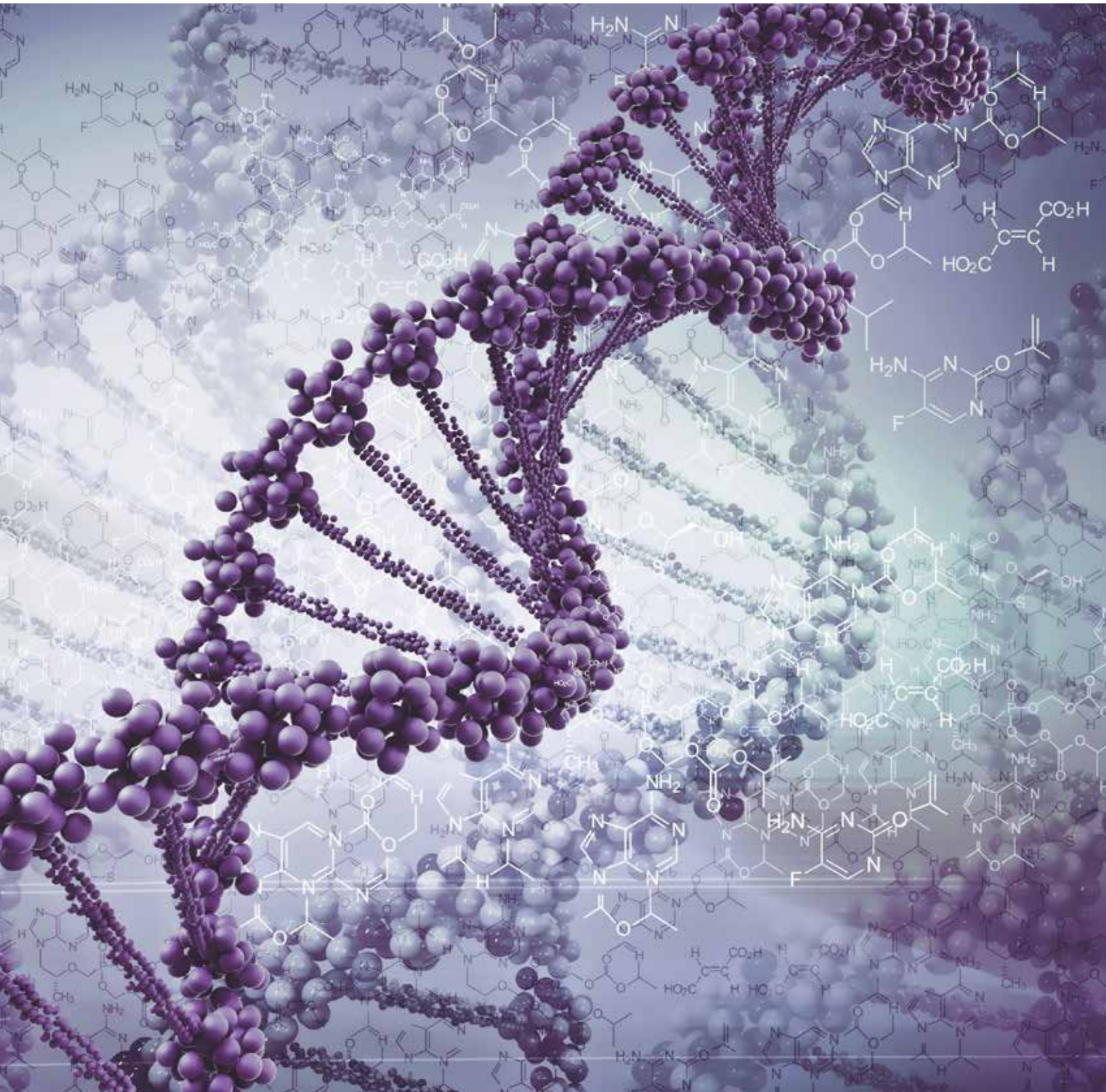
DNA SEQUENCING METHODS COLLECTION

An overview of recent DNA-seq publications featuring Illumina® technology



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INTRODUCTION

This publication is a collection of next-generation sequencing (NGS) methods for DNA sequencing, compiled from the scientific literature. It is both a tribute to the creativity of the users and the versatility of the technology. We hope it will inspire researchers to use these methods or to develop new ones to address new scientific challenges.

A method refers to the processing steps between extracting the nucleic acids (sample preparation) and the addition of oligonucleotide adapters for sequencing (library preparation). With a few extra processing steps, a wide range of scientific questions can be addressed by this technology.

When using this document, consider the following points:

- New methods are continually being developed. For the most up-to-date list of methods, visit: www.illumina.com/science/sequencing-method-explorer.html
- Only the most recent back references are provided, typically for the past 2 years.
- With few exceptions the capitalization, dashes and special characters in the method name are exactly the same as in the original, referenced paper. This is important, because methods such as CapSeq[1] and CAP-seq[2] are quite different and refer to RNA and DNA methods, respectively.
- The methods are arranged according to their similarity, so the most similar methods should be adjacent. To visually compare the methods, refer to http://www.illumina.com/content/dam/illumina-marketing/documents/applications/ngs-library-prep/ForAllYouSeqMethods.pdf
- When methods are essentially identical, and can be represented by a single diagram, they are grouped together, such as GRO-seq[3] and BRIC-Seq.[4]
- The diagrams are stylized depictions of the methods. They may not include every detail of the method.
- These methods were developed by users, so readers should refer to the original publications for detailed descriptions and protocols.

Have we missed anything? Contact us if you are aware of a protocol that should be listed.

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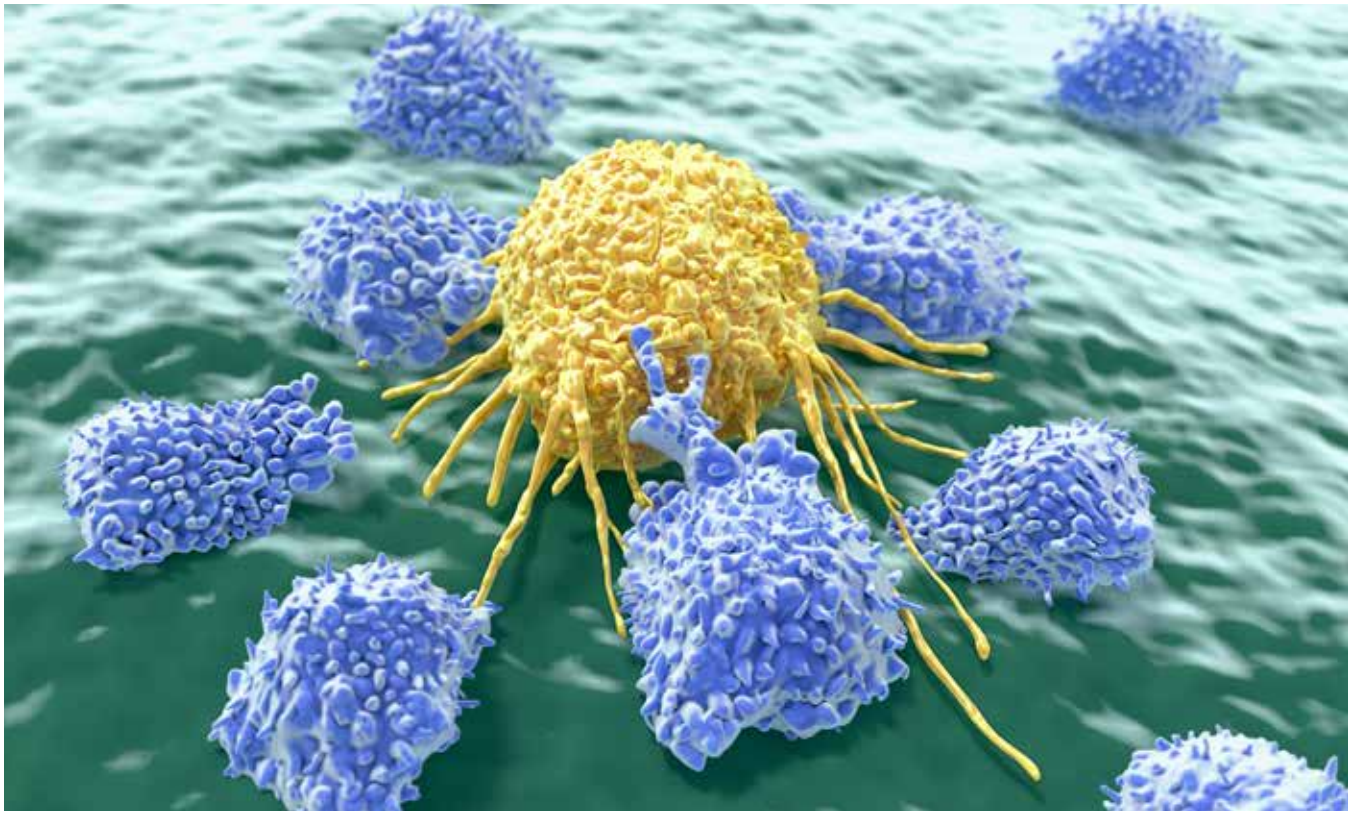
SEQUENCE REARRANGEMENTS

A growing body of evidence suggests that somatic genomic rearrangements, such as retrotransposition and copy-number variants (CNVs), are relatively common in healthy individuals.[5,6,7] Cancer genomes also contain numerous complex rearrangements.[8] While many of these rearrangements can be detected during routine NGS, specific methods are available to study rearrangements such as transposable elements.

Transposable genetic elements (TEs) comprise a vast array of DNA sequences with the ability to move to new sites in genomes, either directly by a cut-and-paste mechanism (transposons) or indirectly through an RNA intermediate (retrotransposons).[9] TEs make up approximately 66–69% of the human genome[10] and play roles in ageing, cancers, brain function, development, embryogenesis, and phenotypic variation in populations and evolution.[11] Along with sequence rearrangements by TEs, chromosome and centromere rearrangements can lead to multiple diseases and disorders.[12]



Genomic rearrangements in somatic cells can lead to the metastasis of cancerous cells.

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RAD and PE RAD-Seq: Restriction-Site Associated DNA Sequencing

RAD-seq is a protocol for genotyping and discovery of single-nucleotide polymorphisms (SNPs).[13] This approach is particularly useful for genotyping when a reference genome is not available, such as in ecological studies.[14] PE RAD-seq, also called RAD-PE, is the same protocol as RAD but uses paired-end sequencing for improved alignments.[15] Several variations, such as ddRADseq,[16] 2b-RAD,[17] SLAF-seq,[18] and hyRAD[19] have been developed to address specific applications, and multiple software packages are available to analyze RAD data.[20,21]

In this method, genomic DNA (gDNA) is first digested with a restriction enzyme and a barcoded P1 adapter is ligated to the fragments. The adapter-ligated fragments from different samples are combined, if samples are multiplexed, and the DNA is sheared. The fragments are size-selected and purified. The P2 adapter-primers are ligated and the fragments are amplified to produce the sequencing library.

A schematic overview of RAD-seq.

Advantages	Disadvantages
• No reference genome required [22] • Relatively inexpensive, compared to whole-genome sequencing • The degree of genome coverage can be adjusted by selecting various restriction enzymes and fragment sizes	• There can be gaps in the genome coverage • Requires high-quality DNA (see hyRAD[23] for low-quality DNA) • Sequence polymorphism at the DNA restriction sites causes a progressive loss of shared restriction sites among diverging clades [24]

13. Baird N. A., Etter P. D., Atwood T. S., et al. Rapid SNP discovery and genetic mapping using sequenced RAD markers. *PLoS One*. 2008;3:e3376.
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The authors used RAD-seq to construct a high-resolution linkage map of the abalone Haliotis diversicolor for growth-related quantitative trait locus (QTL) analysis. They were able to build a reduced-representation library with 3756 loci in more than 95% of the offspring. Based on this map, they identified 15 QTLs for 6 growth-related traits.

Illumina Technology: HiSeq 2000 System

Wang J., Xue D. X., Zhang B. D., et al. Genome-Wide SNP Discovery, Genotyping and Their Preliminary Applications for Population Genetic Inference in Spotted Sea Bass (Lateolabrax maculatus). *PLoS One*. 2016;11:e0157809.

The researchers used PE RAD-seq on 30 individuals from 2 populations to discover 22,648 SNPs across the genome of L. maculatus. The results showed shallow, but significant, genetic differentiation between the 2 populations.

Illumina Technology: HiSeq 2500 System

He T., D'Agui H., Lim S. L., Enright N. J. and Luo Y. Evolutionary potential and adaptation of Banksia attenuata (Proteaceae) to climate and fire regime in southwestern Australia, a global biodiversity hotspot. *Sci Rep*. 2016;6:26315.

This study applied RAD-seq and environmental association analysis to 80 plants and found candidate genes associated with rainfall gradients, temperatures, and fire intervals. The authors discovered that overall population adaptive genetic variation was affected significantly by shortened fire intervals, whereas declining rainfall and rising temperature did not have a detectable influence. Gene annotation further revealed 4 genes with functions in stress tolerance, the regulation of stomatal opening and closure, energy use, and morphogenesis with adaptation to climate and fire intervals.

Illumina Technology: HiSeq 2000 System

Paun O., Turner B., Trucchi E., et al. Processes Driving the Adaptive Radiation of a Tropical Tree (Diospyros, Ebenaceae) in New Caledonia, a Biodiversity Hotspot. *Syst Biol.* 2016;65:212-227.

The authors used RAD-seq to resolve phylogenetic relationships among 21 diploid Diospyros species that radiated recently in New Caledonia. The dataset contained 84 individuals from 39 populations. The 8400 filtered SNPs generally confirmed species delimitations and produced a well-supported phylogenetic tree.

Illumina Technology: HiSeq 2000

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Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

ddRADseq: Double Digest Restriction-Site Associated DNA Marker Generation

ddRADseq,[25] also called ddRAD, is a variation on the RAD sequencing protocol,[26] which is used for SNP discovery and genotyping.[27,28] In this variation, the fragment shearing is replaced with a second restriction digestion to improve the tunability and accuracy of the size-selection step. The protocol also includes a second index to allow combinatorial indexing. Several RAD variations, such as 2b-RAD,[29] SLAF-seq,[30] and hyRAD,[31] have been developed to address specific applications, and multiple software packages are available to analyze RAD data.[32,33]

In this method, gDNA is first digested with a restriction enzyme, and a barcoded P1 adapter is ligated to the fragments. The adapter-ligated fragments from different samples are combined, if samples are multiplexed, and the DNA is digested by a second restriction enzyme. The fragments are size-selected and purified. The P2 adapter-primers are ligated, and the fragments are amplified to produce the sequencing library.

A schematic overview of ddRADseq.

Advantages	Disadvantages
• No reference genome required [34] • Relatively inexpensive, compared to whole-genome sequencing • The degree of genome coverage can be adjusted by selecting various restriction enzymes	• There can be gaps in the genome coverage • Requires high-quality DNA (see hyRAD[35] for low-quality DNA)

Reviews

Andrews K. R., Good J. M., Miller M. R., Luikart G. and Hohenlohe P. A. Harnessing the power of RADseq for ecological and evolutionary genomics. *Nat Rev Genet*. 2016;17:81-92.

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DaCosta J. M. and Sorenson M. D. ddRAD-seq phylogenetics based on nucleotide, indel, and presence-absence polymorphisms: Analyses of two avian genera with contrasting histories. *Mol Phylogenet Evol.* 2016;94:122-135.

This study used ddRADseq for phylogenetic analyses of the Lagonosticta firefinches (family Estrildidae) and the Vidua brood parasitic finches (family Viduidae). The researchers obtained > 1000 homologous loci despite ~20 million years divergence. In addition to nucleotide polymorphisms, the ddRADseq data yielded large sets of indel and locus presence–absence polymorphisms, all of which had higher consistency indices than mitochondrial DNA (mtDNA) sequence data.

Illumina Technology: HiSeq 2000 System

Lal M. M., Southgate P. C., Jerry D. R. and Zenger K. R. Fishing for divergence in a sea of connectivity: The utility of ddRADseq genotyping in a marine invertebrate, the black-lip pearl oyster Pinctada margaritifera. *Mar Genomics.* 2016;25:57-68.

The authors applied ddRADseq to Pinctada margaritifera and detected 5243 high-quality, genome-wide SNP markers. They were able to assess population structure, genome diversity, and perform association testing in 156 individuals belonging to 3 wild populations and 1 hatchery-produced population from the Fiji Islands. They also found shallow, but significant, population structure among the wild populations.

Illumina Technology: HiSeq 2000 System

Brown J. K., Taggart J. B., Bekaert M., et al. Mapping the sex determination locus in the hapuku (Polyprion oxygeneios) using ddRAD sequencing. *BMC Genomics.* 2016;17:448.

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Wu Z., Wang B., Chen X., et al. Evaluation of Linkage Disequilibrium Pattern and Association Study on Seed Oil Content in Brassica napus Using ddRAD Sequencing. *PLoS One.* 2016;11:e0146383.

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Cai G., Yang Q., Yi B., et al. A bi-filtering method for processing single nucleotide polymorphism array data improves the quality of genetic map and accuracy of quantitative trait locus mapping in doubled haploid populations of polyploid Brassica napus. *BMC Genomics.* 2015;16:409.

Davik J., Sargent D. J., Brurberg M. B., et al. A ddRAD Based Linkage Map of the Cultivated Strawberry, Fragaria xananassa. *PLoS One.* 2015;10:e0137746.

Leache A. D., Banbury B. L., Felsenstein J., de Oca A. N. and Stamatakis A. Short Tree, Long Tree, Right Tree, Wrong Tree: New Acquisition Bias Corrections for Inferring SNP Phylogenies. *Syst Biol.* 2015;64:1032-1047.

Leache A. D., Chavez A. S., Jones L. N., et al. Phylogenomics of phrynosomatid lizards: conflicting signals from sequence capture versus restriction site associated DNA sequencing. *Genome Biol Evol.* 2015;7:706-719.

Meik J. M., Streicher J. W., Lawing A. M., Flores-Villela O. and Fujita M. K. Limitations of climatic data for inferring species boundaries: insights from speckled rattlesnakes. *PLoS One.* 2015;10:e0131435.

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

2b-RAD: RAD With Type IIB Restriction Endonucleases

2b-RAD is similar to ddRadseq but uses type IIB restriction enzymes (BsaXI or AlfI), which will cleave upstream and downstream of a recognition site. This shears the target genome into a large number of DNA fragments with a constant length of 33 bp (BsaXI) or 36 bp (AlfI). These short DNA fragments can be sequenced to determine genetic variants.

In this method, gDNA is first digested with a restriction enzyme (BsaXI), and adapters with partial (NNN) overhangs are ligated to the fragments. The adapter-ligated fragments from different samples are combined, and the fragments are amplified to produce the sequencing library.

A schematic overview of 2b-RAD.

Advantages	Disadvantages
• Highly reduced 2b-RAD libraries require much less sequencing for accurate genotyping • High density of markers • No interim purification steps, reducing losses and processing time	• Requires a reference genome • Short tags may not be long enough for efficient locus discrimination in complex genomes

Reviews

Andrews K. R., Good J. M., Miller M. R., Luikart G. and Hohenlohe P. A. Harnessing the power of RADseq for ecological and evolutionary genomics. *Nat Rev Genet*. 2016;.

da Fonseca R. R., Albrechtsen A., Themudo G. E., Ramos-Madrigal J., Sibbesen J. A., et al. Next-generation biology: Sequencing and data analysis approaches for non-model organisms. *Mar Genomics*. 2016;30:3-13.

Jiang N., Zhang F., Wu J., Chen Y., Hu X., et al. A highly robust and optimized sequence-based approach for genetic polymorphism discovery and genotyping in large plant populations. *Theor Appl Genet.* 2016;129:1739-1757.

Kagale S., Koh C., Clarke W. E., Bollina V., Parkin I. A., et al. Analysis of Genotyping-by-Sequencing (GBS) Data. *Methods Mol Biol*. 2016;1374:269-284.

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Pecoraro C., Babbucci M., Villamor A., et al. Methodological assessment of 2b-RAD genotyping technique for population structure inferences in yellowfin tuna (Thunnus albacares). *Mar Genomics*. 2016;25:43-48.

The global population genetic structure of yellowfin tuna (Thunnus albacares) is poorly understood. The authors used 2b-RAD to show shallow but significant population structure among oceans (fixation index (FST) = 0.0273; P < 0.01). Discriminant analysis of principal components supported the presence of genetically discrete yellowfin tuna populations among 3 oceanic pools.

Illumina Technology: HiSeq 2500 System

Fu B., Liu H., Yu X. and Tong J. A high-density genetic map and growth related QTL mapping in bighead carp (Hypophthalmichthys nobilis). *Sci Rep.* 2016;6:28679.

The authors used 2b-RAD sequencing of 117 individuals in an F1 family to construct a map with 3121 SNP markers. Based on this genetic map, they identified 1 genome-wide significant and 37 suggestive QTLs for 5 growth-related traits in 6 linkage groups (LG3, LG11, LG15, LG18, LG19, LG22).

Illumina Technology: HiSeq 2500 System

Associated Kits

TruSeq DNA PCR-Free Library Prep Kit

SLAF-seq: Specific Locus Amplified Fragment Sequencing

SLAF-seq is an optimized version of ddRADseq, specifically intended for large-scale genotyping experiments.[36]

The enzymes and the sizes of the restriction fragments are optimized with training data to ensure even distribution and avoid repeats. The fragments are also selected over a tight range, to optimize PCR amplification. The protocol is similar to ddRAD, with a first digestion with MseI, heat inactivation, and a second digestion with AluI. The resulting fragments are PCR-amplified, adapters are added, and the fragments are purified to produce the sequencing library.

A schematic overview of SLAF-seq.

Advantages	Disadvantages
• Deep sequencing for genotyping accuracy • Reduced-representation strategy to reduce sequencing costs • Predesigned reduced-representation scheme to optimize marker efficiency • Double barcode system for large populations	• Does not cover the whole genome

Reviews

Andrews K. R., Good J. M., Miller M. R., Luikart G. and Hohenlohe P. A. Harnessing the power of RADseq for ecological and evolutionary genomics. *Nat Rev Genet.* 2016;.

References

Zhao X., Huang L., Zhang X., et al. Construction of high-density genetic linkage map and identification of flowering-time QTLs in orchardgrass using SSRs and SLAF-seq. *Sci Rep.* 2016;6:29345.

The authors developed 447,177 markers based on SLAF-seq and used them to perform a comparative genomics analysis between perennial ryegrass and orchardgrass. They found 11 potentially significant QTLs for 2 target traits—heading date (HD) and flowering time (FT).

Illumina Technology: HiSeq 2500 System

Geng X., Jiang C., Yang J., et al. Rapid Identification of Candidate Genes for Seed Weight Using the SLAF-Seq Method in Brassica napus. *PLoS One.* 2016;11:e0147580.

The researchers used SLAF-seq with association analysis and bulked segregant analysis to identify candidate genes in 1000-seed weight (TSW) data. They found a total of 1933 high-quality polymorphic SLAF markers and 4 TSW-associated markers.

Illumina Technology: HiSeq 2500

Zhang J., Yuan H., Li M., et al. A High-Density Genetic Map of Tetraploid Salix matsudana Using Specific Length Amplified Fragment Sequencing (SLAF-seq). *PLoS One*. 2016;11:e0157777.

The authors created an intraspecific F1 hybrid population by crossing the salt-sensitive "Yanjiang" variety of an arbor tree species (Salix matsudana) as the female parent with the salt-tolerant "9901" variety as the male parent. They genotyped this population, along with its parents. Both the parents and offspring were tetraploid, but the authors were able to construct a genetic map with 6737 SLAF markers. Their data will be used to map quantitative trait loci that modulate salt tolerance and resistance in Salix.

Illumina Technology: HiSeq 2500 System

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Xu X., Chao J., Cheng X., et al. Mapping of a Novel Race Specific Resistance Gene to Phytophthora Root Rot of Pepper (Capsicum annuum) Using Bulked Segregant Analysis Combined with Specific Length Amplified Fragment Sequencing Strategy. *PLoS One. 2*016;11:e0151401.

Ye Y., Cai M., Ju Y., et al. Identification and Validation of SNP Markers Linked to Dwarf Traits Using SLAF-Seq Technology in Lagerstroemia. *PLoS One.* 2016;11:e0158970.

Zhang H., Yi H., Wu M., et al. Mapping the Flavor Contributing Traits on "Fengwei Melon" (Cucumis melo L.) Chromosomes Using Parent Resequencing and Super Bulked-Segregant Analysis. *PLoS One.* 2016;11:e0148150.

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Ma J. Q., Huang L., Ma C. L., et al. Large-Scale SNP Discovery and Genotyping for Constructing a High-Density Genetic Map of Tea Plant Using Specific-Locus Amplified Fragment Sequencing (SLAF-seq). *PLoS One.* 2015;10:e0128798.

Qin D., Dong J., Xu F., et al. Characterization and fine mapping of a novel barley Stage Green-Revertible Albino Gene (HvSGRA) by Bulked Segregant Analysis based on SSR assay and Specific Length Amplified Fragment Sequencing. *BMC Genomics.* 2015;16:838.

Shan T., Pang S., Li J., Li X. and Su L. Construction of a high-density genetic map and mapping of a sex-linked locus for the brown alga Undaria pinnatifida (Phaeophyceae) based on large scale marker development by specific length amplified fragment (SLAF) sequencing. *BMC Genomics.* 2015;16:902.

Wang J., Zhang K., Zhang X., et al. Construction of Commercial Sweet Cherry Linkage Maps and QTL Analysis for Trunk Diameter. *PLoS One.* 2015;10:e0141261.

Wang W., Zhang T., Zhang G., et al. Genome-wide association study of antibody level response to NDV and IBV in Jinghai yellow chicken based on SLAF-seq technology. *J Appl Genet.* 2015;56:365-373.

Xu F., Sun X., Chen Y., et al. Rapid identification of major QTLs associated with rice grain weight and their utilization. *PLoS One.* 2015;10:e0122206.

Xu X., Lu L., Zhu B., et al. QTL mapping of cucumber fruit flesh thickness by SLAF-seq. *Sci Rep.* 2015;5:15829.

Xu Y., Huang L., Ji D., et al. Construction of a dense genetic linkage map and mapping quantitative trait loci for economic traits of a doubled haploid population of Pyropia haitanensis (Bangiales, Rhodophyta). *BMC Plant Biol.* 2015;15:228.

Zhang J., Zhang Q., Cheng T., et al. High-density genetic map construction and identification of a locus controlling weeping trait in an ornamental woody plant (Prunus mume Sieb. et Zucc). *DNA Res.* 2015;22:183-191.

Zhang Y., Zhang J., Huang L., et al. A high-density genetic map for P genome of Agropyron Gaertn. based on specific-locus amplified fragment sequencing (SLAF-seq). *Planta*. 2015;242:1335-1347.

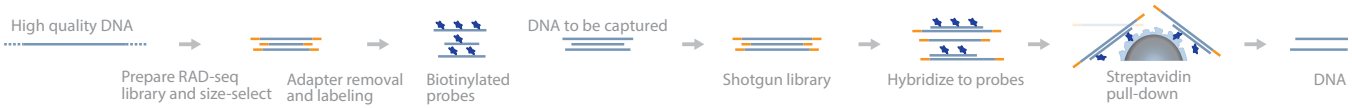
Associated Kits

TruSeq DNA PCR-Free Library Prep Kit

hyRAD: Hybridization RAD for Degraded DNA

hyRAD[37] was developed for use on degraded DNA samples, such as those from museum collections. Museum and preserved samples offer a rich source of valuable specimens, but their degraded DNA is unable to sustain the double-digestion and sample fractionation required by ddRAD.[38] To address this limitation, hyRAD uses biotinylated probes and streptavidin-covered beads to capture and enrich the fragments of interest.

The first step in the process is to generate a ddRAD library from high-quality DNA, usually from an extant specimen. The fragments are size-selected and biotinylated. They can now be used as probes for hybridization capture of shotgun or ddRad libraries.



A schematic overview of hyRAD.

Advantages	Disadvantages
• Can be used on degraded DNA • Can be used on unsequenced genomes	• Coverage not as complete as with high-quality samples

Reviews

Andrews K. R., Good J. M., Miller M. R., Luikart G. and Hohenlohe P. A. Harnessing the power of RADseq for ecological and evolutionary genomics. *Nat Rev Genet.* 2016;.

Holmes M. W., Hammond T. T., Wogan G. O., et al. Natural history collections as windows on evolutionary processes. *Mol Ecol.* 2016;25:864-881.

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Suchan T., Pitteloud C., Gerasimova N. S., Kostikova A., Schmid S., et al. Hybridization Capture Using RAD Probes (hyRAD), a New Tool for Performing Genomic Analyses on Collection Specimens. *PLoS One.* 2016;11:e0151651.

This study describes the hyRAD protocol and demonstrates its performance. The researchers obtained a large set of orthologous loci from fresh and museum samples from a non–model butterfly species. They discovered a high proportion of SNPs present in all 8 analyzed specimens, including 58-year-old museum samples.

Illumina Technology: MiSeq System, HiSeq System

Associated Kits

TruSeq DNA PCR-Free Library Prep Kit

37. Suchan T., Pitteloud C., Gerasimova N. S., et al. Hybridization Capture Using RAD Probes (hyRAD), a New Tool for Performing Genomic Analyses on Collection Specimens. *PLoS One*. 2016;11:e0151651.
38. Peterson B. K., Weber J. N., Kay E. H., Fisher H. S. and Hoekstra H. E. Double digest RADseq: an inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLoS One.* 2012;7:e37135.

Rapture: Restriction-Site Associated DNA Capture

Rapture is a massively parallel, targeted DNA sequencing technique that combines RAD-seq and sequence capture to compare multiple genes of interest among large numbers of samples. The targeted sequencing is based on identifying restriction enzyme sites specifically near the *loci* of interest.

gDNA samples are pooled into individual wells in plates and digested with selected restriction enzymes. Biotinylated RAD adapters with well-specific barcodes are ligated to the sticky ends before pooling all the wells from each plate. The barcoded DNA fragments are randomly sheared and bound to streptavidin beads. Again, using restriction enzymes, fragments are cleaved from the streptavidin beads and used in standard DNA library preparation kits with plate-specific barcode labels. Next, libraries from both plates are pooled and hybridized with biotinylated bait-oligos specific to each RAD tag, before a final streptavidin pull-down. The isolated DNA fragments are sequenced and arranged according to their RAD tags, plate barcodes, and well barcodes.

A schematic overview of Rapture.

Advantages	Disadvantages
• Massively parallel, targeted DNA sequencing for SNP identification • Improved number of mapped fragments and locus coverage compared to RAD-seq	• RAD tags needs to be designated prior to the experiment • Additional cost to synthesize baits • Only a small number of loci are interrogated • Biased toward sequences closer to the restriction cut site

Reviews

Andrews K. R., Good J. M., Miller M. R., Luikart G. and Hohenlohe P. A. Harnessing the power of RADseq for ecological and evolutionary genomics. *Nat Rev Genet.* 2016;.

References

Ali O. A., O'Rourke S. M., Amish S. J., Meek M. H., Luikart G., et al. RAD Capture (Rapture): Flexible and Efficient Sequence-Based Genotyping. *Genetics*. 2016;202:389-400.

This publication describes the original Rapture protocol. To test the method, the researchers analyzed fin clips of 96 rainbow trout individuals. The average coverage at the captured loci was 16x compared, to 0.4x for RAD. In a principal-component analysis, the first principal component separated 2 distinct groups corresponding to individuals born in Bear Creek and the spring-fed spawning locations (Thousands Springs and Spring Creek). The third component separated individuals from Thousand Springs and Spring Creek. These results represent a remarkably fine scale of spatial analysis.

Illumina Technology: HiSeq 2500 System

Associated Kits

TruSeq DNA PCR-Free Library Prep Kit

39. Ali O. A., O'Rourke S. M., Amish S. J., et al. RAD Capture (Rapture): Flexible and Efficient Sequence-Based Genotyping. *Genetics*. 2016;202:389-400.

Digenome-seq: Cas9-Digested Whole-Genome Sequencing

Digenome-seq was designed to profile genome-wide Cas9 off-target effects.[40] A multiplexed version has also been published.[41] It belongs to a family of methods, including HTGTS[42] LAM-HTGTS,[43] and Guide-seq,[44] which are aimed at detecting off-target effects of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 and other RNA-guided engineered nucleases (RGENs).

This method detects off-target mutations, induced by RGENs in a bulk population of cells, by sequencing *in vitro* nuclease-digested genomes (digenomes). These digests should produce many DNA fragments with identical 5' ends, which are vertically aligned at cleavage sites.

A schematic overview of Digenome-seq.

Advantages	Disadvantages
• Relies on DNA cleavage rather than binding • Performed in a genomic context and captures sites with a DNA/RNA bulge • Detects off-target effects with a frequency of 0.1% or lower [45]	• Requires *in vivo* cleavage confirmation46 • High skill requirement for bioinformatic analysis [46]

Reviews

Lee C. M., Cradick T. J., Fine E. J. and Bao G. Nuclease Target Site Selection for Maximizing On-target Activity and Minimizing Off-target Effects in Genome Editing. *Mol Ther.* 2016;24:475-487.

Mei Y., Wang Y., Chen H., Sun Z. S. and Ju X. D. Recent Progress in CRISPR/Cas9 Technology. *J Genet Genomics.* 2016;43:63-75.

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Kim D., Kim J., Hur J. K., et al. Genome-wide analysis reveals specificities of Cpf1 endonucleases in human cells. *Nat Biotechnol.* 2016;34:863-868.

To test whether Cpf1 endonucleases could replace Cas9 to improve the precision of gene editing, the authors used a mismatched CRISPR RNA (crRNA). They found that Cpf1 can tolerate single or double mismatches in the 3'-protospacer-adjacent motif (PAM) distal region, but not in the 5'-PAM proximal region. Next, they used Digenome-seq to perform a genome-wide analysis of cleavage sites for 8 Cpf1 nucleases. Of these, Lachnospiraceae bacterium Cpf1 (LbCpf1) and Acidaminococcus sp Cpf1 (AsCpf1) showed 6 and 12 cleavage sites in the human genome, respectively, while Cas9 nucleases generally cut over 90 sites. Most of the identified Cpf1 cleavage sites did not produce mutations in cells. The authors also found mismatches in either the 3'-PAM-distal region or in the PAM sequence of 12 off-target sites that were validated *in vivo*. The off-target effects were eliminated by using preassembled recombinant Cpf1 ribonucleoproteins.

Illumina Technology: HiSeq X Ten System, TruSeq DNA Library Prep Kit

Associated Kits

TruSeq DNA PCR-Free Library Prep Kit

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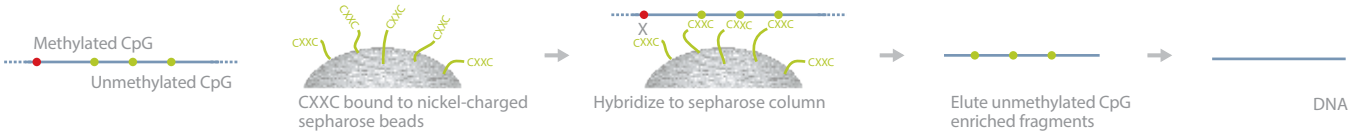
41. Kim D., Kim S., Kim S., Park J. and Kim J. S. Genome-wide target specificities of CRISPR-Cas9 nucleases revealed by multiplex Digenome-seq. *Genome Res.* 2016;26:406-415.
42. Chiarle R., Zhang Y., Frock R. L., et al. Genome-wide translocation sequencing reveals mechanisms of chromosome breaks and rearrangements in B cells. *Cell.* 2011;147:107-119.
43. Hu J., Meyers R. M., Dong J., Panchakshari R. A., Alt F. W. and Frock R. L. Detecting DNA double-stranded breaks in mammalian genomes by linear amplification-mediated high-throughput genome-wide translocation sequencing. *Nat Protoc*. 2016;11:853-871.
44. Kim D., Bae S., Park J., et al. Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nat Methods.* 2015;12:237-243, 231 p following 243.
45. Mei Y., Wang Y., Chen H., Sun Z. S. and Ju X. D. Recent Progress in CRISPR/Cas9 Technology. *J Genet Genomics*. 2016;43:63-75.
46. Hu J., Meyers R. M., Dong J., Panchakshari R. A., Alt F. W. and Frock R. L. Detecting DNA double-stranded breaks in mammalian genomes by linear amplification-mediated high-throughput genome-wide translocation sequencing. *Nat Protoc.* 2016;11:853-871.

CAP-seq: CXXC Affinity Purification Sequencing

CAP-seq maps methylated CpGs in gDNA.[47]

In this method, DNA is incubated with CXXC bound to nickel-charged sepharose beads. Next, DNA is eluted from the complex and sequenced. Deep sequencing provides insights into methylated CpG sites in gDNA.



A schematic overview of CAP-seq.

Advantages	Disadvantages
• Targets CpG islands	• None known

Reviews

Vilborg A., Passarelli M. C., Yario T. A., Tycowski K. T. and Steitz J. A. Widespread Inducible Transcription Downstream of Human Genes. Mol Cell. 2015;59:449-461.

Smallwood S. A., Lee H. J., Angermueller C., et al. Single-cell genome-wide bisulfite sequencing for assessing epigenetic heterogeneity. Nat Methods. 2014;11:817-820.

Suzuki M. M., Yoshinari A., Obara M., et al. Identical sets of methylated and nonmethylated genes in Ciona intestinalis sperm and muscle cells. Epigenetics Chromatin. 2013;6:38.

References

None available yet

Associated Kits

Nextera DNA Library Prep Kit

TruSeq Nano DNA Library Prep Kit

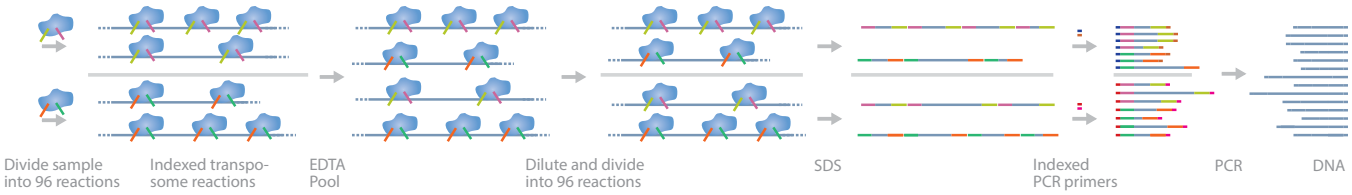
TruSeq DNA PCR-Free Library Prep Kit

47. Illingworth R. S., Gruenewald-Schneider U., Webb S., et al. Orphan CpG islands identify numerous conserved promoters in the mammalian genome. *PLoS Genet*. 2010;6:e1001134.

CPT-seq: Contiguity-Preserving Transposition Sequencing

CPT-seq is a method for genome-wide haplotyping based on contiguity-preserving transposition (CPT) and combinatorial indexing.[48]

Tn5 transposition is used to modify DNA with adapter and index sequences while preserving contiguity. After DNA dilution and compartmentalization, the transposase is removed, and the DNA is separated into individually indexed libraries. The libraries in each compartment are enriched for neighboring genomic elements and are further indexed via PCR. Combinatorial 96-plex indexing at both the transposition and PCR stage enables the construction of phased synthetic reads from each of the nearly 10,000 virtual compartments.



A schematic overview of CPT-seq.

Advantages	Disadvantages
• Highly indexed and efficient • FragScaff reported to be highly effective at scaffolding large genomes from CPT-seq data[49] • Large effective number of virtual compartments per physical compartment could avoid the amplification biases associated with multiple displacement amplification (MDA)[50]	• Not yet adopted widely by the scientific community

Reviews

Mostovoy Y., Levy-Sakin M., Lam J., et al. A hybrid approach for *de novo* human genome sequence assembly and phasing. *Nat Methods.* 2016;13:587-590.

Snyder M. W., Adey A., Kitzman J. O. and Shendure J. Haplotype-resolved genome sequencing: experimental methods and applications. *Nat Rev Genet.* 2015;16:344-358.

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Amini S., Pushkarev D., Christiansen L., Kostem E., Royce T., et al. Haplotype-resolved whole-genome sequencing by contiguity-preserving transposition and combinatorial indexing. *Nat Genet.* 2014;46:1343-1349.

This publication describes the original CPT-seq method. The authors demonstrate the feasibility of this method by assembling >95% of the heterozygous variants in a human genome into long, accurate haplotype blocks (N50 = 1.4–2.3 Mb).

Illumina Technology: HiSeq 2000 System

Associated Kits

Nextera DNA Library Prep Kit

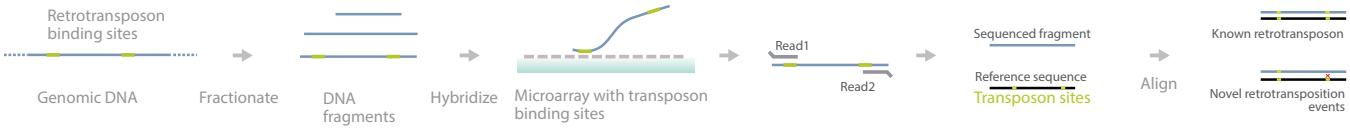
Nextera XT DNA Library Prep Kit

48. Kuleshov V., Snyder M. P. and Batzoglou S. Genome assembly from synthetic long read clouds. *Bioinformatics.* 2016;32:i216-i224.
49. Cao C.-c. and Sun X. Combinatorial pooled sequencing: experiment design and decoding. *Quantitative Biology*. 2016;4:36-46.
50. Amini S., Pushkarev D., Christiansen L., et al. Haplotype-resolved whole-genome sequencing by contiguity-preserving transposition and combinatorial indexing. *Nat Genet.* 2014;46:1343-1349.

