

## AutoCUT&RUN: genome-wide profiling of chromatin proteins in a 96 well format on a Biomek

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dx.doi.org/10.17504/protocols.io.ufeetje

Human Cell Atlas Method Development Community | Henikovian CUT&RUNners

Derek Janssens

### ABSTRACT

CUT&RUN is an antibody-targeted nuclease-cleavage method that profiles the genome-wide occupancy of DNA-binding proteins, histones and chromatin modifying proteins *in situ* with exceptional sensitivity and resolution. Here we provide a protocol to perform CUT&RUN in an automated 96 well format using a Beckman Biomek FX liquid handling robot equipped for magnetic separation and temperature control. To circumvent the need to purify small amounts of DNA prior to library preparation, we developed a method to polish the DNA ends in chromatin fragments for direct ligation of Illumina library adapters. This allows the samples to be kept in a 96 well format throughout the DNA end-polishing and adapter ligation steps. Deproteinated CUT&RUN libraries are purified on the Biomek using Ampure XP magnetic beads both before and after PCR enrichment. This AutoCUT&RUN protocol allows a single operator to generate up to 96 libraries in two days that are ready to be pooled and sequenced. The easy, cost-effective workflow makes AutoCUT&RUN an attractive tool for high-throughput characterization of chromatin features, and because AutoCUT&RUN generates high-quality profiles from frozen tissue samples this method is also suitable for rapidly profiling the epigenetic content of patient samples.

### PROTOCOL STATUS

#### Working

We use this protocol in our group and it is working

### GUIDELINES

#### ▪ WORKFLOW

#### Days 1-3, Cells to Libraries

Steps 1-8, binding cells to beads: 30 min  
Steps 9-13, permeabilize cells and bind (primary) antibody: 2 hr-overnight  
Steps 14-21 (Optional), bind secondary antibody: 1 hr-overnight  
Steps 22-27, bind Protein A-MNase fusion protein on Biomek: ~1.5 hr  
Steps 28-34, targeted chromatin digestion on Biomek: ~1.5 hr  
Steps 35-38, chromatin end repair and dA-tailing: ~2.5 hr  
Steps 39-44, adapter ligation: 1 hr – overnight  
Steps 45-52, Pre-PCR DNA cleanup on the Biomek: ~2 hr  
Steps 53-56, PCR amplification of CUT&RUN Libraries: 1 hr-overnight  
Steps 57-64, Post-PCR DNA cleanup on the Biomek: ~2 hr

#### Days 4-6, Sequencing

Step 65-69, sequencing: 1-2 days

#### Day 7 (variable), Data processing and analysis

Step 70, ≥1 day

#### ▪ TROUBLESHOOTING

Troubleshooting table			
Steps	Problem	Possible reasons	Solutions
Biomek Setup	Liquid volume is inconsistent between wells	The Biomek aspirate and or dispense function is imprecise from well to well potentially due to a clogged line or pump	<ul style="list-style-type: none"> <li>Avoid using problematic wells.</li> <li>Contact Beckman Coulter technician, and possibly replace the P200 head.</li> </ul>
Biomek Setup	Incorrect volume is being aspirated or dispensed in all wells	Aspiration is not occurring at the desired height within the well. The Biomek aspirate and or dispense function is not accurate.	<ul style="list-style-type: none"> <li>Adjust aspiration height in the Method.</li> <li>Adjust volumes for desired result as determined empirically.</li> <li>Contact Beckman Coulter technician and possibly recalibrate the machine.</li> </ul>
13	Beads clump and cannot be disaggregated	Cells lyse	<ul style="list-style-type: none"> <li>Reduce the digitonin concentration. Use Non-stick tubes</li> </ul>
67	No DNA is detected by Tape Station analysis	This indicates the reaction failed and could be due to (1) failed CUT&RUN reaction or (2) failed Library Prep.	<ul style="list-style-type: none"> <li>Run a positive control sample for an abundant epitope, e.g. H3K27me3.</li> <li>Ensure Bioimek is pipetting accurately e.g. no Ethanol remains after 10 min Air Dry.</li> </ul>
67	A prominent peak at ~120bp is detected by Tapestation analysis in a large number of samples	This peak corresponds to self-ligated adapters and can persist after cleanup when relatively little digested chromatin is released (i.e. low cell numbers or low abundance epitopes).	<ul style="list-style-type: none"> <li>Perform additional rounds of Ampure Cleanup.</li> <li>Reduce the ratio of Ampure Beads or HXP Mix to sample.</li> <li>Increase pA-MNase digestion time.</li> </ul>
67	A small peak at ~120bp is detected by Tapestation analysis in a few samples	This is typical and indicates a small amount of self-ligated adapter remains in these libraries.	<ul style="list-style-type: none"> <li>Pool Libraries then perform one round of Ampure Cleanup before sequencing</li> </ul>
67	No DNA <300bp is detected by Tapestation analysis	Sub-nucleosomal particles (often protected by transcription factors) are being denatured during the end repair and ligation or being removed during library cleanup.	<ul style="list-style-type: none"> <li>Ensure the dA-Tailing step is at 58 degrees.</li> <li>Increase the concentration of Adapters.</li> <li>Increase the ratio of Ampure Beads or HXP Mix to sample.</li> </ul>
70	Data quality from a sample of interest is indistinguishable from the IgG control	This indicates the reaction failed possibly due to an antibody failure or over digestion by pA-MNase.	<ul style="list-style-type: none"> <li>Increase antibody concentration.</li> <li>Test antibody binding by adding a fluorescent secondary and imaging. Reduce digestion time.</li> <li>Replace antibody.</li> </ul>

## ■ BIOMEK PROGRAMMING

Labware Type Editor:

Eppendorf 96 Well LoBind PCR Plate, Semi-skirted (PCR 96 Well Plate)																																																						
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Height:	1		cm																																						
Bottom:	N/A																																								

96S Super Magnet Plate (ALPAQUA Magnet Plate)					
Basic Info	X		Y		cm
	Span:	12.7762		8.5471	
Stacking	Height:	1.6		cm	
	X		Y	Z	
	Stack Offset:	0	0	0.1	cm
	Stacking Speed:	100	%		
Stack Offsets Edit	PCR 96 Well Plate:	X	Stack Offset X:	0	cm
			Stack Offset Y:	0.15	cm
			Stack Offset Z:	1.2	cm
Wells	X		Y		cm
	Well Offset:	1.43764		1.12268	
Well Configuration	Well Count:	12		8	
	Well Spacing:	0.9		0.9	cm
	Max Volume:	170		μL	
	Shape:	Round			
	Upper Radius:	0.28		cm	
	Lower Radius:	0.28		cm	
	Height:	0.7		cm	
	Bottom:	N/A			

Aluminum Heat Block for PCR Plates (Cold Block)					
Basic Info	X		Y		cm
	Span:	12.7762		8.5471	
Stacking	Height:	1.4		cm	
	X		Y	Z	
	Stack Offset:	0	0	0.1	cm
	Stacking Speed:	100	%		
	PCR 96 Well Plate:	X	Stack Offset X:	0	cm
			Stack Offset Y:	0	cm
			Stack Offset Z:	0.45	cm
Wells	X		Y		cm
	Well Offset:	1.43764		1.12268	
	Well Count:	12		8	
	Well Spacing:	0.9		0.9	cm
	Max Volume:	180		μL	
	Shape:	Round			
	Upper Radius:	0.3		cm	
	Lower Radius:	0.28		cm	
	Height:	0.5		cm	
	Bottom:	X			
	Shape:	Cone			
	Radius:	0.28		cm	
	Height:	0.7		cm	

96 Well Polystyrene V-Bottom Microplate (V-Bottom Plate)				
Basic Info				
	X	Y		
Span:	12.78	8.56	cm	
Height:	1.41	cm		
Wells	X	Y		
	Well Offset:	1.44	1.12	cm
Well Count:	12	8		
Well Spacing:	0.9	0.9	cm	
Max Volume:	215		μL	
Well Configuration	Shape:	Round		
	Upper Radius:	0.309	cm	
	Lower Radius:	0.2735	cm	
	Height:	0.75	cm	
	Bottom:	X		
	Shape:	Cone		
	Radius:	0.2735	cm	
	Height:	0.2	cm	

96 Deep Well 2 mL Plate (Deep Well Plate)				
Basic Info				
	X	Y		
Span:	12.78	8.56	cm	
Height:	4.4	cm		
Wells	X	Y		
	Well Offset:	1.44	1.13	cm
Well Count:	12	8		
Well Spacing:	0.9	0.9	cm	
Max Volume:	2300		μL	
Well Configuration	Shape:	Rectangle		
	X	Y		
	Upper:	0.82	0.82	cm
	Lower:	0.737	0.737	cm
	Height:	3.732	cm	
	Bottom:	X		
	Shape:	Hemisphere		
	Radius:	0.3685	cm	

Biomek AP96 P250 barrier tips (AP96_200μL)				
Basic Info				
	X	Y		
Span:	12.789	8.56	cm	
Height:	5.6	cm		
Tips	X	Y		
	Tip Offset:	1.447	1.132	cm
	Tip Count:	12	8	
	Tip Spacing:	0.9	0.9	cm
	Tip Load Z Offset:	-0.53	cm	
	Tip Unload Z Offset:	0	cm	
	Tip Type:	P200		

#### Liquid Type Editor:

DigWash							
Aspirate		Dispense					
Trailing Air Gap:	1	μL	Delay: Speed: Cutoff Velocity:	0	ms		
Delay:	0	ms		10	μL/s		
Speed:	25	μL/s		150	μL/s		
Blowout		Tip Touch					
Volume:	20	μL	Height: from: Angle: Speed: Delay:	-1	mm		
Delay:	5000	ms		Top			
Prewet				90			
Overage:	0	μL		100	%		
Delay:	0	ms		0	ms		
Wash		Sensitivity					
Default Cycles:	1	μL	Liquid Level Sensing				
Default Volume:	100%	μL	N/A				

Ligase/PCR Buffer							
Aspirate		Dispense					
Trailing Air Gap:	0	μL	Delay: Speed: Cutoff Velocity:	0	ms		
Delay:	0	ms		10	μL/s		
Speed:	25	μL/s		150	μL/s		
Blowout		Tip Touch					
Volume:	20	μL	Height: from: Angle: Speed: Delay:	-1	mm		
Delay:	5000	ms		Top			
Prewet				90			
Overage:	0	μL		100	%		
Delay:	0	ms		0	ms		
Wash		Sensitivity					
Default Cycles:	1	μL	Liquid Level Sensing				
Default Volume:	100%	μL	N/A				

Ampure Wash							
Aspirate		Dispense					
Trailing Air Gap:	1	μL	Delay: Speed: Cutoff Velocity:	0	ms		
Delay:	0	ms		10	μL/s		
Speed:	1	μL/s		150	μL/s		
Blowout		Tip Touch					
Volume:	20	μL	Height: from: Angle: Speed: Delay:	-1	mm		
Delay:	5000	ms		Top			
Prewet				90			
Overage:	0	μL		100	%		
Delay:	0	ms		0	ms		
Wash		Sensitivity					
Default Cycles:	1	μL	Liquid Level Sensing				
Default Volume:	100%	μL	N/A				

EtOH							
Aspirate		Dispense					
Trailing Air Gap:	1	μL	Delay:	0	ms		
Delay:	0	ms	Speed:	10	μL/s		
Speed:	25	μL/s	Cutoff Velocity:	150	μL/s		
Blowout		Tip Touch					
Volume:	20	μL	Height:	-1	mm		
Delay:	5000	ms	from:	Top			
Prewet		Angle:		90			
Overage:	0	μL	Speed:	100	%		
Delay:	0	ms	Delay:	0	ms		
Wash		Sensitivity					
Default Cycles:	1	μL	Liquid Level Sensing				
Default Volume:	100%	μL	N/A				

Tris-HCl-1							
Aspirate		Dispense					
Trailing Air Gap:	1	μL	Delay:	0	ms		
Delay:	0	ms	Speed:	10	μL/s		
Speed:	25	μL/s	Cutoff Velocity:	150	μL/s		
Blowout		Tip Touch					
Volume:	20	μL	Height:	-1	mm		
Delay:	5000	ms	from:	Top			
Prewet		Angle:		90			
Overage:	0	μL	Speed:	100	%		
Delay:	0	ms	Delay:	0	ms		
Wash		Sensitivity					
Default Cycles:	1	μL	Liquid Level Sensing				
Default Volume:	100%	μL	N/A				

Tris-HCl-2							
Aspirate		Dispense					
Trailing Air Gap:	1	μL	Delay:	0	ms		
Delay:	0	ms	Speed:	10	μL/s		
Speed:	1	μL/s	Cutoff Velocity:	150	μL/s		
Blowout		Tip Touch					
Volume:	20	μL	Height:	-1	mm		
Delay:	5000	ms	from:	Top			
Prewet		Angle:		90			
Overage:	0	μL	Speed:	100	%		
Delay:	0	ms	Delay:	0	ms		
Wash		Sensitivity					
Default Cycles:	1	μL	Liquid Level Sensing				
Default Volume:	100%	μL	N/A				

## Techniques:

Aspirate on Magnet		
Pipetting Template:	Default Template	
<b>Aspirate Tab</b>		
Move within the well at:	25	% speed
Aspirate at:	0	mm from the Bottom
N/A	Follow liquid level when aspirating or dispensing liquid	
N/A	Touch tips on the sides of wells	
N/A	Prewet the tips	
X	Aspirate a leading air gap for blowout	
N/A	Mix prior to aspirating liquid	
X	Aspirate a trailing air gap after leaving the liquid	

Dispense in Waste		
Pipetting Template:	Default Template	
<b>Dispense Tab</b>		
Move within the Well at:	100	% speed
Dispense at:	30	mm from the Bottom
N/A	Follow liquid level when aspirating or dispensing liquid	
X	Touch tips on the sides of the wells	
X	Blowout all leading air gaps	
N/A	Mix after dispensing	

Aspirate from DWP		
Pipetting Template:	Default Template	
<b>Aspirate Tab</b>		
Move within the well at:	50	% speed
Aspirate at:	2	mm from the Bottom
N/A	Follow liquid level when aspirating or dispensing liquid	
N/A	Touch tips on the sides of wells	
N/A	Prewet the tips	
X	Aspirate a leading air gap for blowout	
N/A	Mix prior to aspirating liquid	
X	Aspirate a trailing air gap after leaving the liquid	

Dispense on Magnet		
Pipetting Template:	Default Template	
<b>Dispense Tab</b>		
Move within the Well at:	25	% speed
Dispense at:	2	mm from the Bottom
N/A	Follow liquid level when aspirating or dispensing liquid	
X	Touch tips on the sides of the wells	
X	Blowout all leading air gaps	
N/A	Mix after dispensing	

