**SMARTerV4 (0.5x) Amplification for single-cell or single-nuclei RNASeq**

1. **Scope and Applicability:** Generate full-length cDNA from single cells, or nuclei, using Takara SMARTer V4.
2. **Materials:**
	1. Associated Amplification paperwork
	2. SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara 634898)
		1. SMART-Seq v4 Oligonucleotide (48 μM) (ST0944)
		2. 3’ SMART-Seq CDS Primer II A (12 μM) (ST0947)
		3. PCR Primer II A (12 μM) (ST0945)
		4. 5X Ultra Low First-Strand Buffer (ST0946)
		5. SMARTScribe™ Reverse Transcriptase (100 U/μl) (ST0655)
		6. Nuclease-Free Water (ST0804)
		7. RNase Inhibitor (40 U/μl) (ST0764)
		8. 10X Lysis Buffer (ST0948)
		9. Elution Buffer (10 mM Tris-Cl, pH 8.5) (ST0770)
	3. SeqAmp DNA Polymerase (Takara 638509)
		1. SeqAmp DNA Polymerase (ST0784)
		2. 2X SeqAmp PCR Buffer (4 x 1.25 ml) (ST0665)
	4. Sample strips from -80ºC associated with amplification paperwork.
	5. Control strip from -80ºC associated with amplification paperwork.
	6. PCR Strip Caps (Axygen PCR-02CP-C)
	7. Eppendorf DNA LoBind Tube 2.0 ml, PCR Clean (Eppendorf 022431048)
	8. Eppendorf DNA LoBind Tube 1.5 ml, PCR Clean (VWR 80077-230)
	9. 0.2 ml PCR 8-tube Flex-Free Strip, Attached Clear Flat Caps, Natural (USA Scientific #1402-4700)
	10. Filtered Pipette tips (Rainin GP-L10F, GP-L200F, GP-L1000F)
	11. GripTip 12.5 μl 384 Sterile Filter Tips (Integra Biosciences# 6455)
3. **Equipment:**
	1. C1000 thermocycler
	2. PCR Clean Pre-Amp hood with HEPA filter and UV capabilities
	3. Pre-Amp Hood Vortex Mixer
	4. Pre-Amp Hood Minifuge (with 8-strip and 1.5-2ml tube capabilities)
	5. Pre-Amp ice pan (located above Pre-Amp hood)
	6. Pre-Amp swing-bucket centrifuge with test tube adapters
	7. GeneMate 96-well Aluminum Cooler Block (R-2027-P)
	8. GeneMate Minifuge Tube Block
	9. Single Channel Pipettes (P10, P20, P200, P1000)
	10. 8-Channel Pipettes (P10 and P200)
	11. Automatic heat sealer for PCR plates
	12. Strip Cap Tool for sealing PCR Tubes
	13. Lab Timer
4. **Safety:**
	1. Nitrile Gloves
	2. Eye protection
	3. Lab coat
	4. Disposable laboratory sleeves
5. **Output:**
	1. Amplified full-length cDNA transcripts from single-cells for purification and validation in downstream applications.
6. **Reference Documents:**
	1. SPRI Bead Purification using Agilent Bravo Liquid Handling Robot
	2. HT\_043 How to Clean Hoods

**Warning: Personal Protective Equipment (PPE) should be used at all times while operating this protocol. If you are unsure what PPE you should be using, see your immediate supervisor.**

