Applied Biosystems SOLiD® 4 System

Standard and Barcoded Fragment Library Preparation Using the Tecan Freedom EVO® 75

AB Demonstrated Protocol

life technologies™

For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

TRADEMARKS:

6/2011

Contents

Preface

Applied Biosystems (AB) protocols for the SOLiD® System can be described by the following categories:

Category	Description
AB Supported (S)	Testing and validation have been performed by Applied Biosystems for this protocol on this instrument system. The technical support and field application specialists have been trained to support this protocol.
AB Demonstrated (A)	Applied Biosystems has tested this protocol but no validation was performed for this instrument system. Certain components of the protocol workflow such as reagent kits and other protocols for preparation of reagents may not be available through Applied Biosystems. Supporting documentation such as application notes may be available from Applied Biosystems and/or third parties. Limited support is available from Applied Biosystems.
Customer Demonstrated (C)	The performance of this protocol has not been evaluated on this instrument by Applied Biosystems. However, at least one customer or third party has reported successfully performing this protocol on this instrument. Applied Biosystems cannot guarantee instrument and reagent performance specifications with the use of customer demonstrated protocols. However supporting documentation from Applied Biosystems and/or third parties may be available and Applied Biosystems may provide basic guidelines in connection with this protocol.
Experimental / Not Supported (N)	This protocol has not been evaluated and/or tested on this instrument by either Applied Biosystems or its customers. Applied Biosystems cannot warranty that attempting to utilize this protocol will not adversely affect the functionality of the instrument and other Applied Biosystems products. Data generated by this protocol on this instrument may not be fully representative of typical results. Applied Biosystems does not provide any support for this protocol.

For the latest services and support information, go to:

www.appliedbiosystems.com

Materials and equipment required

In addition to the required equipment, kits, and consumables outlined in Appendix A in the *Applied Biosystems SOLiD® 4 System Library Preparation Guide* (PN 4445673), the following items are required.

Required equipment

Item‡	Quantity	Source
Freedom EVO® 75 base unit	1	Tecan 30025019
Freedom EVOware® Standard software, v2.3 or higher	1	Tecan Included with 30025019
Liquid handling arm (LiHa), 2-tip, EVO 75	1	Tecan 30049817
Safety panel, right side, EVO 75	1	Tecan 30025021
Disposable tip cone assembly, standard	2	Tecan 30019984
Lower disposable tip eject	1	Tecan 30025029
Syringe, 1000-µL, XP diluters, conical cap	2	Tecan 10619532
FastWash option, EVO 75	1	Tecan 30025024
Bottle system liquid, 10-L	1	Tecan 10619677
Bottle waste, 10-L	1	Tecan 10619818
Wash station, LiHa, with DiTi waste chute and trough carrier	1	Tecan 10650037
DiTi Carrier, LiHa, 3-Position	2	Tecan 10613022
Tube Carrier, 16-mm, 16-Position, 1-strip	1	Tecan 30019986
Microplate Carrier, 3-Position, Landscape	2	Tecan 10613031
7-Bar magnet	1	V&P Scientific VP771MM
MicroAmp® 96-Well Base	1	Applied Biosystems N801-0531

‡ Applied Biosystems has tested this protocol using this specific material. Substitution may adversely affect system performance.

Required consumables

Item‡	Source
100-mL disposable trough for reagents (polypropylene, natural)	Tecan 10613048
Disposable Tips, 1000-µL Conductive with Filters	Tecan 30000631
MicroAmp® 8-Tube strip, 0.2-mL	Applied Biosystems N8010580
MicroAmp® 96-well Optical Reaction Plate (0.2-mL)	Applied Biosystems 4306737
MicroAmp® Fast Optical 96-Well Reaction Plate (0.1-mL)	Applied Biosystems 4346906
Optical Adhesive Cover	Applied Biosystems 4360954
15-mL conical tubes	Any Supplier
Nunc Aluminum Seal Tape for 96-Well Plates	Thermo Scientific 232698
Qubit® assay tubes (500/pk)	Invitrogen Q32856
Deep-well plate 96/500µL, DNA LoBind	Eppendorf 951032085
Corning® 96 Well Clear V-Bottom 2-mL Polypropylene Block, sterile	Corning 3960
microTUBE™ (6 x 16 mm), AFA Fiber with snap-caps	Covaris 520045

‡ Applied Biosystems has tested this protocol using this specific material. Substitution may adversely affect system performance, and may damage a consumable(s) and/or spill a reagent.

Required reagents

Item‡	Source
SOLiD® Fragment Library Oligos Kit	Applied Biosystems 4401151
SOLiD® Barcoding Kit Modules 1-96§	Applied Biosystems 4449637
SOLiD® Fragment Library Construction Kit Reagents	Applied Biosystems 4443713
Agencourt AMPure® XP, 60 mL Kit	Beckman Coulter Genomics A63881
SOLiD® Library TaqMan® Quantitation Kit	Applied Biosystems 4449639
Quanti-iT™ dsDNA HS Assay Kits, 500 Assays	Invitrogen Q32854
1x Low TE Buffer	Applied Biosystems 4389764
95% or 100% Ethanol	Any supplier

‡ Applied Biosystems has tested this protocol using this specific material. Substitution may adversely affect system performance, and may damage a consumable(s) and/or spill a reagent.

§ This kit is made up of the following modules: SOLiD® Fragment Library Barcoding Module 1-16 (PN 4444837), SOLiD® Fragment Library Barcoding Module 17-32 (PN 4449636), SOLiD® Fragment Library Barcoding Module 33-48 (PN 4449635), SOLiD® Fragment Library Barcoding Module 49-64 (PN 4449641), SOLiD® Fragment Library Barcoding Module 65-80 (PN 4449462), SOLiD® Fragment Library Barcoding Module 81-96 (PN 4449643)

Required scripts

Item	Source
SOLiD4_Frag_24.exd	Contact your Applied Biosystems field application specialist

Assumptions

This guide assumes that:

- The Tecan Freedom EVO® 75 has the appropriate hardware and software installed, and has been calibrated.
- Users are properly trained in the operation, maintenance, and troubleshooting of the Freedom EVO 75 .
- Users have access to the Freedom EVO operating manual, and Freedom EVOware software manuals, and other applicable Tecan documentation, and the *Applied Biosystems SOLiD® 4 System Library Preparation Guide* (PN 4445673).
- Users have read important safety information related to the use of the Freedom EVO 75.
- Users have read the safety information in Appendix I, “Safety” on page 248 in the *Applied Biosystems SOLiD® 4 System Library Preparation Guide* (PN 4445673).

Introduction

A robotic method for preparing and purifying standard and express fragment libraries has been validated at Applied Biosystems. The Freedom EVO 75 liquid handling systems was chosen for this work due to the small footprint and low cost. This method will ease the burden of multiple pipetting and purification steps.

Prepare a fragment library

This protocol is designed for 10 ng to 5 µg of genomic DNA.

- For Standard Libraries refer to "Prepare a standard fragment library" on page 16 in the *Applied Biosystems SOLiD® 4 System Library Preparation Guide* (PN 4445673) for more information.
- For Express Libraries refer to "Prepare an express fragment library" on page 31 in the *Applied Biosystems SOLiD® 4 System Library Preparation Guide* (PN 4445673) for more information.
- For Barcoded Libraries refer to "Barcoded Fragment Library Preparation" on page 128 in the *Applied Biosystems SOLiD® 4 System Library Preparation Guide* (PN 4445673) for more information.

Workflow

The workflow is comprised of both manual and automation steps. In Figure 1, the automation steps are highlighted in yellow.

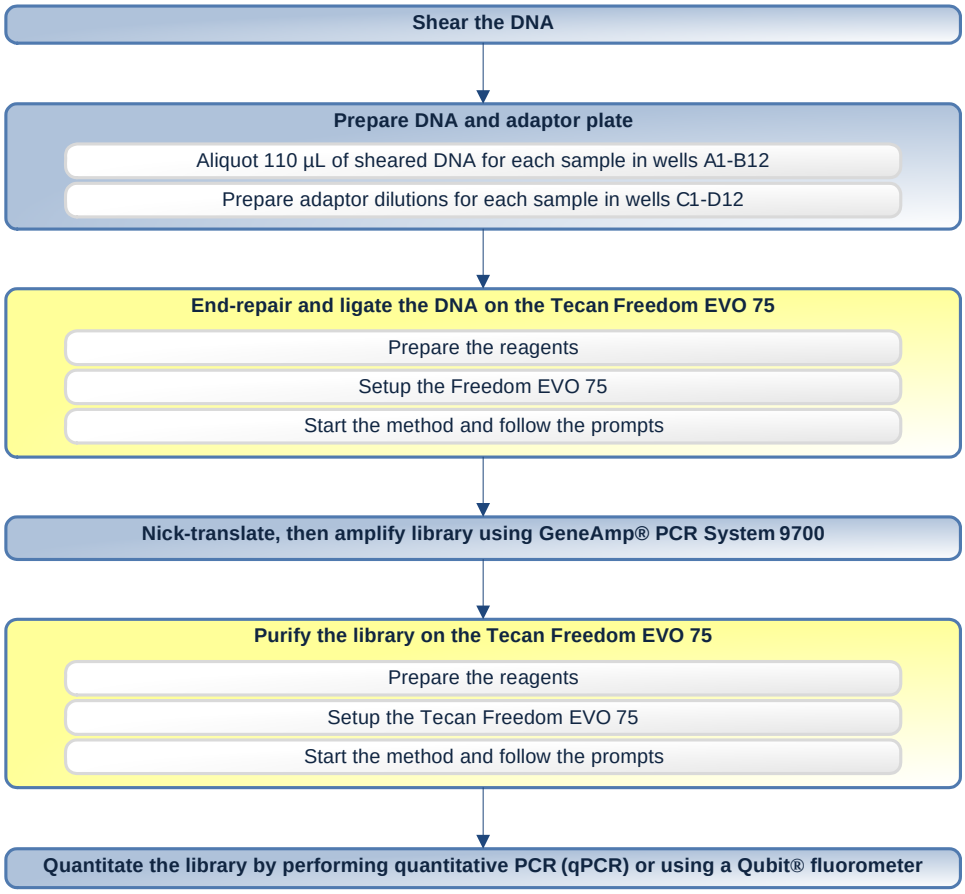


Figure 1 Fragment library preparation workflow with the steps performed on the Freedom EVO 75 displayed in yellow.

Shear the DNA

DNA is sheared by sonication into small fragments with a mean fragment size of 165 bp and a fragment size range of 150 to 180 bp (before adaptor ligation) using the Covaris® S2 System. The conditions have been tested for shearing 10 ng to 5 µg DNA in a total volume of 120 µL. For certain DNA samples, optimizing the shearing protocol may be necessary.

End-repair and ligate DNA on the Freedom EVO 75

This step is performed on the Freedom EVO 75. End Polishing Enzyme 1 and End Polishing Enzyme 2 are used to convert DNA that has damaged or incompatible 5′ -protruding and/or 3′ -protruding ends to 5′ -phosphorylated, blunt-ended DNA. End Polishing Enzyme 1 and ATP are also included for phosphorylation of the 5′ -ends of the blunt-ended DNA to allow for subsequent ligation. AMPure® XP beads are used to size select and purify the DNA after the end-repair. P1 and P2 Adaptors[1] for fragment libraries or Multiplex P1 and P2 Adaptors for barcoded fragment libraries[2] are ligated to the ends of the end-repaired DNA.

The adaptor-ligated DNA is purified twice using AMPure® XP beads. An optional PCR set up step aliquots a PCR master mix for subsequent nick-translation and library amplification performed in a thermal cycler.

Nick-translate, then amplify the library

The library is nick-translated and then amplified using Library PCR Primer 1 and Library PCR Primer 2 (standard fragment library) or Multiplex Library PCR Primer 1 and Multiplex Library PCR Primer 2 (barcoded fragment library) and Platinum® PCR Amplification Mix.