Aspirate from VBP			
Pipetting Template:	Default Template		
<b>Aspirate tab</b>			
Move within the well at:	50	% speed	
Aspirate at:	0.5	mm from the	Bottom
N/A	Follow liquid level when aspirating or dispensing liquid		
N/A	Touch tips on the sides of wells		
N/A	Prewet the tips		
X	Aspirate a leading air gap for blowout		
N/A	Mix prior to aspirating liquid		
X	Aspirate a trailing air gap after leaving the liquid		

Dispense and Mix-1			
Pipetting Template:	Default Template		
<b>Dispense Tab</b>			
Move within the Well at:	25	% speed	
Dispense at:	5	mm from the	Bottom
N/A	Follow liquid level when aspirating or dispensing liquid		
X	Touch tips on the sides of the wells		
X	Blowout all leading air gaps		
X	Mix after dispensing liquid		
Mix:	30	µL	10 times
Aspirate at:	5	mm from the	Bottom at 25 µL/s
Dispense at:	5	mm from the	Bottom at 25 µL/s

Dispense and Mix-2			
Pipetting Template:	Default Template		
<b>Dispense Tab</b>			
Move within the Well at:	25	% speed	
Dispense at:	3	mm from the	Bottom
N/A	Follow liquid level when aspirating or dispensing liquid		
X	Touch tips on the sides of the wells		
X	Blowout all leading air gaps		
X	Mix after dispensing liquid		
Mix:	15	µL	10 times
Aspirate at:	3	mm from the	Bottom at 25 µL/s
Dispense at:	3	mm from the	Bottom at 25 µL/s

Dispense and Mix-3				
Pipetting Template:	Default Template			
Dispense Tab				
Move within the Well at:	25	% speed		
Dispense at:	3	mm from the	Bottom	
N/A	Follow liquid level when aspirating or dispensing liquid			
X	Touch tips on the sides of the wells			
X	Blowout all leading air gaps			
X	Mix after dispensing liquid			
Mix:	25	µL	10	times
Aspirate at:	3	mm from the	Bottom	at 25 µL/s
Dispense at:	3	mm from the	Bottom	at 25 µL/s

Aspirate on Rack				
Pipetting Template:	Default Template			
Aspirate tab				
Move within the well at:	25	% speed		
Aspirate at:	0.5	mm from the	Bottom	
N/A	Follow liquid level when aspirating or dispensing liquid			
N/A	Touch tips on the sides of wells			
N/A	Prewet the tips			
X	Aspirate a leading air gap for blowout			
N/A	Mix prior to aspirating liquid			
N/A	Aspirate a trailing air gap after leaving the liquid			

Dispense and Mix-4				
Pipetting Template:	Default Template			
Dispense Tab				
Move within the Well at:	25	% speed		
Dispense at:	1	mm from the	Bottom	
N/A	Follow liquid level when aspirating or dispensing liquid			
X	Touch tips on the sides of the wells			
X	Blowout all leading air gaps			
X	Mix after dispensing liquid			
Mix:	175	µL	20	times
Aspirate at:	1	mm from the	Bottom	at 25 µL/s
Dispense at:	1	mm from the	Bottom	at 25 µL/s

Dispense and Mix-5					
Pipetting Template:	Default Template				
Dispense Tab					
Move within the Well at:	25	% speed			
Dispense at:	1	mm from the	Bottom		
N/A	Follow liquid level when aspirating or dispensing liquid				
X	Touch tips on the sides of the wells				
X	Blowout all leading air gaps				
X	Mix after dispensing liquid				
Mix:	30	µL	20	times	
Aspirate at:	1	mm from the	Bottom	at	50 µL/s
Dispense at:	1	mm from the	Bottom	at	50 µL/s

Dispense and Mix-6					
Pipetting Template:	Default Template				
Dispense Tab					
Move within the Well at:	25	% speed			
Dispense at:	1	mm from the	Bottom		
N/A	Follow liquid level when aspirating or dispensing liquid				
X	Touch tips on the sides of the wells				
X	Blowout all leading air gaps				
X	Mix after dispensing liquid				
Mix:	110	µL	20	times	
Aspirate at:	1	mm from the	Bottom	at	25 µL/s
Dispense at:	1	mm from the	Bottom	at	25 µL/s

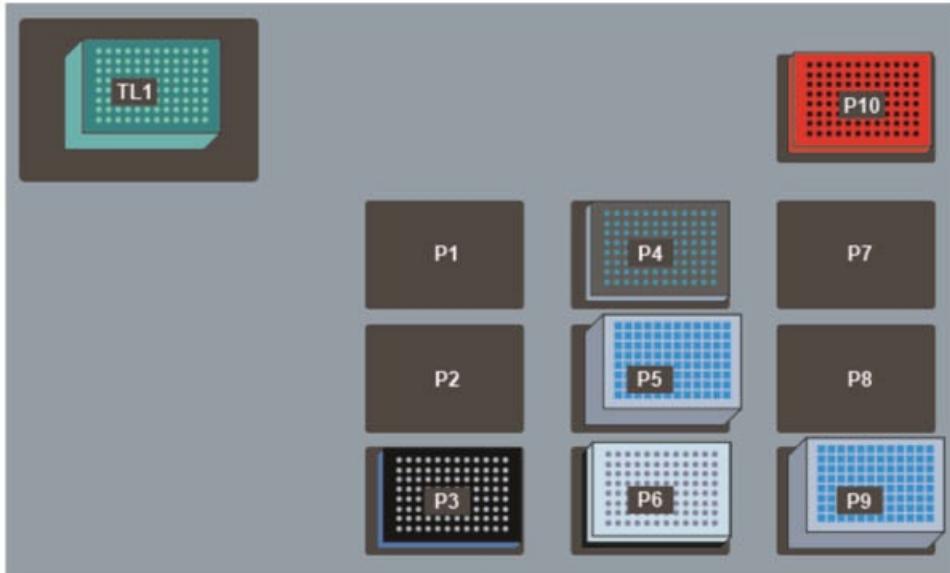
Dispense on Rack					
Pipetting Template:	Default Template				
Dispense Tab					
Move within the Well at:	25	% speed			
Dispense at:	1	mm from the	Bottom		
N/A	Follow liquid level when aspirating or dispensing liquid				
X	Touch tips on the sides of the wells				
X	Blowout all leading air gaps				
N/A	Mix after dispensing				

## Methods:

### ▪ pA-MNase Binding

1) Start

2) Instrument Setup



TL1: Fresh AP96 200  $\mu$ L Tips (double click to increase the # of load times to 10)

P3: ALPAQUA Magnet Plate

P4: V-Bottom Plate preloaded with 175  $\mu$ L pA-MNase solution

P5: Deep Well Plate preloaded with 1 mL of Dig-Wash Buffer

P6: PCR 96 Well Plate preloaded with up to 150  $\mu$ L of ConA bead-bound cells + Antibody stacked on a PCR Plate Rack

P9: Deep Well Plate for receiving liquid waste

P10: Cold Block seated on a Cooling/Heating ALP routed to a Cooling Unit pre-chilled to 0°C

### 3) Move Labware from P6 to P3

Using pod:	Pod1	
Move labware from:	P6	to P3
Move the topmost:	1	piece of labware from the stack

### 4) Pause the whole system for 180 s

Check: Pause	the whole system	for	180	s
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### 5) Load tips from TL1

Tips:	Load
Location:	TL1
Pod:	Pod1

### 6) Aspirate from P3

Labware Type:	Ensure tip height in well is 0.00 mm from bottom		
Position:	PCR 96 Well Plate	Pod:	Pod1
Liquid Type:	P3	N/A	Refresh tips
Volume:	DigWash		
Technique:	150	$\mu$ L	
	Aspirate on Magnet		

7) Dispense in P9			
Labware Type:	Ensure tip height in well is 30.00 mm from bottom		
Position:	Deep Well Plate	Pod:	Pod1
Liquid Type:	P9	N/A	Empty Tips
Volume:	DigWash		
Technique:	150	μL	
	Dispense in Waste		

8) Aspirate from P5			
Labware Type:	Ensure tip height in well is 2.00 mm from bottom		
Position:	Deep Well Plate	Pod:	Pod1
Liquid Type:	P5	N/A	Refresh tips
Volume:	DigWash		
Technique:	150	μL	
	Aspirate from DWP		

9) Dispense in P3			
Labware Type:	Ensure tip height in well is 2.00 mm from bottom		
Position:	PCR 96 Well Plate	Pod:	Pod1
Liquid Type:	P3	N/A	Empty Tips
Volume:	DigWash		
Technique:	150	μL	
	Dispense on Magnet		

10) Pause the whole system for 30 s				
Check:	Pause	the whole system	for	30 s

11) Repeat 6-10 to wash cells a second time

12) Aspirate from P3			
Labware Type:	Ensure tip height in well is 0.00 mm from bottom		
Position:	PCR 96 Well Plate	Pod:	Pod1
Liquid Type:	P3	N/A	Refresh tips
Volume:	DigWash		
Technique:	175	μL	
	Aspirate on Magnet		

13) Dispense in P9			
Labware Type:	Ensure tip height in well is 30.00 mm from bottom		
Position:	Deep Well Plate	Pod:	Pod1
Liquid Type:	P9	N/A	Empty Tips
Volume:	DigWash		
Technique:	175	μL	
	Dispense in Waste		

14) Unload tips to TL1		
Tips:	Unload	
Location:	TL1	
Pod:	Pod1	

15) Move Labware from P3 to P10			
Using pod:	Pod1		
Move labware from:	P3	to	P10
Move the topmost:	1	piece of labware from the stack	

16) Load tips from TL1	
Tips:	Load
Location:	TL1
Pod:	Pod1

17) Aspirate from P4			
Labware Type:	Ensure tip height in well is 1.5 mm from bottom		
V-Bottom Plate	Pod:	Pod1	
Position:	P4	N/A	Refresh tips
Liquid Type:	DigWash		
Volume:	150	μL	
Technique:	Aspirate from VBP		

18) Dispense in P10			
Labware Type:	Ensure tip height in well is 5.00 mm from bottom		
PCR 96 Well Plate	Pod:	Pod1	
Position:	P10	N/A	Empty Tips
Liquid Type:	DigWash		
Volume:	150	μL	
Technique:	Dispense and Mix-1		

19) Pause the whole system for 510 s				
Check:	Pause	the whole system	for	510 s

20) Mix in P10			
		Ensure tip height in well is 5.00 mm from bottom	
Labware Type:	PCR 96 Well Plate	Pod	Pod1
Position:	P10	N/A	Refresh tips
Liquid Type:	DigWash	Mix	25 times
Volume:	30	μL	
Technique:	Custom		
<b>Customize</b>			
Pipetting Template:	Default Template		
<b>Mix Tab</b>			
Move within the well at:	25	% speed	
Aspirate at:	5	mm from the	Bottom
Dispense at:	5	mm from the	Bottom
		at	25 μL/s
		at	25 μL/s
N/A	Follow liquid level when aspirating or dispensing liquid		
X	Touch tips on the sides of the wells		
X	Aspirate a leading air gap prior to mix and blowout after mix is complete		

21) Repeat Steps 19&20 four times in order to mix cells in pA-MNase solution for ~1hr

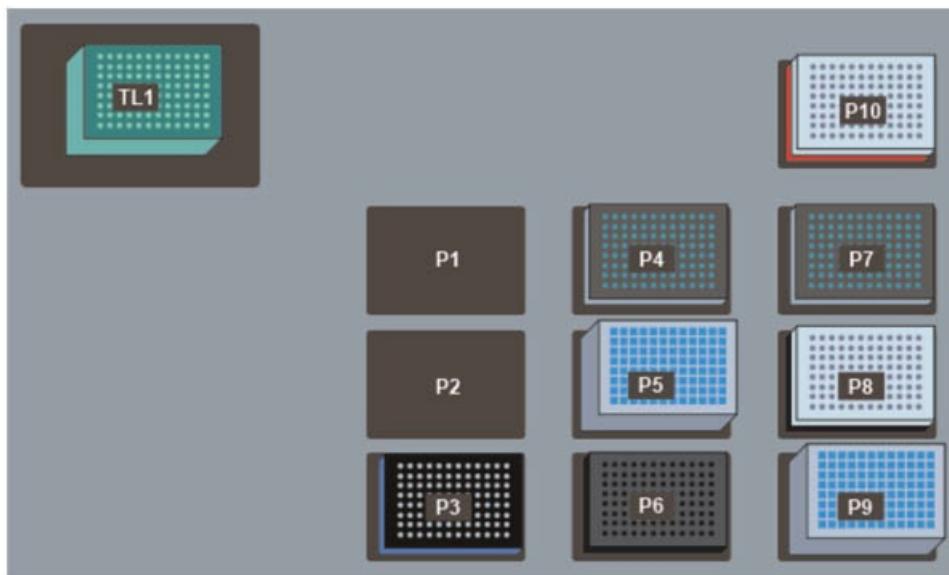
22) Unload tips to TL1	
Tips:	Unload
Location:	TL1
Pod:	Pod1

23) Finish
Check: Clear current instrument setup of all labware after the method completes
Check: Clear current devise setup of all labware after the method completes
Check: Unload disposable tips from all pods after the method completes
Check: Clear all global variables after the method completes

#### ▪ pA-MNase Digest

##### 1) Start

##### 2) Instrument Setup



TL1: Continue with same AP96 200 µL Tips (double click to increase the # of load times to 10)

P3: ALPAQUA Magnet Plate

P4: V-Bottom Plate preloaded with 50 µL pA-MNase Reaction Mix

P5: Deep Well Plate containing leftover Dig-Wash buffer from pA-MNase Binding Method

P6: PCR Plate Rack

P7: V-Bottom Plate preloaded with 25 µL 4X STOP Buffer

P8: PCR 96 Well Plate for accepting digested chromatin stacked on a PCR Plate Rack

P9: Deep Well Plate for receiving liquid waste

P10: 96 Well PCR Plate preloaded with 150 µL Sample in pA-MNase solution on a Cold Block seated on a Cooling/Heating ALP routed to a

Cooling Unit pre-chilled to 0°C

3) Move Labware from P10 to P3				
Using pod:	Pod1			
Move labware from:	P10	to	P3	
Move the topmost:	1		piece of labware from the stack	

4) Pause the whole system for 180 s				
Check: Pause	the whole system	for	180	s

5) Load tips from TL1	
Tips:	Load
Location:	TL1
Pod:	Pod1

6) Aspirate from P3			
Ensure tip height in well is 0.00 mm from bottom			
Labware Type:	PCR 96 Well Plate	Pod:	Pod1
Position:	P3	N/A	Refresh tips
Liquid Type:	DigWash		
Volume:	150	µL	
Technique:	Aspirate on Magnet		

