

**EpiCypher®**

Bringing Epigenetics to Life

**CUTANA™**

**CUT&RUN Library Prep Kit**  
**User Manual Version 1.5**

Powered by NEBNext®  
from New England Biolabs®

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**EpiCypher, Inc.**

PO Box 14453

Durham, NC 27709

**[www.epicypher.com](http://www.epicypher.com)**

Ph: 1-855-374-2461

F: 1-855-420-6111

Email: [info@epicypher.com](mailto:info@epicypher.com)

Tech Support: [techsupport@epicypher.com](mailto:techsupport@epicypher.com)



# CUTANA<sup>TM</sup>

## CUT&RUN Library Prep Kit

Kit Version 1

Catalog No. 14-1001 & 14-1002

48 CUT&RUN Library Prep Reactions

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**Upon receipt, store indicated components  
at **-20°C** and **room temperature (RT)****

Stable for 6 months upon date of receipt.

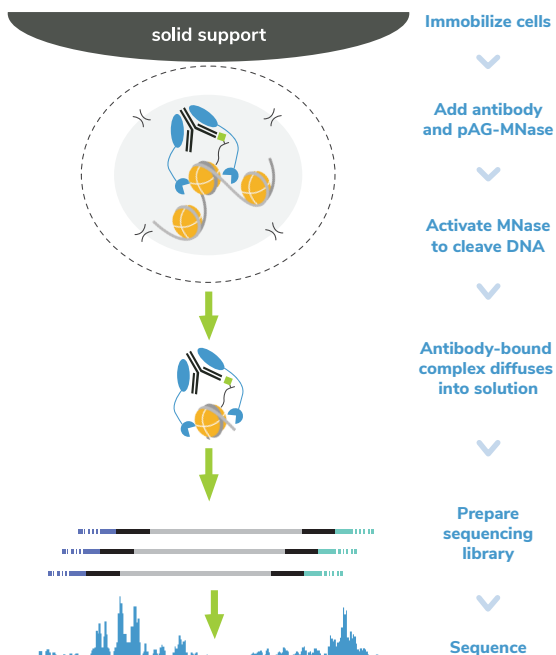
See p. 10 for storage instructions.

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**Cleavage Under Targets & Release Using Nuclease (CUT&RUN)** is an innovative chromatin mapping approach that builds on recent advances in immunotethering technology<sup>1-2</sup>. In CUT&RUN, a Protein AG-Micrococcal Nuclease (pAG-MNase) fusion is used to selectively cleave antibody-bound chromatin in intact cells or nuclei ([Figure 1](#))<sup>3</sup>. Next-generation sequencing of clipped fragments provides high resolution genome-wide profiles of histone post-translational modifications (PTMs) and chromatin-associated proteins (e.g. transcription factors [TFs]).

However, preparation of CUT&RUN DNA for sequencing is far from straightforward. Existing library prep kits are not optimized for low CUT&RUN yields, and often lack key reagents such as indexing primers and DNA purification beads. As a result, CUT&RUN library prep remains a challenge for many users.



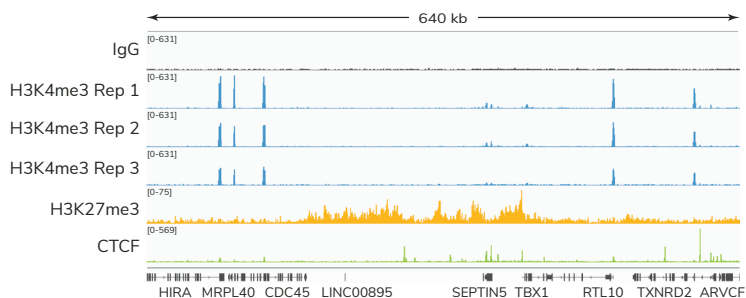
**FIGURE 1**

Overview of the CUTANA™ CUT&RUN protocol.

## Product Description

The CUTANA™ Library Prep Kit is the first library prep kit specifically developed for CUT&RUN assays. Advantages and features include:

- Protocol is uniquely optimized for CUT&RUN, eliminating guesswork surrounding adaptation of multi-purpose or ChIP-seq library prep kits.
- Workflow is robust for the limited inputs generated by CUT&RUN, providing high-quality Illumina® sequencing libraries from 10 to 0.5 ng DNA ([Figures 2 & 3](#)).
- Kit contains everything you need for CUT&RUN library prep: enzymes, indexing primers, DNA purification beads, buffers, and 8-strip tubes.
- Easily pairs with the CUTANA™ CUT&RUN Kit (EpiCypher 14-1048) or CUT&RUN Protocol ([epicypher.com/protocols](http://epicypher.com/protocols)) for seamless workflow integration, increased throughput & reliability, and reduced assay costs.
- **FAQs** detail expected library yields, quality control metrics, and troubleshooting.
- Effective for preparing CUT&RUN DNA libraries for both histone PTMs and chromatin-associated proteins (e.g. TFs; see [Figure 2](#)).

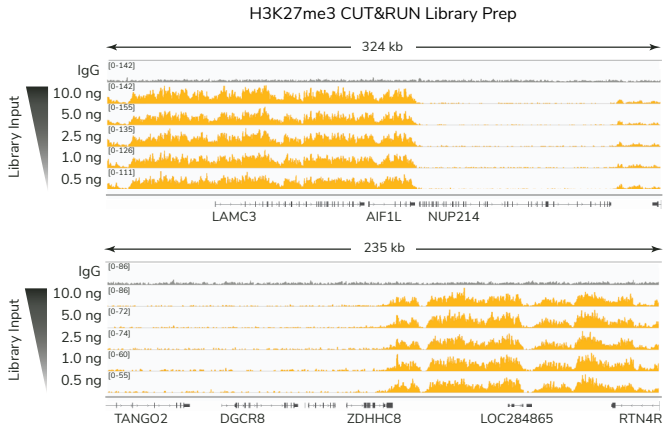


**FIGURE 2**

Representative genome browser tracks show CUTANA™ CUT&RUN Library Prep results for diverse targets in K562 cells. CUT&RUN was performed using the CUTANA CUT&RUN Kit (EpiCypher 14-1048) and antibodies to H3K4me3 (EpiCypher 13-0041; updated to 13-0060), H3K27me3 (ABclonal A16199), CTCF (EpiCypher 13-2014), and IgG (EpiCypher 13-0042). 2-5 ng CUT&RUN-enriched DNA was used for library prep. Three replicates of H3K4me3 (Rep) are shown to highlight assay reproducibility.

Importantly, this kit harnesses the power of New England Biolabs® (NEB®) best-in-class NEBNext® library prep reagents and has optimized them specifically for compatibility with CUT&RUN. Among leading library prep systems, EpiCypher found that NEBNext® reagents consistently delivered the highest quality CUT&RUN libraries across multiple targets and DNA inputs. The final CUTANA Library Prep Kit allows researchers to fully realize the low input capabilities of CUT&RUN at an accessible price point. Although it is recommended to use 5 ng CUT&RUN DNA, comparable data can be generated using as little as 0.5 ng (Figure 3).

The Library Prep Kit contains sufficient materials for the preparation of 48 CUT&RUN sequencing libraries. A combinatorial dual indexing primer strategy enables the entire 48-reaction kit to be multiplexed in a single run, if desired. The two versions of this kit (14-1001 and 14-1002) contain distinct primer sets, allowing up to 96 CUT&RUN libraries to be multiplexed when the kits are used together. **Appendix 2** details our indexing strategy and selection of primer pairs for successful multiplexing.



