spa-ChIP-seq with Bravo

spa-ChIP-seq protocol is based on the following published protocol:
Texari L, Spann NJ, Troutman TD, Sakai M, Seidman JS, Heinz S. 2021. An optimized protocol for rapid, sensitive and robust on-bead ChIP-seq from primary cells. *STAR Protocols* 2: 100358. https://doi.org/10.1016/j.xpro.2021.100358.

Preparing buffers/solutions

- 10% BSA in PBS: Mix 5 grams of BSA with 50 mL of PBS at room temperature.
- 2.625 M Glycine: Mix 9.85 g glycine with 50 mL of UltraPure DNase/RNase-Free Distilled at room temperature.
- Lysis buffer LB3: Freshly prepare before sonication. Table below shows an example of the amount of each reagent required for making 10 mL of LB3.

LB3		
Reagent	**Final concentration**	**Amount**
UltraPure DNase/RNase-Free Distilled Water		8.93 mL
20% N-Lauroylsarcosine	0.5%	250 µL
5 M NaCl	100 mM	200 µL
1 M Tris-HCL pH 7.5	10 mM	100 µL
10% sodium deoxycholate	0.1%	100 µL
0.5 M EDTA	1 mM	20 µL
25× protease inhibitor cocktail (PIC)	1×	400 µL

- 10% Triton X-100: Mix 5 mL of Triton X-100 with 45 mL of UltraPure water to prepare a total of 50 mL.
- ChIP wash buffer 1 (WBI): Make a stock of 500 mL according to the table below. Store at 4°C.

WBI		
Reagent	**Final concentration**	**Amount**
UltraPure DNase/RNase-Free Distilled Water		418 mL
10% Triton X-100	1%	50 mL
5 M NaCl	150 mM	15 mL
1 M Tris-HCL pH 7.5	20 mM	10 mL
10% SDS	0.1%	5 mL
0.5 M EDTA	2 mM	2 mL

- ChIP wash buffer 3 (WBIII): Make a stock of 500 mL according to the table below. Store at 4°C.

WBIII		
Reagent	**Final concentration**	**Amount**
UltraPure DNase/RNase-Free Distilled Water		393.375 mL
10% Triton X-100	1%	50 mL
10% sodium deoxycholate	0.7%	35 mL
8 M LiCl	250 mM	15.625 mL
1 M Tris-HCL pH 7.5	10 mM	5 mL
0.5 M EDTA	1 mM	1 mL

- TET buffer: Make a stock of 500 mL according to the table below. Store at 4°C.

TET		
Reagent	**Final concentration**	**Amount**
UltraPure DNase/RNase-Free Distilled Water		493 mL
1 M Tris-HCL pH 7.5	10 mM	5 mL
0.5 M EDTA	1 mM	1 mL
Tween 20	0.2%	1 mL

- TT buffer: Make a stock of 50 mL according to the table below. Store at 4°C.

TT		
Reagent	**Final concentration**	**Amount**
UltraPure DNase/RNase-Free Distilled Water		49.475 mL
1 M Tris-HCL pH 8.0	10 mM	500 µL
Tween 20	0.05%	25 µL

Crosslink cells

1. Grow cells till ~80%-100% confluency. Count cell numbers before harvest if necessary.
2. Prepare crosslinking reagents for either double or single crosslinking.
    a. **Double crosslinking (DSG+FA)**: For each **1 million cells**, freshly prepare a minimum of **1 mL of a 2 mM DSG solution in PBS**. First warm up the glass vial of 100 mg DSG to room temperature before opening (to avoid water condensation into the vial). Each 1 million cells require at least 0.654 mg DSG dissolved in 3.92 µL anhydrous DMSO (adjust the amount of DMSO to the amount of DSG in the tube). Place 1 mL PBS per 1 million cells in a conical tube. Using a micropipette, swiftly ‘‘shoot’’ **3.92 µL of the DSG/DMSO solution** into the PBS, immediately close the tube, and invert multiple times to rapidly disperse the DSG. If this is done too slowly, the DSG will form a white precipitate, and the DSG/PBS preparation procedure will need to be repeated.
    b. **Single crosslinking**: For each **1 million cells**, freshly prepare a minimum of **1 mL of a 1% formaldehyde solution in PBS**. Place 1 mL PBS per 1 million cells in a conical tube and add 66.67 µL of 16% formaldehyde solution per 1 mL PBS.
3. Prepare cells for crosslinking either on tissue culture plates or in conical tubes.
    a. **Crosslink adherent cells on plate**: Aspirate old media from the plate. Wash cells once with 5-10 mL PBS.
    b. **Crosslink adherent cells in tube**: Harvest adherent cells by trypsinizing and neutralize trypsin with 4 volumes of FBS-containing media, transfer cell suspension to 15 mL or 50 mL conical, depending on volume. Pellet cells by centrifuging for 8 min at 300 × g at 20°C–25°C. Aspirate or decant supernatant. Wash cells once by resuspending in 5-10 mL PBS and centrifugation for 8 min at 300 × g at 20°C–25°C.
    c. **Crosslink suspension cells in tube**: Transfer cell suspension to 15 mL or 50 mL conical, depending on volume. Pellet cells by centrifuging for 8 min at 300 × g at 20°C–25°C. Aspirate or decant supernatant. Wash cells once by resuspending in 5-10 mL PBS and centrifugation for 8 min at 300 × g at 20°C–25°C.
4. **Double crosslinking with DSG and FA**:
    a. Make sure the PBS wash is aspirated.

    b. Per 1 million cells, add at least 1 mL of freshly made 2mM DSG/PBS solution, and incubate at room temperature for 30 minutes while shaking the plates on a platform shaker or rotating the tubes overhead at 8 rpm.
    c. Next, per 1 million cells, add 66.67 µL of 16% formaldehyde to each 1 ml cell suspension in DSG/PBS, and incubate at room temperature for an additional 10 minutes while shaking the plates on a platform shaker or rotating the tubes overhead at 8 rpm.
5. **Single crosslinking with FA only**:
    a. Make sure the PBS wash is aspirated.
    b. Per 1 million cells, add at least 1 mL of freshly made 1% formaldehyde/PBS solution, and incubate at room temperature for 10 minutes while shaking the plates on a platform shaker or rotating the tubes overhead at 8 rpm.
6. Quench the crosslinking reaction by adding 1/20th volume of 2.625 M Glycine and 1/20th volume 10% BSA in PBS (50 µL each per each 1 mL crosslinking reaction).
7. If crosslinking on plate, scrape the cells and transfer the cell slurry into a conical tube. Pellet cells by centrifuging for 5 minutes at 1000–1500 × g at 4°C.
8. Aspirate and discard supernatant, then resuspend fixed cells in 1 mL ice-cold 0.5% BSA/PBS and transfer the cell suspension to a 1.5 mL microcentrifuge tube.
9. Pellet cells by centrifuging for 5 minutes at 1000–1500 × g at 4°C.
10. Aspirate and discard supernatant and resuspend fixed cells in 1 mL ice-cold 0.5% BSA/PBS.
11. Pellet cells by centrifuging for 5 minutes at 1000–1500 × g at 4°C.
12. Aspirate and discard supernatant (can leave behind ~10 µL).
13. If proceeding to next steps immediately, place the cell pellet on ice. If saving the cells for future use, snap-freeze the cell pellet and store at −80°C.

Shearing Crosslinked Cells with PIXUL™ Multi-Sample Sonicator

1. Per 1 million fixed cells, freshly prepare at least 100 µL of lysis buffer LB3 with protease inhibitor cocktail (PIC) added. Mix by vortexing and keep on ice.
2. Add ~100 µL of lysis buffer LB3+PIC into 1 million fixed cell pellet and suspend the mixture thoroughly.
3. Load 100 µL of cells in lysis buffer into each well of the PIXUL shearing plate (~10,000 - 5,000,000 cells per well) and seal the plate with a PCR plate pressure seal.
    - **Note**: All wells lacking sample in the columns being sonicated MUST be filled with liquid (water, coupling buffer, etc.) prior to starting the sonication run. Be sure to keep the outside bottom of the plate clean and free of lint or other debris.
4. Turn on the power switch on the back side of the PIXUL instrument to the ON position. The light around the center power button on the front of the instrument should illuminate.
5. Press the main power button on the front side of the PIXUL instrument. The touchscreen will start initializing and load the home screen.
6. Once PIXUL is on, the first step is to ensure that the PIXUL Coupling Fluid level is about an inch (2.5 cm) below the reservoir top (or just above the circular cut-out in the plastic column within reservoir). The reservoir capacity is approximately 600 mL, and the fluid level should be topped off as needed. Do not overfill.
7. Lift the white external lid of the PIXUL instrument and place the shearing plate on the black grid with well A1 in the upper left corner, secure down the black hold-down pressure

distribution lid on top of the plate with plate-securing tension rods/clamps (fastening both sides at the same time), and close the white external lid.

8. On the touchscreen, press “CIRCULATE” to initiate PIXUL Coupling Fluid cooling and allow PIXUL to cool to at least 15°C, which takes up to 5 minutes. You cannot start the sonication run without circulation active. You can monitor Coupling Fluid temperature in the upper right-hand corner of the touchscreen.
9. On the touchscreen, select the plate columns for which you would like to set sonication parameters. Columns selected together will be outlined in the same color.
10. On the left side of the touchscreen, you can use the left and right arrows to select from saved presets of sonication parameters. You can also use the add and delete buttons to add or remove a row of process settings. Adjust the following parameters:

Columns	Pulse [N]	PRF [kHz]	Process Time	Burst Rate [Hz]
1 - 12	50	1.00	108:00	20.00

    - **Note**: The software will not allow settings that could damage the transducers, and the upper limits are a maximum Pulse of 50 or a maximum PRF of 1.00. Recommended a minimum Burst Rate is 20.00. Varying the Process Time is typically the only parameter that needs adjusting.
11. Once the PIXUL Coupling Fluid has reached approximately 15°C, press “START” on the touchscreen. The run-time to completion will appear in the top left-hand corner of the touchscreen. Do not lift lid while PIXUL runs.
12. Once the sonication run has completed, pop-up appears on screen, click OK, open the external lid. PIXUL Coupling Fluid will drain from underneath the sample plate for the next few seconds (this will happen automatically and will take 5-10 seconds).
13. Unload the sample plate by lifting the pressure distribution plate cover by the lift handle and simply pulling up. Close lid but do not fasten the tension rods.
14. To turn off the PIXUL instrument, press the main power button on the front side of the PIXUL instrument and switch the power switch off on the back side of the PIXUL instrument.
15. After sonication, spin down the plate for at least 1 minute at 1000–1500 × g.