**NOTE: Always use NEW Strip-tube caps when placing caps on tubes - for ALL steps.**

1. **Setup:**
	1. Fill a Pre-Amp ice pan with wet ice.
	2. On top of the ice, place a GeneMate 96-well aluminum cooler block for the samples, another one for reagent aliquoting, and a GeneMate minifuge tube block then allow to cool for 5 minutes.
	3. Retrieve sample strips from -80ºC using associated paperwork as a guide, and place these strips, in order, on the pre-cooled GeneMate 96-well aluminum cooler block in the wet ice pan.
	4. Place another GeneMate 96-well aluminum cooler block at room temperature inside the hood.
	5. Check out the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara 634898) along with the associated SeqAmp DNA Polymerase (Takara 638509) kit.
		1. Record the kit ID #s in the upper-left box of the coversheet on the AMP paperwork.
	6. Retrieve the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara 634898) from -20ºC.
		1. Remove the following items from the kit and allow them to thaw at room temperature:
			1. SMART-Seq v4 Oligonucleotide (48 μM) (ST0944)
			2. 3’ SMART-Seq CDS Primer II A (12 μM) (ST0947)
			3. 5X Ultra Low First-Strand Buffer (ST0946)
			4. Nuclease-Free Water (ST0804)
			5. 10X Lysis Buffer (ST0948)
			6. Elution Buffer (10 mM Tris-Cl, pH 8.5) (ST0770)
		2. Place the following components directly on the pre-chilled GeneMate minifuge tube block on ice:
			1. SMARTScribe™ Reverse Transcriptase (100 U/μl) (ST0655)
			2. RNase Inhibitor (40 U/μl) (ST0764)
		3. Place the kit box containing only the PCR Primer II A (ST0945) in the working 4ºC.
	7. 10X Lysis Buffer (ST0948) and Elution Buffer (ST0770) are not utilized in this SOP, but will be needed for bead cleanup. These reagents may be brought over to the bead cleanup area at this time.
2. **Methodology/Procedures:**
	1. **First Strand DNA Synthesis:**
		1. Spin down the sample strips, two at a time, in the Pre-Amp minifuge for 10 seconds then immediately return them to the GeneMate 96-well aluminum cooler block in the wet ice pan.
		2. With a witness, verify the strip IDs by reading them out loud while the witness confirms the IDs against the paperwork.
			1. Ensure all strips are orientated correctly with the strip ID number in the ‘A’ position, the buffer type and creation date in the ‘G’ position and the FACS date in the ‘H’ position.
			2. Write the column number on the neck of the H-well tube for easy identification later on in the process.
		3. On the thermocycler, select folder “S4 Half vol” to access all 0.5X protocols. Start the “72C\_6uL” protocol on the thermocycler (72°C with 105°C lid, held indefinitely).
		4. Gently pulse vortex (Setting 9 for 10s) the 3’ SMART-Seq CDS Primer II A (ST0947) on the Pre-Amp hood vortex genie.
			1. Spin down the 3’ SMART-Seq CDS Primer II A (ST0947) in the Pre-Amp minifuge to collect it.

Table 1: CDS Primer (diluted to 6 µM) Addition

* + - 1. Dilute CDS primer from 12 µM to 6 µM: Add 220 µl NFdH20 (water from kit) to the 3'SMART-Seq CDS Primer II A (12 µM) tube (stock at 220 µl).
			2. Write 'D' and 4-digit kit number on 6uM diluted CDS cap, and mark a black stripe on the tube. This indicates that it is now at 6uM. This tube will supply 4 x 96 reactions. Cap, vortex to mix, spin down.
		1. Place a reagent 8-strip (0.2 ml PCR 8-tube Flex-Free Strip, Attached Clear Flat Caps, Natural USA Scientific 1402-4700) in the reagent GeneMate 96-well aluminum cooler block.
		2. Using the “μL / 8-well strip” calculation on the AMP paperwork (Table 1), aliquot 16 μl 6 µM 3’SMART-Seq CDS Primer II A (ST0947) into each well of the reagent 8-strip using a P20 @ 16 μl.
			1. Close the caps on this strip and briefly spin it down to collect the volume using the Pre-Amp minifuge then return the reagent strip to its aluminum block on ice.
			2. Cap the diluted CDS primer tube containing the unused portion. Store this in a box for diluted primers at -20ºC. Unused and undiluted CDS go in a separate labeled box at -20ºC.
		3. Begin removing caps from the sample tubes by moving the tubes, one at a time, over to the room temperature Genemate 96-well aluminum cooler block.
		4. Remove the caps from the sample strip and then return it to the GeneMate 96-well aluminum cooler block in the ice pan.
			1. This is done to limit any aerosol contamination since open tubes are nowhere near the tubes being de-capped.
			2. Repeat until all tubes are de-capped.
		5. Using an 8-channel P10 @ 1μl, add 1μl of 6 µM 3’SMART-Seq CDS Primer II A (ST0947) from the reagent 8-strip into each well of the sample strips.
			1. Take care not to touch the sample with the pipette tips, or to rinse the tips after dispensing.
			2. Visually confirm the addition by checking each pipette tip, and later, checking the sides of the wells during tube capping.
		6. Cap each sample strip by transferring them, one at a time, to the room temperature block and using a new strip cap and the strip cap tool to seal them.
			1. Vortex the strips on setting 9 for 10 seconds and then spin them down using the Pre-Amp hood vortex and minifuge.
			2. Label the caps to aid in maintaining strip order.
			3. Return the strips to the GeneMate 96-well aluminum cooler block in the ice pan.
		7. Bring the strips and a timer, set to 3 minutes, over to the thermocycler running the "72C\_6ul" protocol.
		8. Place the strips onto the block, and start the timer as soon as the strips are loaded.
		9. Close and tighten the lid for the remainder of the 3-minute incubation.
		10. Immediately upon completion of the 3-minute incubation, remove strips from the thermocycler and place them back on the GeneMate 96-well aluminum cooler block in the ice pan.
		11. End the "72C\_6ul" protocol on the thermocycler.
		12. Dispose of the reagent 8-strip with 6 µM 3’SMART-Seq CDS Primer II A (ST0947).
	1. **SMARTer RT:**
		1. Start the "10ul\_RT" protocol (42°C hold, 42°C for 90 - 70°C for 10 minutes - 4°C indefinitely) on the thermocycler to pre-heat the lid and bring the block up to 42ºC.