**FIGURE 3**

Representative genome browser tracks for H3K27me3 CUT&RUN experiments in K562 cells. Decreasing amounts of CUT&RUN-enriched DNA were used for library prep to simulate low abundance targets or low cell input experiments. Data are largely indistinguishable across 10 to 0.5 ng DNA input, demonstrating robust preparation of libraries for Illumina® sequencing.

## Outline of Workflow

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The CUTANA Library Prep Kit uses purified CUT&RUN-enriched DNA as input. No fragmentation step is required due to the processive activity of MNase, which produces mononucleosome-sized fragments (~170 bp) for most targets. Here, we review the main steps of the library prep procedure (Figure 4).

### Step 1: End Repair, 5' Phosphorylation, and 3' dA-Tailing

Fragmented CUT&RUN DNA with varying 5' and 3' overhangs is repaired, generating blunt-ended DNA. To maintain 5' and 3' directionality for sequencing, 5' ends are phosphorylated and 3' ends are labeled with a non-templated dAMP. This creates a small 3' overhang, targeted by the 5' adapter during ligation (next step).

### Step 2: Adapter Ligation and Uracil-Specific Excision

End-repaired DNA is ligated to the Adapter for Illumina®, which contains a hairpin loop to connect the distinct 5' and 3' adapter sequences in a single structure. This strategy improves ligation efficiency, reduces adapter dimer contamination, and supports paired-end sequencing analysis. The incorporation of uracil enables digestion of the hairpin loop by the U-Excision Enzyme. The resulting DNA is purified and used for PCR.

### Step 3: Indexing PCR and Sequencing Library Purification

Adapter-ligated DNA is amplified using the Hot Start 2X PCR Master Mix and selected i5 & i7 indexing primers. i5 primers are specific for 5' adapters, while i7 primers anneal to 3' adapters (Figure 4). Each primer incorporates a unique barcode (i.e. index) and standard Illumina® P5/P7 sequences during PCR.

The kit uses a combinatorial dual indexing approach, meaning that each library will contain a unique pair of i5 & i7 barcodes. This strategy enables multiplexing of up to 48 libraries using the eight i5 primers and six i7 primers provided with each kit. For guidance on i5 & i7 primer pair selection, see **Appendix 2**.

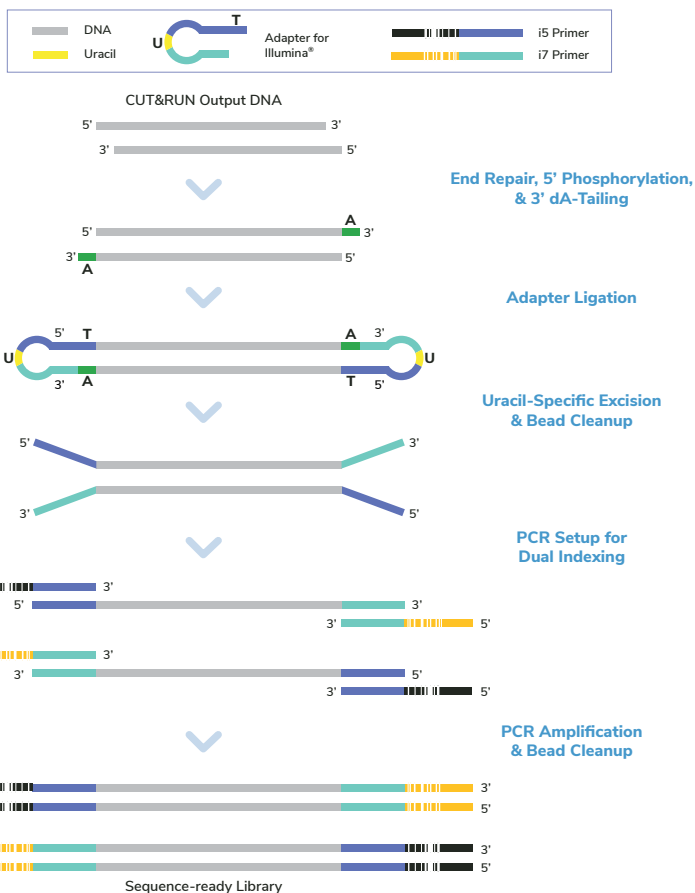
### Step 4: Analysis of Library Quality

Purified CUT&RUN libraries are examined using the Qubit™ Fluorometer and Agilent Bioanalyzer® or TapeStation® to determine library concentration and fragment size distribution. In some cases (e.g. excessive adapter dimer contamination), additional cleanup prior to sequencing may be needed. See **Protocol: Section VI** and **FAQs 7-10** for details about expected results.

### Step 5: Illumina® Next-Generation Sequencing

Once libraries are quantified, they are diluted, pooled, and sequenced on an appropriate Illumina® sequencing platform. See **Protocol: Section VII** and **Appendix 1** for guidance.





**FIGURE 4**

Overview of the CUTANA™ CUT&RUN Library Prep Kit Protocol. CUT&RUN-enriched DNA is repaired and ligated to the Adapter for Illumina®, followed by U-excision to enable PCR amplification. Samples are dual barcoded during PCR using various combinations of i5 & i7 indexing primers. Final libraries are analyzed by capillary electrophoresis (e.g. Bioanalyzer) and Qubit, pooled, and loaded on the desired Illumina® instrument for sequencing.

## Included in the Kit

### Store at room temperature (RT) upon receipt:

Item	Catalog No.	Notes before use
8-strip Tubes	10-0009p	Compatible with multi-channel pipettors and magnetic rack (e.g. EpiCypher 10-0008).
SPRIselect Reagent manufactured by Beckman Coulter, Inc.	21-1405p	<b>NOTE: DO NOT FREEZE.</b> Reagent is slightly viscous. Thoroughly mix prior to use and pipette carefully to ensure correct volumes are transferred. Use to purify DNA.
0.1X TE Buffer	21-1025p	Use to elute CUT&RUN libraries.

### Store at -20°C upon receipt:

Colored bullets (●) are used to denote tube cap color of each reagent.

Item	Catalog No.	Notes before use
(●) End Prep Enzyme	15-1019p	<b>SMALL VOLUME: quick spin before use.</b> For end repair of purified CUT&RUN DNA.
(●) End Prep Buffer	21-1012p	<b>NOTE:</b> Buffer may precipitate. If observed: bring to RT, mix until dissolved. Return to ice.
(●) Adapter for Illumina®	18-1000p	<b>SMALL VOLUME: quick spin before use.</b> 1.5 µM adapter for Illumina® sequencing.
(●) Ligation Mix	15-1020p	<b>NOTE:</b> Buffer is highly viscous, may precipitate. Thoroughly mix to resuspend. Pipette slowly; ensure correct volume is dispensed.
(●) Ligation Enhancer	15-1021p	<b>SMALL VOLUME: quick spin before use.</b>
(●) U-Excision Enzyme	15-1023p	<b>SMALL VOLUME: quick spin before use.</b>
(●) Hot Start 2X PCR Master Mix	15-1022p	For PCR amplification and indexing of CUT&RUN libraries.
(o) i5 Primers	i501-i508	<b>SMALL VOLUME: quick spin before use.</b> <b>Transfer to 8-strip tubes before first use.</b> 14-1001 & 14-1002 come with i501-i508.
(●) i7 Primers and Spare Caps	Kit 14-1001: i701-i706	<b>SMALL VOLUME: quick spin before use.</b> Six spare caps are provided to help prevent cross-contamination of i7 primers.  <b>NOTE:</b> 14-1001 & 14-1002 contain distinct i7 primers. Each kit generates 48 unique pairs of barcodes as detailed in <b>Appendix 2</b> .
	Kit 14-1002: i707-i712	

\* View i5 & i7 catalog numbers and download index sequences at  
[epicypher.com/14-1001](https://epicypher.com/14-1001) under Documents and Resources.