Purify the library on the Freedom EVO 75

This step is performed on the Freedom EVO 75. After amplification, the PCR amplified library is purified using AMPure® XP beads.

Quantitate the library

Quantitate the library by either quantitative PCR (qPCR) or using the Qubit® fluorometer. For qPCR, the SOLiD™ Library TaqMan® Quantitation Kit (PN 4449639) is recommended for accurate library quantitation. For the Qubit® fluorometer, use the dsDNA HS assay or the Quant-iT™ PicoGreen® reagent.

Tips

Reagents

- Thaw reagents on ice before use.
- Always prepare fresh 70% ethanol daily. Ethanol that is old and has absorbed water from the atmosphere, may decrease DNA recovery.

1 The P1 and P2 Adaptors are included in double-stranded form in the SOLiD® Fragment Library Oligos Kit.

2 Multiplex P1 and P2 Adaptors are included in double-stranded form in the SOLiD® Fragment Library Barcoding Modules 1-96, or any of the individual 16-barcode SOLiD Fragment Library Barcoding Modules. You can design experiments to use as few as 4 barcodes, as long as at least one of the following full sets of four barcodes are used: Barcodes 1-4, 5-8, 9-12, 13-16, 17-20, 21-24, 25-28, 29-32, 33-36, 37-40, 41-44, 45-48, 49-52, 53-56, 57-60, 61-64, 65-68, 69-72, 73-76, 77-80, 81-84, 85-88, 89-92, or 93-96.

Tecan Freedom EVO 75

- Always remove the 100-mL reagent troughs and 15-mL conical tubes from the worktable to refill them.
- Enzyme mixes include a 200 µL overfill volume, Buffers include a 500 µL overfill volume and ethanol includes a 5 mL overfill volume.
- Never *stop* a run in the middle of a loop. The software will not restart the loop from the appropriate sample number. Always *pause* to troubleshoot.
- Applied Biosystems has tested this protocol using materials specified in "Materials and equipment required" on page 2. Substitution may adversely affect system performance, and may damage a consumable(s) and/or spill a reagent.

Scripts for the Freedom EVO 75

Available scripts and files

The following scripts and files are available for importing on the Freedom EVO 75:

Table 1 Available scripts and files, and their function

Script name	Use to...
SOLiD4_Frag_Part1.esc	Perform end-repair, size selection, ligation and PCR setup for up to 24 samples
SOLiD4_Frag_Part2.esc	Perform post-PCR cleanup for up to 24 samples
Alignment.esc	Test and correct the alignment of the robot arm
DailyStartUpEVO75.esc	Set up the system for the first run of the day
FlushEVO75.esc	Flush the lines and prime the system
File name	**Use to...**
SOLiD 4 Adaptor Calculator for Tecan EVO75.xls	Calculate adaptor dilutions and reagent volumes

How to obtain the scripts

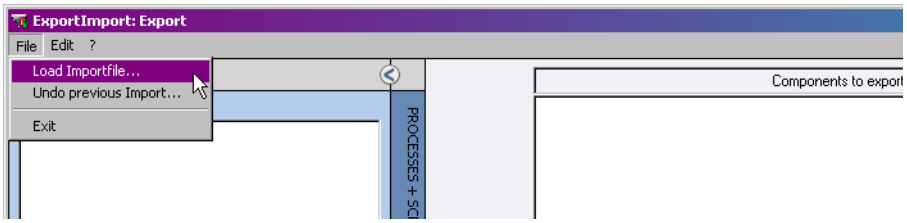
Contact your Applied Biosystems field application specialist to obtain the SOLiD scripts. All scripts are in one file named **SOLiD4_Frag_24.exd**.

Import the scripts

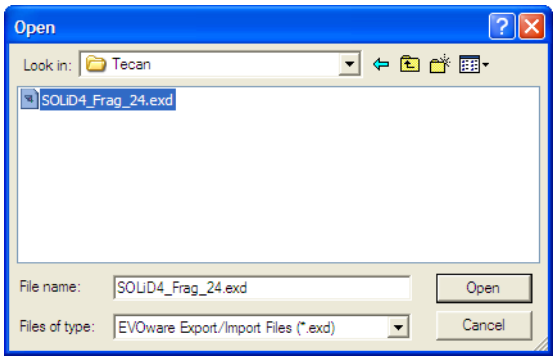
1. Power on the computer.
2. To start the Export/Import Tool, select **Start ▸ All Programs ▸ Tecan EVOware ▸ Export/Import**.

3. Import the scripts.

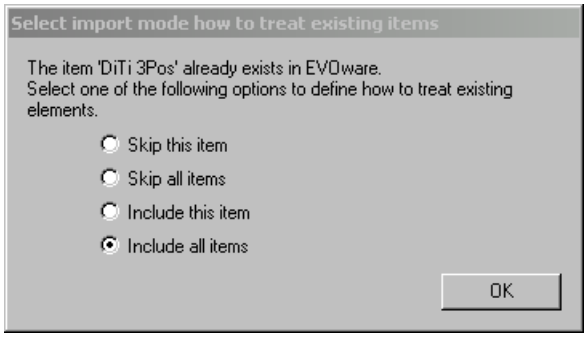
   a. Click **File ▸ LoadImportfile**.



   b. Select **SOLiD4_Frag_24.exd** and click **Open**.

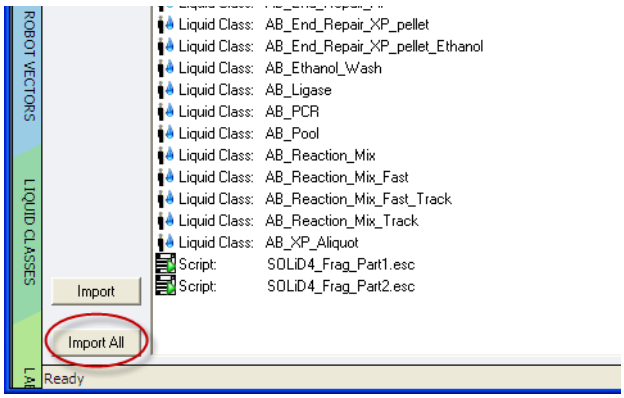


   c. Select **Include all items** and click **OK**.



   All the processes and scripts populate the ExportImport: Import window.

   d. In the ExportImport: Import window, click **Import All**.



   A backup file is created and when import is completed a popup will appear stating that process was successfully completed.

e. Click **OK**.

4. Close the Export/Import tool.

Safety

Refer to the complete safety alert descriptions in Appendix I, “Safety” on page 248 in the *Applied Biosystems SOLiD® 4 System Library Preparation Guide* (PN 4445673).

Understanding how the robotic arm transfers samples and reagents

The Freedom EVO 75 uses a robotic arm equipped with two tips so that two samples can be processed simultaneously. The spacing between the tips is fixed.

The scripts are written so that samples are processed in the most efficient manner. DNA and adaptors are loaded across the columns, the robot rearranges the samples to minimize samples processing time. The samples are returned to the original arrangement following processing. See Appendix B for more detail.

Prepare DNA, adaptors and reagents for end-repair and ligation

Shear the DNA

1. Dilute the desired amount of DNA to 120 µL in 1X Low TE Buffer in a LoBind tube (see Table 2).

Table 2 DNA dilution

Component	Amount
DNA	10 ng to 5 µg
1X Low TE Buffer	Variable
Total	120

Note: Increase volume of DNA to be sheared from 100 µL to 120 µL to decrease chances of an air bubble causing problems with shearing.

2. Follow the method for shearing the DNA as described in Chapter 2 of the *Applied Biosystems SOLiD® 4 System Library Preparation Guide* (PN 4445673).

If preparing...	Then go to page...
Standard fragment library	16
Barcoded fragment library	135

3. Transfer 110 µL for each sample to Eppendorf 96/500-µL DNA LoBind plate as shown in Figure 2.

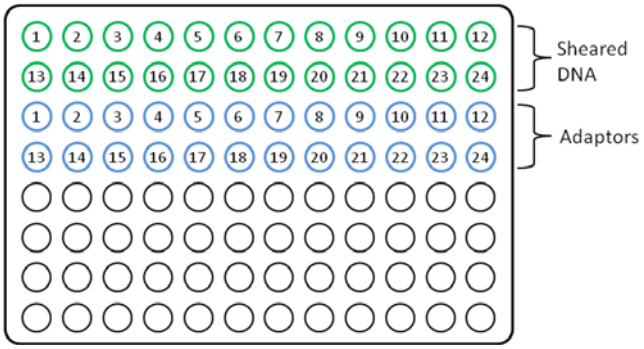


Figure 2 Eppendorf 96/500-µL plate with sheared DNA and Adaptors

Prepare adaptors

1. Open the file SOLiD4 Adaptor Calculator for Tecan EVO 75.xls and **Enable Macros**.

Applied Biosystems® SOLiD™ 4 System

Adaptor Calculator Tool Instructions:
Fill in library information in white cells. Calculated values will automatically appear in yellow cells.

Platform: SOLiD™ 4
Protocol: Standard

Reset Form

Final PCR Reaction Volume: 450

Sample	DNA Position	Adaptor Position	Input DNA (µg)	Adaptor Type	BC #	P1 Adaptor (µL)	P2 Adaptor (µL)	1x Low TE (µL)
1	A1	C1	5	Barcoded	1	2.76	24.80	47.4
2	A2	C2	1	Barcoded	2	1.65	14.88	58.5
3	A3	C3	0.05	Standard		0.50	0.50	74.0
4	A4	C4						
5	A5	C5						
6	A6	C6						
7	A7	C7						
8	A8	C8						
9	A9	C9						
10	A10	C10						
11	A11	C11						
12	A12	C12						
13	B1	D1						
14	B2	D2						
15	B3	D3						
16	B4	D4						
17	B5	D5						
18	B6	D6						
19	B7	D7						
20	B8	D8						
21	B9	D9						
22	B10	D10						
23	B11	D11						
24	B12	D12						

Component	Required Volume (µL)
End Repair	***470***
5x Ligase Buffer	209
dNTP mix, 10 mM	42
End Polishing Enzyme 1, 10 U/µL	21
End Polishing Enzyme 2, 5 U/µL	84
Nuclease-free Water	115
Ligation	***350***
5x Ligase Buffer	280
T4 Ligase, 5 U/µL	70
PCR - *Optional*	***1700***
Platinum PCR Mix	1615
PCR Primer 1, 50µM	43
PCR Primer 2, 50µM	43
AMPure XP, Part 1	1730
Elution Buffer, Part 1	1325
70% Ethanol, Part 1	8600
AMPure XP, Tube 1, Part 2	2525
AMPure XP, Tube 2, Part 2	0
Elution Buffer, Part 2	1100
70% Ethanol, Part 2	7400

2. Select the library type from the drop-down list in cell B6.

3. Enter the starting amount of DNA (in µg) for each library in column D.
4. Enter the adaptor type in column E.

   Note: Both standard and barcoded adaptors may be used in the same run.

5. *Optional:* Enter the barcode used for each sample in column F.
6. *Optional:* Enter the final PCR Reaction Volumes in cell N5. The adaptor dilutions and all master mixes will be automatically calculated for both Part 1 and Part 2 scripts.

   Note: For detailed calculations, see "Appendix E: Calculations for reagents" on page 39.

7. For each DNA sample, add the calculated volume of adaptor and 1X Low TE buffer to the appropriate well of the LoBind plate. Place on ice until ready to use.