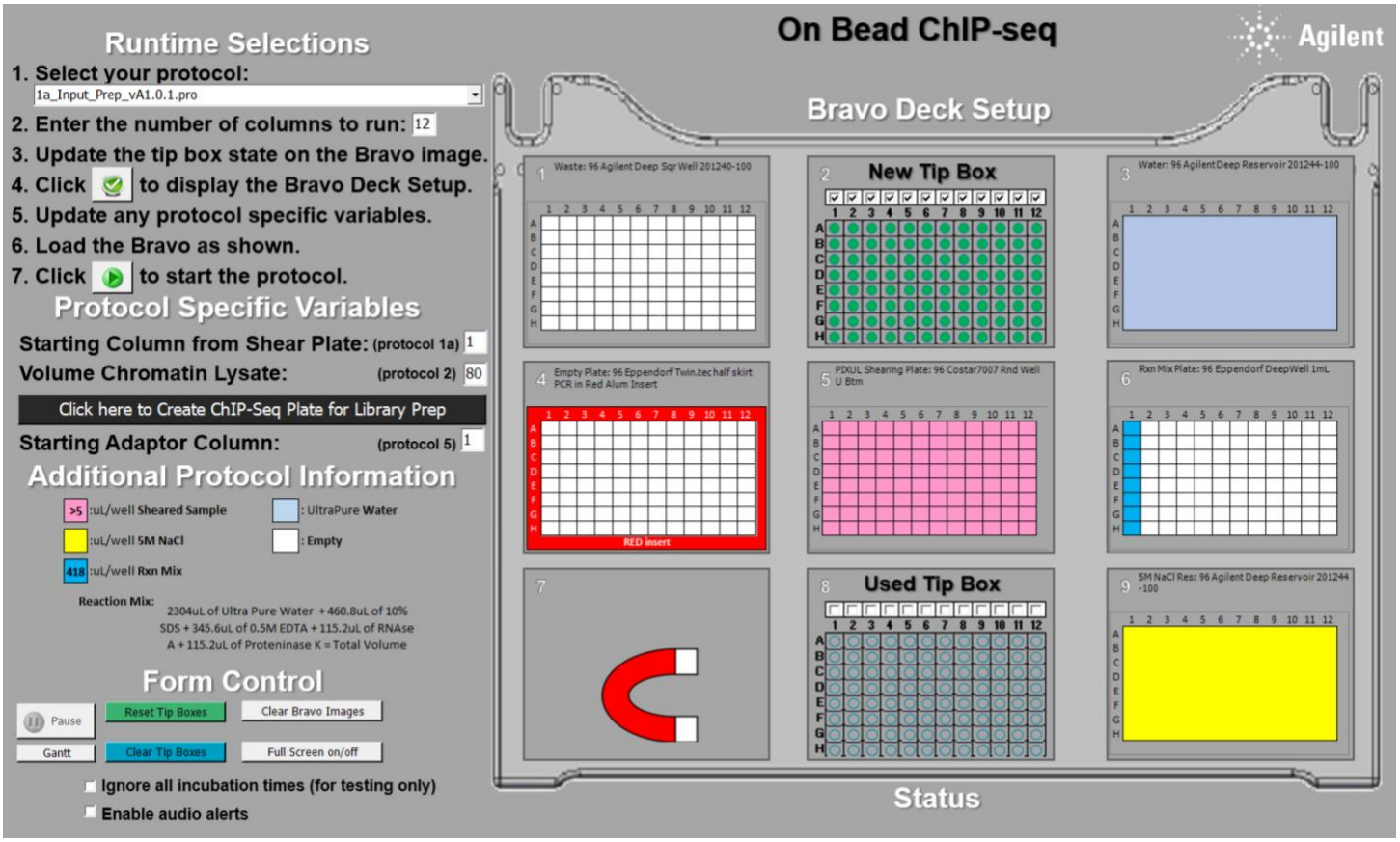
Analysis of Sonicated Chromatin

1. Freshly prepare the following **Reaction Mix** at room temperature. Each sample requires 29 µL and it is recommended to prepare 20% more reaction volumes per sample. Aliquot 1/8 of the total volume of the mastermix to each well in the first column of the **Reaction Mix Plate**. Place the **Reaction Mix Plate** on **Bravo deck 6**. (The example below shows the amount required for a full 96-well plate.)

Bravo 1a Reaction Mix				
Reagent	**Final concentration**	**Amount per sample (µL)**	**Amount required for 96 samples (µL)**	**Amount prepared for 96 samples (µL)**
UltraPure DNase/RNase-Free Distilled Water		20	1920	2304
10% SDS	0.5%	4	384	460.8
0.5 M EDTA	18.75 mM	3	288	345.6
20 mg/mL Proteinase K	250 µg/mL	1	96	115.2

20 mg/mL RNase A	125 µg/mL	1	96	115.2
Mastermix Total		**29**	**2784**	**3340.8**
Amount added to each well of the first column for Bravo (µL)				**417.6**

2. Place the **PIXUL Shearing Plate** on **Bravo deck 5** and **5 M NaCl Reservoir** (filled with ~5-10 mL 5 M NaCl) on **Bravo deck 9**. Ensure the reservoirs contain at least enough liquid to form a thin layer across the bottom.
3. Select Bravo protocol **1a_Input_Prep_vA1.0.1.pro** from the drop down manual in step 1 and click the green check symbol to display the Bravo Deck Setup. Make sure the deck is set up as shown. Once ready, click on the right-pointing triangle (▷) at step 7 to start the protocol.



Bravo will perform the following steps:

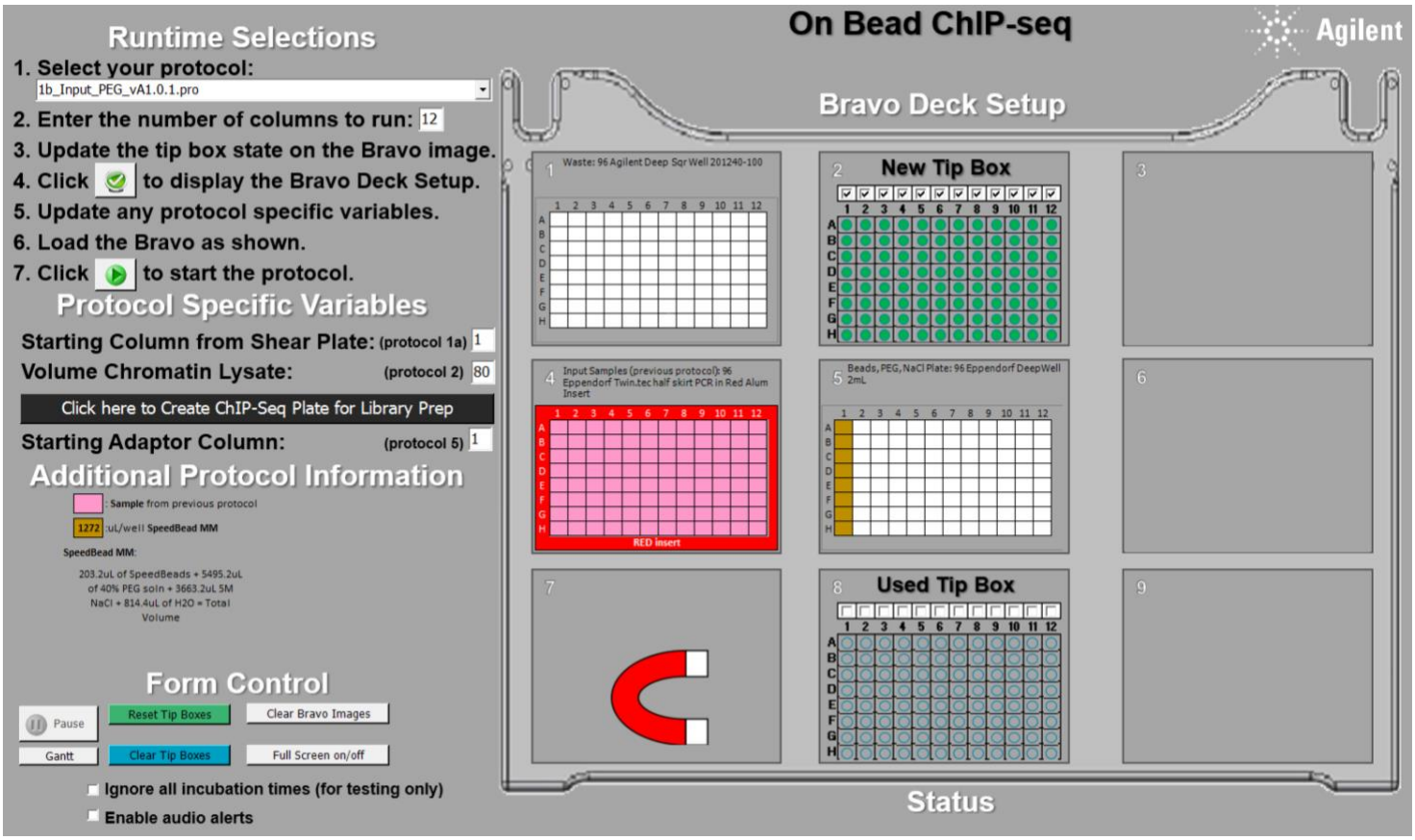
- Combine 5 µL sonicated chromatin lysate from each well and 41.5 µL of UltraPure water to a final volume of 46.5 µL.
- Add 29 µL of the Reaction Mix to each sample.
- Add 4.5 µL of 5 M NaCl to each sample.
- Mix up and down at least 10 times (the mix cycle can be changed in the parameters).

4. **Reverse crosslinking**: Retrieve the plate with samples from **Bravo deck 4**. Incubate the plate at 37°C for 15 minutes and at 55°C for 30 minutes to digest RNA and proteins, then at 65°C for 30 minutes to 1 hour in a PCR cycler with the heated lid set to at least 75°C to prevent evaporation and drying out of the samples. This can be left at 12°C or 4°C overnight.
5. Freshly prepare the following **Speedbeads Mastermix** at room temperature. Each sample requires 80 µL and it is recommended to prepare 20% more reaction volumes per sample. Aliquot 1/8 of the total volume of the mastermix to each well in the first column of the

Speedbeads Mastermix Plate. Place the **Speedbeads Mastermix Plate** on **Bravo deck 5**. (The example below shows the amount required for a full 96-well plate.)