- \* Access to Qubit™ and Bioanalyzer® or TapeStation® instruments is necessary to perform this protocol.

### REAGENTS:

- Molecular biology grade water
- 100% Ethanol (200 proof)

### EQUIPMENT:

- Low-retention filter pipette tips
- Magnetic separation rack for 8-strip tubes (EpiCypher 10-0008)
- Qubit™ 4 Fluorometer (Invitrogen Q33238) and 1X dsDNA HS Kit (Q33230); Older versions of the Qubit™ Fluorometer can also be used
- Capillary electrophoresis machine and required reagents (e.g. Agilent Bioanalyzer® and High Sensitivity DNA Analysis Kit 5067-4626, or Agilent TapeStation® and D1000 ScreenTape 5067-5582 and D1000 Reagents 5067-5583)
- 8-channel multi-channel pipettors: P200 (e.g. VWR 76169-250), P20 (e.g. VWR 76169-248), and P10 (e.g. VWR 76169-246)
- Multi-channel reagent reservoir (e.g. Thermo Fisher 14-387-072)
- Vortex (e.g. Vortex-Genie®, Scientific Industries SI-0236)
- Benchtop centrifuge/mini-centrifuge with an 8-strip tube adapter (e.g. from Fisher Scientific, Benchmark Scientific)
- Thermocycler with heated lid (e.g. from BioRad, Applied Biosystems, Eppendorf)

### NOTES ON KIT DEVELOPMENT

- \* This library prep kit was specifically developed to prepare CUT&RUN-enriched DNA for multiplexed sequencing on Illumina® platforms.
- \* It has been optimized and validated using DNA produced by the CUTANA™ CUT&RUN Kit and Protocol.

### RECOMMENDED INPUT FOR KIT

- 5 ng CUT&RUN-enriched DNA is the recommended input for this library prep kit. However, robust data can be generated with as little as 0.5 ng input ([Figure 3](#)).
- For CUT&RUN reactions with yields between 5 to 0.5 ng DNA (e.g. low abundance targets), use as much DNA as possible for library prep ([FAQ 4](#)).
- If using less than 0.5 ng DNA as input, refer to [FAQ 5](#) for guidance. Note that these libraries often show reduced yields, elevated adapter dimers, and increased read duplication rates, significantly impacting sequencing data quality. However, good enrichment profiles can still be obtained.

### KEY TIPS FOR PROTOCOL & EXPERIMENTAL PLANNING

- Kit components are indicated by **bold text**. Reagents with colored tube caps are further denoted by colored bullets (●) in the protocol.
- The protocol has been optimized for high throughput processing using 8-strip tubes and multi-channel pipettors.
- **Maintaining 4°C temperature in Section II is essential to avoiding adapter dimer formation.** Other tips for successful library prep are noted at the start of each protocol section.
- Read [Appendix 2](#) for guidance on the selection of **i5 & i7 primers** for indexing PCR and multiplexed sequencing. Index sequences are available at [epicypher.com/14-1001](https://epicypher.com/14-1001) under Documents and Resources.
- Paired-end sequencing (2 x 50 bp) is recommended for CUT&RUN to identify sites of MNase cleavage and enable target footprinting analysis (e.g. for TFs). 5-8 million total reads provides adequate coverage for most targets.
- This protocol generates robust nucleosome-resolution data (~170 bp) for TFs. To prep subnucleosomal fragments (<120 bp), see protocol adjustments in [FAQ 6](#).

## SECTION I: END REPAIR (~75 MIN)

## TIPS FOR LIBRARY PREP SUCCESS

- \* Gentle to moderate vortexing is suggested to mix reactions throughout the protocol due to the viscosity of reagents. Briefly, vortex tubes with three 4 sec pulses, flick and invert 8-strip tubes, and quick spin to collect liquid. This method sufficiently mixes reactions, retains enzyme activity, and increases yields vs. pipette mixing.
- \* Thoroughly mix (●) **End Prep Buffer** after thawing. If a white precipitate is observed, bring to room temperature, mix by pipetting/vortexing to dissolve, and return to ice.

1. Thaw kit reagents stored at -20°C and keep on ice (4°C) during the experiment.
2. Transfer 5 ng of CUT&RUN-enriched DNA to a new **8-strip tube** and adjust final volume to 25 µL with **0.1X TE Buffer**.
3. Prepare an End Repair Master Mix for N reactions by combining the following reagents in a fresh 1.5 mL tube on ice. This recipe includes 20% excess volume to account for pipetting error:
 

4.2 µL (●) <b>End Prep Buffer</b>	x	N reactions = _____ µL
1.8 µL (●) <b>End Prep Enzyme</b>	x	N reactions = _____ µL
4. Gently vortex **End Repair Master Mix**. Perform a quick spin in a benchtop centrifuge and return to ice.
5. Add 5 µL **End Repair Master Mix** to 25 µL CUT&RUN DNA in 8-strip tubes. Pipette up and down 5 times to clear tips, gently vortex to mix, and quick spin.
6. Place reactions in a thermocycler and run the following program with heated lid set to ≥75°C:

Step #	Temperature	Time	Cycles	Notes
1	20°C	20 min	1	Reaction temperature
2	65°C	30 min	1	Enzyme inactivation
3	4-12°C	∞	1	Hold temperature

7. Quick spin 8-strip tubes to collect liquid in tube bottom. Place tubes directly on ice or in a pre-chilled aluminum block on ice. Proceed immediately to **Section II**.

## SECTION II: ADAPTER LIGATION AND U-EXCISION (~45 MIN)

### TIPS TO AVOID ADAPTER DIMERS:

- \* Maintaining 4°C temperature during reaction setup is crucial to minimize adapter dimers.
- \* The (●) **Ligation Mix** is highly viscous, and precipitation may occur. To minimize adapter dimers, thoroughly mix before using to ensure reagent homogeneity, and pipette slowly to ensure the correct volume is dispensed.
- \* The concentration of adapter is optimized for 10 ng down to 0.5 ng CUT&RUN DNA.

8. Prepare a Ligation Master Mix for N reactions by combining the following reagents in a fresh 1.5 mL tube on ice. This recipe includes 10% excess volume to account for pipetting error:

16.5 µL (●) **Ligation Mix**                      x        N reactions = \_\_\_\_\_ µL

0.55 µL (●) **Ligation Enhancer**                      x        N reactions = \_\_\_\_\_ µL

Gently vortex Ligation Master Mix, quick spin, and return to ice.

9. Add the following reagents to end-repaired DNA in 8-strip tubes (from Step 7). Keep tubes on ice during while adding reagents.

1.25 µL of 1.5 µM (●) **Adapter for Illumina®**

15.5 µL **Ligation Master Mix**

Thoroughly vortex to mix, quick spin, and return to ice. If processing multiple tube strips, dispense reagents to one 8-tube strip, vortex and quick spin, before continuing to the next strip. Keep all reactions on ice until next step.

10. Incubate tubes in a thermocycler without a heated lid for 15 min at 20°C.
11. Remove tubes from thermocycler to a room temperature (RT) rack. Add 1 µL (●) **U-Excision Enzyme** to each reaction. Pipette up and down 3 times to clear tip. Gently vortex and quick spin. Final volume of each reaction is now 47.75 µL.
12. Place tubes in a thermocycler with a heated lid. Set lid temperature to ≥47°C, block temperature to 37°C, and incubate reactions for 15 min.
13. Remove tubes from thermocycler and quick spin.

**Safe pause point.** Reactions can be stored at -20°C overnight.

### SECTION III: DNA CLEANUP (~15 MIN)

- \* Over drying the beads may result in poor recovery.
- \* Use of multi-channel pipettors is recommended from this point forward.