7) Dispense in P9			
Ensure tip height in well is 30.00 mm from bottom			
Labware Type:	Deep Well Plate	Pod:	Pod1
Position:	P9	N/A	Empty Tips
Liquid Type:	DigWash		
Volume:	150	µL	
Technique:	Dispense in Waste		

8) Aspirate from P5			
Ensure tip height in well is 2.00 mm from bottom			
Labware Type:	Deep Well Plate	Pod:	Pod1
Position:	P5	N/A	Refresh tips
Liquid Type:	DigWash		
Volume:	150	µL	
Technique:	Aspirate from DWP		

9) Dispense in P3			
Ensure tip height in well is 2.00 mm from bottom			
Labware Type:	PCR 96 Well Plate	Pod:	Pod1
Position:	P3	N/A	Empty Tips
Liquid Type:	DigWash		
Volume:	150	µL	
Technique:	Dispense on Magnet		

10) Pause the whole system for 30 s				
Check:	Pause	the whole system	for	30 s

11) Repeat Steps 6-10 to wash cells a second time

12) Aspirate from P3			
Ensure tip height in well is 0.00 mm from bottom			
Labware Type:	PCR 96 Well Plate	Pod:	Pod1
Position:	P3	N/A	Refresh tips
Liquid Type:	DigWash		
Volume:	175	µL	
Technique:	Aspirate on Magnet		

13) Dispense in P9			
Labware Type:	Ensure tip height in well is 30.00 mm from bottom		
Position:	Deep Well Plate	Pod:	Pod1
Liquid Type:	P9	N/A	Empty Tips
Volume:	DigWash		
Technique:	175	μL	
	Dispense in Waste		

14) Unload tips to TL1			
Tips:	Unload		
Location:	TL1		
Pod:	Pod1		

15) Move Labware from P3 to P10			
Using pod:	Pod1		
Move labware from:	P3	to	P10
Move the topmost:	1	piece of labware from the stack	

16) Load tips from TL1			
Tips:	Load		
Location:	TL1		
Pod:	Pod1		

17) Aspirate from P4			
Labware Type:	Ensure tip height in well is 1.5 mm from bottom		
Position:	V-Bottom Plate	Pod:	Pod1
Liquid Type:	P4	N/A	Refresh tips
Volume:	DigWash		
Technique:	37.5	μL	
	Aspirate from VBP		

18) Dispense in P10			
Labware Type:	Ensure tip height in well is 3.00 mm from bottom		
Position:	PCR 96 Well Plate	Pod:	Pod1
Liquid Type:	P10	N/A	Empty Tips
Volume:	DigWash		
Technique:	37.5	μL	
	Dispense and Mix-2		

19) Pause the whole system for 540 - 1800 s				
Check:	Pause	the whole system	for	540 - 1800 s

20) Aspirate from P7			
Labware Type:	Ensure tip height in well is 1.5 mm from bottom		
Position:	V-Bottom Plate	Pod:	Pod1
Liquid Type:	P7	N/A	Refresh tips
Volume:	DigWash		
Technique:	15	μL	
	Aspirate from VBP		

21) Dispense in P10			
Labware Type:	Ensure tip height in well is 3.00 mm from bottom		
Position:	PCR 96 Well Plate	Pod:	Pod1
Liquid Type:	P10	N/A	Empty Tips
Volume:	DigWash		
Technique:	15	μL	
	Dispense and Mix-3		

22) Unload tips to TL1	
Tips:	Unload
Location:	TL1
Pod:	Pod1

23) Move Labware from P10 to P6			
Using pod:	Pod1		
Move labware from:	P10	to	P6
Move the topmost:	1	piece of labware from the stack	

24) Load tips from TL1	
Tips:	Load
Location:	TL1
Pod:	Pod1

25) Pause the whole system for 510 s				
Check:	Pause	the whole system	for	510 s

26) Mix in P6			
Labware Type:	Ensure tip height in well is 3.00 mm from bottom		
Position:	PCR 96 Well Plate	Pod	Pod1
Liquid Type:	P6	N/A	Refresh tips
Volume:	DigWash	Mix:	25 times
Technique:	25	μL	
Customize	Custom		
Pipetting Template:	Default Template		
Mix Tab			
Move within the well at:	25	% speed	
Aspirate at:	3	mm from the	Bottom at 25 μL/s
Dispense at:	3	mm from the	Bottom at 25 μL/s
N/A	Follow liquid level when aspirating or dispensing liquid		
X	Touch tips on the sides of the wells		
X	Aspirate a leading air gap prior to mix and blowout after mix is complete		

27) Repeat Steps 25&26 two more times to release digested chromatin for ~30min.

28) Unload tips to TL1	
Tips:	Unload
Location:	TL1
Pod:	Pod1

29) Move Labware from P6 to P3	
Using pod:	Pod1
Move labware from:	P6
Move the topmost:	1 piece of labware from the stack

30) Pause the whole system for 180 s				
Check:	Pause	the whole system	for	180 s

31) Load tips from TL1	
Tips:	Load
Location:	TL1
Pod:	Pod1

32) Aspirate from P3		
Labware Type:	Ensure tip height in well is 0.0 mm from bottom	
Position:	PCR 96 Well Plate	Pod: Pod1
Liquid Type:	P3	N/A Refresh tips
Volume:	DigWash	μL
Technique:	37.5	
	Aspirate on Magnet	

33) Dispense in P8		
Labware Type:	Ensure tip height in well is 3.00 mm from bottom	
Position:	PCR 96 Well Plate	Pod: Pod1
Liquid Type:	P8	N/A Empty Tips
Volume:	DigWash	μL
Technique:	37.5	
	Dispense on Rack	

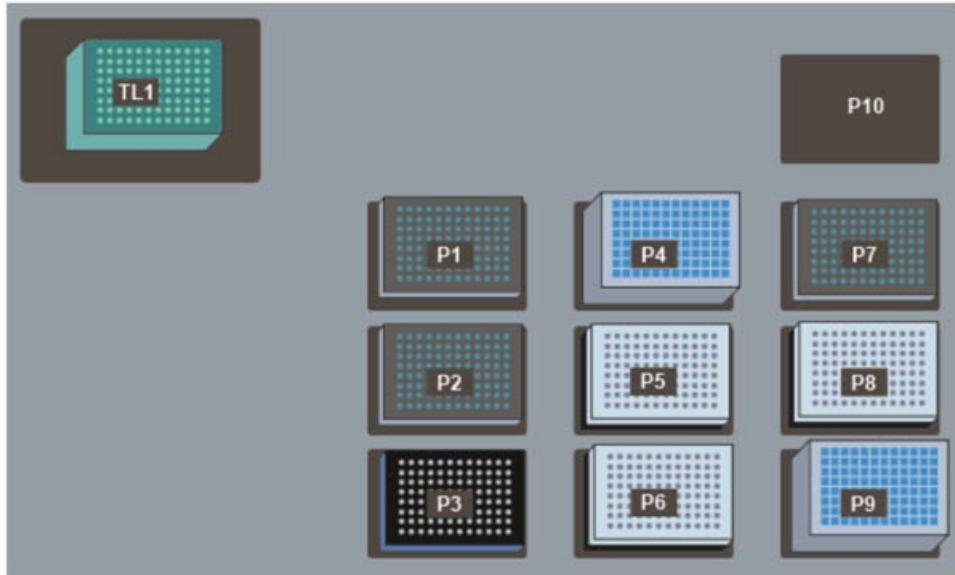
34) Unload tips to TL1	
Tips:	Unload
Location:	TL1
Pod:	Pod1

35) Finish	
Check:	Clear current instrument setup of all labware after the method completes
Check:	Clear current devise setup of all labware after the method completes
Check:	Unload disposable tips from all pods after the method completes
Check:	Clear all global variables after the method completes

## ▪ Pre-PCR DNA Cleanup

### 1) Start

### 2) Instrument Setup



TL1: Fresh AP96 200  $\mu$ L Tips (double click to increase the # of load times to 10)

P1: V-Bottom Plate preloaded with 100  $\mu$ L 10mM Tris-HCl pH 8 (used for washing tips)

P2: V-Bottom Plate preloaded with 100  $\mu$ L 10mM Tris-HCl pH 8 (used for DNA elution)

P3: ALPAQUA Magnet Plate

P4: Deep Well Plate preloaded with 1 mL 80% Ethanol

P5: PCR 96 Well Plate containing 110  $\mu$ L of Adapter Ligated DNA stacked on a PCR Plate Rack

P6: PCR 96 Well Plate preloaded with 90  $\mu$ L of Ampure Beads stacked on a PCR Plate Rack

P7: V-Bottom Plate preloaded with 100  $\mu$ L HXP Mix

P8: PCR 96 Well Plate for accepting cleaned-up DNA stacked on a PCR Plate Rack

P9: Deep Well Plate for receiving liquid waste

3) Load tips from TL1	
Tips:	Load
Location:	TL1
Pod:	Pod1

4) Aspirate from P5			
Labware Type:	Ensure tip height in well is 0.50 mm from bottom		
	PCR 96 Well Plate	Pod:	Pod1
Position:	P5	N/A	Refresh tips
Liquid Type:	Ligase/PCR Buffer		
Volume:	110	$\mu$ L	
Technique:	Aspirate on Rack		

5) Dispense in P6			
Labware Type:	Ensure tip height in well is 1.00 mm from bottom		
	PCR 96 Well Plate	Pod:	Pod1
Position:	P6	N/A	Empty Tips
Liquid Type:	Ligase/PCR Buffer		
Volume:	110	$\mu$ L	
Technique:	Dispense and Mix-4		

6) Mix in P1							
Labware Type:	V-Bottom Plate	Ensure tip height in well is 1.50 mm from bottom					
Position:	P1	N/A	Pod:	Pod1			
Liquid Type:	Tris-HCl-1	Refresh tips		Mix: 5 times			
Volume:	50	$\mu$ L					
Technique:	Custom						
Customize							
Pipetting Template:	Default Template						
Mix Tab							
Move within the well at:	25	% speed					
Aspirate at:	1.5	mm from the	Bottom	at 25 $\mu$ L/s			
Dispense at:	1.5	mm from the	Bottom	at 25 $\mu$ L/s			
N/A	Follow liquid level when aspirating or dispensing liquid						
X	Touch tips on the sides of the wells						
N/A	Aspirate a leading air gap prior to mix and blowout after mix is complete						

7) Pause the whole system for 300 s				
Check:	Pause	the whole system	for 300	s

8) Unload tips to TL1				
Tips:	Unload			
Location:	TL1			
Pod:	Pod1			

9) Move Labware from P6 to P3				
Using pod:	Pod1			
Move labware from:	P6	to	P3	
Move the topmost:	1	piece of labware from the stack		

10) Pause the whole system for 300 s				
Check:	Pause	the whole system	for 300	s

11) Load tips from TL1				
Tips:	Load			
Location:	TL1			
Pod:	Pod1			

12) Aspirate from P3				
Labware Type:	Ensure tip height in well is 0.00 mm from bottom			
Position:	P3	Pod:	Pod1	
Liquid Type:	Ampure Wash		N/A	Refresh tips
Volume:	105	$\mu$ L		
Technique:	Aspirate on Magnet			

13) Dispense in P9			
Labware Type:	Ensure tip height in well is 30.00 mm from bottom		
Position:	Deep Well Plate	Pod:	Pod1
Liquid Type:	P9	N/A	Empty Tips
Volume:	Ampure Wash		
Technique:	105	μL	
	Dispense in Waste		

14) Repeat Steps 12&13 to remove remaining Ampure Buffer.

15) Aspirate from P4			
Labware Type:	Ensure tip height in well is 2.00 mm from bottom		
Position:	Deep Well Plate	Pod:	Pod1
Liquid Type:	P4	N/A	Refresh tips
Volume:	EtOH		
Technique:	150	μL	
	Aspirate from DWP		

16) Dispense in P3			
Labware Type:	Ensure tip height in well is 2.00 mm from bottom		
Position:	PCR 96 Well Plate	Pod:	Pod1
Liquid Type:	P3	N/A	Empty Tips
Volume:	EtOH		
Technique:	150	μL	
	Dispense on Magnet		

17) Pause the whole system for 30 s				
Check:	Pause	the whole system	for	30 s

18) Aspirate from P3			
Labware Type:	Ensure tip height in well is 0.00 mm from bottom		
Position:	PCR 96 Well Plate	Pod:	Pod1
Liquid Type:	P3	N/A	Refresh tips
Volume:	EtOH		
Technique:	155	μL	
	Aspirate on Magnet		

19) Dispense in P9			
Labware Type:	Ensure tip height in well is 30.00 mm from bottom		
Position:	Deep Well Plate	Pod:	Pod1
Liquid Type:	P9	N/A	Empty Tips
Volume:	EtOH		
Technique:	155	μL	
	Dispense in Waste		

20) Repeat Steps 15-19 to wash bead-bound DNA a second time with Ethanol

21) Pause the whole system for 600 s				
Check:	Pause	the whole system	for	600 s

22) Unload tips to TL1	
Tips:	Unload
Location:	TL1
Pod:	Pod1

23) Move Labware from P3 to P6			
Using pod:	Pod1		
Move labware from:	P3	to	P6
Move the topmost:	1	piece of labware from the stack	

24) Load tips from TL1	
Tips:	Load
Location:	TL1
Pod:	Pod1

25) Aspirate from P2			
Labware Type:	Ensure tip height in well is 1.5 mm from bottom		
V-Bottom Plate	Pod:	Pod1	
Position:	P2	N/A	Refresh tips
Liquid Type:	Tris-HCl-1		
Volume:	50	μL	
Technique:	Aspirate from VBP		

26) Dispense in P6			
Labware Type:	Ensure tip height in well is 1.00 mm from bottom		
PCR 96 Well Plate	Pod:	Pod1	
Position:	P6	N/A	Empty Tips
Liquid Type:	Tris-HCl-1		
Volume:	50	μL	
Technique:	Dispense and Mix-5		

27) Mix in P1			
Labware Type:	Ensure tip height in well is 1.50 mm from bottom		
V-Bottom Plate	Pod:	Pod1	
Position:	P1	N/A	Refresh tips
Liquid Type:	Tris-HCl-1	Mix:	5 times
Volume:	50	μL	
Technique:	Custom		
Customize			
Pipetting Template:	Default Template		
Mix Tab			
Move within the well at:	25	% speed	
Aspirate at:	1.5	mm from the	Bottom
Dispense at:	1.5	mm from the	Bottom
N/A	at 25 μL/s		
X	Follow liquid level when aspirating or dispensing liquid		
N/A	Touch tips on the sides of the wells		
N/A	Aspirate a leading air gap prior to mix and blowout after mix is complete		

