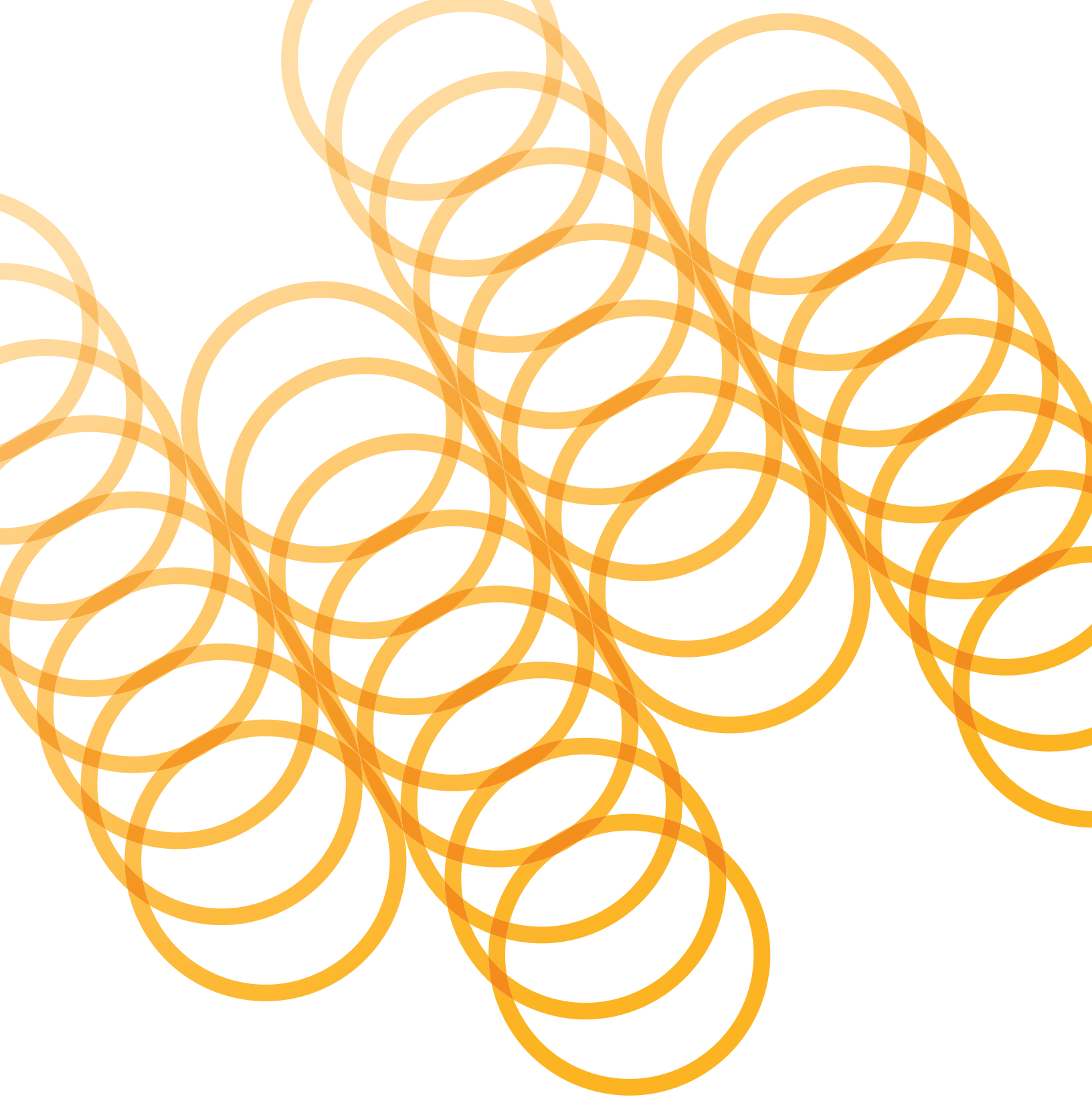

EpiCypher®
Bringing Epigenetics to Life

CUTANA™

CUT&RUN Library Prep Kit Version 1

User Manual Version 1.6

Powered by NEBNext® from New England Biolabs®

Kit Version 1
User Manual Version 1.6

EpiCypher®

CUTANA™

CUT&RUN Library Prep Kit

Catalog No. 14-1001 & 14-1002
48 Reactions

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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24 Hour Emergency Phone Number:
US & Canada: 1-800-535-5053 | International: 1-352-323-3500

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See EpiCypher's Tech Support Center at support.epicypher.com for library prep FAQs and troubleshooting guidance.