- For each reaction, make 1 mL 85% Ethanol (EtOH) by combining 850  $\mu$ L 100% EtOH and 150  $\mu$ L molecular biology grade water. Prepare fresh, mix well, and store at RT. Calculations include 25% excess to account for pipetting error.
  - \* If pausing protocol after Section III, prepare 500  $\mu$ L 85% EtOH per reaction.
- Vortex **SPRIselect** reagent (beads) thoroughly to resuspend. Slowly add 47.75  $\mu$ L **SPRIselect** reagent (1X volume) to each reaction in 8-strip tubes. Ensure pipette tip is free of extra bead droplets before dispensing to reactions.
- Mix well by vortexing and/or pipetting. Quick spin tubes. Incubate 5 min at RT.
- Place tubes on 8-strip tube magnetic rack for 2-5 min at RT, until the solution clears. Pipette to remove supernatant without disturbing beads.
- Keep tubes on magnet. Add 180  $\mu$ L **85% EtOH** directly onto beads. Carefully pipette to remove supernatant. Repeat one time for a total of two washes.
- Remove tubes from magnet and quick spin with caps facing in to avoid dislodging beads. Return to magnet and pipette to remove residual EtOH.
- Remove tubes from magnet, leave caps open, and air dry the beads for 2-3 min. Beads should appear damp matte brown ([Figure 5](#)). If beads are crackly and/or light brown, they are too dry.
- Add 12  $\mu$ L **0.1X TE Buffer** to each reaction to elute target DNA. Pipette and/or vortex to fully resuspend beads and quick spin. Incubate 2 min at RT.
- Place tubes on magnet for 2 min at RT. Transfer 10.5  $\mu$ L eluted DNA to new **8-strip tubes**.



**FIGURE 5**

Elute DNA when beads are "ideal."

**Safe pause point.** Reactions can be stored at -20°C overnight.

## SECTION IV: INDEXING PCR (~30 MIN)

### TIPS TO AVOID PRIMER CONTAMINATION:

- \* Before first use of kit, transfer total volume of (○) i501-i508 primers to one of the provided 8-strip tubes. This step also enables multi-channel pipetting of i5 primers.
- \* When adding primers to reactions, visually inspect tips to confirm that the correct volume was aspirated and change tips between EACH addition to prevent cross-contamination.
- \* If concerned about cross-contamination, discard (●) i7 primer tube caps and replace with fresh caps. Six spare i7 primer tube caps (●) are provided in each kit.

23. Following the primer selection instructions in **Appendix 2**, assign a unique pair of (○) i5 and (●) i7 primers to each reaction. Index sequences are available in Excel format at [epicypher.com/14-1001](https://epicypher.com/14-1001), under Documents and Resources.

Mark the combination as consumed on the **Primer Tracking Table** (provided on the **Quick-Start Card**).

24. To each 10.5 µL CUT&RUN DNA sample (from Step 26), add the following reagents individually and in order:

1 µL assigned (●) i7 primer

1 µL assigned (○) i5 primer

12.5 µL (●) Hot Start 2X PCR Master Mix (mix well before using)

25. Mix reactions by vortexing and then quick spin to collect liquid.

26. Place reactions in a thermocycler with a heated lid set to 105°C and perform PCR using the following parameters:

Step #	Temperature	Time	Cycles	Notes
1	98°C	45 sec	1	Hot start activation of DNA Polymerase
2	98°C	15 sec	14	DNA melting
3	60°C	10 sec		Hybrid annealing/extension
4	72°C	60 sec	1	Final extension
5	4-12°C	∞	1	Hold temperature

\* The PCR cycling parameters are designed to enrich 200-700 bp DNA fragments.



## SECTION V: PCR CLEANUP (~15 MIN)

\* Over drying the beads may result in poor recovery.

27. If performing library prep in a single day: Proceed to the next step.

If starting from DNA that was frozen after **Section III**: Per reaction, make 500  $\mu\text{L}$  85% Ethanol (EtOH) by combining 425  $\mu\text{L}$  100% EtOH and 75  $\mu\text{L}$  molecular biology grade water. Prepare fresh, mix well, and store at RT. Note that these calculations include extra volume to account for pipetting error.

28. Vortex **SPRIselect** reagent (beads) thoroughly to completely resuspend. Slowly add 25  $\mu\text{L}$  **SPRIselect** reagent (1X volume) to PCR reactions in 8-strip tubes. Ensure pipette tip is free of extra bead droplets before dispensing to reactions.

29. Mix well by vortexing and/or pipetting. Quick spin tubes. Incubate 5 min at RT.

30. Place tubes on magnet for 2-5 min at RT, until the solution clears. Pipette to remove supernatant without disturbing beads.

31. Keeping tubes on magnet, add 180  $\mu\text{L}$  **85% EtOH** directly onto beads. Pipette to remove supernatant. Repeat one time for a total of two washes.

32. Remove tubes from magnet and quick spin with caps facing in to avoid dislodging beads. Return to magnet and pipette to remove residual EtOH.

33. Remove tubes from magnet, leave caps open, and air dry the beads for 2-3 min. Beads should appear damp matte brown ([Figure 5](#)). If beads are crackly and/or light brown, they are too dry

34. Add 12  $\mu\text{L}$  **0.1X TE Buffer** to each reaction to elute DNA libraries. Pipette and/or vortex to fully resuspend beads and quick spin. Incubate 2 min at RT.

35. Place tubes on magnet for 2 min at RT. Transfer 10.5  $\mu\text{L}$  eluted CUT&RUN sequencing libraries to new **8-strip tubes**.

**Safe pause point.** Reactions can be stored at  $-20^{\circ}\text{C}$  overnight.

## SECTION VI: ANALYSIS OF LIBRARY FRAGMENT SIZE (~1 HR)

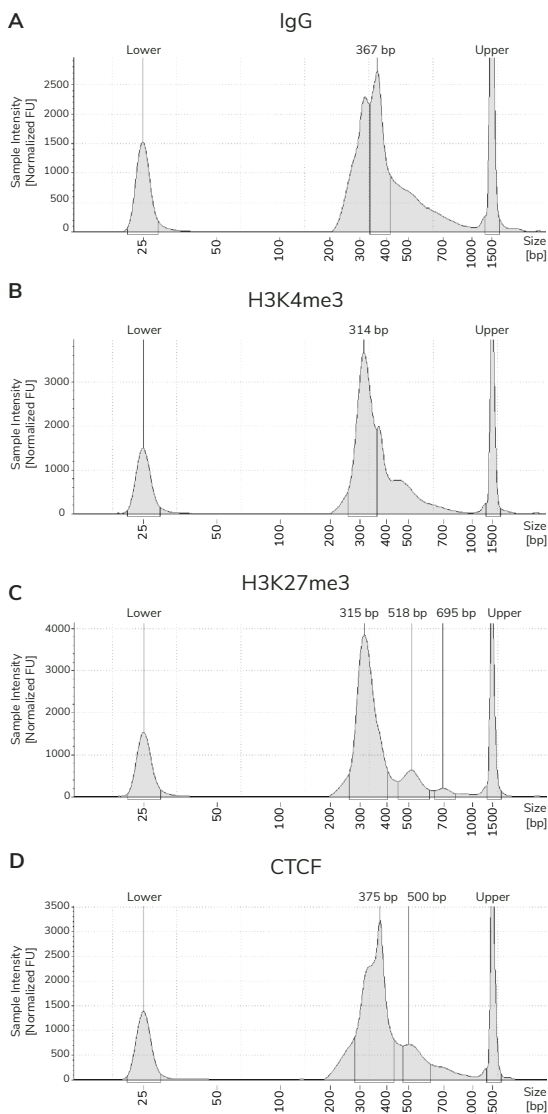
### NOTES ON EXPECTED YIELDS AND FRAGMENT SIZE ENRICHMENT

- \* The **BEST** indicator of CUT&RUN experimental success prior to sequencing is enrichment of mononucleosome-sized fragments (~300 bp = ~170 bp nucleosome + sequencing adapters).
- \* Final CUT&RUN library concentration (200-700 bp region) is usually 100-200 nM. Libraries  $\geq 1$  nM allow pooling at standard concentrations for sequencing, but good data are obtained down to 0.5 nM. If library concentrations are  $< 0.5$  nM, see **FAQ 9**.
- \* Fragment distributions for positive (e.g. H3K4me3, H3K27me3) and negative (e.g. IgG) control reactions can be used to assess yields and validate library prep workflows (**FAQ 7**).
- \* See **FAQs 8-11** for troubleshooting low library yields and/or adapter dimer contamination.