Bravo 1b Speedbeads Mastermix				
Reagent	**Final concentration**	**Amount per sample (µL)**	**Amount required for 96 samples (µL)**	**Amount prepared for 96 samples (µL)**
40% PEG8000	12%	54	5184	6220.8
5 M NaCl	1 M	36	3456	4147.2
Speedbeads	-	2	192	230.4
Water	-	8	768	921.6
	Mastermix Total	**100**	**9600**	**11520**
Amount added to each well of the first column for Bravo (µL)				**1440**
	Sample (µL)	80		
	Final Volume (µL)	180		

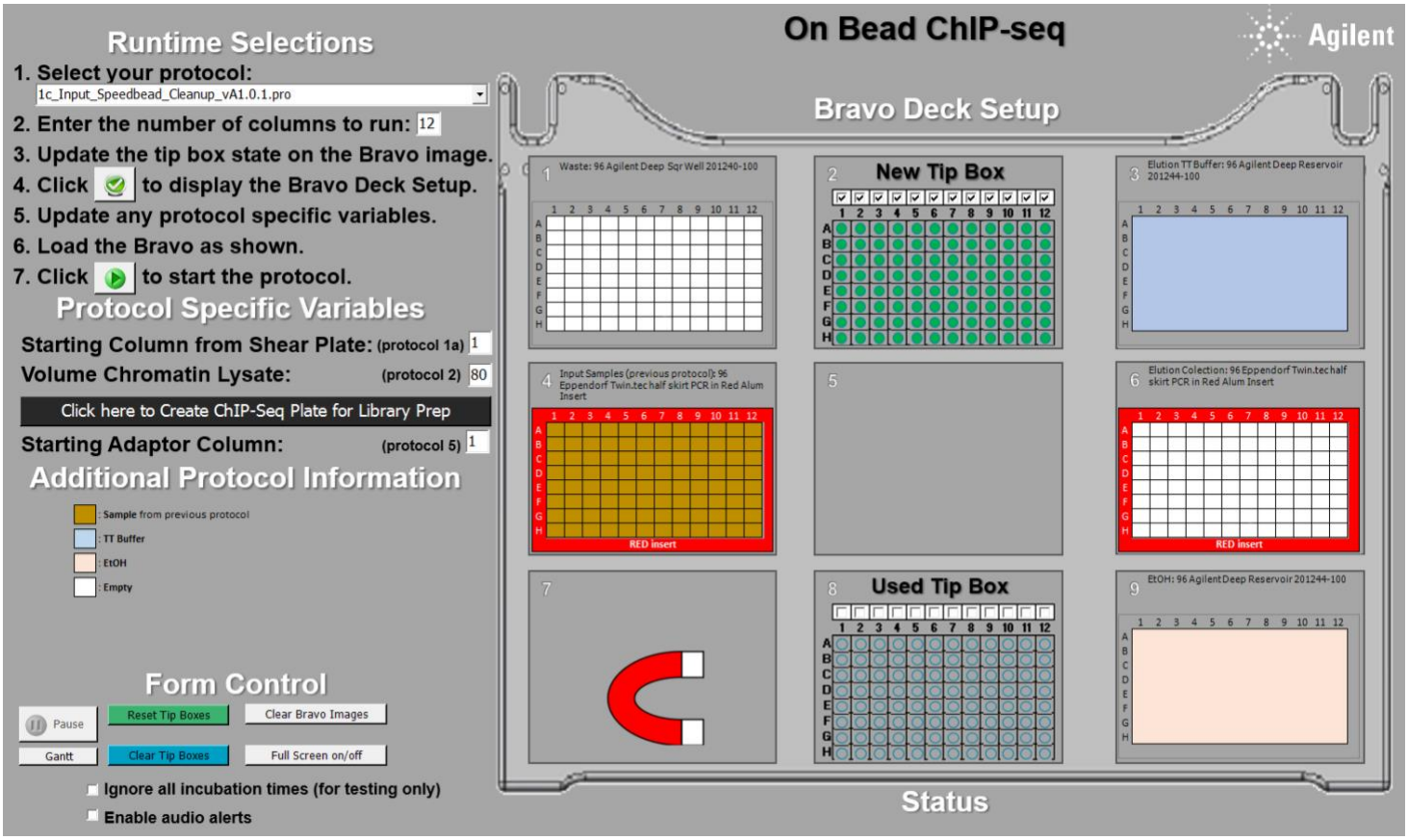
6. Remove the **Sample Plate** from reverse crosslinking incubation and place it back to **Bravo deck 4**.
7. Select Bravo protocol **1b_Input_PEG_vA1.0.1.pro** from the drop down manual in step 1 and click the green check symbol to display the Bravo Deck Setup. Make sure the deck is set up as shown. Once ready, click on the right-pointing triangle (▷) at step 7 to start the protocol.



Bravo will perform the following steps:

- Add 100 µL of SpeedBead/PEG mastermix to each 80 µL ChIP input sample. The final concentration of PEG will be 12% and the final concentration of NaCl will be 1 M.
- Mix the samples to homogeneity by repetitive pipetting. Incubate at room temperature for 10 minutes.

8. During incubation, place the **Waste Plate** on **Bravo deck 1**, **TT Buffer Reservoir** on **Bravo deck 3**, **empty Elution Plate** on **Bravo deck 6**, and **80% Ethanol Reservoir** on **Bravo deck 9**. Ensure the reservoirs contain at least enough liquid to form a thin layer across the bottom.
9. Immediately after protocol 1b is completed, select Bravo protocol **1c_Input_Speedbead_Cleanup_vA1.0.1.pro** from the drop down manual in step 1 and click the green check symbol to display the Bravo Deck Setup. Make sure the deck is set up as shown. Once ready, click on the right-pointing triangle (▷) at step 7 to start the protocol.



Bravo will perform the following steps:

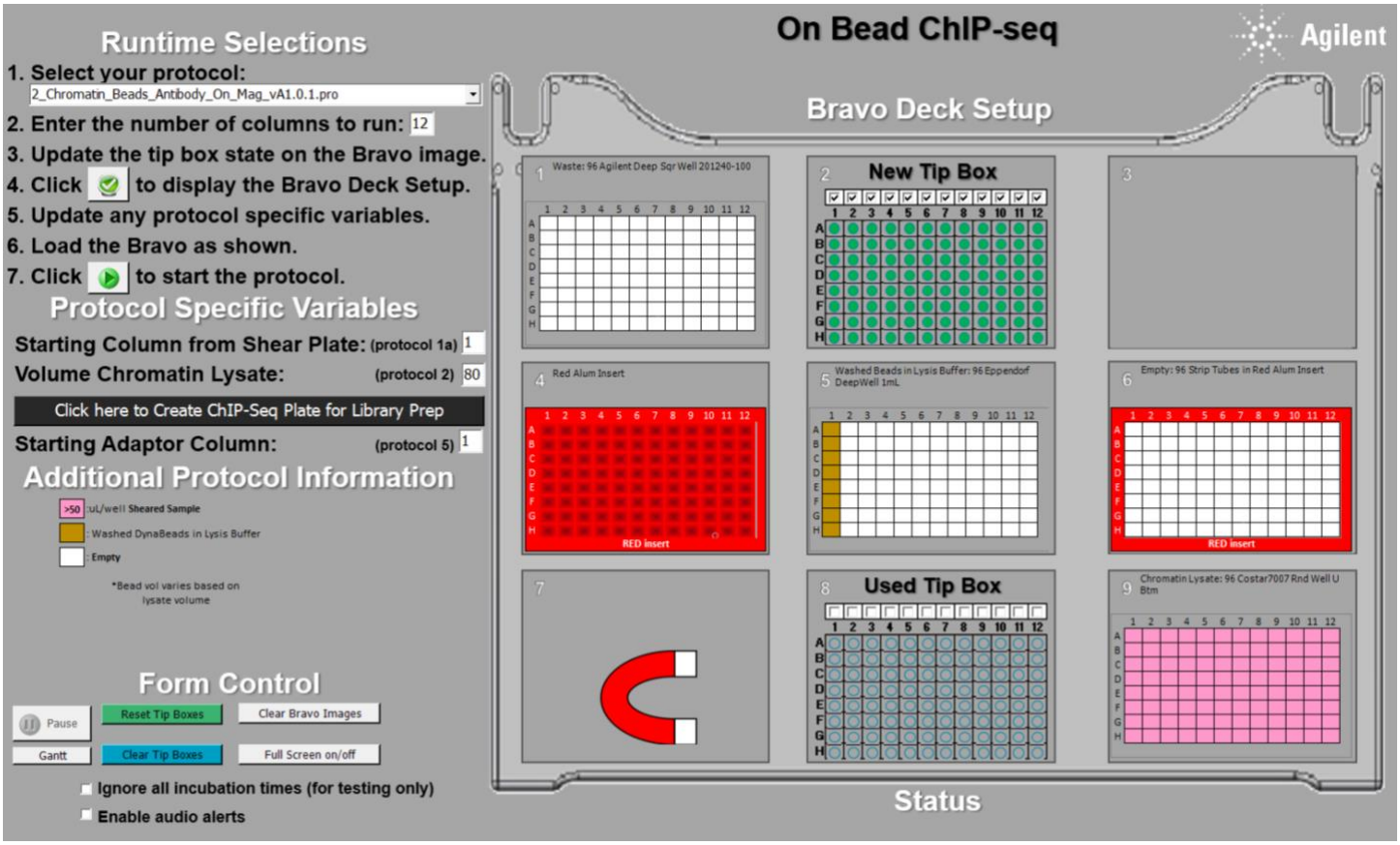
- Place the plate to a magnet deck and aspirate the cleared supernatant.
- Wash the beads by adding 180 µL of 80% ethanol at room temperature and pipetting up and down to mix. Collect the beads on the magnet and discard the wash supernatant once cleared. Repeat this wash one additional time.
- After removing the second ethanol wash, air-dry the beads for ~10 minutes, or until “cracks” just begin to appear in the packed beads.
- Elute DNA by adding 25 µL TT Buffer. Mix then incubate at room temperature for 5–10 minutes.
- Apply the plate to the magnet. Transfer the supernatant to a new plate and discard the Speedbeads.

10. At the end of the protocols, the **Sample Elution Plate** should contain cleaned DNA samples eluted in 25 µL of TT buffer.
11. To check the shearing, run 5 µL of the sample from each well on a 2% agarose gel at 120V for 10 minutes & 30 minutes.
12. The recommended length of shearing chromatin is 200 - 500 bp for ChIP assays. If shearing pattern is as expected, proceed to the next step. If storing the sheared chromatin for future

use, combine the wells of the PIXUL shearing plate with the same cell type/condition into one tube and place at −80°C.