RC-Seq: Retrotransposon Capture Sequencing

RC-seq is a high-throughput protocol to map and study retrotransposon insertions.[51]

In this method, after gDNA is fractionated, retrotransposon binding sites on DNA hybridize to transposon binding sites on a microarray. Deep sequencing provides accurate information that can be aligned to a reference sequence to discover novel retrotransposition events. A single-cell version (scRC-seq) has also been described.[52]



A schematic overview of RC-seq.

Advantages	Disadvantages
• Ability to clearly identify and detect novel retrotransposition events • Can specifically study transposon binding sites of interest • High-throughput protocol • PCR validation rate estimated at 98.5%	• Different types of mobile element insertions (MEIs) require separate PCR experiments with different primers[53] • Hybridization errors can lead to sequencing unwanted DNA fragments • PCR biases can underrepresent GC-rich templates • Similar transposition binding sites can lead to sequence ambiguity and detection for a transposition event

Reviews

Xing J., Witherspoon D. J. and Jorde L. B. Mobile element biology: new possibilities with high-throughput sequencing. *Trends Genet.* 2013;29:280-289.

References

Klawitter S., Fuchs N. V., Upton K. R., et al. Reprogramming triggers endogenous L1 and Alu retrotransposition in human induced pluripotent stem cells. *Nat Commun.* 2016;7:10286.

The researchers used RC-seq to map the genomic integration sites of de novo retrotransposon insertions in human induced pluripotent stem cells (hiPSCs). They wanted to determine if activation of the L1 mobilization machinery produced L1-mediated retrotransposition. They detected 40,608 non–reference retrotransposon insertions and estimated that each hiPSC carried ~1 *de novo* L1 insertion.

Illumina Technology: HiSeq 2000 System

Upton K. R., Gerhardt D. J., Jesuadian J. S., Richardson S. R., Sanchez-Luque F. J., et al. Ubiquitous L1 mosaicism in hippocampal neurons. *Cell*. 2015;161:228-239.

The authors performed scRC-seq on individual human hippocampal neurons and glia, as well as cortical neurons. They estimated that 13.7 somatic L1 insertions occurred per hippocampal neuron and carried the sequence hallmarks of target-primed reverse transcription (RT). This result indicates that L1 mosaicism is pervasive at genomic loci expressed in hippocampal neurons.

Illumina Technology: Unspecified Illumina sequencing system

Solyom S., Ewing A. D., Rahrmann E. P., et al. Extensive somatic L1 retrotransposition in colorectal tumors. *Genome Res*. 2012;22:2328-2338.

Shukla R., Upton K. R., Munoz-Lopez M., et al. Endogenous retrotransposition activates oncogenic pathways in hepatocellular carcinoma. *Cell*. 2013;153:101-111.

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

51. Baillie J. K., Barnett M. W., Upton K. R., et al. Somatic retrotransposition alters the genetic landscape of the human brain. *Nature*. 2011;479:534-537.
52. Upton K. R., Gerhardt D. J., Jesuadian J. S., et al. Ubiquitous L1 mosaicism in hippocampal neurons. *Cell*. 2015;161:228-239.
53. Xing J., Witherspoon D. J. and Jorde L. B. Mobile element biology: new possibilities with high-throughput sequencing. *Trends Genet*. 2013;29:280-289.

Tn-Seq: Transposon Sequencing
INSeq: Insertion Sequencing

Tn-seq[54] and INSeq[55] are nearly identical methods that determine quantitative genetic interactions accurately. INSeq includes a polyacrylamide gel purification step following adapter ligation and PCR, whereas Tn-seq requires agarose gel purification.[56]

In this method, a transposon with flanking MmeI digestion sites is transposed into bacteria which, after culturing, can help detect the frequency of mutations within the transposon. After MmeI digestion and subsequent adapter ligation, PCR amplification and sequencing can provide information about the transposon insertion sites.

A schematic overview of Tn-seq.

Advantages	Disadvantages
• Can study mutational frequency of transposons • Can be used to deduce fitness of genes within microorganisms • Robust, reproducible, and sensitive	• Limited to bacterial studies • Errors during PCR amplification can lead to inaccurate sequence reads

Reviews

None available yet.

References

Hooven T. A., Catomeris A. J., Akabas L. H., et al. The essential genome of Streptococcus agalactiae. *BMC Genomics*. 2016;17:406.

In order to establish the essential genome of group B Streptococcus (GBS), the authors used the Himar1 minitransposon, which inserts at genomic TA dinucleotide sites. Next, they performed Tn-seq on DNA collected from 3 independent mutant libraries. Each library had at least 135,000 mutants after 24 hour outgrowth in rich media. From these data, the authors identified 13.5% of genes as essential and 1.2% as critical.

Illumina Technology: HiSeq 4000 System

Le Breton Y., Belew A. T., Valdes K. M., et al. Essential Genes in the Core Genome of the Human Pathogen Streptococcus pyogenes. *Sci Rep.* 2015;5:9838.

The authors developed a mariner-based system (*Krmit*) for en masse monitoring of complex mutant pools in group A *Streptococcus* (GAS) by Tn-seq. Highly saturated transposant libraries carried *Krmit* insertions at approximately every 25 nucleotides. The authors identified 227 and 241 of those genes in a GAS serotype M1T1 invasive strain 5448 and a GAS nephritogenic serotype M49 strain NZ131, respectively.

Illumina Technology: HiSeq 1500 System

54. van Opijnen T., Bodi K. L. and Camilli A. Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. *Nat Methods.* 2009;6:767-772.
55. Goodman A. L., McNulty N. P., Zhao Y., et al. Identifying genetic determinants needed to establish a human gut symbiont in its habitat. *Cell Host Microbe.* 2009;6:279-289.
56. van Opijnen T. and Camilli A. Transposon insertion sequencing: a new tool for systems-level analysis of microorganisms. *Nat Rev Microbiol*. 2013;11:435-442.

Liu F., Wang C., Wu Z., Zhang Q. and Liu P. A zero-inflated Poisson model for insertion tolerance analysis of genes based on Tn-seq data. *Bioinformatics*. 2016;32:1701-1708.

DeJesus M. A., Ambadipudi C., Baker R., Sassetti C. and Ioerger T. R. TRANSIT--A Software Tool for Himar1 TnSeq Analysis. *PLoS Comput Biol.* 2015;11:e1004401.

Lee S. A., Gallagher L. A., Thongdee M., et al. General and condition-specific essential functions of Pseudomonas aeruginosa. *Proc Natl Acad Sci U S A.* 2015;112:5189-5194.

Meeske A. J., Sham L. T., Kimsey H., et al. MurJ and a novel lipid II flippase are required for cell wall biogenesis in Bacillus subtilis. *Proc Natl Acad Sci U S A.* 2015;112:6437-6442.

Rubin B. E., Wetmore K. M., Price M. N., et al. The essential gene set of a photosynthetic organism. *Proc Natl Acad Sci U S A.* 2015;112:E6634-6643.

Turner K. H., Wessel A. K., Palmer G. C., Murray J. L. and Whiteley M. Essential genome of Pseudomonas aeruginosa in cystic fibrosis sputum. *Proc Natl Acad Sci U S* A. 2015;112:4110-4115.

Wu M., McNulty N. P., Rodionov D. A., et al. Genetic determinants of *in vivo* fitness and diet responsiveness in multiple human gut Bacteroides. *Science*. 2015;350:aac5992.

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

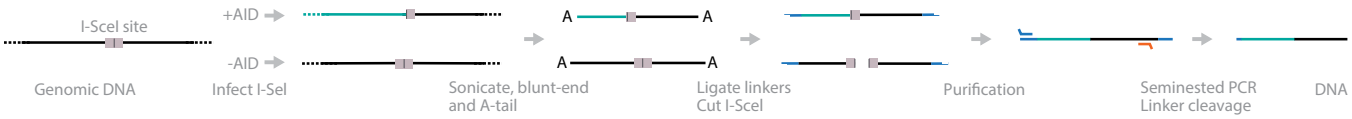
Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

TC-Seq: Translocation-Capture Sequencing

TC-Seq was developed to study chromosomal rearrangements and translocations.[57]

In this method, cells are infected with retrovirus expressing I-SceI sites in cells with and without activation-induced cytidine deaminase (AICDA or AID) protein. gDNA from cells is sonicated, linker-ligated, purified, and amplified via seminested ligation-mediated (LM)-PCR. The linker is cleaved, and the DNA is sequenced. Any AID-dependent chromosomal rearrangement will be amplified by LM-PCR, while AID-independent translocations will be discarded.



A schematic overview of TC-Seq.

Advantages	Disadvantages
• Can study chromosomal translocations within a given model or environment • Random sonication generates unique linker ligation points, and deep sequencing allows reading through rearrangement breakpoints	• Does not resolve junction structures • Relatively higher cost and lower sensitivity compared with LAM-HTGTS[58] • PCR amplification errors • Nonlinear PCR amplification can lead to biases affecting reproducibility • PCR biases can underrepresent GC-rich templates

Reviews

None available yet

References

Robbiani D. F., Deroubaix S., Feldhahn N., et al. Plasmodium Infection Promotes Genomic Instability and AID-Dependent B Cell Lymphoma. *Cell*. 2015;162:727-737.

Plasmodium chabaudi (Pc) induces prolonged expansion of germinal centers (GCs). These centers are unique compartments in which B cells undergo rapid clonal expansion and express AID, a DNA mutator. The authors used TC-Seq to detect the AID-induced damage and found that GC B cells elicited during Pc infection suffer widespread DNA damage, leading to chromosome translocations. Although infection does not change the overall rate, it modifies lymphomagenesis to favor mature AID-dependent B cell lymphomas. As a result, malaria infection appears to favor mature B cell cancers by eliciting protracted AID expression in GC B cells.

Illumina Technology: Unspecified HiSeq system

Qian J., Wang Q., Dose M., et al. B cell super-enhancers and regulatory clusters recruit AID tumorigenic activity. *Cell*. 2014;159:1524-1537.

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

57. Klein I. A., Resch W., Jankovic M., et al. Translocation-capture sequencing reveals the extent and nature of chromosomal rearrangements in B lymphocytes. *Cell*. 2011;147:95-106.
58. Hu J., Meyers R. M., Dong J., Panchakshari R. A., Alt F. W. and Frock R. L. Detecting DNA double-stranded breaks in mammalian genomes by linear amplification-mediated high-throughput genome-wide translocation sequencing. *Nat Protoc.* 2016;11:853-871.

Rep-Seq: Repertoire Sequencing
Ig-seq: DNA Sequencing of Immunoglobulin Genes
MAF: Molecular Amplification fingerprinting

Rep-Seq is a collective term for repertoire sequencing technologies,[59] including Ig-seq[60] and MAF.[61]

Ig-seq is a targeted gDNA amplification method performed with primers complementary to the rearranged V-region gene (VDJ recombinant). Amplification of gDNA is then performed with the appropriate 5' primers. Although throughput is high, information regarding which VH and VL chains were paired in the same cell is lost, as cells are lysed in bulk and VH and VL genes are amplified in separate reactions. MAF (illustrated below) was developed to correct the amplification biases in Ig-seq, by using tagging with unique identifiers (UIDs) before and during the multiplex PCR amplification, as well as a bioinformatics pipeline.

A schematic overview of Rep-Seq.

Advantages	Disadvantages
• Measures antibody frequencies with up to 99% accuracy	• Data analysis is challenging[62] • In bulk analysis, information regarding which V_H and V_L chains were paired in the same cell is lost[61]

Reviews

Hackl H., Charoentong P., Finotello F. and Trajanoski Z. Computational genomics tools for dissecting tumour-immune cell interactions. *Nat Rev Genet.* 2016;17:441-458.

Hou X. L., Wang L., Ding Y. L., Xie Q. and Diao H. Y. Current status and recent advances of next generation sequencing techniques in immunological repertoire. *Genes Immun.* 2016;17:153-164.

Georgiou G., Ippolito G. C., Beausang J., Busse C. E., Wardemann H., et al. The promise and challenge of high-throughput sequencing of the antibody repertoire. *Nat Biotechnol.* 2014;32:158-168.

References

Khan T. A., Friedensohn S., Gorter de Vries A. R., Straszewski J., Ruscheweyh H. J., et al. Accurate and predictive antibody repertoire profiling by molecular amplification fingerprinting. *Sci Adv.* 2016;2:e1501371.

This study describes the original MAF method. The authors found that MAF bias correction, when combined with a bioinformatic pipeline, led to measurements of antibody frequencies with up to 99% accuracy. They also used MAF to correct PCR and sequencing errors, to achieve full-length antibody diversity measurements with 98–100% error correction.

Illumina Technology: MiSeq System

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

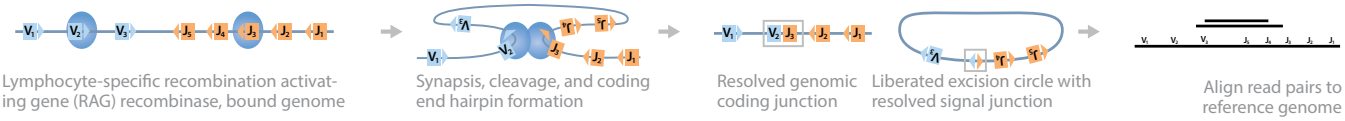
Nextera XT DNA Library Prep Kit

59. Benichou J., Ben-Hamo R., Louzoun Y. and Efroni S. Rep-Seq: uncovering the immunological repertoire through next-generation sequencing. *Immunology*. 2012;135:183-191.
60. Georgiou G., Ippolito G. C., Beausang J., Busse C. E., Wardemann H. and Quake S. R. The promise and challenge of high-throughput sequencing of the antibody repertoire. *Nat Biotechnol.* 2014;32:158-168.
61. Khan T. A., Friedensohn S., Gorter de Vries A. R., Straszewski J., Ruscheweyh H. J. and Reddy S. T. Accurate and predictive antibody repertoire profiling by molecular amplification fingerprinting. *Sci Adv.* 2016;2:e1501371.
62. Georgiou G., Ippolito G. C., Beausang J., Busse C. E., Wardemann H. and Quake S. R. The promise and challenge of high-throughput sequencing of the antibody repertoire. *Nat Biotechnol.* 2014;32:158-168.

EC-seq: Excision Circle Sequencing

EC-seq allows the capture and analysis of immune locus rearrangements from whole thymic and splenic tissues.

Double-stranded cleavage at recombination signal sequence (RSS) sites, mediated by recombination-activating gene recombinase (RAG), precedes genomic deletion and end-processing by the nonhomologous end-joining (NHEJ) complex. Coding ends are covalently hair-pinned and reopened prior to ligation. The excision circle junction is then ligated from unprocessed DNA ends with little modification and used to prepare a sequencing library.



A schematic overview of EC-seq.

Advantages	Disadvantages
• Provides capture and analysis of immune locus rearrangements	• Not yet adopted widely by the scientific community

Reviews

None available yet

References

Parkinson N. J., Roddis M., Ferneyhough B., et al. Violation of the 12/23 rule of genomic V(D)J recombination is common in lymphocytes. *Genome Res.* 2015;25:226-234.

The authors developed EC-seq to study V(D)J recombination in mouse whole thymic and splenic tissues. They found that the 12/23 rule of genomic recombination was frequently violated under physiological conditions, resulting in unanticipated hybrid recombination. These nonclassical excision circles are liberated during the formation of antigen receptor diversity.

Illumina Technology: HiSeq 2000 System

Associated Kits

TruSeq DNA Sample Prep Kit

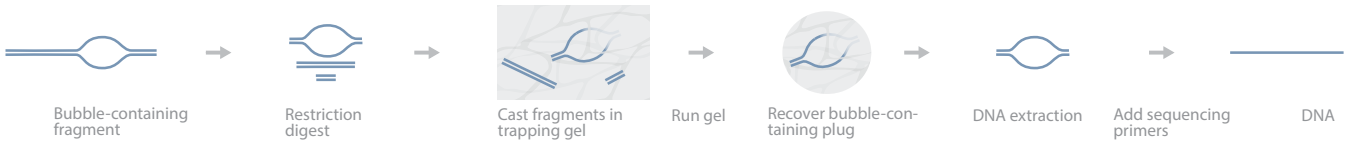
TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Bubble-Seq: Libraries of Restriction Fragments that Contain Replication Initiation Sites (Bubbles)

Bubble-Seq prepares libraries of restriction fragments that contained replication initiation sites (bubbles) *in vivo*.[63]

In this method, DNA from origin libraries is biotinylated by a 3'-end tailing reaction with biotin-16-dUTP. The biotinylated DNA is sonicated and captured with streptavidin-coated beads. The free DNA fragments are purified and used to prepare a sequencing.



A schematic overview of Bubble-Seq.

Advantages	Disadvantages
• Simple protocol	• Not yet adopted widely by the scientific community

Reviews

None available yet

References

Foulk M. S., Urban J. M., Casella C. and Gerbi S. A. Characterizing and controlling intrinsic biases of lambda exonuclease in nascent strand sequencing reveals phasing between nucleosomes and G-quadruplex motifs around a subset of human replication origins. *Genome Res.* 2015;25:725-735.

The authors used Bubble-Seq as an orthogonal mapping technique to confirm that G-quadruplex (G4) structures are not general determinants for origin specification, but they may play a role for a subset.

Illumina Technology: HiSeq System

Mukhopadhyay R., Lajugie J., Fourel N., et al. Allele-specific genome-wide profiling in human primary erythroblasts reveal replication program organization. *PLoS Genet.* 2014;10:e1004319.

Associated Kits

TruSeq DNA PCR-Free Library Prep Kit

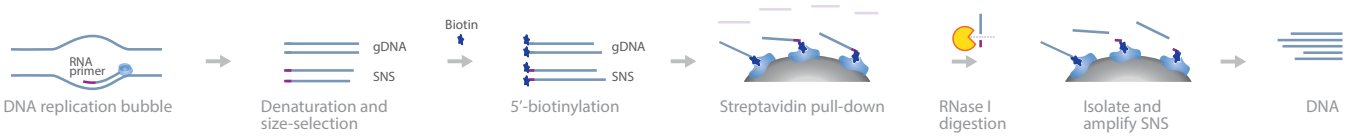
Nextera DNA Library Prep Kit

63. Mesner L. D., Valsakumar V., Cieslik M., Pickin R., Hamlin J. L. and Bekiranov S. Bubble-seq analysis of the human genome reveals distinct chromatin-mediated mechanisms for regulating early- and late-firing origins. *Genome Res.* 2013;23:1774-1788.

NSCR: Nascent Strand Capture and Release

NSCR isolates and sequences the origin of DNA replication by capturing short nascent strands (SNS), which are single-stranded RNA-DNA chimeras.[64] In short, NSCR captures and purifies RNA-DNA chimeric SNS, and releases the DNA part by cutting the strand at the end of the initial RNA primer section.

First, gDNA is denatured, size-selected through a sucrose gradient for 400–2000 bp fragments, and 5'-biotinylated. The biotinylated fragments are isolated by streptavidin pull-down, purifying both SNS and gDNA strands. Next, RNase I is used to separate the DNA from the initial RNA primer section of the single-stranded RNA-DNA chimera. This process leaves RNA primers and gDNA on the streptavidin beads. The released nascent DNA strands are amplified and sequenced. This method is an improvement over widely used SNS purification methods using BrdU and lambda exonuclease (λ-exo).



A schematic overview of NSCR.

Advantages	Disadvantages
• Maps genome-wide DNA replication origins • Higher discovery of nascent strands than λ-exo–based methods (2231 vs 3922 peaks) • RNase I cleaves with minimal sequence specificity • Can be used for systems that use small RNA primers to produce RNA-DNA chimeras	• Less than 10% purification yield (1–2 ng of SNS from 1 mg total DNA) • False reads may arise due to misincorporation of ribonucleotides into the DNA portion of the SNS

Reviews

None available yet

References

Kunnev D., Freeland A., Qin M., Wang J. and Pruitt S. C. Isolation and sequencing of active origins of DNA replication by nascent strand capture and release (NSCR). *J Biol Methods.* 2015;2:.

The authors developed NSCR to isolate SNS and identify the locations of DNA replication origins. They performed NSCR on murine cell lines and obtained 3922 SNS peaks. These peaks showed 79% overlap over the 2231 SNS peaks from a previous experiment using λ-exo–based techniques.

Illumina Technology: HiSeq 2000 System

Associated Kits

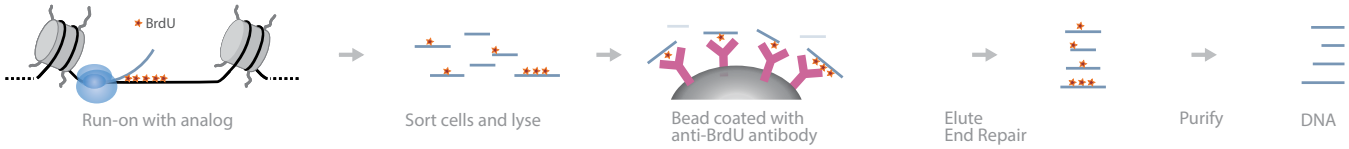
TruSeq ChIP Library Prep Kit

64. Kunnev D., Freeland A., Qin M., Wang J. and Pruitt S. C. Isolation and sequencing of active origins of DNA replication by nascent strand capture and release (NSCR). *J Biol Methods.* 2015;2:

Repli-Seq: Nascent DNA Replication Strand Sequencing

Repli-Seq maps the sequences of nascent DNA replication strands throughout the whole genome during each of the 6 cell-cycle phases.[65]

This process is achieved by growing cells in media containing BrdU to replace thymidine. The cells are sorted to their current state in cell division using fluorescence-activated cell sorting (FACS). BrdU-labeled DNA strands are immunoprecipitated by anti-BrdU antibodies on magnetic beads. These immunoprecipitated strands can be prepared for sequencing following the TruSeq DNA library preparation protocol.



A schematic overview of Repli-Seq.

Advantages	Disadvantages
• Maps sequences of newly replicated DNA to the phases of cell division • Low sample input required (5000 cells) makes it suitable for studying rare cell populations • Streamlined DNA library preparation step	• Limited to cell cultures and other artificial systems, due to the requirement for incubation in the presence of labeled nucleotides

Reviews

Reuter J. A., Spacek D. V. and Snyder M. P. High-Throughput Sequencing Technologies. *Mol Cell.* 2015;58:586-597.

References

Ohbayashi R., Watanabe S., Ehira S., et al. Diversification of DnaA dependency for DNA replication in cyanobacterial evolution. *ISME J.* 2015;.

The authors studied the importance of DNA replication initiation factor DnaA, which is highly conserved in prokaryotes. They applied Repli-Seq to identify replication initiation sites within DnaA disruptants in Synechococcus elongates. The results revealed that deletion of the dnaA gene had no effect in DNA replication or cell growth of the model. The researchers concluded that functional dependency on DnaA was lost in some cyanobacterial lineages during diversification.

Illumina Technology: Genome Analyzer System

Deyle D. R., Hansen R. S., Cornea A. M., et al. A genome-wide map of adeno-associated virus-mediated human gene targeting. *Nat Struct Mol Biol.* 2014;21:969-975.

Barlow J. H., Faryabi R. B., Callen E., et al. Identification of early replicating fragile sites that contribute to genome instability. *Cell*. 2013;152:620-632.

Associated Kits

TruSeq Nano DNA Library Prep Kit

65. Hansen R. S., Thomas S., Sandstrom R., et al. Sequencing newly replicated DNA reveals widespread plasticity in human replication timing. *Proc Natl Acad Sci U S A.* 2010;107:139-144.

NS-Seq: Nascent Strand Sequencing

NS-Seq sequences nascent DNA strands to locate DNA replication origins in the genome. NS-Seq uses λ-exo to digest parental DNA effectively while leaving the RNA primer–protected nascent strands intact. However, λ-exo inefficiently digests G-quadruplex structures (G4) and GC-rich motifs; this bias can be normalized by using λ-exo–digested DNA from nonreplicating cells as a control.[66]

In this method, gDNA is enriched, made single-stranded, and 5'-phosphorylated using T4 PNK. Next, the DNA is digested with λ-exo and purified. The resultant single-stranded nascent strands are converted to double-stranded DNA using random hexamers and fragmented to 100–600 bp. DNA libraries are prepared, using standard kits, and sequenced.

A schematic overview of NS-seq.

Advantages	Disadvantages
• Locates DNA replication origins by sequencing RNA primer–protected nascent DNA strands • λ-exo–digested DNA from nonreplicating cells can be used as a control to normalize for biases in λ-exo digestion • Replacing K^+ with Na^+ during λ-exo digestion reduces digestion inefficiency in G4 regions	• λ-exo does not efficiently digest G4 structures in plasmid and halts upon GC-rich motifs • Purified samples can be contaminated with GC-rich and G4-protected DNA

Reviews

MacAlpine D. M. ORChestrating the human DNA replication program. *Proc Natl Acad Sci U S A.* 2016;113:9136-9138.

Song J., Perreault J.-P., Topisirovic I. and Richard S. RNA G-quadruplexes and their potential regulatory roles in translation. Translation. 2016;4:e1244031.

References

Smith O. K., Kim R., Fu H., et al. Distinct epigenetic features of differentiation-regulated replication origins. *Epigenetics Chromatin.* 2016;9:18.

This study explores the relationship between DNA replication origins and transcriptomic changes, especially in human oncogenic cell lines. The authors performed NS-Seq on short nascent strands isolated using λ-exo digestion and BrdU substitution. They found that cancer and noncancer cells displayed very similar distributions of replication origins. Using NS-Seq data, they discovered that cell type–specific replication origins are replicated during late S phase, while shared origins are replicated throughout the S phase.

Illumina Technology: HiSeq 2000 System, TruSeq SBS Kit v3

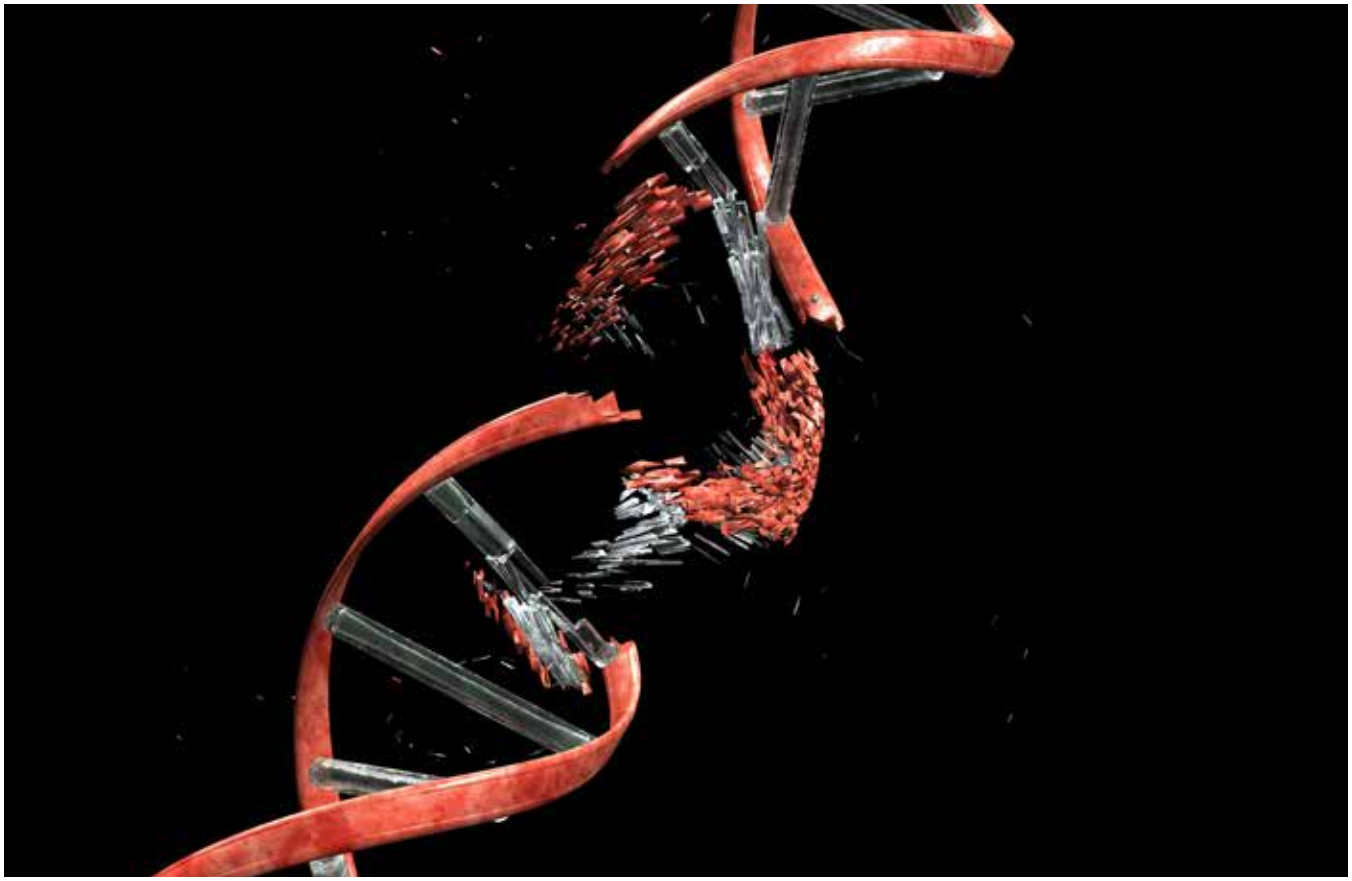
Associated Kits

TruSeq SBS Kit v3

66. Foulk M. S., Urban J. M., Casella C. and Gerbi S. A. Characterizing and controlling intrinsic biases of lambda exonuclease in nascent strand sequencing reveals phasing between nucleosomes and G-quadruplex motifs around a subset of human replication origins. *Genome Res.* 2015;25:725-735.

DNA BREAK MAPPING

DNA strand breaks are intrinsic to various biological processes, such as transcription and the generation of antigen receptor diversification in lymphocytes.[67] They are key substrates for translocations, deletions, and amplifications associated with various cancers.[68,69] With the advent of CRISPR/Cas9 gene editing, there is renewed interest in determining cleavage patterns characteristic of on- and off-target sites.[70]



Accurate identification of DNA strand breaks are notably used in cancer research and CRISPR-mediated gene editing studies.

Reviews

Kim D., Kim S., Kim S., Park J. and Kim J. S. Genome-wide target specificities of CRISPR-Cas9 nucleases revealed by multiplex Digenome-seq. Genome Res. 2016;26:406-415.

Lee C. M., Cradick T. J., Fine E. J. and Bao G. Nuclease Target Site Selection for Maximizing On-target Activity and Minimizing Off-target Effects in Genome Editing. Mol Ther. 2016;24:475-487.

Hu J., Meyers R. M., Dong J., Panchakshari R. A., Alt F. W., et al. Detecting DNA double-stranded breaks in mammalian genomes by linear amplification-mediated high-throughput genome-wide translocation sequencing. Nat Protoc. 2016;11:853-871.

67. Hu J., Meyers R. M., Dong J., Panchakshari R. A., Alt F. W. and Frock R. L. Detecting DNA double-stranded breaks in mammalian genomes by linear amplification-mediated high-throughput genome-wide translocation sequencing. *Nat Protoc.* 2016;11:853-871.
68. Nussenzweig A. and Nussenzweig M. C. Origin of chromosomal translocations in lymphoid cancer. *Cell*. 2010;141:27-38.
69. Alt F. W., Zhang Y., Meng F. L., Guo C. and Schwer B. Mechanisms of programmed DNA lesions and genomic instability in the immune system. *Cell*. 2013;152:417-429.
70. Kim D., Kim S., Kim S., Park J. and Kim J. S. Genome-wide target specificities of CRISPR-Cas9 nucleases revealed by multiplex Digenome-seq. *Genome Res.* 2016;26:406-415.

Map DNA Single-Strand Breaks (SSB-Seq)

SSB-Seq[71] maps *in vivo* DNA single-strand breaks (SSBs) on a genome-wide scale.

In this method, gDNA is isolated and subjected to nick-translation by DNA polymerase I in the presence of deoxynucleotide triphosphates (dNTPs), including digoxigenin-labeled dUTP, and a small amount of dideoxynucleotides. The DNA is sheared, and the labeled fragments are immunoprecipitated with antidigoxigenin antibodies and purified to prepare a sequencing library.

A schematic overview of SSB-Seq.

Advantages	Disadvantages
• Simple protocol	• Locations of breaks cannot be determined accurately • Not yet adopted widely by the scientific community

Reviews

None available yet

References

Baranello L., Kouzine F., Wojtowicz D., Cui K., Przytycka T. M., et al. DNA break mapping reveals topoisomerase II activity genome-wide. *Int J Mol Sci.* 2014;15:13111-13122.

This study describes the original SSB-Seq protocol. The authors tested the method in human colon cancer cells and validated the results using the topoisomerase II (Top2)–poisoning agent, etoposide (ETO). The combination of ETO treatment with break-mapping techniques appears to be a powerful method for elaborating the pattern of Top2 enzymatic activity across the genome.

Illumina Technology: Genome Analyzer System

Associated Kits

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

71. Baranello L., Kouzine F., Wojtowicz D., et al. DNA break mapping reveals topoisomerase II activity genome-wide. *Int J Mol Sci.* 2014;15:13111-13122.

BLESS: Breaks labeling and Enrichment on Streptavidin and Sequencing

BLESS is a genome-wide approach to map DSBs at nucleotide resolution.[72] BLESS is able to detect telomere ends, Sce endonuclease–induced DSBs, and complex genome-wide DSB landscapes.

In this method, DSBs are ligated *in situ* to a proximal linker covalently linked to biotin. The gDNA is extracted and fragmented, and the labeled fragments are captured on streptavidin beads. Next, a distal linker is ligated to the free end of the captured fragments. The fragments are released by linker digestion with I-SceI and PCR-amplified.

A schematic overview of BLESS.

Advantages	Disadvantages
• Detects DSBs at nucleotide resolution • Does not depend on proteins that bind to DSBs—a source of bias • Does not depend on single-stranded DNA (ssDNA)—a source of bias	• High background; only maps unjoined ends[73] • Susceptible to artifacts associated with cell fixation[74]

Reviews

Lee C. M., Cradick T. J., Fine E. J. and Bao G. Nuclease Target Site Selection for Maximizing On-target Activity and Minimizing Off-target Effects in Genome Editing. *Mol Ther.* 2016;24:475-487.

References

Ran F. A., Cong L., Yan W. X., et al. *In vivo* genome editing using Staphylococcus aureus Cas9. *Nature*. 2015;520:186-191.

The authors applied BLESS to capture a snapshot of Cas9-induced DNA DSBs in cells. They were able to identified off-target sites not previously predicted by sequence similarity to the target or by chromatin immunoprecipitation (ChIP).

Illumina Technology: MiSeq System

Associated Kits

TruSeq Nano DNA LT Library Prep Kit

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73. Hu J., Meyers R. M., Dong J., Panchakshari R. A., Alt F. W. and Frock R. L. Detecting DNA double-stranded breaks in mammalian genomes by linear amplification-mediated high-throughput genome-wide translocation sequencing. *Nat Protoc.* 2016;11:853-871.
74. Tsai S. Q., Zheng Z., Nguyen N. T., et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol.* 2015;33:187-197.

DSB-Seq: Map DNA Double-Strand Breaks

DSB-Seq maps *in vivo* DNA DSBs on a genome-wide scale.[75]

In this method, DNA is biotinylated by a 3'-end tailing reaction with biotin-16-dUTP. The biotinylated DNA is sonicated and captured with streptavidin-coated beads. The free DNA fragments are purified and used to prepare a sequencing library.

A schematic overview of DSB-Seq.

Advantages	Disadvantages
• Simple protocol	• Not yet adopted widely by the scientific community

Reviews

None available yet.

References

Baranello L., Kouzine F., Wojtowicz D., Cui K., Przytycka T. M., et al. DNA break mapping reveals topoisomerase II activity genome-wide. *Int J Mol Sci.* 2014;15:13111-13122.

This study describes the original DSB-Seq protocol. The authors tested the method in human colon cancer cells and validated the results using the Top2-poisoning agent, ETO. The combination of ETO treatment with break-mapping techniques appears to be a powerful method for elaborating the pattern of Top2 enzymatic activity across the genome.

Illumina Technology: Genome Analyzer

Associated Kits

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

75. Baranello L., Kouzine F., Wojtowicz D., et al. DNA break mapping reveals topoisomerase II activity genome-wide. *Int J Mol Sci.* 2014;15:13111-13122.

Break-seq: Double-Stranded Break Labeling

Break-seq combines DSB labeling with NGS to map chromosome breaks with improved sensitivity and resolution.[76]

DNA trapped in agarose plugs is end-repaired and labeled with biotin. The agarose is then digested by β-agarase, followed by dilution and fragmentation. The fragmented DNA is purified and end-repaired with unmodified dNTPs, followed by A-tailing. The fragments are purified on magnetic beads, PCR-amplified, purified, and used to prepare a sequencing library.

A schematic overview of Break-seq.

Advantages	Disadvantages
• Simple protocol	• Not replicated in other laboratories

Reviews

None available yet.

References

Hoffman E. A., McCulley A., Haarer B., Arnak R. and Feng W. Break-seq reveals hydroxyurea-induced chromosome fragility as a result of unscheduled conflict between DNA replication and transcription. *Genome Res.* 2015;25:402-412.

The authors used Break-seq to show that DSBs occur preferentially at genes transcriptionally induced by hydroxyurea (HU) in yeast cells. Notably, different subsets of the HU-induced genes produced DSBs in the checkpoint mutant mec1 and MEC1 cells. The authors conclude that replication forks traversed a greater distance in MEC1 cells than in mec1 cells during recovery from HU.

Illumina Technology: MiSeq System

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

76. Hoffman E. A., McCulley A., Haarer B., Arnak R. and Feng W. Break-seq reveals hydroxyurea-induced chromosome fragility as a result of unscheduled conflict between DNA replication and transcription. *Genome Res.* 2015;25:402-412.

GUIDE-seq: Genome-Wide, Unbiased Identification of DSBs Enabled by Sequencing

GUIDE-seq[77] relies on the integration of double-stranded oligodeoxynucleotides (DSOs) into DSBs. It belongs to a family of methods—such as HTGTS,[78] LAM-HTGTS,[79] and Digenome-seq[80]—that are aimed at detecting off-target effects of CRISPR/Cas9 and other RNA-guided nucleases (RGNs).

RGN-induced DSBs are tagged by integration of blunt-ended DSOs in the genomes of living human cells. The DSO integration sites are mapped precisely in the genome at the nucleotide level with unbiased amplification and NGS.

A schematic overview of GUIDE-seq.

Advantages	Disadvantages
• Generates global specificity landscapes for RGNs in living human cells • Targeted sequencing reduces cost	• Can miss some targets[81]

Reviews

Hu J., Meyers R. M., Dong J., Panchakshari R. A., Alt F. W., et al. Detecting DNA double-stranded breaks in mammalian genomes by linear amplification-mediated high-throughput genome-wide translocation sequencing. *Nat Protoc.* 2016;11:853-871.

Lee C. M., Cradick T. J., Fine E. J. and Bao G. Nuclease Target Site Selection for Maximizing On-target Activity and Minimizing Off-target Effects in Genome Editing. *Mol Ther.* 2016;24:475-487.

References

Kleinstiver B. P., Tsai S. Q., Prew M. S., et al. Genome-wide specificities of CRISPR-Cas Cpf1 nucleases in human cells. *Nat Biotechnol.* 2016;.

The authors found that Cpf1 nucleases from Acidaminococcus sp (AsCpf1) and Lachnospiraceae bacterium (LbCpf1) have on-target editing accuracies in human cells that are comparable to that of Streptococcus pyogenes Cas9. Through GUIDE-seq and targeted deep sequencing analysis performed on both nucleases, they found no off-target effects for most of the crRNAs used, suggesting that AsCpf1 and LpCpf1 are highly specific in human cells.

Illumina Technology: MiSeq System

Kleinstiver B. P., Pattanayak V., Prew M. S., et al. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature*. 2016;529:490-495.

In this study, the authors created and studied 15 SpCas9 mutants, each with a single, double, triple, or quadruple combination of 4 substitutions (N497A, R661A, Q695A, and Q926A). Through an assay using a single-guide RNA (sgRNA) targeted to an enhanced green fluorescent protein (EGFP) reporter gene, they identified the quadruple mutant (referred as SpCas9-HFI) as a high fidelity variant that reduces nonspecific DNA contacts. SpCas9-HFI retained on-target activities with wild-type SpCas9 with > 85% of sgRNAs tested in human cells. With sgRNAs targeted at nonrepetitive sequences, Cas9-HF1 rendered all or nearly all off-target events undetectable by GUIDE-seq and by targeted deep sequencing. Even for atypical, repetitive target sites, the majority of off-target mutations induced by wild-type SpCas9 were not detected with SpCas9-HFI.