36. Use 1  $\mu$ L purified CUT&RUN library for quantification. Use the Qubit fluorometer with the 1X dsDNA HS Assay Kit per the manufacturer's instructions.
37. For each library, prepare 5  $\mu$ L at 10 ng/ $\mu$ L for loading onto the Bioanalyzer or TapeStation system. Record the dilution factor, which is needed to calculate library molarity from the results (reported as DNA concentrations in nM for the desired 200 - 700 bp region).
38. Load and analyze 1  $\mu$ L diluted sequencing library using the High Sensitivity DNA Kit (Bioanalyzer) or the D1000 ScreenTape System & Reagents (TapeStation) per the manufacturer's instructions.
39. The final traces should show predominant enrichment of mononucleosome-sized fragments, such as those yielded by H3K4me3, H3K27me3, and CTCF antibodies in **Figure 6** (~300 bp: ~170 bp + sequencing adapters). Adapter dimers, if present, would be observed as a peak at ~150 bp; see **FAQ 10** for an example and troubleshooting information.

### FIGURE 6

Typical TapeStation traces from CUTANA CUT&RUN libraries prepared using antibodies targeting IgG (**A**: EpiCypher 13-0042), H3K4me3 (**B**: EpiCypher 13-0041, updated to EpiCypher 13-0060), H3K27me3 (**C**: EpiCypher 13-0055), and CTCF (**D**: EpiCypher 13-2014). All libraries, including the IgG negative control reaction, contain predominantly mononucleosome-sized fragments (~300 bp peak, represents ~170 bp nucleosomes + sequencing adapters).



## SECTION VII: ILLUMINA® SEQUENCING

### TIPS FOR SEQUENCING CUT&RUN LIBRARIES:

- \* Paired-end sequencing (2 x 50 cycles minimum) is recommended for CUT&RUN to identify both ends of MNase cleavage for target footprinting analysis.
- \* Confirm that each library in a sequencing run has a unique pair of i5 & i7 indexes. Check with other researchers if pooling multiple experiments for sequencing. Libraries with the same pair of indexes must be sequenced in separate lanes/flow cells.
- \* Sequence to a depth of 5-8 million **total** reads per library. The end goal is to generate 3-5 million **unique** reads per library.
- \* See **FAQ 9** for considerations when sequencing low-concentration CUT&RUN libraries.

40. Select appropriate Illumina® sequencing platform based on the number of CUT&RUN libraries and desired sequencing depth. See **Appendix 1**.
41. Pool libraries at desired ratios using the molarity calculations from **Section VI** (200-700 bp region) and load onto Illumina® sequencer. General steps:
  - a. Dilute each library to the same nM concentration, depending on final yields. For NextSeq 2000 and NextSeq 500/550, dilute to 1-4 nM.
  - b. Pool equimolar libraries into one tube.
  - c. Dilute pooled libraries to appropriate concentration and in the volume required for Illumina® platform. Follow guidelines from specific Illumina® kit to load onto sequencer ([support.illumina.com](https://support.illumina.com)).
  - d. When setting up a multiplexed sequencing run, make sure dual i5 & i7 indexes are correctly assigned for each library (see Tips, above). For a full list of sequencing indexes in an easy-to-copy format, see the CUTANA Library Prep Multiplexing Primers Excel spreadsheet at [epicypher.com/14-1001](https://epicypher.com/14-1001) under Documents and Resources.
42. For guidance with CUT&RUN sequencing analysis, see **FAQ 13**. See the CUTANA CUT&RUN Kit Manual ([epicypher.com/protocols](https://epicypher.com/protocols)) for discussion of success metrics and expected sequencing results.

- \* The CUTANA™ Library Prep Kit is compatible with Illumina® platforms, including benchtop models (e.g. MiSeq) and high throughput systems (e.g. NextSeq 2000).
- \* Paired-end sequencing (2 x 50 bp minimum) is recommended for CUT&RUN.
- \* Only 5-8 million total reads are needed to generate high-quality sequencing data.

The table below outlines Illumina® sequencing platforms and kits compatible with the multiplexing capabilities of the CUTANA CUT&RUN Library Prep Kit. The number of reactions per run assumes ~5 million total reads per CUT&RUN library. Note that these are estimates, and additional user optimization may be required.

- \* Other Illumina sequencing kits (e.g. 300 cycles) can be used for CUT&RUN sequencing.

Platform	Cartridge	Cat#	Expected Read Depth	# CUT&RUN Reactions
MiSeq	v3 150 cycles	MS-102-3001	25 million	~5
MiniSeq	High Output Kit (150 cycles)	FC- 420-1002	25-40 million	~8
HiSeq 3000/4000	SBS Kit (150 cycles) & PE Cluster Kit	FC-410-1002 & PE-410-1001	350 million per lane	~70 per lane
NextSeq 500/550	Mid Output v2.5 (150 cycles)	20024904	130 million	~26
	High Output v2.5 (150 cycles)	20024907	400 million	~80
NextSeq 1000/2000	P2 (100 cycles) v3	20046811	400 million	~80
	P2 (200 cycles) v3	20046812		
NovaSeq 6000	SP v1.5 100-cycle	20028401	650 million	~130
NextSeq 2000	P3 100 cycles	20040559	1 billion	~220
	P3 200 cycles	20040560		

## Appendix 2: Primer Selection Guide

- \* Each CUT&RUN library must be assigned a unique pair of i5 & i7 primers, included with each kit. Primers are used to add dual barcodes to libraries during indexing PCR.
- \* The **Primer Selection Guide** will help ensure i5 & i7 indexes are properly balanced for successful multiplexed sequencing on Illumina® sequencing systems.
- \* It is recommended to multiplex at least five CUT&RUN libraries to ensure adequate i5 & i7 index sequence diversity.
- \* Index (barcode) sequences are available in an easy-to-copy Excel spreadsheet at [epicypher.com/14-1001](http://epicypher.com/14-1001), under Documents and Resources.

To enable multiplexed sequencing on Illumina® systems, this kit uses a **combinatorial dual indexing primer strategy**, meaning that each CUT&RUN library is prepared with a distinct combination of two 8 bp barcodes, or indexes – one at the 5' end (the i5 index), and the second at the 3' end (the i7 index; see [Figure 7](#)).

Dual i5 & i7 indexes, as well as the respective P5 and P7 sequences required by Illumina® flow cells, are added to libraries during the **Indexing PCR (Section IV)**. Each kit comes with eight (o) i5 primers and six (•) i7 primers, which can be mixed and matched in various combinations to generate 48 unique pairs of dual barcodes.

This approach allows up to 48 libraries to be multiplexed in a single sequencing run. Note that EpiCypher 14-1001 comes with i7 primers i701-i706, while EpiCypher 14-1002 comes with i7 primers i707-i712 ([Figure 8](#)). Combining the two kits increases the number of distinct dual barcode pairs to 96, maximizing flexibility for large-scale projects.