Immunoprecipitation

1. Prepare 10 µL of Dynabeads Protein A or Dynabeads Protein G per sample into a microcentrifuge tube.
    - **Note**: The choice of protein A and/or protein G Dynabeads, and the volume to use, is dictated by the antibody used for ChIP. Protein A binds rabbit antibodies with highest affinity, while protein G has higher affinity for mouse, rat, goat, and sheep antibodies.
2. Wash Dynabeads twice on magnet with equal volume of lysis buffer LB3/1% Triton X-100 + PIC.
3. For each antibody, prepare a mixture of antibody and Dynabeads Protein A/G in LB3 + PIC and 1% Triton X-100. Add the mixture to **Antibody/Dynabeads Mix Plate**.
4. Place **Antibody/Dynabeads Mix Plate** on **Bravo deck 5**, **empty tube strips** (with detached lid strips, without the lids) on **Bravo deck 6**, and **Chromatin Lysate Plate** on **Bravo deck 9**.
5. Select Bravo protocol **2_Chromatin_Beads_Antibody_On_Mag_vA1.0.1.pro** from the drop down manual in step 1 and click the green check symbol to display the Bravo Deck Setup. Update the protocol specific variables if necessary. The default volume of chromatin lysate is 80. Make sure the deck is set up as shown. Once ready, click on the right-pointing triangle (▷) at step 7 to start the protocol.



Bravo will perform the following steps:

- Distribute antibody/Dynabeads mix to each tube of an 8-tube strip accordingly.
- Aliquot appropriate amount (50-150µl) of sheared chromatin into each tube with the antibody/Dynabeads mix.

- Mix by pipetting up and down.

6. Incubate IP tube strips overnight (up to 16 h) at 4°C by rotating on a HulaMixer (setting below) in the cold room.

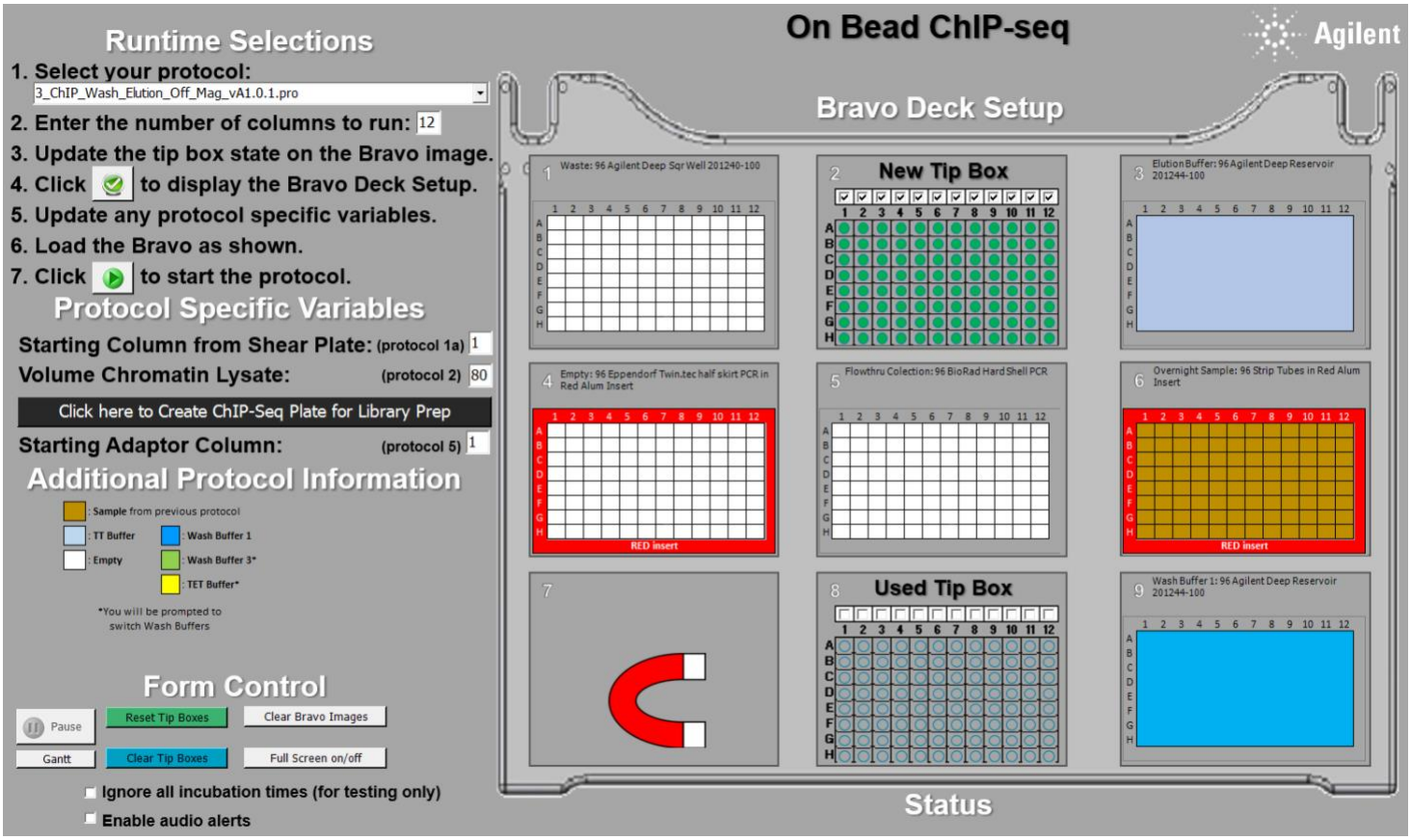
Orbital (rpm)	Reciprocal (deg.)	Vibro/pause	Time (hr:min)
6	90°	5°	0:00
10	10	5	Vertical
Time (sec.)			Vertical/Horizontal

Wash ChIP Samples

1. Freshly prepare the following amounts of ChIP Wash Buffer 1 (WBI), ChIP Wash Buffer 3 (WBIII), and TET Buffer by adding 25× protease inhibitor cocktail (PIC). Mix by vortexing and keep on ice. (The example below shows the amount required for a full 96-well plate.)

Buffer	**Amount per sample (µL)**	**Amount for 96 samples (µL)**	**Total volume prepared (µL)**	**25× PIC to add (µL)**
WBI	540	51840	55000	2200
WBIII	540	51840	55000	2200
TET	360	34560	40000	1600

2. Place **Waste Plate** on **Bravo deck 1**, **TT Buffer Reservoir** on **Bravo deck 3**, **empty ChIP Plate** on **Bravo deck 4**, **IP tube strips** (without the lids) on **Bravo deck 6**, and **Wash Buffer Reservoir** (with WBI + PIC first) on Bravo deck 9. Ensure the reservoirs contain at least enough liquid to form a thin layer across the bottom.
3. Select Bravo protocol **3_ChIP_Wash_Elution_Off_Mag_vA1.0.1.pro** from the drop down manual in step 1 and click the green check symbol to display the Bravo Deck Setup. Make sure the deck is set up as shown. Once ready, click on the right-pointing triangle (▷) at step 7 to start the protocol.



Bravo will perform the following steps:

- Wash the ChIP samples 3 times with 180 µL WBI + PIC
- Wash the ChIP samples 3 times with 180 µL WBIII + PIC
- Wash the ChIP samples 2 times with 180 µL TET + PIC
- Resuspend beads in 25 µL of TT buffer

4. At the end of the protocol, each well of the **ChIP plate** (deck 4) should contain 25µL of ChIP sample eluted in TT buffer.

Library Preparation

1. Add each of the 25 µL of diluted input chromatin sample to the **ChIP Plate** manually or run Bravo protocol **4_prePrep_Combine_Samples_with_DilutedInputControls_vA1.0.1.pro**

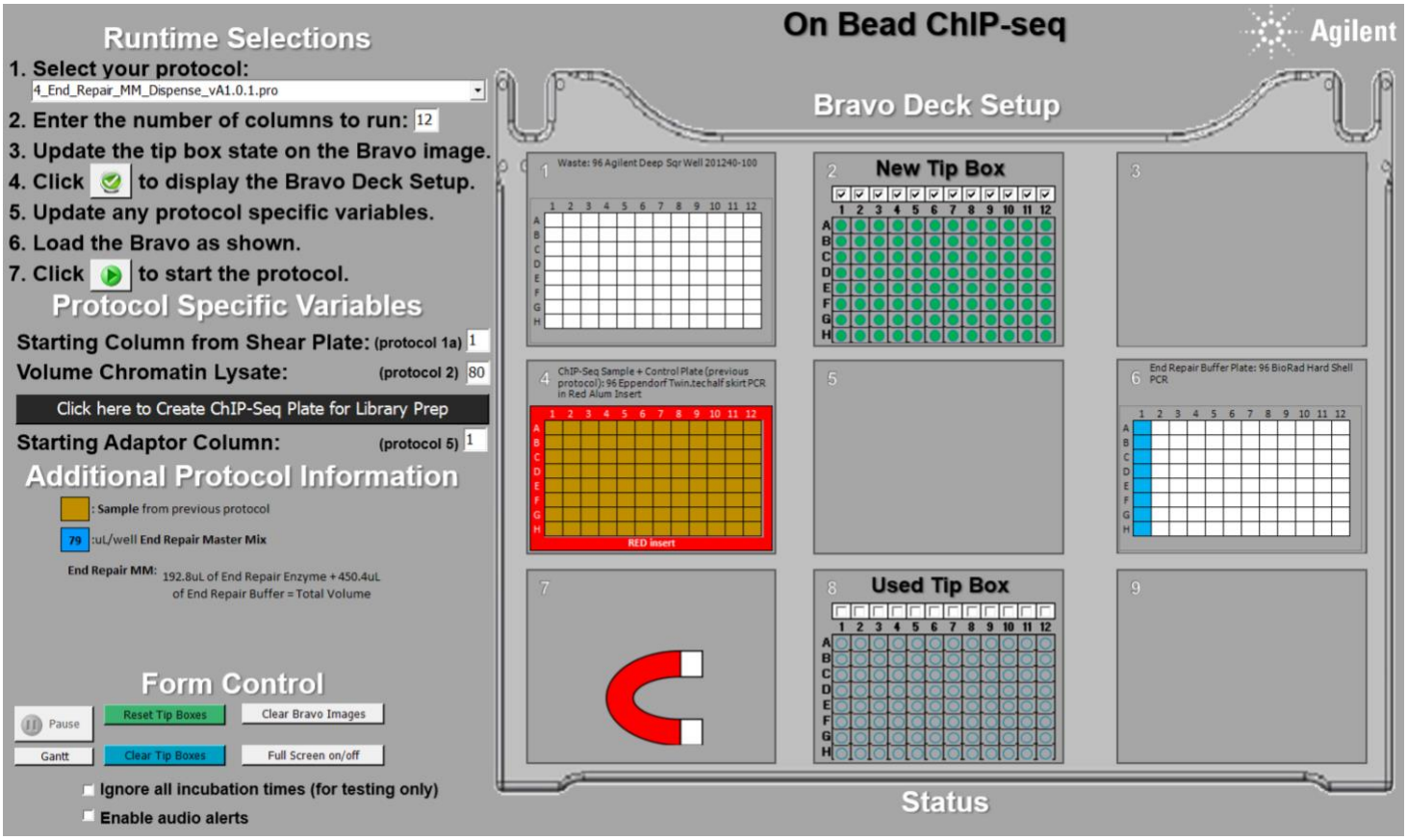
   Manual steps for diluting chromatin lysate to use as input: For each condition, take 4 µL of the sheared chromatin lysate and add 46 µL of TT buffer to make a 1:12.5 dilution. Take 5 µL of the 1:12.5 diluted chromatin lysate and add 20 µL of TT buffer to make another 1:5 dilution. The total dilution of the chromatin lysate is 1:62.5.

