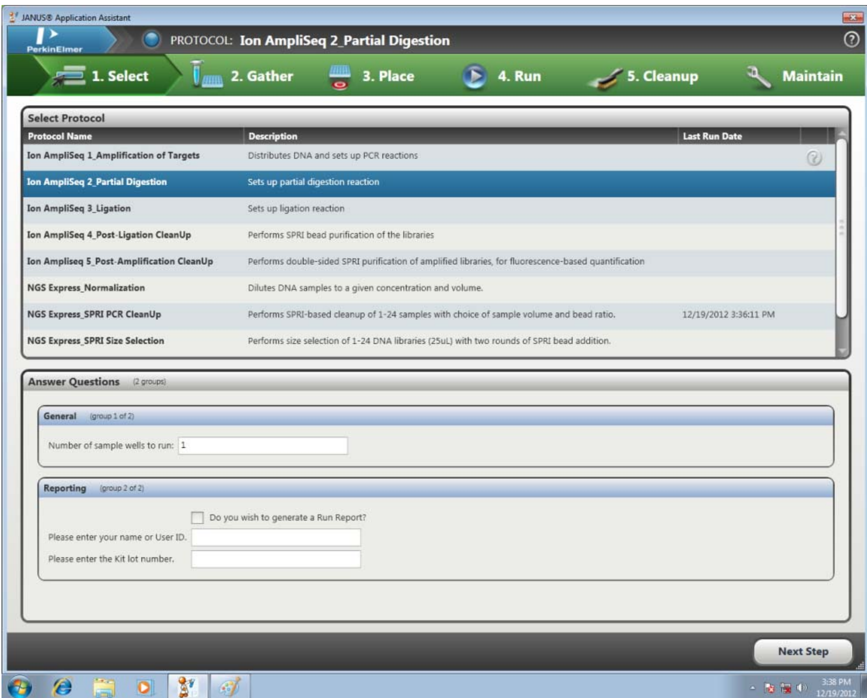


Figure 17. Select Protocol Screen with AmpliSeq 2 Partial Digestion selected and related questions shown.

6. Place the labware and reagents on the deck.
    a. Click on the Place icon in the top green bar.

b. Follow the instructions in the Place tab of the software for placing support tiles, tips, and plates on the deck. Note that your sample plate must be in the basket in the front left position on the deck, as shown in Figure 18.

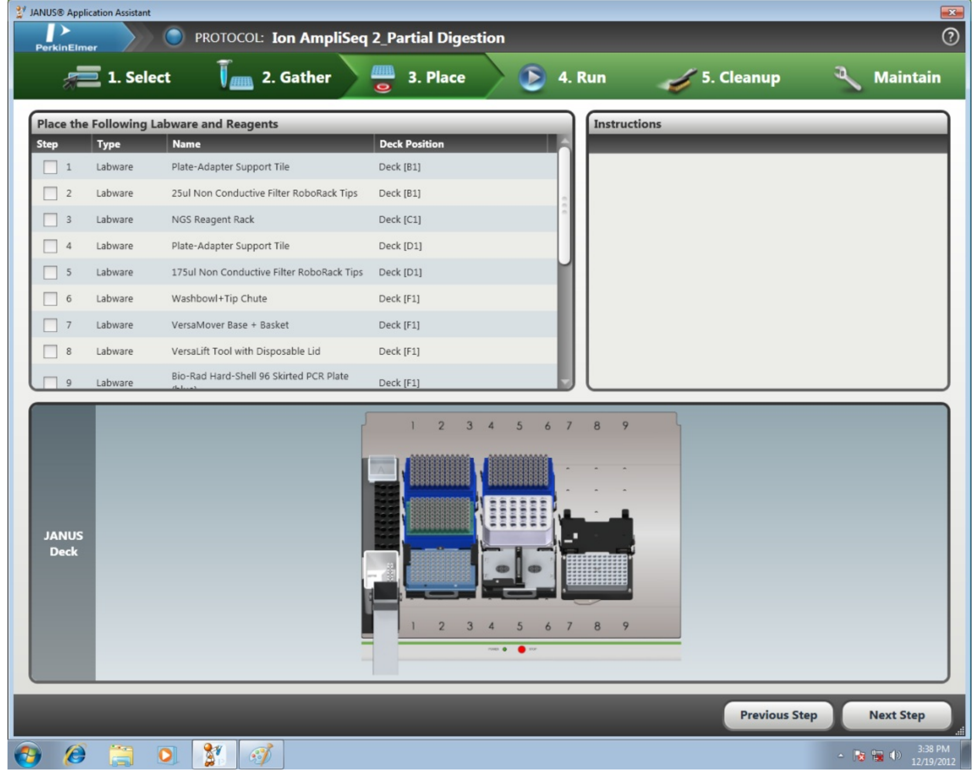


Figure 18. Place page of NGS Express software showing the location of the sample plate on the deck for Ion AmpliSeq Partial Digestion protocol.

c. Make sure the 0.5mL tube of FuPa reagent in the IsoRack chilling block according to the diagram in Figure 16.

Run the Protocol

d. Click on the Run icon in the top green bar.
e. Click the Start button.
f. You will be asked to verify/change tip counts. If you are placing full tip boxes on the deck, make sure that each box shows 96/0 tips in the software. If you have full tip boxes on the deck but not in the software, click the Fill button for each tip box that is full on the deck.
g. Click OK to commence the run.

Consult the Workflow Chart for details on the steps being performed by the NGS Express.

Your samples will be in the plate on the magnet tile, in the wells shown below:

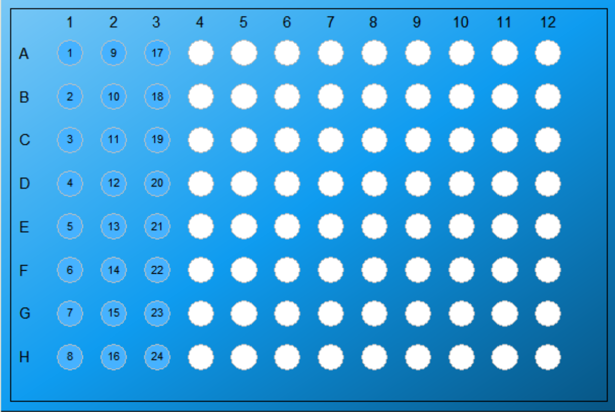


Figure 19. Sample locations at the completion of protocol 2.

The plate should be sealed and moved to the thermal cycler to run the temperature protocol as indicated in the in the Ion AmpliSeq™ Library Kit 2.0 Users Guide (MAN0006735).

Ligation Setup

In the Ligation Setup the initial screen asks if Barcoded Adapters are to be used. This protocol performs the following steps:

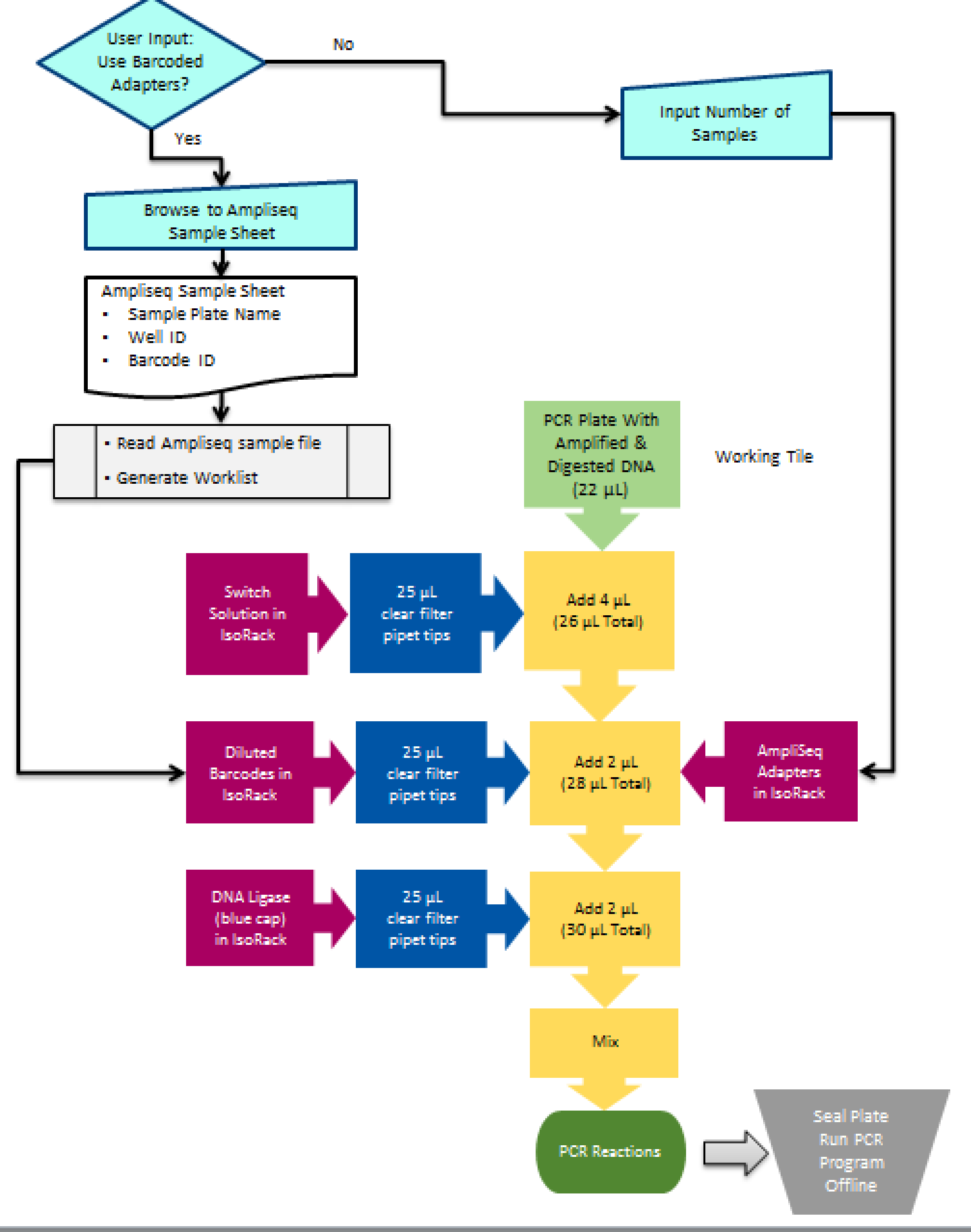
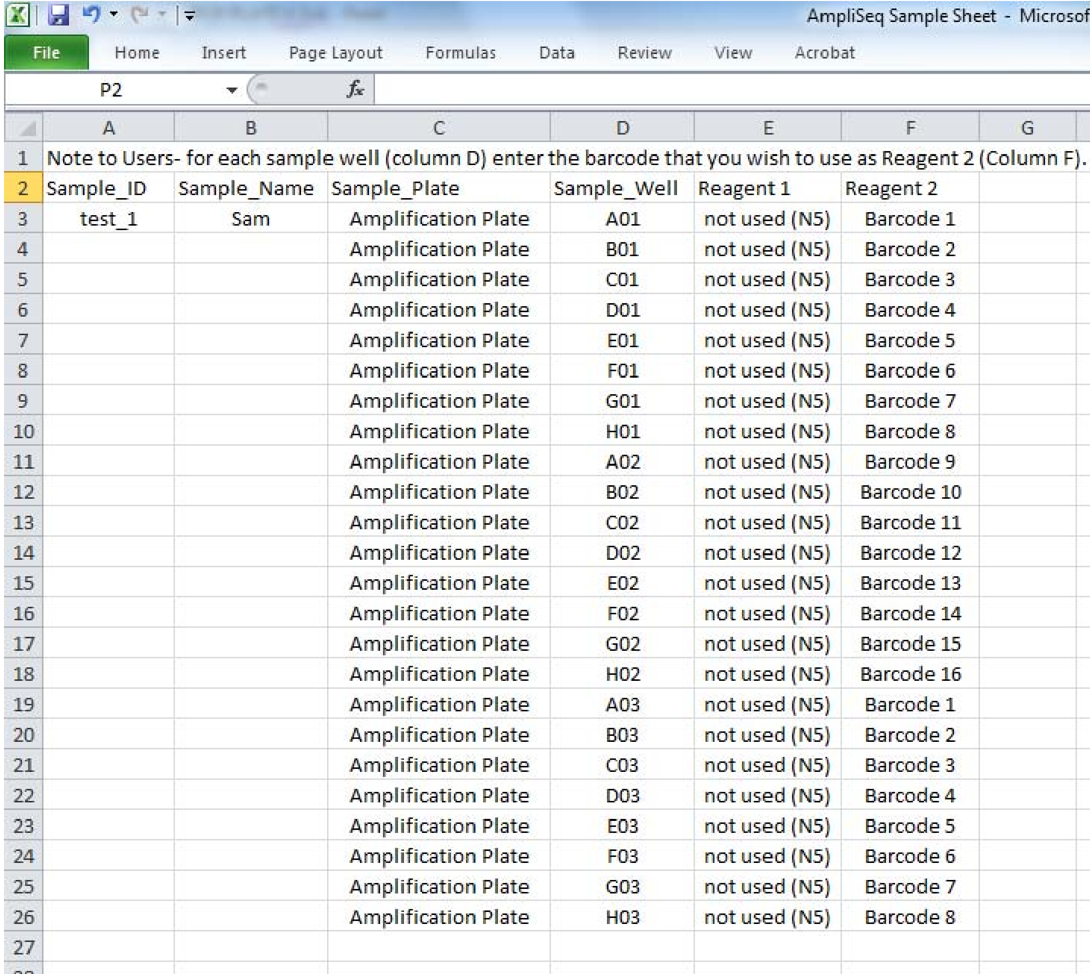


Figure 20: Flow Chart for Ligation Setup in the Ion AmpliSeq protocol on the NGS Express

Barcoded Adapter Worksheet

In order to perform the barcode addition if selected, a .csv file is required to determine which barcodes are dispensed into each sample well. The NGS Express automatically generates a worklist to perform this task from the provided AmpliSeq Sample Sheet file. It is recommended that you edit the provided template AmpliSeq Sample Sheet.csv and change the Barcode used in column F and save the file. The template is located in C:\Packard\Janus\Bin and is shown below:



	A	B	C	D	E	F	G
1	Note to Users- for each sample well (column D) enter the barcode that you wish to use as Reagent 2 (Column F).						
2	Sample_ID	Sample_Name	Sample_Plate	Sample_Well	Reagent 1	Reagent 2	
3	test_1	Sam	Amplification Plate	A01	not used (N5)	Barcode 1	
4			Amplification Plate	B01	not used (N5)	Barcode 2	
5			Amplification Plate	C01	not used (N5)	Barcode 3	
6			Amplification Plate	D01	not used (N5)	Barcode 4	
7			Amplification Plate	E01	not used (N5)	Barcode 5	
8			Amplification Plate	F01	not used (N5)	Barcode 6	
9			Amplification Plate	G01	not used (N5)	Barcode 7	
10			Amplification Plate	H01	not used (N5)	Barcode 8	
11			Amplification Plate	A02	not used (N5)	Barcode 9	
12			Amplification Plate	B02	not used (N5)	Barcode 10	
13			Amplification Plate	C02	not used (N5)	Barcode 11	
14			Amplification Plate	D02	not used (N5)	Barcode 12	
15			Amplification Plate	E02	not used (N5)	Barcode 13	
16			Amplification Plate	F02	not used (N5)	Barcode 14	
17			Amplification Plate	G02	not used (N5)	Barcode 15	
18			Amplification Plate	H02	not used (N5)	Barcode 16	
19			Amplification Plate	A03	not used (N5)	Barcode 1	
20			Amplification Plate	B03	not used (N5)	Barcode 2	
21			Amplification Plate	C03	not used (N5)	Barcode 3	
22			Amplification Plate	D03	not used (N5)	Barcode 4	
23			Amplification Plate	E03	not used (N5)	Barcode 5	
24			Amplification Plate	F03	not used (N5)	Barcode 6	
25			Amplification Plate	G03	not used (N5)	Barcode 7	
26			Amplification Plate	H03	not used (N5)	Barcode 8	
27							

Figure 21: AmpliSeq Sample Sheet.csv for use in Ligation with Barcoded adapters

To edit the template

- Open the file in Microsoft Excel
- Edit the barcode used for each sample in column F. If you are running less than 24 samples, delete the rows for the samples you are not running.

Save the file in a csv (Microsoft Excel Comma Separated Values File) format. It is not required that you keep the same file name, just the format. At the beginning of the run you will browse to select this file when starting the Ligation Run.

Setting up the reagents- refer to the Sample & Reagent Volume Calculator worksheet to determine the volume of reagents needed for the number of samples to be run. The reagents should be placed in the Eppendorf IsoRack with the chiller block as shown below:

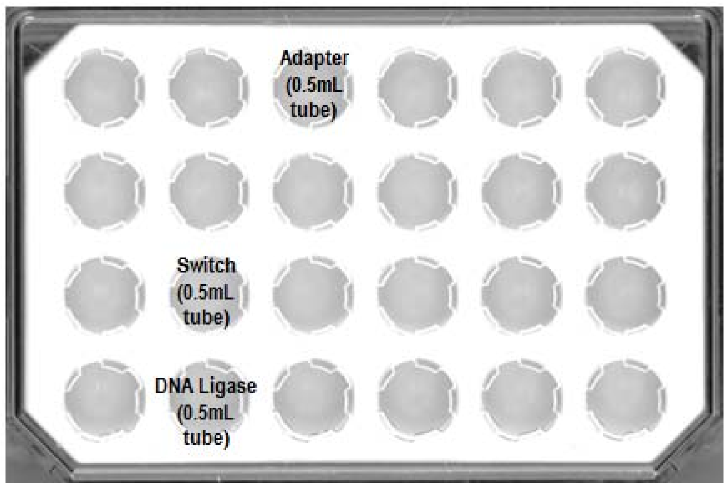


Figure 22: Well Map of the Eppendorf IsoRack-Reagents, with Switch Solution, DNA Ligase and Adapter for the Ligation Setup step.

If Barcoded adapters are used, 2 Eppendorf IsoRacks must be loaded on the deck; the second rack will contain the diluted barcodes. The figure below illustrates the well map using barcodes 1-24.