Prepare the reagents

1. Prepare the end-repair mix, ligation mix, and optional PCR master mix in 15-mL conical tubes.

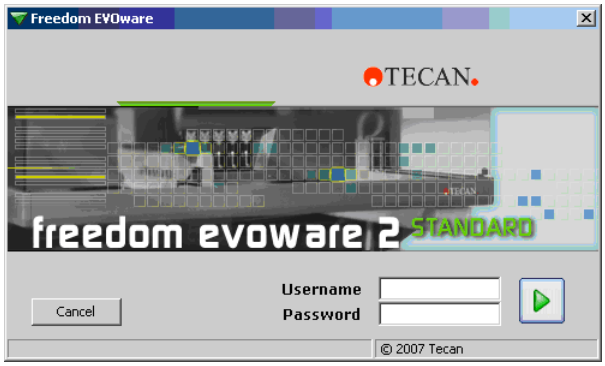
   IMPORTANT! Keep the tubes on ice until prompted by the script to place each tube on the worktable. The PCR efficiency is greatly decreased if the master mix sits at room temperature for extended times.

2. Prepare the AMPure XP beads and Elution buffer in 15-mL conical tubes.
3. Prepare the 70% Ethanol in a 100-mL trough.

End-repair and purify the DNA using the Freedom EVO 75 and SOLiD4_Frag_Part1 Script

Setup the Freedom EVO 75

1. Power on the instrument and computer.
2. On your desktop, click  to launch the Freedom EVOware 2 Standard software, then enter your user name and password.

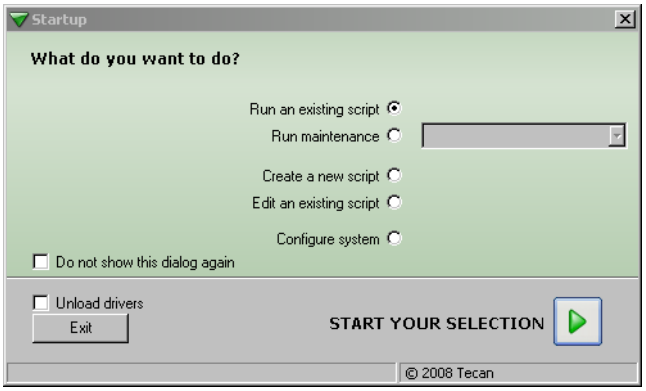


3. Click  .
4. If necessary, run one of the following scripts (see "Appendix D: Maintenance scripts" on page 35 for instructions).

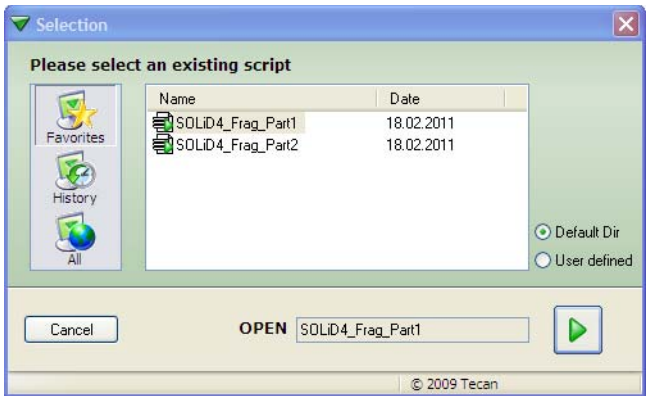
If the Freedom EVO 75 has not been used...	Then run the...
Today	DailyStartUpEVO75 script
For several hours	FlushEVO75 script, and if necessary, refill the DiTi racks

Start script 1

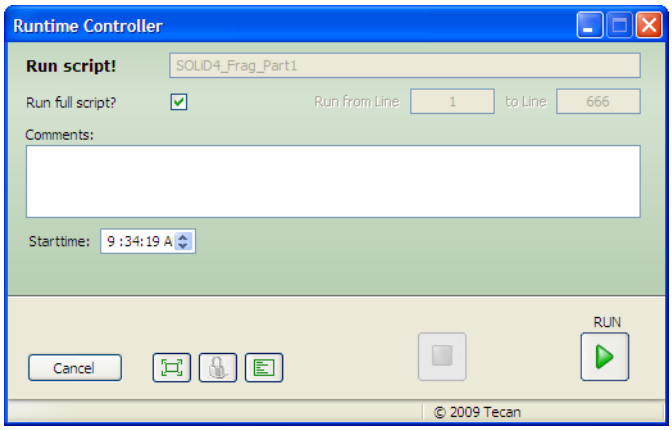
1. Select and start the **SOLiD4_Frag_Part1** script.
   a. Select **Run an existing script**, then click  .



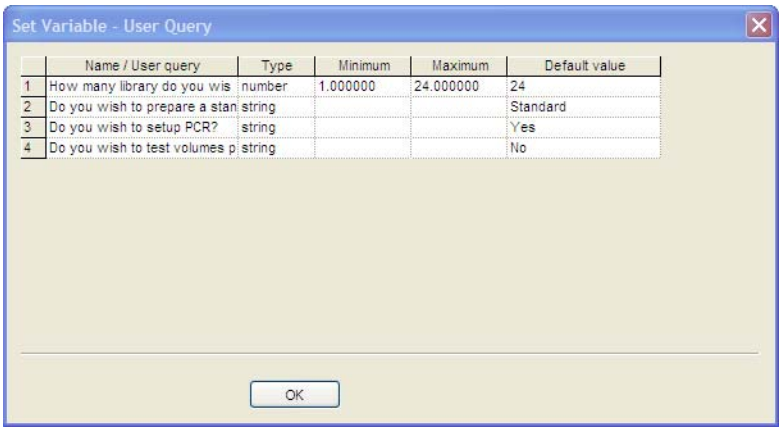
   b. In the Selection dialog box, select **SOLiD4_Frag_Part1** and click  to open the selected script



c. In the Runtime Controller dialog box, click  to run the script. Wait for the system to complete the initialization.



d. In the Set Variable - User Query dialog box:



- Enter the number of samples in the Default value column. 1 to 24 samples can be processed at one time.
- Enter the library type. Enter Standard for size selected libraries or Express for express libraries.

- If you wish to setup PCR, enter Yes, otherwise enter No.

  Determining the appropriate PCR vessel:

Starting Amount of DNA	Number of Cycles
2 µg to 5 µg, standard adaptors	2 to 3 cycles
2 µg to 5 µg, barcoded adaptors	3 to 6 cycles
1 µg to 2 µg	4 to 6 cycles
100 ng to 1 µg	6 to 8 cycles
10 ng to 100 ng	8 to 10 cycles

  Plan PCR vessel: Do one of the following depending on whether or not all the DNA samples will be amplified using the same cycle number as determined above.

If the amplification cycle number is the…	Then…
Same	Use a 96-well plate or an 8-well strip tube in a MicroAMP® base (place at grid 22, site 2) depending on user preference. PCR will be setup according to figure 4. Each sample requires 4 reaction tubes.
Different	Use 8-well strip tubes (or parts of a strip) in a MicroAMP® base (place at grid 22, site 2). PCR will be setup according to figure 4. Each sample requires 4 reaction tubes.

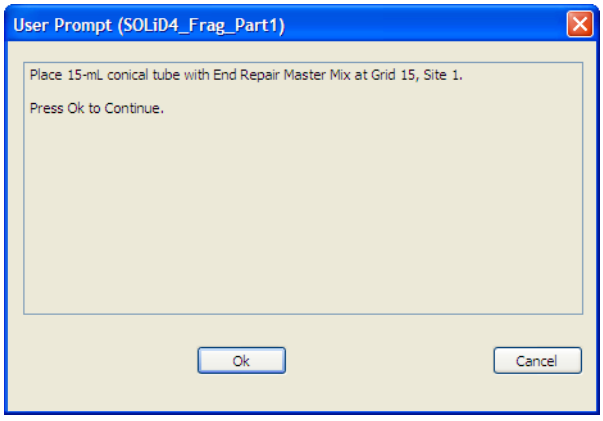
- To perform a liquid volume check prior to starting the method enter Yes otherwise enter No.

  Note: Do not change the default status to Yes in the script. This will cause an endless loop error. Only change to Yes when prompted at the beginning of the run.

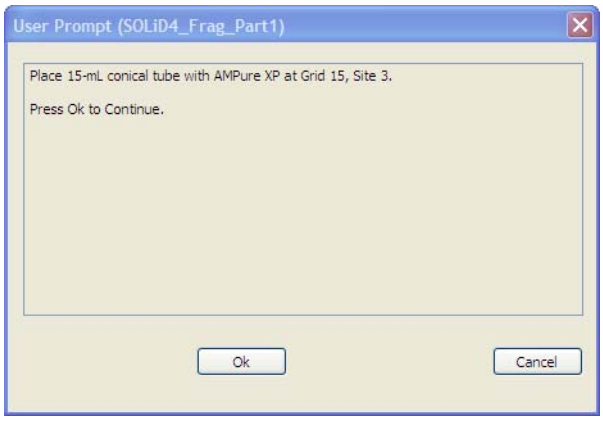
Setup the worktable

1. Follow the prompts to setup the worktable (refer to Figure 3 on page 29.)

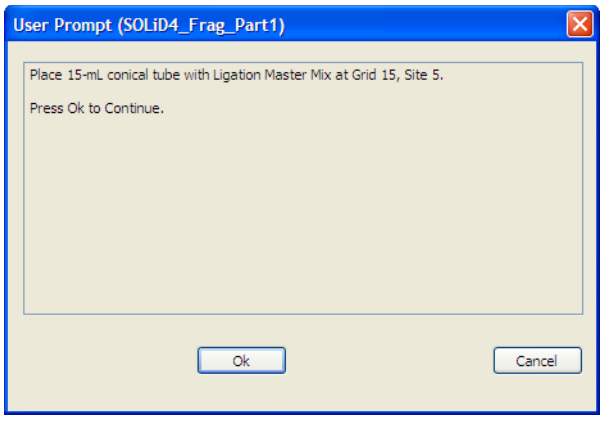
   a. Place a 15-mL conical tube with End Repair master mix at Grid 15, site 1, then click **Ok**.



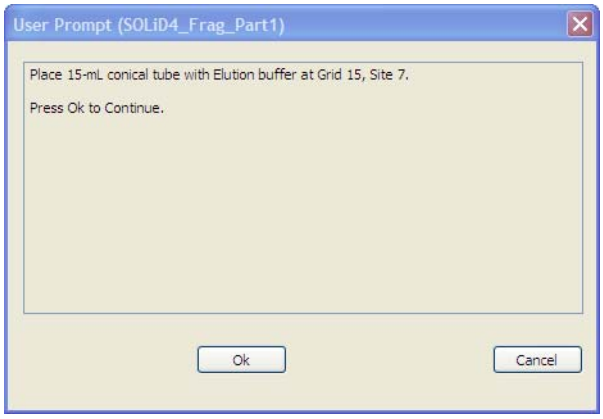
   b. Place a 15-mL conical tube with AMPure® XP at Grid 15, site 3, then click **Ok**.



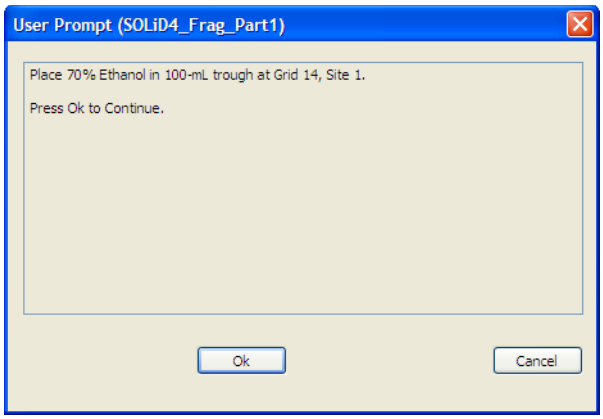
   c. Place a 15-mL conical tube with Ligation master mix at Grid 15, site 5, then click **Ok**.