Background

Cleavage **U**nder **T**argets & **R**elease **U**sing **N**uclease (CUT&RUN) is an innovative chromatin mapping approach that builds on recent advances in immunotethering technology[1-2]. 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)[3]. 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.

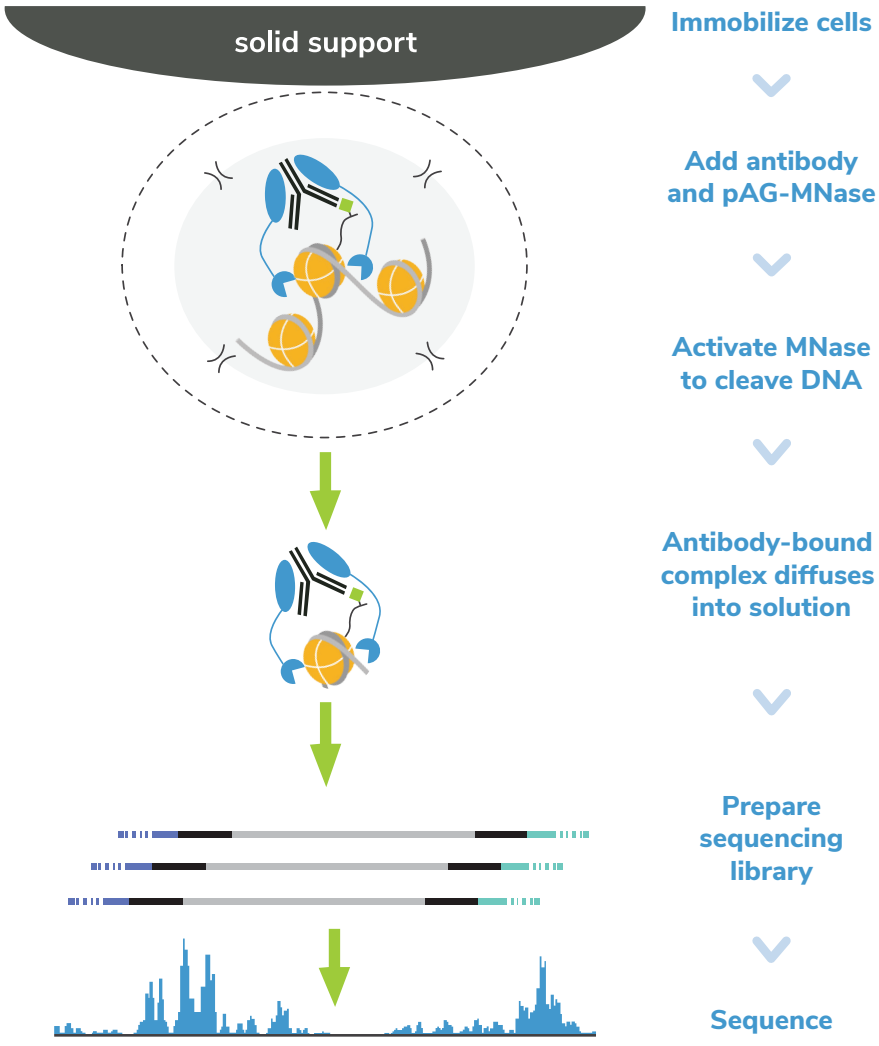


FIGURE 1
Overview of the CUTANA™ CUT&RUN workflow.

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) for increased throughput, improved reliability, and reduced assay costs.
- A **Tech Support Center** (support.epicypher.com) with detailed FAQs and troubleshooting guidance.
- Effective for preparing CUT&RUN DNA libraries for both histone PTMs and chromatin-associated proteins, including transcription factors (Figure 2).