<b>28) Pause the whole system for 300 s</b>				
Check:	Pause	the whole system	for	300 s

<b>29) Aspirate from P7</b>				
Labware Type:	Ensure tip height in well is 1.5 mm from bottom			
V-Bottom Plate	Pod:	Pod1		
Position:	P7	N/A	Refresh tips	
Liquid Type:	Ampure Wash			
Volume:	55	µL		
Technique:	Aspirate from VBP			

<b>30) Dispense in P6</b>				
Labware Type:	Ensure tip height in well is 1.00 mm from bottom			
PCR 96 Well Plate	Pod:	Pod1		
Position:	P6	N/A	Empty Tips	
Liquid Type:	Ampure Wash			
Volume:	55	µL		
Technique:	Dispense and Mix-6			

<b>31) Mix in P1</b>				
	Ensure tip height in well is 1.50 mm from bottom			
Labware Type:	V-Bottom Plate	Pod:	Pod1	
Position:	P1	N/A	Refresh tips	
Liquid Type:	Tris-HCl-1	Mix:	5	times
Volume:	50	µL		
Technique:	Custom			
Customize				
Pipetting Template:	Default Template			
Mix Tab				
Move within the well at:	25	% speed		
Aspirate at:	1.5	mm from the	Bottom	at 25 µL/s
Dispense at:	1.5	mm from the	Bottom	at 25 µL/s
N/A	Follow liquid level when aspirating or dispensing liquid			
X	Touch tips on the sides of the wells			
N/A	Aspirate a leading air gap prior to mix and blowout after mix is complete			

<b>32) Pause the whole system for 300 s</b>				
Check:	Pause	the whole system	for	300 s

<b>33) Unload tips to TL1</b>				
Tips:	Unload			
Location:	TL1			
Pod:	Pod1			

<b>34) Move Labware from P6 to P3</b>				
Using pod:	Pod1			
Move labware from:	P6	to	P3	
Move the topmost:	1	piece of labware from the stack		

35) Pause the whole system for 300 s				
Check:	Pause	the whole system	for	300 s

36) Load tips from TL1	
Tips:	Load
Location:	TL1
Pod:	Pod1

37) Aspirate from P3				
Labware Type:	Ensure tip height in well is 0.00 mm from bottom			
Position:	PCR 96 Well Plate		Pod:	Pod1
Liquid Type:	P3		N/A	Refresh tips
Volume:	Ampure Wash			
Technique:	110 $\mu$ L			
	Aspirate on Magnet			

38) Dispense in P9				
Labware Type:	Ensure tip height in well is 30.00 mm from bottom			
Position:	Deep Well Plate	Pod:	Pod1	
Liquid Type:	P9	N/A	Empty Tips	
Volume:	Ampure Wash			
Technique:	110 $\mu$ L			
	Dispense in Waste			

39) Perform Steps 15-22 again to wash bead-bound DNA twice with Ethanol and then allow to air dry.

40) Move Labware from P3 to P6				
Using pod:	Pod1			
Move labware from:	P3	to	P6	
Move the topmost:	1	piece of labware from the stack		

41) Load tips from TL1	
Tips:	Load
Location:	TL1
Pod:	Pod1

42) Aspirate from P2						
Labware Type:	Ensure tip height in well is 1.5 mm from bottom					
Position:	V-Bottom Plate	Pod:	Pod1			
Liquid Type:	P2	N/A	Refresh tips			
Volume:	Tris-HCl-1					
Technique:	37.5 $\mu$ L					
	Aspirate from VBP					

43) Dispense in P6			
Labware Type:	Ensure tip height in well is 1.00 mm from bottom		
Position:	PCR 96 Well Plate	Pod:	Pod1
Liquid Type:	P6	N/A	Empty Tips
Volume:	Tris-HCl-1		
Technique:	37.5	μL	
	Dispense and Mix-5		

44) Mix in P1			
Labware Type:	Ensure tip height in well is 1.50 mm from bottom		
Position:	V-Bottom Plate	Pod:	Pod1
Liquid Type:	P1	N/A	Refresh tips
Volume:	Tris-HCl-1	Mix:	5 times
Technique:	50	μL	
Customize	Custom		
Pipetting Template:	Default Template		
Mix Tab			
Move within the well at:	25	% speed	
Aspirate at:	1.5	mm from the	Bottom at 25 μL/s
Dispense at:	1.5	mm from the	Bottom at 25 μL/s
N/A	Follow liquid level when aspirating or dispensing liquid		
X	Touch tips on the sides of the wells		
N/A	Aspirate a leading air gap prior to mix and blowout after mix is complete		

45) Pause the whole system for 300 s				
Check:	Pause	the whole system	for	300 s

46) Unload tips to TL1		
Tips:	Unload	
Location:	TL1	
Pod:	Pod1	

47) Move Labware from P6 to P3			
Using pod:	Pod1		
Move labware from:	P6	to	P3
Move the topmost:	1	piece of labware from the stack	

48) Pause the whole system for 300 s				
Check:	Pause	the whole system	for	300 s

49) Load tips from TL1		
Tips:	Load	
Location:	TL1	
Pod:	Pod1	

50) Aspirate from P3			
Labware Type:	PCR 96 Well Plate	Pod:	Pod1
Position:	P3	N/A	Refresh tips
Liquid Type:	Tris-HCl-2		
Volume:	32.5	µL	
Technique:	Aspirate on Magnet		

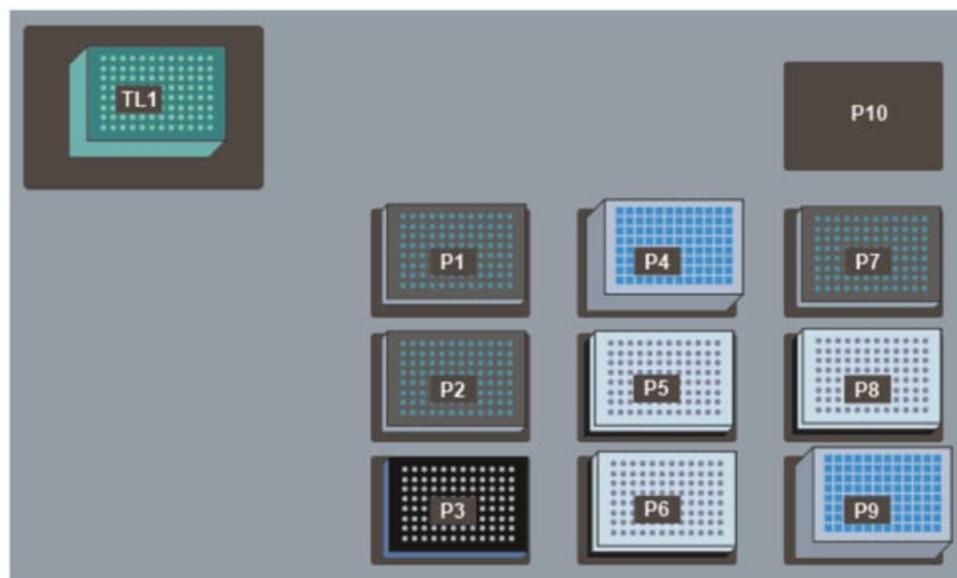
52) Unload tips to TL1	
Tips:	Unload
Location:	TL1
Pod:	Pod1

53) Finish	
Check:	Clear current instrument setup of all labware after the method completes
Check:	Clear current devise setup of all labware after the method completes
Check:	Unload disposable tips from all pods after the method completes
Check:	Clear all global variables after the method completes

#### ▪ Post-PCR DNA Cleanup

##### 1) Start

##### 2) Instrument Setup



TL1: Fresh AP96 200 µL Tips (double click to increase the # of load times to 10)

P1: V-Bottom Plate preloaded with 100 µL 10mM Tris-HCl pH 8 (used for washing tips)

P2: V-Bottom Plate preloaded with 100 µL 10mM Tris-HCl pH 8 (used for DNA elution)

P3: ALPAQUA Magnet Plate

P4: Deep Well Plate preloaded with 1 mL 80% Ethanol

P5: PCR 96 Well Plate containing 50 µL of PCR product stacked on a PCR Plate Rack

P6: PCR 96 Well Plate preloaded with 55 µL of Ampure Beads stacked on a PCR Plate Rack

P7: V-Bottom Plate preloaded with 100 µL HXP Mix

P8: PCR 96 Well Plate for accepting cleaned-up DNA stacked on a PCR Plate Rack

P9: Deep Well Plate for receiving liquid waste

3) Load tips from TL1		
Tips:	Load	
Location:	TL1	
Pod:	Pod1	

4) Aspirate from P5		
Labware Type:	Ensure tip height in well is 0.50 mm from bottom	
Position:	PCR 96 Well Plate	Pod: Pod1
Liquid Type:	P5	N/A Refresh tips
Volume:	Ligase/PCR Buffer	μL
Technique:	55	
	Aspirate on Rack	

5) Dispense in P6		
Labware Type:	Ensure tip height in well is 1.00 mm from bottom	
Position:	PCR 96 Well Plate	Pod: Pod1
Technique:	P6	N/A Empty Tips
Liquid Type:	Ligase/PCR Buffer	μL
Volume:	55	
Technique:	Dispense and Mix-6	

6) Mix in P1		
Labware Type:	V-Bottom Plate	Ensure tip height in well is 1.50 mm from bottom
Position:	P1	Pod: Pod1
Liquid Type:	Tris-HCl-1	N/A Refresh tips
Volume:	50	Mix: 5 times
Technique:	Custom	μL
Customize		
Pipetting Template:	Default Template	
Mix Tab		
Move within the well at:	25	% speed
Aspirate at:	1.5	mm from the Bottom at 25 μL/s
Dispense at:	1.5	mm from the Bottom at 25 μL/s
N/A		Follow liquid level when aspirating or dispensing liquid
X		Touch tips on the sides of the wells
N/A		Aspirate a leading air gap prior to mix and blowout after mix is complete

7) Pause the whole system for 300 s				
Check:	Pause	the whole system	for	300 s

8) Unload tips to TL1		
Tips:	Unload	
Location:	TL1	
Pod:	Pod1	

9) Move Labware from P6 to P3			
Using pod:	Pod1		
Move labware from:	P6	to	P3
Move the topmost:	1	piece of labware from the stack	

10) Pause the whole system for 300 s			
Check: Pause	the whole system	for	300 s

11) Load tips from TL1			
Tips:	Load		
Location:	TL1		
Pod:	Pod1		

12) Aspirate from P3			
Labware Type:	PCR 96 Well Plate	Pod:	Pod1
Position:	P3	N/A	Refresh tips
Liquid Type:	Ampure Wash		
Volume:	120	µL	
Technique:	Aspirate on Magnet		

13) Dispense in P9			
Labware Type:	Deep Well Plate	Pod:	Pod1
Position:	P9	N/A	Empty Tips
Liquid Type:	Ampure Wash		
Volume:	120	µL	
Technique:	Dispense in Waste		

14) Aspirate from P4			
Labware Type:	Deep Well Plate	Pod:	Pod1
Position:	P4	N/A	Refresh tips
Liquid Type:	EtOH		
Volume:	150	µL	
Technique:	Aspirate from DWP		

15) Dispense in P3			
Labware Type:	PCR 96 Well Plate	Pod:	Pod1
Position:	P3	N/A	Empty Tips
Liquid Type:	EtOH		
Volume:	150	µL	
Technique:	Dispense on Magnet		

16) Pause the whole system for 30 s			
Check: Pause	the whole system	for	30 s

17) Aspirate from P3			
Labware Type:	PCR 96 Well Plate	Pod:	Pod1
Position:	P3	N/A	Refresh tips
Liquid Type:	EtOH		
Volume:	155	µL	
Technique:	Aspirate on Magnet		

18) Dispense in P9			
Labware Type:	Ensure tip height in well is 0.00 mm from bottom		
Position:	Deep Well Plate	Pod:	Pod1
Liquid Type:	P9	N/A	Empty Tips
Volume:	EtOH		
Technique:	155	µL	
	Dispense in Waste		

19) Repeat Steps 14-18 to wash bead-bound DNA a second time with Ethanol

20) Pause the whole system for 600 s				
Check:	Pause	the whole system	for	600 s

21) Unload tips to TL1	
Tips:	Unload
Location:	TL1
Pod:	Pod1

22) Move Labware from P3 to P6			
Using pod:	Pod1		
Move labware from:	P3	to	P6
Move the topmost:	1	piece of labware from the stack	

23) Load tips from TL1	
Tips:	Load
Location:	TL1
Pod:	Pod1

24) Aspirate from P2			
Labware Type:	Ensure tip height in well is 1.5 mm from bottom		
Position:	V-Bottom Plate	Pod:	Pod1
Liquid Type:	P2	N/A	Refresh tips
Volume:	Tris-HCl-1		
Technique:	50	µL	
	Aspirate from VBP		

26) Mix in P1					
Labware Type:	V-Bottom Plate	Ensure tip height in well is 1.50 mm from bottom			
Position:	P1	Pod:	Pod1		
Liquid Type:	Tris-HCl-1	N/A	Refresh tips		
Volume:	50	Mix:	5	times	
Technique:	Custom	μL			
Customize					
Pipetting Template:	Default Template				
Mix Tab					
Move within the well at:	25	% speed			
Aspirate at:	1.5	mm from the	Bottom	at 25 μL/s	
Dispense at:	1.5	mm from the	Bottom	at 25 μL/s	
	N/A	Follow liquid level when aspirating or dispensing liquid			
	X	Touch tips on the sides of the wells			
	N/A	Aspirate a leading air gap prior to mix and blowout after mix is complete			

27) Pause the whole system for 300 s				
Check: Pause	the whole system	for	300	s

28) Aspirate from P7				
Labware Type:	Ensure tip height in well is 1.5 mm from bottom			
V-Bottom Plate	Pod:	Pod1		
Position:	P7	N/A	Refresh tips	
Liquid Type:	Ampure Wash			
Volume:	55	μL		
Technique:	Aspirate from VBP			

29) Dispense in P6				
Labware Type:	Ensure tip height in well is 1.00 mm from bottom			
PCR 96 Well Plate	Pod:	Pod1		
Position:	P6	N/A	Empty Tips	
Liquid Type:	Ampure Wash			
Volume:	55	μL		
Technique:	Dispense and Mix-6			

30) Mix in P1					
Labware Type:	V-Bottom Plate	Ensure tip height in well is 1.50 mm from bottom			
Position:	P1	Pod:	Pod1		
Liquid Type:	Tris-HCl-1	N/A	Refresh tips		
Volume:	50	Mix:	5	times	
Technique:	Custom	μL			
Customize					
Pipetting Template:	Default Template				
Mix Tab					
Move within the well at:	25	% speed			
Aspirate at:	1.5	mm from the	Bottom	at 25 μL/s	
Dispense at:	1.5	mm from the	Bottom	at 25 μL/s	
N/A	Follow liquid level when aspirating or dispensing liquid				
X	Touch tips on the sides of the wells				
N/A	Aspirate a leading air gap prior to mix and blowout after mix is complete				

31) Pause the whole system for 300 s				
Check:	Pause	the whole system	for	300 s

32) Unload tips to TL1	
Tips:	Unload
Location:	TL1
Pod:	Pod1

33) Move Labware from P6 to P3			
Using pod:	Pod1		
Move labware from:	P6	to	P3
Move the topmost:	1	piece of labware from the stack	

34) Pause the whole system for 300 s				
Check:	Pause	the whole system	for	300 s

35) Load tips from TL1	
Tips:	Load
Location:	TL1
Pod:	Pod1

36) Aspirate from P3			
Labware Type:	PCR 96 Well Plate	Pod:	Pod1
Position:	P3	N/A	Refresh tips
Liquid Type:	Ampure Wash		
Volume:	110	μL	
Technique:	Aspirate on Magnet		

37) Dispense in P9			
Labware Type:	Ensure tip height in well is 30.00 mm from bottom		
Position:	Deep Well Plate	Pod:	Pod1
Liquid Type:	P9	N/A	Empty Tips
Volume:	Ampure Wash		
Technique:	110	μL	
	Dispense in Waste		

38) Perform Steps 14-21 again to wash bead-bound DNA twice with Ethanol and then allow to air dry.