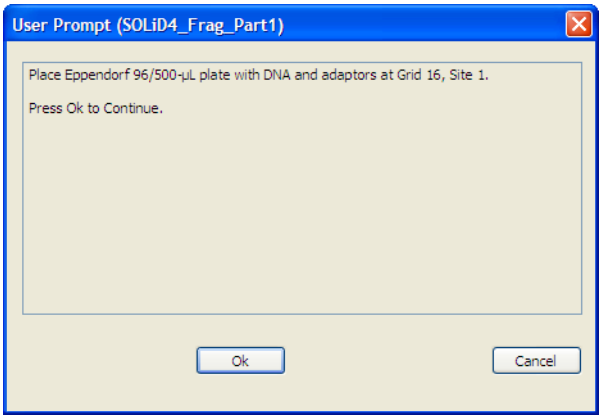
d. Place a 15-mL conical tube with Elution buffer at Grid 15, site 7, then click **Ok**.



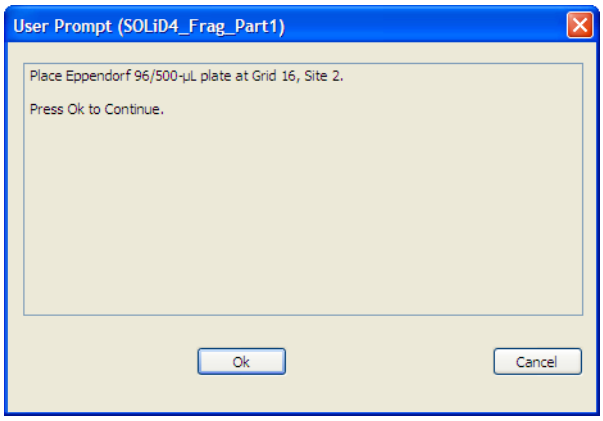
e. Place a 100-mL trough with 70% Ethanol at Grid 14, site 1, then click **Ok**.



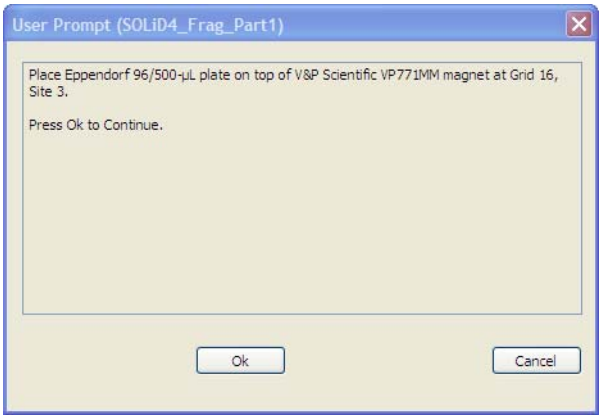
f. Place Eppendorf® 96/500-µL plate with DNA and adaptors at Grid 16, site 1, then click **Ok**.



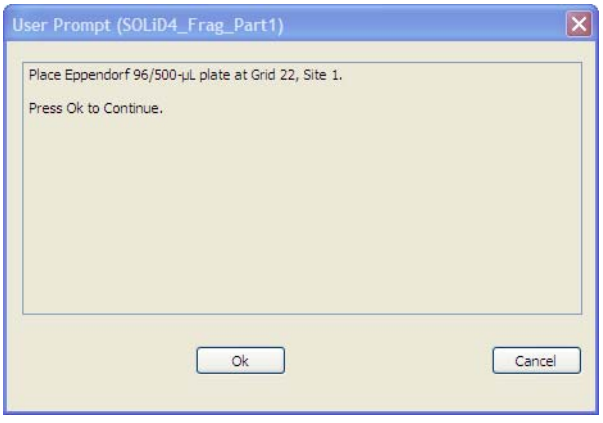
g. Place Eppendorf 96/500-µL plate Grid 16, site 2, then click **Ok**.



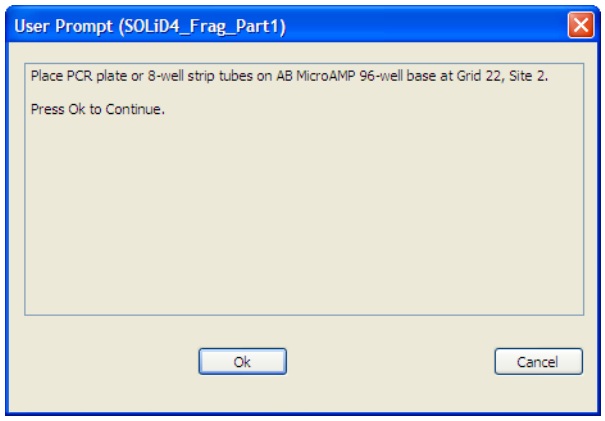
h. Place Eppendorf 96/500-µL plate on top of VP771MM magnet at Grid 16, site 3, then click **Ok**.



i. If PCR is not selected, place Eppendorf 96/500-µL plate at Grid 22, site 1, then click **Ok**.



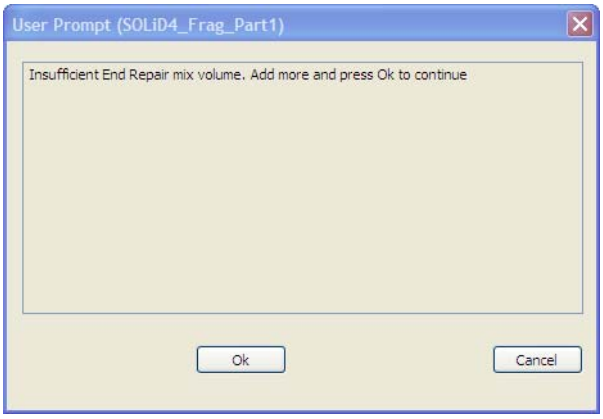
j. If PCR is selected, place PCR plate/tubes at Grid 22, site 2, then click **Ok**.



2. Reagent Volume Test, Optional

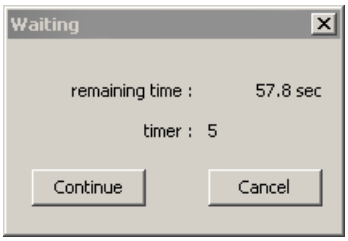
   a. Prior to beginning the method, and optional volume test can be performed to ensure sufficient reagents have been added for each process.

   b. An error message will appear if a reagent volume is too low. Add more reagent to the tube to proceed, the volume will be rechecked.



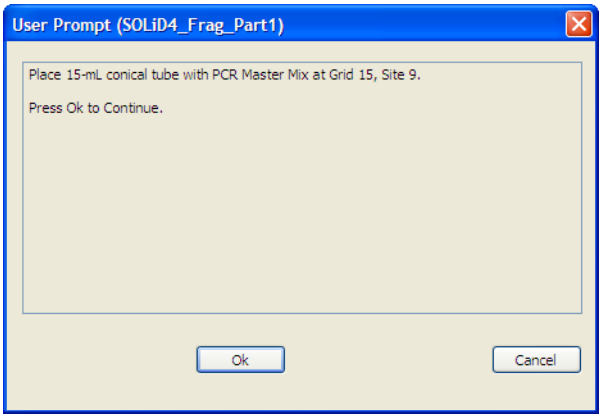
Note: When preparing less than 8 samples, the script may underestimate the volume in the 15-mL conical tube. If this occurs, lift the tubes up in the carrier2-3 mm and retry liquid detection.

3. Throughout the script, various timers are displayed for the incubation steps, The times vary depending on the process.



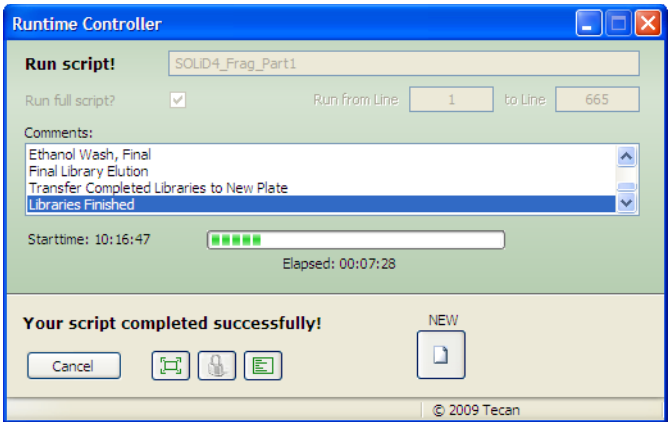
IMPORTANT! Let the timer run its course. Do NOT click Continue or Cancel. The script continues once the timer finishes.

4. If PCR is selected, place a 15-mL conical tube containing PCR master mix at Grid 15, site 9, then click **Ok**.



Note: This prompt will appear near the end of the method. Depending on the number of samples being processed this will occur after 2.5 - 4 hours.

5. When the run is completed, click **Cancel** to exit the dialog box.



6. Select File ▸ Exit, unload drivers.

IMPORTANT! Exit the Freedom EVOware software and unload drivers at least once daily when running the scripts.

7. Remove and properly dispose of leftover reagents and used plasticware.

8. Do one of the following:

If PCR set up was...	Then go to...
selected	"Nick-translate and amplify the DNA" on page 20
not selected	proceed to step 9

9. If PCR set up was not selected, remove finished libraries from Grid 22, Site 1, cover with foil tape. Store at 4°C until ready to use or proceed to page 27 (standard adaptors) or page 140 (barcodes adaptors) of the *Applied Biosystems SOLiD™ 4 System Library Preparation Guide* (PN 4445673) to set up the PCR reactions.

Nick-translate and amplify the DNA

IMPORTANT! If the PCR was not set up in the last method, proceed to page 27 (standard adaptors) or page 140 (barcodes adaptors) of the *Applied Biosystems SOLiD™ 4 System Library Preparation Guide* (PN 4445673) to set up the PCR reactions.

1. Program the thermal cycler(s) for the conditions in Table 3. Determine the number of cycles based on the amount of starting input DNA.

   IMPORTANT! Minimize the number of cycles to avoid over amplification and production of redundant molecules.

Table 3 PCR conditions to nick-translate and amplify the library

Stage	Step	Temp	Time
Holding	Nick translation	72°C	20 min
Holding	Denature	95°C	5 min
Cycling: • Standard: 2 to 10 cycles‡ • Barcoded: 3 to 10 cycles§	Denature	95°C	15 sec
	Anneal	62°C	15 sec
	Extend	70°C	1 min
Holding	Extend	70°C	5 min
Holding	Holding	4°C	∞

‡ Starting amount of DNA: number of cycles for standard samples:
10 ng to 100 ng: 10 cycles
100 ng to 1 µg: 6 to 8 cycles
1 µg to 2 µg: 4 to 6 cycles
2 µg to 5 µg: 2 to 3 cycles

§ Starting amount of DNA: number of cycles for barcoded samples:
10 ng to 100 ng: 10 cycles
100 ng to 1 µg: 6 to 8 cycles
1 µg to 2 µg: 4 to 6 cycles
2 µg to 5 µg: 3 to 6 cycles

2. Transfer the 96-well plates or strip tubes to the thermal cycler(s) programmed in step 1.

3. Start the run(s). Set the ramp speed to 9600 and the volume to 50 µL.

STOPPING POINT. When the PCR reaction is complete, store the plate at 4 °C until ready to use or proceed to "Purify amplified DNA using the Freedom EVO 75 and SOLiD4_Frag_Part2 Script".

Purify amplified DNA using the Freedom EVO 75 and SOLiD4_Frag_Part2 Script

Prepare the reagents

1. Use the volumes calculated using SOLiD4 Adaptor Calculator for Tecan EVO 75 spreadsheet from Part 1 to prepare the reagents for Part 2.
   a. Prepare the AMPure XP beads and Elution buffer in 15-mL conical tubes.
   b. Prepare the 70% Ethanol in a 100-mL trough.