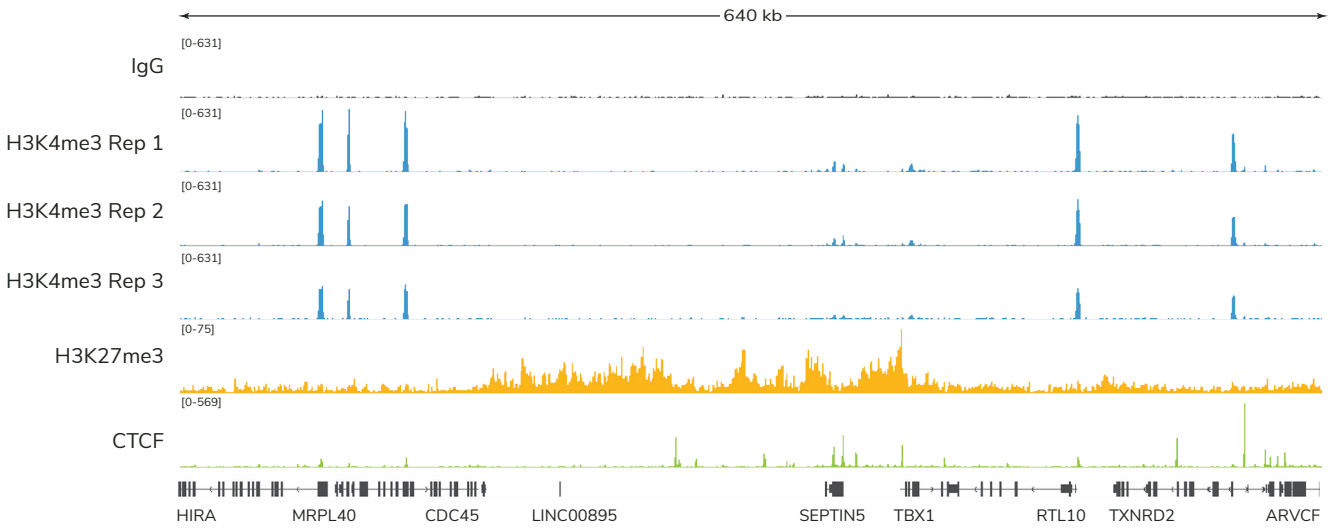


FIGURE 2

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 pooled in a single run. The two versions of this kit (14-1001 and 14-1002) contain distinct primer sets, allowing up to 96 reactions to be multiplexed when kits are used together.