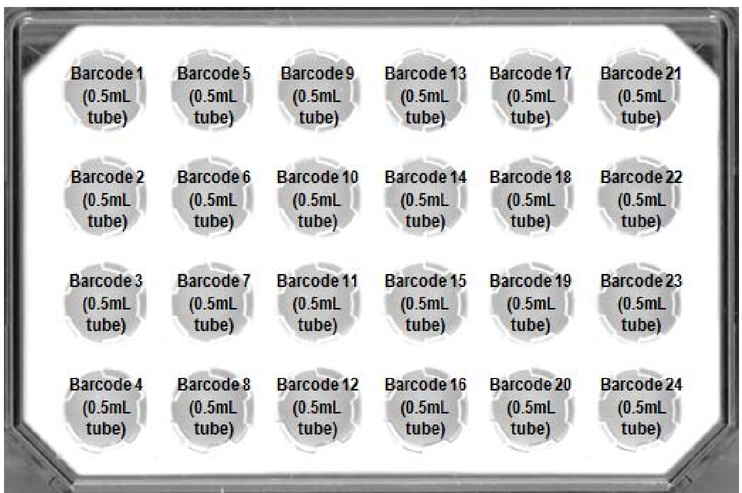


Figure 23: Well Map of the Eppendorf IsoRack-Barcodes, with diluted Barcodes for the optional Barcode Ligation Setup.

Protocol 3- Ligate Adapters

This protocol will add 4µL of Switch Solution to each sample. Next, for non-barcodes samples 2µL of the AmpliSeq Adapter is added. For Barcoded samples, 2µL of the appropriate barcode is added based on the worklist generated. Finally, 2µL of DNA ligase is added to each sample and the sample is mixed.

To Start the Protocol,

1. Open the NGS Express software by clicking on the Janus Application Assistant (JAA) icon.
2. The list of available protocols will appear.
3. Select the protocol “Ion AmpliSeq 3_Ligate Adapters” by clicking on it once.
4. Questions related to this protocol will appear in the lower panel.
    a. Select if you want to run Barcode Adapters or Non-barcoded adapters.
    b. If you wish to generate a Run Report, check the Run Report box. If checked, a report will be generated including the following:
        i. Name or User ID- this optional file allows you to record which operator is running the instrument.
        ii. Kit Barcode- use the handheld barcode scanner or type in the kit barcode. This information will be saved in the Run Report.

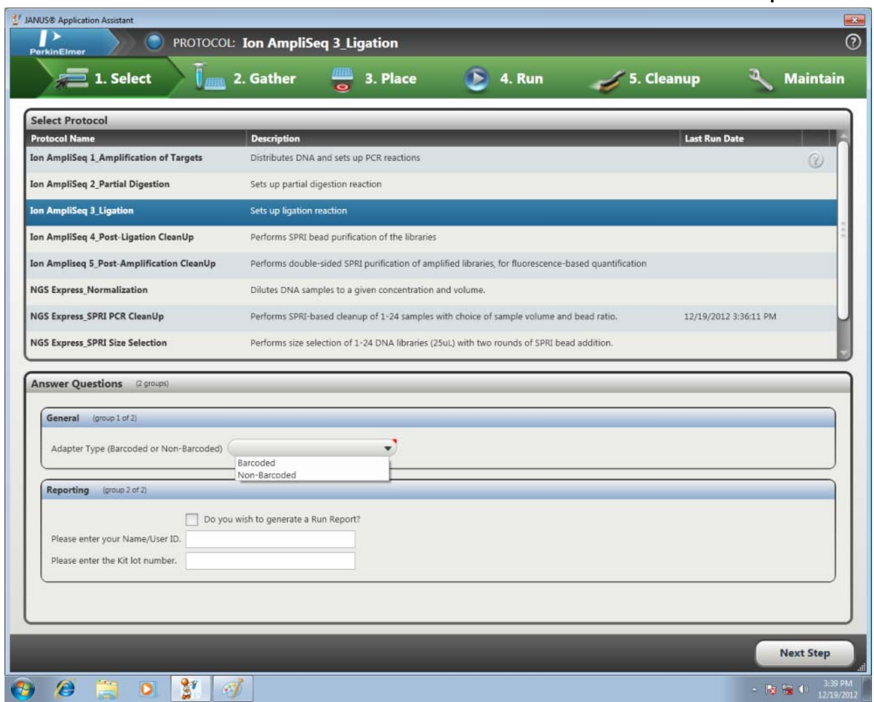


Figure 24. Select Protocol Screen with AmpliSeq 3 Ligation selected and related questions shown.

5. Place the labware and reagents on the deck.
    a. Click on the Place icon in the top green bar.

b. Follow the instructions in the Place tab of the software for placing support tiles, tips, and plates on the deck. Note that your sample plate must be in the basket in the front left position on the deck, as shown below:

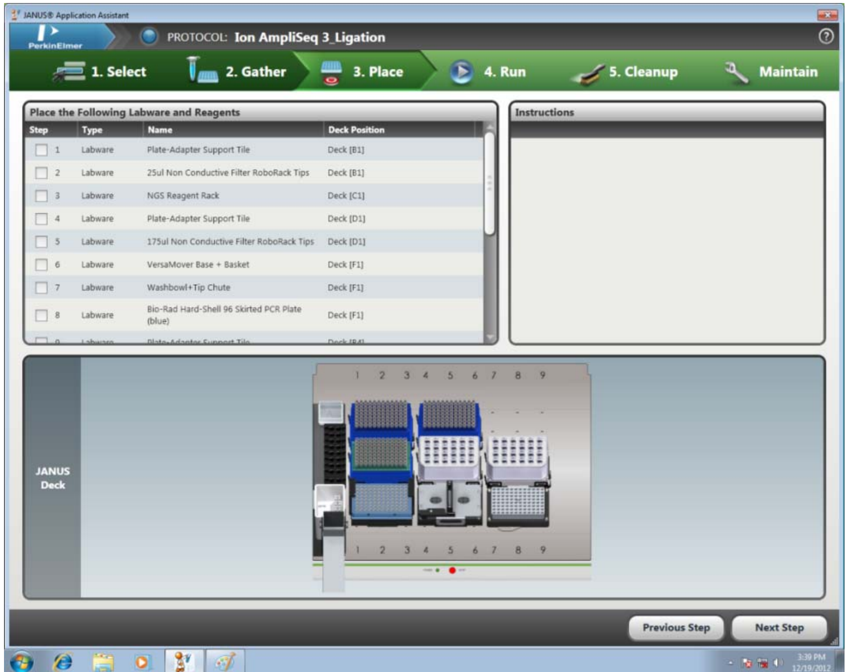


Figure 25. Place page of NGS Express software showing the location of the sample plate on the deck for Ion AmpliSeq Ligate Adapters protocol.

c. Make sure the 0.5mL tubes of Switch Solution, barcoded or non-barcoded adapters and DNA Ligase are the IsoRack chilling blocks according to the reagent setup diagram in Figure 22 and Figure 23 (for bar coded adapters).

Run the Protocol

d. Click on the Run icon in the top green bar.
e. Click the Start button.
f. You will be asked to verify/change tip counts. If you are placing full tip boxes on the deck, make sure that each box shows 96/0 tips in the software. If you have full tip boxes on the deck but not in the software, click the Fill button for each tip box that is full on the deck.
g. Click OK to commence the run.

Consult the Workflow Chart for details on the steps being performed by the NGS Express.

Your samples will be in the plate on the magnet tile, in the wells shown below:

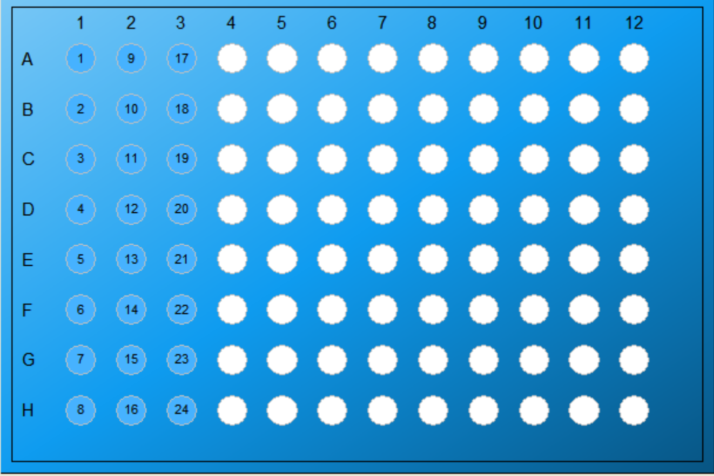


Figure26. Sample locations at the completion of protocol 3.

The plate should be sealed and moved to the thermal cycler to run the temperature protocol as indicated in the in the Ion AmpliSeq™ Library Kit 2.0 Users Guide (MAN0006735).