Illumina Technology: MiSeq System

77. Tsai S. Q., Zheng Z., Nguyen N. T., et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol.* 2015;33:187-197.
78. Chiarle R., Zhang Y., Frock R. L., et al. Genome-wide translocation sequencing reveals mechanisms of chromosome breaks and rearrangements in B cells. *Cell*. 2011;147:107-119.
79. Hu J., Meyers R. M., Dong J., Panchakshari R. A., Alt F. W. and Frock R. L. Detecting DNA double-stranded breaks in mammalian genomes by linear amplification-mediated high-throughput genome-wide translocation sequencing. *Nat Protoc.* 2016;11:853-871.
80. Kim D., Bae S., Park J., et al. Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nat Methods.* 2015;12:237-243, 231 p following 243.
81. Kim D., Kim S., Kim S., Park J. and Kim J. S. Genome-wide target specificities of CRISPR-Cas9 nucleases revealed by multiplex Digenome-seq. *Genome Res.* 2016;26:406-415.

Yin H., Song C. Q., Dorkin J. R., et al. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components *in vivo*. *Nat Biotechnol.* 2016;34:328-333.

In this study, the authors combined lipid nanoparticle-mediated delivery of Cas9 mRNA with adeno-associated viruses (AAV) encoding a sgRNA and a repair template in a mouse model of human hereditary tyrosinemia. They showed that the treatment generated fumarylacetoacetate hydrolase (Fah)-positive hepatocytes by correcting the causative Fah-splicing mutation. They used GUIDE-seq in cultured liver cells and targeted deep sequencing to check for off-target effects, and found that the *in vivo* off-target rate was low for the proposed method.

Illumina Technology: NextSeq 500 System, MiSeq System, Nextera XT DNA Library Prep Kit

Bolukbasi M. F., Gupta A., Oikemus S., et al. DNA-binding-domain fusions enhance the targeting range and precision of Cas9. *Nat Methods.* 2015; 12:1150-1156.

To improve the editing precision of Cas9, the authors fused a programmable DNA binding domain (pDBD) to Cas9 and reduced the inherent DNA-binding affinity of Cas9. They used GUIDE-seq and discovered that the Cas9-pDBD chimera had a significantly improved editing precision, providing a flexible system that can achieve extremely precise genome-editing results.

Illumina Technology: MiSeq System, TruSeq DNA Library Prep Kit

Friedland A. E., Baral R., Singhal P., et al. Characterization of Staphylococcus aureus Cas9: a smaller Cas9 for all-in-one adeno-associated virus delivery and paired nickase applications. *Genome Biol*. 2015;16:257.

The authors used GUIDE-seq to compare the specificity of Streptococcus aureus Cas9 (SaCas9) with Streptococcus pyogenes Cas9 (SpCas9), using a guide RNA (gRNA) that has known off-target sites. A substantial number of the SpCas9 off-target sites could be reproduced. By comparison, SaCas9 showed a high number of on-target reads but only single-digit read counts for comparatively fewer off-target sites.

Illumina Technology: MiSeq System

Kleinstiver B. P., Prew M. S., Tsai S. Q., et al. Broadening the targeting range of Staphylococcus aureus CRISPR-Cas9 by modifying PAM recognition. *Nat Biotechnol.* 2015;33:1293-1298.

The researchers used GUIDE-seq to study the gene-editing specificity of wild-type SaCas9 and a series of mutants with modifications in the protospacer adjacent motif (PAM). One mutant, termed KKH, showed robust genome editing activities and increased the SaCas9 targeting range by 2- to 4-fold. Both wild-type and KKH SaCas9 induced comparable numbers of off-target effects in human cells.

Illumina Technology: MiSeq System

Doench J. G., Fusi N., Sullender M., et al. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat Biotechnol*. 2016;34:184-191.

Yin H., Song C. Q., Dorkin J. R., Zhu L. J., Li Y., et al. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components *in vivo*. *Nat Biotechnol.* 2016;34:328-333.

Chari R., Mali P., Moosburner M. and Church G. M. Unraveling CRISPR-Cas9 genome engineering parameters via a library-on-library approach. *Nat Methods.* 2015;12:823-826.

Kleinstiver B. P., Prew M. S., Tsai S. Q., et al. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature*. 2015;523:481-485.

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

HTGTS: High-Throughput Genome-Wide Translocation Sequencing

HTGTS was developed to study translocation mechanisms in mammalian cells.[82] This approach is particularly suitable for studying for AID-dependent IgH class-switching (HTGTS-Rep-seq)[83] and CRISPR/Cas9 genome modifications.[84]

In HTGTS-Rep-seq, genomic DNA from B-cell populations is sonicated and linearly amplified with a biotinylated primer that anneals downstream of a J segment. The biotin-labeled single-stranded DNA products are enriched with streptavidin beads, and the 3' ends are ligated to a bridge adaptor containing a 6-nucleotide UMI.

A schematic overview of HTGTS-Rep-seq.

Advantages	Disadvantages
• Higher efficiency compared to whole-genome sequencing	• Underestimates the frequency of DSBs[85] • Limited by chromatin accessibility[86]

Reviews

None available yet.

References

Schwer B., Wei P. C., Chang A. N., et al. Transcription-associated processes cause DNA double-strand breaks and translocations in neural stem/progenitor cells. *Proc Natl Acad Sci U S A.* 2016;113:2258-2263.

The authors used HTGTS to determine if breakpoint junctions are enriched around transcription start sites (TSSs) that were determined to be active by global run-on sequencing analyses of primary neural stem/progenitor cells (NSPCs). All actively transcribed genes containing translocation junctions in NSPCs within 2 kb of the TSS showed a significantly higher transcription rate, on average, than genes at distances greater than 2 kb from the TSS.

Illumina Technology: MiSeq System

Frock R. L., Hu J., Meyers R. M., et al. Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. *Nat Biotechnol.* 2015;33:179-186.

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

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84. Mei Y., Wang Y., Chen H., Sun Z. S. and Ju X. D. Recent Progress in CRISPR/Cas9 Technology. *J Genet Genomics.* 2016;43:63-75.
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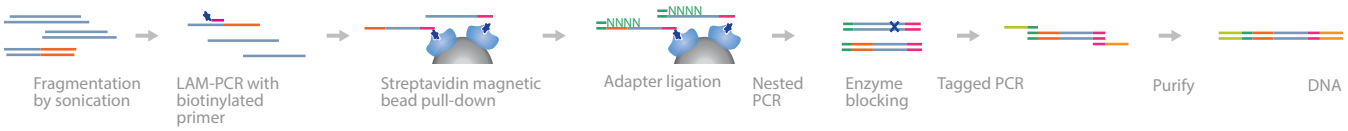
86. Kim D., Bae S., Park J., et al. Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nat Methods.* 2015;12:237-243, 231 p following 243.

LAM-HTGTS: Linear Amplification-Mediated High-Throughput Genome-Wide Sequencing

LAM-HTGTS is a method for the genome-wide detection of "prey" DSBs via their translocation to a fixed "bait" DSB in cultured mammalian cells.[87] Bait-prey junctions are cloned directly from isolated gDNA using LAM-PCR and ligated unidirectionally to bridge adapters. Subsequent PCR steps amplify the single-stranded DNA junction library in preparation for sequencing.



A schematic overview of LAM-HTGTS.

Advantages	Disadvantages
• Sensitive detection of DSBs within chromosomes	• Not applicable to limited amounts of material

Reviews

None available yet.

References

Hu J., Meyers R. M., Dong J., Panchakshari R. A., Alt F. W., et al. Detecting DNA double-stranded breaks in mammalian genomes by linear amplification-mediated high-throughput genome-wide translocation sequencing. Nat Protoc. 2016;11:853-871.

The authors used LAM-HTGTS to study the on-target and off-target activities of the recombination-activating gene (RAG) endonuclease during V(D)J recombination, using endogenous RAG-generated DSBs as bait. Although prior studies detected only a handful of off-target RAG-generated DSBs, the LAM-HTGTS studies identified thousands of RAG off-target sites, which were restricted tightly within chromosomal loop domains. This result suggests a linear RAG tracking model to explain the generation of most RAG off-target events.

Illumina Technology: MiSeq System

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

87. Hu J., Meyers R. M., Dong J., Panchakshari R. A., Alt F. W. and Frock R. L. Detecting DNA double-stranded breaks in mammalian genomes by linear amplification-mediated high-throughput genome-wide translocation sequencing. *Nat Protoc.* 2016;11:853-871.

LOW-LEVEL DNA DETECTION

Single-cell genomics can be used to identify and study circulating tumor cells, cell-free DNA, microbes, and uncultured microbes. In addition, it can aid preimplantation diagnosis and help us better understand tissue-specific cellular differentiation.[88,89] DNA replication during cell division is not perfect. As a result, progressive generations of cells accumulate unique somatic mutations. Consequently, each cell in the body has a unique genomic signature, which allows the reconstruction of cell-lineage trees with high precision.[90] These cell-lineage trees can predict the existence of small populations of stem cells. This information is important for diverse fields, from cancer development[91,92] to preimplantation and genetic diagnosis.[93,94]

Single-cell genomics can help characterize and identify circulating tumor cells as well as microbes.

Reviews

Gawad C., Koh W. and Quake S. R. Single-cell genome sequencing: current state of the science. *Nat Rev Genet.* 2016;17:175-188.

Huang L., Ma F., Chapman A., Lu S. and Xie X. S. Single-Cell Whole-Genome Amplification and Sequencing: Methodology and Applications. *Annu Rev Genomics Hum Genet.* 2015;16:79-102.

Voet T. and Van Loo P. SNES makes sense? Single-cell exome sequencing evolves. *Genome Biol.* 2015;16:86.

Wang Y. and Navin N. E. Advances and applications of single-cell sequencing technologies. *Mol Cell*. 2015;58:598-609.

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89. Blainey P. C. The future is now: single-cell genomics of bacteria and archaea. *FEMS Microbiol Rev.* 2013;37:407-427.
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smMIP: Single-Molecule Molecular Inversion Probes

The smMIP method uses single-molecule tagging and molecular inversion probes to detect and quantify genetic variations occurring at low frequencies.[95] In this method, probes are used to detect targets in gDNA. After the probed targets are copied, exonuclease digestion leaves the target with a tag, which subsequently undergoes PCR amplification. Sequencing allows for high-resolution sequence reads of targets, while greater depth allows for better alignment of every unique molecular tag.

A schematic overview of smMIPs.

Advantages	Disadvantages
• Detects low-frequency targets • Can perform single-cell sequencing or sequencing for samples with limited starting material • Per-base error of 2.6 x 10^{-5} in clinical samples[96]	• PCR amplification errors • PCR biases can underrepresent GC-rich templates • Targets smaller than 500 bp are amplified preferentially by polymerases during PCR

Reviews

Eboreime J., Choi S. K., Yoon S. R., Arnheim N. and Calabrese P. Estimating Exceptionally Rare Germline and Somatic Mutation Frequencies via Next Generation Sequencing. *PLoS One.* 2016;11:e0158340.

References

Luks V. L., Kamitaki N., Vivero M. P., et al. Lymphatic and other vascular malformative/overgrowth disorders are caused by somatic mutations in PIK3CA. *J Pediatr.* 2015;166:1048-1054 e1041-1045.

To test if mutations in somatic phosphatidylinositol-4,5-bisphospate 3-kinase, catalytic subunit alpha gene (PIK3CA) would be found in patients with more common disorders, including isolated lymphatic malformation (LM) and Klippel-Trenaunay syndrome (KTS), the authors screened a small cohort of patients with these diseases. In 8 patients, no PIK3CA mutations were detected by whole-exome sequencing, targeted capture sequencing, or droplet digital polymerase chain reaction (ddPCR). The researchers used smMIPs to screen the entire PIK3CA coding sequence for novel mutations. They identified likely disease-causing PIK3CA mutations in 4 of the 8 affected tissue samples, with mutant allele frequencies ranging from 2.8% (8/275) to 37.5% (314/837).

Illumina Technology: MiSeq System

Mirzaa G. M., Conti V., Timms A. E., et al. Characterisation of mutations of the phosphoinositide-3-kinase regulatory subunit, PIK3R2, in perisylvian polymicrogyria: a next-generation sequencing study. *Lancet Neurol.* 2015;14:1182-1195.

This study was intended to identify additional genetic causes of bilateral perisylvian polymicrogyria (BPP) and characterize their frequency in the study population. The researchers used targeted sequencing of the entire PIK3R2 gene by smMIPs on 38 patients with BPP with normal to large head sizes. They found constitutional and mosaic PIK3R2 mutations in 17 additional children.

Illumina Technology: HiSeq 2000 System

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96. Eboreime J., Choi S. K., Yoon S. R., Arnheim N. and Calabrese P. Estimating Exceptionally Rare Germline and Somatic Mutation Frequencies via Next Generation Sequencing. *PLoS One.* 2016;11:e0158340.

Carlson K. D., Sudmant P. H., Press M. O., et al. MIPSTR: a method for multiplex genotyping of germline and somatic STR variation across many individuals. *Genome Res.* 2015;25:750-761.

Carvill G. L., McMahon J. M., Schneider A., et al. Mutations in the GABA Transporter SLC6A1 Cause Epilepsy with Myoclonic-Atonic Seizures. *Am J Hum Genet.* 2015;96:808-815.

Kang H. C., Baek S. T., Song S. and Gleeson J. G. Clinical and Genetic Aspects of the Segmental Overgrowth Spectrum Due to Somatic Mutations in PIK3CA. *J Pediatr.* 2015;167:957-962.

Weren R. D., Ligtenberg M. J., Kets C. M., et al. A germline homozygous mutation in the base-excision repair gene NTHL1 causes adenomatous polyposis and colorectal cancer. *Nat Genet.* 2015;47:668-671.

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

Nextera Rapid Capture Exome/Custom Enrichment Kit

MIPSTR: Targeted Capture of STR Loci by smMIPs

MIPSTR is a method for multiplex genotyping of germline and somatic short tandem repeat (STR) variation across many individuals.[97] This method is a variation of the smMIP[98] approach and uses a novel mapping strategy.

The method uses a smMIP with a common backbone for a PCR primer, sequencing adapters, 12 bp degenerate tag, and targeting arms with locus-specific and STR-flanking sequences. Capture across genetically diverse individuals identifies germline STR variation. The use of degenerate tags identifies technical variation, and STR variation across tag-defined read groups is considered somatic variation.

A schematic overview of MIPSTR.

Advantages	Disadvantages
• Capable of distinguishing technical error from somatic STR mutations	• Requires high-quality reference genome

Reviews

Estivill X. Genetic variation and alternative splicing. Nat Biotechnol. 2015;33:357-359.

References

Carlson K. D., Sudmant P. H., Press M. O., Eichler E. E., Shendure J., et al. MIPSTR: a method for multiplex genotyping of germline and somatic STR variation across many individuals. *Genome Res*. 2015;25:750-761.

The authors used MIPSTR to determine germline STR genotypes for 102 STR loci across diverse populations of the plant Arabidopsis thaliana. They showed that putatively functional STRs may be identified by deviation from predicted STR variation and by association with quantitative phenotypes. They also used DNA mixing experiments, with a mutant deficient in DNA repair, to demonstrate that MIPSTR can detect low-frequency somatic STR variants.

Illumina Technology: MiSeq System

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

Nextera Rapid Capture Exome/Custom Enrichment Kit

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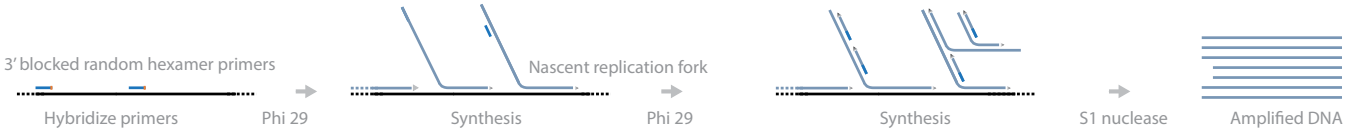
MDA: Multiple Displacement Amplification

MDA is a method commonly used for sequencing microbial genomes due to its ability to amplify templates larger than 0.5 Mbp, but it can also be used to study genomes of other sizes.[99]

In this method, 3'-blocked random hexamer primers are hybridized to the template, followed by strand-displacement DNA synthesis with Phi 29 polymerase. The method allows for efficient and rapid DNA amplification. Deep sequencing of the amplified DNA provides accurate representation of reads, while sequencing depth provides better alignment and consensus for sequences.

Several variations on the original MDA method—such as MIDAS,[100] ddMDA,[101] SNES,[102] and IMS-MDA[103]—have been developed to improve the amplification bias and throughput.[104]



A schematic overview of MDA.

Advantages	Disadvantages
• Templates can be circular DNA (eg, plasmids, bacterial DNA) • Can sequence large templates • Can perform single-cell sequencing or sequencing for samples with limited amounts of starting material	• Strong amplification bias; genome coverage as low as ~6%[105] • PCR biases can underrepresent GC-rich templates • Contaminated reagents can impact results[106]

Reviews

Sauvage V. and Eloit M. Viral metagenomics and blood safety. *Transfus Clin Biol.* 2016;23:28-38.

Zhang X., Marjani S. L., Hu Z., et al. Single-Cell Sequencing for Precise Cancer Research: Progress and Prospects. *Cancer Res.* 2016;76:1305-1312.

Hou Y., Wu K., Shi X., et al. Comparison of variations detection between whole-genome amplification methods used in single-cell resequencing. *Gigascience*. 2015;4:37.

Wang Y. and Navin N. E. Advances and applications of single-cell sequencing technologies. *Mol Cell*. 2015;58:598-609.

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Newton I. L., Clark M. E., Kent B. N., et al. Comparative Genomics of Two Closely Related Wolbachia with Different Reproductive Effects on Hosts. *Genome Biol Evol.* 2016;8:1526-1542.

The authors compared the genomes of 2 closely related Wolbachia pipientis strains (with 0.57% genome-wide synonymous divergence) that differ in their reproductive effects on hosts. wVitA induces a sperm–egg incompatibility (also known as cytoplasmic incompatibility) in the parasitoid insect Nasonia vitripennis, whereas wUni causes parthenogenetic development in a different parasitoid, Muscidifurax uniraptor. The authors performed MDA on DNA from both strains. They identified a set of genes whose loss or pseudogenization in the wUni lineage implicates them in the phenotypic shift from cytoplasmic incompatibility to induction of parthenogenesis.

Illumina Technology: Genome Analyzer System

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100. Gole J., Gore A., Richards A., et al. Massively parallel polymerase cloning and genome sequencing of single cells using nanoliter microwells. *Nat Biotechnol.* 2013;31:1126-1132.
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Salman V., Berben T., Bowers R. M., et al. Insights into the single cell draft genome of "Candidatus Achromatium palustre". *Stand Genomic Sci*. 2016;11:28.

Candidatus Achromatium palustre belong to the group of large sulfur bacteria that can grow to nearly 100 µm in size and store elemental sulfur (S0) intracellularly. As a unique feature, Achromatium spp. can accumulate colloidal calcite (CaCO3) inclusions in great amounts. All Achromatium spp. are unculturable. The authors used MDA and sequencing to produce a draft genome at 83% completion of a single Candidatus Achromatium palustre cell.

Illumina Technology: MiSeq System

Troell K., Hallstrom B., Divne A. M., et al. Cryptosporidium as a testbed for single cell genome characterization of unicellular eukaryotes. *BMC Genomics.* 2016;17:471.

To explore the potential use of single-cell genomics methodology for revealing genome-level variation in clinical samples from Cryptosporidium-infected hosts, the authors sorted individual oocysts for subsequent MDA and full-genome sequencing. The genomics workflow, starting directly from fecal samples, had an 80% success rate with 81% genome coverage.

Illumina Technology: MiSeq System

Ning L., Li Z., Wang G., et al. Quantitative assessment of single-cell whole genome amplification methods for detecting copy number variation using hippocampal neurons. *Sci Rep.* 2015;5:11415.

In this study, the authors compared MDA, multiple annealing and looping–based amplification cycles (MALBAC), and GenomePlex amplification methods in sequencing of individual hippocampal neurons. They amplified gDNA from individual hippocampal neurons with these methods, followed by sequencing at shallow depth on a HiSeq 2000 system. Their results showed that single-cell sequencing results from MALBAC and GenomePlex methods were highly reproducible and had high success rates. MALBAC did display significant GC bias, but it was overcome by using bioinformatics tools. Overall, the authors determined that MALBAC and GenomePlex had better performance in detecting CNVs compared to MDA.

Illumina Technology: HiSeq 2000 System

Brito I. L., Yilmaz S., Huang K., et al. Mobile genes in the human microbiome are structured from global to individual scales. *Nature*. 2016;535:435-439.

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Ottolini C. S., Capalbo A., Newnham L., et al. Generation of meiomaps of genome-wide recombination and chromosome segregation in human oocytes. *Nat Protoc.* 2016;11:1229-1243.

Peterson S. W., Knox N. C., Golding G. R., et al. A Study of the Infant Nasal Microbiome Development over the First Year of Life and in Relation to Their Primary Adult Caregivers Using cpn60 Universal Target (UT) as a Phylogenetic Marker. *PLoS One.* 2016;11:e0152493.

Thoendel M., Jeraldo P. R., Greenwood-Quaintance K. E., et al. Comparison of microbial DNA enrichment tools for metagenomic whole genome sequencing. *J Microbiol Methods.* 2016;127:141-145.

Bigdeli S., Dettloff R. O., Frank C. W., Davis R. W. and Crosby L. D. A simple method for encapsulating single cells in alginate microspheres allows for direct PCR and whole genome amplification. *PLoS One.* 2015;10:e0117738.

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Zhang C. Z., Spektor A., Cornils H., et al. Chromothripsis from DNA damage in micronuclei. *Nature*. 2015;522:179-184.

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

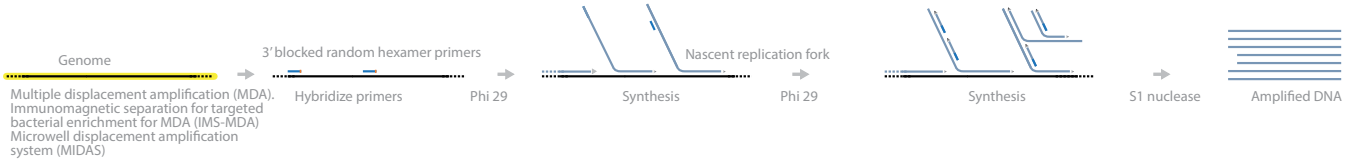
Nextera XT DNA Library Prep Kit

Nextera Rapid Capture Exome/Custom Enrichment Kit

MIDAS: Microwell displacement amplification system
IMS-MDA: Immunomagnetic Separation for Targeted Bacterial Enrichment for MDA
ddMDA: Digital Droplet MDA

Several variations on the original MDA method[107]—such as MIDAS,[108] ddMDA,[109] SNES,[110] and IMS-MDA[111]—have been developed to improve the amplification bias and throughput.[112]

Microwell and droplet displacement amplification systems are protocol variations of MDA in which single cells are randomly distributed into hundreds to thousands of nanoliter wells or droplets, and their genetic material is simultaneously amplified.[113] Sequencing individual cells simplifies the genomic diversity and reduces inherent amplification biases among cells. It results in improved representation and sensitivity in heterogeneous cell populations. In an alternative approach to simplify genomic diversity, IMS-MDA uses immunomagnetic separation for targeted bacterial enrichment.[114]



A schematic overview of MIDAS.

Advantages	Disadvantages
• Templates used for this method can be circular DNA (eg, plasmids, bacterial DNA) • Can sequence large templates • Can perform single-cell sequencing or sequencing for samples with limited amounts of starting material	• PCR biases can underrepresent GC-rich templates

Reviews

Zhang X., Marjani S. L., Hu Z., Weissman S. M., Pan X., et al. Single-Cell Sequencing for Precise Cancer Research: Progress and Prospects. *Cancer Res*. 2016;76:1305-1312.

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Rhee M., Light Y. K., Meagher R. J. and Singh A. K. Digital Droplet Multiple Displacement Amplification (ddMDA) for Whole Genome Sequencing of Limited DNA Samples. *PLoS One.* 2016;11:e0153699.

The researchers amplified *E. coli* genomes by performing tube MDA (20 µl total volume) and ddMDA (42 µl total volume, partitioned into ~300,000 droplets of 150 pl volume), with template DNA concentrations varying from 0.1 pg/µl to 100 pg/µl. They sequenced the same mass (1 ng) of amplicon prepared from each method. They found that ddMDA had a markedly improved quality of amplification compared to tube MDA at the same concentrations. This effect was most pronounced at low concentrations of template DNA.

Illumina Technology: MiSeq System, Nextera XT DNA Sample Prep Kit

Sidore A. M., Lan F., Lim S. W. and Abate A. R. Enhanced sequencing coverage with digital droplet multiple displacement amplification. *Nucleic Acids Res.* 2016;44:e66.

The authors demonstrated the ability to amplify and sequence the genomes of single *E. coli* cells with 4.7 fg of starting DNA. They obtained sequence coverage distributions similar to unamplified material.

Illumina Technology: MiSeq System

Lan F., Haliburton J. R., Yuan A. and Abate A. R. Droplet barcoding for massively parallel single-molecule deep sequencing. *Nat Commun*. 2016;7:11784.

To demonstrate the ability to sequence large DNA molecules, the researchers obtained synthetic read-lengths up to 10 kb from the *E. coli* genome. Single-molecule droplet barcoding (SMDB) was able to detect 457 SNPs in 81 haplotypes, while conventional short-read sequencing could only detect 1 SNP and could not generate haplotypes.

Illumina Technology: MiSeq System

Nishikawa Y., Hosokawa M., Maruyama T., et al. Monodisperse Picoliter Droplets for Low-Bias and Contamination-Free Reactions in Single-Cell Whole Genome Amplification. *PLoS One.* 2015;10:e0138733.

Whole-genome amplification (WGA) is a critical component of single-cell sequencing pipelines, and MDA is the most common WGA method in single-cell sequencing. Despite its widespread use, MDA typically produces uneven genome coverage due to amplification bias and the formation of DNA chimeras. To overcome this problem, the authors developed a droplet MDA method that minimizes these technical artifacts. They used microfluidics to compartmentalize extracted DNA fragments into 67 pl droplets, in which the individual fragments were amplified using MDA. This approach was validated by sequencing the droplet MDA products of *E. coli* cells, with genome recovery improving to 89% compared to 59% using traditional MDA.

Illumina Technology: MiSeq System, Nextera XT DNA Library Prep Kit

Bigdeli S., Dettloff R. O., Frank C. W., Davis R. W. and Crosby L. D. A simple method for encapsulating single cells in alginate microspheres allows for direct PCR and whole genome amplification. *PLoS One*. 2015;10:e0117738.

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Fu Y., Li C., Lu S., et al. Uniform and accurate single-cell sequencing based on emulsion whole-genome amplification. *Proc Natl Acad Sci U S A*. 2015;112:11923-11928.

Associated Kits

TruSeq Nano DNA Library Prep Kit

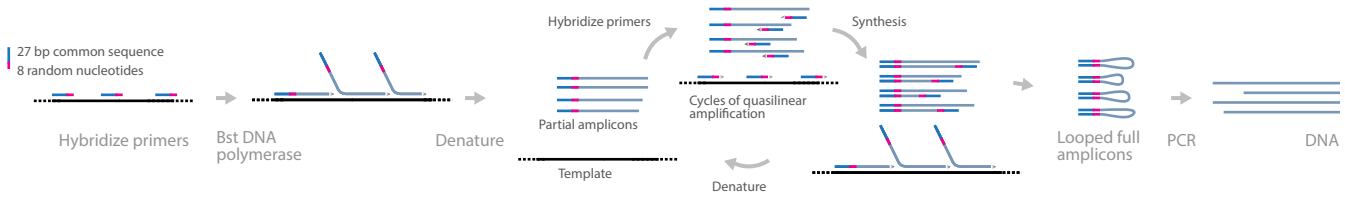
Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

MALBAC: Multiple Annealing and Looping–Based Amplification Cycles

MALBAC is intended to address some of the shortcomings of MDA.[115]

In this method, MALBAC primers randomly anneal to a DNA template. A polymerase with displacement activity at elevated temperatures amplifies the template, generating "semiamplicons." As the amplification and annealing process is repeated, the semiamplicons are amplified into full amplicons that have a 3' end complementary to the 5' end. As a result, full-amplicon ends hybridize to form a looped structure, inhibiting further amplification of the looped amplicon, while only the semiamplicons and gDNA undergo amplification. Deep sequencing of the full-amplicon sequences allows for accurate representation of reads, while sequencing depth provides improved alignment for consensus sequences. This method can also be applied to cDNA for transcriptome analysis. [116]



A schematic overview of MALBAC.

Advantages	Disadvantages
• Can sequence large templates • Can perform single-cell sequencing or sequencing for samples with limited amounts of starting material • Full-amplicon looping inhibits overrepresentation of templates, reducing PCR bias • Can amplify GC-rich regions • Provides uniform genome coverage • Lower allele dropout rate compared to MDAI	• Polymerase is relatively error prone, compared to Phi 29[117] • Temperature-sensitive protocol • Provides genome coverage up to ~90%,[118] but some regions of the genome are underrepresented consistently[119]

Reviews

Leung M. L., Wang Y., Kim C., et al. Highly multiplexed targeted DNA sequencing from single nuclei. *Nat Protoc*. 2016;11:214-235.

Zhang X., Marjani S. L., Hu Z., Weissman S. M., Pan X., et al. Single-Cell Sequencing for Precise Cancer Research: Progress and Prospects. *Cancer Res*. 2016;76:1305-1312.

Bowers R. M., Clum A., Tice H., et al. Impact of library preparation protocols and template quantity on the metagenomic reconstruction of a mock microbial community. *BMC Genomics.* 2015;16:856.

Grun D. and van Oudenaarden A. Design and Analysis of Single-Cell Sequencing Experiments. *Cell*. 2015;163:799-810.

Hou Y., Wu K., Shi X., Li F., Song L., et al. Comparison of variations detection between whole-genome amplification methods used in single-cell resequencing. *Gigascience*. 2015;4:37.

Li N., Wang L., Wang H., Ma M., Wang X., et al. The Performance of Whole Genome Amplification Methods and Next-Generation Sequencing for Pre-Implantation Genetic Diagnosis of Chromosomal Abnormalities. *J Genet Genomics.* 2015;42:151-159.

Saadatpour A., Lai S., Guo G. and Yuan G. C. Single-Cell Analysis in Cancer Genomics. *Trends Genet.* 2015;31:576-586.

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Snyder M. W., Adey A., Kitzman J. O. and Shendure J. Haplotype-resolved genome sequencing: experimental methods and applications. *Nat Rev Genet.* 2015;16:344-358.

Sun H. J., Chen J., Ni B., Yang X. and Wu Y. Z. Recent advances and current issues in single-cell sequencing of tumors. *Cancer Lett.* 2015;365:1-10.

Wang Y. and Navin N. E. Advances and applications of single-cell sequencing technologies. *Mol Cell.* 2015;58:598-609.

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Gui B., Yang P., Yao Z., et al. A New Next-Generation Sequencing-Based Assay for Concurrent Preimplantation Genetic Diagnosis of Charcot-Marie-Tooth Disease Type 1A and Aneuploidy Screening. *J Genet Genomics.* 2016;43:155-159.

Charcot-Marie-Tooth (CMT) disease is the most common hereditary neuropathy, with a population prevalence of 1 in 2500. CMT disease type 1A (CMT1A), accounting for ~70% of CMT1 cases and ~50% of all CMT cases, is transmitted in an autosomal dominant manner. The authors used a MALBAC single-cell WGA method for amplifying gDNA from a single cell. They showed that it is possible to detect the CMT1A 30 duplication, combined with screening of aneuploidy, in a single test.

Illumina Technology: HiSeq 2500 System

Mehetre G. T., Paranjpe A. S., Dastager S. G. and Dharne M. S. Complete metagenome sequencing based bacterial diversity and functional insights from basaltic hot spring of Unkeshwar, Maharashtra, India. *Genom Data.* 2016;7:140-143.

In this study, the authors analyzed the microbial community of the Unkeshwar basaltic hot springs, located in the South East Deccan Continental region of Maharashtra, India, using whole metagenome shotgun sequencing. They used MALBAC to enrich the low-abundant metagenomic DNA and created a complete representation of both abundant and rare members of the microbiome.

Illumina Technology: HiSeq 2500 System

Yan L., Huang L., Xu L., et al. Live births after simultaneous avoidance of monogenic diseases and chromosome abnormality by next-generation sequencing with linkage analyses. *Proc Natl Acad Sci U S A.* 2015;112:15964-15969.

NGS methods have improved the precision of preimplantation genetic screening and diagnosis. Although the precision has been limited by false-positive and false-negative SNVs, linkage analysis can overcome this challenge. In this study, the authors developed MARSALA, a method that combines NGS using the HiSeq System with single-cell WGA. The method allows for embryo diagnosis with single-molecule precision and reduces false-positive and false-negative errors significantly. Their method is the first integrated NGS-based preimplantation genetic diagnostic procedure that simultaneously detects disease-causing mutations and chromosome abnormalities, and performs linkage analyses.

Illumina Technology: HiSeq 2500 System

Briese M., Saal L., Appenzeller S., Moradi M., Baluapuri A., et al. Whole transcriptome profiling reveals the RNA content of motor axons. *Nucleic Acids Res.* 2016;44:e33.

Huang J., Yan L., Lu S., et al. Validation of a next-generation sequencing-based protocol for 24-chromosome aneuploidy screening of blastocysts. *Fertil Steril.* 2016;105:1532-1536.

Chapman A. R., He Z., Lu S., et al. Single cell transcriptome amplification with MALBAC. *PLoS One.* 2015;10:e0120889.

Dey S. S., Kester L., Spanjaard B., Bienko M. and van Oudenaarden A. Integrated genome and transcriptome sequencing of the same cell. *Nat Biotechnol.* 2015;33:285-289.

Ning L., Li Z., Wang G., Hu W., Hou Q., et al. Quantitative assessment of single-cell whole genome amplification methods for detecting copy number variation using hippocampal neurons. *Sci Rep.* 2015;5:11415.

Yan L., Huang L., Xu L., Huang J., Ma F., et al. Live births after simultaneous avoidance of monogenic diseases and chromosome abnormality by next-generation sequencing with linkage analyses. *Proc Natl Acad Sci U S A*. 2015;112:15964-15969.

Zhang C. Z., Adalsteinsson V. A., Francis J., et al. Calibrating genomic and allelic coverage bias in single-cell sequencing. *Nat Commun.* 2015;6:6822.

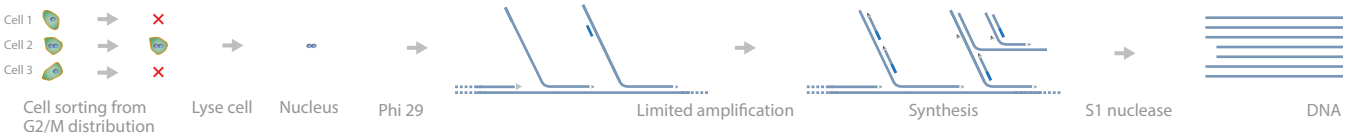
Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

nuc-seq: Single G2/M Nucleus Sequencing of Cells in S Phase
SNES: Single Nucleus Exome Sequencing

A modified MDA protocol, nuc-seq takes advantage of the fact that a single cell in the G2–M stage of the cell cycle has 4 copies of the genome. This property allows the cells to be isolated with a cell sorter, and it also significantly increases the genome coverage of single cells.[120] SNES is an additional variation that includes targeted selection and sequencing of the exomes.[121] Div-Seq is a variation that combines nuc-seq with pulse labeling of proliferating cells by 5-ethynyl-2'-deoxyuridine (EdU).[122]



A schematic overview of nuc-seq.

Methods	Advantages	Disadvantages
nuc-seq	• Improves physical coverage performance to more than 90% for single-cell sequencing	• Cannot be applied to cells with low proliferation rates
SNES	• 95.94% exome coverage in single cells • Detection efficiencies of 92.37% for SNVs in an isogenic population	• Limited to exomes

Reviews

Zhang X., Marjani S. L., Hu Z., Weissman S. M., Pan X., et al. Single-Cell Sequencing for Precise Cancer Research: Progress and Prospects. *Cancer Res.* 2016;76:1305-1312.

Leung M. L., Wang Y., Waters J. and Navin N. E. SNES: single nucleus exome sequencing. *Genome Biol.* 2015;16:55.

Saadatpour A., Lai S., Guo G. and Yuan G. C. Single-Cell Analysis in Cancer Genomics. *Trends Genet.* 2015;31:576-586.

Szulwach K. E., Chen P., Wang X., et al. Single-Cell Genetic Analysis Using Automated Microfluidics to Resolve Somatic Mosaicism. *PLoS One.* 2015;10:e0135007.

Voet T. and Van Loo P. SNES makes sense? Single-cell exome sequencing evolves. *Genome Biol.* 2015;16:86.

Wang Y. and Navin N. E. Advances and applications of single-cell sequencing technologies. *Mol Cell.* 2015;58:598-609.

References

Leung M. L., Wang Y., Waters J. and Navin N. E. SNES: single nucleus exome sequencing. *Genome Biol*. 2015;16:55.

Despite the great potential of single-cell sequencing methods to increase the understanding of tissue heterogeneity, current single-cell DNA-Seq methods are challenged by technical errors and poor physical coverage data. In this study, the authors developed SNES, a single-cell DNA-Seq method that combines flow-sorting of G1/0 or G2/M nuclei, time-limited MDA, exome capture using the TruSeq Exome Enrichment Kit, and sequencing on the HiSeq 2000 system. They validated SNES by sorting and sequencing single nuclei from a fibroblast cell line. The method generated 96% coverage of individual cells and demonstrated 92% detection efficiency for SNVs and 85% for indels in single cells.

Illumina Technology: HiSeq 2000 System, TruSeq Exome Enrichment Kit

120. Wang Y., Waters J., Leung M. L., et al. Clonal evolution in breast cancer revealed by single nucleus genome sequencing. Nature. 2014;512:155-160.
121. Leung M. L., Wang Y., Waters J. and Navin N. E. SNES: single nucleus exome sequencing. Genome Biol. 2015;16:55.
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Leung M. L., Wang Y., Kim C., Gao R., Jiang J., et al. Highly multiplexed targeted DNA sequencing from single nuclei. *Nat Protoc.* 2016;11:214-235.

This study describes a refinement to SNES and includes the addition of DNA barcoding to allow multiplexing of 48–96 individual cells into single sequencing reactions. Compared to SNES,[123] this new technique has higher throughput and reduced cost. The authors suggest that it is more suitable for detecting point mutations and indels at base-pair resolution.

Illumina Technology: HiSeq 2000 System

Habib N., Li Y., Heidenreich M., Swiech L., Trombetta J. J., et al. Div-Seq: A single nucleus RNA-Seq method reveals dynamics of rare adult newborn neurons in the CNS. *bioRxiv.* 2016;.

Associated Kits

TruSeq Nano DNA Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

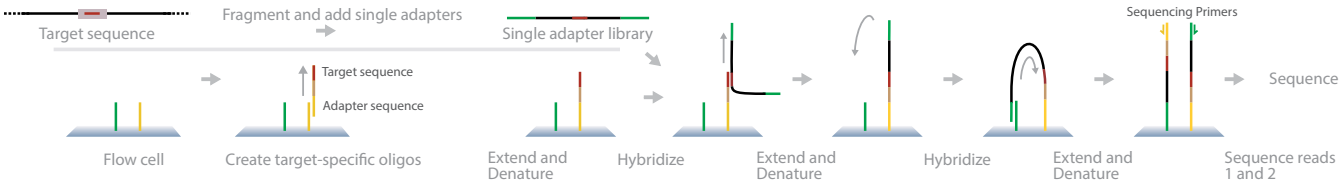
Nextera Rapid Capture Exome/Custom Enrichment Kit

123. Leung M. L., Wang Y., Waters J. and Navin N. E. SNES: single nucleus exome sequencing. *Genome Biol.* 2015;16:55.

OS-Seq: Oligonucleotide-Selective Sequencing

OS-Seq[124] was developed to improve targeted resequencing by capturing and sequencing gene targets directly on the flow cell.

In this method, target sequences with adapters are used to modify the flow cell primers. Targets in the template are captured onto the flow cell with the modified primers. Further extension, denaturation, and hybridization provide sequence reads for the target genes. Deep sequencing provides accurate representation of reads.



A schematic overview of OS-Seq.

Advantages	Disadvantages
• Can resequence multiple targets at a time • No gel excision or narrow size-purification required • Rapid, single-day protocol • Samples can be multiplexed • Reduced PCR bias due to removal of amplification steps • Avoids loss of material	• Primers may interact with similar target sequences, leading to sequence ambiguity

Reviews

None available yet.

References

Akinrinade O., Ollila L., Vattulainen S., et al. Genetics and genotype-phenotype correlations in Finnish patients with dilated cardiomyopathy. *Eur Heart J.* 2015;36:2327-2337.

The authors used OS-Seq to target the coding regions and splice junctions of 101 genes associated with cardiomyopathies in 145 unrelated Finnish patients with dilated cardiomyopathy (DCM). The diagnostic yield was 35.2% (familial 47.6% and sporadic 25.6%; P = 0.004) when both pathogenic and likely pathogenic variants are considered as disease-causing. Of these, 53% were titin (*TTN*) truncations affecting all *TTN* transcripts.

Illumina Technology: MiSeq System

Vattulainen S., Aho J., Salmenpera P., et al. Accurate genetic diagnosis of Finnish pulmonary arterial hypertension patients using oligonucleotide-selective sequencing. *Mol Genet Genomic Med.* 2015;3:354-362.