2. Place the ChIP Plate on Bravo deck 4.
3. Freshly prepare the following **End Prep Mix** on ice. Each sample requires 5 µL and it is recommended to prepare at least 20% more reaction volumes per sample. Aliquot 1/8 of the total volume of the mastermix to each well in the first column of the End Prep Mix Plate. Place the **End Prep Mix Plate** on **Bravo deck 6**. (The example below shows the amount required for a full 96-well plate.)

Bravo 4 End Prep Mix

Reagent	Amount per sample (µL)	Amount required for 96 samples (µL)	Amount prepared for 96 samples (µL)
NEBNext Ultra II End Prep Enzyme Mix	1.5	144	192.8
NEBNext Ultra II End Prep Reaction Buffer	3.5	336	450.4
Mastermix Total	**5**	**480**	**576**
Amount added to each well of the first column for Bravo (µL)			**80.4**

4. Select Bravo protocol **4_End_Repair_MM_Dispense_vA1.0.1.pro** from the drop down manual in step 1 and click the green check symbol to display the Bravo Deck Setup. Make sure the deck is set up as shown. Once ready, click on the right-pointing triangle (▷) at step 7 to start the protocol.



Bravo will perform the following steps:

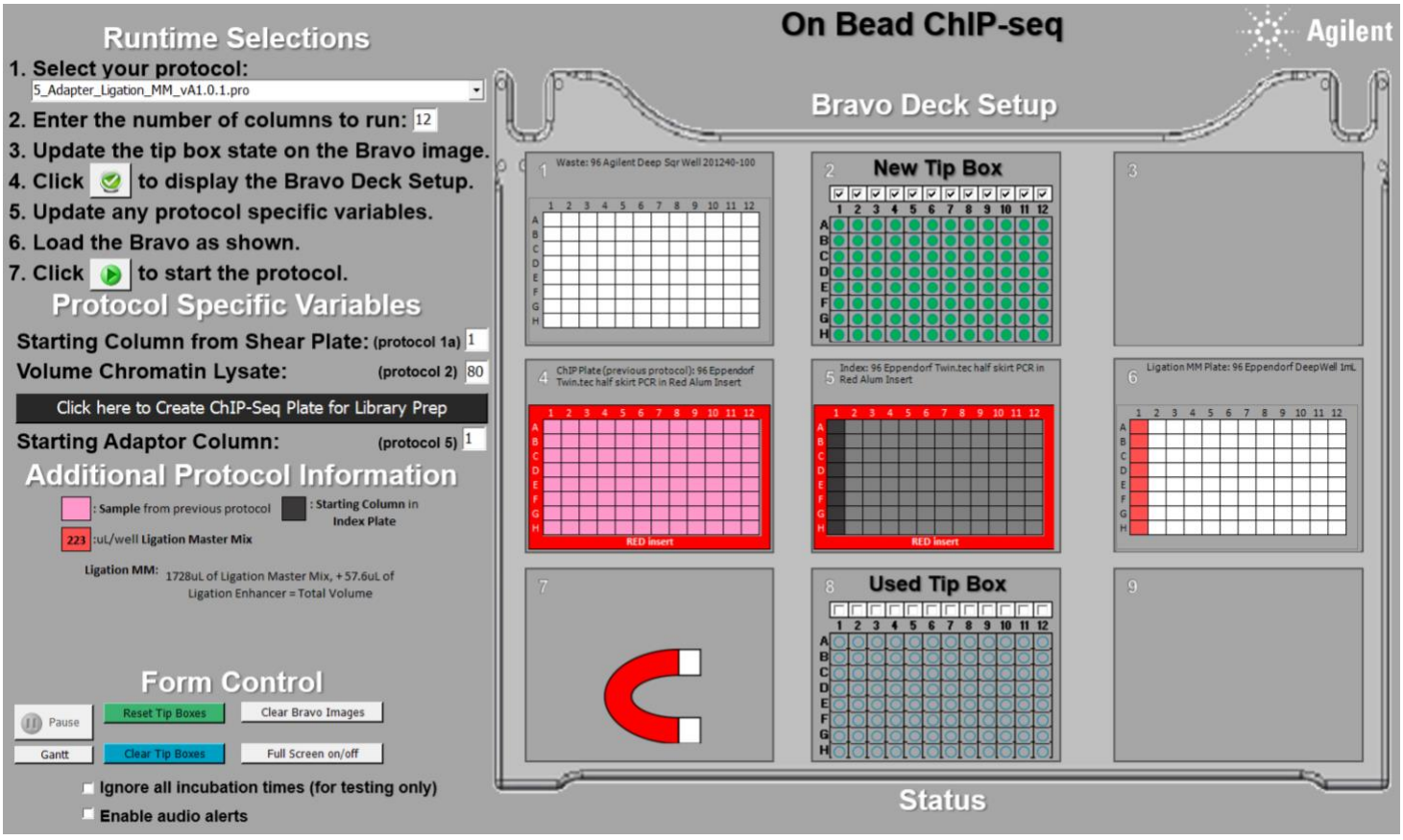
- Add 5 uL End Prep Mix to each sample.
- Mix up and down at least 10 times (the mix cycle can be changed in the parameters).

5. Incubate for 30 minutes at 20°C, then 30 minutes at 65°C, then hold at 4°C in a PCR cycler with the lid set to 75°C (can leave at 4°C for ~ 1 hour with no problems).
6. Freshly prepare the following **Ligation Mix** on ice. Each sample requires 15.5 µL and it is recommended to prepare 20% more reaction volumes per sample. Aliquot 1/8 of the total volume of the mastermix to each well in the first column of the **Ligation Mix Plate**. Place the **Ligation Mix Plate** on **Bravo deck 6**. (The example below shows the amount required for a full 96-well plate.)

Bravo 5 Ligation Mix

Reagent	Amount per sample (µL)	Amount required for 96 samples (µL)	Amount prepared for 96 samples (µL)
NEBNext Ultra II End Prep Enzyme Mix	15	1440	1728
NEBNext Ultra II End Prep Reaction Buffer	0.5	48	57.6
Mastermix Total	**15.5**	**1488**	**1785.6**
Amount added to each well of the first column for Bravo (µL)			**223.2**

7. After incubation, place the **ChIP Plate** on **Bravo deck 4** and the **Index Plate** on **Bravo deck 5**. The indexing adapters we usually use are from NEXTflex® Unique Dual Index Barcodes (Set B: UDI#97-192) (Catalog #NOVA-514151) (1:100 diluted 0.25 µM). Adapters can be diluted with 1× T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, pH 7.5 at 25°C).
8. Select Bravo protocol **5_Adapter_Ligation_MM_vA1.0.1.pro** from the drop down manual in step 1 and click the green check symbol to display the Bravo Deck Setup. Make sure the deck is set up as shown. Once ready, click on the right-pointing triangle (▷) at step 7 to start the protocol.



Bravo will perform the following steps:

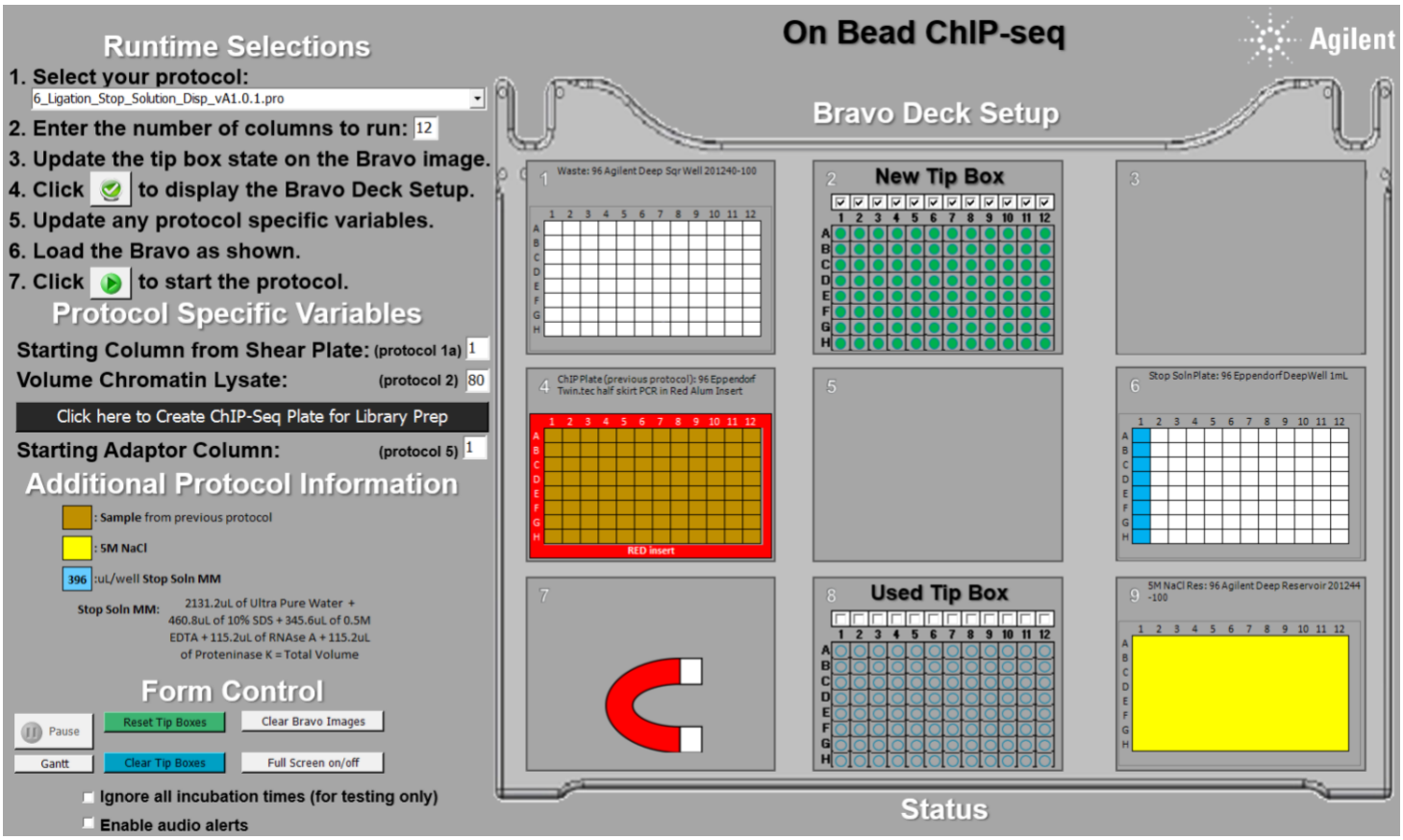
- Add 15.5 µL of the Ligation Mix to each sample.
- Add 2.5 µL of Index Barcodes (1:100 diluted 0.25µM) to each sample.
- Mix up and down at least 10 times (the mix cycle can be changed in the parameters).