39) Move Labware from P3 to P6			
Using pod:	Pod1		
Move labware from:	P3	to	P6
Move the topmost:	1	piece of labware from the stack	

40) Load tips from TL1		
Tips:	Load	
Location:	TL1	
Pod:	Pod1	

41) Aspirate from P2			
Labware Type:	Ensure tip height in well is 1.5 mm from bottom		
V-Bottom Plate	Pod:	Pod1	
Position:	P2	N/A	Refresh tips
Liquid Type:	Tris-HCl-1		
Volume:	37.5	μL	
Technique:	Aspirate from VBP		

42) Dispense in P6			
Labware Type:	Ensure tip height in well is 1.00 mm from bottom		
PCR 96 Well Plate	Pod:	Pod1	
Position:	P6	N/A	Empty Tips
Liquid Type:	Tris-HCl-1		
Volume:	37.5	μL	
Technique:	Dispense and Mix-5		

43) Mix in P1				
Labware Type:	V-Bottom Plate	Ensure tip height in well is 1.50 mm from bottom		
Position:	P1	N/A	Pod:	Pod1
Liquid Type:	Tris-HCl-1	Mix:	5	times
Volume:	50	μL		
Technique:	Custom			
Customize				
Pipetting Template:	Default Template			
Mix Tab				
Move within the well at:	25	% speed		
Aspirate at:	1.5	mm from the	Bottom	at 25 μL/s
Dispense at:	1.5	mm from the	Bottom	at 25 μL/s
N/A	Follow liquid level when aspirating or dispensing liquid			
X	Touch tips on the sides of the wells			
N/A	Aspirate a leading air gap prior to mix and blowout after mix is complete			

44) Pause the whole system for 300 s				
Check:	Pause	the whole system	for 300	s

45) Unload tips to TL1				
Tips:	Unload			
Location:	TL1			
Pod:	Pod1			

46) Move Labware from P6 to P3				
Using pod:	Pod1			
Move labware from:	P6	to	P3	
Move the topmost:	1	piece of labware from the stack		

47) Pause the whole system for 300 s				
Check:	Pause	the whole system	for 300	s

48) Load tips from TL1				
Tips:	Load			
Location:	TL1			
Pod:	Pod1			

49) Aspirate from P3				
Labware Type:	Ensure tip height in well is 0.00 mm from bottom			
Position:	PCR 96 Well Plate	Pod:	Pod1	
Liquid Type:	P3	N/A	Refresh tips	
Volume:	Tris-HCl-2			
Technique:	32.5	μL		
	Aspirate on Magnet			

50) Dispense in P8			
Labware Type: Position: Liquid Type: Volume: Technique:	PCR 96 Well Plate	Pod: Pod1	Ensure tip height in well is 1.00 mm from bottom
	P8	N/A	Empty Tips
	Tris-HCl-2	μL	
	32.5		
	Dispense on Rack		

51) Unload tips to TL1	
Tips:	Unload
Location:	TL1
Pod:	Pod1

52) Finish	
Check: Clear current instrument setup of all labware after the method completes	
Check: Clear current devise setup of all labware after the method completes	
Check: Unload disposable tips from all pods after the method completes	
Check: Clear all global variables after the method completes	

#### MATERIALS

NAME	CATALOG #	VENDOR
T4 Polynucleotide Kinase - 500 units	M0201S	New England Biolabs
Microtube, 1.5ml, 1000/bag	V1231	Promega
10 mM Adenosine 5-Triphosphate (ATP)	P0756S	New England Biolabs
Agencourt Ampure XP	A63880	Beckman Coulter
1 M Tris/HCl Stock Solution (dissolved Tris base adjusted to pH 8.0 with HCl)		
Concanavalin-coated magnetic beads	BP531	Bangs Laboratories
Antibody to an epitope of interest. For example, rabbit $\alpha$ -CTCF polyclonal antibody (Millipore 07-729) for mapping 1D and 3D interactions by CUT&RUN		
Positive control antibody to an abundant epitope, e.g. $\alpha$ -H3K27me3 rabbit monoclonal antibody (Cell Signaling Technology, cat. no. 9733)		
Negative control antibody to an absent epitope, e.g. guinea pig $\alpha$ -rabbit antibody		
5% Digitonin	300410	Emd Millipore
Spike-in DNA (e.g., from <i>Saccharomyces cerevisiae</i> micrococcal nuclease-treated chromatin, provided by authors upon request)		
Distilled, deionized or RNase-free H2O (dH2O e.g., Promega, cat. no. P1197)	P1197	Promega
1 M Manganese Chloride (MnCl2)	203734	Sigma Aldrich
1 M Calcium Chloride (CaCl2)	BP510	Fisher Scientific
1 M Potassium Chloride (KCl)	P3911	Sigma Aldrich
1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.5 (HEPES (Na ))	H3375	Sigma Aldrich
1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.9 (HEPES (K ))	H3375	Sigma Aldrich

NAME	CATALOG #	VENDOR
5 M Sodium chloride (NaCl)	S5150-1L	Sigma Aldrich
0.5 M Ethylenediaminetetraacetic acid (EDTA)	3002E	Research Organics
0.2 M Ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N-tetraacetic acid (EGTA)	E3889	Sigma Aldrich
2 M Spermidine	S2501	Sigma Aldrich
Roche Complete Protease Inhibitor EDTA-Free tablets	5056489001	Sigma Aldrich
2 mg/ml Glycogen (1:10 dilution)	10930193001	Sigma Aldrich
10% Sodium dodecyl sulfate (SDS)	L4509	Sigma Aldrich
Proteinase K	E00492	Thermo Fisher Scientific
Ethanol	2716	Decon Labs
Vortex-Genie 2	SI-0236	Scientific Industries, Inc.
Protein A-Micrococcal Nuclease (pA-MNase) fusion protein (provided in 50% glycerol by the authors upon request). Store at -20 oC.	<a href="#">View</a>	
RNase A, DNase and protease-free	EN0531	Thermo Fisher Scientific
10mM dNTPs	KK1017	Kapa Biosystems
Taq DNA polymerase	EP0401	Thermo Scientific
2X Rapid ligase buffer	B101L	
Enzymatics DNA ligase	L6030-HC-L	
5X KAPA buffer	KK2502	Kapa Biosystems
KAPA HS HIFI polymerase	KK2502	Kapa Biosystems
Microcentrifuge		
10X NEB T4 DNA ligase buffer		 New England Biolabs
Cell suspension. We have used human K562 and H1 cells as well as several human brain tumor lines propagated both in cell culture as well as in the brains of immuno-compromised mice before being resected and frozen Drosophila S2 cells and dissected Drosophila tissues such as brains and imaginal disks and spheroplastic yeast.	<a href="#">View</a>	
Qubit 1X dsDNA HS Assay Kit	Q33230	Thermo Fisher Scientific
40% PEG 4000	81242	Sigma-aldrich
40% PEG 8000	202452	Sigma-aldrich
Centrifuge 5810 swinging bucket	022625004	Eppendorf Centrifuge
Centrifuge 5424 R refrigerated with Rotor FA-45-24-11 rotary knobs 120 V/50Hz-60 Hz (US)	5404000537	Eppendorf Centrifuge
MACSiMAG Separator	130-092-168	Miltenyi Biotec
2 mL Snaplock Microcentrifuge Tube	MCT-200-C	Axygen
0.6 mL low-retention microcentrifuge tubes	3446	Thermo Fisher Scientific
BD Clay Adams™ Nutator Mixer BD Diagnostics	15172-203	Vwr
Heater block with wells for 1.5-mL microcentrifuge tubes (set to 100 °C)	<a href="#">View</a>	

NAME	CATALOG #	VENDOR
Capillary electrophoresis instrument (e.g. Agilent Tapestation 4200)	<a href="#">View</a>	
Thermal cycler with 3 degree C/sec ramp rate that is compatible with a 96 well PCR plate	<a href="#">View</a>	
Qubit Fluorometer	Q33216	Life Technologies
Biomek FX or FXP equipped with a 96-channel pod and P200 head	<a href="#">View</a>	Beckman Coulter
One 1 x 1 Tip Loader ALP	C02867	Beckman Coulter
Three 1 x 3 Static ALPs	B87478	Beckman Coulter
One Single Position Cooling/Heating ALP	719361	Beckman Coulter
96S Super Magnet Plate	A001322	Alpaqua
Aluminum Heat Block Insert for PCR Plates	VP74116A	V&P Scientific
Recirculating Cooling Unit filled with antifreeze (e.g Thermo Neslab RTE-7 Digital One Recirculating Chiller Mfr # 271103200000)	<a href="#">View</a>	
MicroAmp Support Bases	N801-0531	Thermofisher
96 well LoBind PCR plates Semi-skirted	0030129504	Eppendorf
MicroAmp Clear Adhesive Film	4306311	Applied Biosystems
Biomek AP96 P250 Pre-Sterile Tips with barrier	717253	Beckman Coulter
96 well Polystyrene V-Bottom Microplates	651101	greiner bio-one
• MASTERBLOCK™ 96 Deep Well Conical Bottom 2 mL Storage Plates	780271	greiner bio-one
2-20 µL 8-Channel Multi Pipette (e.g. Rainin 17013803)	<a href="#">View</a>	
20-200 µL 8-Channel Multi Pipette (e.g. Rainin 17013805)	<a href="#">View</a>	
Reagent Reservoirs (e.g. Thermo Scientific 8095)	<a href="#">View</a>	

#### MATERIALS TEXT

In the current format this protocol is optimized for use with TruSeq-Y Adapters with a free 3'T overhang. The following oligos can be ordered from any company that provides custom oligo synthesis (e.g. IDT or Sigma-Aldrich):

TruSeq Universal Adapter (PAGE purification):

AATGATAACGGCGACCACCGAGATCTACACTTTCCCTACACGACGCTTCCGATC\*T

TruSeq Indexed Adapters (PAGE purification):

P-GATCGGAAGAGCACACGTCTGAACCTCCAGTCAC(INDEX)ATCTCGTATGCCGTCTCTGCTT\*G

Adapter master stocks should be prepared by annealing the TruSeq Universal adapter to each of the TruSeq Indexed Adapters individually by mixing them at a concentration of 25 µM, and then heating them to 100 °C and allowing them to slowly cool either at RT on a bench top or in a thermocycler (1 degree per minute).

P5 primer (HPLC purification):

AATGATAACGGCGACCACCGA\*G

P7 primer (HPLC purification):

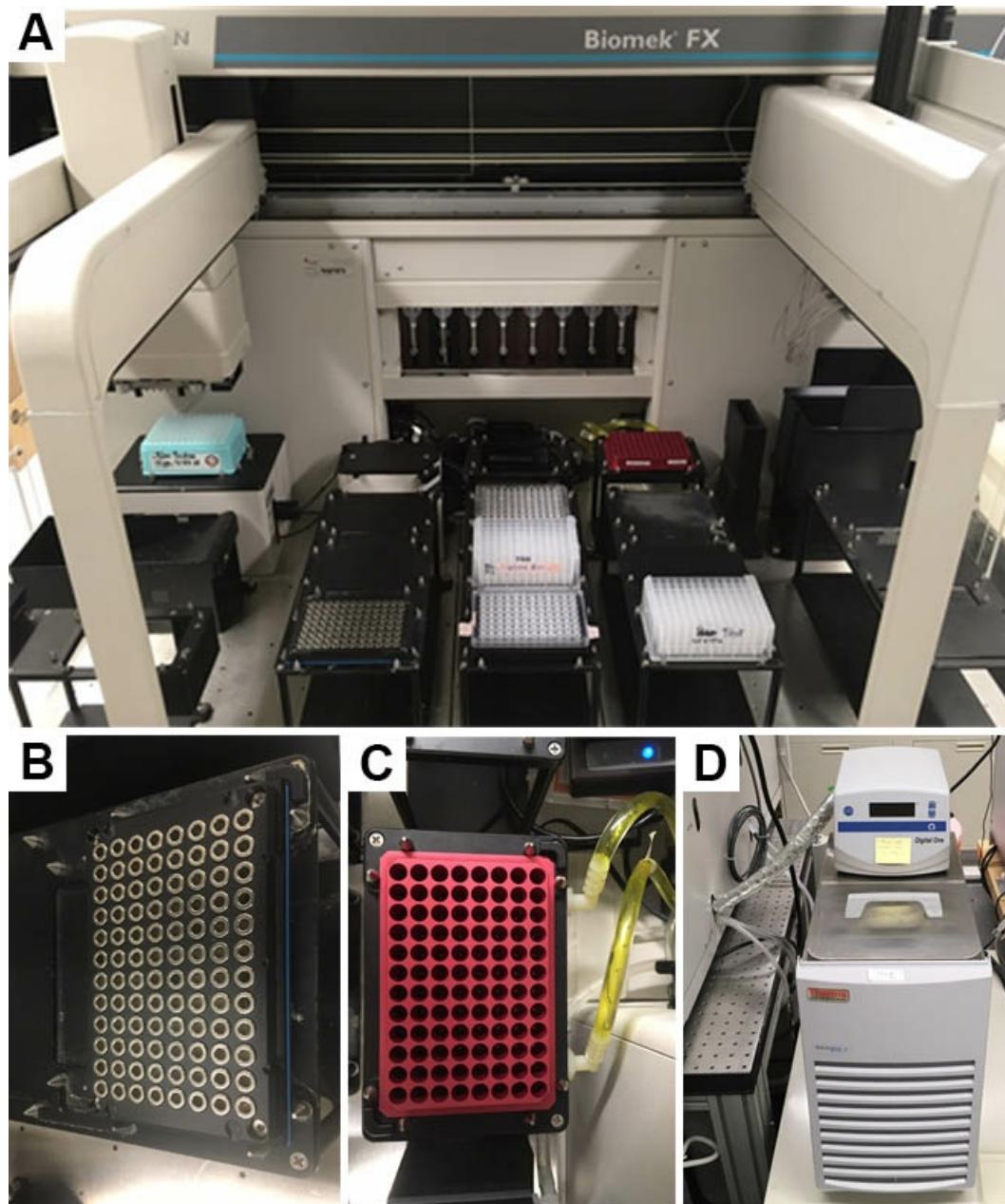
CAAGCAGAACGGCATACGA\*G

(\* = phosphorothioate bond, P = phosphate group, INDEX = 6 nucleotide barcode)

#### SAFETY WARNINGS

Digitonin is toxic and care should be taken especially when weighing out the powder, for these steps PPE including a mask, labcoat and gloves are recommended. A digitonin stock may be prepared by dissolving in dimethylsulfoxide (DMSO), but be aware that DMSO can absorb through the skin.