DNA samples from 21 Finnish patients with pulmonary arterial hypertension (PAH) with BMPR2 and ACVRL1 mutation status had been previously studied using Sanger sequencing. In this study, the authors used OS-Seq to screen for a panel of 7 PAH genes (*BMPR2, BMPR1B, ACVRL1, ENG, SMAD9, CAV1,* and *KCNK3*). The sequencing panel covered 100% of the targeted base pairs with > 15x sequencing depth. The authors identified pathogenic base substitutions in the *BMPR2* gene in 29% of the Finnish PAH cases. Two of the pathogenic variant–positive patients previously had tested negative using Sanger sequencing.

Illumina Technology: MiSeq System

Hopmans E. S., Natsoulis G., Bell J. M., et al. A programmable method for massively parallel targeted sequencing. *Nucleic Acids Res.* 2014;42:e88.

Associated Kits

TruSeq Nano DNA Library Prep Kit

124. Myllykangas S., Buenrostro J. D., Natsoulis G., Bell J. M. and Ji H. P. Efficient targeted resequencing of human germline and cancer genomes by oligonucleotide-selective sequencing. *Nat Biotechnol.* 2011;29:1024-1027.

Safe-SeqS: Safe-Sequencing System is a Unique Molecular Identifier (UMI) Approach to Detect Rare Variants

Safe-SeqS (or, more commonly, Safe-Seq) is an approach that uses unique molecular identifiers (UMIs) to detect rare variants.[125] In the time after the publication of the method in 2011, the use of UMIs has become ubiquitous, particularly in single-cell sequencing approaches, and the name of the method fell into disuse.

Safe-Seq assigns a UMI to each template molecule and amplifies each uniquely tagged template molecule to create UMI families. The abundance of each UMI can be used to distinguish between rare mutations and technical errors, as well as to correct for PCR amplification bias.

A schematic overview of Safe-SeqS.

Advantages	Disadvantages
• Distinguishes between rare mutations and technical errors • Detects 1 mutant template among 5000 to 1,000,000 wild-type templates[126] • Corrects for PCR amplification bias	• Can introduce spurious cross-hybridization • Complex protocol that requires a gel-purification step[127] • Uses 2 or 4 PCR cycles for barcode addition and does not satisfy the basic principle of labeling each molecule with a single, unique barcode[128]

Reviews

Gregory M. T., Bertout J. A., Ericson N. G., et al. Targeted single molecule mutation detection with massively parallel sequencing. *Nucleic Acids Res.* 2016;44:e22.

Wang K., Ma X., Zhang X., et al. Using ultra-sensitive next generation sequencing to dissect DNA damage-induced mutagenesis. *Sci Rep*. 2016;6:25310.

Belic J., Koch M., Ulz P., et al. Rapid Identification of Plasma DNA Samples with Increased ctDNA Levels by a Modified FAST-SeqS Approach. *Clin Chem*. 2015;61:838-849.

Do H. and Dobrovic A. Sequence artifacts in DNA from formalin-fixed tissues: causes and strategies for minimization. *Clin Chem.* 2015;61:64-71.

Heitzer E., Ulz P. and Geigl J. B. Circulating tumor DNA as a liquid biopsy for cancer. *Clin Chem.* 2015;61:112-123.

Maslov A. Y., Quispe-Tintaya W., Gorbacheva T., White R. R. and Vijg J. High-throughput sequencing in mutation detection: A new generation of genotoxicity tests? *Mutat Res.* 2015;776:136-143.

Patel K. M. and Tsui D. W. The translational potential of circulating tumour DNA in oncology. Clin Biochem. 2015;48:957-961.

125. Kinde I., Wu J., Papadopoulos N., Kinzler K. W. and Vogelstein B. Detection and quantification of rare mutations with massively parallel sequencing. *Proc Natl Acad Sci U S A.* 2011;108:9530-9535.
126. Kinde I., Bettegowda C., Wang Y., et al. Evaluation of DNA from the Papanicolaou test to detect ovarian and endometrial cancers. *Sci Transl Med.* 2013;5:167ra164.
127. Stahlberg A., Krzyzanowski P. M., Jackson J. B., Egyud M., Stein L. and Godfrey T. E. Simple, multiplexed, PCR-based barcoding of DNA enables sensitive mutation detection in liquid biopsies using sequencing. *Nucleic Acids Res*. 2016;44:e105.
128. Kukita Y., Matoba R., Uchida J., et al. High-fidelity target sequencing of individual molecules identified using barcode sequences: de novo detection and absolute quantitation of mutations in plasma cell-free DNA from cancer patients. *DNA Res*. 2015;22:269-277.

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Dal Molin M., Zhang M., de Wilde R. F., et al. Very Long-term Survival Following Resection for Pancreatic Cancer Is Not Explained by Commonly Mutated Genes: Results of Whole-Exome Sequencing Analysis. *Clin Cancer Res.* 2015;21:1944-1950.

The authors sequenced the *BRAF, CDKN2A, GNAS, KRAS, PIK3CA, RNF43, SMAD4, TP53,* and *VHL* genes using Safe-SeqS in a panel of 27 additional surgically resected ductal adenocarcinomas of the pancreas, obtained from very long-term survivors. *KRAS* was the most commonly mutated gene, as alterations were found in 27 of 27 (100%) of these validation cancers.

Illumina Technology: Genome Analyzer$_{IIx}$ System

Eboreime J., Choi S. K., Yoon S. R., Arnheim N. and Calabrese P. Estimating Exceptionally Rare Germline and Somatic Mutation Frequencies via Next Generation Sequencing. *PLoS One.* 2016;11:e0158340.

The authors used Safe-SeqS to measure ultra-rare de novo mutation frequencies in the human male germline. The results showed that the average background frequency of some mutation types was at 1.5 x 10^{-6} per base, which far exceeded the well-documented human genome average frequency per base pair (~10^{-8}). This result suggested a nonbiological explanation for the data. The authors suggest that most of the base-dependent variation in background frequency is due to a mixture of deamination and oxidation during the first 2 PCR cycles.

Illumina Technology: HiSeq 2000 system

Wang Y., Springer S., Mulvey C. L., et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. Sci Transl Med. 2015;7:293ra104.

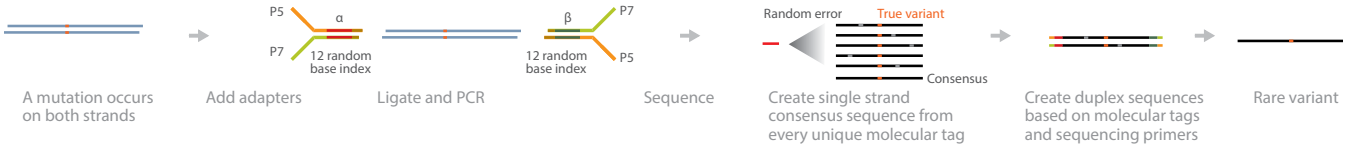
Associated Kits

TruSeq Nano DNA Library Prep Kit

Duplex-Seq: Duplex Sequencing

Duplex-Seq is a tag-based, error-correction method to improve sequencing accuracy.[129]

In this method, adapters (with primer sequences and random 12 bp indexes) are ligated onto the template and amplified using PCR. Deep sequencing provides consensus sequence information from every unique molecular tag. Based on the molecular tags and sequencing primers, duplex sequences are aligned, determining the true sequence on each DNA strand. The method is estimated to be > 10,000-fold more accurate than conventional NGS.[130] A targeted version of duplex sequencing includes 2 rounds of capture to yield read depth as high as 1,000,000x.[131]



A schematic overview of Duplex-Seq.

Advantages	Disadvantages
• Can detect a single point mutation among > 107 sequenced nucleotides[132] • Very low error rate due to duplex tagging • PCR amplification errors can be detected and removed from analysis • No additional library preparation steps after addition of adapters	• Complex library construction protocols[133]

Reviews

Gregory M. T., Bertout J. A., Ericson N. G., Taylor S. D., Mukherjee R., et al. Targeted single molecule mutation detection with massively parallel sequencing. *Nucleic Acids Res.* 2016;44:e22.

Do H. and Dobrovic A. Sequence artifacts in DNA from formalin-fixed tissues: causes and strategies for minimization. *Clin Chem.* 2015;61:64-71.

Maslov A. Y., Quispe-Tintaya W., Gorbacheva T., White R. R. and Vijg J. High-throughput sequencing in mutation detection: A new generation of genotoxicity tests? *Mutat Res.* 2015;776:136-143.

129. Schmitt M. W., Kennedy S. R., Salk J. J., Fox E. J., Hiatt J. B. and Loeb L. A. Detection of ultra-rare mutations by next-generation sequencing. *Proc Natl Acad Sci U S A.* 2012;109:14508-14513.
130. Ahn E. H., Hirohata K., Kohrn B. F., Fox E. J., Chang C. C. and Loeb L. A. Detection of Ultra-Rare Mitochondrial Mutations in Breast Stem Cells by Duplex Sequencing. *PLoS One*. 2015;10:e0136216.
131. Schmitt M. W., Fox E. J., Prindle M. J., et al. Sequencing small genomic targets with high efficiency and extreme accuracy. *Nat Methods.* 2015;12:423-425.
132. Schmitt M. W., Kennedy S. R., Salk J. J., Fox E. J., Hiatt J. B. and Loeb L. A. Detection of ultra-rare mutations by next-generation sequencing. *Proc Natl Acad Sci U S A.* 2012;109:14508-14513, Schmitt M. W., Fox E. J., Prindle M. J., et al. Sequencing small genomic targets with high efficiency and extreme accuracy. *Nat Methods*. 2015;12:423-425.
133. Stahlberg A., Krzyzanowski P. M., Jackson J. B., Egyud M., Stein L. and Godfrey T. E. Simple, multiplexed, PCR-based barcoding of DNA enables sensitive mutation detection in liquid biopsies using sequencing. *Nucleic Acids Res.* 2016;44:e105.

References

Krimmel J. D., Schmitt M. W., Harrell M. I., et al. Ultra-deep sequencing detects ovarian cancer cells in peritoneal fluid and reveals somatic TP53 mutations in noncancerous tissues. *Proc Natl Acad Sci U S A.* 2016;113:6005-6010.

The authors used Duplex-Seq to analyze TP53 mutations in 17 peritoneal fluid samples from women with high-grade serous ovarian carcinomas (HGSOCs) and 20 samples from women without cancer. The tumor TP53 mutation was detected in 94% (16/17) of peritoneal fluid samples from women with HGSOC, at a frequency as low as 1 mutant per 24,736 normal genomes.

Illumina Technology: HiSeq 2500 System

Ahn E. H., Hirohata K., Kohrn B. F., Fox E. J., Chang C. C., et al. Detection of Ultra-Rare Mitochondrial Mutations in Breast Stem Cells by Duplex Sequencing. *PLoS One.* 2015;10:e0136216.

mtDNA is vulnerable to damage mediated by reactive oxygen species (ROS), and it is more prone to accumulating mutations than nuclear DNA. This study presents an ultra-deep mutation analysis for the whole mtDNA genome in human breast normal epithelial cells (non–stem cells vs stem cells) using Duplex-Seq. The results indicated that stochastic rare mutation frequency was lower in stem cells than in the corresponding non–stem cells. The authors conclude that the mitochondrial genome is maintained with greater fidelity in stem cells than in non–stem cells.

Illumina Technology: HiSeq 2500 System

Schmitt M. W., Fox E. J., Prindle M. J., Reid-Bayliss K. S., True L. D., et al. Sequencing small genomic targets with high efficiency and extreme accuracy. *Nat Methods.* 2015;12:423-425.

To detect rare mutations, the researchers adapted the Duplex-Seq protocol to include 2 rounds of capture, essentially creating a single-gene targeted duplex sequencing method. The double-capture approach resulted in extremely high depth and uniformity of coverage up to 1,000,000x depth, with average and minimum depths of 830,000x and 250,000x, respectively. This approach yielded an average of more than 1000 unique DNA molecules sampled at every nucleotide position within the ABL1 gene target.

The authors used their protocol to sequence the ABL1 gene from an individual with chronic myeloid leukemia who relapsed after treatment with the targeted therapy, imatinib. This individual did not show any ABL1 mutations with conventional high-throughput sequencing. In contrast, Duplex-Seq revealed a single mutation with a mutant fraction of 1%. This mutation, E279K, confers imatinib resistance.

Illumina Technology: HiSeq 2500 System

Associated Kits

TruSeq Nano DNA Library Prep Kit

DR-Seq: DNA-mRNA Sequencing

DR-Seq studies the genomic and transcriptomic relationship in single cells.[134] Nucleic acid amplification prior to physical separation reduces sample loss and the risk of contamination. DR-Seq involves multiple amplification steps, including a quasilinear amplification technique similar to MALBAC.

First, mRNA from lysed single-cells is reverse-transcribed using poly(dT) primers with Ad-1x adapters, producing single-stranded cDNA. The Ad-1x adapter sequence contains cell-identifying barcodes, 5' Illumina adaptors, and a T7 promoter. Next, both gDNA and sscDNA are simultaneously amplified via quasilinear WGA with Ad-2 primers. These primers are similar to MALBAC adapters, containing an 8 nt random sequence for random priming followed by a constant 27 nt tag at the 5' end. The products of this amplification step are split into 2 batches. One half is prepared for genome sequencing, in which gDNA is PCR-amplified and liberated from the Ad-2 adapters before DNA library preparation and sequencing. The other half is used for transcriptome sequencing, where double-stranded cDNA is synthesized and RNA is amplified by *in vitro* transcription. The resulting amplified RNA is produced only from cDNA fragments flanked with Ad-1x and Ad-2, suppressing amplification of the gDNA fragments. The RNA library is prepared for sequencing following the Illumina small-RNA protocol. Sequencing gDNA and mRNA from the same cell preserves information between the genome and its expression levels.

A schematic overview of DR-Seq.

Advantages	Disadvantages
• Interrogates genomic and transcriptomic behavior from a single cell • Amplification prior to separation reduces sample loss and contamination • Length-based identifier used to remove duplicate reads • Quasilinear amplification reduces PCR bias	• Manual single-cell isolation prevents high-throughput adaptation • Quasilinear amplification is temperature-sensitive • RNA reads are 3'-end-biased[135]

Reviews

Zhang X., Marjani S. L., Hu Z., Weissman S. M., Pan X., et al. Single-Cell Sequencing for Precise Cancer Research: Progress and Prospects. *Cancer Res*. 2016;76:1305-1312.

Saadatpour A., Lai S., Guo G. and Yuan G. C. Single-Cell Analysis in Cancer Genomics. *Trends Genet*. 2015;31:576-586.

References

Dey S. S., Kester L., Spanjaard B., Bienko M. and van Oudenaarden A. Integrated genome and transcriptome sequencing of the same cell. *Nat Biotechnol*. 2015;33:285-289.

In this study, the authors describe the original DR-Seq method. They amplified gDNA and cDNA from mouse embryonic stem cells (ESCs), subsequently divided the nucleic acids for further amplification and library construction, and sequenced both libraries using a HiSeq 2500 system. They demonstrated that genes with high cell-to-cell variability in transcript numbers have low CNVs, and *vice versa*.

Illumina Technology: HiSeq 2500 System

Associated Kits

TruSeq Small RNA Library Prep Kit

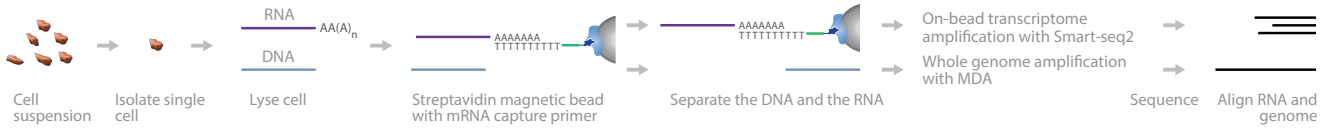
TruSeq Nano DNA Library Prep Kit

134. Dey S. S., Kester L., Spanjaard B., Bienko M. and van Oudenaarden A. Integrated genome and transcriptome sequencing of the same cell. *Nat Biotechnol*. 2015;
135. Macaulay I. C., Haerty W., Kumar P., et al. G&T-seq: parallel sequencing of single-cell genomes and transcriptomes. *Nat Methods*. 2015;

G&T-Seq: Genome and Transcriptome Sequencing

G&T-seq can separate and sequence gDNA and full-length mRNA from single cells.[136]

In this method, single cells are isolated and lysed. RNA is captured using biotinylated oligo(dT) capture primers and separated from DNA using streptavidin-coated magnetic beads. Smart-seq2 is used to amplify captured RNA on the bead, while MDA is used to amplify DNA. After sequencing, integrating the DNA and RNA sequences provides insights into the gene expression profiles of single cells.



A schematic overview of G&T-seq.

Advantages	Disadvantages
• Compatible with any WGA method • No 3'-end bias in sequence reads because full-length transcripts are captured • Because DNA and RNA are physically separated and amplified independently, there is no need to mask coding sequences during analysis	• Physical separation of DNA and RNA can increase risk of sample loss or contamination • Increased handling time

Reviews

Zhang X., Marjani S. L., Hu Z., Weissman S. M., Pan X., et al. Single-Cell Sequencing for Precise Cancer Research: Progress and Prospects. *Cancer Res*. 2016;76:1305-1312.

Saadatpour A., Lai S., Guo G. and Yuan G. C. Single-Cell Analysis in Cancer Genomics. *Trends Genet.* 2015;31:576-586.

References

Macaulay I. C., Haerty W., Kumar P., et al. G&T-seq: parallel sequencing of single-cell genomes and transcriptomes. *Nat Methods.* 2015;12:519-522.

The authors performed G&T-seq-enabled transcriptome analysis using a modified Smart-Seq2 protocol,[137,138] and they automated the method on a robotic liquid-handling platform. The authors sequenced human cancer cells, reversine-treated mouse embryo blastomeres, and neurons derived from induced pluripotent stem cells (iPSCs). Notably, G&T-seq analysis of aneuploid blastomeres demonstrated that chromosomal gains/losses led to increases/losses in chromosome-wide relative gene expression, during a single cell division.

Illumina Technology: MiSeq System, HiSeq 2500 System, HiSeq X Ten System, Nextera XT DNA Library Prep Kit

Associated Kits

TruSeq RNA Library Prep Kit v2

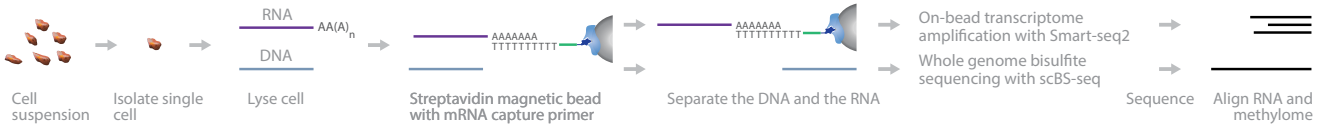
TruSeq Nano DNA Library Prep Kit

136. Macaulay I. C., Haerty W., Kumar P., et al. G&T-seq: parallel sequencing of single-cell genomes and transcriptomes. *Nat Methods*. 2015;
137. Picelli S., Bjorklund A. K., Faridani O. R., Sagasser S., Winberg G. and Sandberg R. Smart-seq2 for sensitive full-length transcriptome profiling in single cells. *Nat Methods*. 2013;10:1096-1098.
138. Picelli S., Faridani O. R., Bjorklund A. K., Winberg G., Sagasser S. and Sandberg R. Full-length RNA-seq from single cells using Smart-seq2. *Nat Protoc.* 2014;9:171-181.

scM&T-Seq: Single-Cell Methylome and Transcriptome Sequencing

scM&T-seq allows parallel analysis of both epigenetic and gene expression patterns from single cells using Smart-seq2 and scBS-seq.[139] scM&T-Seq is based on G&T-seq, but instead of using MDA for DNA sequencing, it uses scBS-Seq to determine DNA methylation patterns.

Single cells are isolated and individually lysed. The mRNAs are captured with streptavidin-coupled mRNA capture primers to separate them physically from the DNA strands. Smart-seq2 uses RT with template switching and tagmentation to generate cDNA libraries from the mRNA. DNA libraries are prepared via scBS-seq, which involves bisulfite conversion of DNA strands to identify methylated cytosines. Both libraries are ready for sequencing.



A schematic overview of scM&T-seq.

Advantages	Disadvantages
• Investigates links between epigenetic and transcriptional heterogeneity in single cells • Because DNA and RNA are physically separated and amplified independently, there is no need to mask coding sequences during analysis	• Smart-seq2 is not strand-specific and applicable to only poly(A)$^+$ RNA • Does not distinguish between 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC)

Reviews

Clark S. J., Lee H. J., Smallwood S. A., Kelsey G. and Reik W. Single-cell epigenomics: powerful new methods for understanding gene regulation and cell identity. *Genome Biol.* 2016;17:72.

Wen L. and Tang F. Single-cell sequencing in stem cell biology. *Genome Biol.* 2016;17:71.

References

Angermueller C., Clark S. J., Lee H. J., Macaulay I. C., Teng M. J., et al. Parallel single-cell sequencing links transcriptional and epigenetic heterogeneity. *Nat Methods.* 2016;13:229-232.

The authors used the G&T-seq protocol to purify single-cell DNA that was then subjected to single-cell bisulfite sequencing (scBS-seq).[140] They performed single-cell methylome and transcriptome sequencing (scM&T-seq) on 61 mouse ESCs. They found that gene expression levels of many pluripotency factors were negatively associated with DNA methylation. These data demonstrate that epigenetic heterogeneity is an important mechanism of fluctuating pluripotency in ESCs. They also demonstrate that scM&T-seq can reveal the relationship between transcriptional and DNA-methylation heterogeneity in single cells.

Illumina **Technology**: HiSeq 2000 System, Nextera XT DNA Library Prep Kit

Associated Kits

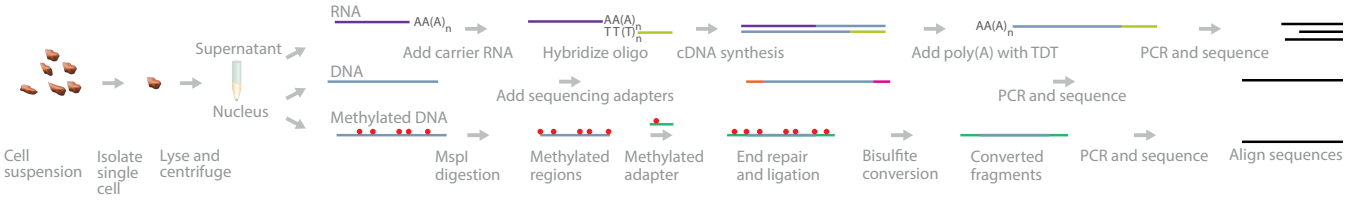
Nextera XT DNA Library Preparation Kit

139. Angermueller C., Clark S. J., Lee H. J., et al. Parallel single-cell sequencing links transcriptional and epigenetic heterogeneity. *Nat Methods*. 2016;13:229-232.
140. Smallwood S. A., Lee H. J., Angermueller C., et al. Single-cell genome-wide bisulfite sequencing for assessing epigenetic heterogeneity. *Nat Methods*. 2014;11:817-820.

scTrio-seq: Single-Cell Triple Omics Sequencing

scTrio-seq can analyze genomic CNVs, the DNA methylome, and the transcriptome of an individual mammalian cell simultaneously.[141] This approach is an extension of previous methods, such as scMT-seq.[142]

A single cell is lysed with a mild lysis buffer and the lysis product centrifuged. The supernatant is transferred to a new tube for transcriptome sequencing analyses, while the pellet (containing the nucleus) is bisulfite-converted for genome and epigenome sequencing.



A schematic overview of scTrio-seq.

Advantages	Disadvantages
• Accurately analyzes the mechanism by which the transcriptome, genome, and DNA methylome regulate each other • CNVs can be identified reliably using single-cell reduced-representation bisulfite sequencing (scRRBS) data	• Lower transcriptome coverage than scMT-seq[141] • Results in 3'-biased transcriptome

Reviews

None available yet.

References

Hou Y., Guo H., Cao C., Li X., Hu B., et al. Single-cell triple omics sequencing reveals genetic, epigenetic, and transcriptomic heterogeneity in hepatocellular carcinomas. Cell Res. 2016;26:304-319.

The authors used scTrio-seq to analyze 25 single cells derived from a human hepatocellular carcinoma (HCC) tissue sample. They found 2 subpopulations distinct in DNA copy numbers, DNA methylation, and RNA expression levels. By comparing the differences between the 2 HCC subpopulations, the researchers discovered that the minor subpopulation I, which harbored more copy-gain CNVs, expressed more invasive cell markers and was more likely to evade immune surveillance.

Illumina Technology: HiSeq 2000 System, HiSeq 2500 System

Associated Kits

TruSeq Nano DNA Library Prep Kit

Nextera DNA Library Prep Kit

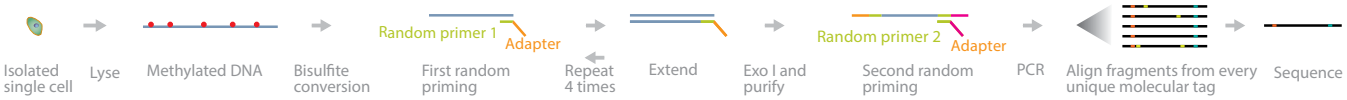
Nextera XT DNA Library Prep Kit

141. Hou Y., Guo H., Cao C., et al. Single-cell triple omics sequencing reveals genetic, epigenetic, and transcriptomic heterogeneity in hepatocellular carcinomas. *Cell Res.* 2016;26:304-319.
142. Hu Y., Huang K., An Q., et al. Simultaneous profiling of transcriptome and DNA methylome from a single cell. *Genome Biol*. 2016;17:88.

scBS-Seq: Single-Cell Bisulfite Sequencing

scBS-seq is a version of the well-established bisulfite sequencing (BS-seq) and post-bisulfite adapter tagging (PBAT) protocols, modified to detect methylated cytosines in gDNA from single cells.[143]

In this method, after single cells are isolated, gDNA is treated with sodium bisulfite, which fragments the DNA. The converted DNA then undergoes random priming several times and is PCR-amplified for sequencing. Deep sequencing provides single-nucleotide resolution mapping of methylated cytosines.



A schematic overview of scBS-seq.

Advantages	Disadvantages
• Measure DNA methylation of up to 48% (10.1 M) CpG sites within a single cell	• DNA degradation after bisulfite step[144]

Reviews

Pisanic T. R., 2nd, Athamanolap P. and Wang T. H. Defining, distinguishing and detecting the contribution of heterogeneous methylation to cancer heterogeneity. *Semin Cell Dev Biol.* 2016;.

Yong W. S., Hsu F. M. and Chen P. Y. Profiling genome-wide DNA methylation. *Epigenetics Chromatin*. 2016;9:26.

Greenleaf W. J. Assaying the epigenome in limited numbers of cells. *Methods*. 2015;72:51-56.

Liang J., Cai W. and Sun Z. Single-cell sequencing technologies: current and future. *J Genet Genomics.* 2014;41:513-528.

Plongthongkum N., Diep D. H. and Zhang K. Advances in the profiling of DNA modifications: cytosine methylation and beyond. *Nat Rev Genet.* 2014;15:647-661.

References

Smallwood S. A., Lee H. J., Angermueller C., Krueger F., Saadeh H., et al. Single-cell genome-wide bisulfite sequencing for assessing epigenetic heterogeneity. *Nat Methods.* 2014;11:817-820.

The authors tested the performance of scBS-seq on ovulated metaphase II mouse oocytes. They obtained methylation scores on an average of 3.7 million CpG dinucleotides, corresponding to 17.7% of all CpGs.

Illumina Technology: HiSeq 2500 System

Associated Kits

TruSeq DNA Methylation Kit

TruSeq Nano DNA Library Prep Kit

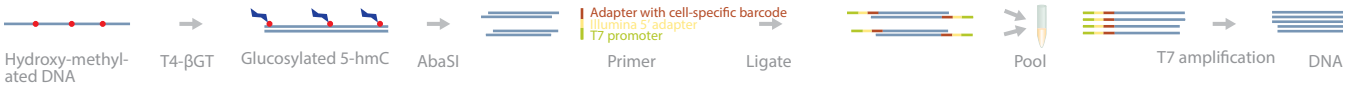
TruSeq DNA PCR-Free Library Prep Kit

143. Smallwood S. A., Lee H. J., Angermueller C., et al. Single-cell genome-wide bisulfite sequencing for assessing epigenetic heterogeneity. *Nat Methods*. 2014;11:817-820.
144. Yong W. S., Hsu F. M. and Chen P. Y. Profiling genome-wide DNA methylation. *Epigenetics Chromatin*. 2016;9:26.

scAba-seq: Detect 5hmC Marks in Single Cells With AbaSI Nuclease

scAba-seq is a single-cell, genome-wide, and strand-specific 5hmC sequencing technology.[145]

5hmC marks in DNA from individual cells are glucosylated with T4 phage β-glucosyltransferase (T4-βGT), and the DNA is digested with the restriction endonuclease AbaSI. The digested DNA is ligated to an adapter containing a cell-specific barcode, an Illumina 5' adapter, and a T7 promoter. The ligated DNA from different cells is pooled and amplified using *in vitro* transcription mediated by T7 RNA polymerase.



A schematic overview of scAba-seq.

Advantages	Disadvantages
• Determines strand-specific 5hmC marks in individual cells	• Ambiguity can exist in 13% of the cleaved molecules[146] • Sequence bias in AbaSI activity[147]

Reviews

None available yet.

References

Mooijman D., Dey S. S., Boisset J. C., Crosetto N. and van Oudenaarden A. Single-cell 5hmC sequencing reveals chromosome-wide cell-to-cell variability and enables lineage reconstruction. *Nat Biotechnol.* 2016;34:852-856.

In this study, the authors describe the development of the scAba-seq method. They applied it in 2- and 4-cell mouse embryos to reconstruct their lineage. Their analysis of individual chromosomes in the sister cells of the 2-cell embryos showed a strong anticorrelation between the bias of the chromosomes from sister cell 1 and sister cell 2. This observation is consistent with each sister cell receiving an old strand, containing a high level of 5hmC marks, and a new strand containing fewer 5hmC marks.

Illumina Technology: NextSeq 500 System

Associated Kits

TruSeq Nano DNA Library Prep Kit

145. Mooijman D., Dey S. S., Boisset J. C., Crosetto N. and van Oudenaarden A. Single-cell 5hmC sequencing reveals chromosome-wide cell-to-cell variability and enables lineage reconstruction. *Nat Biotechnol.* 2016;34:852-856.
146. Serandour A. A., Avner S., Mahe E. A., et al. Single-CpG resolution mapping of 5-hydroxymethylcytosine by chemical labeling and exonuclease digestion identifies evolutionarily unconserved CpGs as TET targets. *Genome Biol.* 2016;17:56.

Single-cell RC-Seq: Single-Cell Retrotransposon Capture Sequencing

Single-cell RC-seq uses sequence capture to enrich DNA for the junctions between retrotransposon termini and adjacent genomic regions, followed by paired-end sequencing.[148]

In this method, nuclei are purified first by fluorescence-activated cell sorting (FACS) and then picked with a micromanipulator. The DNA is extracted from nuclei and subjected to linear WGA, followed by exponential PCR in 2 separate reactions for each nucleus, using different enzymes. The products are combined and analyzed via WGS to assess genome coverage. The libraries prepared are enriched by hybridization to locked nucleic acid (LNA) probes and sequenced.[147]

A schematic overview of single-cell RC-seq.

Advantages	Disadvantages
• Detects somatic mutations in single cells	• Low throughput due to manual single-nucleus isolation

Reviews

None available yet.

References

Upton K. R., Gerhardt D. J., Jesuadian J. S., Richardson S. R., Sanchez-Luque F. J., et al. Ubiquitous L1 mosaicism in hippocampal neurons. *Cell*. 2015;161:228-239.

The authors carried out single-cell RC-seq on individual human hippocampal neurons and glia, as well as cortical neurons. They found that an estimated 13.7 somatic long interspersed nuclear elements (LINE-1 or L1) insertions occurred per hippocampal neuron and carried the sequence hallmarks of target-primed reverse transcription. Hippocampal neuron L1 insertions were specifically enriched in transcribed neuronal stem cell enhancers and hippocampus genes, which may indicate that they are functionally relevant.

Illumina Technology: MiSeq System

Associated Kits

TruSeq Nano DNA Library Prep Kit

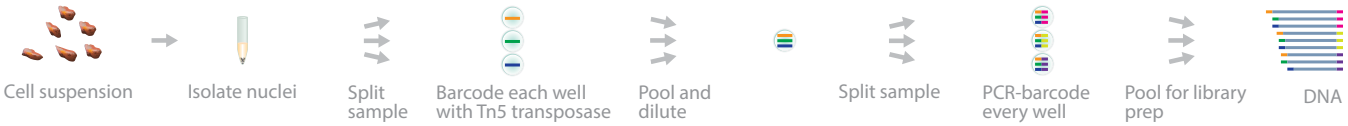
Nextera DNA Library Prep Kit

147. Sun Z., Dai N., Borgaro J. G., et al. A sensitive approach to map genome-wide 5-hydroxymethylcytosine and 5-formylcytosine at single-base resolution. *Mol Cell*. 2015;57:750-761.
148. Upton K. R., Gerhardt D. J., Jesuadian J. S., et al. Ubiquitous L1 mosaicism in hippocampal neurons. *Cell*. 2015;161:228-239.

scATAC-Seq (Cell index variation): Single-Cell Assay for Transposase-Accessible Chromatin

This version of scATAC-seq uses combinatorial cellular indexing to measure chromatin accessibility in thousands of single cells per assay. This method avoids the need for compartmentalization of individual cells, which makes the system scalable to analyze thousands of cells at a time.[149]

In this method, nuclei are isolated and molecularly tagged in bulk with barcoded Tn5 transposases in wells. Next, the nuclei are pooled and a limited number redistributed into a second set of wells. A second barcode is introduced during the PCR amplification step, and the fragments are pooled for library preparation and sequencing.



A schematic overview of scATAC-seq: combinatorial indexing version.

Advantages	Disadvantages
• High throughput and scalable	• None reported

Reviews

Liu S. and Trapnell C. Single-cell transcriptome sequencing: recent advances and remaining challenges. *F1000Res.* 2016;5:.

Lu F., Liu Y., Inoue A., et al. Establishing Chromatin Regulatory Landscape during Mouse Preimplantation Development. *Cell*. 2016;165:1375-1388.

References

Corces M. R., Buenrostro J. D., Wu B., et al. Lineage-specific and single-cell chromatin accessibility charts human hematopoiesis and leukemia evolution. *Nat Genet.* 2016;48:1193-1203.

This study used an improved scATAC-seq protocol, named Fast-ATAC, to create a comprehensive map of chromatin accessibility and transcriptional landscapes of 13 human primary blood cell types in a total of 137 samples. The authors found that single acute myeloid leukemia (AML) cells exhibited distinctive mixed regulome profiles, corresponding to disparate developmental stages, possibly caused by HOX factors. This result could provide insights into hematopoietic development and disease.

Illumina Technology: HiSeq 2000 System, HiSeq 2500 System, NextSeq 500 System

Cusanovich D. A., Daza R., Adey A., Pliner H. A., Christiansen L., et al. Multiplex single cell profiling of chromatin accessibility by combinatorial cellular indexing. *Science.* 2015;348:910-914.

The researchers applied combinatorial cellular indexing with scATAC-seq to measure chromatin accessibility in thousands of single cells per assay and reported chromatin accessibility maps for > 15,000 single cells. They predicted that their combinatorial cellular indexing scheme could be scaled feasibly to collect data from ~17,280 cells per experiment by using 384-by-384 barcoding and sorting 100 nuclei per well.

Illumina Technology: MiSeq System

Associated Kits

Nextera DNA Library Prep Kit

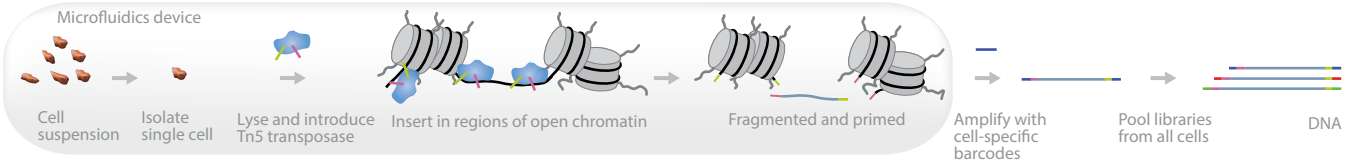
Nextera XT DNA Library Prep Kit

149. Cusanovich D. A., Daza R., Adey A., et al. Multiplex single cell profiling of chromatin accessibility by combinatorial cellular indexing. *Science*. 2015;348:910-914.

scATAC-Seq (Microfluidics variation): Single-Cell Assay for Transposase-Accessible Chromatin

This version of scATAC-seq is integrated into a programmable microfluidics platform.[150] The method is similar to scATAC-seq (combinatorial indexing version), which relies on indexing to identify single cells.[151]

In this protocol the cells are captured in a fluidics device, lysed, and treated with Tn5 transposases. The ends of the Tn5 fragments are extended, and the fragments are PCR-amplified with dual-index primers, pooled, purified, and sequenced.



A schematic overview of scATAC-seq: microfluidics version.

Advantages	Disadvantages
• Maps the accessible genome of individual cells	• Requires dedicated fluidics

Reviews

Liu S. and Trapnell C. Single-cell transcriptome sequencing: recent advances and remaining challenges. *F1000Res*. 2016;5:.

Lu F., Liu Y., Inoue A., Suzuki T., Zhao K., et al. Establishing Chromatin Regulatory Landscape during Mouse Preimplantation Development. *Cell*. 2016;165:1375-1388.

References

Buenrostro J. D., Wu B., Litzenburger U. M., Ruff D., Gonzales M. L., et al. Single-cell chromatin accessibility reveals principles of regulatory variation. Nature. 2015;523:486-490.

Using scATAC-seq (microfluidics version), the researchers investigated single-cell epigenomic heterogeneity and were able to link cis- and trans-effectors to variability in accessibility profiles within individual epigenomes. They also identified trans-factors associated with increased accessibility variance. Other trans-factors, such as CTCF, appeared to buffer variability, perhaps by providing a stable anchor for chromatin accessibility or an insulator function that dampens potential fluctuations.

Illumina Technology: MiSeq System, HiSeq 2500 System, NextSeq 500 System

Associated Kits

Nextera DNA Library Prep Kit

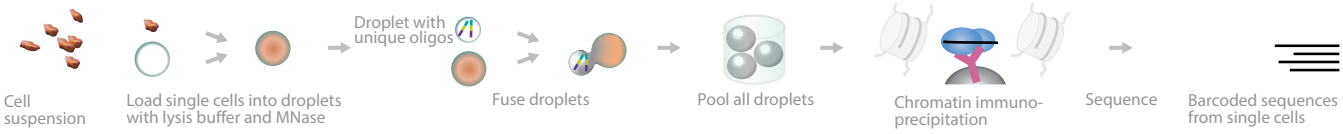
Nextera XT DNA Library Prep Kit

150. Buenrostro J. D., Wu B., Litzenburger U. M., et al. Single-cell chromatin accessibility reveals principles of regulatory variation. *Nature*. 2015;523:486-490.
151. Cusanovich D. A., Daza R., Adey A., et al. Multiplex single cell profiling of chromatin accessibility by combinatorial cellular indexing. *Science*. 2015;348:910-914.

Drop-ChIP or scChIP-seq: Droplet-Based Single-Cell Chromatin Immunoprecipitation Sequencing

Drop-ChIP or scChIP-seq analyzes the chromatin states of single cells by using microfluidics, unique molecular barcodes, and NGS.[152]

In this method, single cells are isolated into droplets containing lysis buffer and micrococcal nuclease (MNase), and then fused to a droplet carrying distinct oligonucleotides. These oligonucleotides contain the sequences for cell-specific barcodes, sequencing adapters, and restriction sites. In addition, DNA ligase is fused with the droplet to complete the tagging process. Next, carrier chromatin is introduced into the pooled droplets before chromatin immunoprecipitation. Library preparation is carried out according to standard ChIP-Seq procedures before sequencing.



A schematic overview of Drop-ChIP.

Advantages	Disadvantages
• Analyzes chromatin states from single cells in a highly parallel manner • Unique molecular barcoding reduces the risk posed by unspecific antibodies	• Data from each cell are sparse

Reviews

Clark S. J., Lee H. J., Smallwood S. A., Kelsey G. and Reik W. Single-cell epigenomics: powerful new methods for understanding gene regulation and cell identity. *Genome Biol*. 2016;17:72.

Wen L. and Tang F. Single-cell sequencing in stem cell biology. *Genome Biol.* 2016;17:71.

Shin J., Ming G. L. and Song H. Decoding neural transcriptomes and epigenomes via high-throughput sequencing. *Nat Neurosci.* 2014;17:1463-1475.

References

Rotem A., Ram O., Shoresh N., Sperling R. A., Goren A., et al. Single-cell ChIP-seq reveals cell subpopulations defined by chromatin state. *Nat Biotechnol.* 2015;.

The authors demonstrated the utility of scChIP-seq by assaying thousands of individual cells. They used the data to deconvolute a mixture of ESCs, fibroblasts, and hematopoietic progenitors into high-quality chromatin state maps for each cell type. By assaying thousands of ESCs, they were able to identify a spectrum of subpopulations defined by differences in chromatin signatures of pluripotency and differentiation priming.