Post-Ligation Cleanup

This protocol performs the following steps:

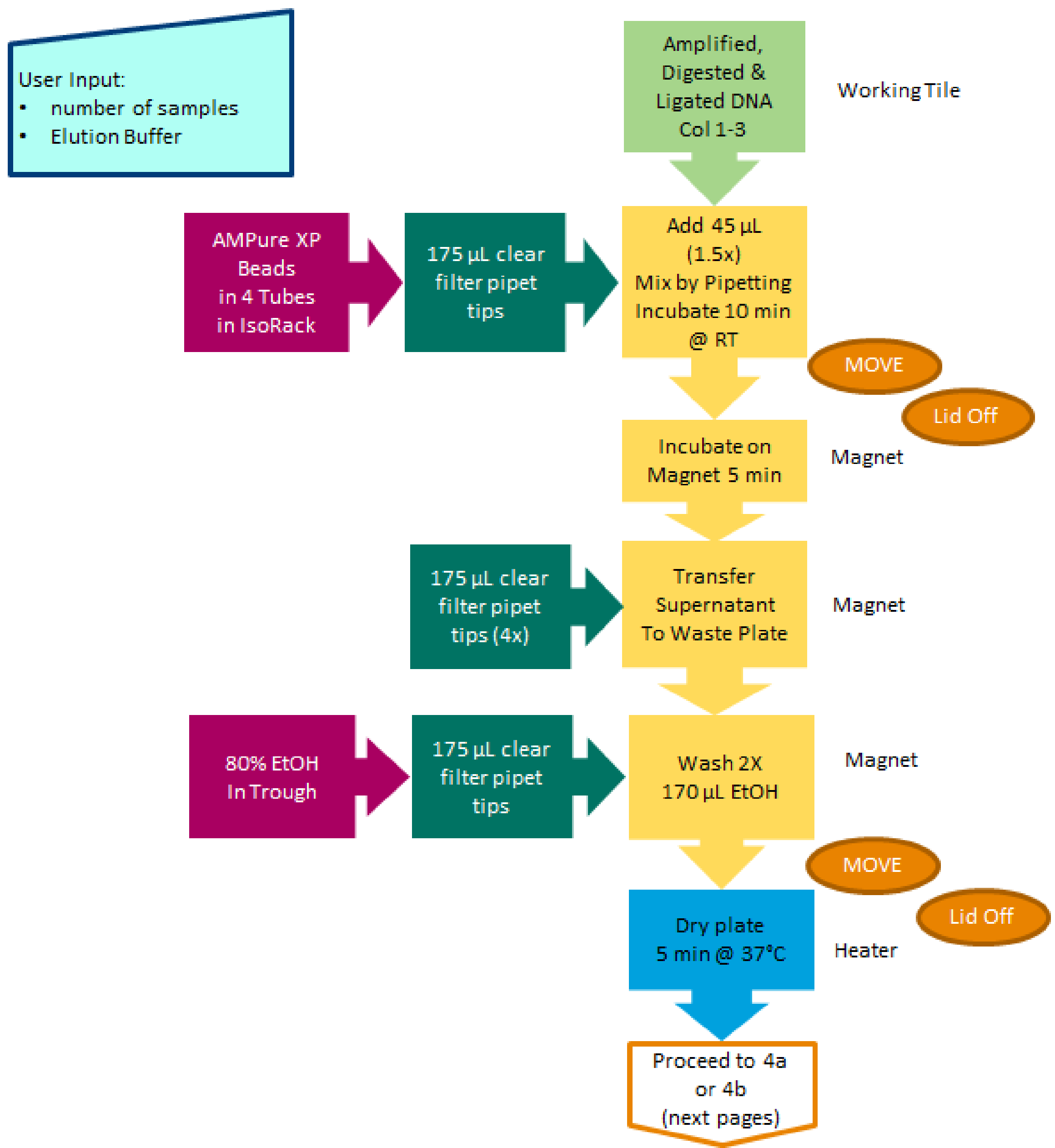


Figure 27: Flow Chart for Post-Ligation CleanUp in the Ion AmpliSeq protocol on the NGS Express

Setting up the reagents- refer to the Sample & Reagent Volume Calculator worksheet to determine the volume of reagents needed for the number of samples to be run. At the beginning of the run, the software will prompt the user to select if they will be eluting for Quantitation for qPCR or Eluting and Preparing for Amplification. Both elution steps require the

80% Ethanol and SPRI beads. For qPCR the Low TE is required for elution. For preparing for amplification the with Platinum® PCR SuperMix High Fidelity with Library Amplification Primer Mix is required. The reagents should be placed in the Eppendorf IsoRack with the chiller block and NGS Reagent Rack as shown below:

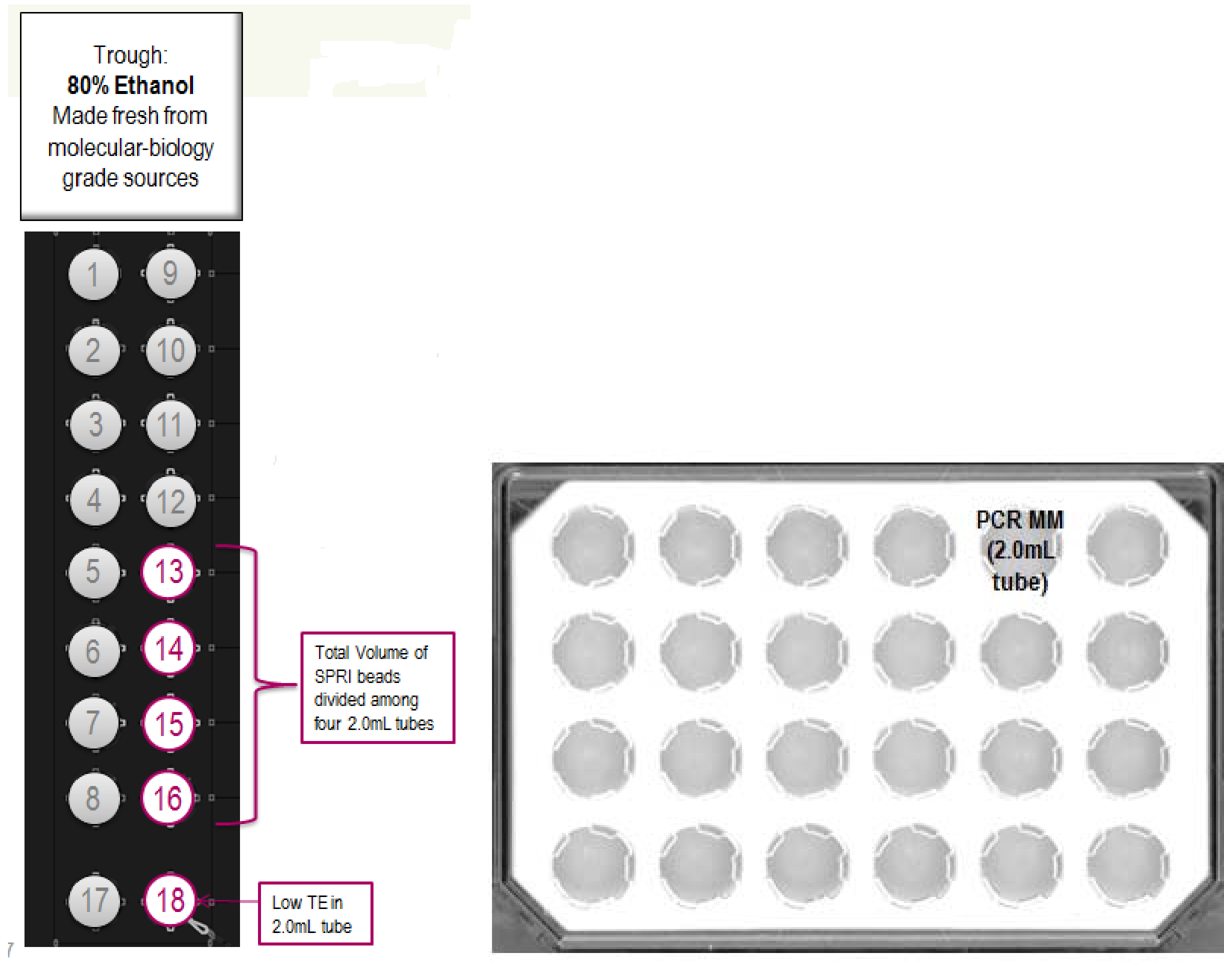


Figure 28: The NGS reagent rack (left) with SPRI beads and Low TE. On the right the Eppendorf IsoRack with Chiller block in place, with Platinum® PCR SuperMix High Fidelity with Library Amplification Primer Mix for the Post-Ligation CleanUp step.