9. Incubate the plate at 20°C for 15 minutes in a PCR cycler with the heated lid set to “off.”

10. Freshly prepare the following **Ligation Stop Solution** at room temperature. Each sample requires 27.5 µL and it is recommended to prepare 1.2 reaction volumes per sample. Aliquot 1/8 of the total volume of the mastermix to each well in the first column of the **Stop Solution Plate**. Place the **Stop Solution Plate** on **Bravo deck 6**. (The example below shows the amount required for a full 96-well plate.)

Bravo 6 Ligation Stop Solution				
Reagent	**Final concentration**	**Amount per sample (µL)**	**Amount required for 96 samples (µL)**	**Amount prepared for 96 samples (µL)**
UltraPure DNase/RNase-Free Distilled Water		17.5	1680	2016
10% SDS	0.5%	4	384	460.8
0.5 M EDTA	18.75 mM	3	288	345.6
20 mg/mL Proteinase K	250 µg/mL	1	96	115.2
20 mg/mL RNase A	125 µg/mL	1	96	115.2
	Mastermix Total	**26.5**	**2544**	**3052.8**
Amount added to each well of the first column for Bravo (µL)				**381.6**

11. After incubation, place the **ChIP Plate** on **Bravo deck 4** and **5M NaCl Reservoir** on **Bravo deck 9**. Ensure the reservoirs contain at least enough liquid to form a thin layer across the bottom.
12. Select Bravo protocol **6_Ligation_Stop_Solution_Disp_vA1.0.1.pro** from the drop down manual in step 1 and click the green check symbol to display the Bravo Deck Setup. Make sure the deck is set up as shown. Once ready, click on the right-pointing triangle (▷) at step 7 to start the protocol.



Bravo will perform the following steps:

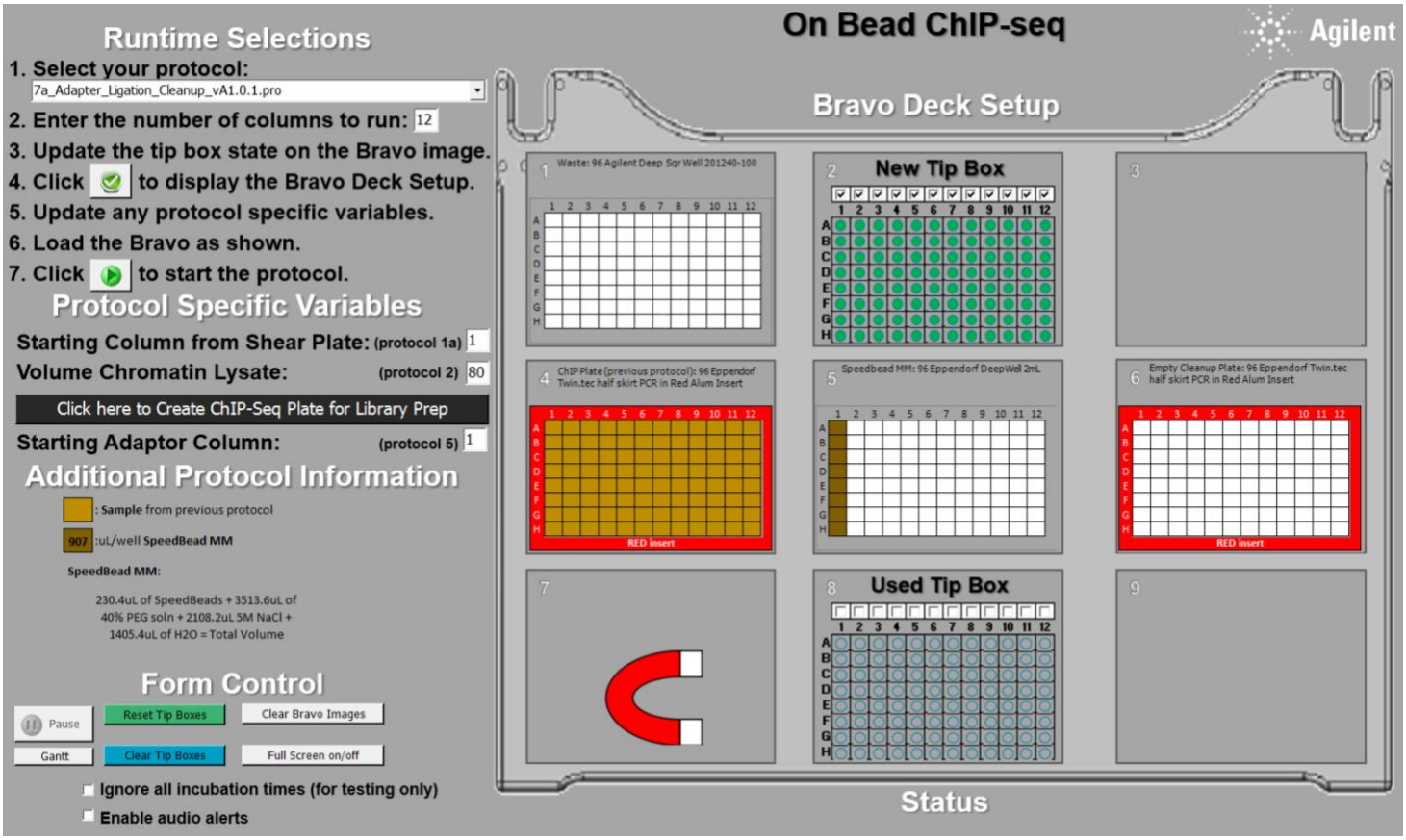
- Add 27.5 µL of the Ligation Stop Solution to each sample.
- Add 4.5 µL of 5 M NaCl to each sample.
- Mix up and down at least 10 times (the mix cycle can be changed in the parameters).

13. **Reverse crosslinking**: Incubate at 55°C for 1 hour to digest RNA and proteins, then shift the incubation temperature to 65°C for at least 2 hours (up to 16 hours) in a PCR cycler with the heated lid set to at least 75°C to prevent evaporation and drying out of the samples. This can be left at 12°C or 4°C overnight.
14. Freshly prepare the following **Speedbeads Mastermix** at room temperature. Each sample requires 63 µL and it is recommended to prepare 20% more reaction volumes per sample. Aliquot 1/8 of the total volume of the mastermix to each well in the first column of the **Speedbeads Mastermix Plate**. Place the **Speedbeads Mastermix Plate** on **Bravo deck 5**. (The example below shows the amount required for a full 96-well plate.)

Bravo 7a Speedbeads Mastermix				
Reagent	**Final concentration**	**Amount per sample (µL)**	**Amount required for 96 samples (µL)**	**Amount prepared for 96 samples (µL)**
40% PEG8000	8.6%	30.5	2928	3513.6
5M NaCl	0.8M	18.3	1756.8	2108.16
Speedbeads	-	2	192	230.4
Water	-	12.2	1171.2	1405.44
	Mastermix Total	**63**	**6048**	**7257.6**
Amount added to each well of the first column for Bravo (µL)				**907.2**
	Sample (µL)	79		