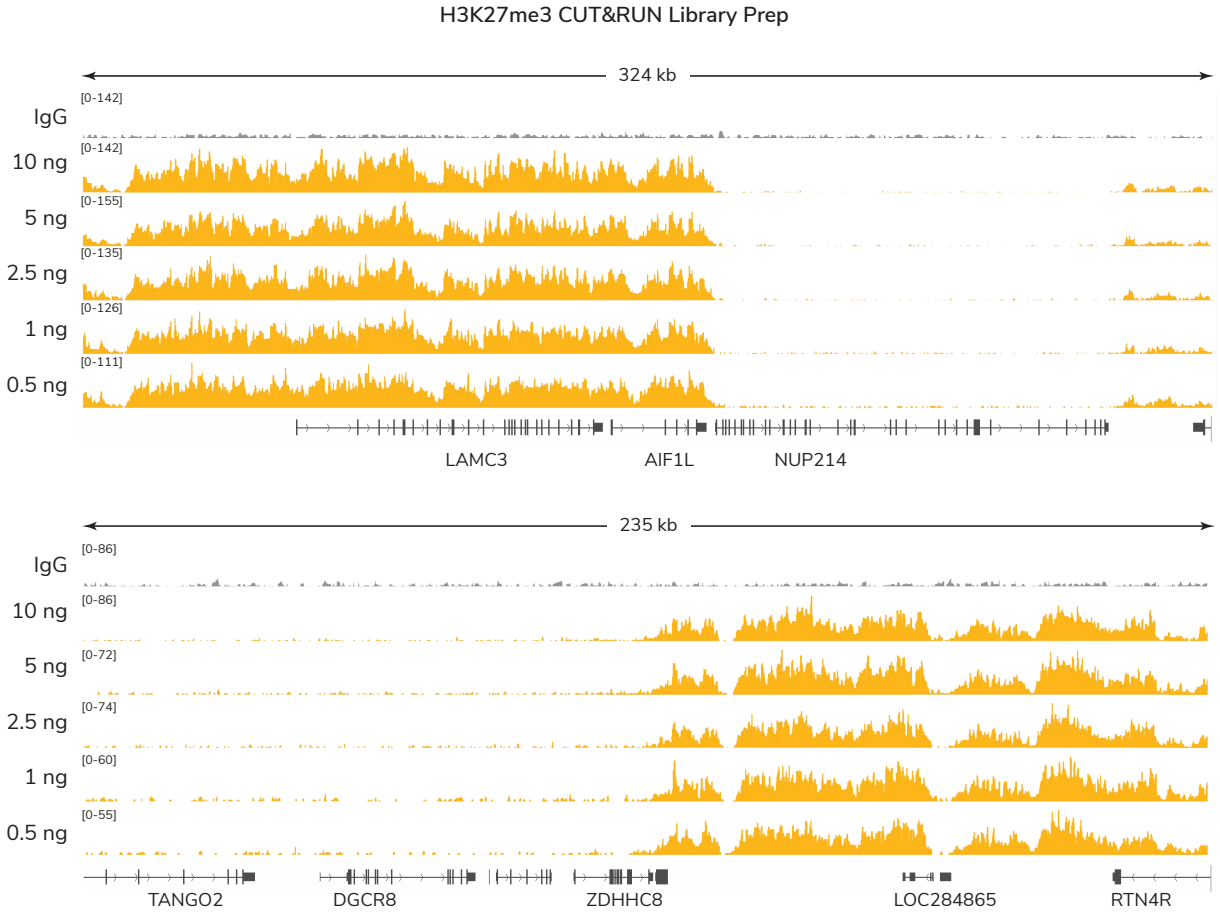


FIGURE 3

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

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 is end-repaired to create blunt ends. 5' ends are phosphorylated, and 3' ends are dA-tailed to generate a small overhang, enabling directional adpter ligation.

Step 2: Adapter Ligation and Uracil-Specific Excision

Adapters with a hairpin structure are ligated to DNA, promoting efficient ligation and reducing adapter dimers. The hairpin is removed via uracil-specific digestion, followed by DNA purification.

Step 3: Indexing PCR and Sequencing Library Purification

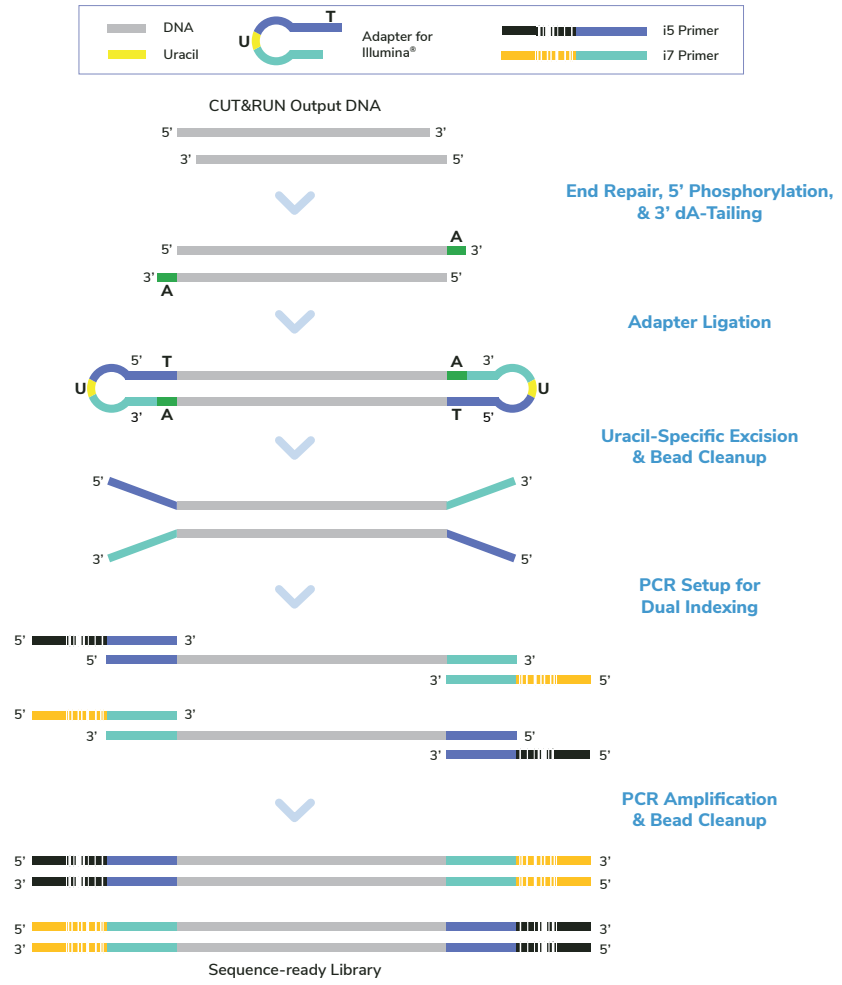
Libraries are amplified with a unique pair of i5 & i7 primers, each incorporating a distinct barcode (or index) and Illumina® P5/P7 sequences. This combinatorial dual indexing approach allows multiplexing of up to 48 libraries.