Table 2: RT Master Mix Preparation

Using the Pre-Amp vortex and minifuge, vortex all the reagents listed in Table 2 on setting 9 for 10 sec and then spin them down to collect the volume.

* + - 1. 5x Ultra Low First-Strand Buffer (ST0946) contains DTT, which is difficult to get into solution. Check the tube after spinning it down to make sure all the white precipitate is completely dissolved.
			2. Repeat vortexing as needed until the solution is free from any precipitate.
		1. On ice, prepare the RT Master Mix in a 1.5 ml Eppendorf LoBind tube (VWR 80077-230) by adding the black-text colored reagents in the order and volume listed at right of the RT Master Mix Preparation table.
			1. Add 210 μl of 5x Ultra Low First Strand Buffer (ST0946) using a P1000 @ 210 μl.
			2. Add 52.5 μl of SMART-Seq V4 Oligonucelotide (48 µM) (ST0944) using a P200 @ 52.5 μl.
			3. Add 26.25 μl of RNase Inhibitor (40U/μl) (ST0764) using a P200 @ 26.25 μl.
			4. **Do not add the SMART Scribe Reverse Transcriptase (100 U/μl) (ST0655) at this point.**
		2. Using the Pre-Amp vortex and minifuge, vortex the RT Master Mix tube on setting 9 for 5 seconds and then spin it down to collect the volume.
		3. Begin removing caps from the sample tubes by moving the tubes, one at a time, over to the room temperature Genemate 96-well aluminum cooler block.
		4. Remove the caps from the sample strip and then return it to the GeneMate 96-well aluminum cooler block in the ice pan.
			1. This is done to limit any aerosol contamination since open tubes are nowhere near the tubes being de-capped.
			2. Repeat until all tubes are de-capped.
		5. Add the 105 μl of SMARTScribe Reverse Transcriptase (100U/μl) (ST0655) to the RT Master Mix tube using a P200 @ 105 μl.
		6. Using the Pre-Amp vortex and minifuge, vortex the RT Master Mix tube on setting 9 for 10 seconds and then spin it down to collect the volume.
		7. Aliquot 46.875 μl of the RT Master Mix into a reagent 8-strip (USA Scientific 1402-4700) using a P200 @ 46.875 μl and following the volume instructions listed in the “μl / 8-well strip” box on Table 2.
		8. Add 3.75 μl of RT Master Mix to the sample strips using an 8-channel P10 @ 3.75 μl, taking care not to touch the pipette tips to the sample.
		9. Cap each sample strip by transferring them, one at a time, to the room temperature block and using a new strip cap and the strip cap tool to seal them.
			1. Vortex the strips on setting 9 for 10 seconds, and then spin them down using the Pre-Amp hood vortex and minifuge.
			2. Label the caps to aid in maintaining strip order.
			3. Return the strips to the GeneMate 96-well aluminum cooler block in the ice pan.
		10. Bring the sample strips over to the thermocycler running the “10ul RT” protocol and load them onto the thermocycler block.
		11. Once all strips are loaded and the lid is closed and tightened, advance the thermocycler to begin the RT protocol.
			1. Make sure the protocol has successfully advanced before leaving the instrument.
	1. **cDNA Amplification by LD PCR using SeqAmp DNA Polymerase**
		1. Approximately 20 minutes before the RT protocol is complete, retrieve the SeqAmp DNA Polymerase (Takara 638509) kit from the -20°C working freezer.
			1. Place the enzyme (ST0784) on ice while letting the buffer thaw at room temp for 3 minutes.
		2. Take the PCR Primer II A (12 μM) (ST0945) placed in the working 4°C refrigerator earlier, and place it in the ice pan.
		3. Using the Pre-Amp vortex and minifuge, vortex all the reagents on setting 9 for 10 seconds, and spin them down to collect the volume.
			1. Place all reagents on ice after being spun down.
		4. On ice, prepare the PCR Master Mix in a 2 ml Eppendorf LoBind tube by adding the reagents in the volume and order listed in table 3 below.