Illumina Technology: HiSeq 2500 System

Associated Kits

TruSeq ChIP Library Prep Kit

152. Rotem A., Ram O., Shoresh N., et al. Single-cell ChIP-seq reveals cell subpopulations defined by chromatin state. *Nat Biotechnol.* 2015;

SMDB: Single-Molecule Droplet Barcoding

SMDB is a method to leverage short-read sequencing to obtain long and accurate reads.[153]

Using a microfluidics system, the method isolates, amplifies, fragments, and barcodes single DNA molecules in aqueous picoliter droplets. This approach allows the full-length molecules to be sequenced with multi-fold coverage, using short-read sequencing.

A schematic overview of SMDB.

Advantages	Disadvantages
• Limit of detection scales with the number of molecules sequenced and can be orders of magnitude more sensitive than conventional NGS	• Requires specialized hardware

Reviews

None available yet.

References

Lan F., Haliburton J. R., Yuan A. and Abate A. R. Droplet barcoding for massively parallel single-molecule deep sequencing. *Nat Commun*. 2016;7:11784.

To demonstrate the ability of SMDB to detect rare SNPs confidently, the authors generated a population of DNA templates via 35 cycles of PCR with a bacterial plasmid extracted from a culture grown from a single colony. In a population such as this, every sequence shares significant homology, but rare variants exist. The authors identified 457 high-confidence SNPs in ~10% of templates, whereas ~90% of the templates contained no SNPs compared to the reference. A conventional analysis without barcodes identified only 1 SNP.

Illumina Technology: MiSeq System

Associated Kits

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

153. Lan F., Haliburton J. R., Yuan A. and Abate A. R. Droplet barcoding for massively parallel single-molecule deep sequencing. *Nat Commun*. 2016;7:11784.

EPIGENETICS

DNA methylation and hydroxymethylation are involved in development, X-chromosome inactivation, cell differentiation, tissue-specific gene expression, plant epigenetic variation, imprinting, and cancers and other diseases.[154-157] Methylation usually occurs at the 5' position of cytosines, and it plays a crucial role in gene regulation and chromatin remodeling.

The active agouti gene in mice codes for yellow coat color. When pregnant mice with the active agouti gene are fed a diet rich in methyl donors, the offspring are born with the agouti gene turned off. This effect has been used as an epigenetic biosensor for nutritional and environmental alterations of the fetal epigenome.[159]

Most cytosine methylation occurs on cytosines located near guanines, called CpG sites. These CpG sites are often located upstream of promoters, or within the gene body. CpG islands are defined as regions that are greater than 500 bp in length with greater than 55% GC and an expected/observed CpG ratio of > 0.65.

While cytosine methylation (5mC) is known as a silencing mark that represses genes, cytosine hydroxymethylation (5hmC) is shown to be an activating mark that promotes gene expression and is a proposed intermediate in the DNA demethylation pathway.[1,4,6] Similar to 5mC, 5hmC is involved during development, cell differentiation, and cancers and other diseases.[160]

5mC and/or 5hmC can be a diagnostic tool to help identify the effects of nutrition, carcinogens,[161] and environmental factors in relation to diseases. The impact of these modifications on gene regulation depends on their locations within the genome. It is therefore important to determine the exact position of the modified bases.

154. Smith Z. D. and Meissner A. DNA methylation: roles in mammalian development. *Nat Rev Genet*. 2013;14:204-220.
155. Jullien P. E. and Berger F. DNA methylation reprogramming during plant sexual reproduction? *Trends Genet.* 2010;26:394-399.
156. Schmitz R. J., He Y., Valdes-Lopez O., et al. Epigenome-wide inheritance of cytosine methylation variants in a recombinant inbred population. *Genome Res.* 2013;23:1663-1674.
157. Koh K. P. and Rao A. DNA methylation and methylcytosine oxidation in cell fate decisions. *Curr Opin Cell Biol*. 2013;25:152-161.
158. Dolinoy D. C., Weidman J. R., Waterland R. A. and Jirtle R. L. Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. *Environ Health Perspect.* 2006;114:567-572.
159. Dolinoy D. C. The agouti mouse model: an epigenetic biosensor for nutritional and environmental alterations on the fetal epigenome. *Nutr Rev*. 2008;66 Suppl 1:S7-11, Dolinoy D. C. and Faulk C. Introduction: The use of animals models to advance epigenetic science. *ILAR J.* 2012;53:227-231.
160. Pfeifer G. P., Kadam S. and Jin S. G. 5-hydroxymethylcytosine and its potential roles in development and cancer. *Epigenetics Chromatin.* 2013;6:10.
161. Thomson J. P., Lempiainen H., Hackett J. A., et al. Non-genotoxic carcinogen exposure induces defined changes in the 5-hydroxymethylome. *Genome Biol*. 2012;13:R93.

Sequencing Reads Created by Various Bisulfite Conversion Methods

Base	Sequence	BS Sequence	oxBS Sequence	TAB Sequence	RRBS Sequence
C	C	T	T	T	T
5mC	C	C	C	T	C
5hmC	C	C	T	C	C

BS, bisulfite sequencing; oxBS, oxidative bisulfite sequencing; TAB, TET-assisted bisulfite sequencing; RRBS, reduced-representation bisulfite sequencing.

Reviews

Devall M., Roubroeks J., Mill J., Weedon M. and Lunnon K. Epigenetic regulation of mitochondrial function in neurodegenerative disease: New insights from advances in genomic technologies. *Neurosci Lett.* 2016;625:47-55.

Yan H., Tian S., Slager S. L., Sun Z. and Ordog T. Genome-Wide Epigenetic Studies in Human Disease: A Primer on -Omic Technologies. *Am J Epidemiol.* 2016;183:96-109.

Yong W. S., Hsu F. M. and Chen P. Y. Profiling genome-wide DNA methylation. *Epigenetics Chromatin*. 2016;9:26.

References

Bak M., Boonen S. E., Dahl C., et al. Genome-wide DNA methylation analysis of transient neonatal diabetes type 1 patients with mutations in ZFP57. *BMC Med Genet*. 2016;17:29.

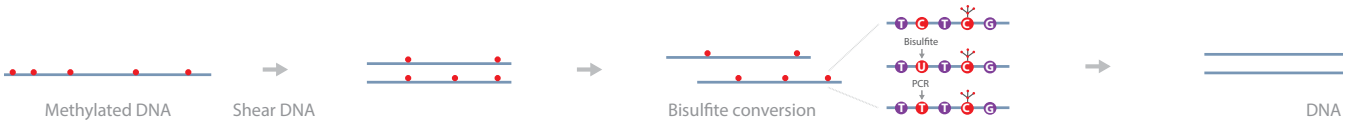
Prezza N., Vezzi F., Kaller M. and Policriti A. Fast, accurate, and lightweight analysis of BS-treated reads with ERNE 2. *BMC Bioinformatics.* 2016;17 Suppl 4:69.

BS-Seq, Bisulfite-seq, and WGBS: Whole-Genome Bisulfite Sequencing (WGBS)

BS-Seq/Bisulfite-seq or WGBS is a well-established protocol to detect methylated cytosines in gDNA.[162]

In this method, gDNA is treated with sodium bisulfite and then sequenced, providing single-base resolution of methylated cytosines in the genome. Upon bisulfite treatment, unmethylated cytosines are deaminated to uracils which, upon sequencing, are converted to thymidines. Simultaneously, methylated cytosines resist deamination and are read as cytosines. The location of the methylated cytosines can then be determined by comparing treated and untreated sequences. Bisulfite treatment of DNA converts unmethylated cytosines to thymidines, leading to reduced sequence complexity. Very accurate deep sequencing serves to mitigate this loss of complexity.

The TruSeq DNA Methylation Kit (previously known as the EpiGnome Methyl-Seq Kit) uses a unique library construction method that incorporates bisulfite conversion as the first step.



A schematic overview of BS-Seq.

A schematic overview of the TruSeq DNA Methylation Kit procedure.

Advantages	Disadvantages
BS-Seq or WGBS • CpG and non-CpG methylation throughout the genome is covered at single-base resolution • Covers 5mCs in dense and less dense repeat regions	• Bisulfite converts unmethylated cytosines to thymidines, reducing sequence complexity, which can make it difficult to create alignments • SNPs where a cytosine is converted to thymidine will be missed upon bisulfite conversion • Bisulfite conversion does not distinguish between 5mC and 5hmC
TruSeq DNA Methylation Kit • Pre-library bisulfite conversion • Low input gDNA (50 ng) • Provides uniform CpG, CHG, and CHH coverage • No fragmentation and no methylated adapters • Retains sample diversity	• Bisulfite converts unmethylated cytosines to thymidines, reducing sequence complexity, which can make it difficult to create alignments • SNPs where a cytosine is converted to thymidine will be missed upon bisulfite conversion • Bisulfite conversion does not distinguish between 5mC and 5hmC • Higher duplicate percentage

Reviews

Devall M., Roubroeks J., Mill J., Weedon M. and Lunnon K. Epigenetic regulation of mitochondrial function in neurodegenerative disease: New insights from advances in genomic technologies. *Neurosci Lett.* 2016;625:47-55.

Yong W. S., Hsu F. M. and Chen P. Y. Profiling genome-wide DNA methylation. *Epigenetics Chromatin*. 2016;9:26.

162. Feil R., Charlton J., Bird A. P., Walter J. and Reik W. Methylation analysis on individual chromosomes: improved protocol for bisulphite genomic sequencing. *Nucleic Acids Res.* 1994;22:695-696.

References

Derks M. F., Schachtschneider K. M., Madsen O., et al. Gene and transposable element methylation in great tit (Parus major) brain and blood. *BMC Genomics.* 2016;17:332.

The authors used WGBS of whole blood and brain tissue in the great tit (Parus major) to correlate gene features, CpG islands (CGIs), TEs, and their functional roles. They found that 6877 (~21%) of the CGIs were differentially methylated between blood and brain, of which 1186 and 2055 were annotated to promoter and intragenic regions, respectively. The dominant sequence motifs for brain non-CpG methylation were similar to those found in mammals, suggesting that a conserved non-CpG regulatory mechanism was already present in a common ancestor.

Illumina Technology: HiSeq 2000 System, TruSeq Stranded RNA Sample Preparation Kit

Lu Y. C., Feng S. J., Zhang J. J., et al. Genome-wide identification of DNA methylation provides insights into the association of gene expression in rice exposed to pesticide atrazine. *Sci Rep.* 2016;6:18985.

The authors used BS-seq to identify the pattern of cytosine methylation in atrazine (ATR)-exposed rice and detected altered DNA methylation patterns in the genome. They profiled the transcriptome of ATR-exposed rice to reveal the link between DNA methylation and gene expression.

Illumina Technology: Genome Analyzer$_{IIx}$ System, HiSeq 2000 System

Wang X., Werren J. H. and Clark A. G. Allele-Specific Transcriptome and Methylome Analysis Reveals Stable Inheritance and Cis-Regulation of DNA Methylation in Nasonia. *PLoS Biol.* 2016;14:e1002500.

In this study, the authors quantified allele-specific expression and methylation genome-wide in the parasitic wasp species Nasonia vitripennis and Nasonia girault, as well as their reciprocal F1 hybrids. They found no parent-of-origin effect in allelic expression for > 8000 covered genes, suggesting a lack of genomic imprinting in adult Nasonia. All 178 differentially methylated genes were also differentially methylated between the 2 alleles in F1 hybrid offspring, recapitulating the parental methylation status with nearly 100% fidelity. The authors suggest that this effect may be due to the presence of strong cis-elements driving the target of gene-body methylation.

Illumina Technology: HiSeq 2000 System, TruSeq RNA Sample Prep Kit v2

Zhang Y., Zhang D., Li Q., et al. Nucleation of DNA repair factors by FOXA1 links DNA demethylation to transcriptional pioneering. *Nat Genet.* 2016;48:1003-1013.

The researchers performed WGBS in human mammary carcinoma MCF-7 cells and obtained methylomes at single-base resolution in 2 replicates, with a combined total of 886 million mapped reads (100 bp paired-end reads). Genome-wide DNA methylomes demonstrated that the FOXA1 DNA repair complex was functionally linked to DNA demethylation in a lineage-specific fashion. Depletion of FOXA1 resulted in localized reestablishment of methylation in a large portion of FOXA1-bound regions.

Illumina Technology: HiSeq 2500/4000 System

Bogdanovic O., Smits A. H., de la Calle Mustienes E., et al. Active DNA demethylation at enhancers during the vertebrate phylotypic period. *Nat Genet.* 2016;48:417-426.

Groth M., Moissiard G., Wirtz M., et al. MTHFD1 controls DNA methylation in Arabidopsis. *Nat Commun.* 2016;7:11640.

Jeong Y. H., Lu H., Park C. H., et al. Stochastic anomaly of methylome but persistent SRY hypermethylation in disorder of sex development in canine somatic cell nuclear transfer. *Sci Rep.* 2016;6:31088.

Kaaij L. J., Mokry M., Zhou M., et al. Enhancers reside in a unique epigenetic environment during early zebrafish development. *Genome Biol*. 2016;17:146.

Qu W., Tsukahara T., Nakamura R., et al. Assessing Cell-to-Cell DNA Methylation Variability on Individual Long Reads. *Sci Rep.* 2016;6:21317.

Rehan S. M., Glastad K. M., Lawson S. P. and Hunt B. G. The Genome and Methylome of a Subsocial Small Carpenter Bee, Ceratina calcarata. *Genome Biol Evol.* 2016;8:1401-1410.

Thienpont B., Steinbacher J., Zhao H., et al. Tumour hypoxia causes DNA hypermethylation by reducing TET activity. *Nature*. 2016;537:63-68.

Wallner S., Schroder C., Leitao E., et al. Epigenetic dynamics of monocyte-to-macrophage differentiation. *Epigenetics Chromatin*. 2016;9:33.

Yang Y., Zhou R., Mu Y., et al. Genome-wide analysis of DNA methylation in obese, lean, and miniature pig breeds. *Sci Rep.* 2016;6:30160.

Zhang X., Su J., Jeong M., et al. DNMT3A and TET2 compete and cooperate to repress lineage-specific transcription factors in hematopoietic stem cells. *Nat Genet.* 2016;48:1014-1023.

Associated Kits

TruSeq DNA Methylation Kit

Infinium HumanMethylation450 Arrays

PBAT: Post-bisulfite Adapter Tagging

In PBAT, bisulfite treatment precedes adaptor tagging to avoid the bisulfite-induced fragmentation of adaptor-tagged template DNAs.[163]

Bisulfite treatment is followed by adapter tagging and 2 rounds of random-primer extension. This procedure generates a substantial number of unamplified reads from subnanogram quantities of DNA.[164]

A schematic overview of PBAT.

Advantages	Disadvantages
• Requires only 100 ng of DNA for amplification-free WGBS of mammalian genomes	• Bisulfite converts unmethylated cytosines to thymidines, reducing sequence complexity, which can make it difficult to create alignments • SNPs where a cytosine is converted to thymidine will be missed upon bisulfite conversion • Bisulfite conversion does not distinguish between 5mC and 5hmC

Reviews

Yong W. S., Hsu F. M. and Chen P. Y. Profiling genome-wide DNA methylation. *Epigenetics Chromatin*. 2016;9:26.

References

Guo F., Yan L., Guo H., et al. The Transcriptome and DNA Methylome Landscapes of Human Primordial Germ Cells. *Cell*. 2015;161:1437-1452.

Human primordial germ cells (PGCs) show unique transcription patterns involving the simultaneous expression of both pluripotency genes and germline-specific genes, with a subset of them displaying developmental stage–specific features. The authors used PBAT to analyze the DNA methylome of human PGCs. Approximately 10–11 weeks after gestation, the PGCs were nearly devoid of any DNA methylation, with only 7.8% and 6.0% of the median methylation levels in male and female PGCs, respectively. The study paves the way toward deciphering the complex epigenetic reprogramming of the germline, with the aim of restoring totipotency in fertilized oocytes.

Illumina Technology: HiSeq 2500 System

Sakashita A., Kawabata Y., Jincho Y., et al. Sex Specification and Heterogeneity of Primordial Germ Cells in Mice. *PLoS One*. 2015;10:e0144836.

The authors used PBAT to determine the impact of demethylation on mouse PGC development. They found that, except for the retrotransposon regions, PGCs were uniformly hypomethylated at embryonic day 13.5.

Illumina Technology: MiSeq System

Associated Kits

TruSeq DNA Methylation Kit

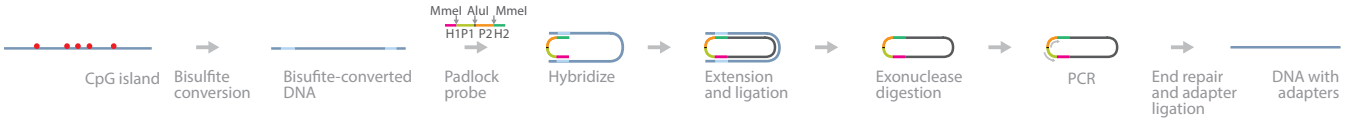
Infinium HumanMethylation450 Arrays

163. Miura F., Enomoto Y., Dairiki R. and Ito T. Amplification-free whole-genome bisulfite sequencing by post-bisulfite adaptor tagging. *Nucleic Acids Res.* 2012;40:e136.
164. Smallwood S. A., Lee H. J., Angermueller C., et al. Single-cell genome-wide bisulfite sequencing for assessing epigenetic heterogeneity. *Nat Methods.* 2014;11:817-820.

BSPP: Bisulfite Sequencing With Padlock Probes

BSPP is a targeted method that isolates selected locations for methylation profiling.[165,166]

Padlock probes are ~100 nt DNA fragments designed to hybridize to genomic DNA targets in a horseshoe manner. After the gap between the 2 hybridized, locus-specific arms of a padlock probe is filled and ligated, the circular strand of DNA can be amplified and sequenced.[165]



A schematic overview of BSPP.

Advantages	Disadvantages
• Requires only 100 ng of DNA for amplification-free WGBS of mammalian genomes	• Fails to capture areas with lower CpG densities[167]

Reviews

Shull A. Y., Noonepalle S. K., Lee E. J., Choi J. H. and Shi H. Sequencing the cancer methylome. *Methods Mol Biol.* 2015;1238:627-651.

References

None available yet.

Associated Kits

TruSeq DNA Methylation Kit

Infinium HumanMethylation450 Arrays

165. Deng J., Shoemaker R., Xie B., et al. Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming. *Nat Biotechnol.* 2009;27:353-360.
166. Ball M. P., Li J. B., Gao Y., et al. Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. *Nat Biotechnol.* 2009;27:361-368.
167. Tanaka T., Reilly B., Diep D., Zhang K. and Bejar R. Cytosine Methylation Patterns As Biomarkers in MDS. *Blood*. 2015;126:5226-5226.

RRBS-Seq: Reduced-Representation Bisulfite Sequencing

RRBS uses one or multiple restriction enzymes on the genomic DNA to produce sequence-specific fragmentation.[168] The fragmented genomic DNA is treated with bisulfite and sequenced. This is the method of choice to study specific regions of interest. It is particularly effective where methylation levels are high, such as in promoters and repeat regions.

A schematic overview of RRBS.

Advantages	Disadvantages
• Provides genome-wide coverage of CpGs in islands at single-base resolution • Covers CpG methylation in dense regions	• Restriction enzymes cut at specific sites, providing biased sequence selection • Method measures 10-15% of all CpGs in the genome • Cannot distinguish between 5mC and 5hmC • Does not cover non-CpG areas, genome-wide CpGs, and CpGs in areas without the enzyme restriction site

Reviews

Devall M., Roubroeks J., Mill J., Weedon M. and Lunnon K. Epigenetic regulation of mitochondrial function in neurodegenerative disease: New insights from advances in genomic technologies. *Neurosci Lett*. 2016;625:47-55.

Yong W. S., Hsu F. M. and Chen P. Y. Profiling genome-wide DNA methylation. *Epigenetics Chromatin*. 2016;9:26.

References

Auclair G., Borgel J., Sanz L. A., et al. EHMT2 directs DNA methylation for efficient gene silencing in mouse embryos. *Genome Res*. 2016;26:192-202.

The authors used RRBS in a model of knockout mice to explore the role of the lysine methyltransferase, EHMT2, in DNA methylation during mouse embryogenesis. Their results showed that DNA methylation was instrumental in EHMT2-mediated gene silencing during embryogenesis. This result implies that EHMT2 is a critical factor that facilitates repressive DNA methylation at specific genomic loci during mammalian development.

Illumina Technology: HiSeq 2500 System

Day S. E., Coletta R. L., Kim J. Y., et al. Next-generation sequencing methylation profiling of subjects with obesity identifies novel gene changes. *Clin Epigenetics.* 2016;8:77.

The authors studied the role of skeletal muscle DNA methylation, in combination with transcriptomic changes in obesity. They obtained skeletal muscle biopsies basally from lean (n = 12; body mass index (BMI) = 23.4 ± 0.7 kg/m2) and obese (n = 10; BMI = 32.9 ± 0.7 kg/m2) study participants. They found 13,130 differentially methylated cytosines (DMCs) (uncorrected $P < 0.05$) that were altered in the promoter and 5'- and 3'-untranslated regions (UTRs) in the obese vs lean analysis. They also observed a negative relationship between gene expression and DNA methylation.

Illumina Technology: HiSeq 2000 System

Heller G., Topakian T., Altenberger C., et al. Next-generation sequencing identifies major DNA methylation changes during progression of Ph+ chronic myeloid leukemia. *Leukemia*. 2016;30:1861-1868.

The authors used RRBS and RNA-Seq to analyze samples from chronic myeloid leukemia (CML) patients at chronic phase (CP), accelerated phase (AP), and blast crisis (BC) for differences in CpG site methylation and expression. They demonstrated that CpG site methylation increases dramatically during the progression from CP-CML to AP-CML/BC-CML, and they identified genes that are transcriptionally regulated by methylation in BC-CML samples. Some of these genes are involved in the pathogenesis of various malignancies.

Illumina Technology: HiSeq 2000 System

168. Meissner A., Gnirke A., Bell G. W., Ramsahoye B., Lander E. S. and Jaenisch R. Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. *Nucleic Acids Res.* 2005;33:5868-5877.

Baheti S., Kanwar R., Goelzenleuchter M., et al. Targeted alignment and end repair elimination increase alignment and methylation measure accuracy for reduced representation bisulfite sequencing data. *BMC Genomics.* 2016;17:149.

Flinders C., Lam L., Rubbi L., et al. Epigenetic changes mediated by polycomb repressive complex 2 and E2a are associated with drug resistance in a mouse model of lymphoma. *Genome Med.* 2016;8:54.

Orlanski S., Labi V., Reizel Y., et al. Tissue-specific DNA demethylation is required for proper B-cell differentiation and function. *Proc Natl Acad Sci U S A.* 2016;113:5018-5023.

Page A., Paoli P., Moran Salvador E., et al. Hepatic stellate cell transdifferentiation involves genome-wide remodeling of the DNA methylation landscape. *J Hepatol.* 2016;64:661-673.

Pegoraro M., Bafna A., Davies N. J., Shuker D. M. and Tauber E. DNA methylation changes induced by long and short photoperiods in Nasonia. *Genome Res.* 2016;26:203-210.

Yuan X. L., Gao N., Xing Y., et al. Profiling the genome-wide DNA methylation pattern of porcine ovaries using reduced representation bisulfite sequencing. *Sci Rep*. 2016;6:22138.

Associated Kits

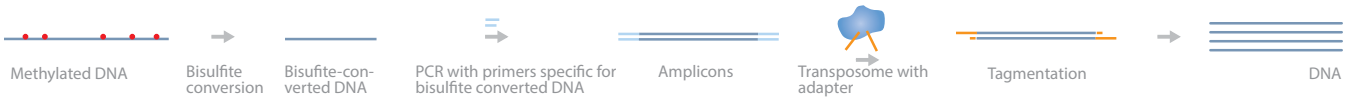
TruSeq DNA Methylation Kit

Infinium HumanMethylation450 Arrays

BSAS: Bisulfite Amplicon Sequencing

BSAS[169] is a targeted BS-Seq method that uses PCR enrichment of targeted regions and transposome-mediated library construction for rapid generation of sequencing libraries, from low (1 ng) sample input.

Genomic DNA is bisulfite-converted and subjected to PCR, using primers specific for bisulfite-converted DNA. The amplicons are subjected to Nextera XT library preparation, including dual indexing. The final libraries consist of a random insert of bisulfite-converted amplified DNA, capture probes, and specific index sequences. These libraries are multiplexed and sequenced.



A schematic overview of BSAS.

Advantages	Disadvantages
• Can be applied to any genomic region from any DNA source, including tissue and cell culture. • Rapid and highly quantitative	• Does not cover the whole genome • Genome and target must be known

Reviews

None available yet.

References

Sun L., Wang J., Yin X., et al. Identification of a 5-Methylcytosine Site that may Regulate C/EBPbeta Binding and Determine Tissue-Specific Expression of the BPI Gene in Piglets. Sci Rep. 2016;6:28506.

Bactericidal/permeability-increasing protein (BPI) plays an important role in innate immune defense in mammals. The authors used BSAS to quantify CpG islands in the BPI gene promoter, in 11 different tissue types from weaned Yorkshire piglets. They confirmed that methylation of the mC-15 residue could inhibit the ability of C/EBPβ binding to the BPI promoter and affect gene expression. They suggest that this mechanism plays a role in the tissue-specificity of BPI gene expression in weaned piglets.

Illumina Technology: MiSeq System

Ou X., Thakali K. M., Shankar K., Andres A. and Badger T. M. Maternal adiposity negatively influences infant brain white matter development. Obesity (Silver Spring). 2015;23:1047-1054.

Associated Kits

TruSeq DNA Methylation Kit

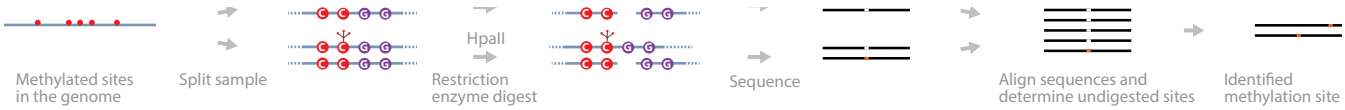
Infinium HumanMethylation450 Arrays

169. Masser D. R., Berg A. S. and Freeman W. M. Focused, high accuracy 5-methylcytosine quantitation with base resolution by benchtop next-generation sequencing. *Epigenetics Chromatin.* 2013;6:33.

MRE-Seq and Methyl-Seq: Methylation-Sensitive Restriction Enzyme Sequencing

MSRE/MRE-seq and Methyl-seq are protocols that use methylation-sensitive restriction enzymes (MSREs) on genomic DNA to study DNA methylation.[170,171] MRE-seq enriches unmethylated DNA and can cover 1.7 million CpG sites across the human genome.[172]

In this method, gDNA is first separately digested with different MSREs. The library is prepared from size-selected restriction fragments and sequenced. Deep sequencing allows for accurate detection of methylation sites in the genome.



A schematic overview of MSRE.

Advantages	Disadvantages
• Allows the estimation of relative DNA methylation levels	• Relatively low coverage of the genome, because CpG-containing recognition sites are limited

Reviews

Shull A. Y., Noonepalle S. K., Lee E. J., Choi J. H. and Shi H. Sequencing the cancer methylome. Methods Mol Biol. 2015;1238:627-651.

References

Lee H. J., Lowdon R. F., Maricque B., et al. Developmental enhancers revealed by extensive DNA methylome maps of zebrafish early embryos. Nat Commun. 2015;6:6315.

To understand how DNA methylation changes during zebrafish embryogenesis, the researchers generated high-resolution DNA methylome maps of 6 developmental stages. For each developmental stage, they constructed 2 sequencing libraries using methylated DNA immunoprecipitation sequencing (MeDIP-seq) and MRE-seq. Overall, the average genic DNA methylation levels of each developmental stage were almost identical. The authors observed a negative correlation in the methylation level and the proximity to the TSS. The DNA methylation level increased in the 3'-UTR and stayed high in exons, which resembled the DNA methylation pattern over genic regions in human ESCs.

Illumina Technology: HiSeq System

Chen K., Chen Z., Wu D., et al. Broad H3K4me3 is associated with increased transcription elongation and enhancer activity at tumor-suppressor genes. Nat Genet. 2015;.

Elliott G., Hong C., Xing X., et al. Intermediate DNA methylation is a conserved signature of genome regulation. Nat Commun. 2015;6:6363.

Gascard P., Bilenky M., Sigaroudinia M., et al. Epigenetic and transcriptional determinants of the human breast. Nat Commun. 2015;6:6351.

Lee H. J., Lowdon R. F., Maricque B., Zhang B., Stevens M., et al. Developmental enhancers revealed by extensive DNA methylome maps of zebrafish early embryos. Nat Commun. 2015;6:6315.

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

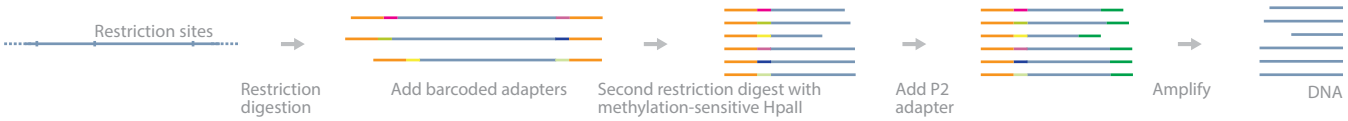
Infinium HumanMethylation450 BeadChip Kit

170. Maunakea A. K., Nagarajan R. P., Bilenky M., et al. Conserved role of intragenic DNA methylation in regulating alternative promoters. *Nature*. 2010;466:253-257.
171. Brunner A. L., Johnson D. S., Kim S. W., et al. Distinct DNA methylation patterns characterize differentiated human embryonic stem cells and developing human fetal liver. *Genome Res.* 2009;19:1044-1056.
172. Shull A. Y., Noonepalle S. K., Lee E. J., Choi J. H. and Shi H. Sequencing the cancer methylome. *Methods Mol Biol*. 2015;1238:627-651.

EpiRADseq: Double-Digest Restriction Site–Associated DNA Marker Generation with a Methylation-Sensitive Restriction Enzyme

EpiRADseq is similar to the widely used double-digest RAD-seq method (ddRADseq), except that it uses an MSRE.[173]

DNA samples are digested with PstI and HpaII followed by purification. Double-stranded sequencing adapters with unique barcodes are ligated to each sample. Size-selected libraries are then amplified with indexed primers.



A schematic overview of EpiRADseq.

Advantages	Disadvantages
• Does not need a reference genome	• Measures a subset of methylation sites

Reviews

None available yet.

References

Schield D. R., Walsh M. R., Card D. C., Andrew A. L., Adams R. H., et al. EpiRADseq: scalable analysis of genomewide patterns of methylation using next-generation sequencing. *Methods in Ecology and Evolution.* 2016;7:60-69.

The researchers tested the utility of EpiRADseq by identifying shifts in the epigenome of clonal water fleas (Daphnia ambigua) in response to exposure to fish predator cues. These cues are known to induce transgenerational changes in life-history traits. In response to predator cues, the authors found significant transgenerational shifts in the methylation states of the sampled loci within treatment groups.

Illumina Technology: MiSeq System

Associated Kits

TruSeq DNA Methylation Kit

Infinium HumanMethylation450 Arrays

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

TruSeq Nano DNA Library Prep Kit

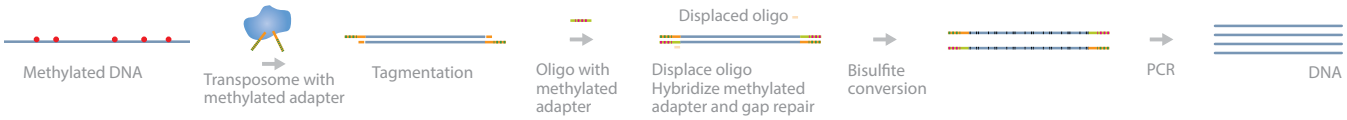
TruSeq DNA PCR-Free Library Prep Kit

173. Schield D. R., Walsh M. R., Card D. C., et al. EpiRADseq: scalable analysis of genomewide patterns of methylation using next-generation sequencing. *Methods in Ecology and Evolution.* 2016;7:60-69.

T-WGBS: Tagmentation-Based Whole-Genome Bisulfite Sequencing

T-WGBS uses the Tn5 transposome and bisulfite conversion to study 5mC.[174]

In this method, DNA is incubated with Tn5 transposome containing methylated primers, which fragments the DNA and ligates adapters. The tagged DNA first undergoes oligonucleotide displacement, followed by methylated oligonucleotide replacement and gap repair, ensuring the addition of methylated adapters to the tagmented DNA. Next, the DNA is treated with sodium bisulfite, PCR-amplified, and sequenced. Deep sequencing provides single-base resolution of 5mC in the genome.



A schematic overview of T-WGBS.

Advantages	Disadvantages
• Can sequence samples with very limited starting material (~20 ng) • Fast protocol with few steps • Elimination of multiple steps minimizes loss of DNA	• Bisulfite converts unmethylated cytosines to thymidines, reducing sequence complexity, which can make it difficult to create alignments • SNPs where a cytosine is converted to thymidine will be missed upon bisulfite conversion • Bisulfite conversion does not distinguish between 5mC and 5hmC

Reviews

Tang J., Fang F., Miller D. F., et al. Global DNA methylation profiling technologies and the ovarian cancer methylome. *Methods Mol Biol.* 2015;1238:653-675.

Plongthongkum N., Diep D. H. and Zhang K. Advances in the profiling of DNA modifications: cytosine methylation and beyond. *Nat Rev Genet.* 2014;15:647-661.

Weichenhan D. and Plass C. The evolving epigenome. *Hum Mol Genet.* 2013;22:R1-6.

References

Wang Q., Gu L., Adey A., Radlwimmer B., Wang W., et al. Tagmentation-based whole-genome bisulfite sequencing. *Nat Protoc.* 2013;8:2022-2032.

Associated Kits

TruSeq DNA Methylation Kit

Infinium HumanMethylation450 Arrays

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

Nextera Rapid Capture Exome/Custom Enrichment Kit

174. Wang Q., Gu L., Adey A., et al. Tagmentation-based whole-genome bisulfite sequencing. *Nat Protoc*. 2013;8:2022-2032.

JBP1-seq: J-Binding Protein 1 Sequencing

JBP1-seq is a method for genome-wide profiling of 5hmC. It relies on the strong affinity of the J-binding protein 1 (JBP1) for glucosylated 5hmC. The method uses a recombinant JBP1 protein with an additional His tag and Avi tag that is biotinylated and subsequently conjugated to streptavidin-coated magnetic beads.[175]

The JBP1-seq workflow involves 4 main steps: (1) gDNA is tagmented to fragment it and add adapter sequences, (2) 5hmC sites are glucosylated by T4-bGT, (3) JBP1-magnetic beads are used to enrich for DNA fragments containing b-glu- 5hmC, and finally (4) enriched library is amplified to add P5 and P7 adapters and barcodes for sequencing.

A schematic overview of JBP1-seq.

Advantages	Disadvantages
• Streamlined protocol	• Regions that contain high levels of 5hmC can be overrepresented • Optimal resolution is approximately 50 bp

Reviews

Shull A. Y., Noonepalle S. K., Lee E. J., Choi J. H. and Shi H. Sequencing the cancer methylome. *Methods Mol Biol.* 2015;1238:627-651.

Plongthongkum N., Diep D. H. and Zhang K. Advances in the profiling of DNA modifications: cytosine methylation and beyond. *Nat Rev Genet*. 2014;15:647-661.

References

Cui L., Chung T. H., Tan D., Sun X. and Jia X. Y. JBP1-seq: a fast and efficient method for genome-wide profiling of 5hmC. *Genomics*. 2014;104:368-375.

This paper provides a detailed description of the protocol.

Illumina Technology: HiSeq System, Nextera DNA Sample Prep Kit

Associated Kits

TruSeq DNA Methylation Kit

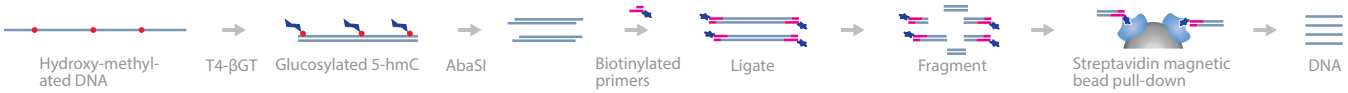
TruSeq DNA PCR-Free Library Prep Kit

175. Cui L., Chung T. H., Tan D., Sun X. and Jia X. Y. JBP1-seq: a fast and efficient method for genome-wide profiling of 5hmC. *Genomics*. 2014;104:368-375.

Aba-seq: AbaSI Coupled with Sequencing

Aba-seq is a method for high-resolution mapping of the 5hmC methylome that provides sensitive detection of 5hmC at low-occupancy regions.[176]

The method relies on the unique property of the restriction enzyme AbaSI to recognize glucosylated 5hmC with high specificity, compared to 5mC and C. A single-cell variation, scAba-seq, is also available.[177] A related method uses PvuRts1I to overcome the sequence bias in AbaSI.[178]



A schematic overview of Aba-seq.

Advantages	Disadvantages
• Covers CpG and non-CpG methylation throughout the genome at single-base resolution • Covers 5mC in dense and less dense repeat regions • Clearly differentiates between 5mC and 5hmC	• Ambiguity can exist in 13% of the cleaved molecules in the Aba-seq assay[179] • Sequence bias in AbaSI activity[180]

Reviews

Plongthongkum N., Diep D. H. and Zhang K. Advances in the profiling of DNA modifications: cytosine methylation and beyond. *Nat Rev Genet.* 2014;15:647-661.

References

Sun Z., Terragni J., Borgaro J. G., Liu Y., Yu L., et al. High-resolution enzymatic mapping of genomic 5-hydroxymethylcytosine in mouse embryonic stem cells. *Cell Rep*. 2013;3:567-576.

The authors used Aba-seq to generate a high-resolution hydroxymethylome map of mouse E14 ESCs. The results indicated that 5hmC was enriched at poised enhancers with monomethylated histone H3 lysine 4 (H3K4me1), but not at active enhancers with acetylated histone H3 lysine 27 (H3K27Ac). The authors also observed that the mitochondrial genome contained non-CG hydroxymethylated sites.

Illumina Technology: HiSeq System

Associated Kits

TruSeq DNA Methylation Kit

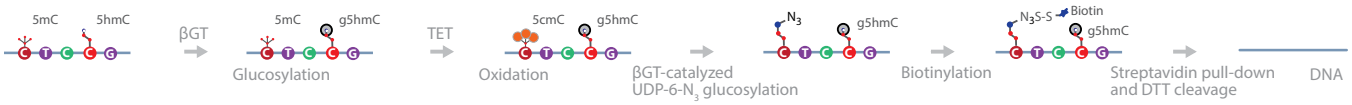
TruSeq DNA PCR-Free Library Prep Kit

176. Sun Z., Terragni J., Borgaro J. G., et al. High-resolution enzymatic mapping of genomic 5-hydroxymethylcytosine in mouse embryonic stem cells. *Cell Rep*. 2013;3:567-576.
177. Mooijman D., Dey S. S., Boisset J. C., Crosetto N. and van Oudenaarden A. Single-cell 5hmC sequencing reveals chromosome-wide cell-to-cell variability and enables lineage reconstruction. *Nat Biotechnol.* 2016;34:852-856.
178. Sun Z., Dai N., Borgaro J. G., et al. A sensitive approach to map genome-wide 5-hydroxymethylcytosine and 5-formylcytosine at single-base resolution. *Mol Cell*. 2015;57:750-761.
179. Serandour A. A., Avner S., Mahe E. A., et al. Single-CpG resolution mapping of 5-hydroxymethylcytosine by chemical labeling and exonuclease digestion identifies evolutionarily unconserved CpGs as TET targets. *Genome Biol.* 2016;17:56.
180. Sun Z., Dai N., Borgaro J. G., et al. A sensitive approach to map genome-wide 5-hydroxymethylcytosine and 5-formylcytosine at single-base resolution. *Mol Cell*. 2015;57:750-761.

TAmC-Seq: Tet-Assisted 5-Methylcytosine Sequencing

TAmC-Seq selectively tags 5mC with an azide residue that can be further labeled with biotin for affinity purification.[181] The method relies on the properties of the Ten-eleven translocation (Tet) family of iron(II)/α-ketoglutarate-dependent dioxygenases that are involved in the demethylation of 5mC.

In this method, the endogenous 5hmC in genomic DNA is protected by glucosylation with normal glucose. The 5mC residues are converted to 5hmC via mTet1-catalyzed oxidation. The 5hmC is then labeled via β-GT-mediated glucosylation with a modified glucose moiety (6-N_3-glucose) to generate 6-N_3-β-glucosyl-5-hydroxymethylcytosine (N_3-5gmC). This molecule can be labeled further, using click chemistry, for detection, affinity purification, and sequencing.



A schematic overview of TAmC-Seq.

Advantages	Disadvantages
• Captures a larger fraction of methylated CpGs with less density bias	• Not yet adopted widely by the scientific community

Reviews

None available yet.

References

Zhang L., Szulwach K. E., Hon G. C., Song C. X., Park B., et al. Tet-mediated covalent labelling of 5-methylcytosine for its genome-wide detection and sequencing. *Nat Commun.* 2013;4:1517.