BEFORE STARTING



#### ■ BIOMEK PROGRAMMING

**1) Set up the Biomek Deck.** You will need a 96-channel pod and P200 head installed as Pod1, as well as one Tip Loader ALP (TL1), three 1 x 3 Static ALPs (P1-P9), and one Single Position Cooling/Heating ALP routed to a circulating Cooling Unit filled with anti-freeze (P10). ALPs can be installed in the positions indicated below, or according to user preference, however this will require changing the ALP settings. A Static Peltier ALP equipped with a 96 well adapter can be used in place of the Single Position Cooling/Heating ALP (P10), however, running the digestion reaction at the minimum temperature setting for the Static Peltier ALP (4 degrees Celsius) may result in slightly higher background cleavage during the CUT&RUN digestion reaction. To set up the deck layout open the Deck Editor under the Instrument tab. Create new deck named "AutoCnR" that includes all of the equipment installed on your instrument. The following chart indicates the minimum number of ALP positions for AutoCUT&RUN, but there is no need to delete additional unused equipment from your deck layout:



Tip Loader ALP Position Properties				
Name	TL1	ALP Type: TipLoader		
	X(cm)	Y(cm)	Z(cm)	Precision
Pod 1 Coordinates	-7.296	-9.398	-15.676	Position Framed
Pod 2 Coordinates	N/A	N/A	N/A	Deck Framed

1 X 3 Static ALP (1)				
Name	P1	ALP Type: OneByThree		
	X(cm)	Y(cm)	Z(cm)	Precision
Pod 1 Coordinates	24.698	7.258	-15.683	Position Framed
Pod 2 Coordinates	N/A	N/A	N/A	Position Framed
Name	P2	ALP Type: OneByThree		
	X(cm)	Y(cm)	Z(cm)	Precision
Pod 1 Coordinates	24.707	18.67	-15.677	ALP Framed
Pod 2 Coordinates	N/A	N/A	N/A	Position Framed
Name	P3	ALP Type: OneByThree		
	X(cm)	Y(cm)	Z(cm)	Precision
Pod 1 Coordinates	24.726	30.126	-15.683	Position Framed
Pod 2 Coordinates	N/A	N/A	N/A	Position Framed

1 X 3 Static ALP (2)				
Name	P4	ALP Type: OneByThree		
	X(cm)	Y(cm)	Z(cm)	Precision
Pod 1 Coordinates	43.763	7.288	-15.67	Position Framed
Pod 2 Coordinates	N/A	N/A	N/A	Position Framed
Name	P5	ALP Type: OneByThree		
	X(cm)	Y(cm)	Z(cm)	Precision
Pod 1 Coordinates	43.765	18.753	-15.673	Position Framed
Pod 2 Coordinates	N/A	N/A	N/A	Position Framed
Name	P6	ALP Type: OneByThree		
	X(cm)	Y(cm)	Z(cm)	Precision
Pod 1 Coordinates	43.762	30.191	-15.678	Position Framed
Pod 2 Coordinates	N/A	N/A	N/A	Position Framed

1 X 3 Static ALP (3)				
Name	P7	ALP Type: OneByThree		
	X(cm)	Y(cm)	Z(cm)	Precision
Pod 1 Coordinates	62.869	7.296	-15.663	Position Framed
Pod 2 Coordinates	N/A	N/A	N/A	Position Framed
Name	P8	ALP Type: OneByThree		
	X(cm)	Y(cm)	Z(cm)	Precision
Pod 1 Coordinates	62.851	18.741	-15.673	Position Framed
Pod 2 Coordinates	N/A	N/A	N/A	Position Framed
Name	P9	ALP Type: OneByThree		
	X(cm)	Y(cm)	Z(cm)	Precision
Pod 1 Coordinates	62.824	30.169	-15.673	Position Framed
Pod 2 Coordinates	N/A	N/A	N/A	Position Framed

Single Position Cooling/Heating ALP Position Properties				
Name	P10	ALP Type: OneByOne		
	X(cm)	Y(cm)	Z(cm)	Precision
Pod 1 Coordinates	62.805	-6.682	-15.676	Position Framed
Pod 2 Coordinates	N/A	N/A	N/A	Deck Framed

**2) Input the AutoCUT&RUN Methods.** Start a new project named "AutoCUT&RUN." This will avoid overwritting other equipment etc. that has the same name.

**Option 1:** The various Labware, Liquid Types, Techniques and Methods for AutoCUT&RUN are available below for download. the files can be imported into the AutoCUT&RUN project. Be sure to check the Instrument Setup. If your deck layout varies from what is suggested above you may need to "map to an existing deck," and confirm all the labware shows up at the correct positions according to the **Instrument Setup** diagrams provided in below in **Figures 2-5** as well as in the **Guidelines** section (e.g. AP96\_200uL at TL1).

- Note: These methods files were generated using Biomek v4, and cannot be imported into Biomek v5 and are unlikely to be backwards compatible with Biomek v3.

- [1-pA-MNase Binding.bmf](#)
- [2-pA-MNase Digest.bmf](#)
- [3-Pre-PCR Cleanup.bmf](#)
- [4-Post-PCR Cleanup.bmf](#)

**Option 2:** The Labware, Liquid Types, Techniques and Methods for AutoCUT&RUN can be input manually according to the specifications listed in the **Guidelines** section.

- **CRITICAL STEP:** In order for the Biomek to perform AutoCUT&RUN as expected the labware must be properly calibrated and zeroed. The equipment specs provided should be empirically tested on your specific instrument by setting up a short method in which the Biomek transfers liquid from each of the plate types with the aspirate and dispense height set at 0.5 mm from the bottom. Run the method using single step mode to carefully examine the tip heights within the wells in each of the plate types (i.e PCR 96 Well Plate, Deep Well Plate, and the V-Bottom Plate). The PCR 96 Well Plate should be zeroed while stacked on the PCR Plate Rack, ALPAQUA Magnet Stand and Cold Block, if necessary the Stack Offset Z parameter can be adjusted for each type of Rack independently. Properly zeroing the PCR 96 Well Plate on the ALPAQUA Magnet Stand should engage the spring without causing the stand to bottom out. Reducing the movement speed within the well during these initial calibration steps will minimize the possibility of the instrument becoming damaged if a crash occurs. In addition, prior to performing an AutoCUT&RUN reaction on biological samples it is recommended to test each method by pre-loading the labware with H<sub>2</sub>O containing a small amount of food coloring to improve visibility. The 96 well LoBind PCR plates, Semi-skirted (Eppendorf # 0030129504) are highly recommended because their dimensions are consistent enough from plate to plate to ensure the Biomek will also move and stack the plates in a consistent manner. To prevent the PCR Plate Racks from catching and being carried along with the PCR 96 Well Plate during movement steps the PCR Plate Racks can be taped down to the stationary ALPs (this issue has not been observed for

the ALPAQUA Magnet Stand or the Cold Block). Finally, the operator should remain present throughout each method for the first several runs to ensure the instrument performs as expected. In the event of a mishap the operator can then pause or stop the procedure and intervene before the experiment is compromised.

## REAGENT SETUP

**5% Digitonin:** 1 mL of 5% digitonin is sufficient for up to 48 AutoCUT&RUN reactions, to perform up to 96 reactions prepare 2 mL of 5% digitonin. Weigh out the digitonin powder in a 2 mL microcentrifuge tube, boil water in a small beaker in a microwave oven. Pipette the hot water into the tube with the digitonin powder to make 5% (wt/vol), close the cap and quickly vortex on full. If digitonin is not completely dissolved seal with a tube-lock to prevent the cap from opening and place the tube in a 100°C heat block for 1-5 min. If saved and refrigerated, this stock can be used up to a week, but will need reheating as the digitonin slowly precipitates.

- **CAUTION:** Digitonin is toxic and PPE including a mask and gloves should be worn when weighing out the powder. A digitonin stock may be prepared by dissolving in dimethylsulfoxide (DMSO) but be aware that DMSO can absorb through the skin.

**Binding buffer:** Mix 20 mL of Binding Buffer in a 50 mL conical tube. Store the buffer at 4 °C for up to 6 months.

Binding Buffer		
Component	Amount	Final concentration
ddH <sub>2</sub> O	19.36 mL	-
1M HEPES-KOH pH 7.9	400 µL	20 mM
1M KCl	200 µL	10 mM
1M CaCl <sub>2</sub>	20 µL	1 mM
1M MnCl <sub>2</sub>	20 µL	1 mM

**Activate Concanavalin A-coated beads in Binding Buffer:** Gently resuspend and withdraw enough of the bead suspension such that there will be 10 µL for each final sample. Transfer Concanavalin A-coated beads into 1.5 mL Binding Buffer in a 2 mL tube. For more than 48 reactions use a second 2 mL tube. Place tube(s) on a magnet stand to clear (30 s to 2 min). Withdraw the liquid and remove from the magnet stand. Add 1.5 mL Binding buffer, mix by inversion or gentle pipetting, remove liquid from the cap and side with a quick pulse on a micro-centrifuge. Place tube(s) on a magnet stand to clear (30 s to 2 min). Withdraw the liquid, then wash Concanavalin A-coated beads a second time with 1.5 mL of Binding buffer. After removing liquid from the second wash on a magnet stand, resuspend in a volume of Binding Buffer equal to the volume of bead suspension (10 µL per final sample).

**Wash Buffer:** 50 mL of Wash Buffer is sufficient for up to 24 AutoCUT&RUN reactions. This buffer can be stored at 4 °C for up to 1 week, however, Roche Complete Protease Inhibitor tablet should be added fresh on the day of use.

- Note: A concentration of salt that is in the physiological range avoids stress when washing the cells and mixing with beads. Spermidine in the wash buffer is intended to compensate for removal of Mg<sup>2+</sup> during incubation in the Antibody Buffer, which might otherwise affect chromatin properties.

Wash Buffer		
Component	Amount per 24 samples	Final
ddH <sub>2</sub> O	47 mL	-
1M HEPES pH 7.5	1 mL	20 mM
5 M NaCl	1.5 mL	150 mM
2 M Spermidine	12.5 µL	0.5 mM
Roche Complete Protease Inhibitor EDTA-Free	1 tablet	-

**Digitonin Buffer:** For up to 24 AutoCUT&RUN reactions mix 150-600 µL 5% (wt/vol) digitonin with 30 mL Wash Buffer for a final concentration of digitonin between 0.025% and 0.1% (wt/vol). Store this buffer on ice or at 4 °C for up to 1 day, and vortex before use.

- Note: The effectiveness of digitonin varies between batches, so testing for full permeability of Trypan blue is recommended to

determine the concentration to use for a cell type. We have obtained excellent results for H1 and K562 cells with 0.05% digitonin (300 µL 5% (wt/vol) digitonin in 30 mL Wash Buffer). For simplicity, we use this same buffer for all steps starting from the incubation in primary antibody until the chromatin digestion.

**Antibody Buffer:** For up to 24 reactions (150 µL/reaction) mix 4 mL Digitonin Buffer with 16 µL 0.5 M EDTA and place on ice.

- Note: The presence of EDTA during antibody treatment removes excess divalent cations used to activate the Concanavalin A-coated beads, as well as endogenous cations from the cells of interest. This serves to halt metabolic processes, stop endogenous DNase activity, and prevent carry-over of Ca<sup>2+</sup> from the Binding Buffer that might prematurely initiate strand cleavage after addition of pA-MNase. Washing out the EDTA before pA-MNase addition avoids inactivating the enzyme.

### Binding cells to beads

- 1 Harvest fresh culture(s) at room temperature and count cells. The AutoCUT&RUN protocol is recommended for 50,000 to 1,000,000 mammalian cells per sample. Tissue samples can also be profiled using AutoCUT&RUN and should be processed either manually or enzymatically into a homogenous suspension of intact cells prior to binding Concanavalin A-coated beads.



**SECTION TIMING:** 30 min



**CRITICAL STEP:** All steps prior to the addition of antibody are performed at room temperature (~22 °C) to minimize stress on the cells. Because it is crucial that DNA breakage is minimized throughout the protocol, we recommend that cavitation during resuspension and vigorous vortexing be avoided.



**PAUSE POINT:** If necessary, cells can be cryopreserved in 10% (vol/vol) DMSO using a Mr. Frosty isopropyl alcohol chamber. Alternatively, cells can simply be washed with PBS, pelleted at 600 X g and then snap frozen following aspiration of all liquid. Similarly, dissected tissue can be snap frozen in a cryogenic vial for processing at a later date.

- 2 Centrifuge 3 min 600 x g at room temperature and withdraw liquid.

- 3 Resuspend in 1.5 mL room temperature Wash Buffer by gently pipetting and transfer if necessary to a 2 mL tube.

- 4 Centrifuge 3 min 600 x g at room temperature and withdraw liquid.

- 5 Repeat steps 3 and 4 two more times.



**CRITICAL STEP:** Thorough washing removes free sugars and other molecules that can compete for binding to the Concanavalin A coated-beads, ensuring efficient binding and recovery of the cells of interest.

- 6 Resuspend in 1 mL room temperature Wash Buffer by gently pipetting.
- 7 While gently vortexing the cells at room temperature, add the Concanavalin A coated-bead suspension.
- 8 Place on tube nutator at room temperature for 5-10 min.