Step 4: Analysis of Library Quality and Next-Generation Sequencing

Purified libraries are quantified and examined for proper fragment size distibution. Indexed libraries are diluted, pooled, and sequenced on an appropriate Illumina® platform.

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

Kit components are stable for 6 months upon date of receipt. Store as outlined below.

Store at room temperature (RT) upon receipt:

Item	Cat. No.	Notes before use
8-strip Tubes	10-0009p	Enables use of multi-channel pipettors.
FluidX Caps	10-0015p	Fresh caps for i7 Primers to avoid cross-contamination.
CUTANA™ DNA Purification Beads	21-1407p	**DO NOT FREEZE.** Reagent is slightly viscous. Thoroughly mix and pipette carefully to ensure correct volume is 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	Cat. No.	Notes before use
(●) End Prep Enzyme	15-1019p	**SMALL VOLUME: quick spin before use.** For end repair of purified CUT&RUN DNA.
(●) End Prep Buffer	21-1012p	**NOTE:** Buffer may precipitate. If observed: bring to RT, mix until dissolved. Return to ice.
(●) Adapter for Illumina®	18-1000p	**SMALL VOLUME: quick spin before use.** 1.5 µM adapter for Illumina® sequencing.
(●) Ligation Mix	15-1020p	**NOTE:** Buffer is highly viscous, may precipitate. Thoroughly mix and pipette carefully to ensure correct volume.
(●) Ligation Enhancer	15-1021p	**SMALL VOLUME: quick spin before use**.
(●) U-Excision Enzyme	15-1023p	**SMALL VOLUME: quick spin before use**
(●) Hot Start 2X PCR Master Mix	15-1022p	For PCR amplification and indexing of CUT&RUN libraries.
(○) i5 Primers, 10 µM i501-i508	18-1001 to 18-1008	**SMALL VOLUME: quick spin before use.** Transfer to 8-strip tubes before first use.
Kit 14-1001 (●) i7 Primers, 10 µM i701-i706	18-1101 to 18-1106	**SMALL VOLUME: quick spin before use.** 14-1001 and 14-1002 contain distinct i7 primers. Each kit generates 48 unique pairs of barcodes; combined, they generate up to 96 uniquely barcoded libraries. ***NOTE**: Download i5 and i7 index sequences at epicypher.com/14-1001 under Documents and Resources
Kit 14-1002 (●) i7 Primers, 10 µM i707-i712	18-1107 to 18-1112	

Materials Required But Not Supplied

REAGENTS:

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

EQUIPMENT:

- Low-retention filter pipette tips
- Magnetic separation rack for 8-strip tubes (EpiCypher 10-0008)
- 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)
- Qubit™ 4 Fluorometer (Invitrogen Q33238 or previous version) and 1X dsDNA High Sensitivity Kit (Invitrogen Q33230)
- Capillary electrophoresis machine and required reagents, e.g. Agilent TapeStation® with D1000 ScreenTape (5067-5582) and D1000 reagents (5067-5583) or Agilent Bioanalyzer® with High Sensitivity DNA Kit (5067-4626)

Notes Before Starting

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. Below are EpiCypher's general recommendations for success:

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.
- 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. In these cases, we recommend increasing the number of indexing PCR cycles and increasing sequencing depth; read this article for more information.

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.
- This kit uses a dual indexing strategy. Each library must contain a unique pair of i5 & i7 barcodes, or indexes, to ensure successful multiplex sequencing. Indexes can be selected using indexoligo.neb.com. Select "Dual Index" from the Multiplex Oligo dropdown, and "E7600S Dual Index Set 1" from the Oligo Set dropdown. Select your sequencing platform and plexity, and use the tool to generate dual indexes compatible with the experiment.
- Paired-end sequencing (2 x 50 bp) is recommended for CUT&RUN. 3-8 million uniquely aligned reads provides adequate coverage for most targets.

See EpiCypher's Tech Support Center at support.epicypher.com for library prep FAQs and troubleshooting guidance.