Table 3: PCR Master Mix Preparation



* + - 1. Add 318 μl of nuclease-free water (ST0804) using a P1000 @ 159 μl.
			2. Add 1325 μl of 2x SeqAMP PCR Buffer (ST0665) by using a P1000 @ 1000 μl and a P1000 @ 325 μl.
			3. Add 53 μl of PCR Primer II A (12 µM) (ST0945) by using a P200 @ 53 μl.
			4. Vortex the 2 ml Eppendorf LoBind tube for 10seconds on setting 9, and briefly spin down in a Pre-Amp minifuge to collect the volume.
			5. Add 53 μl of SeqAMP DNA Polymerase (ST0784) to the master mix using a P200 @ 53 μl.
		1. Vortex the 2 ml Eppendorf LoBind tube for 10 seconds on setting 9, and briefly spin down in a Pre-Amp minifuge to collect the volume.
		2. Aliquot out the master mix into a new reagent 8 strip (USA Scientific 1402-4700) using a P200 @ 190 μl. Close the caps and spin the strip in a minifuge. This volume is sufficient for all 12 strips.
		3. Begin removing caps from the sample tubes by moving the tubes, one at a time, over to the room temperature Genemate 96-well aluminum cooler block.
		4. Remove the caps from the sample strip and then return it to the GeneMate 96-well aluminum cooler block in the ice pan.
			1. This is done to limit any aerosol contamination since open tubes are nowhere near the tubes being de-capped.
			2. Repeat until all tubes are de-capped.
		5. Using an 8-channel P20 @ 15μl, add 15μl of PCR master mix to each of the sample strips, taking care to not touch the pipette tips to the sample. Use fresh tips when adding master mix to each strip.
		6. Cap each sample strip by transferring them, one at a time, to the room temperature block and using a new strip cap and the strip cap tool to seal them.
			1. Vortex the strips on setting 9 for 10 seconds and then spin them down using the Pre-Amp hood vortex and minifuge.
			2. Label the caps to aid in maintaining strip order.
			3. Return the strips to the GeneMate 96-well aluminum cooler block in the ice pan.
		7. Load the sample strips onto a thermocycler and run the following protocol (Table 4), using the coversheet of the associated amplification paperwork to inform cycling conditions. Make sure to select “S4 Half vol” folder in thermocycler to access all 0.5X PCR programs.