#### Permeabilize cells and bind (primary) antibodies

- 9 Mix well by vigorous inversion to ensure the bead-bound cells are in a homogenous suspension and divide into aliquots in 0.6-mL low-bind tubes, one for each antibody to be used.



**SECTION TIMING: 2 hrs–overnight**

- 10 Place on the magnet stand to clear and pull off and discard the liquid.

- 11 Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and squirt 150 µL of the Antibody Buffer per sample along the side while gently vortexing to allow the solution to dislodge most or all of the beads. Tap to dislodge the remaining beads.



**CRITICAL STEP:** Permeabilizing the cells with digitonin and chelating divalent cations with EDTA serves to quickly halt metabolic processes and prevent endogenous DNase activity. This helps to preserve the native chromatin state and reduce background noise in the final CUT&RUN libraries. Thus, it is recommended to work quickly to get cells into Antibody Buffer.

- 12 Mix in the primary antibody to a final concentration of 1:100 or to the manufacturer's recommended concentration for immunofluorescence.



**CRITICAL STEP:** To evaluate success of the procedure without requiring sequencing, include in parallel a positive control antibody (*e.g.* anti-H3K27me3) and a negative control antibody (*e.g.* anti-mouse IgG). Do not include a no-antibody control, as the lack of tethering may allow any unbound pA-MNase to act as a “time-bomb” and digest accessible DNA, resulting in a background of DNA-accessible sites.

- 13 Place on the tube nutator at room temperature for 2 hrs.



**PAUSE POINT:** Antibody incubation may proceed overnight at 4 °C.



**? TROUBLESHOOTING:** See Troubleshooting Table in **Guidelines**

#### Bind secondary antibody (as required)

- 14 Remove liquid from the cap and side with a quick pulse on a micro-centrifuge.



**SECTION TIMING: 1 hr–overnight**

 **NOTE**

**CRITICAL STEP:** The binding efficiency of Protein A to the primary antibody depends on host species and IgG isotype. For example, Protein A binds well to rabbit and guinea pig IgG but poorly to mouse and goat IgG, and so for these latter antibodies a secondary antibody, such as rabbit anti-mouse is recommended.

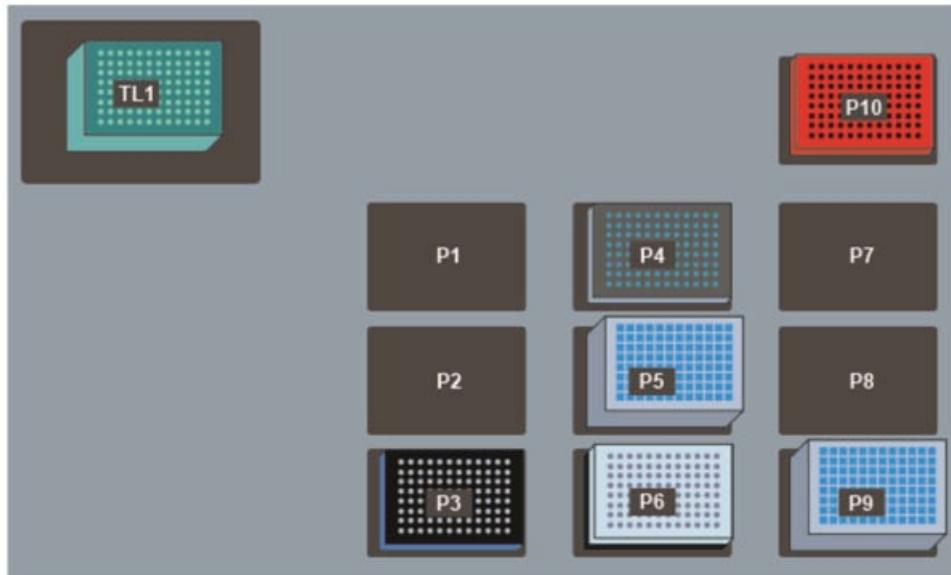
 **NOTE**

**CRITICAL STEP:** After mixing, but before placing a tube on the magnet stand, a very quick spin on a micro-centrifuge (no more than 100 x g) will minimize carry-over of antibody and pA-MN that could result in overall background cleavages during the digestion step.

- 15 Place on the magnet stand to clear (~30 s) and pull off all of the liquid.
- 16 Add 150 µL Digitonin Buffer to wash, mix by inversion, or by gentle pipetting using a 1 mL tip if clumps persist, and remove liquid from the cap and side with a quick pulse on a micro-centrifuge.
- 17 Repeat Digitonin Buffer wash steps 14-16.
- 18 Place on the magnet stand to clear and pull off all of the liquid.
- 19 Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and squirt 150 µL of the Digitonin Buffer (per sample and/or digestion time point) along the side while gently vortexing to allow the solution to dislodge most or all of the beads. Tap to dislodge the remaining beads.
- 20 Mix in the secondary antibody to a final concentration of 1:100 or to the manufacturer's recommended concentration.
- 21 Place on the tube nutator at 4 °C for ~1 hr or overnight.

Bind Protein A-MNase fusion protein on Biomek

22



Set Cooling Unit (filled with antifreeze and routed to Heating/Cooling ALP with Aluminum Cooling Block at position P10) to 0 °C.

**NOTE**

**SECTION TIMING:** ~1.5 hr

**NOTE**

**CRITICAL STEP:** Performing the steps up until this point by hand increases the versatility of the platform, allowing individual users to harvest their cells or tissue of interest and bind any antibody of their choosing. Because the antibody incubation is not time sensitive, samples from multiple users can be synchronized at this step and arrayed on a single plate, allowing the remaining steps to be performed in unison on the Biomek by a single operator.

23

Prepare 4.5 mL of pA-MNase solution per 24 AutoCUT&RUN reactions by mixing the pA-MNase (supplied upon request) to a final concentration of ~700 ng/mL in Digitonin Buffer. Transfer pA-MNase solution into a reservoir and dispense 175 µL into each of the active wells of a labeled V-Bottom Plate using a Multi-Channel Pipette. Place at position P4 on the Biomek deck.

**NOTE**

**CRITICAL STEP:** CUT&RUN is relatively insensitive to the concentration of pA-MNase as is evident from the titration test of two different batches, where increasing the concentration of pA-MNase above ~100 ng/mL resulted in little additional release of H3K27me3-bound nucleosomes from 600,000 human cells after 30 min digestion in a 500 µL volume.

24

Using wide bore 200 µL tips, resuspend Concanavalin A bead-bound cells + Antibodies and array them in a PCR 96 Well Plate. Be sure to record the position of each sample in the plate and stack it on a PCR Plate Rack at position P6 on the Biomek deck.

**NOTE**

To prevent the PCR Plate Racks from catching and being carried along with the PCR 96 Well Plate during movement steps, the PCR Plate Racks can be taped down to the stationary ALPs (this issue has not been observed for the ALPAQUA Magnet Stand or the Cold Block).

25

Dispense 1 mL of Digitonin Buffer into each of the active wells of a labeled Deep Well Plate and place it at position P5 on the Biomek deck.

26

Place fresh AP96 200 µL tips at position TL1, the ALPAQUA Magnet Plate at position P3, and an empty labeled Deep Well Plate for collecting liquid waste at position P9 on the Biomek deck.

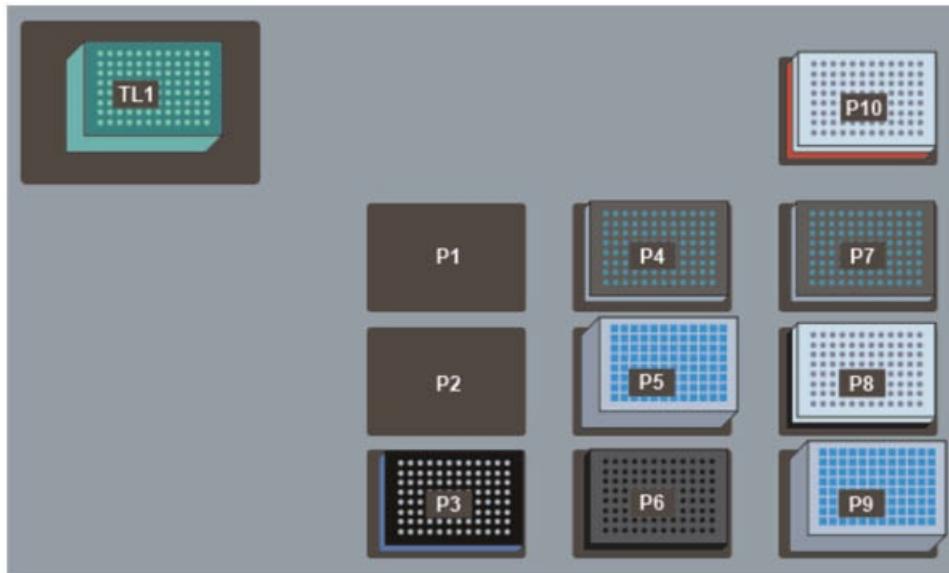
27 Start the pA-MNase Binding Method.

**NOTE**

During the 1 hr mixing step continue on to prepare pA-MNase Reaction Mix and 4X Stop Buffer solutions and keep on Ice for the next step of the reaction.

Targeted chromatin digestion on the Biomek

28



Prepare pA-MNase Reaction Mix (50 µL/sample). Keep on Ice until use.

pA-MNase Reaction Mix				
Component	Amount per 24 samples (µL)	Added for Reservoir (µL)	Final volume per 24 samples (µL)	Final concentration
Digitonin Buffer	1176	441	1617	
100mM CaCl <sub>2</sub> (diluted 1:10 from a 1 M stock)	24	9	33	2mM

**NOTE**

**SECTION TIMING:** ~1.5 hr

29 Prepare 4X Stop Buffer (25 µL/sample). Keep on Ice until use.

4X Stop Buffer				
Component	Amount per 24 samples ( $\mu$ L)	Added for Reservoir ( $\mu$ L)	Final volume per 24 samples ( $\mu$ L)	Final concentration
ddH <sub>2</sub> O	346.8	260.1	606.9	-
0.2M EGTA	240	180	420	80 mM
5% Digitonin	6	4.5	10.5	0.05%
RNase A (10 mg/mL)	6	4.5	10.5	100 $\mu$ g/mL
Heterologous spike-in DNA (0.1 ng/ $\mu$ L)	1.2	0.9	2.1	0.2 pg/ $\mu$ L

 **NOTE**

**CRITICAL STEP:** Heterologous spike-in DNA is highly recommended for calibration, as there is too little background cleavage for normalization of samples. Spike-in DNA should be fragmented down to ~200 bp mean size, for example an MNase-treated sample of mononucleosome-sized fragments. As we use the total number of mapped reads as a normalization factor only, very little spike-in DNA is needed. For example, addition of 1.5 pg results in 1,000-10,000 mapped spike-in reads for 1-10 million mapped experimental reads (in inverse proportion).

- 30 When the pA-MNase Binding Method is complete, remove the labeled V-Bottom plate containing residual pA-MNase solution and wash well with DI water (this plate can be reused in subsequent experiments). Then empty the liquid waste from the labeled Deep Well Plate, rinse it well with DI water and again place it at position P9 on the Biomek deck. The recirculating water bath filled with antifreeze should remain at 0 degrees celcius for this method.
- 31 Using a Reservoir and Multi-Channel Pipette dispense 50  $\mu$ L of pA-MNase Reaction Mix into the active wells of a labeled V-Bottom Plate and place it at position P4 on the Biomek deck. Dispense 25  $\mu$ L of 4X STOP Buffer in the active wells of a labeled V-Bottom Bottom Plate and place it at position P7 on the Biomek deck. Gently shake the Deep Well Plate containing Digitonin Buffer to ensure digitonin remains suspended in solution and again place it at position P5 on the Biomek deck.
- 32 Place a fresh PCR 96 Well Plate on a PCR Plate Rack at position P8 for accepting digested chromatin. The AP96 200  $\mu$ L Tips at TL1 can be used again for this method, the ALPAQUA Magnet Plate should remain at position P3, and a free PCR Plate Rack should remain at position P6. The PCR 96 Well Plate containing Concanavalin A bead-bound cells + Antibodies + pA-MNase should remain on the Cooling Block set to 0°C at position P10 on the Biomek deck.
- 33 Ensure the Pause at Step 19 of the pA-MNase Digest Method (after addition of MNase Reaction Mix and before addition of 4X STOP Buffer) is set for the desired digestion time.

 **NOTE**

**CRITICAL STEP:** Longer digestion times tend to increase the yield of digested chromatin from each reaction, and can be critical to obtain quality signal from low abundance epitopes (e.g. transcription factors). However, for some antibodies (e.g. anti-H3K27ac) longer digestion times have been observed to increase the amount of non-specific or “background” DNA that is recovered. Because the current platform adds 4X STOP Buffer to all the wells synchronously, setting individual digestion lengths for each reaction is currently not an option, therefore a digestion time of 9-30 min is recommended.

- 34 Start the pA-MNase Digest Method.

 **NOTE**

During the chromatin release step, when the plate is mixing and incubating at room temperature for about half an hour, prepare 4X End Repair and A-Tailing Buffer and keep on Ice for the next step of the reaction.

**Chromatin end repair and dA-tailing**

35

Prepare 4X End Repair and A-tailing (ERA) buffer (12.5 µL/sample). Keep on Ice until use.

4X End Repair and A-tailing (ERA) Buffer				
Component	Amount per 24 samples (µL)	Added for Reservoir (µL)	Final volume per 24 samples (µL)	Final concentration
T4 DNA ligase Buffer (10X)	120	180	300	4X
dNTPs (10mM each)	60	90	150	2 mM each
ATP (10mM)	30	45	75	1 mM
40% PEG 4000	75	112.5	187.5	10%
T4 PNK (10 U/µL)	15	22.5	37.5	0.5 U/µL
T4 DNA Polymerase (5U/µL)	3	4.5	7.5	0.05 U/µL
Taq DNA Polymerase (5U/µL)	3	4.5	7.5	0.05 U/µL

**NOTE**

**SECTION TIMING:** ~2.5 hr

36 When the pA-MNase Digestion Method is complete, remove the labeled V-Bottom plates containing residual pA-MNase Reaction Mix and 4X Stop Buffer, as well as the labeled Deep Well Plates containing residual Digitonin Buffer and Liquid Waste from the Biomek deck. Empty the plates and wash well with DI water (these plates can be reused in subsequent experiments). Seal the PCR 96 Well Plate containing the digested Con-A bead-bound cells (position P3) with an adhesive cover and store at -20 °C for potential troubleshooting. Turn off the recirculating water bath.