Setup the Freedom EVO 75

1. On your desktop, click  to launch the Freedom EVOware 2 Standard software, then enter your user name and password.

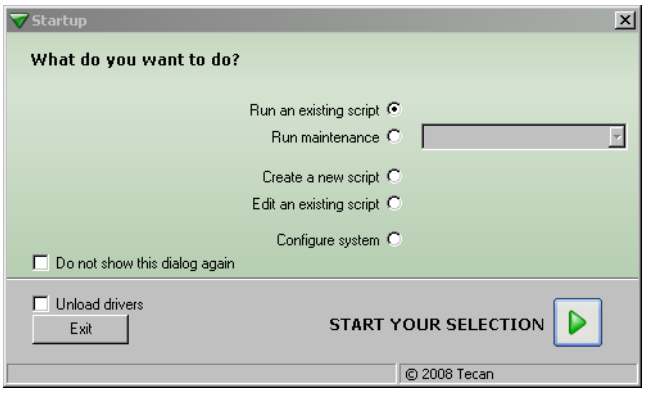
2. Click  .

3. If necessary, run one of the following scripts (see “Appendix D: Maintenance scripts” on page 35 for instructions).

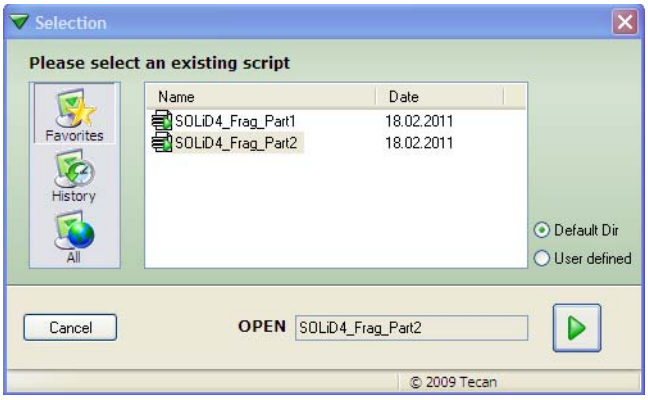
If the Freedom EVO 75 has not been used...	Then run the...
Today	DailyStartUpEVO75 script
For several hours	FlushEVO75 script, and if necessary, refill the DiTi racks

Start Script 2

1. Select and start the **SOLiD4_Frag_Part2** script.
   a. Select **Run an existing script**, then click  .

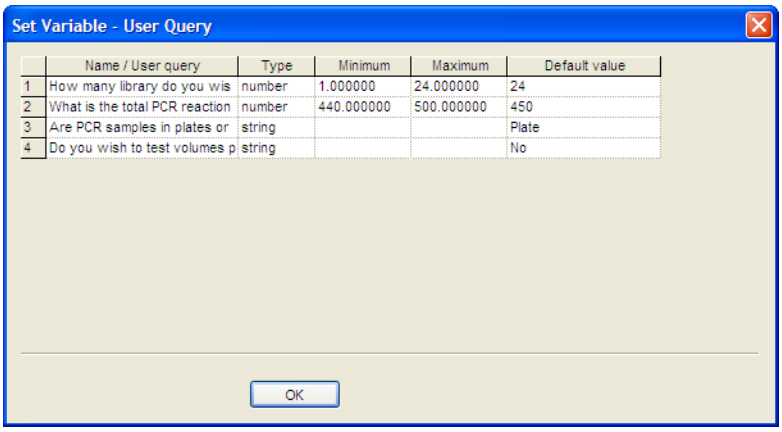


b. In the Selection dialog box, select **SOLiD4_Frag_Part2,** and click ▶ to open the selected script.



c. In the Runtime Controller dialog box, click ▶ to run the script. Wait for the system to complete the initialization.

d. In the Set Variable - User Query dialog box:



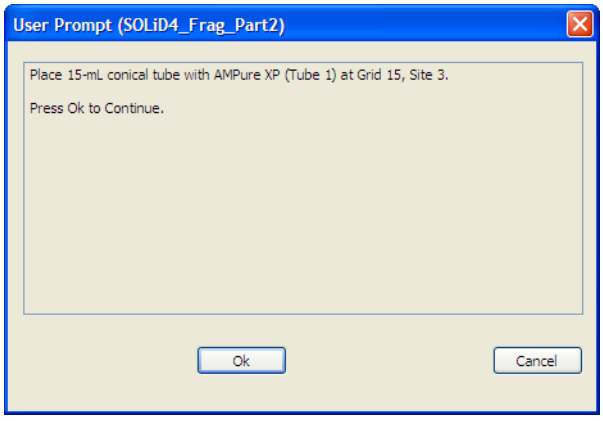
- Enter the PCR volume reaction size. If PCR is manually setup, the reaction volume can be 440 to 500 µL, divided equally in 4 tubes. The default value is 450 µL.
- Enter the reaction vessel type. Enter Plate for plates or Strip for 8-well striptubes.
- To perform a liquid volume check prior to starting the method enter Yes otherwise enter No.

Note: Do not change the default status to Yes in the script. This will cause an endless loop error. Only change toes when prompted at the beginning of the run.

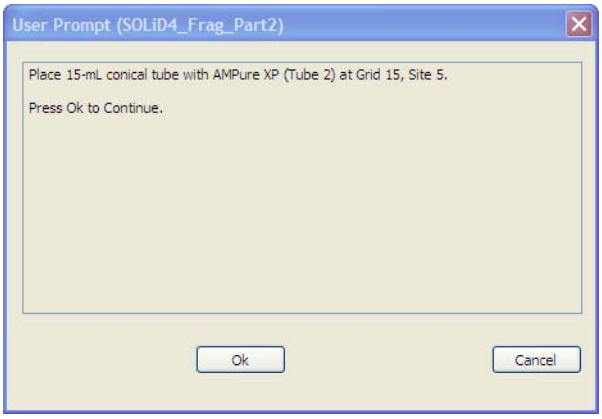
Setup the worktable

1. Follow the prompts to setup the worktable (refer to Figure 4 on page 30):

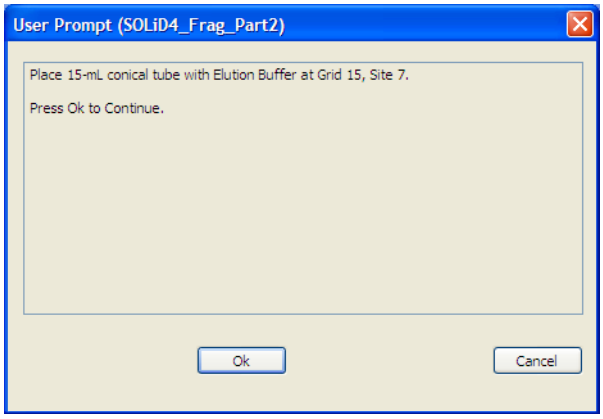
a. Place AMPure® XP (Tube 1) at Grid 15, site 3, then click **Ok**.



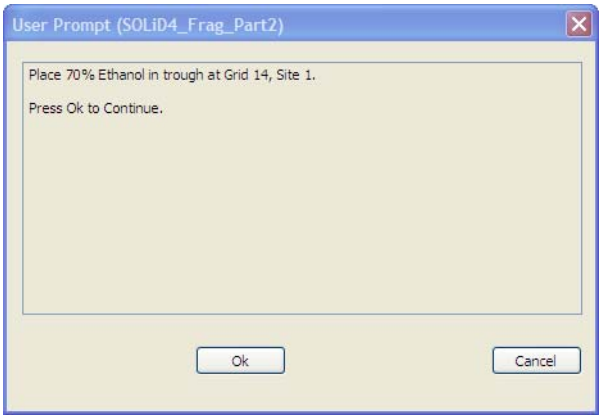
b. If more than 12 samples are being processed, place AMPure® XP (Tube 2) at Grid 15, site 5, then click **Ok**.



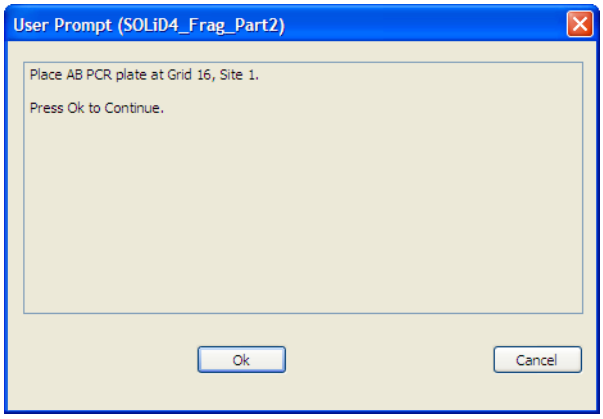
c. Place Elution buffer at Grid 15, site 7, then click **Ok**.



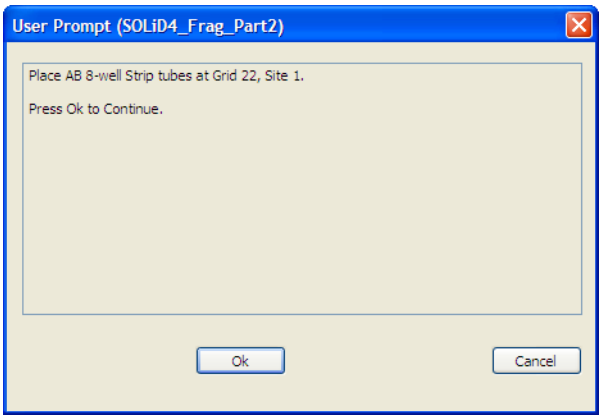
d. Place 70% Ethanol at Grid 14, site 1, then click **Ok**.



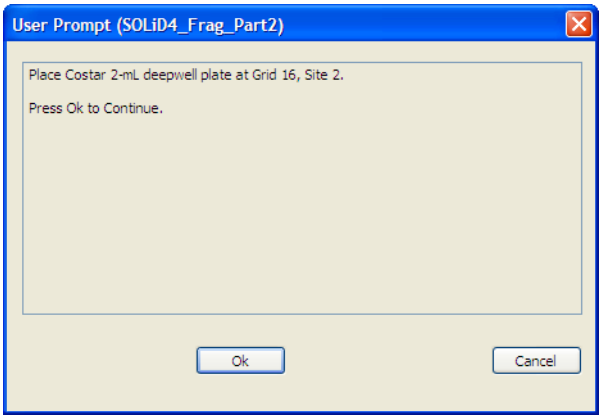
e. If PCR was performed in a plate, place PCR plate in a MicroAMP® base at Grid 16, site 1, then click **Ok**.



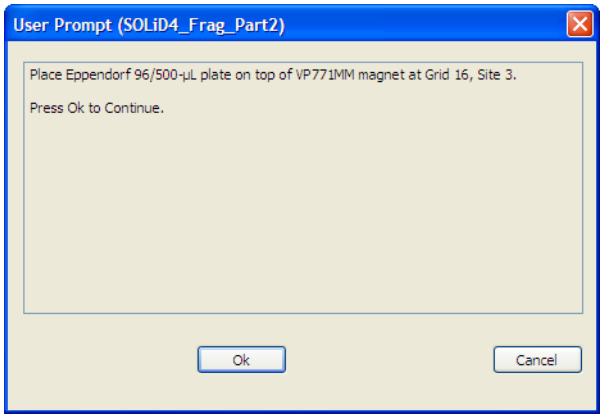
f. If PCR was performed in 8-well strips, place tubes in a MicroAMP® base at Grid 22, site 1, then click **Ok**.