Table 4: PCR Program

* + 1. After PCR is complete, remove the strips and end the thermocycler protocol.
			1. Process strips through the SPRI bead cleanup immediately, or store at 4°C for up to 24 hours.
	1. Follow SPRI bead purification on Agilent Bravo robot instructions: See section 10.0 for list of steps.
1. **Take Down:**
	1. **Cleanup:**
		1. Dispose of all used tips and tubes.
		2. Since this is a 0.5X SMARTerV4 protocol, only half the amount of reagents are used for every 96 samples processed. Leftovers from reagents such as diluted CDS primer, 5X SeqAMP buffer, SeqAMP DNA polymerase should be saved and stored in labeled boxes at -20ºC. These will be used for another 0.5X SMARTer reaction of 96 samples.
		3. Clean the ice pan with RNase Away followed by a 70% EtOH cleaning.
			1. Make sure ice pan is dry before storage.
		4. Follow the HowTo document (HT\_043) detailing daily RSeq hood cleaning
		5. Sign the associated amplification paperwork and file in its designated lab area.
2. **Amplification product purification with SPRI beads**
	1. **Materials:**
		1. Associated Amplification paper work
		2. Amplified cDNA in 12 x 8-strip tubes (Amplification strips), or cDNA arrayed and transferred into a 96-well PCR plate.
		3. 10X Lysis Buffer (SMART-Seq4 kit: Clontech 634892)
		4. Reservoir, 25 ml divided (VWR 41428-958)
		5. Eppendorf twin.tec® 96 well PCR plates (VWR 47744-106)
		6. SPRI Beads (GE Healthcare 2934057)
		7. 1 ml Nunc Deep-well plates (VWR 73520-120)
		8. Elution Buffer (SMART-Seq4 kit: Clontech 634892)
		9. 1.2 ml Square-well storage plate (Thermo Scientific #AB-1127)
		10. 100% ABS Grade Ethanol, pint (AAPER/Pharmco E200G-P)
		11. 500 ml Reagent Bottles (VWR 28199-760)
		12. 2 ml deep well sterile plate (VWR 10755-250)
		13. Printed labels for Elution/Collection Plate, PicoGreen aliquot plate, and FA plate
		14. Axygen Velocity 165 µl Filtered Bravo Tips (Axygen VTF-165-R-S)
		15. Rainin 200 µl filtered pipette tips (GP-L200F)
		16. 50 ml disposable serological pipet (VWR 53106-441)
		17. FA marker plate ready for sample addition. (4ºC)
		18. 12.5 µl Filtered Integra GripTips (Integra 6455)
	2. **Equipment:**
		1. Pre-AMP Rainin E4-XLS+ 8-channel Electronic p200 Pipette (Rainin E8-200XLS+)
		2. Hirschmann Pipetus serological pipette
		3. Rainin P20 8-channel pipette (Rainin L8-20XLS+)
		4. Pre-AMP PCR clean hood
		5. Post-AMP PCR clean hood
		6. Plate sealer with aluminum seals for long-term cold storage
		7. VWR plate spinner (VWR 89184-608)
		8. VWR Vortex Mixer
		9. Agilent Technologies BRAVO Liquid Handling Robot in Post-AMP dead air box
		10. Post-AMP Integra ViaFLO 384 Liquid Handler in Post-AMP hood
		11. Post-AMP designated Integra 96-well aluminum blocks
		12. Disposable Integra tip receptacles
		13. 2x Agilent red aluminum inserts
		14. Pre-AMP designated swing bucket centrifuge
		15. Post-AMP designated swing bucket centrifuge
		16. Working 4ºC Refrigerator for short term storage
		17. 250 ml glass graduated cylinder for Ethanol use only
	3. **Amplified cDNA SPRI Bead Cleaning Setup**
		1. Ensure 10X Lysis Buffer (SMART-Seq4 kit: Clontech 634892) has thawed and equilibrated to room temperature.
		2. Pour into a 25 ml divided reservoir (VWR 41428-958) using the long side.
		3. Aliquot 50 µl 10X Lysis Buffer into each well of Eppendorf twin.tec® 96 well PCR plate (VWR 47744-106), using the E4-XLS+ 8-channel pipette with multi-dispense settings of 50 µl, 4 aliquots, at aspirate and dispense speed 5.
		4. Seal the plate and briefly centrifuge in the VWR plate spinner to collect.
			1. This plate will cover one working week of AMP cleanups and should always be thrown away after that, or if any contamination is suspected.
		5. Remove the 12 cDNA amplification strips from either the C1000 thermocycler or the working 4ºC refrigerator.
			1. PatchSeq samples will be arrayed in plastic racks holding 0.2 ml PCR tubes in two separate thermocyclers.
				1. For PatchSeq samples, follow steps 10.3.5.1.1 – 10.3.5.1.5
				2. Label a Eppendorf twin.tec® 96 well PCR plate (VWR 47744-106) with the amplification name from the associated paperwork.
				3. Together with a witness, follow the paperwork and transfer the entire contents of the PatchSeq tubes to their associated wells in the PCR plate, using an 8-channel P200 @ 60 µl.
				4. Visually confirm the transfer of the entire volume, and transfer any remaining volume with a single channel P20, under witness, if needed.
				5. Seal the plate and spin it down to collect the volume.
		6. Use the Post-AMP Integra to add 0.5 µl of 10X Lysis Buffer to the strips or plate containing amplified cDNA OR add 0.5 µl manually with p2 8-channel.
			1. This brings the final volume to 25.5 µl.
		7. On the Coversheet, initial the box indicating10X Lysis Buffer added to plate then encircle “0.5 µl (0.5X)” next to it.
		8. Load the amplified cDNA in strips or a PCR plate onto the red metal Bravo 96-well block.
			1. Orient the red block so that A1 is in upper left and H12 is in lower right.
			2. Referencing the CoverSheet, place the amplification strips according to well names: strips will have the Strip Number on A01 and BufferCode on G01.
			3. Strips should also carry a column ID number written on the collar of their “H” wells. Visually confirm the numbers correspond to their correct column placement.
		9. Proceed directly to cleanup.
	4. **SPRI XP setup:**