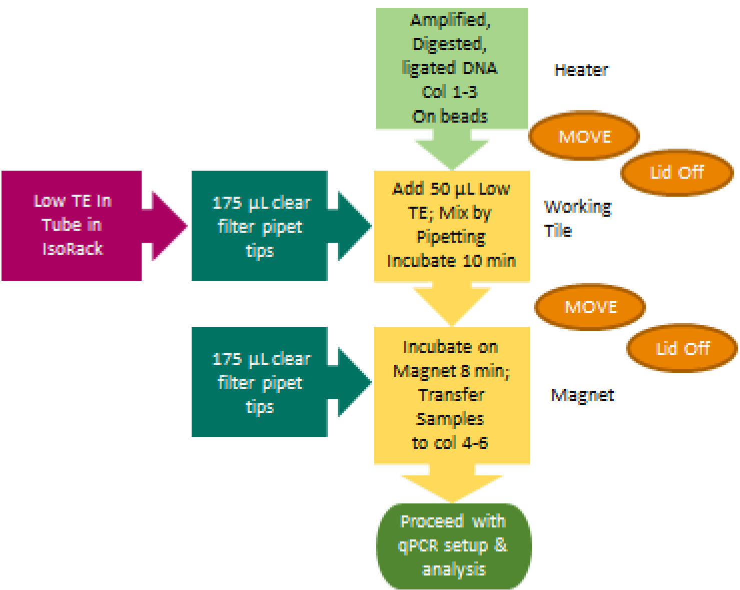


Figure 29: Flow Chart for Post-Ligation CleanUp – Elution for quantitation by qPCR in the Ion AmpliSeq protocol on the NGS Express

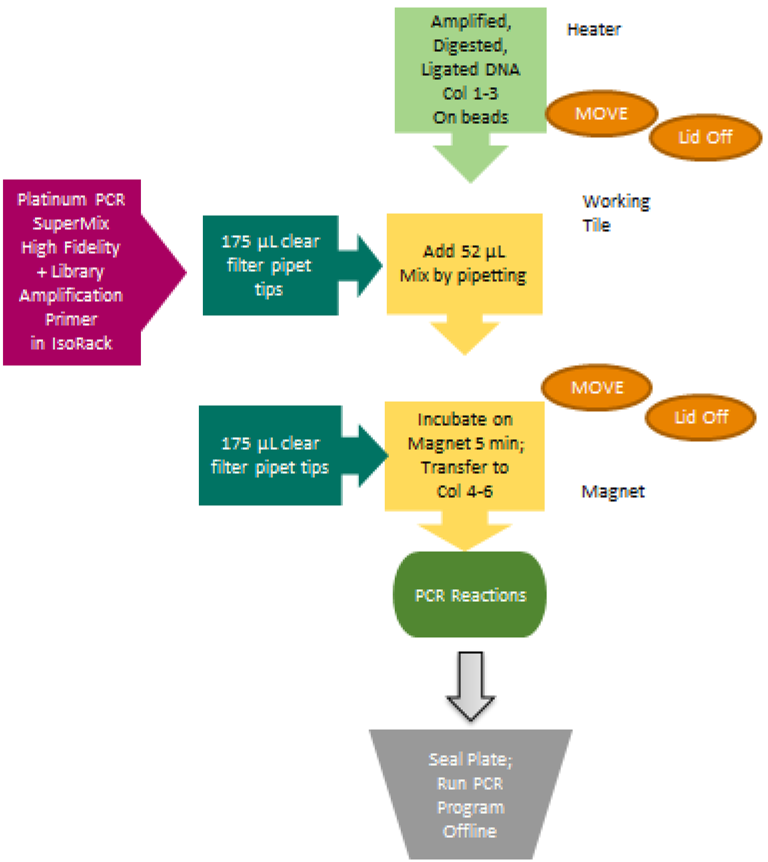


Figure 30: Flow Chart for Post-Ligation CleanUp –elute and prepare for Amplification in the Ion AmpliSeq protocol on the NGS Express

Protocol 4- Post-Ligation Cleanup

This protocol will perform a single SPRI sample cleanup at 1.5X sample volume. Finale elution is done in Low TE for sample to be quantitated by qPCR. For final quantification by a fluorometric method, the sample must be first amplified and again cleaned up before quantitation.

To Start the Protocol,

1. Open the NGS Express software by clicking on the Janus Application Assistant (JAA) icon.
2. The list of available protocols will appear.
3. Select the protocol “Ion AmpliSeq 4_Post-Ligation Cleanup” by clicking on it once.
4. Questions related to this protocol will appear in the lower panel.
   a. Enter the number of samples you want to process.
   b. Select either Low TE or PCR master mix for Amplification.
   c. Check the box if you wish for the Janus to mix the SPRI beads prior to transfer.

d. If you wish to generate a Run Report, check the Run Report box. If checked, a report will be generated including the following:
    i. Name or User ID- this optional file allows you to record which operator is running the instrument.
    ii. Kit Barcode- use the handheld barcode scanner or type in the kit barcode. This information will be saved in the Run Report.

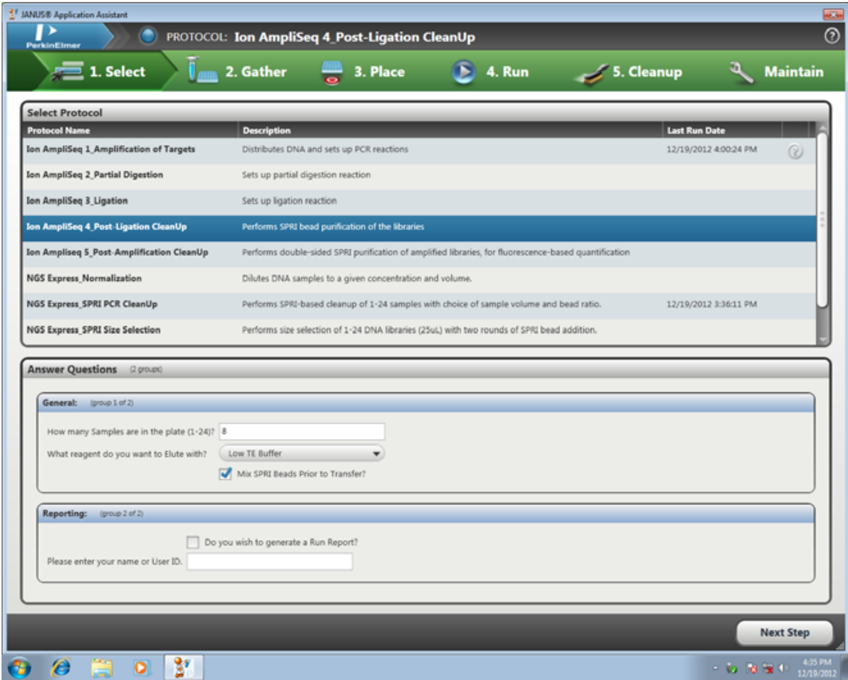


Figure 31. Select Protocol Screen with AmpliSeq 4 Post-Ligation CleanUp selected and related questions shown.

5. Place the labware and reagents on the deck.
    a. Click on the Place icon in the top green bar.

b. Follow the instructions in the Place tab of the software for placing support tiles, tips, and plates on the deck. Note that your sample plate must be in the basket in the front left position on the deck, as shown in Figure 32.

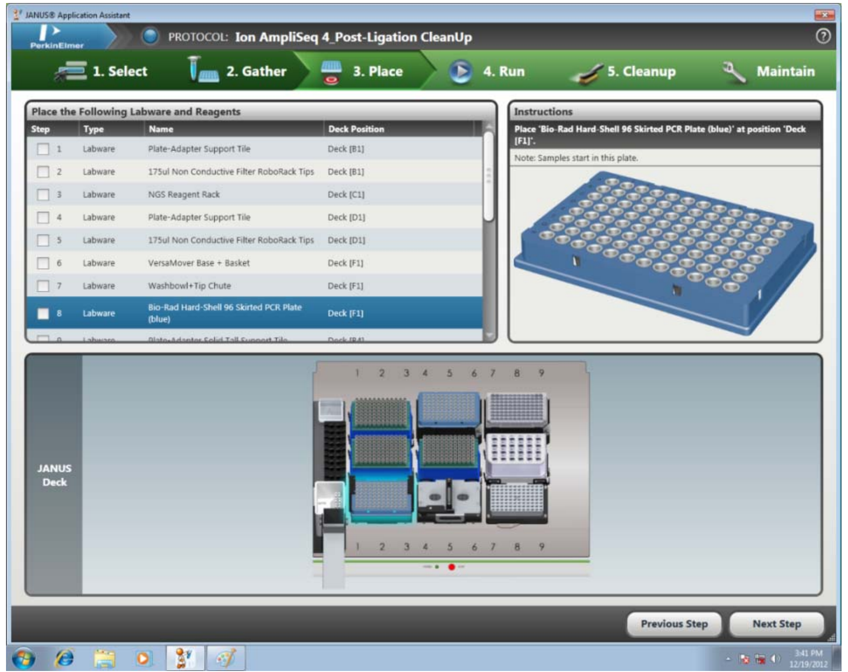


Figure 32. Place page of NGS Express software showing the location of the sample plate on the deck for Ion AmpliSeq Post-Ligation Cleanup protocol.

c. Make sure the SPRI beads and Low TE or PCR Mater mix tubes are located on the deck according to the diagram in Figure 28.

Run the Protocol

d. Click on the Run icon in the top green bar.
e. Click the Start button.
f. You will be asked to verify/change tip counts. If you are placing full tip boxes on the deck, make sure that each box shows 96/0 tips in the software. If you have full tip boxes on the deck but not in the software, click the Fill button for each tip box that is full on the deck.
g. Click OK to commence the run.

Consult the Workflow Chart for details on the steps being performed by the NGS Express.