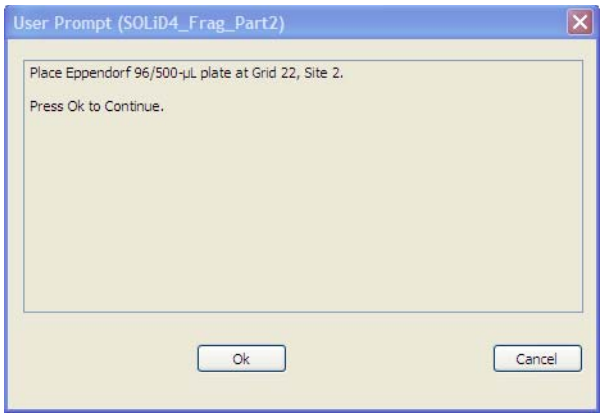
g. Place Corning 2-mL deepwell plate at Grid 16, site 2, then click **Ok**.



h. Place Eppendorf 96/500-µL plate on top of VP771MM magnet at Grid 16, site 3, then click **Ok**.



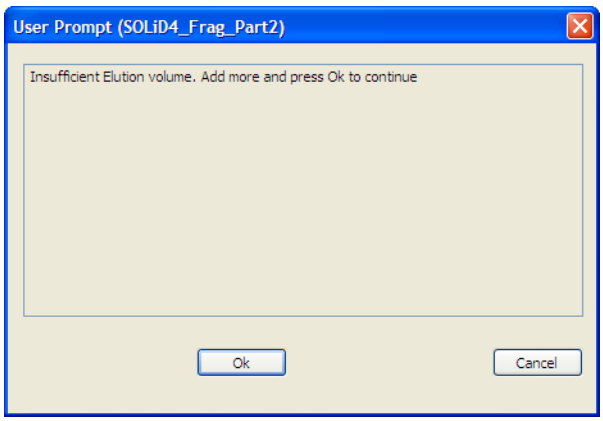
i. Place Eppendorf 96/500-µL plate at Grid 22, site 2, then click **Ok**.



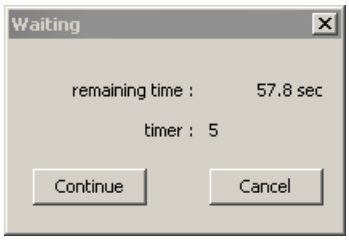
2. Reagent Volume Test, Optional

a. Prior to beginning the method, and optional volume test can be performed to ensure sufficient reagents have been added for each process.

b. An error message will appear if a reagent volume is too low. Add more reagent to the tube to proceed, the volume will be rechecked.

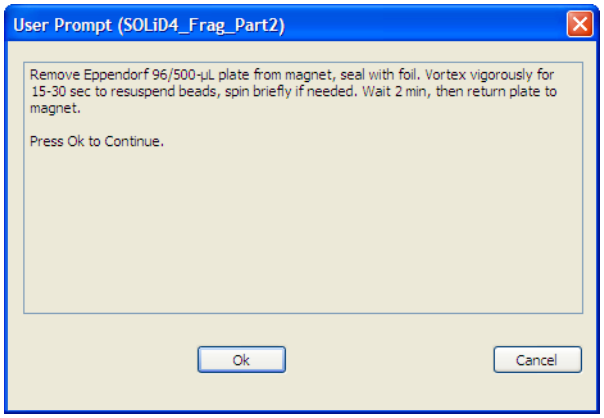


3. Throughout the script, various timers are displayed for the incubation steps, The times vary depending on the process.



IMPORTANT! Let the timer run its course. Do NOT click Continue or Cancel. The script continues once the timer finishes.

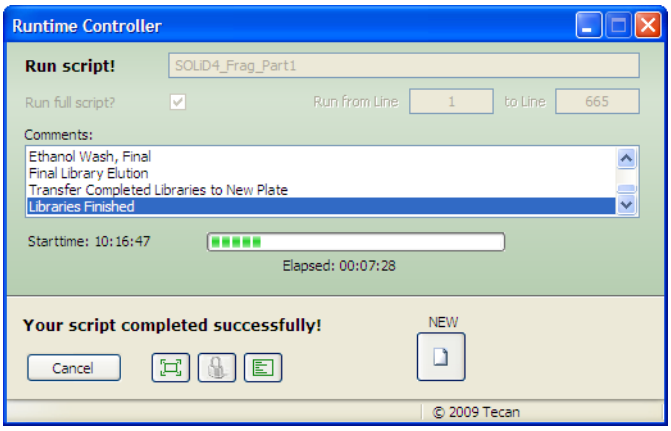
4. After elution buffer is added to the bead pellet:



a. Remove the Eppendorf 96/500-µL plate from the magnet.
b. Vortex vigorously for 15-30 seconds, spin briefly if needed.
c. Wait 2 minutes, then return to magnet.
d. Press **Ok** to finish the script.

IMPORTANT! Due to the large bead volume in this step, elution efficiency is very low if beads are not thoroughly resuspended. Elution efficiency is not affected with smaller bead volumes used in other scripts.

5. When the run is completed, click **Cancel** to exit the dialog box.



6. Select File ▸ Exit, unload drivers.

   IMPORTANT! Exit the Freedom EVOware software and unload drivers at least once daily when running the scripts.

7. Remove and properly dispose of leftover reagents and used plasticware.

8. Remove Purified Libraries at Grid 22, site 2, seal with foil.

STOPPING POINT. Store the purified DNA at 4 ºC, or proceed to "Quantitate the purified DNA."

Quantitate the purified DNA

1. Quantitate the library by either quantitative PCR (qPCR) or using the Qubit® fluorometer. For qPCR, the SOLiD™ Library TaqMan® Quantitation Kit (PN 4449639) is recommended for accurate library quantitation. For Qubit fluorometer, use 1 µL of the library and the dsDNA HS assay or the Quant-iT™ PicoGreen® reagent.

   STOPPING POINT. Store the purified DNA at – 20 °C, or proceed directly to emulsion PCR.

Appendix A: Worktable layouts for samples and reagents

Layout for SOLiD4_Frag_Part 1

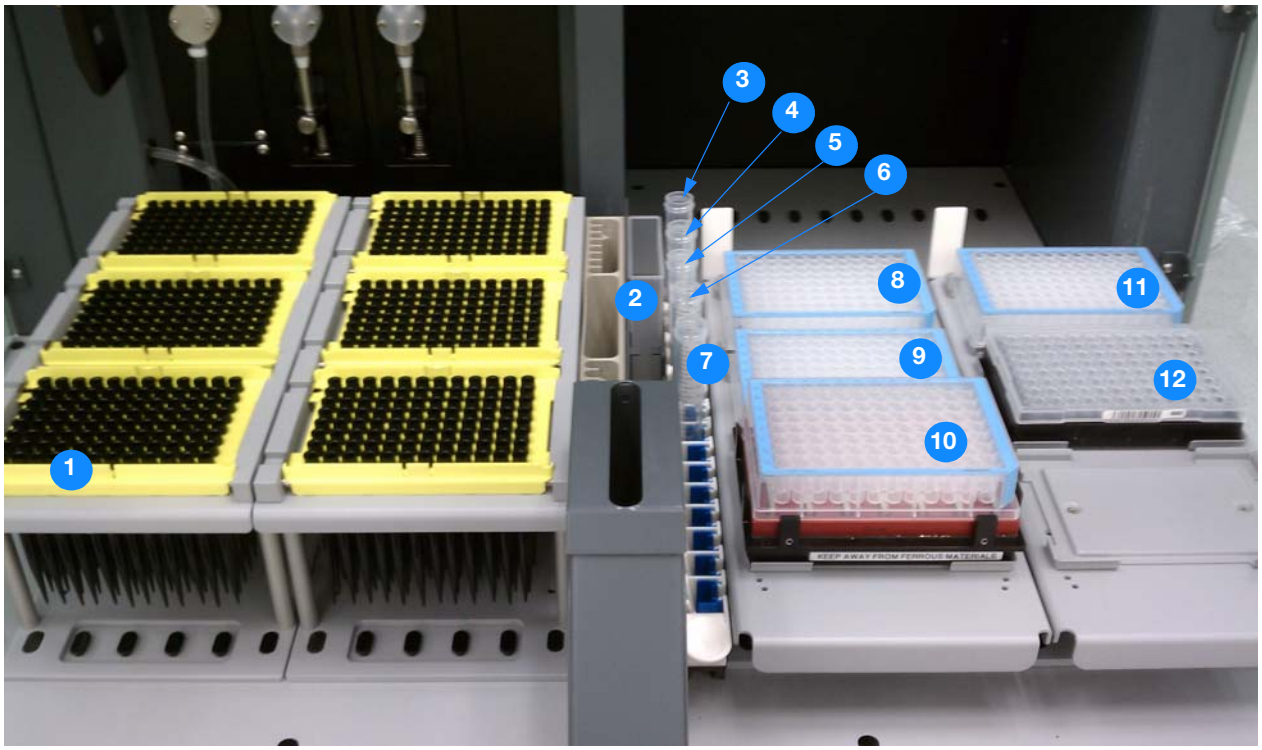


Figure 3 Layout of the worktable for Layout for SOLiD4_Frag_Part 1

For each position identified in Figure 3, the table below lists the reagents, and their worktable grid and site numbers.

Number	Item	Grid(s)	Site(s)
1	1000-µL Tecan Conductive Tips	1 7	1-3 1-3
2	100-mL trough containing 70% ethanol	14	1
3	15-mL conical tube containing End Repair Master Mix	15	1
4	15-mL conical tube containing AMPure® XP	15	3
5	15-mL conical tube containing Ligation Master Mix	15	5
6	15-mL conical tube containing Elution buffer	15	7
7	15-mL conical tube containing PCR Master Mix	15	9
8	Eppendorf® 96/500-µL plate with DNA and Adaptors	16	1
9	Eppendorf® 96/500-µL plate	16	2
10	Eppendorf® 96/500-µL plate on top of VP771MM magnet	16	3
11	Eppendorf® 96/500-µL plate	22	1
12	AB PCR plate or 8-well strips on MicroAMP® base	22	2

Layout for SOLiD4_Frag_Part 2

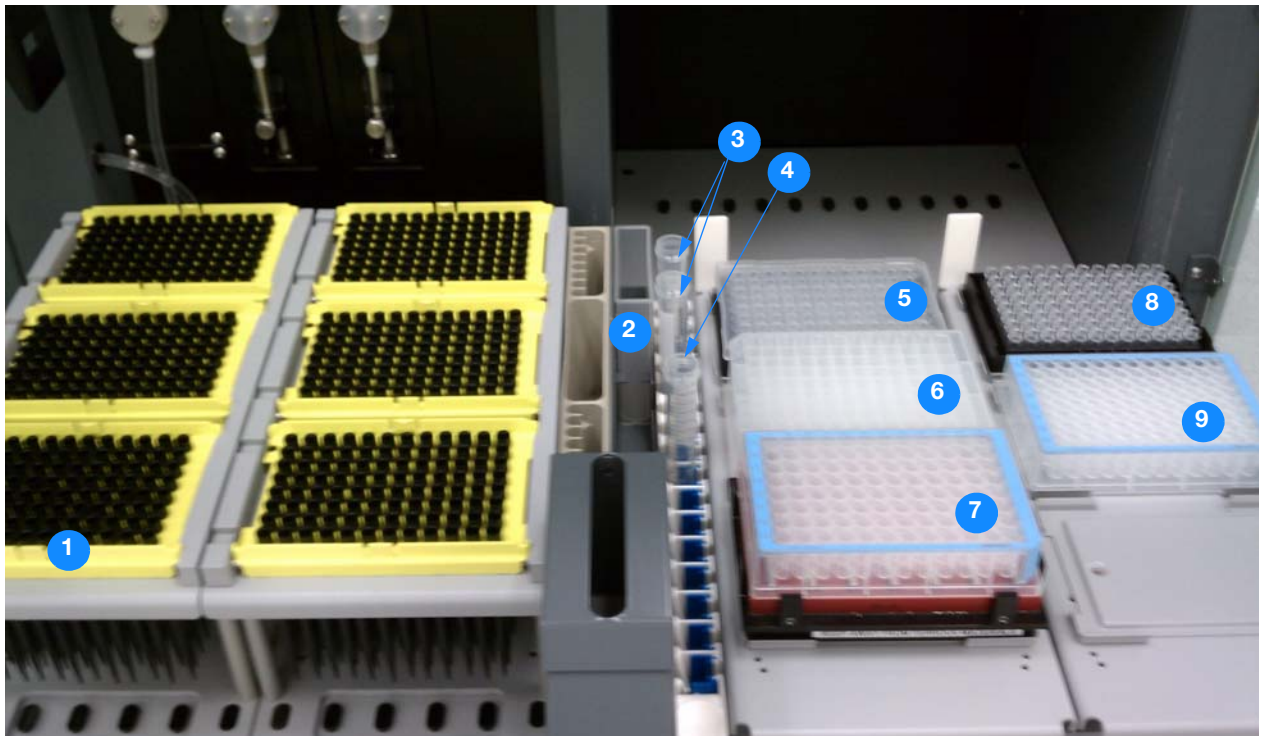


Figure 4 Layout of the worktable for Layout for SOLiD4_Frag_Part 2

For each position identified in Figure 4, the table below lists the reagents, and their worktable grid and site numbers.