Final Volume (µL)	142		

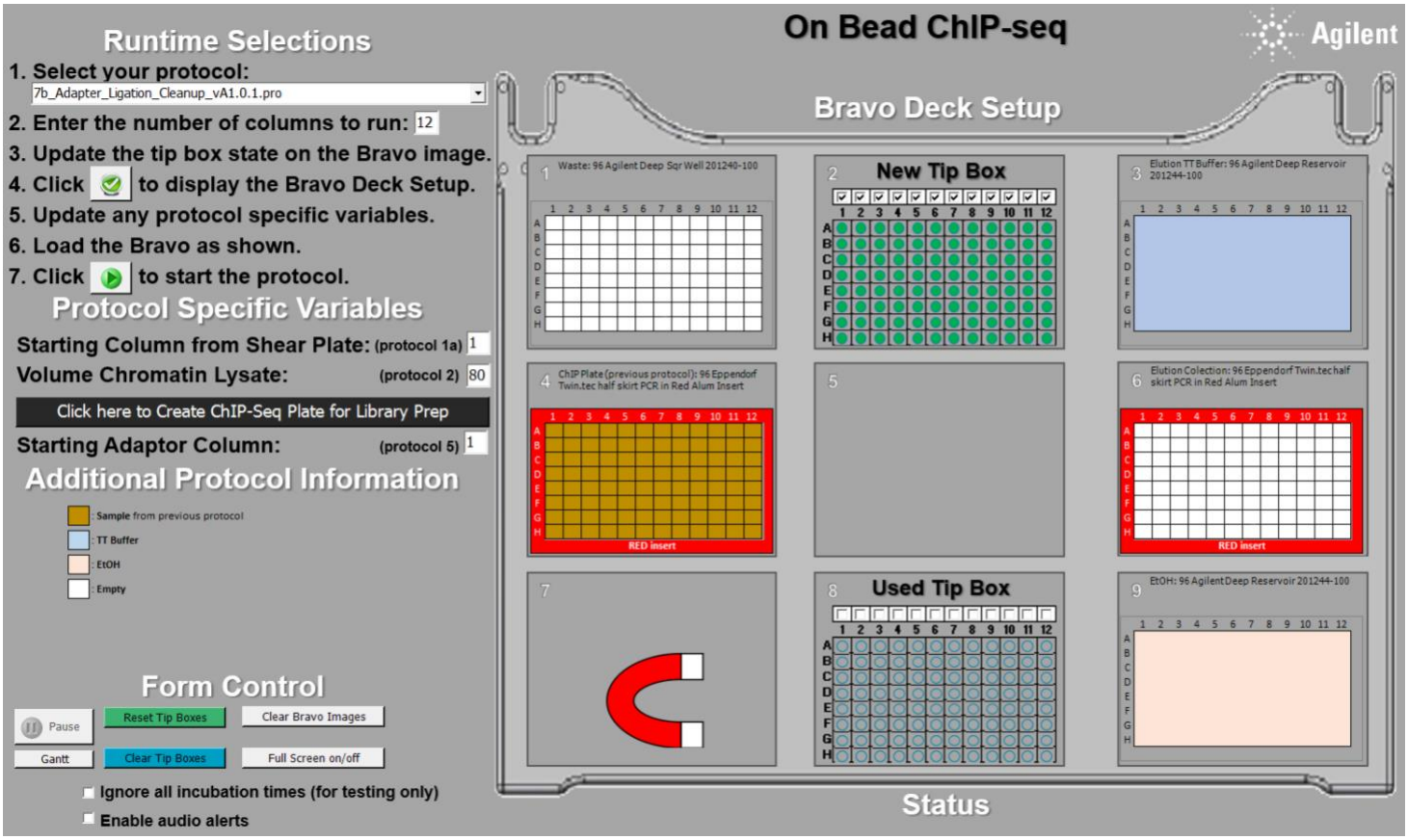
15. After incubation, place the **ChIP Plate** back to **Bravo deck 4**.
16. Select Bravo protocol **7a_Adapter_Ligation_Cleanup_vA1.0.1.pro** from the drop down manual in step 1 and click the green check symbol to display the Bravo Deck Setup. Make sure the deck is set up as shown. Once ready, click on the right-pointing triangle (▷) at step 7 to start the protocol.



Bravo will perform the following steps:

- Transfer the supernatant from the ChIP plate with Dynabeads to the empty ChIP Cleanup Plate.
- Add 63 µL of Speedbeads mastermix to each 79 µL ChIP sample. The final concentration of PEG will be 8.6% and the final concentration of NaCl will be 0.8 M.
- Mix the samples to homogeneity by vortexing or repetitive pipetting. Incubate at room temperature for 10 minutes.

17. During incubation, place the **Waste Plate** on **Bravo deck 1**, **TT Buffer Reservoir** on **Bravo deck 3**, **empty Elution Plate** on **Bravo deck 6**, and **80% Ethanol Reservoir** on **Bravo deck 9**. Ensure the reservoirs contain at least enough liquid to form a thin layer across the bottom.
18. Immediately after protocol 7a is completed, select Bravo protocol **7b_Adapter_Ligation_Cleanup_vA1.0.1.pro** from the drop down manual in step 1 and click the green check symbol to display the Bravo Deck Setup. Make sure the deck is set up as shown. Once ready, click on the right-pointing triangle (▷) at step 7 to start the protocol.



Bravo will perform the following steps:

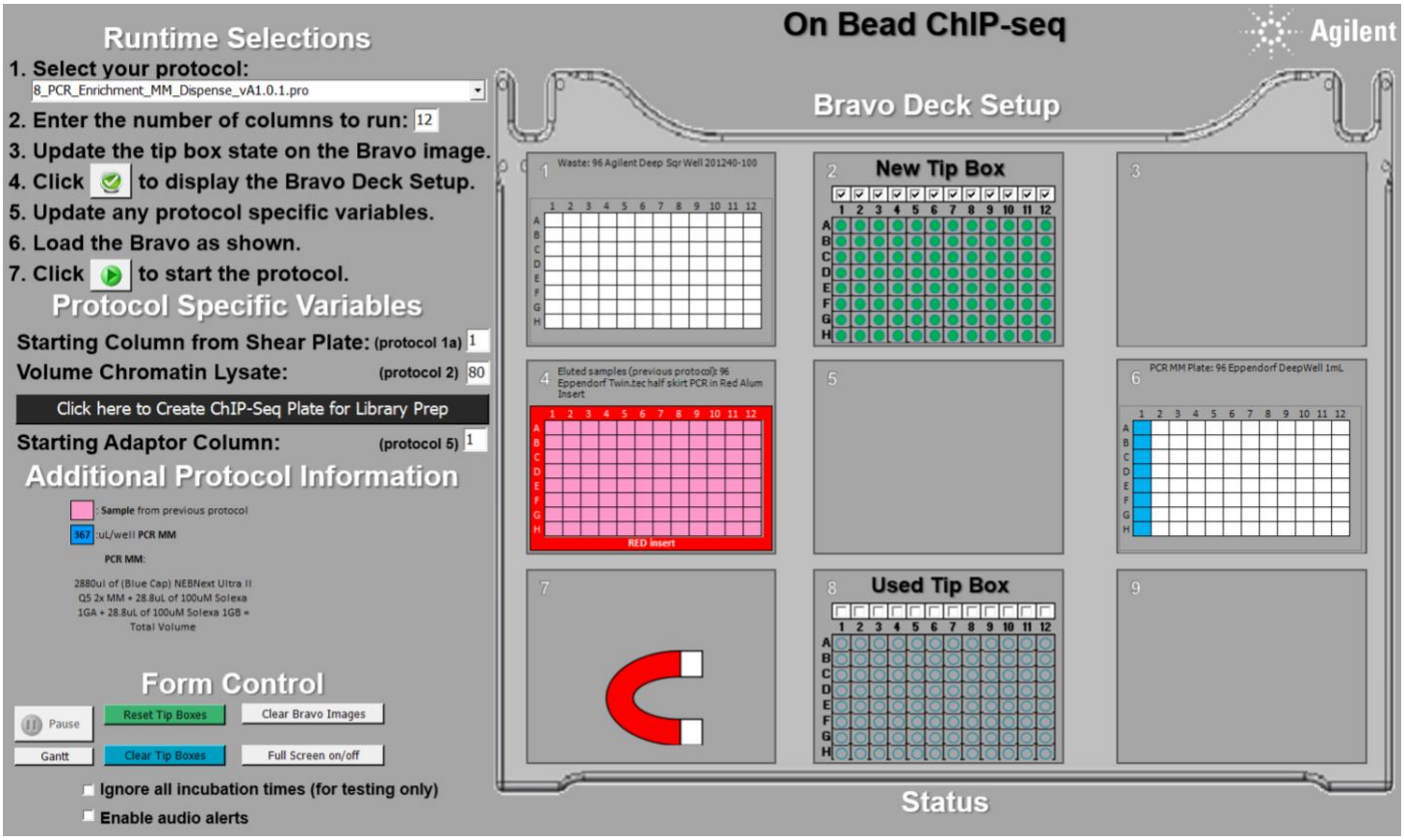
- Apply the plate to a magnet and aspirate the cleared supernatant.
- Wash the beads by adding 180 µL of 80% ethanol at room temperature and moving the plate to either side of the magnet six times. Collect the beads on the magnet and discard the supernatant once cleared. Repeat this wash one additional time.
- After removing the second ethanol wash, air-dry the beads for 10 minutes, or until “cracks” just begin to appear in the packed beads.
- Elute DNA by adding 25 µL TT Buffer. Mix then incubate at room temperature for 5–10 minutes.
- Apply the plate to the magnet. Transfer the supernatant to a new plate and discard the Speedbeads.

19. At the end of the protocols, the **ChIP Plate** (deck 6) should contain ChIP samples eluted in 25 µL of TT buffer. Move the **ChIP Plate** to **Bravo deck 4** before proceeding to the next protocol.
20. Freshly prepare the following **PCR Mastermix**. Each sample requires 25.5 µL and it is recommended to prepare 20% more reaction volumes per sample. Aliquot appropriate amount to each well in the first column of the **PCR Mastermix Plate.** Place the **PCR Mastermix Plate** on **Bravo deck 6**. (The example below shows the amount required for a full 96-well plate.)

Bravo 8 PCR Mastermix			
Reagent	**Amount per sample (µL)**	**Amount required for 96 samples (µL)**	**Amount prepared for 96 samples (µL)**
NEBNext Ultra II Q5 2x Mastermix	25	2400	2880
Forward Primer: 100 µM Solexa 1GA	0.25	24	28.8

Reverse Primer: 100 µM Solexa 1GB	0.25	24	28.8
Mastermix Total	**25.5**	**2448**	**2937.6**
Amount added to each well of the first column for Bravo (µL)			**367.2**
Sample (µL)	24.5		
Final Volume (µL)	50		

21. Select Bravo protocol **8_PCR_Enrichment_MM_Dispense_vA1.0.1.pro** from the drop down manual in step 1 and click the green check symbol to display the Bravo Deck Setup. Make sure the deck is set up as shown. Once ready, click on the right-pointing triangle (▷) at step 7 to start the protocol.



Bravo will perform the following steps:

- Add 25.5 µL of the PCR Mastermix to each sample.
- Mix up and down at least 10 times (the mix cycle can be changed in the parameters).