**FIGURE 7**

CUTANA™ CUT&RUN Libraries are prepared using a combinatorial dual-indexing primer strategy. Each library is prepared using unique pairs of i5 & i7 primers to add indexes and P5/P7 sequences.

## INDEXING PRIMER ORGANIZATION

Sequencing runs must contain libraries with a sufficient mixture of i5 and i7 indexes to ensure proper diversity for Illumina® systems. To enable proper selection of primer pairs, i5 primers and i7 primers are grouped into **Sequential Primer Sets**, or **i5 Sets** and **i7 Sets** for brevity.

- Each Sequential Primer Set comprises an odd-numbered primer and the subsequent even-numbered primer. Sets are denoted by dashed lines in Figure 8.
- For example: i701 & i702 are an i7 Set. However, i702 & i703 are NOT an i7 Set because they begin with an even-numbered i7 primer.

**At least one i5 Set and one i7 Set must be represented in each sequencing run.** This requires multiplexing a minimum of two libraries (blue wells in Figure 8), although five is recommended.

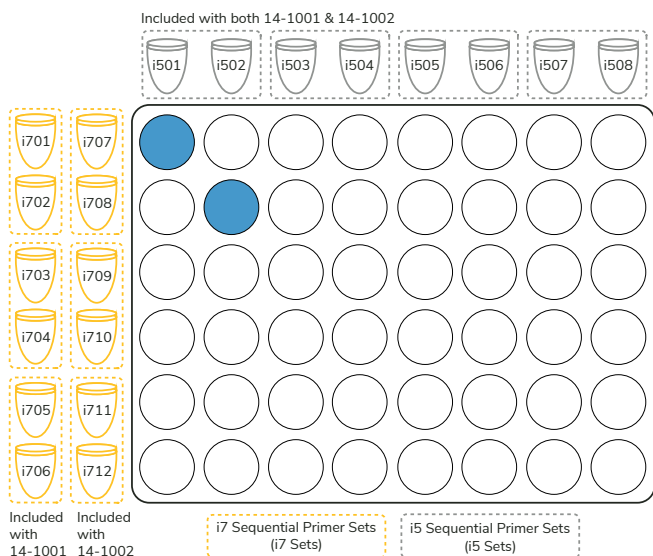


FIGURE 8

i5 & i7 primers are organized to guide primer pair selection and ensure successful multiplexed sequencing. Dashed lines indicate i5 Sets (grey) and i7 Sets (orange). Tubes colors reflect i5 & i7 primer tube caps. Blue wells illustrate the selection of two primer pairs for multiplexing that incorporate a full i5 Set and a full i7 Set, ensuring adequate sequencing diversity for Illumina® systems.

## SELECTION OF i5 & i7 PRIMER PAIRS FOR MULTIPLEXING:

### TIPS FOR SUCCESSFUL MULTIPLEXING:

- \* Ensure a complete i5 Set and a complete i7 Set are included in each sequencing run.
- \* Multiplex at least five CUT&RUN libraries to ensure proper index sequence diversity across Illumina® sequencing platforms. Index sequences are available at [epicypher.com/14-1001](https://epicypher.com/14-1001).
- \* Follow Tips in Section IV (and described below) to avoid primer cross-contamination.

1. Before first use, transfer entire volume of (o) **i501-i508 primers** to new **8-strip tubes**. This prevents cross-contamination and enables multi-channel pipetting.

2. Determine the number of CUT&RUN libraries to be multiplexed.

Example: Sequencing Run 1 in [Figure 9](#) contains 11 pooled libraries.

3. Select i7 Sets. At least complete one i7 Set (orange dashed lines in [Figure 9](#)) must be included in each sequencing run.

Example: For Run 1, we select the i701 & i702 Set.

- \* It is recommended to work through all possible i5 primer combinations with a given i7 Set before using another i7 Set.

4. Select i5 primers to pair with the chosen i7 Set(s). At least one complete i5 Set (grey dashed lines in [Figure 9](#)) must be included in each sequencing run.

5. Assign i5 & i7 primer pairs to reactions. Assign primer pairs in a manner that simplifies the workflow and allows multi-channel pipetting.

Example: Reactions 1-6 are assigned i701 & paired with i501-i506. Reactions 7-11 are assigned i702 & paired with i501-i505 ([Figure 9](#)).

6. Add primers to indexing PCR reactions (**Section IV**). Visually inspect tips to confirm that the correct volume was aspirated and change tips between each addition to prevent cross-contamination.

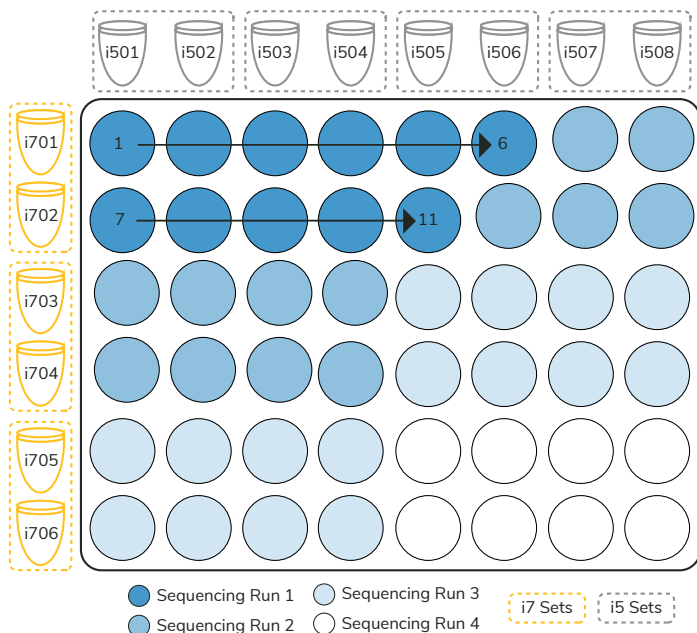
Add 1 µL assigned (•) **i7 primer** to each reaction.

Add 1 µL assigned (o) **i5 primer** to each reaction.

7. If (•) i7 primer contamination is a concern, discard tube caps and replace with fresh caps. Six spare i7 primer tube caps (•) come with each kit.

8. Continue indexing PCR setup in **Protocol: Section IV**. Mark the selected i5 & i7 primer pairs as consumed on the **Protocol Quick-Start Card**.





**FIGURE 9**

Four sequencing runs using appropriate i5 & i7 primer pairs for successful dual-indexing\*. Each run contains at least one i5 Sequential Primer Set (grey dashed lines) and one i7 Sequential Primer Set (orange dashed lines). Tubes colors reflect i5 & i7 primer tube caps.

Sequencing Run 1 is used as an example in the instructions on the opposite page.

Reactions 1 through 6 use i701 paired with i501 to i506.

Reactions 7 through 11 use i702 paired with i501 to i505.

\* Primers for kit 14-1001 are shown. However, the same rules apply for i5 & i7 primers included with kit 14-1002.

## Appendix 3: Frequently Asked Questions (FAQs)

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### 1. What is the recommended DNA input for CUTANA™ CUT&RUN Library Prep?

The validated input range for the kit is 10 to 0.5 ng (10,000 to 500 pg) CUT&RUN-enriched DNA, although 5 ng provides the optimal balance between low DNA input and good library quality. The metrics used to determine the input range for CUT&RUN library prep were library complexity (i.e. read duplication rate), adapter dimer formation, and sequencing data quality (i.e. signal-to-noise, observation of expected peak structures).