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**. For CUT&RUN reactions with yields below 5 ng DNA, use as much DNA as possible for library prep.
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 (•) **End Prep Buffer** x N reactions = _____ µL

   1.8 µL (•) **End Prep Enzyme** x N reactions = _____ µL
4. Gently vortex **End Repair Master Mix**. Quick spin in a benchtop centrifuge and place on 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)

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

14. For each reaction, make 1 mL 85% Ethanol (EtOH) by combining 850 µL 100% EtOH and 150 µL 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 µL 85% EtOH per reaction.
15. Vortex **DNA Purification Beads** thoroughly to resuspend. Slowly add 47.75 µL **DNA Purification Beads** (1X volume) to each reaction in 8-strip tubes. Ensure pipette tip is free of extra bead droplets before dispensing to reactions.
16. Mix well by vortexing and/or pipetting. Quick spin tubes. Incubate 5 min at RT.
17. 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.
18. Keep tubes on magnet. Add 180 µL **85% EtOH** directly onto beads. Carefully pipette to remove supernatant. Repeat one time for a total of two washes.
19. Remove tubes from magnet and quick spin with caps facing in to avoid dislodging beads. Return to magnet and pipette to remove residual EtOH.
20. 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.
21. Add 12 µL **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.
22. Place tubes on magnet for 2 min at RT. Transfer 10.5 µL 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. Assign a unique pair of **i5 and i7 Primers** to each reaction. Indexes can be selected using indexoligo.neb.com. Select "Dual Index" from the Multiplex Oligo dropdown, and "E7600S Dual Index Set 1" from the Oligo Set dropdown. Select your sequencing platform and plexity, and use the tool to generate dual indexes compatible with the experiment. Index sequences are also available in Excel format at epicypher.com/14-1001, under Resources.
24. To each 10.5 µL CUT&RUN DNA sample (from Step 22), 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)

* Overdrying 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 µL 85% Ethanol (EtOH) by combining 425 µL 100% EtOH and 75 µL 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 **DNA Purification Beads** thoroughly to resuspend. Slowly add 25 µL **DNA Purification Beads** (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 µL **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 µL **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 µL eluted CUT&RUN sequencing libraries to new **8-strip tubes**.

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

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

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).
* 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 (Figure 7).
* Libraries ≥1 nM allow pooling at standard concentrations for sequencing, but good data are obtained down to 0.5 nM.
* See the Tech Support Center for questions regarding yields, adapter dimers, and other issues.

36. Use 1 µL purified CUT&RUN library for quantification. Use the Qubit fluorometer with the 1X dsDNA HS Assay Kit.
37. For each library, prepare 5 µL at 10 ng/µL 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 µL diluted sequencing library using the High Sensitivity DNA Kit (Bioanalyzer) or the D1000 ScreenTape System & Reagents (TapeStation).
39. The final traces should show predominant enrichment of mononucleosome-sized fragments, such as those yielded by H3K4me3, H3K27me3, and CTCF antibodies in Figure 7 on the next page (~300 bp: ~170 bp + sequencing adapters). Adapter dimers, if present, would be observed as a peak at ~150 bp (Figure 6). Read this article for an in-depth discussion on adapter dimers.

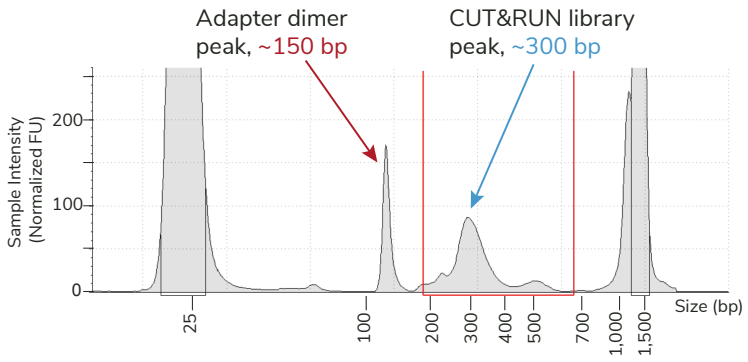


FIGURE 6

Adapter dimers observed in an H3K27me3 CUT&RUN sequencing library. The TapeStation trace shows an adapter dimer peak at ~150 bp (red arrow) and a mononucleosome-sized library peak at ~300 bp (blue arrow). 8 picograms of CUT&RUN-enriched DNA was used for library prep, highlighting the risk of adapter dimers with low library prep inputs.