Your samples will be in the plate on the magnet tile, in the wells shown below:

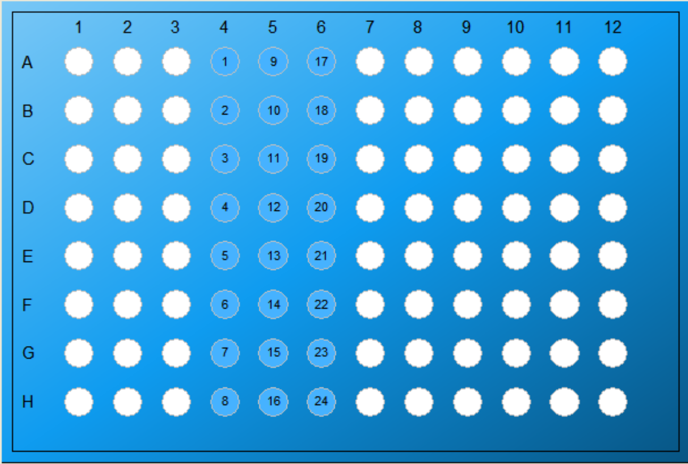


Figure33. Sample locations at the completion of protocol 2.

For amplification, the plate should be sealed and moved to the thermal cycler to run the temperature protocol as indicated in the in the Ion AmpliSeq™ Library Kit 2.0 Users Guide (MAN0006735). If you are running qPCR for quantitation, set-up the plate for qPCR in accordance with the reagents and qPCR Instrument used.

Post-Amplification Cleanup (Optional)

This protocol is only required if an additional amplification is done to quantitate samples by a fluorometric method. The protocol performs the following steps:

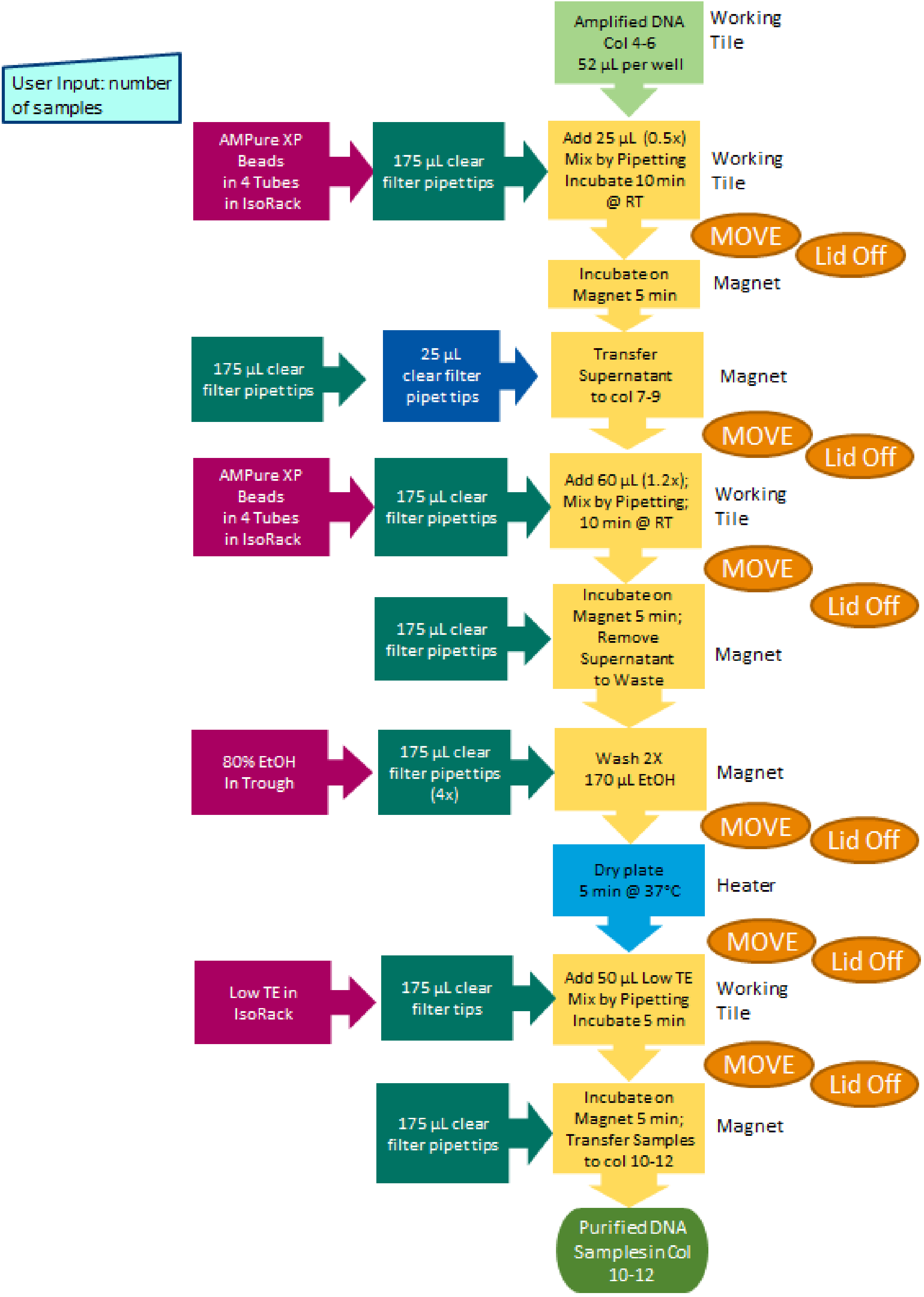


Figure34. Flow Chart for Post-Amplification CleanUp

Setting up the reagents- refer to the Sample & Reagent Volume Calculator worksheet to determine the volume of reagents needed for the number of samples to be run. For the final cleanup the 80% Ethanol, SPRI beads and Low TE are all placed in the NGS reagent Rack on the left side of the NGS Express as shown below:

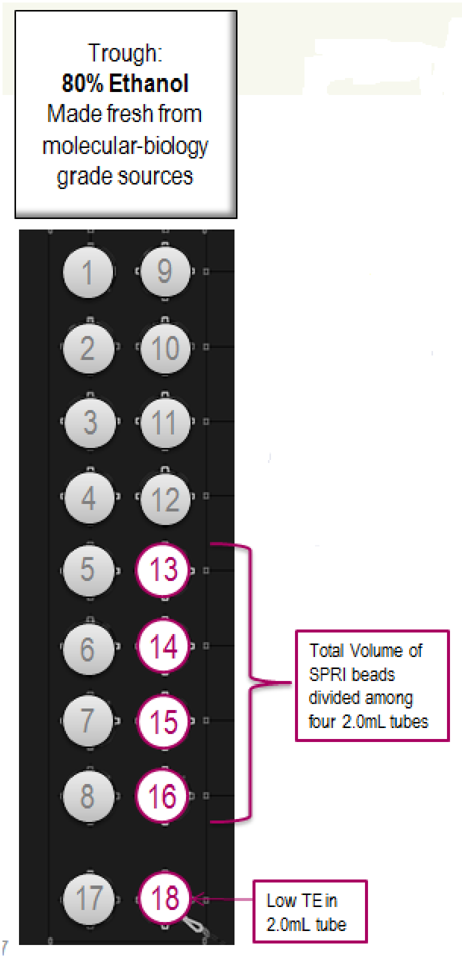


Figure 35: The NGS reagent rack (left) with SPRI beads and Low TE for the Post-Amplification CleanUp step.

Protocol 5- Post-Amplification Cleanup

This protocol will perform a double SPRI sample cleanup. The first SPRI is at 0.5X sample volume. The supernatant from this purification is then used for the second SPRI. The second SPRI is at a 1.2X sample volume. After the 80% ethanol wash the final elution is done in Low TE.

To Start the Protocol,

1. Open the NGS Express software by clicking on the Janus Application Assistant (JAA) icon.
2. The list of available protocols will appear.
3. Select the protocol “Ion AmpliSeq 5_Post-Amplification Cleanup” by clicking on it once.

4. Questions related to this protocol will appear in the lower panel.
    a. Enter the number of samples you want to process.
    b. If you wish to generate a Run Report, check the Run Report box. If checked, a report will be generated including the following:
        i. Name or User ID- this optional file allows you to record which operator is running the instrument.
        ii. Kit Barcode- use the handheld barcode scanner or type in the kit barcode. This information will be saved in the Run Report.

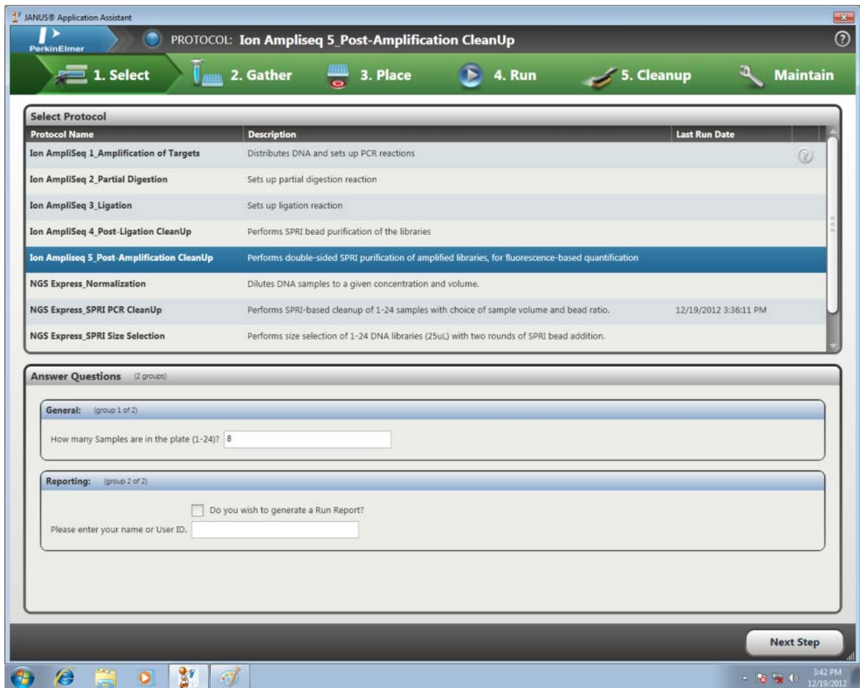


Figure 36. Select Protocol Screen with AmpliSeq 5 Post-Amplification CleanUp selected and related questions shown.