The authors used TAmC-Seq to screen for 5mC in several mouse and human cell lines. The results showed that TAmC-Seq provided a wider coverage of CpG-containing genomic regions. Further, it captured a larger fraction of methylated CpGs with less density bias than other affinity-enrichment methods.

Illumina Technology: HiSeq System, cBot System

Associated Kits

TruSeq DNA Methylation Kit

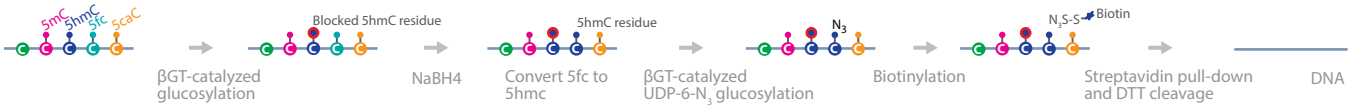
TruSeq DNA PCR-Free Library Prep Kit

181. Zhang L., Szulwach K. E., Hon G. C., et al. Tet-mediated covalent labelling of 5-methylcytosine for its genome-wide detection and sequencing. *Nat Commun*. 2013;4:1517.

fC-Seal: A 5-Formylcytosine-Selective Chemical Labeling

In demethylation pathways, the TET family of proteins oxidize 5mC to 5hmC, 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) in a stepwise fashion. Conversely, *in vivo*, 5fmC residues can be converted to cytosine by base-excision repair.

In fC-Seal, 5hmC residues in the genome are blocked with regular glucose using βGT, and 5fC residues are reduced to 5hmC by $NaBH_4$. The newly generated 5hmC residues are modified, enriched, and sequenced.[182]



A schematic overview of fC-Seal.

Advantages	Disadvantages
• Does not require antibodies	• Cannot map 5fC and 5caC at single-nucleotide resolution and determine their relative abundance[183]

Reviews

Neri F., Incarnato D., Krepelova A., Parlato C. and Oliviero S. Methylation-assisted bisulfite sequencing to simultaneously map 5fC and 5caC on a genome-wide scale for DNA demethylation analysis. *Nat Protoc.* 2016;11:1191-1205.

Plongthongkum N., Diep D. H. and Zhang K. Advances in the profiling of DNA modifications: cytosine methylation and beyond. *Nat Rev Genet.* 2014;15:647-661.

References

Song C. X., Szulwach K. E., Dai Q., Fu Y., Mao S. Q., et al. Genome-wide profiling of 5-formylcytosine reveals its roles in epigenetic priming. *Cell*. 2013;153:678-691.

In this study, genome-wide mapping of 5fC in mouse ESCs revealed that 5fC preferentially occurs at poised enhancers, among other gene regulatory elements. The researchers resolved 5fC at single-base resolution by hydroxylamine-based protection from bisulfite-mediated deamination, thereby confirming sites of 5fC accumulation.

Illumina Technology: HiSeq System

Associated Kits

TruSeq DNA Methylation Kit

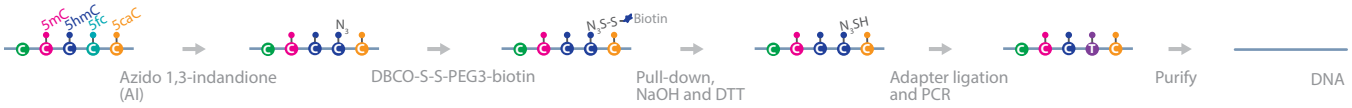
TruSeq DNA PCR-Free Library Prep Kit

182. Song C. X., Szulwach K. E., Dai Q., et al. Genome-wide profiling of 5-formylcytosine reveals its roles in epigenetic priming. *Cell*. 2013;153:678-691.
183. Neri F., Incarnato D., Krepelova A., Parlato C. and Oliviero S. Methylation-assisted bisulfite sequencing to simultaneously map 5fC and 5caC on a genome-wide scale for DNA demethylation analysis. *Nat Protoc.* 2016;11:1191-1205.

fC-CET: 5fC Chemical Labeling and C-to-T Transition During PCR

fC-CET is a bisulfite-free method for whole-genome analysis of 5fC. The method is based on selective chemical labeling of 5fC and subsequent C-to-T transition during PCR.[184]

In this method, gDNA is labeled sequentially with an azido derivative of 1,3-indandione (AI) and then conjugated to biotin using click chemistry. The gDNA is enriched by streptavidin pull-down and ligated to adapters, followed by PCR and sequencing. C-to-T transitions during PCR amplification are searched specifically to define 5fC sites in the genome.



A schematic overview of fC-CET.

Advantages	Disadvantages
• Results are in agreement with fC-Seal • Sequences with a single 5fC are enriched ~100-fold • No noticeable DNA degradation; ideal for analyses of precious DNA samples	• Provides only relative enrichment of cytosines marked by 5fC[185]

Reviews

None available yet.

References

Xia B., Han D., Lu X., Sun Z., Zhou A., et al. Bisulfite-free, base-resolution analysis of 5-formylcytosine at the genome scale. *Nat Methods*. 2015;12:1047-1050.

Using fC-CET, the researchers generated a base-resolution map of 5fC in the whole genome of wild-type mouse ESCs. With a requirement of positive hits in both replicates, they identified 32,685 and 139,027 high-confidence 5fC sites in Tdgfl/fl and Tdg–/– mouse ESCs, respectively.

Illumina Technology: HiSeq 2500 System

Associated Kits

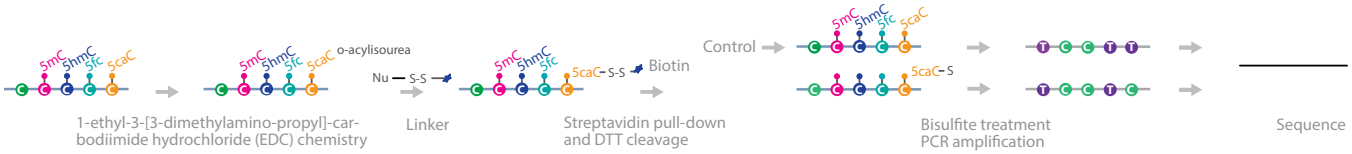
TruSeq DNA Methylation Kit

TruSeq DNA PCR-Free Library Prep Kit

184. Xia B., Han D., Lu X., et al. Bisulfite-free, base-resolution analysis of 5-formylcytosine at the genome scale. *Nat Methods*. 2015;12:1047-1050.
185. Wu H., Wu X. and Zhang Y. Base-resolution profiling of active DNA demethylation using MAB-seq and caMAB-seq. *Nat Protoc.* 2016;11:1081-1100.

CAB-Seq: Chemical Modification–Assisted Bisulfite Sequencing

CAB-Seq can detect 5caC with single-base resolution in DNA.[186] It is based on the observation that chemically modified 5caC can survive bisulfite treatment without deamination.



A schematic overview of CAB-Seq.

Advantages	Disadvantages
• Single-base resolution	• Relatively low protection rate of 5caC deamination (50–60%)[187] • Requires the sequencing of a nontreated DNA control, and the calling of the modified cytosines is based on a subtractive analysis; requires high sequencing depth to reduce the false-discovery rate

Reviews

Plongthongkum N., Diep D. H. and Zhang K. Advances in the profiling of DNA modifications: cytosine methylation and beyond. *Nat Rev Genet.* 2014;15:647-661.

References

Lu X., Song C. X., Szulwach K., Wang Z., Weidenbacher P., et al. Chemical modification-assisted bisulfite sequencing (CAB-Seq) for 5-carboxylcytosine detection in DNA. *J Am Chem Soc.* 2013;135:9315-9317.

Associated Kits

TruSeq DNA Methylation Kit

TruSeq DNA PCR-Free Library Prep Kit

186. Lu X., Song C. X., Szulwach K., et al. Chemical modification-assisted bisulfite sequencing (CAB-Seq) for 5-carboxylcytosine detection in DNA. *J Am Chem Soc.* 2013;135:9315-9317.
187. Wu H., Wu X., Shen L. and Zhang Y. Single-base resolution analysis of active DNA demethylation using methylase-assisted bisulfite sequencing. *Nat Biotechnol*. 2014;32:1231-1240.

oxBS-Seq: Oxidative Bisulfite Sequencing

oxBS-Seq differentiates between 5mC and 5hmC.[188]

In this method, 5hmC is oxidized to 5fC with a selective chemical agent, while 5mC remains unchanged. Sodium bisulfite treatment of 5fC results in its deamination to uracil which, upon sequencing, is read as a thymidine. Deep sequencing of oxBS-treated DNA and sequence comparison of treated vs untreated libraries can identify 5hmC locations at single-base resolution.

A schematic overview of oxBS-Seq.

Advantages	Disadvantages
• Covers CpG and non-CpG methylation throughout the genome at single-base resolution • Covers 5mC in dense and less dense repeat regions • Clearly differentiates between 5mC and 5hmC	• Harsh oxidation conditions lead to a substantial loss of DNA (99.5%) • Must be combined with BS-Seq in order to distinguish and quantify C, 5mC, and 5hmC

Reviews

Devall M., Roubroeks J., Mill J., Weedon M. and Lunnon K. Epigenetic regulation of mitochondrial function in neurodegenerative disease: New insights from advances in genomic technologies. *Neurosci Lett.* 2016;625:47-55.

Marx V. Genetics: profiling DNA methylation and beyond. *Nat Methods.* 2016;13:119-122.

Yong W. S., Hsu F. M. and Chen P. Y. Profiling genome-wide DNA methylation. *Epigenetics Chromatin*. 2016;9:26.

Meldi K. M. and Figueroa M. E. Cytosine modifications in myeloid malignancies. *Pharmacol Ther.* 2015;152:42-53.

Shull A. Y., Noonepalle S. K., Lee E. J., Choi J. H. and Shi H. Sequencing the cancer methylome. *Methods Mol Biol.* 2015;1238:627-651.

References

Hadad N., Masser D. R., Logan S., et al. Absence of genomic hypomethylation or regulation of cytosine-modifying enzymes with aging in male and female mice. *Epigenetics Chromatin*. 2016;9:30.

The authors used oxBS-Seq to quantify 5mC and 5hmC genome-wide in the hippocampus of young and old male and female mice. They showed that specific levels of 5mC and 5hmC differ with aging and by sex, depending on the chromosomal, promoter, and CG context.

Illumina Technology: NextSeq System

Feng J., Shao N., Szulwach K. E., et al. Role of Tet1 and 5-hydroxymethylcytosine in cocaine action. *Nat Neurosci*. 2015;18:536-544.

The researchers identified 5hmC induction in putative enhancers and coding regions of genes that have pivotal roles in drug addiction. Such induction of 5hmC, which occurred similarly following TET1 knockdown alone, correlated with increased expression of these genes as well as with their alternative splicing in response to cocaine administration. In addition, 5hmC alterations at certain loci persisted for at least 1 month after cocaine exposure. The researchers used oxBS-Seq to confirm induction of 5hmC at each of these genes in an independent cohort of animals.

Illumina Technology: HiSeq 2000 System

188. Booth M. J., Branco M. R., Ficz G., et al. Quantitative sequencing of 5-methylcytosine and 5-hydroxymethylcytosine at single-base resolution. *Science*. 2012;336:934-937.

Aijo T., Huang Y., Mannerstrom H., et al. A probabilistic generative model for quantification of DNA modifications enables analysis of demethylation pathways. *Genome Biol.* 2016;17:49.

Page A., Paoli P., Moran Salvador E., White S., French J., et al. Hepatic stellate cell transdifferentiation involves genome-wide remodeling of the DNA methylation landscape. *J Hepatol.* 2016;64:661-673.

Wallner S., Schroder C., Leitao E., Berulava T., Haak C., et al. Epigenetic dynamics of monocyte-to-macrophage differentiation. *Epigenetics Chromatin.* 2016;9:33.

Associated Kits

TruSeq DNA Methylation Kit

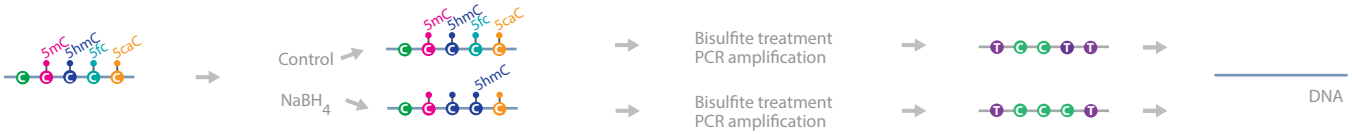
TruSeq DNA PCR-Free Library Prep Kit

TruSeq Nano DNA Library Prep Kit

redBS-Seq: Reduced Bisulfite Sequencing
caMAB-seq: 5-Carboxylcytosine Methylase-Assisted Bisulfite Sequencing

redBS-Seq relies on the chemical reduction of 5fC to 5hmC by NaBH4. The 5hmC is detected in the same manner as 5mC.[189]

In caMAB-seq, 5fC is first reduced by NaBH4 to 5hmC. Owing to the combination of NaBH4 reduction with M.SssI treatment, 5caC is sequenced as thymidine after bisulfite conversion, whereas 5mC, 5hmC, and 5fC are read as cytosine.[190]



A schematic overview of redBS-Seq.

Advantages	Disadvantages
• Base-resolution approach to map 5fC and 5caC	• Requires a high sequencing depth to reduce the false-discovery rate[191]

Reviews

Iatrou A., Kenis G., Rutten B. P., Lunnon K. and van den Hove D. L. Epigenetic dysregulation of brainstem nuclei in the pathogenesis of Alzheimer's disease: looking in the correct place at the right time? *Cell Mol Life Sci.* 2017;74:509-523.

Neri F., Incarnato D., Krepelova A., Parlato C. and Oliviero S. Methylation-assisted bisulfite sequencing to simultaneously map 5fC and 5caC on a genome-wide scale for DNA demethylation analysis. *Nat Protoc.* 2016;11:1191-1205.

References

Van Haute L., Dietmann S., Kremer L., et al. Deficient methylation and formylation of mt-tRNA(Met) wobble cytosine in a patient carrying mutations in NSUN3. *Nat Commun*. 2016;7:12039.

The authors used whole-exome sequencing to identify loss-of-function mutations in the gene for a 5mC methyltransferase, NSUN3, in a patient presenting with combined mitochondrial respiratory chain complex deficiency. They showed that NSun3 is required for deposition of 5mC at the anticodon loop in the mitochondrially encoded transfer RNA methionine (mt-tRNAMet). They also showed that 5mC deficiency in mt-tRNAMet results in the lack of 5fC at the same tRNA position. The authors used RedBS-Seq to detect 5fC in mitochondrial RNA from wt/wt and mut/mut samples.

Illumina Technology: HiSeq 2500 System

Associated Kits

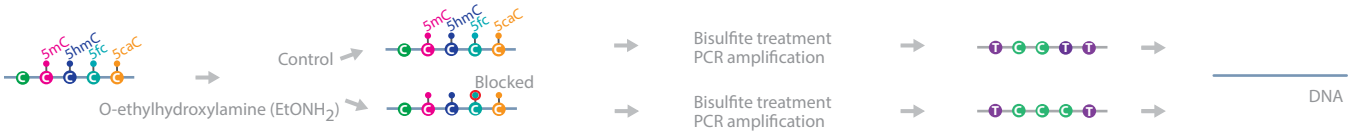
TruSeq DNA Methylation Kit

TruSeq DNA PCR-Free Library Prep Kit

189. Booth M. J., Marsico G., Bachman M., Beraldi D. and Balasubramanian S. Quantitative sequencing of 5-formylcytosine in DNA at single-base resolution. *Nat Chem.* 2014;6:435-440.
190. Wu H., Wu X. and Zhang Y. Base-resolution profiling of active DNA demethylation using MAB-seq and caMAB-seq. *Nat Protoc.* 2016;11:1081-1100.
191. Neri F., Incarnato D., Krepelova A., Parlato C. and Oliviero S. Methylation-assisted bisulfite sequencing to simultaneously map 5fC and 5caC on a genome-wide scale for DNA demethylation analysis. *Nat Protoc*. 2016;11:1191-1205.

fCAB-seq: 5-Formylcytosine Chemical Modification–Assisted Bisulfite Sequencing

fCAB-Seq is a method for EtONH2-modified bisulfite sequencing for base-resolution detection of 5fC in gDNA.[192] It is complementary to fC-Seal, which was developed as a highly selective chemical labeling approach for the affinity purification and genome-wide profiling of 5fC.[191]



A schematic overview of fCAB-Seq.

Advantages	Disadvantages
• Single-base resolution	• Requires the sequencing of a matched nontreated DNA control [193]

Reviews

Balasubramanian S. Chemical biology on the genome. *Bioorg Med Chem*. 2014;22:4356-4370.

Booth M. J., Marsico G., Bachman M., Beraldi D. and Balasubramanian S. Quantitative sequencing of 5-formylcytosine in DNA at single-base resolution. *Nat Chem*. 2014;6:435-440.

Rivera C. M. and Ren B. Mapping human epigenomes. *Cell*. 2013;155:39-55.

References

Wang L., Zhang J., Duan J., et al. Programming and inheritance of parental DNA methylomes in mammals. *Cell*. 2014;157:979-991.

The authors used fCAB-Seq to generate a base-resolution 5fC map of 2-cell mouse embryos. The distribution of CpGs at different genomic elements showed that the highly formylated elements are also the demethylated elements.

Illumina Technology: HiSeq 2000 System

Van Haute L., Dietmann S., Kremer L., Hussain S., Pearce S. F., et al. Deficient methylation and formylation of mt-tRNA(Met) wobble cytosine in a patient carrying mutations in NSUN3. *Nat Commun*. 2016;7:12039.

Xia B., Han D., Lu X., Sun Z., Zhou A., et al. Bisulfite-free, base-resolution analysis of 5-formylcytosine at the genome scale. *Nat Methods*. 2015;12:1047-1050.

Becker D., Lutsik P., Ebert P., et al. BiQ Analyzer HiMod: an interactive software tool for high-throughput locus-specific analysis of 5-methylcytosine and its oxidized derivatives. *Nucleic Acids Res*. 2014;42:W501-507.

Associated Kits

TruSeq DNA Methylation Kit

TruSeq DNA PCR-Free Library Prep Kit

192. Song C. X., Szulwach K. E., Dai Q., et al. Genome-wide profiling of 5-formylcytosine reveals its roles in epigenetic priming. *Cell*. 2013;153:678-691.
193. Neri F., Incarnato D., Krepelova A., Parlato C. and Oliviero S. Methylation-assisted bisulfite sequencing to simultaneously map 5fC and 5caC on a genome-wide scale for DNA demethylation analysis. *Nat Protoc.* 2016;11:1191-1205.

MAB-seq: M.SssI Methylase-Assisted Bisulfite Sequencing

MAB-seq allows simultaneous and quantitative mapping of both 5fC and 5caC at single-base resolution.[194] This method is complementary to caMAB-seq, a method for direct 5caC mapping.[193]

In this method, gDNA is treated with the bacterial CpG methyltransferase M.SssI, which methylates CpG dinucleotides. Next, bisulfite conversion of methylase-treated DNA leads to deamination of only 5fC and 5caC; originally unmodified CpGs are protected as 5mCpG. After sequencing, 5fC and 5caC are read as thymidine, while 5mC is read as cytosine.

A schematic overview of MAB-seq.

Advantages	Disadvantages
• Can provide a quantitative measurement of the abundance of 5fC/5caC within CpG dyads	• Unable to distinguish 5fC/5caC from unmodified C within a non-CpG context

Reviews

Meldi K. M. and Figueroa M. E. Cytosine modifications in myeloid malignancies. *Pharmacol Ther.* 2015;152:42-53.

References

Aijo T., Huang Y., Mannerstrom H., Chavez L., Tsagaratou A., et al. A probabilistic generative model for quantification of DNA modifications enables analysis of demethylation pathways. *Genome Biol*. 2016;17:49.

Neri F., Incarnato D., Krepelova A., Parlato C. and Oliviero S. Methylation-assisted bisulfite sequencing to simultaneously map 5fC and 5caC on a genome-wide scale for DNA demethylation analysis. *Nat Protoc.* 2016;11:1191-1205.

Neri F., Incarnato D., Krepelova A., et al. Single-Base Resolution Analysis of 5-Formyl and 5-Carboxyl Cytosine Reveals Promoter DNA Methylation Dynamics. *Cell Rep*. 2015;10:674-683.

Associated Kits

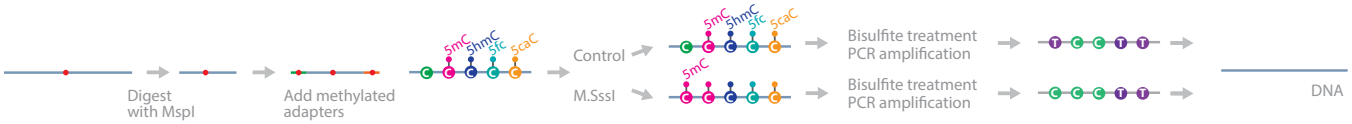
TruSeq DNA Methylation Kit

TruSeq DNA PCR-Free Library Prep Kit

194. Wu H., Wu X., Shen L. and Zhang Y. Single-base resolution analysis of active DNA demethylation using methylase-assisted bisulfite sequencing. *Nat Biotechnol.* 2014;32:1231-1240.

RRMAB-seq: Reduced-Representation M.SssI Methylase-Assisted Bisulfite Sequencing

RRMAB-seq is based on the pretreatment of gDNA with the enzyme MspI, which recognizes and cuts the CCGG consensus to enrich CpG-rich regions, like gene promoters.[195] This method is an adapted version of MAB-seq.[196]



A schematic overview of RRMAB-seq.

Advantages	Disadvantages
• Increased coverage of CpG-rich regions reduces sequencing costs and increases read depth • Reduced number of chemical or enzymatic DNA treatments required before bisulfite treatment	• Unable to distinguish 5fC/5caC from unmodified C within a non-CpG context[195]

Reviews

None available yet.

References

Neri F., Incarnato D., Krepelova A., Parlato C. and Oliviero S. Methylation-assisted bisulfite sequencing to simultaneously map 5fC and 5caC on a genome-wide scale for DNA demethylation analysis. *Nat Protoc.* 2016;11:1191-1205.

Associated Kits

TruSeq DNA Methylation Kit

TruSeq DNA PCR-Free Library Prep Kit

195. Neri F., Incarnato D., Krepelova A., et al. Single-Base Resolution Analysis of 5-Formyl and 5-Carboxyl Cytosine Reveals Promoter DNA Methylation Dynamics. *Cell Rep.* 2015;10:674-683.
196. Wu H., Wu X., Shen L. and Zhang Y. Single-base resolution analysis of active DNA demethylation using methylase-assisted bisulfite sequencing. *Nat Biotechnol.* 2014;32:1231-1240.

TAB-Seq: Tet-Assisted Bisulfite Sequencing

TAB-Seq is a novel method that uses bisulfite conversion and Tet proteins to study 5hmC.[197]

In this method, 5hmC is first protected selectively with a glucose moiety, followed by subsequent oxidation of 5mC to 5caC by Tet proteins. Next, the oxidized gDNA is treated with bisulfite; 5hmC remains unchanged and is read as a cytosine, while 5mC and unmethylated cytosines are deaminated to uracil and read as thymidines upon sequencing. Deep sequencing of TAB-treated DNA compared with untreated DNA provides accurate representation of 5hmC localization in the genome.

A schematic overview of TAB-Seq.

Advantages	Disadvantages
• Covers CpG and non-CpG hydroxymethylation throughout the genome at single-base resolution • Covers 5hmC in dense and less dense repeat regions • Clearly differentiates between 5hmC and 5mC, specifically identifying 5hmC	• Harsh oxidation can lead to substantial loss of DNA5

Reviews

Aijo T., Huang Y., Mannerstrom H., Chavez L., Tsagaratou A., et al. A probabilistic generative model for quantification of DNA modifications enables analysis of demethylation pathways. *Genome Biol.* 2016;17:49.

Yong W. S., Hsu F. M. and Chen P. Y. Profiling genome-wide DNA methylation. *Epigenetics Chromatin*. 2016;9:26.

Shull A. Y., Noonepalle S. K., Lee E. J., Choi J. H. and Shi H. Sequencing the cancer methylome. *Methods Mol Biol.* 2015;1238:627-651.

References

Bogdanovic O., Smits A. H., de la Calle Mustienes E., Tena J. J., Ford E., et al. Active DNA demethylation at enhancers during the vertebrate phylotypic period. *Nat Genet.* 2016;48:417-426.

The authors generated single base–resolution maps of 5hmC in zebrafish, Xenopus, and mouse embryos using TAB-Seq. The 5hmC signal in zebrafish, Xenopus, and mouse coincided with developmental demethylation taking place in both early and late differentially methylated regions.

Illumina Technology: HiSeq 1500 System

Chen K., Zhang J., Guo Z., et al. Loss of 5-hydroxymethylcytosine is linked to gene body hypermethylation in kidney cancer. *Cell Res*. 2016;26:103-118.

The authors used TAB-Seq to show no global loss of 5mC in kidney tumors compared with matched normal tissues. However, they observed global loss of 5hmC in virtually all kidney tumor tissues. Restoring 5hmC levels attenuated the invasion capacity of tumor cells and suppressed tumor growth in a xenograft model.

Illumina Technology: HiSeq 2000 System

Greco C. M., Kunderfranco P., Rubino M., et al. DNA hydroxymethylation controls cardiomyocyte gene expression in development and hypertrophy. *Nat Commun.* 2016;7:12418.

The authors studied the hydroxymethylome in embryonic, neonatal, adult, and hypertrophic mouse cardiomyocytes. They found that DNA hydroxymethylation was highly dynamic during cardiac development and disease. TAB-Seq analysis showed strong hydroxymethylation of LINE-1, accompanied CG demethylation, in hypertrophic cardiomyocytes. The authors suggest that, in hypertrophic heart, 5hmC may play a role in rendering chromatin structure more permissive in LINE-1 regions.

Illumina Technology: HiSeq 2000 System

197. Yu M., Hon G. C., Szulwach K. E., et al. Base-resolution analysis of 5-hydroxymethylcytosine in the mammalian genome. *Cell*. 2012;149:1368-1380.

Yu P., Ji L., Lee K. J., et al. Subsets of Visceral Adipose Tissue Nuclei with Distinct Levels of 5-Hydroxymethylcytosine. *PLoS One*. 2016;11:e0154949.

The authors used TAB-Seq analysis to demonstrate that 5hmC levels were remarkably dynamic in gene bodies of various classes of visceral adipose tissue (VAT) nuclei. These levels dropped 3.8-fold from the highest quintile of expressed genes to the lowest. Their results suggest that VAT-derived adipocytes are more active in remodeling chromatin than non-adipocytes.

Illumina Technology: NextSeq 500 System

Mooijman D., Dey S. S., Boisset J. C., Crosetto N. and van Oudenaarden A. Single-cell 5hmC sequencing reveals chromosome-wide cell-to-cell variability and enables lineage reconstruction. *Nat Biotechnol.* 2016;34:852-856.

Serandour A. A., Avner S., Mahe E. A., Madigou T., Guibert S., et al. Single-CpG resolution mapping of 5-hydroxymethylcytosine by chemical labeling and exonuclease digestion identifies evolutionarily unconserved CpGs as TET targets. *Genome Biol.* 2016;17:56.

Thienpont B., Steinbacher J., Zhao H., D'Anna F., Kuchnio A., et al. Tumour hypoxia causes DNA hypermethylation by reducing TET activity. *Nature*. 2016;537:63-68.

Guo F., Yan L., Guo H., Li L., Hu B., et al. The Transcriptome and DNA Methylome Landscapes of Human Primordial Germ Cells. *Cell*. 2015;161:1437-1452.

Li Q., Suzuki M., Wendt J., et al. Post-conversion targeted capture of modified cytosines in mammalian and plant genomes. *Nucleic Acids Res.* 2015;43:e81.

Xia B., Han D., Lu X., Sun Z., Zhou A., et al. Bisulfite-free, base-resolution analysis of 5-formylcytosine at the genome scale. *Nat Methods.* 2015;12:1047-1050.

Associated Kits

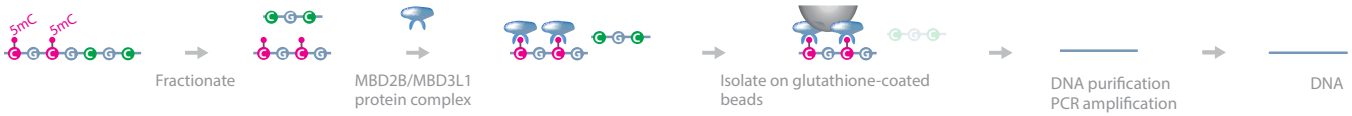
TruSeq DNA Methylation Kit

Infinium HumanMethylation450 Arrays

MIRA: Methylated-CpG Island Recovery Assay (MIRA)

MIRA uses the high affinity of a methylated-CpG-binding protein complex (MBD2B and MBD3L1) to enrich regions with methylated CpG dinucleotides.[198] This approach can be applied to both array-based DNA analysis and NGS, which are sometimes distinguished as MIRA-chip[199] and MIRA-seq.[200]

In this procedure, the fragmented gDNA is incubated with purified GST-MBD2B and His-MBD3L1 proteins. The high-affinity MBD2B/MBD3L1 complex binds to the methylated gDNA templates. The methylated gDNA fragments are captured on glutathione-coated magnetic beads. The enriched methylated DNA fraction is amplified, labeled, and analyzed by NGS.



A schematic overview of MIRA.

Advantages	Disadvantages
• High specificity and sensitivity, requiring as little as 1 ng input gDNA • Does not require DNA denaturation • Independent of restriction sites	• Resolution limited to 100 bases • Requires a minimum of 2 methylated CpGs in the captured fragment[201]

Reviews

None available yet.

References

Benjamin A. L., Green B. B., Crooker B. A., McKay S. D. and Kerr D. E. Differential responsiveness of Holstein and Angus dermal fibroblasts to LPS challenge occurs without major differences in the methylome. *BMC Genomics*. 2016;17:258.

The authors established fibroblast cultures from dairy (Holstein) and beef (Angus) cattle and challenged them with lipopolysaccharide (LPS) to examine breed-dependent differences in the innate immune response. Based on RNA-Seq, the Holstein breed displayed a more robust response to LPS than the Angus breed. MIRA-seq showed similar patterns of genome-wide DNA methylation between breeds, with overall hypomethylation of gene promoters. However, the authors discovered 49 regions of differential methylation between Holstein and Angus fibroblasts.

Illumina Technology: HiSeq 1000 System, cBot System

Jin S. G., Xiong W., Wu X., Yang L. and Pfeifer G. P. The DNA methylation landscape of human melanoma. *Genomics*. 2015;106:322-330.

The authors used MIRA-seq to characterize the DNA methylome of normal melanocytes and 27 metastatic melanoma samples. They found 179 tumor-specific methylation peaks present in all (27/27) melanomas and suggest that these peaks may act as potential disease biomarkers.

Illumina Technology: HiSeq System

Yoneda A., Henson W. R., Goldner N. K., et al. Comparative transcriptomics elucidates adaptive phenol tolerance and utilization in lipid-accumulating Rhodococcus opacus PD630. *Nucleic Acids Res.* 2016;44:2240-2254.

Associated Kits

TruSeq DNA Methylation Kit

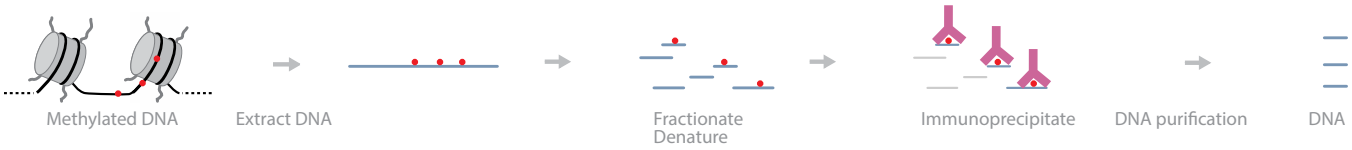
Infinium HumanMethylation450 Arrays

198. Rauch T. A. and Pfeifer G. P. DNA methylation profiling using the methylated-CpG island recovery assay (MIRA). *Methods*. 2010;52:213-217.
199. Jung M., Jin S. G., Zhang X., et al. Longitudinal epigenetic and gene expression profiles analyzed by three-component analysis reveal down-regulation of genes involved in protein translation in human aging. *Nucleic Acids Res.* 2015;43:e100.
200. Benjamin A. L., Green B. B., Crooker B. A., McKay S. D. and Kerr D. E. Differential responsiveness of Holstein and Angus dermal fibroblasts to LPS challenge occurs without major differences in the methylome. *BMC Genomics*. 2016;17:258.
201. Rauch T. A. and Pfeifer G. P. Methods for Assessing Genome-wide DNA Methylation. 2011;135-147.

MeDIP-Seq: Methylated DNA Immunoprecipitation
DIP-seq: DNA Immunoprecipitation Followed by High-Throughput Sequencing

MeDIP-seq is used to study 5mC modification.[202] It is based on MeDIP, originally developed as an approach for immunocapturing methylated DNA followed by microarray analysis.[203] A variation on this method detects 5hmC to provide a more complete view of cytosine modifications (see hMeDIP-seq).[204]

In this method, anti-5mC antibodies are used to isolate methylated DNA from fragmented gDNA via immunoprecipitation. The isolated DNA fragments are purified and used to prepare a sequencing library. Deep sequencing provides greater genome coverage, representing the majority of immunoprecipitated methylated DNA.



A schematic overview of MeDIP-seq.

Advantages	Disadvantages
• Covers CpG and non-CpG 5mC throughout the genome • Covers 5mC in dense and less dense repeat regions • Antibody-based selection is independent of sequence and does not enrich for 5hmC due to antibody specificity • Can be a cost-effective approach when single-base resolution is not desired[205]	• Base-pair resolution is lower (~150 bp), as opposed to single-base resolution with other methods • Antibody specificity and selectivity must be tested to avoid nonspecific interaction • Biased toward hypermethylated regions[204]

Reviews

Sharma G., Sowpati D. T., Singh P., et al. Genome-wide non-CpG methylation of the host genome during M. tuberculosis infection. *Sci Rep.* 2016;6:25006.

Soto J., Rodriguez-Antolin C., Vallespin E., de Castro Carpeno J. and Ibanez de Caceres I. The impact of next-generation sequencing on the DNA methylation-based translational cancer research. *Transl Res.* 2016;169:1-18 e11.

Yan H., Tian S., Slager S. L., Sun Z. and Ordog T. Genome-Wide Epigenetic Studies in Human Disease: A Primer on -Omic Technologies. *Am J Epidemiol.* 2016;183:96-109.

Yong W. S., Hsu F. M. and Chen P. Y. Profiling genome-wide DNA methylation. *Epigenetics Chromatin*. 2016;9:26.

202. Weber M., Davies J. J., Wittig D., et al. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet.* 2005;37:853-862.
203. Weber M., Davies J. J., Wittig D., et al. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet.* 2005;37:853-862.
204. Xu Y., Wu F., Tan L., et al. Genome-wide regulation of 5hmC, 5mC, and gene expression by Tet1 hydroxylase in mouse embryonic stem cells. *Mol Cell.* 2011;42:451-464.
205. Yong W. S., Hsu F. M. and Chen P. Y. Profiling genome-wide DNA methylation. *Epigenetics Chromatin*. 2016;9:26.

References

Staunstrup N. H., Starnawska A., Nyegaard M., et al. Genome-wide DNA methylation profiling with MeDIP-seq using archived dried blood spots. *Clin Epigenetics.* 2016;8:81.

There has been an increased interest in epigenome-wide association studies using dried blood spots (DBS) routinely collected in perinatal screening programs. In this study, the authors demonstrated the feasibility of using MeDIP-seq to interrogate the methylome from a single 3.2 mm DBS punch (60 ng DNA) from filter cards archived for up to 16 years. The authors conclude that the enrichment profile, sequence quality, and distribution of reads across genetic regions were comparable among samples archived 16 years, 4 years, and a freshly prepared control.

Illumina Technology: HiSeq 2000 System

Tang A., Huang Y., Li Z., et al. Analysis of a four generation family reveals the widespread sequence-dependent maintenance of allelic DNA methylation in somatic and germ cells. *Sci Rep.* 2016;6:19260.

To evaluate transgenerational DNA methylation patterns in humans, the authors used MeDIP-seq to analyze the DNA methylomes of somatic and germ cells in a 4-generation family. They found that the allelic methylation patterns for the vast majority of cis-regulated loci were shared between the somatic and germ cells from the same individual.

Illumina Technology: Genome Analyzer$_{IIx}$ System

Han B., Li W., Chen Z., et al. Variation of DNA Methylome of Zebrafish Cells under Cold Pressure. *PLoS One.* 2016;11:e0160358.

The authors performed MeDIP-seq to reveal changes in the genome-wide methylation profile of zebrafish (Danio rerio) embryonic fibroblast cells (ZF4) under cold pressure. They discovered that 21% of the methylation peaks were significantly altered after cold treatment. Approximately 8% of the altered DNA methylation peaks were located in promoter regions, while the majority of them were located in noncoding regions. The methylation of multiple genes involved in biological processes (such as development and the immune response) were significantly affected, suggesting that these processes are responsive to cold stress through the regulation of DNA methylation.

Illumina Technology: HiSeq 2000 System

Halder R., Hennion M., Vidal R. O., et al. DNA methylation changes in plasticity genes accompany the formation and maintenance of memory. *Nat Neurosci*. 2016;19:102-110.

To examine the epigenetic processes underlying short- and long-term memory, the authors studied chromatin modification changes in 2 distinct mouse brain regions, 2 cell types, and 3 time points before and after contextual learning. Using ChIP-Seq and MeDIP-seq, they found that histone modifications predominantly changed during memory acquisition and correlated surprisingly little with changes in gene expression. The data provide evidence for a molecular framework of memory acquisition and maintenance, wherein DNA methylation could alter the expression and splicing of genes involved in functional plasticity and synaptic wiring.

Illumina Technology: HiSeq 2000 System

Chowdhury B., Seetharam A., Wang Z., et al. A Study of Alterations in DNA Epigenetic Modifications (5mC and 5hmC) and Gene Expression Influenced by Simulated Microgravity in Human Lymphoblastoid Cells. *PLoS One*. 2016;11:e0147514.

Lucas E. S., Dyer N. P., Murakami K., et al. Loss of Endometrial Plasticity in Recurrent Pregnancy Loss. *Stem Cells*. 2016;34:346-356.

Pheiffer C., Erasmus R. T., Kengne A. P. and Matsha T. E. Differential DNA methylation of microRNAs within promoters, intergenic and intragenic regions of type 2 diabetic, pre-diabetic and non-diabetic individuals. *Clin Biochem*. 2016;49:433-438.

Su Y., Fan Z., Wu X., et al. Genome-wide DNA methylation profile of developing deciduous tooth germ in miniature pigs. *BMC Genomics*. 2016;17:134.

Sun L. X., Wang Y. Y., Zhao Y., et al. Global DNA Methylation Changes in Nile Tilapia Gonads during High Temperature-Induced Masculinization. *PLoS One.* 2016;11:e0158483.

Yang Y., Zhou R., Mu Y., Hou X., Tang Z., et al. Genome-wide analysis of DNA methylation in obese, lean, and miniature pig breeds. *Sci Rep*. 2016;6:30160.

Zeng Y., Yao B., Shin J., et al. Lin28A Binds Active Promoters and Recruits Tet1 to Regulate Gene Expression. *Mol Cell.* 2016;61:153-160.

Associated Kits

Infinium HumanMethylation450 Arrays

Nextera DNA Library Prep Kit

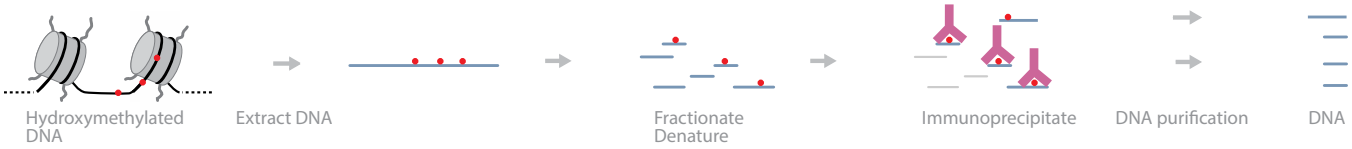
Nextera XT DNA Library Prep Kit

Nextera Rapid Capture Exome/Custom Enrichment Kit

hMeDIP-seq: Hydroxymethylated DNA Immunoprecipitation and Sequencing

hMeDIP-Seq is used to study 5hmC modifications.[206] It is a slight variation of MeDIP-seq, which is based on the original MeDIP method described by Weber *et al.*[207] Although this method is technically almost identical to MeDIP-seq, it is treated as a separate method due to the substantially different biological insight it provides. For a comprehensive insight into epigenetic changes, both 5mC and 5hmC modifications should be measured. [208]

Methylated DNA is isolated from genomic DNA via immunoprecipitation. Anti-5hmC antibodies are incubated with fragmented gDNA and precipitated, followed by DNA purification and preparation of a sequencing library. Deep sequencing provides greater genome coverage, representing the majority of immunoprecipitated hydroxymethylated DNA.



A schematic overview of hMeDIP-seq.