Number	Item	Grid(s)	Site(s)
1	1000-µL Tecan Conductive Tips	1 7	1-3 1-3
2	100-mL trough containing 70% ethanol	14	1
3	15-mL conical tube containing AMPure® XP	15	3, 5
4	15-mL conical tube containing Elution buffer	15	7
5	AB PCR plate on MicroAMP base	16	1
6	Corning 2-mL Deepwell plate	16	2
7	Eppendorf® 96/500-µL plate on top of VP771MM magnet	16	3
8	8-well strips on MicroAMP base	22	1
9	Eppendorf® 96/500-µL plate	22	2

Appendix B: Sample locations during a script

Sample locations during SOLiD4_Frag_Part1

The scripts are written so that samples are processed in the most efficient manner. DNA and adaptors are loaded across the columns, the robot rearranges the samples to minimize samples processing time. The samples are returned to the original arrangement following processing.

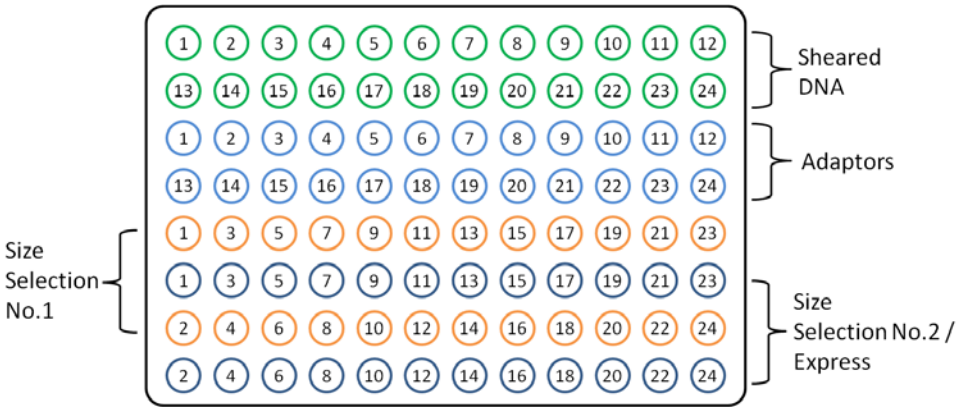


Figure 5 Eppendorf 96/500-µL plate at Grid 16, Site 1.

Figure 6 Eppendorf 96/500-µL plate at Grid 16, Site 2.

Figure 7 Eppendorf 96/500-µL plate on VP771M magnet at Grid 16, Site 3.

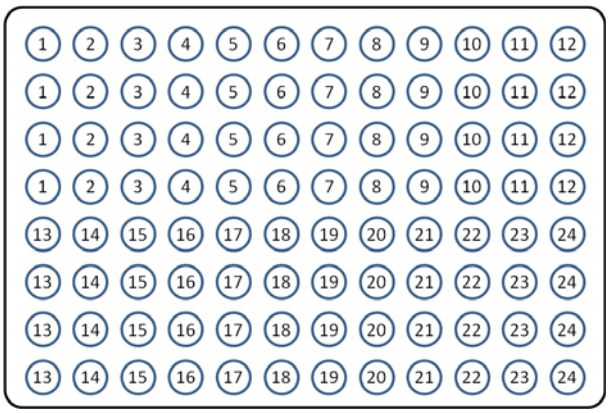


Figure 8 AB PCR plate at Grid 22, Site 2. Output format if PCR is selected.

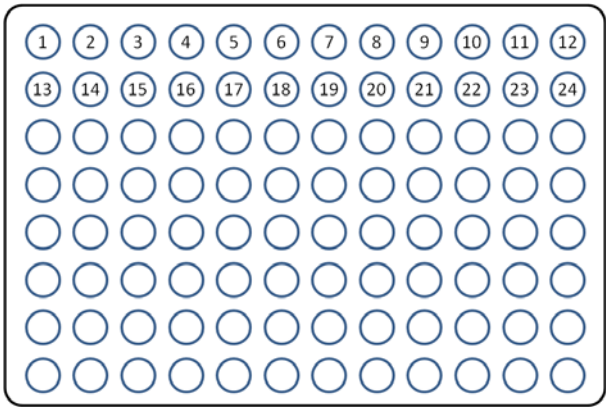


Figure 9 Eppendorf 96/500-µL plate at Grid 22, Site 1. Output format if PCR is not selected.

Sample locations during SOLiD4_Frag_Part2

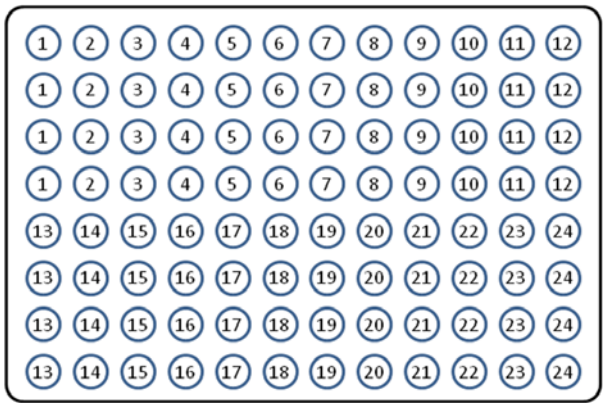


Figure 10 PCR layout for input.

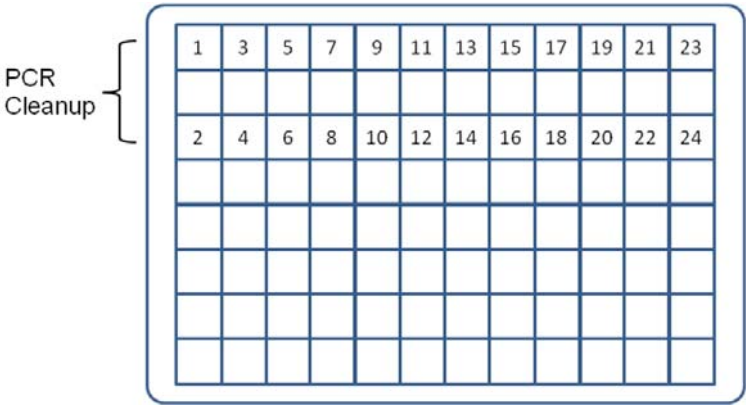


Figure 11 Corning 2-mL Deepwell plate during PCR reaction pooling and cleanup

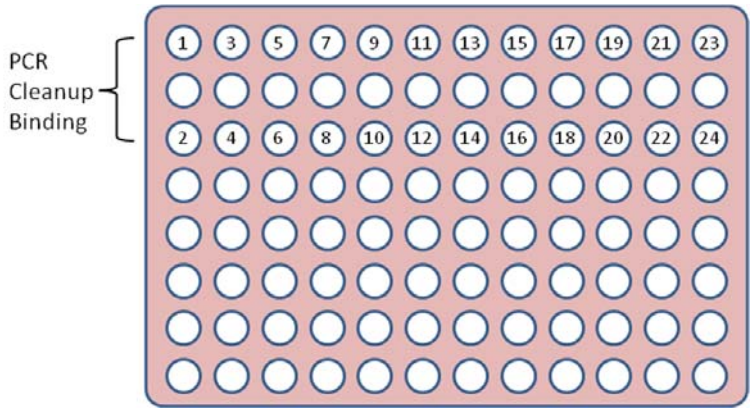


Figure 12 Eppendorf 96/500-µL plate on VP771MM magnet at Grid 16, Site 3

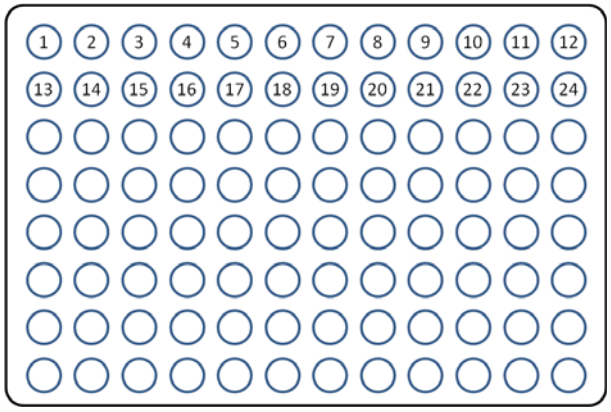


Figure 13 Eppendorf 96/500-µL plate at Grid 22, Site 2. Libraries are output in 100 µL elution buffer.

Appendix C: Number of required Tips for the Scripts

Script	Tips Use	Maximum (*n* = 24)
SOLiD4_Frag_Part1 (Standard Library)	(19 x *n*) + 19	475
SOLiD4_Frag_Part1 (Express Library)	(17 x *n*) + 19	427
SOLiD4_Frag_Part2	(8 x *n*) + 7	199

Appendix D: Maintenance scripts

There are four maintenance scripts:

Script name	Use to...
DailyStartUpEVO75.esc	Set up the system for the first run of the day
FlushEVO75.esc	Flush the lines and prime the system
Alignment.esc	Test and correct the alignment of the robot arm

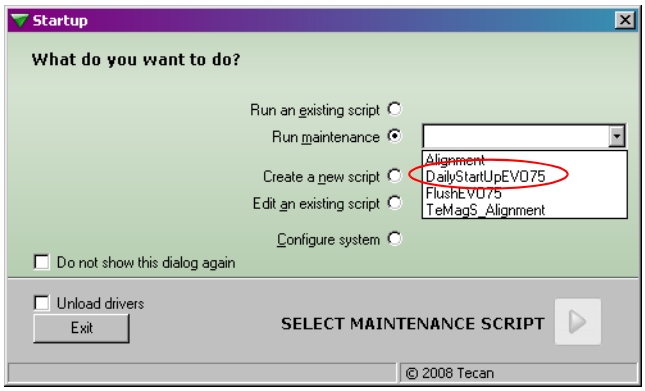
Freedom EVO 75 daily start up script

Prior to using the Freedom EVO 75 for the first time each day, run the start up script.