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| Table 5: Cleanup Reagents |

* + 1. Ensure that SPRI beads (GE Healthcare 29343057) have equilibrated to room temperature for at least 30 minutes before using them for sample cleanup.
		2. Vortex SPRI beads until evenly mixed. Referring to the table above, found in the paperwork, pipette the required bead volume into a 25 ml divided reagent trough (VWR 41428-958) using the long side of the reservoir.
		3. Add 25.0 µl of SPRI Beads (0.9x volume) to each well of a 1 ml deep-well Nunc plate (VWR 73520-120) using the 8-channel E4-XLS+ pipette and multi-dispense settings of: 25 µl volume, 6 dispenses, aspiration and dispense speeds of 2.
		4. Seal the bead plate and briefly spin down (1000 x g for 15s), using the PRE-AMP swing-bucket plate centrifuge.
		5. Label the bead plate with a matching ID to the associated library plate.
		6. Pour 2 bottles (at least 6 ml total) of Elution Buffer (SMART-Seq4 kit: Clontech 634892) into a new 25 ml divided reagent trough, using the long side.
		7. Using an 8-channel P200 at 120 µl, dispense 120 µl of Elution Buffer into each well of a new 1.2 ml Square-well storage plate (Thermo Scientific AB1127). This will supply Elution Buffer for up to 5 plates.
		8. Seal the elution reagent plate, label it as containing Amplification EB, and briefly spin down (1000 x g for 15s) using PRE-AMP swing-bucket plate centrifuge.
		9. Label a Twin-Tec 96-well PCR plate with the pre-printed label associated with the correct cleaned and eluted library ID.
		10. Consult Table 5 above (also found in the paperwork) for the volume of 80% Ethanol to prepare.
		11. Prepare this in a Pre-AMP hood, using the graduated cylinder adding 100% ABS grade Ethanol (AAPER E200G-P) to a new 500 ml reagent bottle (VWR 28199-760).
			1. Volume should reflect what is listed next to the EtOH box on the paperwork. (34.4 ml / plate)
		12. Use a serological pipette to add the listed volume of NFdH2O to the 500 ml reagent bottle (VWR 28199-760) containing the 100% Ethanol to make the final solution 80%. (8.6ml / plate)
			1. Label this 500 ml reagent bottle (VWR 28199-760) “80% EtOH”
	1. **Bead Purification using Agilent BRAVO liquid handler**
		1. Open the "Multi-Source SPRI protocol" on V-works, located in the “Production” folder.

**Bravo script for SPRI: BV-001\_Multi\_Source\_SPRI\_V2.61\_(Axygen)\_190314**

* + - 1. The protocol name is found on the coversheet next to the Bravo Purification box.
		1. From the drop-down menu, select the appropriate source plate from the container list (8 strip in Red Alum Insert).
		2. Place the red Bravo plate containing the amplification strips on position 6 of the Bravo deck.
		3. Referring to table found on the paperwork, enter the source volume, bead volume, and elution recovery volume.
			1. SPRI Bead Volume = 25 µl
			2. DNA Volume =25.60 µl
			3. Elution Volume = 17 µl
		4. Enter the number of columns of sample you wish to purify (use 12 for a full plate).
		5. Make sure the "tip selection" box matches the current tip and deck loadout. Update if necessary.
		6. Check the deck layout on V-works and ensure it matches:

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| --- |
|  1) 2 ml Waste plate |
| 2) 165 µl Filtered Tips (new) |
| 3) Elution/Collection Plate on red aluminum insert |
| 4) Empty  |
| 5) Shaker with sealed NUNC bead plate |
| 6) Samples in pre-selected container resting on red aluminum insert |
| 7) Magnet (empty) |
| 8) Used tip container |
| 9) Sterile tip box lid (80% EtOH) |