Advantages	Disadvantages
• Covers 5hmC in dense and less dense repeat regions • Antibody-based selection is independent of sequence and does not enrich for 5mC, due to antibody specificity	• Base-pair resolution is lower (~150 bp), as opposed to single-base resolution with other methods • Antibody specificity and selectivity must be tested to avoid nonspecific interaction • Biased toward hypermethylated regions

Reviews

Kamdar S. N., Ho L. T., Kron K. J., Isserlin R., van der Kwast T., et al. Dynamic interplay between locus-specific DNA methylation and hydroxymethylation regulates distinct biological pathways in prostate carcinogenesis. *Clin Epigenetics*. 2016;8:32.

Devall M., Roubroeks J., Mill J., Weedon M. and Lunnon K. Epigenetic regulation of mitochondrial function in neurodegenerative disease: New insights from advances in genomic technologies. *Neurosci Lett*. 2016;625:47-55.

Shull A. Y., Noonepalle S. K., Lee E. J., Choi J. H. and Shi H. Sequencing the cancer methylome. *Methods Mol Biol.* 2015;1238:627-651.

References

Chowdhury B., Seetharam A., Wang Z., Liu Y., Lossie A. C., et al. A Study of Alterations in DNA Epigenetic Modifications (5mC and 5hmC) and Gene Expression Influenced by Simulated Microgravity in Human Lymphoblastoid Cells. *PLoS One*. 2016;11:e0147514.

Although microgravity has a wide variety of physiological effects, its influence on the epigenome has not been characterized. In this study, the authors used MeDIP-seq, hMeDIP-seq, and RNA-Seq to examine the effects of induced changes on DNA methylation (5mC), hydroxymethylation (5hmC), and gene expression in cultured human lymphoblastoid cells subjected to simulated microgravity. They found that simulated microgravity induced alterations in the methylomes: ~60% of differentially methylated regions (DMRs) were hypomethylated and ~92% of differentially hydroxymethylated regions (DHMRs) were hyperhydroxymethylated. These induced changes occurred both in promoter regions and gene bodies.

Illumina Technology: HiSeq 2000 System

206. Xu Y., Wu F., Tan L., et al. Genome-wide regulation of 5hmC, 5mC, and gene expression by Tet1 hydroxylase in mouse embryonic stem cells. *Mol Cell.* 2011;42:451-464.
207. Weber M., Davies J. J., Wittig D., et al. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet.* 2005;37:853-862.
208. Kamdar S. N., Ho L. T., Kron K. J., et al. Dynamic interplay between locus-specific DNA methylation and hydroxymethylation regulates distinct biological pathways in prostate carcinogenesis. *Clin Epigenetics*. 2016;8:32.

Devall M., Roubroeks J., Mill J., Weedon M. and Lunnon K. Epigenetic regulation of mitochondrial function in neurodegenerative disease: New insights from advances in genomic technologies. *Neurosci Lett*. 2016;625:47-55.

Changes in mtDNA methylation have been implicated in a number of neurodegenerative diseases. The researchers used MeDIP-seq, hMeDIP-seq, BS-Seq, ChIP-Seq and RNA-Seq to determine the structure and function of Dnmt3a and Dnmt3b adult stem cells. They found the genes differ in their mechanisms of enhancer regulation: Dnmt3a associates with p63 to maintain high levels of DNA hydroxymethylation at the center of enhancers in a Tet2-dependent manner, whereas Dnmt3b promotes DNA methylation along the body of the enhancer. Depletion of either protein inactivates their target enhancers and profoundly affects epidermal stem cell function.

Illumina Technology: HiSeq 2000 System

Kamdar S. N., Ho L. T., Kron K. J., Isserlin R., van der Kwast T., et al. Dynamic interplay between locus-specific DNA methylation and hydroxymethylation regulates distinct biological pathways in prostate carcinogenesis. *Clin Epigenetics*. 2016;8:32.

The authors studied functional pathways and genes regulated by epigenetic modifications in prostate cancer. They used hMeDIP-seq on cancer-derived cells and found disruption of hydroxymethylation distribution, with global loss and highly specific gain in promoter and CpG island regions. They observed locus-specific retention of hydroxymethylation marks in specific intronic and intergenic regions. The authors suggest that these modifications may play a novel role in the regulation of gene expression in critical functional pathways, such as BARD1 signaling and steroid hormone receptor signaling, in cancer.

Illumina Technology: HiSeq 2000 System, HiSeq 2500 System

Bogdanovic O., Smits A. H., de la Calle Mustienes E., Tena J. J., Ford E., et al. Active DNA demethylation at enhancers during the vertebrate phylotypic period. *Nat Genet.* 2016;48:417-426.

Uribe-Lewis S., Stark R., Carroll T., et al. 5-hydroxymethylcytosine marks promoters in colon that resist DNA hypermethylation in cancer. *Genome Biol.* 2015;16:69.

Zhu L., Lv R., Kong L., et al. Genome-Wide Mapping of 5mC and 5hmC Identified Differentially Modified Genomic Regions in Late-Onset Severe Preeclampsia: A Pilot Study. *PLoS One*. 2015;10:e0134119.

Associated Kits

Infinium HumanMethylation450 Arrays

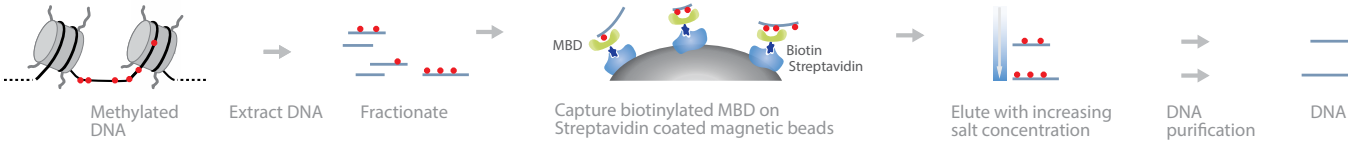
Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

MBDCap-seq: Methyl-CpG Binding Domain (MBD)–Based Capture and Sequencing
MethylCap-Seq: Capture of Methylated DNA Using the Methyl-CpG Binding Domain MBD domain of MeCP2
MiGS: Methyl-CpG Binding Domain–Isolated Genome Sequencing

MBDCap[209,210] and MethylCap[211,212] enrich methylated DNA by affinity purification. These methods are particularly suitable to investigate regional blocks of hypermethylation. The term MiGS is easily confused with the common term MIGS (minimum information about a genome sequence), and is rarely used.

In these methods, gDNA is first sonicated and incubated with tagged MBD proteins that can bind methylated cytosines. Next, the protein-DNA complex is precipitated with antibody-conjugated beads that are specific to the protein tag. The DNA is purified and used to prepare a sequencing library. Deep sequencing provides greater genome coverage, representing the majority of MBD-bound methylated DNA.



A schematic overview of MBDCap-seq.

Advantages	Disadvantages
• Interrogates regional blocks of hypermethylation • Compared to HM450K arrays, MBDCap-seq regional coverage of shores (77% vs 28%), enhancers (12% vs 2%), and insulators (11% vs 1%) is much greater[213] • MBDCap-Seq from formalin-fixed paraffin-embedded (FFPE) tissues provides equivalent methylation data to fresh-frozen DNA[212] • MDB proteins can discriminate between 5mC and 5hmC[214]	• Base-pair resolution is lower (~150 bp), as opposed to single-base resolution with other methods • Biased toward hypermethylated regions

Reviews

Soto J., Rodriguez-Antolin C., Vallespin E., de Castro Carpeno J. and Ibanez de Caceres I. The impact of next-generation sequencing on the DNA methylation-based translational cancer research. *Transl Res*. 2016;169:1-18 e11.

Tang J., Fang F., Miller D. F., Pilrose J. M., Matei D., et al. Global DNA methylation profiling technologies and the ovarian cancer methylome. *Methods Mol Biol.* 2015;1238:653-675.

Yong W. S., Hsu F. M. and Chen P. Y. Profiling genome-wide DNA methylation. *Epigenetics Chromatin.* 2016;9:26.

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Boers A., Wang R., van Leeuwen R. W., et al. Discovery of new methylation markers to improve screening for cervical intraepithelial neoplasia grade 2/3. *Clin Epigenetics*. 2016;8:29.

The authors used MethylCap-seq to identify new methylation markers for the detection of high-grade cervical intraepithelial neoplasia (CIN): CIN2/3, or CIN2 and higher (CIN2+). They found that methylation levels of 8/9 genes were significantly higher in carcinoma compared to normal scrapings of cervical tissue. For all 8 genes, methylation levels increased with the severity of the underlying histological lesion in scrapings from patients referred with an abnormal Pap smear.

Illumina Technology: Genome Analyzer II System

Stirzaker C., Zotenko E., Song J. Z., Qu W., Nair S. S., et al. Methylome sequencing in triple-negative breast cancer reveals distinct methylation clusters with prognostic value. *Nat Commun.* 2015;6:5899.

The authors used MBDCap-seq to analyze samples of FFPE tissues from triple-negative breast cancer (TNBC) and matched normal tissues. They identified regional methylation profiles specific to TNBC, which they validated using methylation data extracted from The Cancer Genome Atlas (TCGA) breast cancer cohort. They also reported the first potential TNBC-specific methylation signature of survival and suggest that it can have prognostic value in larger cohorts.

Illumina Technology: Genome Analyzer$_{IIx}$ System

Wang Y., Jadhav R. R., Liu J., et al. Roles of Distal and Genic Methylation in the Development of Prostate Tumorigenesis Revealed by Genome-wide DNA Methylation Analysis. *Sci Rep*. 2016;6:22051.

The authors used MBDCap-seq analysis on prostate cancer tissue specimens classified into low, high, and very high risk groups, based on Gleason score. They also analyzed normal and tumor-adjacent tissue samples. The results, correlated with RNA-Seq data from TCGA, suggest that DNA methylation in distal and genic regions plays critical roles in contributing to prostate tumorigenesis. It may act either positively or negatively with TSS to alter gene regulation in tumors.

Illumina Technology: Genome Analyzer$_{II}$ System

Clausen M. J., Melchers L. J., Mastik M. F., et al. Identification and validation of WISP1 as an epigenetic regulator of metastasis in oral squamous cell carcinoma. *Genes Chromosomes Cancer.* 2016;55:45-59.

Zhang X. L., Wu J., Wang J., et al. Integrative epigenomic analysis reveals unique epigenetic signatures involved in unipotency of mouse female germline stem cells. *Genome Biol.* 2016;17:162.

Locke W. J., Zotenko E., Stirzaker C., et al. Coordinated epigenetic remodelling of transcriptional networks occurs during early breast carcinogenesis. *Clin Epigenetics.* 2015;7:52.

Associated Kits

Infinium HumanMethylation450 Arrays

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

Nextera Rapid Capture Exome/Custom Enrichment

BisChIP-seq: Bisulfite-Treated Chromatin-Immunoprecipitated DNA
ChIP-BS-seq: ChIP of Bisulfite-treated chromatin-Bisulfite Sequencing
ChIP-BMS: Chromatin Immunoprecipitation with Bisulfite Methylation Sequencing Assay

BisChIP-seq, ChIP-BS-seq, and ChIP-BMS all refer to essentially the same method.[215] It is a direct, quantitative approach to assess DNA methylation patterns associated with chromatin modifications or chromatin-associated factors.[216]

The ChIP-capturing step is used to obtain a restricted representation of the genome occupied by the epigenetic feature of interest. The captured DNA fragments are subjected to end-repair, adapter ligation using methylated adapters, bisulfite conversion, PCR amplification, and NGS.

A schematic overview of BisChIP-seq.

Advantages	Disadvantages
• Genome-wide coverage of 5mC in dense CpG areas and repeat regions • MBD proteins do not interact with 5hmC	• Does not cover genome-wide CpGs and non-CpG methylation; misses areas with less dense 5mC • Base-pair resolution is lower (~150 bp), as opposed to single-base resolution with other methods • Protein-based selection is biased toward hypermethylated regions

Reviews

Plongthongkum N., Diep D. H. and Zhang K. Advances in the profiling of DNA modifications: cytosine methylation and beyond. *Nat Rev Genet.* 2014;15:647-661.

Shull A. Y., Noonepalle S. K., Lee E. J., Choi J. H. and Shi H. Sequencing the cancer methylome. *Methods Mol Biol*. 2015;1238:627-651.

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Jin J., Lian T., Gu C., et al. The effects of cytosine methylation on general transcription factors. *Sci Rep*. 2016;6:29119.

The authors used BisChIP-seq to study the effect of non-CpG methylation on DNA/protein interactions in 3 human transcription factors. They found that the glucocorticoid receptor could recognize highly methylated sites within chromatin in cells. They conclude that non-CpG methylation of DNA can provide a mechanism for regulating gene expression by directly affecting the binding of transcription factors.

Illumina Technology: HiSeq 2000 System

215. Plongthongkum N., Diep D. H. and Zhang K. Advances in the profiling of DNA modifications: cytosine methylation and beyond. *Nat Rev Genet.* 2014;15:647-661.
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Varshney D., Vavrova-Anderson J., Oler A. J., et al. SINE transcription by RNA polymerase III is suppressed by histone methylation but not by DNA methylation. *Nat Commun*. 2015;6:6569.

The authors studied the methylation of short interspersed nuclear elements (SINEs), such as Alu, to examine the effects on retrotransposition of these elements. They used ChIP-BS-seq to assess the methylation status of Alu SINEs bound to RNA polymerase III (RNAPIII) and found high levels of CpG methylation. This DNA methylation did not deter the binding of RNAPIII to Alu SINEs, and loss of DNA methylation had little effect on accessibility of SINEs to the transcriptional machinery. The authors conclude that methylation of histones, rather than DNA, plays a dominant role in suppressing SINE transcription.

Illumina Technology: HiSeq System

Associated Kits

Infinium HumanMethylation450 Arrays

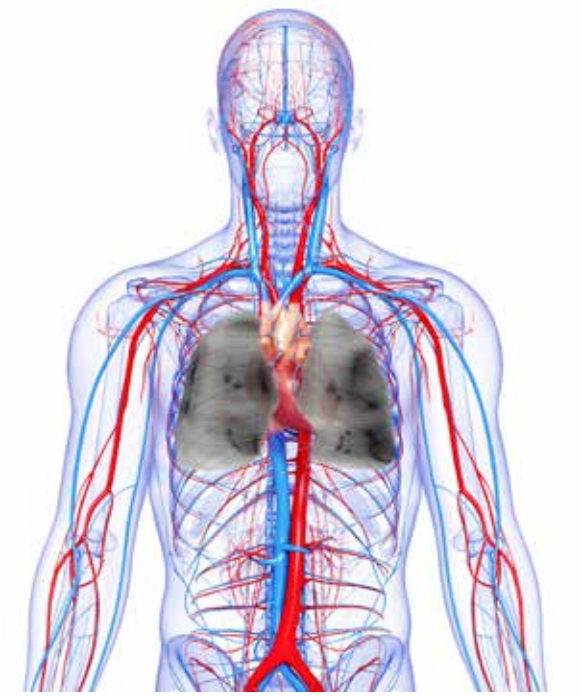
Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

Nextera Rapid Capture Exome/Custom Enrichment

DNA-PROTEIN INTERACTIONS

Chromatin remodeling is a dynamic process driven by factors that change DNA-protein interactions. These epigenetic factors can involve protein modifications, such as histone methylation, acetylation, phosphorylation, and ubiquitination.[217] Histone modifications affect gene activation by recruiting regulatory factors and maintaining an open or closed chromatin state. Epigenetic factors play roles in tissue development,[218] embryogenesis, cell fate, immune response, and diseases such as cancer.[219] Bacterial pathogens can elicit transcriptional repression of immune genes by chromatin remodeling.[220] The study of protein-DNA interactions has also demonstrated that chromatin remodeling can respond to external factors, such as excessive alcohol-seeking behaviors,[221] cigarette smoking,[222] and clinical drugs.



Cigarette smoking disrupts DNA-protein interactions, leading to the development of cancers or pulmonary diseases.

Reviews

Chaitankar V., Karakulah G., Ratnapriya R., et al. Next generation sequencing technology and genomewide data analysis: Perspectives for retinal research. *Prog Retin Eye Res.* 2016;55:1-31.

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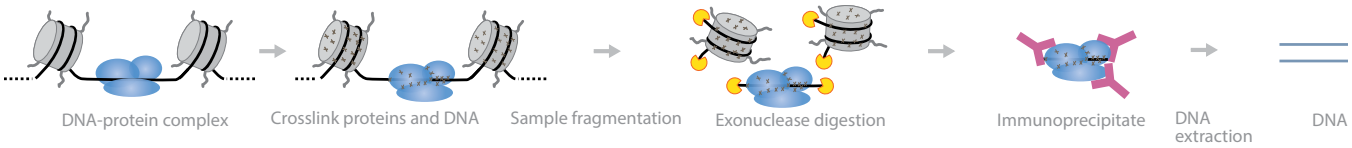
ChIP-Seq: Chromatin Immunoprecipitation Sequencing
HT-ChIP: High-Throughput ChIP
ChIP-exo: Exonuclease trimmed ChIP
Mint-ChIP: Multiplexed ChIP

ChIP-Seq is a well-established method to map specific protein-binding sites.[223] It has given rise to a vast number of derivatives, such as AHT-ChIP-Seq,[224] BisChIP-Seq,[225] CAST-ChIP,[226] ChIP-BMS,[227] ChIP-BS-seq,[228] ChIPmentation,[229] Drop-ChIP,[230] Mint-ChIP,[231] PAT–ChIP,[232] reChIP-seq,[233] scChIP-seq,[234] and X-ChIP.[235] Sequential ChIP-seq (reChIP) can also show the association of different proteins on the chromatin.[236]

In this method, DNA-protein complexes are crosslinked *in vivo*. Next, samples are fragmented and treated with an exonuclease to trim unbound oligonucleotides. Protein-specific antibodies are used to immunoprecipitate the DNA-protein complex. The DNA is extracted, purified, and sequenced, giving high-resolution sequences of the protein-binding sites.



A schematic overview of ChIP-Seq.

Advantages	Disadvantages
• Base-pair resolution of protein-binding sites • Can map specific regulatory factors or proteins • Exonuclease use eliminates contamination by unbound DNA[237]	• Nonspecific antibodies can dilute the pool of DNA-protein complexes of interest • Target protein must be known and be able to raise an antibody

Reviews

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The endogenous function of DNA methyltransferases Dnmt3a and Dnmt3b in adult stem cells are unknown. In this study, the authors used ChIP-Seq in human epidermal stem cells to show that Dnmt3a and Dnmt3b bind in a histone H3K36me3-dependent manner to the most active enhancers and are required to produce their associated enhancer RNAs. Depletion of either protein inactivates their target enhancers and profoundly affects epidermal stem cell function.

Illumina Technology: HiSeq 2000 System

Woolnough J. L., Atwood B. L., Liu Z., Zhao R. and Giles K. E. The Regulation of rRNA Gene Transcription during Directed Differentiation of Human Embryonic Stem Cells. *PLoS One.* 2016;11:e0157276.

To compare the timing of the downregulation of rRNA synthesis with the binding of the RNA polymerase I transcription factor, UBTF, the authors performed ChIP-Seq on untreated H9 ESCs. They compared the binding to H9 ESCs after 6 hours of treatment with the TGBF-β family member, ACTIVIN A. The overall binding of UBTF to the rRNA gene was reduced roughly 50% after a 6 hour ACTIVIN A treatment. This result was consistent with the reduction in total rRNA synthesis measured by 32P metabolic labeling.

Illumina Technology: HiSeq System

Schmidt S. F., Madsen J. G., Frafjord K. O., et al. Integrative Genomics Outlines a Biphasic Glucose Response and a ChREBP-RORgamma Axis Regulating Proliferation in beta Cells. *Cell Rep.* 2016;16:2359-2372.

Nelson D. M., Jaber-Hijazi F., Cole J. J., et al. Mapping H4K20me3 onto the chromatin landscape of senescent cells indicates a function in control of cell senescence and tumor suppression through preservation of genetic and epigenetic stability. *Genome Biol.* 2016;17:158.

Wu T. P., Wang T., Seetin M. G., et al. DNA methylation on N(6)-adenine in mammalian embryonic stem cells. *Nature*. 2016;532:329-333.

Zwart W., Flach K. D., Rudraraju B., et al. SRC3 Phosphorylation at Serine 543 Is a Positive Independent Prognostic Factor in ER-Positive Breast Cancer. *Clin Cancer Res.* 2016;22:479-491.

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De S., Mitra A., Cheng Y., Pfeifer K. and Kassis J. A. Formation of a Polycomb-Domain in the Absence of Strong Polycomb Response Elements. *PLoS Genet.* 2016;12:e1006200.

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Weiner A., Hsieh T. H., Appleboim A., et al. High-resolution chromatin dynamics during a yeast stress response. *Mol Cell.* 2015;58:371-386.

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

DNaseI Seq or DNase-Seq: DNase I Hypersensitive Sites Sequencing

DNase I footprinting was first published in 1978[238] and predates both Sanger sequencing and NGS. The first published use with NGS was published by Boyle et al. 2008[239] and later optimized for sequencing.[240] A high-sensitivity protocol is also available (scDNase-seq).[241]

In this method, DNA-protein complexes are treated with DNase I, followed by DNA extraction and sequencing. Sequences bound by regulatory proteins are protected from DNase I digestion. Deep sequencing provides accurate representation of the location of regulatory proteins in the genome. In a variation on this approach, the DNA-protein complexes are stabilized by formaldehyde crosslinking before DNase I digestion. The crosslinking is reversed before DNA purification. In an alternative modification, called GeF-seq, both the crosslinking and the DNase I digestion are carried out *in vivo*, within permeabilized cells.[242]

A schematic overview of DNase-seq.

Advantages	Disadvantages
• Can detect "open" chromatin[243] • No prior knowledge of the sequence or binding protein is required • Compared to formaldehyde-assisted isolation of regulatory elements and sequencing (FAIRE-seq), has greater sensitivity at promoters[244]	• DNase I is sequence-specific and hypersensitive sites might not account for the entire genome[245] • DNA loss through the multiple purification steps limits sensitivity[246] • Integration of DNase I with ChIP data is necessary to identify and differentiate similar protein-binding sites

Reviews

Chaitankar V., Karakulah G., Ratnapriya R., Giuste F. O., Brooks M. J., et al. Next generation sequencing technology and genomewide data analysis: Perspectives for retinal research. *Prog Retin Eye Res.* 2016;55:1-31.

Yan H., Tian S., Slager S. L., Sun Z. and Ordog T. Genome-Wide Epigenetic Studies in Human Disease: A Primer on -Omic Technologies. *Am J Epidemiol*. 2016;183:96-109.

References

Qiu Z., Li R., Zhang S., et al. Identification of Regulatory DNA Elements Using Genome-wide Mapping of DNase I Hypersensitive Sites during Tomato Fruit Development. *Mol Plant.* 2016;9:1168-1182.

The authors studied the development and ripening of tomato fruit, events that are controlled precisely by the accessibility of regulatory elements to transcription factors. They used DNase-seq to generate stage-specific, genome-wide, high-resolution DNase I hypersensitive site maps from fruit tissues of the tomato cultivar "Moneymaker." They combined the data with gene expression data sets at 20 days after anthesis and break stage, to characterize chromatin accessibility and expression changes to fruit developmental cues.

Illumina Technology: HiSeq 2500 System

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246. Lu F., Liu Y., Inoue A., Suzuki T., Zhao K. and Zhang Y. Establishing Chromatin Regulatory Landscape during Mouse Preimplantation Development. *Cell*. 2016;165:1375-1388.

Frank C. L., Manandhar D., Gordan R. and Crawford G. E. HDAC inhibitors cause site-specific chromatin remodeling at PU.1-bound enhancers in K562 cells. *Epigenetics Chromatin*. 2016;9:15.

The authors used DNase-seq and RNA-Seq to examined changes in response of K562 cells to sub-lethal treatment with of histone deacetylase inhibitors (HDACi). As cell proliferation slowed, chromatin accessibility increased or decreased in several thousand gene regulatory elements. These changes coincided with nearby gene expression changes and likely represent enhancer element activation or deactivation events.

Illumina Technology: HiSeq 2000 System

Lu F., Liu Y., Inoue A., Suzuki T., Zhao K., et al. Establishing Chromatin Regulatory Landscape during Mouse Preimplantation Development. *Cell*. 2016;165:1375-1388.

The authors used DNase-seq to generate DNase I hypersensitive site maps of mouse preimplantation embryos from 1-cell to the morula stage. Their data revealed the dynamics of chromatin accessibility during mouse preimplantation development. They identified key transcription factors involved in the establishment of the regulatory landscape early in mammalian life.

Illumina Technology: HiSeq 2500 System

Badal S. S., Wang Y., Long J., et al. miR-93 regulates Msk2-mediated chromatin remodelling in diabetic nephropathy. *Nat Commun*. 2016;7:12076.

The authors used DNase I digestion to assess miR-93–modulated DNase1 hypersensitivity changes in podocytes cultured in high-glucose (HG) conditions. A number of HG-related DNase I hypersensitive sites were reversed upon miR-93 overexpression. Some of these hypersensitive sites were located at the TSS of several genes critical in the development of diabetic nephropathy.

Illumina Technology: HiSeq 2000 System

Adar S., Hu J., Lieb J. D. and Sancar A. Genome-wide kinetics of DNA excision repair in relation to chromatin state and mutagenesis. *Proc Natl Acad Sci U S A.* 2016;113:E2124-2133.

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Schmidt S. F., Madsen J. G., Frafjord K. O., Poulsen L., Salo S., et al. Integrative Genomics Outlines a Biphasic Glucose Response and a ChREBP-RORgamma Axis Regulating Proliferation in beta Cells. *Cell Rep.* 2016;16:2359-2372.

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Thompson B., Varticovski L., Baek S. and Hager G. L. Genome-Wide Chromatin Landscape Transitions Identify Novel Pathways in Early Commitment to Osteoblast Differentiation. *PLoS One.* 2016;11:e0148619.

Yang R., Kerschner J. L., Gosalia N., et al. Differential contribution of cis-regulatory elements to higher order chromatin structure and expression of the CFTR locus. *Nucleic Acids Res.* 2016;44:3082-3094.

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

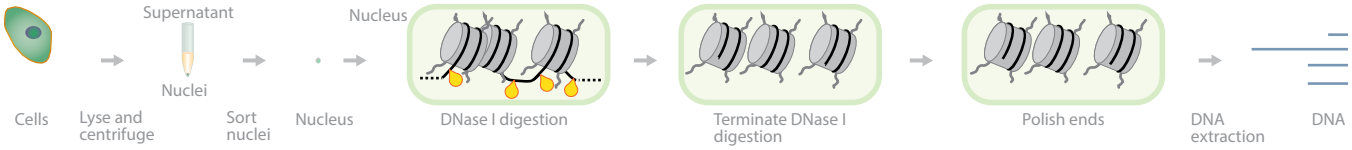
TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

DNase I SIM: DNase I Simplified in-Nucleus Method for plants

This method is a simplified DNase I protocol specifically intended for plants. It contains an additional step of nuclei purification in Percoll gradients prior to DNase I digestion, in order to remove cellular debris and starch granules more efficiently. A DNA end-polishing step, using T4 DNA polymerase, is performed directly in the nuclei following DNase I digestion. These additional steps obviate the need for gel purification and its attendant loss of material.



A schematic overview of DNase I SIM.

Advantages	Disadvantages
• Does not require gel purification	• Optimized for plants only

Reviews

None available yet.

References

Cumbie J. S., Filichkin S. A. and Megraw M. Improved DNase-seq protocol facilitates high resolution mapping of DNase I hypersensitive sites in roots in Arabidopsis thaliana. *Plant Methods.* 2015;11:42.

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

MNase-Seq: Micrococcal Nuclease Sequencing
MAINE-Seq: Micrococcal Nuclease–Assisted Isolation of Nucleosomes
Nucleo-Seq: Isolated Nucleosome Sequencing
Nuc-seq: Isolated Nucleosome Sequencing

Micrococcal nuclease (MNase) is derived from *Staphylococcus aureus*, and its first use to determine chromatin structure dates back to 1975, when the method was called, variously, staphylococcal nuclease or micrococcal nuclease digestion of nuclei or chromatin.[247,248] With the advent of NGS, MNase digestion[249] became more popular and the term MNase-Seq was coined finally.[250] The terms MNase-assisted isolation of nucleosomes sequencing (MAINE-seq),[251,252] Nucleo-Seq, [254] and Nuc-seq are not commonly used. MNase, fused to the protein of interest, has been also been used for calcium-dependent cleavage to study specific genomic loci *in vivo* (ChEC-seq).[255]

In MNase-Seq, gDNA is treated with MNase. Sequences bound by chromatin proteins are protected from MNase digestion. Next, the DNA from the DNA-protein complexes is extracted and used to prepare a sequencing library. Deep sequencing provides accurate representation of the location of regulatory DNA-binding proteins in the genome. [256]

A schematic overview of MNase-Seq.

Advantages	Disadvantages
• Can map nucleosomes and other DNA-binding proteins • Can footprint subnucleosomal particles protecting as little as ~25 bp[258] • Identifies location of various regulatory proteins in the genome • Covers a broad range of regulatory sites	• MNase sites might not account for the entire genome • AT-dependent sequence bias[259] • Integration of MNase with ChIP data is necessary to identify and differentiate similar protein-binding sites

Reviews

Yan H., Tian S., Slager S. L., Sun Z. and Ordog T. Genome-Wide Epigenetic Studies in Human Disease: A Primer on -Omic Technologies. *Am J Epidemiol.* 2016;183:96-109.

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259. Kensche P. R., Hoeijmakers W. A., Toenhake C. G., et al. The nucleosome landscape of Plasmodium falciparum reveals chromatin architecture and dynamics of regulatory sequences. *Nucleic Acids Res.* 2016;44:2110-2124.

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Lavender C. A., Cannady K. R., Hoffman J. A., et al. Downstream Antisense Transcription Predicts Genomic Features That Define the Specific Chromatin Environment at Mammalian Promoters. *PLoS Genet*. 2016;12:e1006224.

The authors performed MNase-Seq on T47D/A1-2 cells to investigate the interplay between antisense transcription and nucleosome positioning. The results showed that nucleosomes were regularly positioned relative to all 3 classes of identified TSSs. Like gene TSSs, MNase-Seq read density was consistent with "+1" nucleosomes placed immediately downstream of daTSSs and uaTSSs, though MNase-Seq peaks were less sharp when centered on daTSS positions.

Illumina Technology: HiSeq System, MiSeq System

Rube H. T., Lee W., Hejna M., et al. Sequence features accurately predict genome-wide MeCP2 binding *in vivo*. *Nat Commun*. 2016;7:11025.

The authors used MNase-Seq to investigate genomic locations of nucleosomes in wild-type olfactory epithelial tissue *in vivo*. The expressed genes had deep nucleosome-depleted regions around the TSS and distinct +1 nucleosomes. The transcription termination sites were also depleted of nucleosomes. Overlaying the MNase-Seq and methyl-CpG binding protein 2 (MeCP2) ChIP-Seq data showed preferential colocalization of MeCP2 peaks with nucleosomes.

Illumina Technology: HiSeq 2000 System

de Dieuleveult M., Yen K., Hmitou I., et al. Genome-wide nucleosome specificity and function of chromatin remodellers in ES cells. *Nature*. 2016;530:113-116.

This study used MNase-Seq to develop genome-wide remodeler–nucleosome interaction profiles for the chromatin remodelers Chd1, Chd2, Chd4, Chd6, Chd8, Chd9, Brg1, and Ep400 in mouse ESCs. After Ep400 depletion, the authors detected an increased MNase resistance, particularly at the –1 nucleosome, where Ep400 was enriched at both positively and negatively regulated genes. This effect was most evident at H3K4me3 promoters, which are bound by high Ep400 levels, compared to bivalent promoters. The authors suggest that Ep400 may therefore alter the structure of the –1 nucleosome.

Illumina Technology: Genome Analyzer$_{IIx}$ System

Cole H. A., Cui F., Ocampo J., et al. Novel nucleosomal particles containing core histones and linker DNA but no histone H1. *Nucleic Acids Res.* 2016;44:573-581.

Deniz O., Flores O., Aldea M., Soler-Lopez M. and Orozco M. Nucleosome architecture throughout the cell cycle. *Sci Rep.* 2016;6:19729.

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Kensche P. R., Hoeijmakers W. A., Toenhake C. G., Bras M., Chappell L., et al. The nucleosome landscape of Plasmodium falciparum reveals chromatin architecture and dynamics of regulatory sequences. *Nucleic Acids Res.* 2016;44:2110-2124.

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Matveeva E., Maiorano J., Zhang Q., et al. Involvement of PARP1 in the regulation of alternative splicing. *Cell Discov.* 2016;2:15046.

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Ramakrishnan S., Pokhrel S., Palani S., et al. Counteracting H3K4 methylation modulators Set1 and Jhd2 co-regulate chromatin dynamics and gene transcription. *Nat Commun*. 2016;7:11949.

Wang M., Wang P., Tu L., et al. Multi-omics maps of cotton fibre reveal epigenetic basis for staged single-cell differentiation. *Nucleic Acids Res*. 2016;44:4067-4079.

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

FiT-Seq: Fixed-Tissue Chromatin Immunoprecipitation Sequencing

FiT-seq is a method to extract soluble chromatin from FFPE tissue samples for the detection of histone-binding sites.[260] The distinguishing feature of this method is a proteinase K digestion step to reverse the effects of heavily crosslinked fixed chromatin in FFPE tissues, before the sonication step.

A schematic overview of FiT-seq.

Advantages	Disadvantages
• Works on FFPE samples • Higher resolution and sensitivity than pathology tissue chromatin immunoprecipitation (PAT-ChIP)	• Not tested in other laboratories • Sonication may introduce sequence bias[261]

Reviews

None available yet.

References

Cejas P., Li L., O'Neill N. K., Duarte M., Rao P., et al. Chromatin immunoprecipitation from fixed clinical tissues reveals tumor-specific enhancer profiles. Nat Med. 2016;22:685-691.

The authors tested FiT-seq on human clinical samples that had been preserved between 2 and 15 years (median 8 years). They were able to assess various histone marks readily and to generate chromatin-state maps comparable to those from cell lines and fresh-frozen tumors.

Illumina Technology: NextSeq 500 System

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

260. Cejas P., Li L., O'Neill N. K., et al. Chromatin immunoprecipitation from fixed clinical tissues reveals tumor-specific enhancer profiles. *Nat Med.* 2016;22:685-691.
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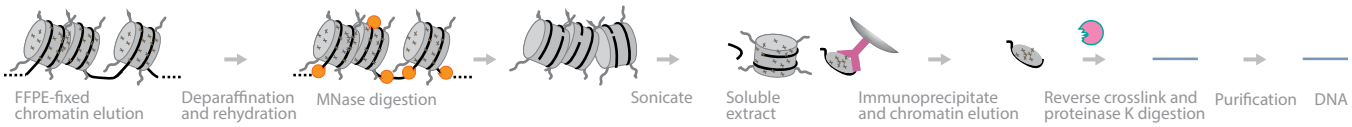
PAT-ChIP: Pathology Tissue Chromatin Immunoprecipitation

PAT-ChIP is a variation of ChIP-Seq, optimized for the analysis of chromatin derived from FFPE samples. The distinguishing features of this method are dewaxing with a xylene substitute (Histolemon, Carlo Erba) followed by both MNase digestion and sonication.[262] FiT-seq introduces further refinements to this method.[263]



A schematic overview of PAT-ChIP.

Advantages	Disadvantages
• Works on FFPE samples	• Long protocol takes 4 days • Low yield[259] • Sonication may introduce sequence bias[264]

Reviews

Mimura I., Kanki Y., Kodama T. and Nangaku M. Revolution of nephrology research by deep sequencing: ChIP-seq and RNA-seq. *Kidney Int.* 2014;85:31-38.

References

Cejas P., Li L., O'Neill N. K., Duarte M., Rao P., et al. Chromatin immunoprecipitation from fixed clinical tissues reveals tumor-specific enhancer profiles. *Nat Med.* 2016;22:685-691.

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

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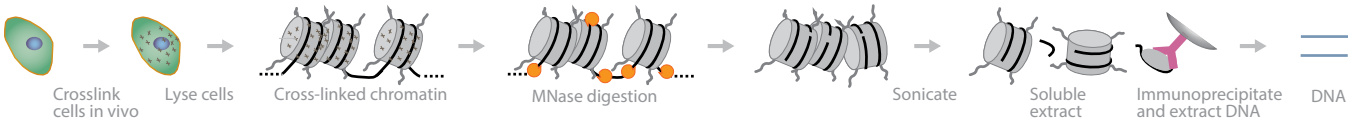
263. Cejas P., Li L., O'Neill N. K., et al. Chromatin immunoprecipitation from fixed clinical tissues reveals tumor-specific enhancer profiles. *Nat Med.* 2016;22:685-691.
264. Teytelman L., Ozaydin B., Zill O., et al. Impact of chromatin structures on DNA processing for genomic analyses. *PLoS One.* 2009;4:e6700.

X-ChIP-seq: High-Resolution Crosslinking Chromatin Immunoprecipitation Sequencing

Crosslinking chromatin immunoprecipitation (X-ChIP) is a foundational technique in chromatin research.[265,266] With the advent of NGS this simple technique, now called X-ChIP-seq, is able produce high-resolution results.[267]

The method involves crosslinking chromatin-bound DNA in cells with 1% formaldehyde for 10 minutes at room temperature. The cells are washed and resuspended in lysis buffer. After MNase digestion, the chromatin is solubilized by brief sonication and then subjected to chromatin immunoprecipitation. The DNA is extracted, enriched for short fragments, and used to prepare a sequencing library.



A schematic overview of X-ChIP-seq.

Advantages	Disadvantages
• Single-base resolution	• Sonication may introduce sequence bias[268] • Captures protein-DNA interactions regardless of duration[269]

Reviews

Zentner G. E. and Henikoff S. High-resolution digital profiling of the epigenome. *Nat Rev Genet.* 2014;15:814-827.

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Schmidt S. V., Krebs W., Ulas T., et al. The transcriptional regulator network of human inflammatory macrophages is defined by open chromatin. *Cell Res*. 2016;26:151-170.

To determine how prolonged exposure to typical macrophage stimuli would alter transcription through long-term changes in histone modifications in human macrophages, the authors performed transcriptome analysis and ChIP-Seq. They used X-ChIP-seq to study 4 histone modifications: H3K4me1 (enhancers), H3K4me3 (promoters), H3K27ac (active chromatin states), and H3K27me3 (poised enhancers and promoters). They were able to define a unique network of transcriptional and epigenetic regulators (TRs), which was characterized by accessible promoters, independent of the activation signal. The results support a model where macrophage activation during inflammation is regulated transcriptionally mainly by a predefined TR network.

Illumina Technology: HiSeq 1000 System

Elsasser S. J., Noh K. M., Diaz N., Allis C. D. and Banaszynski L. A. Histone H3.3 is required for endogenous retroviral element silencing in embryonic stem cells. *Nature*. 2015;522:240-244.

The authors used both native ChiP-Seq and X-ChIP-seq in mouse ESCs to study the silencing of a subset of TEs—endogenous retroviral elements (ERVs)—mediated by histone modifications. They identified 79,532 regions of H3.3 enrichment across the entire mouse genome, including repetitive regions. H3.3 was associated with both active and repressed chromatin states. While most H3.3 peaks localized to genic regions and intergenic regulatory regions such as enhancers, 23% intersected with heterochromatic regions. The authors conclude that their results establish an important role for H3.3 in control of ERV retrotransposition in the mouse genome.

Illumina Technology: HiSeq 2000 System

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

TruSeq Nano DNA Library Prep Kit

Nextera DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

Nextera XT DNA Library Prep Kit

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269. Zentner G. E., Kasinathan S., Xin B., Rohs R. and Henikoff S. ChEC-seq kinetics discriminates transcription factor binding sites by DNA sequence and shape *in vivo*. *Nat Commun*. 2015;6:8733.

ORGANIC: Occupied Regions of Genomes from Affinity-Purified Naturally Isolated Chromatin

ORGANIC is a gentle protocol that avoids crosslinking and sonication.[270] The method is a combination of MNase-Seq and native ChIP that provides accurate maps of chromatin-occupied regions in complex eukaryotic genomes.

A schematic overview of ORGANIC.

Advantages	Disadvantages
• Avoids sonication bias[271] • Avoids crosslinking artifacts[272]	• Potentially poor solubilization of proteins[273] • MNase sequence bias[274] • Nuclei isolation may introduce artifacts • Not replicated in other laboratories

Reviews

Zentner G. E. and Henikoff S. High-resolution digital profiling of the epigenome. *Nat Rev Genet.* 2014;15:814-827.

References

Kasinathan S., Orsi G. A., Zentner G. E., Ahmad K. and Henikoff S. High-resolution mapping of transcription factor binding sites on native chromatin. *Nat Methods.* 2014;11:203-209.

Zentner G. E. and Henikoff S. Mot1 redistributes TBP from TATA-containing to TATA-less promoters. *Mol Cell Biol*. 2013;33:4996-5004.