This script is a series of 9 prompts that cover the following tasks:

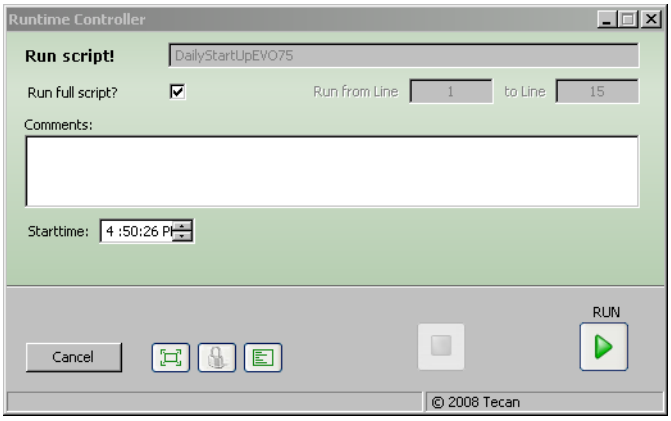
- Check system liquid volume level and refill, if necessary
- Check waste level and empty, if necessary
- Check the Diluter connections
- Replenish DiTis, if necessary
- Verify DiTi gold cones are tight and finger-tighten, if necessary
- Check carriers and labware positions
- Verify system tubing is not kinked and that the wash/waste station drains properly
- Flush the system
- Repeat flush, if air bubbles are still present

1. Select **Run maintenance**, then select **DailyStartUpEVO75** from the drop-down list.



2. Click  to open the script.

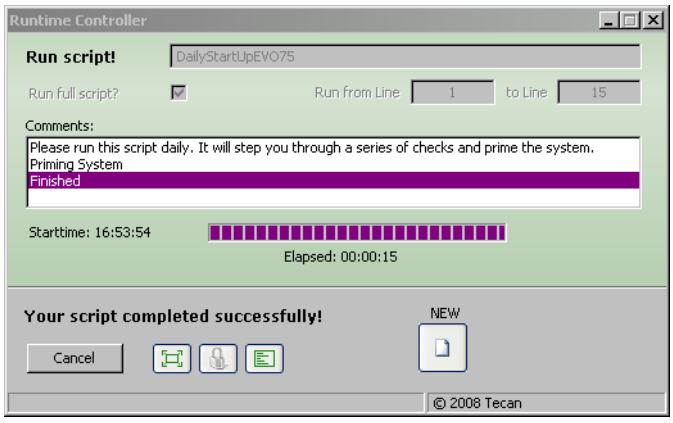
3. In the Runtime Controller dialog box, click  to run the script. Wait for the system to complete the initialization.



4. For each of the 9 prompts, perform the task, then click **Ok**.

   Note: If air bubbles are still present in the lines after Prompt 8, click **Ok** in Prompt 9 to finish the script, then run the flush script in the next section.

5. When the run is completed, click **Cancel** to exit the dialog box.



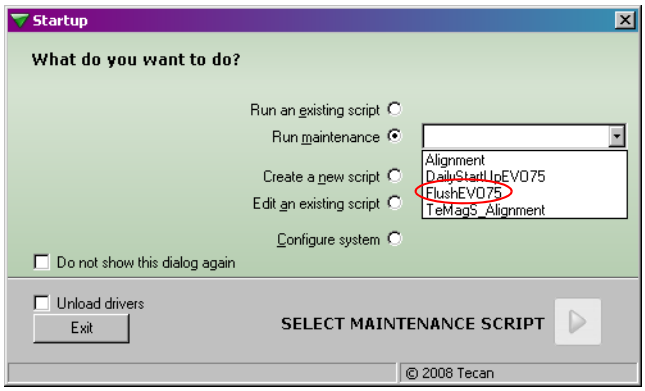
Freedom EVO 75 flush script

Run the flush script if the Freedom EVO 75 has been sitting for several hours or if the system flush performed in the start up script was not successful.

This script is a series of 2 prompts that cover the following tasks:

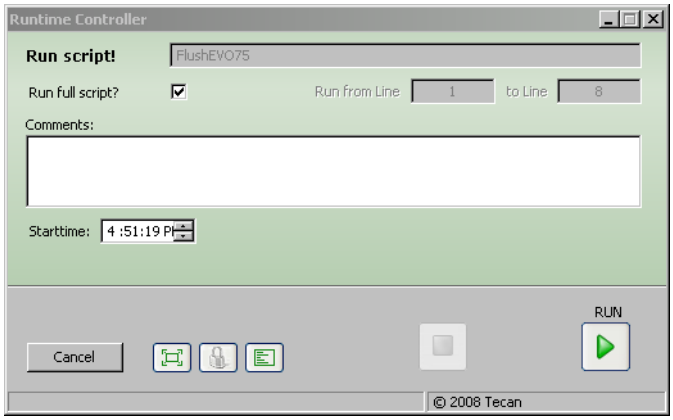
- Flush the liquid system with/without FastWash
- Reflush, if necessary

1. Select **Run maintenance**, then select **FlushEVO75** from the drop-down list.



2. Click  to open the script.

3. In the Runtime Controller dialog box, click  to run the script. Wait for the system to complete the initialization.



4. For each of the 2 prompts, perform the task, then click **Ok**.

   Note: If air bubbles are still present in the lines after Prompt 1, click Ok in Prompt 2 to finish the script, then run the flush script again or use the direct command.

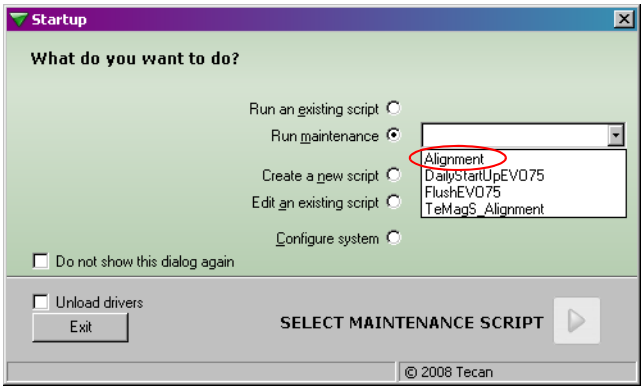
5. When the run is completed, click **Cancel** to exit the dialog box.

Freedom EVO 75 alignment script

Run the alignment script once a week or if you encounter problems with the alignment of the tips to any plasticware on the worktable.

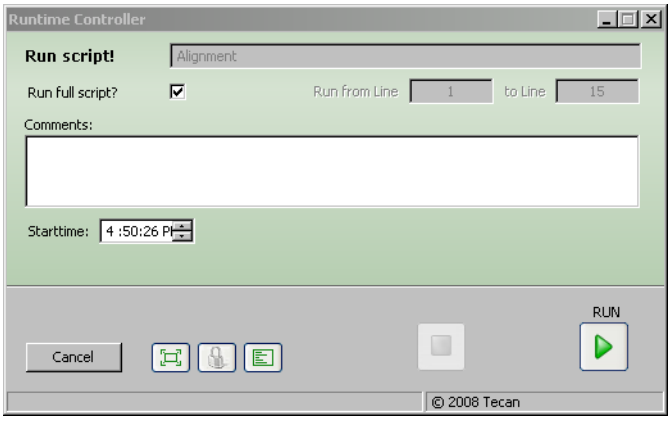
This script is a series of prompts for checking and/or adjusting the alignment of the tips. Refer to the *Tecan Freedom EVOware® Software Manual* (PN 393804) for instructions on how to adjust the tip position.

1. Select **Run maintenance**, then select **Alignment** from the drop-down list.



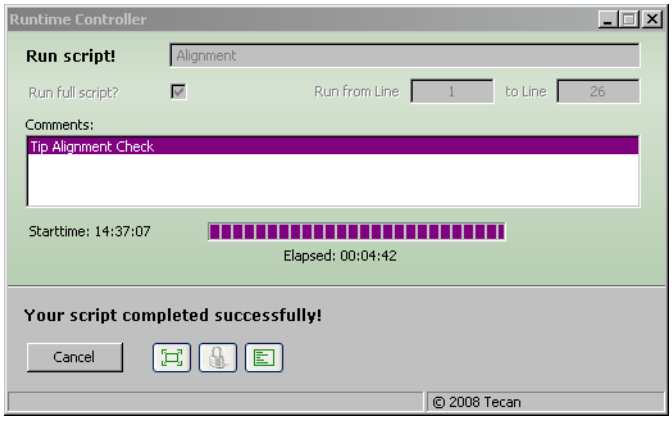
2. Click  to open the script.

3. In the Runtime Controller dialog box, click  to run the script. Wait for the system to complete the initialization.



4. For each prompt, perform the task, then click **Ok**.

5. When the run is completed, click **Cancel** to exit the dialog box.



Appendix E: Calculations for reagents

Prepare adaptors for Part 1

1. Prepare adaptor mix for each sample in a total volume of 75 µL of 1X Low TE Buffer.

   a. Calculate the amount of P1 Adaptor (*Y*) needed:

_Y_ µL P1 Adaptor needed = # µg DNA x A x (9.2 pmol/1 µg DNA) x B x (1 µL P1 Adaptor needed/50 pmol)

where:

A = (value below), if...	Library type
0.3	Standard fragment
0.66	Express fragment
B = (value below), if...	**Amount DNA**
35 - (5 x n µg DNA)	n = 1-5 µg
30	300 ng-1 ng
Y µL P1 Adaptor = (value below) if...	
0.5 µL	<300 ng (standard fragment library)
1.1 µL	<300 ng (express fragment library)

   b. Calculate the amount of P2 Adaptor required:

P2 = if...	Adaptor type
P1	Standard
9 x P1	Barcoded

   c. Calculate the amount of 1X Low TE Buffer required based on the volumes of P1 and P2:

µL 1X Low TE Buffer need = 75 – P1 – P2

2. Store at 4 °C until ready for use.

Prepare enzyme and PCR mixes for Part 1

1. Calculate the required amount of end-repair mix, then prepare the end-repair mix in a 15-mL conical tube.

Total volume = (90 x *n*) + 200

Reagent	Calculated Volume
5X Ligase Buffer	0.4444 x Total volume
dNTP Mix, 10 mM	0.0889 x Total volume
End Polishing Enzyme 1, 10 U/µL	0.0444 x Total volume
End Polishing Enzyme 2, 5 U/µL	0.1777 x Total volume
Nuclease-free water	0.2444 x Total volume

2. Calculate the required amount of ligation mix, then prepare the ligation mix in a 15-mL conical tube.

   Total volume = (50 x *n*) + 200

Reagent	Calculated Volume
5X Ligase Buffer	0.8 x Total volume
T4 DNA Ligase, 5 U/µL	0.2 x Total volume

3. *Optional,* calculate the required amount of PCR mix, then prepare PCR mix in a 15-mL conical tube.

   Total volume = (400 x *n*) + 200

Reagent	Calculated Volume
Platinum PCR mix	0.95 x Total volume
PCR Primer 1, 50 µM	0.025 x Total volume
PCR Primer 2, 50 µM	0.025 x Total volume

Prepare buffer, AMPure® XP beads and 70% ethanol for Part 1

1. Calculate the required amount of Elution Buffer (10 mM Tris HCl, pH 8.5), then add to a 15-mL conical tube.

   Total volume = (275 x *n*) + 500

2. Calculate the required amount of AMPure® XP beads, then add to a 15-mL conical tube.

   Total volume = (410 x *n*) + 500

3. Calculate the required amount of 70% Ethanol, then add to a 100-mL trough.

   Total volume = (1200 x *n*) +10000

Prepare reagents for Part 2

1. Calculate the required amount of AMPure® XP beads, then add to a 15-mL conical tube.

   a. For up to 12 samples, prepare 1 tube

      Total volume = (1.5 x n x PCR reaction volume) + 500

   b. For more than 12 samples, prepare 2 tubes

      Total volume (Tube 1) = (18 x PCR reaction volume) + 500

      Total volume (Tube 2) = (1.5 x (*n* – 12) x PCR reaction volume) + 500

2. Calculate the required amount of Elution buffer, then add to a 15-mL conical tube.

   Total volume = (100 x *n*) + 500

3. Calculate the required amount of 70% Ethanol, then add to a 100-mL trough.

   Total volume = (400 x *n*) + 10000