These features are shown in [Figures 10 & 3](#), in which varying amounts of H3K27me3 CUT&RUN DNA were used for library prep. Inputs from 10 to 0.5 ng generated consistent and robust yields following PCR ([Figure 10A](#)), with low read duplication rates and minimal adapter dimer contamination ([Figure 10B-D](#)). TapeStation analysis confirmed enrichment of mononucleosome-sized fragments ([Figure 10D-E](#)), and sequencing data showed high signal, low background, and expected peaks ([Figure 3](#)).

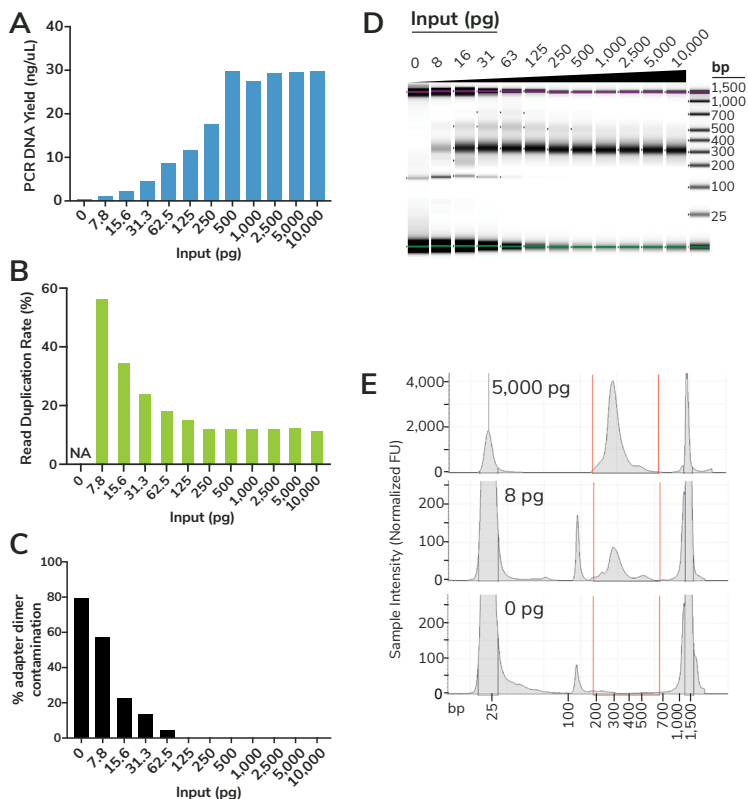
### 2. How important is it to use equal inputs across reactions for library prep?

The amount of DNA used for CUT&RUN library prep is crucial to ensure high-quality sequencing data and should be prioritized over normalizing inputs. In fact, normalizing library inputs to the least-concentrated sample often puts CUT&RUN users at a disadvantage due to the low yields from CUT&RUN (vs. ChIP-seq). These points are illustrated in [Figure 10](#): DNA inputs from 10 to 0.5 ng (10,000 to 500 pg) produced similar yields and high-quality libraries, while inputs below 0.5 ng had lower yields, elevated adapter dimers, and reduced read diversity.

### 3. Should I size select or fragment my CUT&RUN DNA before library prep?

Shearing and/or size selection of CUT&RUN-enriched DNA is **NOT** required nor recommended for several reasons:

- The output of CUT&RUN is mainly mononucleosome-sized DNA fragments, making it unnecessary to shear DNA.
- CUT&RUN DNA yields are very low (vs. ChIP). Additional shearing and size selection risks loss of target-enriched DNA.
- The library prep PCR parameters specifically amplify adapter-ligated fragments from 200-700 bp, eliminating larger DNA fragments from the library.
- Large fragments generated by CUT&RUN and observed in final libraries are typically nonspecific background. Shearing DNA at earlier steps would include them in library prep, diluting on-target signal and increasing background.



**FIGURE 10**

Quality control analysis of the CUTANA CUT&RUN Library Prep Kit workflow. **(A)** Library yields using decreasing amounts of H3K27me3-enriched CUT&RUN DNA as input. **(B)** Sequence read duplication rate determined using Picard. Read duplication rates <20% tend to be of biological origin, but rates >20% indicate poor library complexity. **(C)** The percent of adapter dimers (~150 bp peak) relative to the total library signal for varying input DNA amounts, as quantified by TapeStation analysis. **(D-E)** TapeStation analysis of libraries presented in **(A-C)**. **(D)** Gel representation of the DNA fragment size distribution for H3K27me3 CUT&RUN libraries. **(E)** Signal traces for representative libraries at the recommended starting input (5,000 pg), ultra-low input (8 pg), and no input DNA. Mononucleosomes are visible at ~300 bp and adapter dimers at ~150 bp in both the gel image **(D)** and signal traces **(E)**. Orange lines indicate the 200-700 bp range, which is used to determine the library concentration for sequencing.

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#### 4. What should I do if my CUT&RUN yields are below 5 ng?

If the CUT&RUN experiment yields less than 5 ng DNA, it is recommended to use the total volume of CUT&RUN-enriched DNA for library prep. Low CUT&RUN DNA yields are common for low abundance targets (e.g. H3K4me3), but also depend on the number of starting cells, cell type, quality of sample prep, and antibody binding specificity and efficiency. See the CUTANA™ CUT&RUN Kit Manual for details ([epicypher.com/14-1048](http://epicypher.com/14-1048)).

#### 5. Can I use less than 0.5 ng CUT&RUN-enriched DNA for library prep? What caveats and modifications should I consider?

Although library prep inputs below 0.5 ng can generate useful data, these libraries often have low yields with elevated adapter dimers, reduced read diversity, and low signal over background, all of which impact sequencing quality (Figure 10). Follow these tips to optimize library prep and sequencing for ultra-low input CUT&RUN assays:

- Remove adapter dimers (see **FAQs 10-11**), which take up valuable sequencing bandwidth.
- Increase the number of cycles used for indexing PCR (**Protocol: Section IV**), which will improve library yields for fragment distribution analysis and enable pooling at standard concentrations for sequencing.
- Increase sequencing depth. Additional PCR cycles may increase read duplication rates, requiring deeper sequencing to capture read diversity.
- Filter out duplicate reads from fastq.gz files using Picard (<http://broadinstitute.github.io/picard>).

#### 6. Can I use this kit to prep CUT&RUN libraries for transcription factors?

This kit produces nucleosome-level resolution data and has been successfully applied for transcription factor motif analysis (e.g. HOMER). Additional protocol modifications are not recommended.

## 7. How can positive and negative controls be used to validate library prep workflows?

It is recommended to include positive and negative control reactions in every CUT&RUN experiment. EpiCypher H3K4me3 (EpiCypher 13-0060), H3K27me3 (13-0055), and IgG (13-0042) antibodies are validated for robust performance in CUT&RUN assays. If libraries for control reactions show enrichment of ~300 bp fragments and yield 100-200 nM (for 5 ng input DNA; see [Figure 6](#)), the workflow is validated.

## 8. What yields are typical using this kit? What is required for Illumina® sequencing?

See **Section VI-VII**. Note that EpiCypher does **NOT** recommend using library yields to determine assay success, as yields can vary by cell type, number of cells, target abundance, and antibody quality.

The typical yield for a purified CUT&RUN sequencing library using 5 ng input DNA and following the PCR parameters in this manual is ~300-500 ng (~30-50 ng/μL in a 10.5 μL volume; [Figure 10A](#)). Library molarity values are typically 100-200 nM, but good sequencing data can be obtained down to 0.5 nM. EpiCypher routinely uses 1-4 nM of each library for pooling, and then dilutes to the desired concentration for loading onto the sequencer. See [support.illumina.com](http://support.illumina.com) for dilution guidelines for Illumina® sequencing platforms.