EXAMPLE FRAGMENT DISTRIBUTION ANALYSIS OF CUT&RUN SEQUENCING LIBRARIES

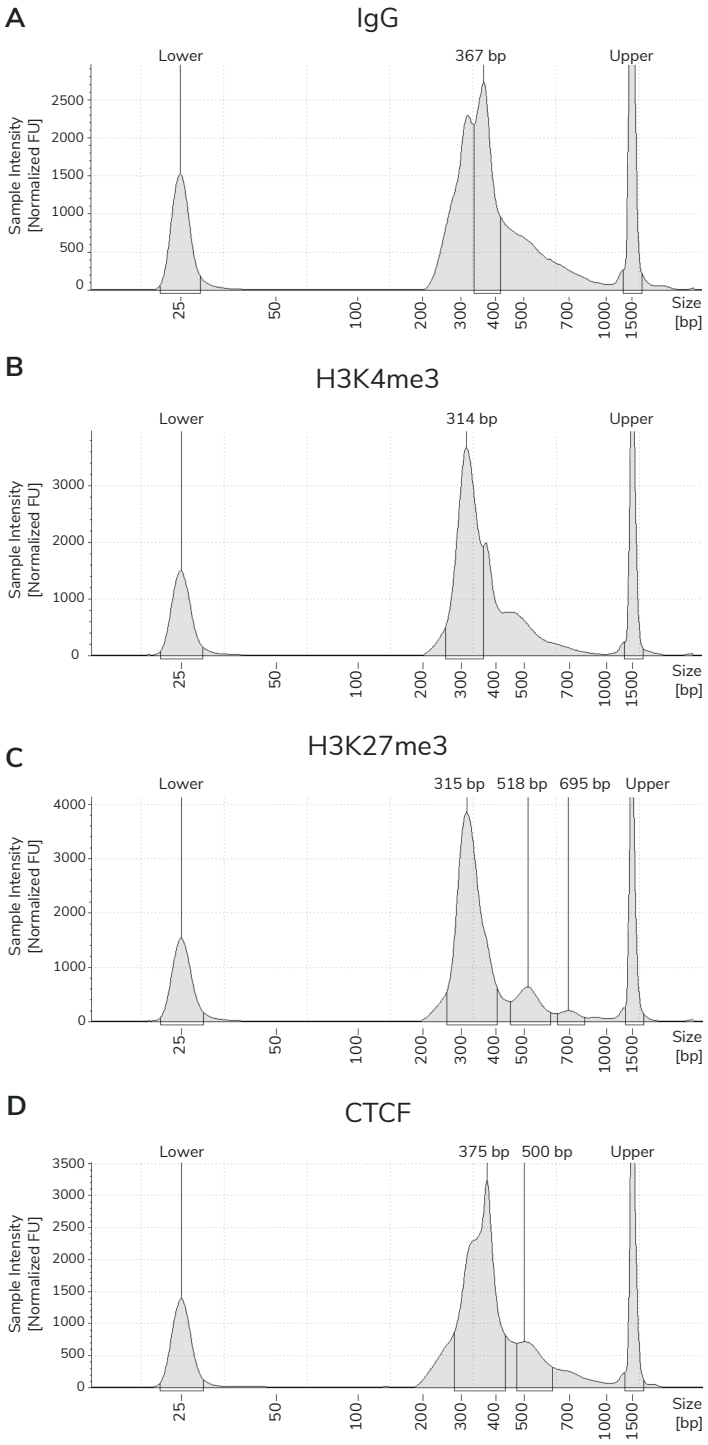


FIGURE 7

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.
* 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 to is generate 3-5 million **unique** reads per library.
* If sequencing low-concentration libraries, follow this guidance on our Tech Support Center.

41. Pool libraries at desired ratios using the molarity calculations from **Section VI** (200-700 bp region). Follow guidelines from specific Illumina® kit to load onto sequencer (support.illumina.com). 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, following guidelines for your kit.
    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 under Documents and Resources.
42. For guidance with CUT&RUN sequencing analysis, see this article on the Tech Support Center. The Tech Support Center also has articles about success metrics when using the CUTANA™ CUT&RUN Kit or Protocol.

Appendix 1: Primer Selection Guide

* Index sequences are available in an easy-to-copy spreadsheet at epicypher.com/14-1001 under Documents & Resources.

This kit uses a combinatorial dual indexing primer strategy. Each CUT&RUN library is prepared with a distinct combination of two 8 bp barcodes, or indexes — one at the 5' end (i5 index), and the second at the 3' end (i7 index). Each kit comes with eight (○) i5 Primers and six (●) i7 Primers, which can be mixed and matched in various combinations to generate 48 unique pairs of dual barcodes.

Note that Kit 14-1001 contains i701-i706, while 14-1002 comes with i707-i712 (Figure 8). Combining the two kits increases the number of unique dual index pairs to 96, maximizing flexibility for large-scale projects.

Figure 8 illustrates proper primer organization to facilitate successful i5 and i7 Primer pair selection and pipetting. Do **NOT** repeat pairs of i5 and i7 Primers in a sequencing run. If an experiment will be combined with others on a single lane or flow cell, ensure that there is no overlap of primer pairs.