* + 1. After confirming the setup, remove plate seals and have a witness confirm all the selections, form entries and deck layout positions.
		2. Initial and fill out the coversheet of the paperwork for:
			1. Person conducting the Bravo purification.
			2. Witness initials on “Strip Order Witness” and “Bravo Deck Witness” boxes
			3. Circle which Bravo instrument was used.
			4. Note the purification date.
			5. Note the purification start time.
		3. Run the program by clicking on the “Run MultiSource SPRI Protocol” button on the form.
			1. **Do not click on the “Run” button on the menu bar above the protocol.**
		4. Table 6 lists each BRAVO protocol step and approximate times.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Process (Bravo)** | **Time Taken** | **Elapsed Time** |
| OFF magnet | Start | 0:00 | 0:00 |
| ON magnet | DNA Addition (to beads) | 0:00 | 0:01 |
|  | Mixing | 0:02 | 0:03 |
|  | Shaking | 0:00 | 0:03 |
|  | 8 minute incubation | 0:08 | 0:11 |
|  | 5 minute magnet | 0:05 | 0:16 |
|  | Remove Supernatant 1 | 0:02 | 0:18 |
|  | Ethanol Wash 1 1 | 0:01 | 0:19 |
|  | 1 min incubation | 0:01 | 0:20 |
|  | Ethanol Aspiration 1 1 | 0:01 | 0:21 |
|  | Ethanol Wash 2 1 | 0:02 | 0:23 |
|  | 1 min incubation | 0:01 | 0:24 |
|  | Ethanol Aspiration 2 1 | 0:01 | 0:25 |
|  | **5 min Bead Drying @ 45C** | 0:05 | 0:30 |
|  | Elution 1 | 0:01 | 0:31 |
|  | Mixing | 0:02 | 0:33 |
|  | Shaking | 0:00 | 0:33 |
|  | 5 minute incubation | 0:05 | 0:38 |
|  | 2 minute magnet | 0:02 | 0:40 |
|  | Aspirate / Finish | 0:01 | 0:41 |
|  | Table 6: BRAVO Protocol Steps |

* + 1. Switch out tips as needed and directed (alarm will briefly sound).
			1. Reset the tip boxes on the V-works form, the run will not continue without this.
		2. Once directed, after the addition of Ethanol for the second wash, replace the 80% EtOH reagent trough with the Elution Buffer (EB) plate and press “continue” on the Bravo computer.
		3. Upon completion, remove the Library Elution plate, seal it. Spin down at 1000 x g using POST-AMP swing-bucket plate centrifuge.
		4. Visually confirm the presence of Elution Buffer in all the wells as the Bravo will sometimes short aspirate, or mis-aspirate completely, from one-or-more wells.
			1. Under witness, manually recover any volume from the bead plate on a magnet to the elution plate.
			2. Note the affected wells and lab personnel on the coversheet of the paperwork.
			3. Some volume will remain in the bead plate as the Bravo adds 2ul to the stated elution volume listed on the form.
		5. Take the spun-down library elution plate over to the Post-AMP Integra.
		6. Use the pre-printed plate labels to label a new Eppendorf twin.tec 96-well PCR plate (VWR 47744-106) with the associated “PG\_” plate name.
		7. Use a pre-printed FA plate label to label one of the marker plates from the 4ºC with the associated library name.
		8. Place the library elution plate onto the Post-AMP Integra in position “AB” on top of an Integra 96-well aluminum block.
		9. Place the labeled PG plate onto deck position B.
		10. Using the Post-AMP Integra, run the 3ul TWINTEK program on the Post-AMP Integra.
			1. Follow the on-screen prompts.
		11. Seal the PG plate and cover the library elution plate before ejecting the used tips into a disposable tip waste receptacle.
		12. Load the labeled FA plate onto the Post-AMP Integra deck in position “B”.
		13. Run the 1ul FA Plate protocol and follow the on-screen prompts.
		14. Seal both the amplification elution plate and the FA plate.
1. **Post-cleanup take down:**
	1. Run Shut Down protocol on BRAVO. Close BRAVO software. Switch off robot and BRAVO room plate sealer.
	2. Discard bead cleanup waste into chemical waste container.
	3. Cap the waste container closed, but do not over tighten.
	4. Discard used labware and wipe BRAVO deck clean.
	5. Close the BRAVO hood and turn on UV light.
	6. Switch off Post-AMP Integra liquid handler.
	7. Wipe the equipment clean.
	8. Close the Post-AMP hood and turn on UV light.
	9. Ensure that all sample plates and aliquots have been sealed and stored appropriately before leaving the lab.