22. PCR amplify using a PCR cycler with a heated lid set to 105°C.

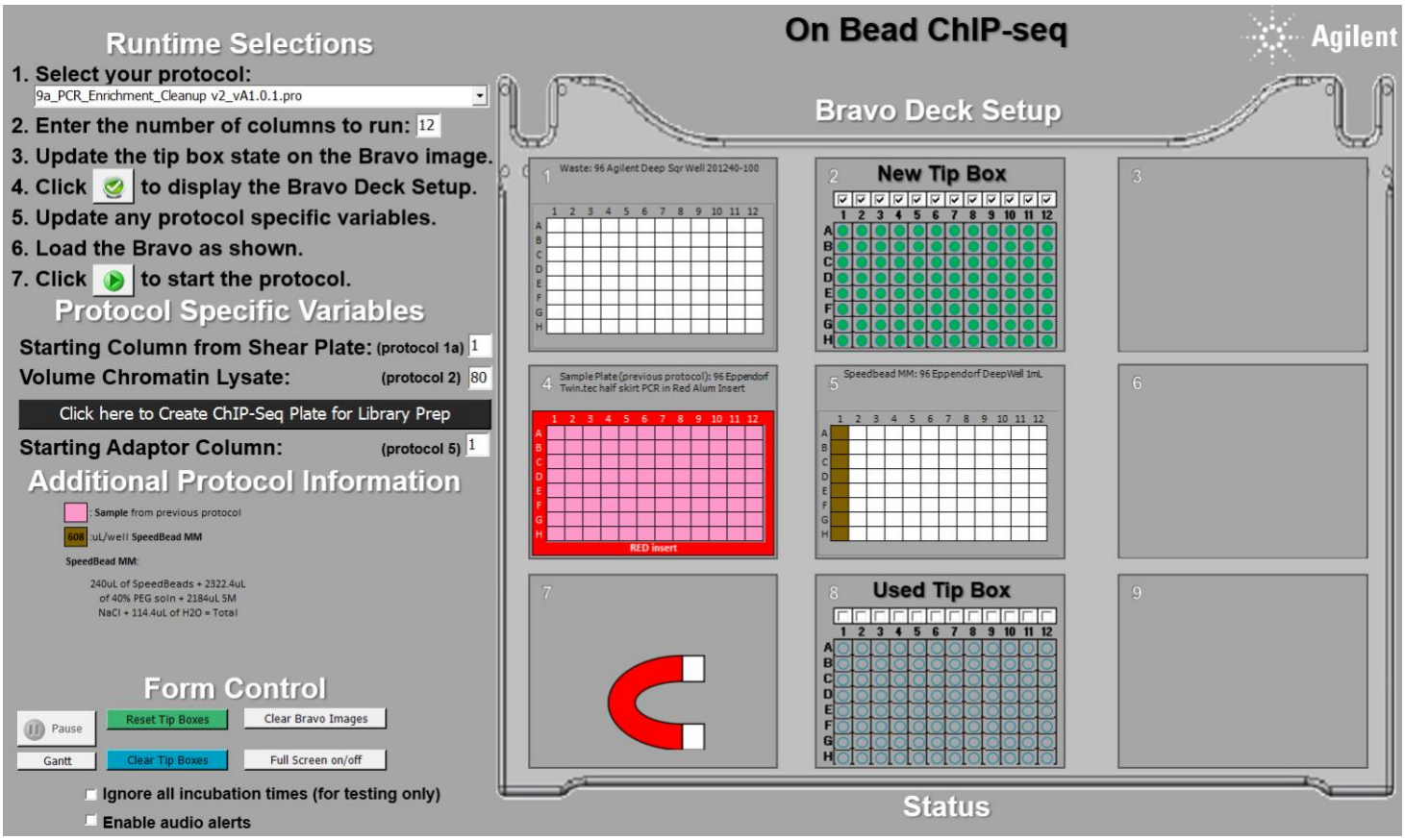
Steps	Temperature	Time	Cycle
Initial denaturation	98°C	30 s	1
Denaturation	98°C	10 s	9–16 cycles (empirically determined, 12 cycles is a good default)
Annealing	60°C	15 s	
Extension	72°C	30 s	

Final extension	72°C	2 min	1
Hold	4°C	Forever	

23. Freshly prepare the following **Speedbeads Mastermix** at room temperature. Each sample requires 40.5 µL and it is recommended to prepare 20% more reaction volumes per sample. Aliquot 1/8 of the total volume of the mastermix to each well in the first column of the **Speedbeads Mastermix Plate**. Place the **Speedbeads Mastermix Plate** on **Bravo deck 5**. (The example below shows the amount required for a full 96-well plate.)
    - Note: The low (8.5%) PEG8000 amount size selects against short adapter dimers (~125 bp size).

Bravo 9a Speedbeads Mastermix				
Reagent	**Final concentration**	**Amount per sample (µL)**	**Amount required for 96 samples (µL)**	**Amount prepared for 96 samples (µL)**
40% PEG8000	8.5%	19.35	1857.6	2229.12
5M NaCl	1M	18.2	1747.2	2096.64
Speedbeads	-	2	192	230.4
Water	-	0.95	91.2	1109.44
	Mastermix Total	**40.5**	**3888**	**4665.6**
Amount added to each well of the first column for Bravo (µL)				**583.2**
	Sample (µL)	50		
	Final Volume (µL)	90.5		

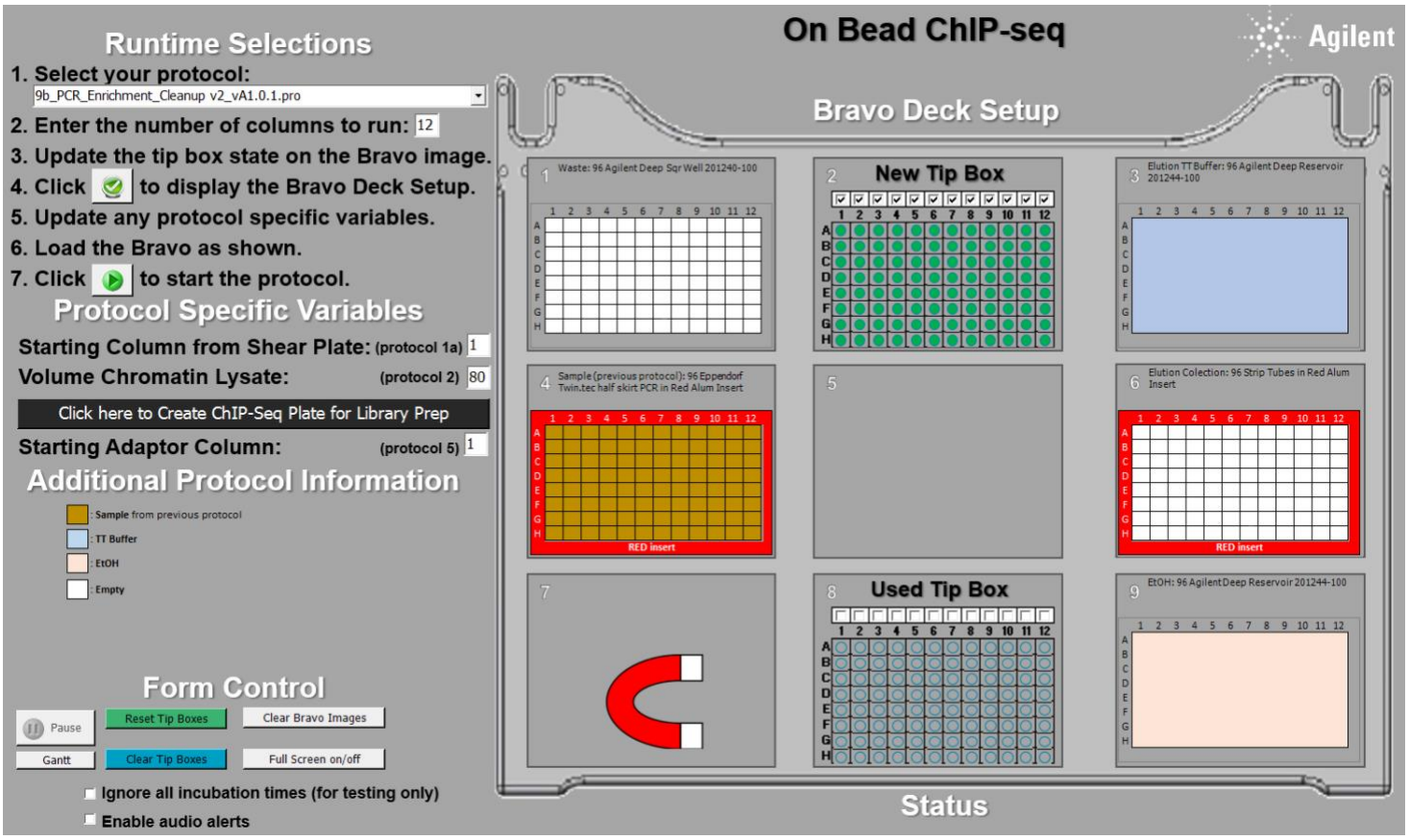
24. After PCR, place the **ChIP Plate** back to **Bravo deck 4**.
25. Select Bravo protocol **9a_PCR_Enrichment_Cleanup v2_vA1.0.1.pro** from the drop down manual in step 1 and click the green check symbol to display the Bravo Deck Setup. Make sure the deck is set up as shown. Once ready, click on the right-pointing triangle (▷) at step 7 to start the protocol.



Bravo will perform the following steps:

- Add 40.5 µL of Speedbead/PEG mastermix to each 50 µL ChIP sample. The final concentration of PEG8000 will be 8.5% and the final concentration of NaCl will be 1 M.
- Mix the samples to homogeneity by vortexing or repetitive pipetting. Incubate at room temperature for 10 min.

26. During incubation, place the **Waste Plate** on **Bravo deck 1**, **TT Buffer Reservoir** on **Bravo deck 3**, **empty Elution Plate** on **Bravo deck 6**, and **80% Ethanol Reservoir** on **Bravo deck 9**. Ensure the reservoirs contain at least enough liquid to form a thin layer across the bottom.
27. Immediately after protocol 9a is completed, select Bravo protocol **9b_PCR_Enrichment_Cleanup v2_vA1.0.1.pro** from the drop down manual in step 1 and click the green check symbol to display the Bravo Deck Setup. Make sure the deck is set up as shown. Once ready, click on the right-pointing triangle (▷) at step 7 to start the protocol.



Bravo will perform the following steps:

- Apply the plate to a magnet and aspirate the cleared supernatant.
- Wash the beads by adding 180 µL of 80% ethanol at room temperature and moving the plate to either side of the magnet six times. Collect the beads on the magnet and discard the supernatant once cleared. Repeat this wash one additional time.
- After removing the second ethanol wash, air-dry the beads for ~10 minutes, or until “cracks” just begin to appear in the packed beads.
- Elute DNA by adding 25 µL TT Buffer. Mix then incubate at room temperature for 5–10 minutes.
- Apply the plate to the magnet. Transfer the supernatant to a new plate and discard the Speedbeads.

28. At the end of the protocols, the **ChIP Elution Plate** (deck 6) should contain ChIP library samples eluted in 25 µL TT buffer.

DNA Quantification

1. Size: Run 5 µL of DNA library + 1 µL of 6X TriTrack DNA Loading Dye on a 2% agarose gel pre-stained with GelGreen.
2. Concentration: Use 1 µL of each library with Qubit HS DNA buffer and standards.
3. Store the remaining DNA library samples at -20°C until ready for sequencing.