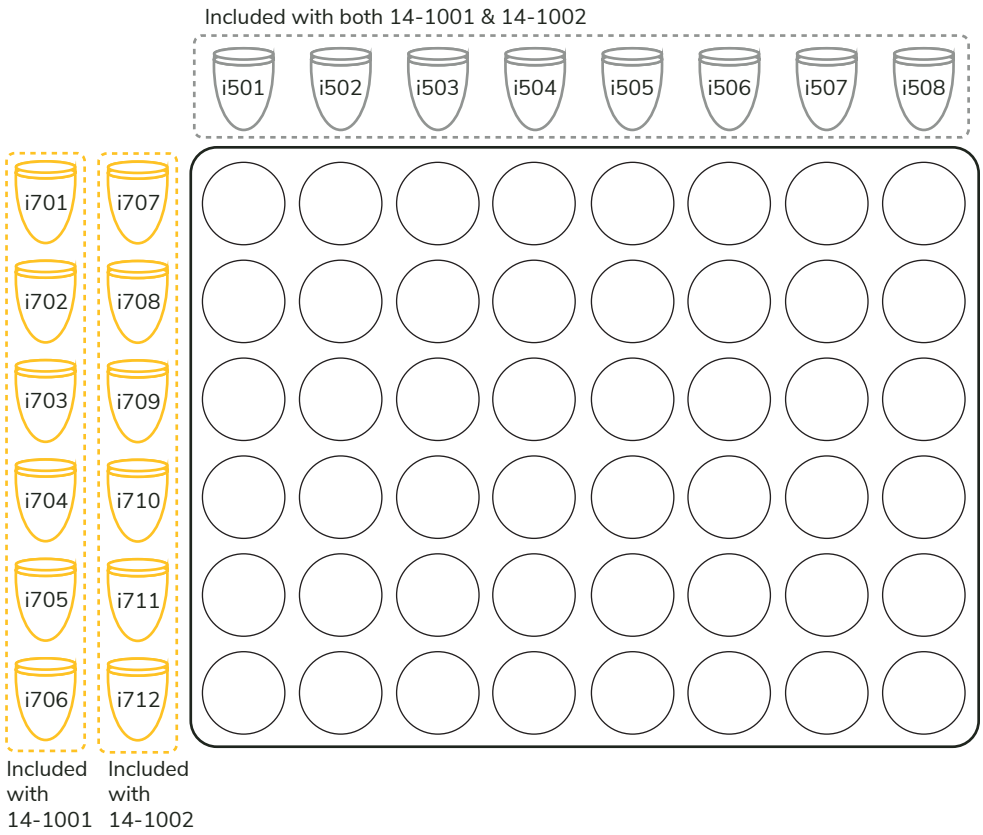


FIGURE 8

i5 and i7 Primers are organized to guide primer pair selection for successful multiplexed sequencing.

Appendix 1: Primer Selection Guide

SELECTION OF I5 AND I7 PRIMER PAIRS

Assign a unique pair of **i5 and i7 Primers** to each reaction using indexoligo.neb.com. Briefly:

- Visit using indexoligo.neb.com.
- Select **Dual Index** from the NEBNext® Multiplex Oligo dropdown menu.
- Select your **sequencing machine** from the Sequencer dropdown menu.
- Select the **number of libraries** you are sequencing under the Plexity dropdown menu.
- Select **E7600S Dual Index Set 1** from the Oligo Set dropdown menu.
- Select primer pairs manually by checking boxes, and the tool will show you the compatibility with your machine and plexity. Alternatively, click **Auto Fill** and the tool will generate ideal dual indexes that are compatible with your sequencing machine and multiplexing needs.
- The tool allows you to download a sheet of selected multiplexing indexes, if desired. Index sequences are also available in Excel format at epicypher.com/14-1001, under Resources.

References

1. Skene & Henikoff. An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites. ***eLife*** 6 (2017).
2. Schmid et *al.* ChIC and ChEC; genomic mapping of chromatin proteins. ***Mol Cell*** 16, 147-157 (2004).
3. Skene et *al.* Targeted *in situ* genome-wide profiling with high efficiency for low cell numbers. **Nat *Prot*** 13, 1006-1019 (2018).

Revision History

Kit Manual Version #	Date	Notes
1.6	6.06.2025	• Updated DNA purification bead reagent name. • Updated i5 & i7 Primer Selection Strategy. • Moved FAQs to Tech Support Center: support.epicypher.com.