5. Place the labware and reagents on the deck.
    a. Click on the Place icon in the top green bar.

b. Follow the instructions in the Place tab of the software for placing support tiles, tips, and plates on the deck. Note that your sample plate must be in the basket in the front left position on the deck, as shown in Figure 37.

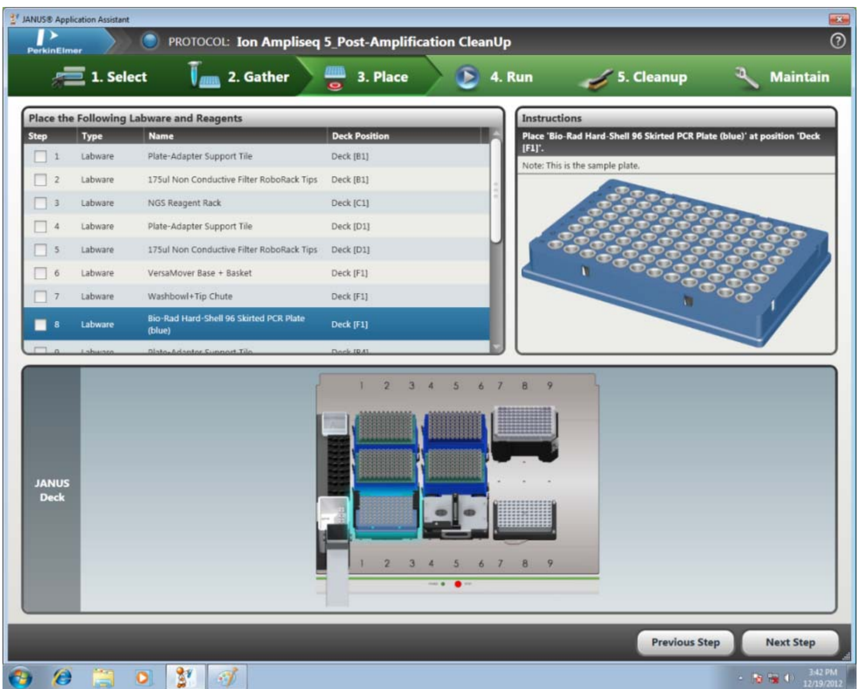


Figure 37. Place page of NGS Express software showing the location of the sample plate on the deck for Ion AmpliSeq Post-Amplification Cleanup protocol.

c. Make sure the SPRI beads and Low TE tubes are located in the NGS Reagent rack on the left side of the deck according to the diagram in Figure 37.

Run the Protocol

d. Click on the Run icon in the top green bar.
e. Click the Start button.
f. You will be asked to verify/change tip counts. If you are placing full tip boxes on the deck, make sure that each box shows 96/0 tips in the software. If you have full tip boxes on the deck but not in the software, click the Fill button for each tip box that is full on the deck.
g. Click OK to commence the run.

Consult the Workflow Chart for details on the steps being performed by the NGS Express.

Your final samples will be in the plate on the magnet tile, in the wells shown below:

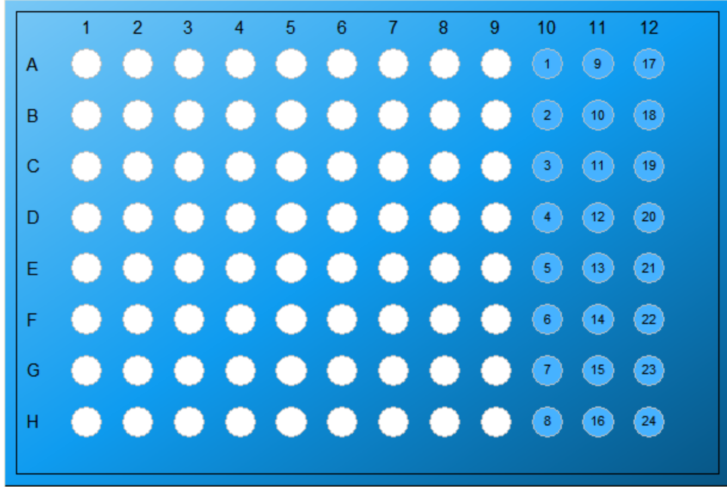


Figure38. Sample locations at the completion of protocol 5.

Proceed to quantitation by the appropriate fluorometric method.

Expected Results

Samples prepared via the automated Ion AmpliSeq method on the NGS Express were analyzed with a DNA High-Sensitivity LabChip running on a LabChip GX Reader. The material between 150-250 bp is the desired profile representing regions of interest after amplification with the Cancer Primer Pool.

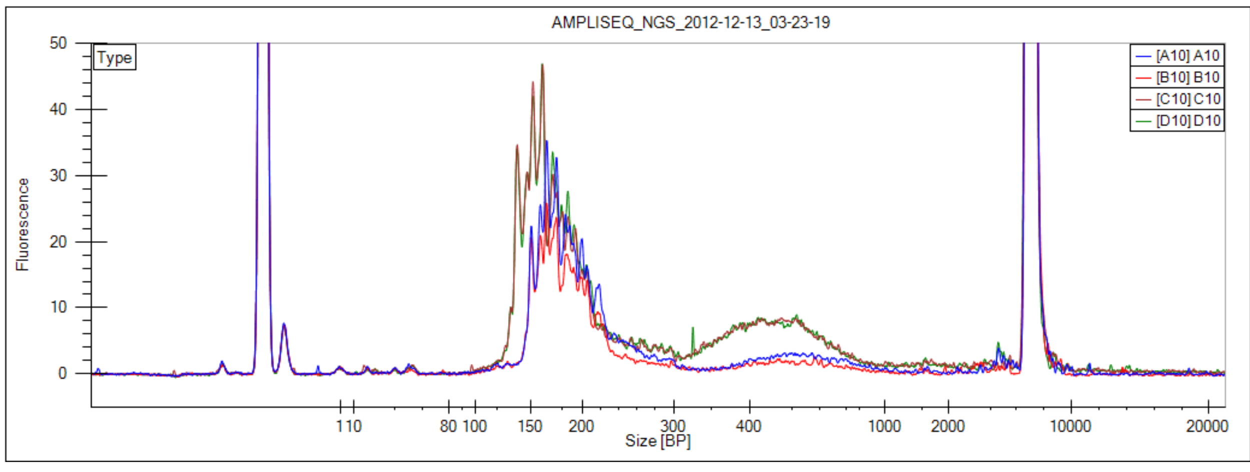


Figure 39. LabChip DNA trace of AmpliSeq libraries generated on the NGS Express using the Cancer Primer Pool in two separate 2-sample runs. Broad peak at 400-500bp in two of the samples is due to a delay between ligation and post-ligation cleanup, unrelated to the automation.

Appendix

Step by Step guide to the "Ion AmpliSeq Library Preparation" application

Step 1: Ion AmpliSeq 1_Amplification of Targets

1. Load 25µL filter tips. DNA Sample transferred from columns 10-12 to Columns 1-3 based on the Amplicon Panel used, Eject tips.
2. Load 25µL filter tips. Aspirate 4µL of HiFi Master Mix from tube in the IsoRack and dispense into DNA Sample in Columns 1-3, Eject tips.
3. Load 25µL filter tips. Aspirate 10µL of Primer Pool from tube in the IsoRack and dispense into DNA Sample in Columns 1-3, mix and Eject tips.
4. Seal Plate, and move to the thermocycler.

Step 2: Ion AmpliSeq 2_Partially Digest Primer Sequences

1. Load 25µL filter tips. Aspirate 2µL of Fu Pa reagent from tube in the IsoRack and dispense into DNA Sample in Columns 1-3, mix and Eject tips.
2. Seal Plate, and move to the thermocycler.

Step 3: Ligate Adapters

1. The user will input if barcoded adapters are used. If Barcoded adapters are sued, they will browse to select the sample sheet providing the sample plate names, well ID and Barcode ID. From this the AmpliSeq sample file is read, and a worklist generated.
2. If Barcoded adapters are not used, the protocol will request the number of samples to be run.
3. Load 25µL filter tips. Aspirate 25µL of Switch Solution from tube in the IsoRack and dispense into DNA Sample in Columns 1-3, Eject tips.
4. For Barcoded Samples: Load 25µL filter tips. Aspirate 2µL of the appropriate diluted barcode from the IsoRack as indicated in the barcode Worklist and dispense into DNA Sample in Columns 1-3, Eject tips.
5. For Non-Barcoded Samples: Load 25µL filter tips. Aspirate 2µL of the AmpliSeq Adapter from the IsoRack and dispense into DNA Sample in Columns 1-3, Eject tips.
6. Load 25µL filter tips. Aspirate 2µL of DNA Ligase from tube in the IsoRack and dispense into DNA Sample in Columns 1-3, Mix and Eject tips.
7. Seal Plate, and move to the thermocycler.