37 Remove the PCR 96 Well Plate containing digested chromatin (position P8) from the Biomek deck. Using a Reservoir and Multi-Channel Pipette add 12.5 µL 4X ERA buffer to each sample and mix by pipetting up and down 5 times.

38 Seal the PCR 96 Well Plate using an adhesive cover and place in a thermocycler that has been pre-cooled to 12°C and run the following program with heated lid for temp >20°C:

End Repair Program				
Cycle number	End Polishing	5' End phosphorylation	dA-Tailing	Final
1	12°C, 15 min			
2		37° C, 15 min		
3			58° C, 1.5 hour	
4				8° C, hold

**Adapter Ligation**

39 Remove pre-annealed 0.15 µM TruSeq adapters from freezer and allow to thaw on ice.

**NOTE**

**SECTION TIMING:** 1 hr-overnight

**NOTE**

**CRITICAL STEP:** To facilitate multiplexing during sequencing each adapter includes a six-nucleotide index or "barcode".

For subsequent data analysis make sure to keep track of which adapter is used for each sample. To allow for addition of adapters using a Multi-Channel Pipette 0.15 µM pre-annealed TruSeq adapters can be arrayed in a 96 Well PCR plate.

40 Prepare 2X Rapid Ligase solution (50 µL/ sample). Keep on Ice until use.

2X Rapid Ligase Solution				
Component	Amount per 24 samples (µL)	Added for Reservoir (µL)	Final volume per 24 samples (µL)	Final concentration
Enzymatics 2X Rapid DNA ligase buffer	1080	405	1485	~2 X
Enzymatics DNA Ligase	120	45	165	

41 Using a Multi-Channel Pipette, add 5 µL of 0.15 µM pre-annealed TruSeq adapters to each sample and mix by pipetting up and down 5 times.

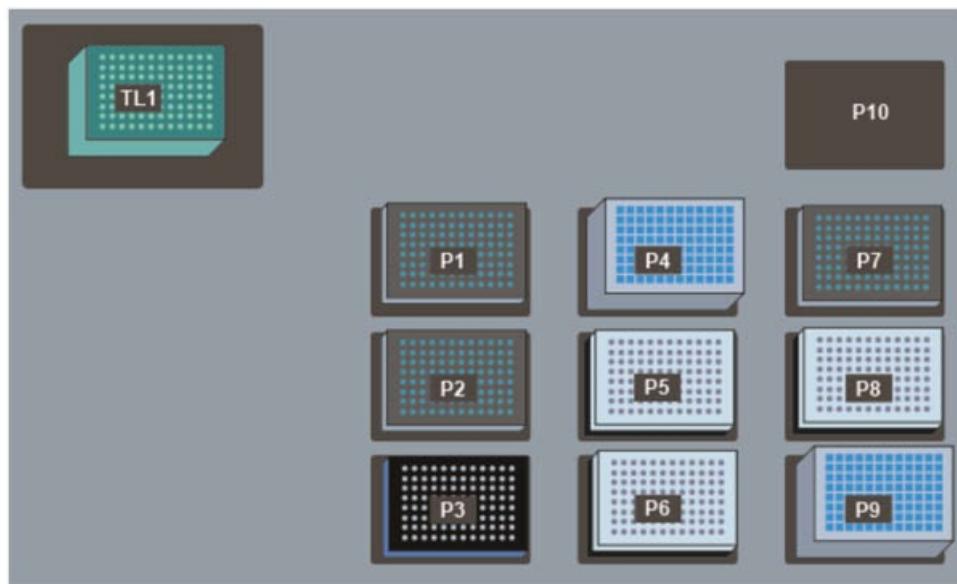
42 Using a Reservoir and Multi-Channel Pipette, add 50 µL 2X Rapid Ligase solution to each sample and mix well by pipetting up and down 10 times.

43 Seal the PCR 96 Well Plate using an adhesive cover and place in a thermocycler that has been pre-cooled to 20°C and incubate at 20°C for 20 min. Following incubation proceed with Proteinase K digestion immediately.

44 Using two Reservoirs and a Multi-Channel Pipette, add 2 µL 10% SDS and 2 µL Proteinase K (20 mg/mL) to each sample. Seal the PCR 96 Well Plate using an adhesive cover and mix well by vortexing. Spin down solution @ 2,000 rpm for 1 min and incubate in a thermocycler at 37°C for 30 min-overnight.

#### Pre-PCR DNA Cleanup on the Biomek

45



Remove Ampure Bead Slurry from refrigerator, resuspend beads by vortexing and allow to equilibrate to room temperature before using.

##### NOTE

**SECTION TIMING:** ~2 hrs

##### NOTE

**CRITICAL STEP:** Two rounds of Ampure Bead Cleanup are performed prior to PCR amplification to remove un-ligated, and

self-ligated adapter as well as unwanted protein, PEG, and salt.

- 46 Spin PCR 96 Well Plate containing Adapter Ligated DNA samples @ 2,000 rpm for 1 min. Remove seal and stack on a PCR Plate Rack positioned at P5 on the Biomek deck.
- 47 Using a Reservoir and Multi-Channel Pipette distribute 90 µL of the Ampure Bead Slurry into the active wells of a PCR 96 Well Plate and stack on a PCR Plate Rack positioned at P6 on the Biomek deck.
- 48 Dispense 1 mL of 80% Ethanol into each of the active wells of a labeled Deep Well Plate and place at position P4 on the Biomek deck.
- 49 For up to 24 AutoCUT&RUN reactions prepare 6 mL of HXP Mix (20% PEG 8000, 2.5M NaCl). Using a Reservoir and Multi-Channel Pipette distribute 100 µL of the HXP Mix into the active wells of a V-Bottom Plate and place at position P7 on the Biomek deck.

 **NOTE**

**CRITICAL STEP:** HXP Mix is light sensitive and will degrade over time once mixed. Therefore, the HXP Mix should either be prepared fresh for each use or stored in the dark at -20°C.

- 50 Using a Reservoir and Multi-Channel Pipette distribute 100 µL of 10mM Tris-HCl pH 8 into the active wells of two labeled V-Bottom Plates (one for tip washes and one for DNA elution) and place at positions P1 and P2 on the Biomek deck.
- 51 Place Fresh AP96 200 µL Tips at TL1, a labeled Deep Well Plate for accepting liquid waste at position P9, and a fresh PCR 96 Well Plate stacked on a PCR Plate Rack for accepting clean Adapter Ligated DNA in position P8 on the Biomek deck.
- 52 Start the Pre-PCR DNA Cleanup Method.

 **NOTE**

After about 1hr continue on to prepare KAPA PCR Master Mix and keep on Ice for the next step of the reaction.

### PCR Amplification of CUT&RUN Libraries.

- 53 Prepare KAPA PCR Master Mix.

KAPA PCR Master Mix				
Component	Amount per 24 samples (µL)	Added for Reservoir (µL)	Final volume per 24 samples (µL)	Final Concentration
5X KAPA buffer	240	225	465	~2.5 X
10 mM dNTPs	36	33.75	69.75	0.75 mM
20 µM P5 primer	120	112.5	232.5	5 µM
20 µM P7 primer	120	112.5	232.5	5 µM
KAPA HS HIFI polymerase	24	22.5	46.5	

 **NOTE**

**SECTION TIMING: 1 hr-overnight**

**54** When the Pre-PCR DNA Cleanup Method is complete, remove the labeled V-Bottom Plates containing residual 10mM Tris-HCl pH 8 and HXP Mix, as well as the labeled Deep Well Plates containing residual Ethanol and Liquid Waste from the Biomek deck. Empty the plates and wash well with DI water (these plates can be reused in subsequent experiments).

**55** Remove PCR 96 Well Plate containing clean Adapter Ligated DNA (position P8) from the Biomek deck. Using a Reservoir and Multi-Channel Pipette add 20 µL KAPA PCR Master Mix to each sample and mix well by pipetting up and down 10 times.

**56** Seal the PCR plate using an adhesive cover, place in thermocycler and run the following program with heated lid:

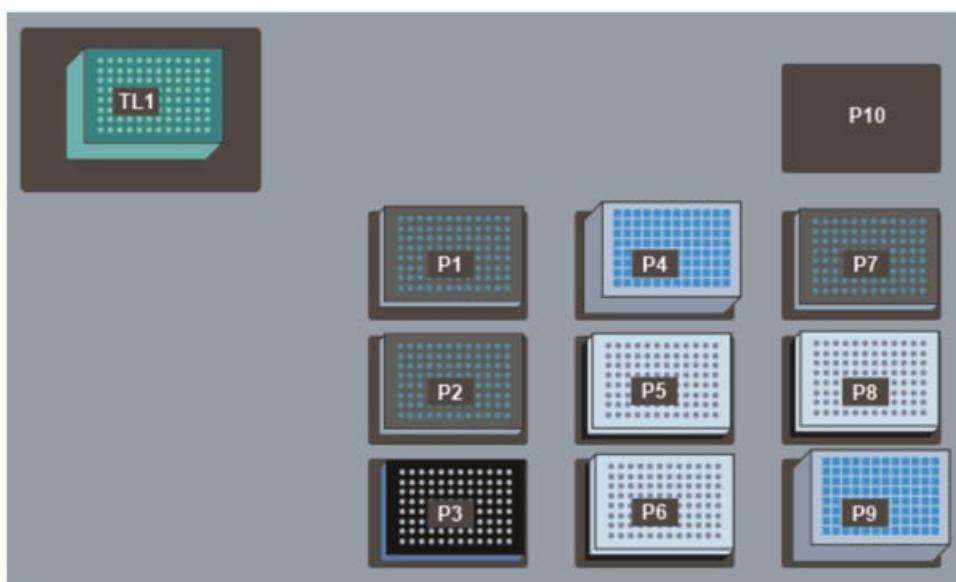
PCR Program				
Cycle number	Denature	Anneal	Extend	Final
1	98° C, 45 s			
2-15	98° C, 15 s	60° C, 10 s		
16			72° C, 1 min	
17				8° C, hold

**NOTE**

**CRITICAL STEP:** Libraries should be amplified with 12-14 PCR cycles, preferably with a 10 s 60°C combined annealing/extension step to minimize the contribution of large DNA fragments to the library. Good results have been obtained with the Hyper-prep kit (KAPA Biosystems).

Post-PCR DNA Cleanup on the Biomek

**57**



Remove Ampure Bead Slurry from refrigerator, resuspend beads by vortexing and allow to equilibrate to room temperature before using.

**NOTE**

**SECTION TIMING: ~2 hr**

**NOTE**

**CRITICAL STEP:** Two rounds of Ampure Bead Cleanup are performed following PCR amplification to remove unused P5 and P7 primers, amplified self-ligated adapters and unwanted protein and salt. Once amplified, the CUT&RUN library can serve as template for subsequent PCR reactions using the P5 and P7 primers, making cross-contamination of Pre-PCR samples a serious concern. Therefore, all Post-PCR reagents should be stored separately from Pre-PCR reagents, and prepared in a separate area. If labware is to be reused, separate plates should be clearly labeled for use in Post-PCR reactions only.

- 
- 58 Spin PCR 96 Well Plate containing PCR amplified CUT&RUN libraries @2,000 rpm for 1 min. Remove seal and stack on a PCR Plate Rack positioned at P5 on the Biomek deck.
- 59 Using a Reservoir and Multi-Channel Pipette distribute 55 µL of the Ampure Bead Slurry into the active wells of a PCR 96 Well Plate and stack on a PCR Plate Rack positioned at P6 on the Biomek deck.
- 60 Dispense 1 mL of 80% Ethanol into each of the active wells of a labeled Deep Well Plate and place at position P4 on the Biomek Deck.
- 61 Using a Reservoir and Multi-Channel Pipette distribute 100 µL of the remaining HXP Mix into the active wells of a V-Bottom Plate and place at position P7 on the Biomek deck.
- 62 Using a Reservoir and Multi-Channel Pipette distribute 100 µL of 10mM Tris-HCl pH 8 into the active wells of two V-Bottom Plates (one for tip washes and one for DNA elution) and place at positions P1 and P2 on the Biomek deck.
- 63 Place Fresh AP96 200 µL Tips at TL1, a labeled Deep Well Plate for accepting liquid waste at position P9, and a fresh PCR 96 Well Plate stacked on a PCR Plate Rack for accepting clean CUT&RUN DNA Libraries at position P8 on the Biomek deck.
- 64 Start the Post-PCR DNA Cleanup Method.

### Sequencing

- 65 When the Post-PCR DNA Cleanup Method is complete, remove the labeled V-Bottom plates containing residual 10mM Tris-HCl pH 8 and HXP Buffer, as well as the labeled Deep Well Plates containing residual Ethanol and Liquid Waste from the Biomek deck. Empty the plates and wash extremely well with DI water (these plates can be reused in subsequent experiments).
- NOTE**  
**SECTION TIMING: 1-2 days**
- 66 Remove PCR 96 Well Plate containing clean CUT&RUN Libraries (position P8) from the Biomek deck and seal with an adhesive cover for subsequent analysis.
- 67 Determine the size distribution of libraries by Agilent 4200 TapeStation analysis.
- NOTE**  
**? TROUBLESHOOTING:** See Troubleshooting Table in **Guidelines**
- 68 Quantify library yield using dsDNA-specific assay, such as Qubit.
- 69 Pool indexed CUT&RUN libraries and perform paired-end Illumina sequencing following the manufacturer's instructions.

**NOTE**

**CRITICAL STEP:** Because of the very low background with CUT&RUN, typically 5 million paired-end reads suffices for transcription factors or nucleosome modifications, even for the human genome. For maximum economy, we mix up to 24 barcoded samples per lane at equimolar concentration (proved a similar number of reads is desired for each sample) and perform paired-end 25x25 bp sequencing on a 2-lane flow cell. Single-end sequencing is not recommended for CUT&RUN, as

it sacrifices resolution and discrimination between transcription factors and neighboring nucleosomes.

## Data processing and analysis

70 We align paired-end reads using Bowtie2 version 2.2.5 with options: --local --very-sensitive-local --no-unal --no-mixed --no-discordant --phred33 -I 10 -X 700. For mapping spike-in fragments, we also use the --no-overlap --no-dovetail options to avoid cross-mapping of the experimental genome to that of the spike-in DNA.

 NOTE

**SECTION TIMING: (variable)**

 NOTE

**CRITICAL STEP:** Separation of sequenced fragments into  $\leq 120$  bp and  $\geq 150$  bp size classes provides mapping of the local vicinity of a DNA-binding protein, but this can vary depending on the steric access to the DNA by the tethered MNase.

 NOTE

**? TROUBLESHOOTING:** See Troubleshooting Table in *Guidelines*



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