## 9. What should I do if my library yields are low (less than 0.5 nM)?

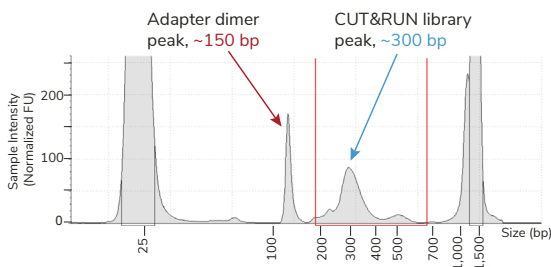
Low library yields are unavoidable in some experiments (e.g. mapping a low abundance target and/or using low cell numbers). For low-concentration libraries, consider the following options:

- **Repeat library prep:** Use more CUT&RUN-enriched DNA input and/or increase the number of PCR cycles used for indexing PCR (**Protocol: Section IV**) to improve yields for sequencing. Note that increasing the number of PCR cycles may lead to higher read duplication rates and require deeper sequencing to capture read diversity.
- **Concentrate library for sequencing:** If library prep cannot be repeated, use a Speedvac to increase the library concentration and add as much of the library as possible to the sequencing pool. Deeper sequencing is recommended to ensure sufficient read depth for the low-concentration library.
- **In both cases, deeper sequencing is strongly recommended.**

## 10. What are adapter dimers? How can I avoid them?

Adapter dimers result from the self-ligation of sequencing adapters and are preferentially amplified due to their small size (~150 bp; see [Figure 11](#)). High levels of adapter dimers occupy valuable sequencing bandwidth and should be avoided. Factors that influence adapter dimer formation and solutions offered by the CUT&RUN Library Prep Kit include:

Potential causes of adapter dimers	Kit solutions
<p><b>⚠ Low input DNA (e.g. <a href="#">Figure 11</a>)</b></p> <p>Combining low input DNA with standard adapter concentrations (designed for higher yield assays; e.g. ChIP-seq) increases adapter-self interaction and dimer formation.</p>	<ul style="list-style-type: none"><li>• Adapter concentrations and ligation steps are optimized for minimal adapter dimers down to 0.5 ng DNA.</li></ul>
<p><b>⚠ Inefficient homogenization of adapter ligation reaction</b></p> <p>Ligation reactions <b>MUST</b> be assembled, mixed, and kept on ice until ligation begins. If reactions and reagents are not kept at 4°C during ligation setup, adapter dimers are more likely to form.</p>	<ul style="list-style-type: none"><li>• Keep adapter ligation reagents and reactions on ice during ligation setup.</li><li>• Remove adapter dimers comprising &gt;5% of a library (FAQ 11).</li></ul>
<p><b>⚠ Excess SPRIselect reagent</b></p> <p>A high ratio of beads:DNA increases retention of short adapter dimer fragments in library prep.</p>	<ul style="list-style-type: none"><li>• SPRIselect reagent ratios are optimized for minimal adapter dimer contamination using 10 to 0.5 ng input DNA.</li></ul>



**FIGURE 11**

Ultra-low DNA inputs increase risk of adapter dimer formation in CUT&RUN sequencing libraries. An H3K27me3 CUT&RUN library was prepared using 8 pg enriched DNA (K562 cells). The TapeStation trace shows an adapter dimer peak (~150 bp peak; red arrow) and library peak (~300 bp, blue arrow). Orange lines denote 200-700 bp, used to determine library concentration.

### 11. What should I do if I have excessive adapter dimers in my library prep?

If more than 5% of the CUT&RUN library is adapter dimers, perform library cleanup using the CUTANA™ Quick Cleanup DNA Purification Kit (EpiCypher 14-0052; [epicypher.com/14-0052](http://epicypher.com/14-0052)). This bead-based kit includes EpiCypher's library cleanup protocol to selectively remove DNA fragments smaller than 180 bp. Our strategy avoids the use of gel-extraction and columns, which streamlines CUT&RUN workflows and maximizes recovery. Note that library cleanup is performed on the entire multiplexed library pool (from **Protocol: Section VII**) rather than individual libraries, further minimizing the risk of library loss. Following library pool cleanup, reassess concentration and fragment distribution and proceed to sequencing.

### 12. Where are index/barcode sequences?

i5 and i7 index sequences are available at [epicypher.com/14-1001](http://epicypher.com/14-1001). See the Excel Spreadsheet under Documents and Resources. This spreadsheet contains index sequences for both 14-1001 & 14-1002.

### 13. How do I analyze CUT&RUN sequencing data?

CUT&RUN analysis methods are similar to those used for ChIP-seq datasets, with key differences. Our most up-to-date recommendations are available at [support.epicypher.com](http://support.epicypher.com). Briefly:

- Align raw reads to a reference genome using Bowtie 2<sup>6</sup>. The Integrative Genomics Viewer (IGV)<sup>7</sup> and/or deepTools<sup>8</sup> can be used to visualize enrichment (e.g. bigWig files graphed over a genome browser).
- For peak calling, EpiCypher frequently uses MACS2<sup>9</sup> and SICER<sup>10</sup>, programs for ChIP-seq that work well for CUT&RUN<sup>11</sup>. SICER can be adjusted for analysis of sharp enrichment peaks (e.g. H3K4me3) vs. broad areas of enrichment (e.g. H3K27me3)<sup>12</sup>. Other options include SEACR<sup>13</sup>, a peak caller designed for CUT&RUN data, and the CUT&RUNTools 2.0 pipeline, which is designed for CUT&RUN and CUT&Tag data, including analysis of single cells<sup>14</sup>. It is recommended to test several programs and select the one that faithfully represents the target of interest.
- To determine signal over background, EpiCypher uses bedTools to calculate fractions of reads in peaks (FRiP) and compare FRiP scores from experimental samples vs. controls<sup>15</sup>. Other tools can be applied for differential analysis and heatmap generation (e.g. DESeq2<sup>16</sup>, deepTools<sup>8</sup>).

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#### 14. Should I use a PhiX spike-in control when sequencing CUT&RUN libraries?

PhiX spike-in control is used to improve diversity for low-complexity libraries (i.e. avoid color saturation during sequencing) and to monitor sequencing run quality. It is added to pooled libraries prior to loading onto the sequencer.

EpiCypher does not include PhiX spike-in for CUT&RUN sequencing, as genomic DNA is of sufficient complexity to avoid saturation, and we rely on other metrics that don't sacrifice read depth to validate sequencing runs (e.g. positive and negative control reactions; SNAP-CUTANA™ K-MetStat Panel, EpiCypher 19-1002; *E. coli* Spike-in DNA, EpiCypher 18-1401).

For guidance on using PhiX controls, see [support.illumina.com](https://support.illumina.com).

#### 15. Can I use indexing primers from this library prep kit for CUT&Tag assays?

The primers included in this kit should **NOT** be used for PCR amplification of CUT&Tag DNA. The adapter sequences used in the CUTANA CUT&RUN Library Prep Kit are distinct from those in used CUT&Tag. Thus, the i5 & i7 primers in this kit will not amplify Tn5 tagmented DNA.

For help with CUT&Tag library prep, including validated indexing primer sequences, see the CUTANA™ CUT&Tag Kit Manual at [epicypher.com/14-1102](https://epicypher.com/14-1102) and our CUT&Tag Protocol at [epicypher.com/protocols](https://epicypher.com/protocols).



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## 24 Hour Emergency Phone Number:

US & Canada: 1-800-535-5053

International: 1-352-323-3500

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### ***EpiCypher, Inc.***

6 Davis Dr. Ste 755

Durham, NC 27709 USA

**[www.epicypher.com](http://www.epicypher.com)**

Ph: 1-855-374-2461    F: 1-855-420-6111

Email: [info@epicypher.com](mailto:info@epicypher.com)    Tech Support: [techsupport@epicypher.com](mailto:techsupport@epicypher.com)