Step 4: Post-Ligation Cleanup

1. The user will input the number of samples, and whether they are going to elute for qPCR or elute for PCR.
2. Load 175µL filter tips. Transfer 45µL of SPRI beads and dispense into sample. Mix by pipetting. Eject tips.
3. Incubate beads and sample for 10 min.
4. Move beads/sample from Working Tile to magnet with the VersaMover plate mover.
5. Incubate in the magnet for 5 minutes.
6. Load a 175µL filter tip and aspirate supernatant to waste plate. Eject tips.
7. Wash beads with 150µL 80% EtOH two times, removing waste wash solution to waste plate.
8. Move plate to Inheco to dry at 55C for 7 minutes.
9. Move plate back to the working tile using the VersaMover.

Step 4a: Elute for Quantitation by qPCR

1. Load 175µL filter tips. Aspirate 50µL of Low TE from tube in the NGS Reagent Rack and dispense into DNA Sample in Columns 1-3, Mix and Eject tips.
2. Incubate for 10 minutes.
3. Move beads/sample from Working Tile to magnet with the VersaMover plate mover.
4. Incubate on the magnet 8 minutes.

5. Load 175µL filter tips and Transfer 30µL sample from beads to wells 4-6..
6. Proceed with qPCR setup & analysis

Step 4b: Elute & Prepare for Amplification

1. Load 175µL filter tips. Aspirate 52µL of Platinum PCR SuperMix High Fidelity with Library Amplification Primer from tube in the IsoRack and dispense into DNA Sample in Columns 1-3, Mix and Eject tips.
2. Move beads/sample from Working Tile to magnet with the VersaMover plate mover.
3. Incubate on the magnet 5 minutes.
4. Load 175µL filter tips and Transfer sample from beads to wells 4-6.
5. Seal plate & proceed with PCR.

Step 5: Post-Amplification Cleanup

1. The user will input the number of samples.
2. Load 175µL filter tips. Transfer 25µL of SPRI beads and dispense into sample. Mix by pipetting. Eject tips.
3. Incubate beads and sample for 10 min.
4. Move beads/sample from Working Tile to magnet with the VersaMover plate mover.
5. Incubate in the magnet for 5 minutes.
6. Load a 175µL filter tip and aspirate supernatant to column 7-9. Eject tips.
7. Load 175µL filter tips. Transfer 60µL of SPRI beads and dispense into sample. Mix by pipetting. Eject tips.
8. Incubate beads and sample for 10 min.
9. Move beads/sample from Working Tile to magnet with the VersaMover plate mover.
10. Incubate in the magnet for 8 minutes.
11. Load a 175µL filter tip and aspirate supernatant to waste. Eject tips.
12. Wash beads with 170µL 80% EtOH two times, removing waste wash solution to waste plate.
13. Move plate to Inheco to dry at 55C for 7 minutes.
14. Move plate back to the working tile using the VersaMover.
15. Load 175µL filter tips. Aspirate 50µL of Low TE from tube in the NGS Reagent Rack and dispense into DNA Sample in Columns 7-9, Mix and Eject tips.
16. Incubate for 10 minutes.
17. Move beads/sample from Working Tile to magnet with the VersaMover plate mover.
18. Incubate on the magnet 5 minutes.
19. Load 175µL filter tips and Transfer 50µL sample from beads to wells 10-12.

List of Files Installed/Modified by the AmpliSeq Option Installer

New files have been added to the Bin, Labware Files, and Performance Files folders.

Option-Specific Protocol Files Added:

- Ion AmpliSeq 1_Amplification of Targets.MPT
- Ion AmpliSeq 2_Partial Digestion.MPT
- Ion AmpliSeq 3_Ligation.MPT
- Ion AmpliSeq 4_Post-Ligation CleanUp.MPT
- Ion Amplise 5_Post-Amplification CleanUp.MPT
- ReportConfig_Ampliseq1.csv
- ReportConfig_Ampliseq2.csv
- ReportConfig_Ampliseq3.csv
- ReportConfig_Ampliseq4.csv
- ReportConfig_Ampliseq5.csv

Common Files Added:

- AlignTips_NGS.MPT
- DailyClean_NGS.MPT
- FlushSysLiq_NGS.MPT
- JAA AlignTips_NGS.MPT
- JAA Clean_NGS.MPT
- JAA Prime_NGS.MPT
- PickupTipTest_NGS.MPT
- Random XY Test_NGS.MPT
- Left Rack Test.MPT
- Versa Mover Library.MPT
- Versa Mover Test.mpt
- NGS.s
- NGSReporting.s
- NGS Express_Normalization.MPT
- NGS Express_SPRI PCR CleanUp.MPT
- NGS Express_SPRI Size Selection.MPT
- NGSExpress_NormalizationWorklist.csv
- ReportConfig_NGS_Normalization.csv
- ReportConfig_NGS_PCR_Cleanup.csv
- ReportConfig_NGS_SizeSelection.csv
- NGS_PCR 96_WinPREP_Template.MPT
- NGS_StorPlate 96-V_WinPREP_Template.MPT
- NGSReportingTemplate.MPT

Labware Files- Consumables:

- IsoRack 24 Custom_Ion Ampliseq Barcodes.lab
- IsoRack 24 Custom_Ion AmpliSeq Reagents.lab
- NGS Reagent Rack Custom_Ion AmpliSeq (left edge).lab
- Bio-Rad Hard-Shell 96 PCR for Magnet.lab
- Bio-Rad Hard-Shell 96 PCR Skirted (blue).lab
- Invitrogen MicroAmp 96-PCR with base.lab
- IsoRack 24-well Cooling Block with 1500uL tube.lab
- IsoRack 24-well Cooling Block with 2000uL tube.lab
- IsoRack 24-well Cooling Block with 500uL tube.lab
- IsoRack 24-Well Custom Cooling Block.lab
- PCR tubes in Bio-Rad Hard-Shell Plate.lab
- PerkinElmer StorPlate 96-V (PP).lab
- Seahorse 10mL Square 24-Pyr (PP).lab
- 175ul Conductive Filter RoboRack Tips.lab
- 175ul Non Conductive Filter RoboRack Tips.lab
- 25ul Conductive Filter RoboRack Tips.lab
- 25ul Non Conductive Filter RoboRack Tips.lab

Labware Files- Custom Hardware:

- Inheco 96-PCR Plate Support 33mm.lab
- Inheco 96-V Plate Support.lab
- Rack_18 Custom Microtube+Trough (left edge).lab

- VM_Base_LiftSupport.lab
- VM_Base_PlateSupport.lab
- VM_Inheco96PCR_LiftSupport.lab
- VM_Inheco96PCR_PlateSupport.lab
- VM_Inheco96V_LiftSupport.lab
- VM_Inheco96V_PlateSupport.lab
- VM_Mag_LiftSupport.lab
- VM_Mag_PlateSupport.lab
- VM_VersaLift.lab
- Washbowl+Tip Chute (left edge).lab
- Tip alignment plate.lab

Plate Mover Files- Do Not Edit:

- HeaterToMagnet.csv
- HeaterToReaction.csv
- HeaterToReactionLidatHeater.csv
- LidFromMagnetToHeater.csv
- LidFromMagnetToHeaterDropLid.csv
- LiftZ20.csv
- LiftZ24.csv
- LowerZ24.csv
- MagnetToWorking.csv
- ParkLidHeaterToMagnet.csv
- ParkLidHeaterToWorking.csv
- ParkLidMagnetToWorking.csv
- ParkLidWorkingToMagnet.csv
- PlateMagnetToHeaterLidatR.csv
- ReactionToHeater.csv
- ReactionToHeaterLidOn.csv
- ReactionToMagnet.csv

Performance Files

The following performance files have been created expressly for the NGS Express and were validated with 250-µL syringes.

- AMPure XP_Blowout_DT-175uL_S250.prf
- AMPure XP_Blowout_DT-25uL_S250.prf
- AMPure XP_Waste_DT-175uL_S250.prf
- Ethanol 80%_Blowout_DT-175uL_S250.prf
- Ethanol 80%_Waste_DT-900uL_S250.prf
- Glycerol 10%_Blowout_DT-25uL_S250.prf
- Glycerol 10%_Waste_DT-25uL_S250.prf
- PEG 20%_Blowout_DT-25uL_S250.prf
- PEG 20%_Waste_DT-175uL_S250.prf
- PEG 20%_Waste_DT-900uL_S250.prf
- Water_Blowout_DT-175uL_S250.prf
- Water_Blowout_FT_S250.prf
- Water_Waste_DT-175uL_S250.prf
- Water_Waste_DT-900uL_S250.prf
- Water_Waste_FT_S250.prf

Modified WinPREP Files

The following common WinPREP files (in Bin unless otherwise noted) are modified by the installer, and are backed up prior to modification:

- Categories.xml
- CleanupActivityList.xml
- DiagnosticTests.csv
- LabwareImagesInfo.XML
- MaintainActivityList.xml
- Msl.ini
- MSLEXT.csv
- C:\Packard\JANUS\InstrumentLayout\InstrumentLayout.xml