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

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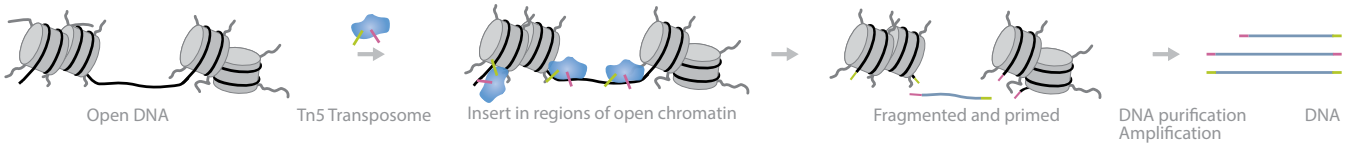
ATAC-Seq: Assay for Transposase-Accessible Chromatin Sequencing
Fast-ATAC: ATAC-seq Optimized for Blood Cells

ATAC-Seq uses the Tn5 transposome to detect nucleosome-free regions of the genome.[275] The method is commonly used, and optimized protocols are available for tissues, such as blood (Fast-ATAC),[276] neurons,[277] biobank specimens,[278] and single cells (scATAC-seq[279] and single-cell ATAC-seq[280]).

In this method, gDNA is incubated with Tn5 transposomes, which fragments it and adds adapters simultaneously, in open chromatin regions. Deep sequencing of the purified regions provides base-pair resolution of nucleosome-free regions in the genome.



A schematic overview of ATAC-seq.

Advantages	Disadvantages
• Two-step protocol with no adapter ligation steps, gel purification, or crosslink reversal • High signal-to-noise ratio compared to FAIRE-Seq	• During mechanical sample processing, bound chromatin regions might open and be tagged by the transposome • Only half of the molecules contain the adapters in the orientation required for PCR amplification • Distance between adapter sites may not be optimal for PCR amplification[281]

Reviews

Chaitankar V., Karakulah G., Ratnapriya R., Giuste F. O., Brooks M. J., et al. Next generation sequencing technology and genomewide data analysis: Perspectives for retinal research. *Prog Retin Eye Res.* 2016;55:1-31.

Yan H., Tian S., Slager S. L., Sun Z. and Ordog T. Genome-Wide Epigenetic Studies in Human Disease: A Primer on -Omic Technologies. *Am J Epidemiol.* 2016;183:96-109.

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Bogdanovic O., Smits A. H., de la Calle Mustienes E., Tena J. J., Ford E., et al. Active DNA demethylation at enhancers during the vertebrate phylotypic period. *Nat Genet.* 2016;48:417-426.

The authors studied epigenetic modifications during the phylotypic stage in zebrafish embryos. They observed widespread, Tet-dependent DNA demethylation of thousands of enhancers associated with conserved regulatory pathways. They used ATAC-seq and whole-genome methylome profiling of tet1-tet2-tet3 morphant zebrafish embryos and demonstrated an upstream regulatory role for DNA methylation in these conserved genomic elements.

Illumina Technology: HiSeq 1500 System

Corces M. R., Buenrostro J. D., Wu B., Greenside P. G., Chan S. M., et al. Lineage-specific and single-cell chromatin accessibility charts human hematopoiesis and leukemia evolution. *Nat Genet.* 2016;48:1193-1203.

This study used Fast-ATAC to create a comprehensive map of chromatin accessibility and transcriptional landscapes of 13 human primary blood cell types in a total of 137 samples. Single-cell ATAC-seq of acute myeloid leukemia (AML) cells showed distinctive, mixed-regulome profiles corresponding to disparate developmental stages, possibly caused by HOX factors. The authors suggest that these results could provide insights into hematopoietic development and disease.

Illumina Technology: HiSeq 2000 System, HiSeq 2500 System, NextSeq 500 System

Miller C. L., Pjanic M., Wang T., et al. Integrative functional genomics identifies regulatory mechanisms at coronary artery disease loci. *Nat Commun*. 2016;7:12092.

The authors examined the regulatory roles of epigenetic mechanisms in human coronary artery smooth muscle cells (HCASMCs) to gain insight into the development of coronary artery disease. They used ATAC-seq to generate epigenomic profiles in primary cultured HCASMCs stimulated with various growth factors, as well as in normal and atherosclerotic human coronary artery tissues. They integrated the data with transcription-factor binding and H3K27ac ChIP-Seq data to define HCASMC-enriched cis-regulatory mechanisms.

Illumina Technology: HiSeq 2500 System

Wu J., Huang B., Chen H., et al. The landscape of accessible chromatin in mammalian preimplantation embryos. *Nature*. 2016;534:652-657.

The authors used ATAC-seq with CRISPR/Cas9-assisted mtDNA depletion to produce a genome-wide map of accessible chromatin in mouse preimplantation embryos. They found that, despite extensive parental asymmetry in DNA methylomes, the chromatin accessibility between the parental genomes is globally comparable after major zygotic genome activation.

Illumina Technology: HiSeq 1500 System, HiSeq 2500 System

Ackermann A. M., Wang Z., Schug J., Naji A. and Kaestner K. H. Integration of ATAC-seq and RNA-seq identifies human alpha cell and beta cell signature genes. *Mol Metab.* 2016;5:233-244.

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Koues O. I., Collins P. L., Cella M., et al. Distinct Gene Regulatory Pathways for Human Innate versus Adaptive Lymphoid Cells. *Cell*. 2016;165:1134-1146.

Lu F., Liu Y., Inoue A., Suzuki T., Zhao K., et al. Establishing Chromatin Regulatory Landscape during Mouse Preimplantation Development. *Cell*. 2016;165:1375-1388.

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Shih H. Y., Sciume G., Mikami Y., et al. Developmental Acquisition of Regulomes Underlies Innate Lymphoid Cell Functionality. *Cell*. 2016;165:1120-1133.

Smith J. D., Suresh S., Schlecht U., et al. Quantitative CRISPR interference screens in yeast identify chemical-genetic interactions and new rules for guide *RNA design.* Genome Biol. 2016;17:45.

Wang L., Siegenthaler J. A., Dowell R. D. and Yi R. Foxc1 reinforces quiescence in self-renewing hair follicle stem cells. *Science*. 2016;351:613-617.

Wang W., Org T., Montel-Hagen A., et al. MEF2C protects bone marrow B-lymphoid progenitors during stress haematopoiesis. *Nat Commun.* 2016;7:12376.

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

THS-seq: Transposome Hypersensitive Sites Sequencing

THS-seq is a method for highly sensitive characterization of chromatin accessibility. This variation on ATAC-seq uses linear amplification of accessible DNA ends, *in vitro* transcription, and an engineered Tn5 super-mutant.[282]

A schematic overview of THS-seq.

Advantages	Disadvantages
• Requires very little input material • Can detect small regions near distal enhancers	• Requires an engineered transposase

Reviews

None available yet.

References

Sos B. C., Fung H. L., Gao D. R., Osothprarop T. F., Kia A., et al. Characterization of chromatin accessibility with a transposome hypersensitive sites sequencing (THS-seq) assay. *Genome Biol.* 2016;17:20.

The authors developed THS-seq to address the limitations of ATAC-seq. They demonstrated the improved sensitivity of THS-seq in 100 GM12878 lymphoblastoid cells, which yielded approximately 110,000 unique transposition events per cell. THS-seq showed an approximate 500-fold improvement in sensitivity over published ENCODE ATAC-seq data.

Illumina Technology: MiSeq System, HiSeq 2500 System

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

282. Sos B. C., Fung H. L., Gao D. R., et al. Characterization of chromatin accessibility with a transposome hypersensitive sites sequencing (THS-seq) assay. *Genome Biol.* 2016;17:20.

CATCH-IT: Covalent Attachment of Tags to Capture Histones and Identify Turnover

CATCH-IT is a direct method for measuring nucleosome turnover dynamics genome-wide.[283]

In this method, cells are treated briefly with the methionine surrogate azidohomoalanine (Aha), which couples biotin to nucleosomes containing newly incorporated histones. The labeled chromatin is affinity-purified with streptavidin, washed stringently to remove nonhistone proteins, and analyzed using tiling microarrays.

A schematic overview of CATCH-IT.

Advantages	Disadvantages
• Can determine differences in nucleosome turnover across the genome	• Potential artifacts

Reviews

Zentner G. E. and Henikoff S. High-resolution digital profiling of the epigenome. Nat Rev Genet. 2014;15:814-827.

References

Skene P. J., Hernandez A. E., Groudine M. and Henikoff S. The nucleosomal barrier to promoter escape by RNA polymerase II is overcome by the chromatin remodeler Chd1. *Elife*. 2014;3:e02042.

This study examined the interactions of the chromatin-remodeling protein Chd1 with regions of active transcription in mouse embryonic fibroblasts. The authors used CATCH-IT to show that histone turnover was most rapid at the nucleosomes flanking either side of the promoter and progressively decreased toward the gene body. Upon expression of a dominant-negative mutant (K510R-Chd1), nucleosome turnover was significantly reduced on either side of the TSS but significantly increased within the gene body. The authors conclude that, through both positively and negatively impacting histone dynamics, Chd1 can regulate pluripotency and reprogramming.

Illumina Technology: HiSeq 2000 System

Weber C. M., Ramachandran S. and Henikoff S. Nucleosomes are context-specific, H2A.Z-modulated barriers to RNA polymerase. *Mol Cell.* 2014;53:819-830.

The authors used CATCH-IT to determine how the histone variant H2A.Z could modulate RNAPII kinetics. They found that H2A.Z occupancy anticorrelated with complete nucleosome turnover. CATCH-IT measured nucleosome turnover as the replacement of H3 and H4, but not H2A or H2B, which suggests that H2A.Z-H2B dimers facilitate retention of (H3-H4)2 tetramers.

Illumina Technology: HiSeq 2000 System

Perez-Lluch S., Blanco E., Tilgner H., et al. Absence of canonical marks of active chromatin in developmentally regulated genes. *Nat Genet*. 2015;47:1158-1167.

Teves S. S. and Henikoff S. Transcription-generated torsional stress destabilizes nucleosomes. *Nat Struct Mol Biol.* 2014;21:88-94.

Yildirim O., Hung J. H., Cedeno R. J., et al. A system for genome-wide histone variant dynamics in ES cells reveals dynamic MacroH2A2 replacement at promoters. *PLoS Genet.* 2014;10:e1004515.

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

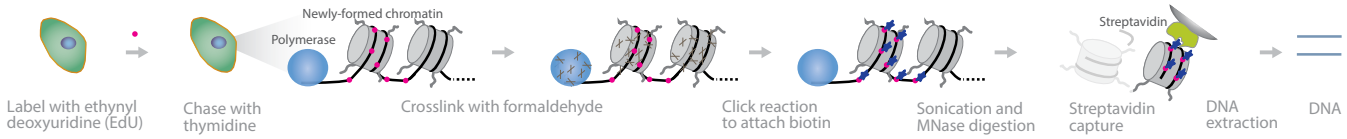
Nextera XT DNA Library Prep Kit

283. Deal R. B., Henikoff J. G. and Henikoff S. Genome-wide kinetics of nucleosome turnover determined by metabolic labeling of histones. *Science*. 2010;328:1161-1164.

MINCE-seq: Mapping *in Vivo* Nascent Chromatin with EdU

MINCE-seq was developed to characterize the genome-wide location of nucleosomes and other chromatin proteins behind replication forks at high temporal and spatial resolution.[284]

In this method, newly replicated DNA is labeled with the nucleotide analog EdU, which is coupled with biotin using click chemistry. Coupling with biotin ensures highly specific purification of newly replicated DNA from asynchronous cells, even if it is only a fraction of a percentage of total DNA. Subsequent MNase treatment recovers DNA fragments bound both by nucleosomes and by nonhistone DNA-binding proteins, which enables the mapping of newly replicated chromatin at near base-pair resolution.[283]



A schematic overview of MINCE-seq.

Advantages	Disadvantages
• Maps newly replicated chromatin at near base-pair resolution	• Robustness unknown

Reviews

None available yet.

References

Ramachandran S. and Henikoff S. Transcriptional Regulators Compete with Nucleosomes Post-replication. *Cell*. 2016;165:580-592.

The authors used MINCE-seq to show that the characteristic chromatin landscape at Drosophila promoters and enhancers is lost upon replication. Promoters that had high levels of RNAPII stalling and DNA accessibility showed specific enrichment for the Brahma (BRM) remodeling factor. Enhancer chromatin was also disrupted during replication, suggesting a role for transcription factor competition in nucleosome re-establishment.

Illumina Technology: HiSeq 2500 System

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

284. Ramachandran S. and Henikoff S. Transcriptional Regulators Compete with Nucleosomes Post-replication. *Cell*. 2016;165:580-592.

FAIRE-seq: Formaldehyde-Assisted Isolation of Regulatory Elements
Sono-Seq: Sonication of Crosslinked Chromatin

FAIRE-seq[285,286] and Sono-Seq[287] are based on differences in crosslinking efficiencies between DNA and nucleosomes or sequence-specific DNA-binding proteins.

In this method, DNA-protein complexes are crosslinked briefly *in vivo* using formaldehyde. The sample is then lysed and sonicated. After phenol/chloroform extraction, the DNA in the aqueous phase is purified and sequenced. Sequencing provides information for regions of DNA that are not occupied by histones.[283]

A schematic overview of FAIRE-seq.

Advantages	Disadvantages
• Simple and highly reproducible protocol • Does not require antibodies • Does not require enzymes, such as DNase or MNase, avoiding the optimization and extra steps necessary for enzymatic processing • Does not require a single-cell suspension or nuclear isolation, so it is easily adapted for use on tissue samples[288]	• Cannot identify regulatory proteins bound to DNA • DNase-Seq may be better at identifying nucleosome-depleted promoters of highly expressed genes[289]

Reviews

Yan H., Tian S., Slager S. L., Sun Z. and Ordog T. Genome-Wide Epigenetic Studies in Human Disease: A Primer on -Omic Technologies. *Am J Epidemiol.* 2016;183:96-109.

References

Lavender C. A., Cannady K. R., Hoffman J. A., Trotter K. W., Gilchrist D. A., et al. Downstream Antisense Transcription Predicts Genomic Features That Define the Specific Chromatin Environment at Mammalian Promoters. *PLoS Genet.* 2016;12:e1006224.

The authors used FAIRE-seq to characterize accessible regions of DNA in T47D/A1-2 cells and revealed an open genomic region at downstream antisense transcription start site (daTSS) positions. ChIP-Seq data revealed that the daTSS coincides with the binding of trans-regulatory factors, consistent with these areas being open and enriched in protein-binding motifs.

Illumina Technology: HiSeq 2500 System, MiSeq System

Behura S. K., Sarro J., Li P., et al. High-throughput cis-regulatory element discovery in the vector mosquito Aedes aegypti. *BMC Genomics.* 2016;17:341.

The authors used FAIRE-seq to assess genetic variation in the regulatory elements of mosquito strains susceptible (Moyo-S) and refractory (Moyo-R) to dengue virus. Known transcription factor consensus binding sites were enriched in the FAIRE peaks and, of these, FoxA1, Hunchback, Gfi, Klf4, MYB/ph3, and Sox9 were the most predominant. All of the elements tested *in vivo* were confirmed to drive gene expression in transgenic Drosophila reporter assays. Of the > 13,000 SNPs recently identified in Moyo-S and Moyo-R mosquito strains, 3365 mapped to FAIRE peaks.

Illumina Technology: HiSeq 2000 System

285. Giresi P. G. and Lieb J. D. Isolation of active regulatory elements from eukaryotic chromatin using FAIRE (Formaldehyde Assisted Isolation of Regulatory Elements). *Methods*. 2009;48:233-239.
286. Hogan G. J., Lee C. K. and Lieb J. D. Cell cycle-specified fluctuation of nucleosome occupancy at gene promoters. *PLoS Genet.* 2006;2:e158.
287. Auerbach R. K., Euskirchen G., Rozowsky J., et al. Mapping accessible chromatin regions using Sono-Seq. *Proc Natl Acad Sci U S A*. 2009;106:14926-14931.
288. Simon J. M., Giresi P. G., Davis I. J. and Lieb J. D. Using formaldehyde-assisted isolation of regulatory elements (FAIRE) to isolate active regulatory DNA. *Nat Protoc.* 2012;7:256-267.
289. Song L., Zhang Z., Grasfeder L. L., et al. Open chromatin defined by DNaseI and FAIRE identifies regulatory elements that shape cell-type identity. *Genome Res.* 2011;21:1757-1767.

Verfaillie A., Imrichova H., Atak Z. K., et al. Decoding the regulatory landscape of melanoma reveals TEADS as regulators of the invasive cell state. *Nat Commun.* 2015;6:6683.

To determine if cis-regulatory regions underlie invasive and proliferative transcriptional states in melanoma, the authors used FAIRE-seq and ChIP-Seq against activated (H3K27ac) and repressed (H3K27me3) chromatin marks in melanoma cell cultures. Clustering on the basis of H3K27ac and FAIRE-seq tracks indicated an active and open SOX10 promoter in the 9 proliferative samples with high SOX10 expression. In the 2 invasive cultures, the SOX10 promoter lacked activating marks but carried repressing H3K27me3 marks.

Illumina Technology: HiSeq 2000 System

Ackermann A. M., Wang Z., Schug J., Naji A. and Kaestner K. H. Integration of ATAC-seq and RNA-seq identifies human alpha cell and beta cell signature genes. *Mol Metab.* 2016;5:233-244.

de Dieuleveult M., Yen K., Hmitou I., Depaux A., Boussouar F., et al. Genome-wide nucleosome specificity and function of chromatin remodellers in ES cells. *Nature*. 2016;530:113-116.

Du J., Leung A., Trac C., et al. Chromatin variation associated with liver metabolism is mediated by transposable elements. *Epigenetics Chromatin*. 2016;9:28.

Fabrizius A., Andre D., Laufs T., et al. Critical re-evaluation of neuroglobin expression reveals conserved patterns among mammals. *Neuroscience*. 2016;337:339-354.

Nevedomskaya E., Stelloo S., van der Poel H. G., et al. Androgen receptor DNA binding and chromatin accessibility profiling in prostate cancer. *Genom Data.* 2016;7:124-126.

Bicker A., Brahmer A. M., Meller S., et al. The Distinct Gene Regulatory Network of Myoglobin in Prostate and Breast Cancer. *PLoS One.* 2015;10:e0142662.

Davie K., Jacobs J., Atkins M., et al. Discovery of transcription factors and regulatory regions driving *in vivo* tumor development by ATAC-seq and FAIRE-seq open chromatin profiling. *PLoS Genet.* 2015;11:e1004994.

Naval-Sanchez M., Potier D., Hulselmans G., Christiaens V. and Aerts S. Identification of Lineage-Specific Cis-Regulatory Modules Associated with Variation in Transcription Factor Binding and Chromatin Activity Using Ornstein-Uhlenbeck Models. *Mol Biol Evol.* 2015;32:2441-2455.

Reschen M. E., Gaulton K. J., Lin D., et al. Lipid-induced epigenomic changes in human macrophages identify a coronary artery disease-associated variant that regulates PPAP2B Expression through Altered C/EBP-beta binding. *PLoS Genet.* 2015;11:e1005061.

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

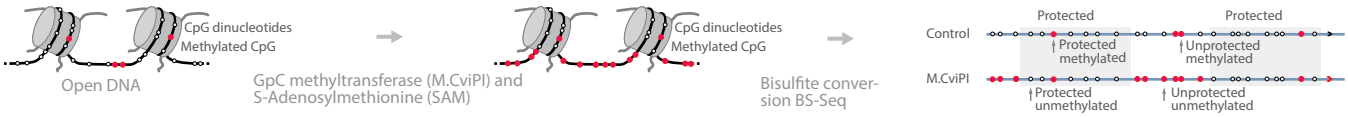
Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

NOMe-Seq: Nucleosome Occupancy Methylome-Sequencing

NOMe-Seq is a single-molecule, high-resolution nucleosome positioning assay.[290] This method is based on the ability of the GpC methyltransferase M.CviPI to methylate GpC sites that are not bound by nucleosomes, to create a digital footprint of nucleosome positioning. M.CviPI can map nucleosome positions at CpG-poor promoters, irrespective of their endogenous methylation status.

In this method, native chromatin is treated with M.CviPI, following which the DNA is treated with sodium bisulfite and subjected to WGBS. From these data, CpG methylation patterns as well as nucleosome-free regions (GpC methylation) can be identified.[291]



A schematic overview of NOMe-Seq.

Advantages	Disadvantages
• High resolution	• Relies on the presence of GpC residues[292]

Reviews

Shull A. Y., Noonepalle S. K., Lee E. J., Choi J. H. and Shi H. Sequencing the cancer methylome. *Methods Mol Biol.* 2015;1238:627-651.

References

Wallner S., Schroder C., Leitao E., Berulava T., Haak C., et al. Epigenetic dynamics of monocyte-to-macrophage differentiation. *Epigenetics Chromatin.* 2016;9:33.

The authors used NOMe-Seq to investigate whether the chromatin accessibility of DMRs change during differentiation. Monocytes had 89,212 NOMe-Seq peaks covering ~21 Mbp, and macrophages had 127,267 peaks covering ~42 Mbp, which demonstrates that macrophages had more nucleosome-depleted regions. More detailed analysis of DMRs and NOMe-Seq peaks that were gained or lost during differentiation indicated that most DMRs become nucleosome-depleted during differentiation.

Illumina Technology: HiSeq 2000 System

Lay F. D., Liu Y., Kelly T. K., et al. The role of DNA methylation in directing the functional organization of the cancer epigenome. *Genome Res.* 2015;25:467-477.

Statham A. L., Taberlay P. C., Kelly T. K., Jones P. A. and Clark S. J. Genome-wide nucleosome occupancy and DNA methylation profiling of four human cell lines. *Genom Data.* 2015;3:94-96.

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

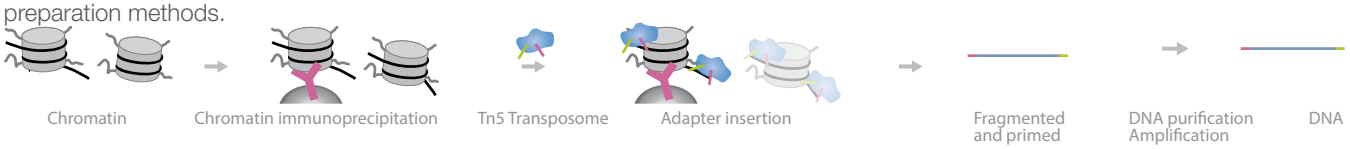
Nextera XT DNA Library Prep Kit

290. Han H., Cortez C. C., Yang X., Nichols P. W., Jones P. A. and Liang G. DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter. *Hum Mol Genet.* 2011;20:4299-4310.
291. Wallner S., Schroder C., Leitao E., et al. Epigenetic dynamics of monocyte-to-macrophage differentiation. *Epigenetics Chromatin.* 2016;9:33.
292. Ishii H., Kadonaga J. T. and Ren B. MPE-seq, a new method for the genome-wide analysis of chromatin structure. *Proc Natl Acad Sci U S A.* 2015;112:E3457-3465.

ChIPmentation: Chromatin Immunoprecipitation with Sequencing Library Preparation by Tn5 Transposase

ChIPmentation combines ChIP with sequencing library preparation by Tn5 transposase (tagmentation).[293] Both ATAC-seq and ChIPmentation can be combined usefully in the same experiment to assay open chromatin and protein binding, respectively.[294] This combination of methods takes advantage of the flexibility and efficiency provided by using Tn5 transposase in library preparation.

Tagmentation is performed directly on bead-bound, immunoprecipitated chromatin, followed by standard library preparation methods.



A schematic overview of ChIPmentation.

Advantages	Disadvantages
• Can generated accurate profiles from as little as 10,000 cells • Simple, one-step reaction	• None reported

Reviews

None available yet.

References

Rendeiro A. F., Schmidl C., Strefford J. C., Walewska R., Davis Z., et al. Chromatin accessibility maps of chronic lymphocytic leukaemia identify subtype-specific epigenome signatures and transcription regulatory networks. *Nat Commun.* 2016;7:11938.

The authors studied epigenetic deregulation in chronic lymphocytic leukemia (CLL). Chromatin profiles created by ChIPmentation and RNA-Seq accurately predicted CLL. The authors also examined the mutation status of IGHV genes, a clinical biomarker for CLL. Gene regulatory networks inferred for IGHV-mutated vs IGHV-unmutated samples identified characteristic differences between less aggressive and more aggressive CLL subtypes, respectively.

Illumina Technology: HiSeq 3000/4000 System

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

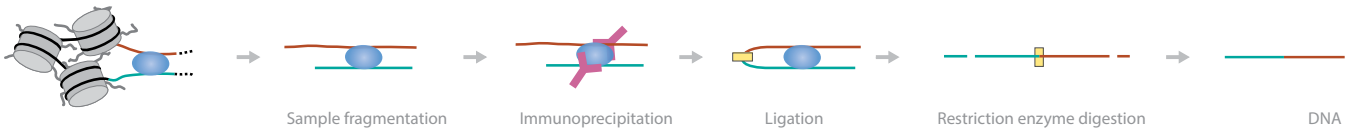
293. Schmidl C., Rendeiro A. F., Sheffield N. C. and Bock C. ChIPmentation: fast, robust, low-input ChIP-seq for histones and transcription factors. *Nat Methods.* 2015;12:963-965.

294. Chaitankar V., Karakulah G., Ratnapriya R., Giuste F. O., Brooks M. J. and Swaroop A. Next generation sequencing technology and genomewide data analysis: Perspectives for retinal research. *Prog Retin Eye Res.* 2016;55:1-31.

ChIA-PET: Chromatin Interaction Analysis by Paired-End Tag Sequencing

ChIA-PET features an immunoprecipitation step to map long-range DNA interactions, similar to Hi-C.[295,296] In this method, DNA-protein complexes are crosslinked and fragmented. Specific antibodies are used to immunoprecipitate proteins of interest. Two sets of linkers, with unique barcodes, are ligated to the ends of the DNA fragments in separate aliquots, which then self-ligate based on proximity. The DNA aliquots are precipitated, digested with restriction enzymes, and sequenced. Deep sequencing provides base-pair resolution of the ligated fragments. Hi-C and ChIA-PET currently provide the best balance of resolution and reasonable coverage in the human genome to map long-range interactions.[297]

A modified protocol, called advanced or long-read ChIA-PET, has been published by Tang *et al*. [298] This method replaces the 2 separate ligation reactions with 2 half linkers and a single biotinylated linker ligation. Next, the de-crosslinked, purified DNA is fragmented and adapters are ligated using Tn5 transposase in a single step. Finally, the DNA is PCR-amplified and sequenced.[299]



A schematic overview of ChIA-PET.

Advantages	Disadvantages
• Suitable for detecting a large number of both long-range and short-range chromatin interactions globally[300] • Studies the interactions made by specific proteins or protein complexes • Public ChIA-PET datasets are available through the ENCODE Project[301] • Removes background generated during traditional ChIP assays • Immunoprecipitation step reduces data complexity[113]	• Requires a large amount of starting material required, generally at least 100 million cells[302] • Nonspecific antibodies can pull down unwanted protein complexes and contaminate the pool • Linkers can self-ligate, generating ambiguity about true DNA interactions • Limited sensitivity; may detect as little as 10% of interactions[113]

Reviews

Sati S. and Cavalli G. Chromosome conformation capture technologies and their impact in understanding genome function. *Chromosoma*. 2016;.

References

Ricano-Ponce I., Zhernakova D. V., Deelen P., et al. Refined mapping of autoimmune disease associated genetic variants with gene expression suggests an important role for non-coding RNAs. *J Autoimmun.* 2016;68:62-74.

The authors performed a systematic analysis of transcriptomic data from 629 blood samples and linked 460 SNPs that are associated with 14 autoimmune diseases. The SNPs were linked to functional regulatory elements, which suggests a model where autoimmune disease genes are regulated by a network of chromatin-looping/noncoding RNA interactions. Data from the ChIA-PET assay in B lymphoblastoid cells indicated that multigene SNPs were more often (70%) involved in looping interactions in B cells than single-gene SNPs (55%).

Illumina Technology: HiSeq 2000 System

295. Li G., Fullwood M. J., Xu H., et al. ChIA-PET tool for comprehensive chromatin interaction analysis with paired-end tag sequencing. *Genome Biol.* 2010;11:R22.
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297. Dekker J., Marti-Renom M. A. and Mirny L. A. Exploring the three-dimensional organization of genomes: interpreting chromatin interaction data. *Nat Rev Genet.* 2013;14:390-403.
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300. Sajan S. A. and Hawkins R. D. Methods for identifying higher-order chromatin structure. *Annu Rev Genomics Hum Genet*. 2012;13:59-82.
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Chang H., Liu Y., Xue M., et al. Synergistic action of master transcription factors controls epithelial-to-mesenchymal transition. *Nucleic Acids Res.* 2016;44:2514-2527.

Darabi H., Beesley J., Droit A., et al. Fine scale mapping of the 17q22 breast cancer locus using dense SNPs, genotyped within the Collaborative Oncological Gene-Environment Study (COGs). *Sci Rep.* 2016;6:32512.

Fujimoto A., Furuta M., Totoki Y., et al. Whole-genome mutational landscape and characterization of noncoding and structural mutations in liver cancer. *Nat Genet*. 2016;48:500-509.

Tewhey R., Kotliar D., Park D. S., et al. Direct Identification of Hundreds of Expression-Modulating Variants using a Multiplexed Reporter Assay. *Cell*. 2016;165:1519-1529.

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

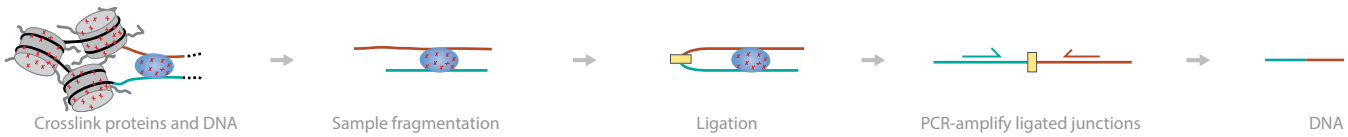
Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

3-C, Capture-C and Hi-C: Chromatin Conformation Capture Sequencing

3C-Seq,[303] Capture-C, and Hi-C[304] comprise a family of methods for analyzing chromatin interactions. Capture-C adds an additional pull-down of the biotinylated fragments with magnetic beads to the 3C method. A new refinement of the Capture-C method (NG Capture-C) is available.[305] The Hi-C approach extends 3C-Seq to map chromatin contacts genome-wide, and it has also been applied to studying *in situ* chromatin interactions.[306,307]

In this method, DNA-protein complexes are crosslinked with formaldehyde. The sample is fragmented, and the DNA is extracted, ligated, and digested with restriction enzymes. The resulting DNA fragments are PCR-amplified and sequenced. Deep sequencing provides base-pair resolution of the ligated fragments.



A schematic overview of 3C-Seq.

Advantages	Disadvantages
• Allows detection of long-range DNA interactions • High-throughput method	• Detection may result from random chromosomal collisions • Less than 1% of DNA fragments actually yield ligation products[308] • Due to multiple steps, the method requires large amounts of starting material

Reviews

Sati S. and Cavalli G. Chromosome conformation capture technologies and their impact in understanding genome function. *Chromosoma*. 2016;.

Turaev D. and Rattei T. High definition for systems biology of microbial communities: metagenomics gets genome-centric and strain-resolved. *Curr Opin Biotechnol*. 2016;39:174-181.

References

Criscione S. W., De Cecco M., Siranosian B., et al. Reorganization of chromosome architecture in replicative cellular senescence. *Sci Adv.* 2016;2:e1500882.

The authors used Hi-C, fluorescence in situ hybridization (FISH), and in silico modeling methods to characterize the 3D architecture of interphase chromosomes in proliferating, quiescent, and senescent cells. Direct measurements of distances between genetic loci, chromosome volumes, and chromatin accessibility suggested that the Hi-C interaction changes were caused by a significant reduction of the volumes occupied by individual chromosome arms. In contrast, centromeres opposed this overall compaction trend and increased in volume.

Illumina Technology: HiSeq 2500 System

Darrow E. M., Huntley M. H., Dudchenko O., et al. Deletion of DXZ4 on the human inactive X chromosome alters higher-order genome architecture. *Proc Natl Acad Sci U S A.* 2016;113:E4504-4512.

During interphase, the inactive X chromosome (Xi) is largely silent transcriptionally and adopts an unusual 3D configuration known as the “Barr body.” The authors constructed a diploid Hi-C map of human GM12878 cells and showed that the Xi chromosome had a distinctive superstructure. It contained superdomains, which are unusually large contact domains. It also contained unusually large chromatin loops called “superloops.” Both superdomains and superloops can span dozens of megabases of the genome. Superloop anchors, like the macrosatellite repeat DXZ4, tended to colocate simultaneously. Deleting DXZ4 on Xi led to the disappearance of superdomains and superloops, changes in compartmentalization patterns, and changes in the distribution of chromatin marks.

Illumina Technology: HiSeq 2000 System

303. Duan Z., Andronescu M., Schutz K., et al. A genome-wide 3C-method for characterizing the three-dimensional architectures of genomes. *Methods*. 2012;58:277-288.
304. Lieberman-Aiden E., van Berkum N. L., Williams L., et al. Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science*. 2009;326:289-293.
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306. Sati S. and Cavalli G. Chromosome conformation capture technologies and their impact in understanding genome function. *Chromosoma*. 2016;
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Krijger P. H., Di Stefano B., de Wit E., et al. Cell-of-Origin-Specific 3D Genome Structure Acquired during Somatic Cell Reprogramming. *Cell Stem Cell.* 2016;18:597-610.

The authors used Hi-C contact maps for each of 4 founder cell types and their respective p3 and p20 iPSC derivatives to investigate how nuclear organization changes during reprogramming. They found that early passage iPSCs carried topological hallmarks that enabled recognition of their cell of origin. These hallmarks were not remnants of somatic chromosome topologies. Instead, the distinguishing topological features were acquired during reprogramming.

Illumina Technology: HiSeq 2000 System

Veluchamy A., Jegu T., Ariel F., et al. LHP1 Regulates H3K27me3 Spreading and Shapes the Three-Dimensional Conformation of the Arabidopsis Genome. *PLoS One.* 2016;11:e0158936.

Like Heterochromatin Protein 1 (LHP1) controls actively transcribed genes and is a member of the plant-specific polycomb-group (PcG) family originally identified in Drosophila. The authors used Hi-C to map the spatial contacts and distribution of genes in chromatin between different parts of the Arabidopsis genome for wild-type and lhp1 plants. Chromosomal contact maps at 100 kb resolution showed significant changes between wt and lhp1 plants. Additional experiments showed that LHP1 was responsible for the spreading of H3K27me3 toward the 3' end of the gene body. The authors also identified a subset of LHP1-activated genes that shape local chromatin topology to control transcriptional coregulation.

Illumina Technology: HiSeq 2500 System

Acemel R. D., Tena J. J., Irastorza-Azcarate I., et al. A single three-dimensional chromatin compartment in amphioxus indicates a stepwise evolution of vertebrate Hox bimodal regulation. *Nat Genet.* 2016;48:336-341.

Bigot P., Colli L. M., Machiela M. J., et al. Functional characterization of the 12p12.1 renal cancer-susceptibility locus implicates BHLHE41. *Nat Commun.* 2016;7:12098.

Capurso D., Bengtsson H. and Segal M. R. Discovering hotspots in functional genomic data superposed on 3D chromatin configuration reconstructions. *Nucleic Acids* Res. 2016;44:2028-2035.

Gunnell A., Webb H. M., Wood C. D., et al. RUNX super-enhancer control through the Notch pathway by Epstein-Barr virus transcription factors regulates B cell growth. *Nucleic Acids Res.* 2016;44:4636-4650.

Kim K. D., Tanizawa H., Iwasaki O. and Noma K. Transcription factors mediate condensin recruitment and global chromosomal organization in fission yeast. *Nat Genet.* 2016;48:1242-1252.

Petryk N., Kahli M., d'Aubenton-Carafa Y., et al. Replication landscape of the human genome. *Nat Commun*. 2016;7:10208.

Pichugina T., Sugawara T., Kaykov A., et al. A diffusion model for the coordination of DNA replication in Schizosaccharomyces pombe. *Sci Rep.* 2016;6:18757.

Putnam N. H., O'Connell B. L., Stites J. C., et al. Chromosome-scale shotgun assembly using an *in vitro* method for long-range linkage. *Genome Res.* 2016;26:342-350.

Rocha P. P., Raviram R., Fu Y., et al. A Damage-Independent Role for 53BP1 that Impacts Break Order and Igh Architecture during Class Switch Recombination. *Cell Rep.* 2016;16:48-55.

Tjong H., Li W., Kalhor R., et al. Population-based 3D genome structure analysis reveals driving forces in spatial genome organization. *Proc Natl Acad Sci U S A.* 2016;113:E1663-1672.

Ulianov S. V., Khrameeva E. E., Gavrilov A. A., et al. Active chromatin and transcription play a key role in chromosome partitioning into topologically associating domains. *Genome Res.* 2016;26:70-84.

Associated Kits

TruSeq Nano DNA Library Prep Kit

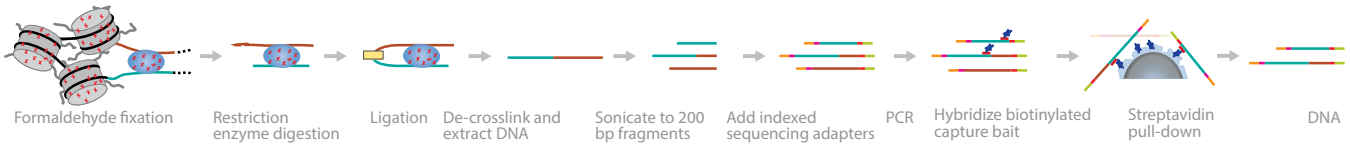
TruSeq DNA Sample Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

NG Capture-C: Next-Generation Capture-C

NG Capture-C is a refinement of 3C-Seq[309] and Hi-C.[310] It represents a family of methods used to analyze chromatin interactions. NG Capture-C adds multiple pull-down steps of the biotinylated fragments with magnetic beads to the 3C method.

The protocol uses formaldehyde fixation followed by restriction enzyme digestion and ligation to form ~10 kb concatamers. The DNA is extracted and sonicated. Indexing adapters are added, and the samples are pooled, purified by pull-down, and PCR-amplified. The pull-down and PCR steps can be repeated to yield up to a million-fold enrichment.[311]



A schematic overview of NG Capture-C.

Advantages	Disadvantages
• High sensitivity to detect cis and trans interactions • Low sample input requirements • Sonicated capture fragments reduces cost compared to capture-C • Sonicated capture fragments act as UMIs to reduce PCR bias	• None reported

Reviews

Sati S. and Cavalli G. Chromosome conformation capture technologies and their impact in understanding genome function. *Chromosoma*. 2016;.

References

Davies J. O., Telenius J. M., McGowan S. J., Roberts N. A., Taylor S., et al. Multiplexed analysis of chromosome conformation at vastly improved sensitivity. *Nat Methods*. 2016;13:74-80.

The authors used NG Capture-C to detect ligation junctions, equivalent to the detection of interactions present in 1 in 10,000 cells, at single-restriction-fragment resolution (~250 bp). This result exceeds the sensitivity and resolution of detection of current 3C methods and complementary methods, such as FISH.

Illumina Technology: HiSeq System, MiSeq System

Hay D., Hughes J. R., Babbs C., Davies J. O., Graham B. J., et al. Genetic dissection of the alpha-globin super-enhancer in vivo. *Nat Genet*. 2016;48:895-903.

The authors used homologous recombination to generate 7 mouse models in which each constituent of the proposed α-globin superenhancer was deleted, individually and in informative pairs, to dissect its function. NG Capture-C detected the only statistically significant changes, which occurred at the α-globin promoters.

Illumina Technology: NextSeq System

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

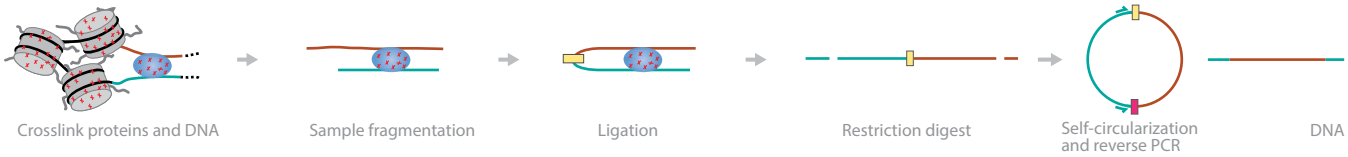
Nextera XT DNA Library Prep Kit

309. Duan Z., Andronescu M., Schutz K., et al. A genome-wide 3C-method for characterizing the three-dimensional architectures of genomes. *Methods*. 2012;58:277-288.
310. Lieberman-Aiden E., van Berkum N. L., Williams L., et al. Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science*. 2009;326:289-293.
311. Davies J. O., Telenius J. M., McGowan S. J., et al. Multiplexed analysis of chromosome conformation at vastly improved sensitivity. *Nat Methods*. 2016;13:74-80.

4C-seq: Circular Chromatin Conformation Capture

4C[312], also called 4C-seq, is a method similar to 3C and is sometimes called circular 3C. It allows the unbiased detection of all genomic regions that interact with a particular region of interest.[313]

In this method, DNA-protein complexes are crosslinked using formaldehyde. The sample is fragmented, and the DNA is ligated and digested. The resulting DNA fragments self-circularize, followed by reverse PCR and sequencing. Deep sequencing provides base-pair resolution of the ligated fragments.



A schematic overview of 4C.

Advantages	Disadvantages
• Preferred strategy to assess the DNA contact profile of individual genomic sites • Highly reproducible data	• Will miss local interactions (< 50 kb) from the region of interest • Large circles do not amplify efficiently

Reviews

None available yet.

References

Cai M., Kim S., Wang K., et al. 4C-seq revealed long-range interactions of a functional enhancer at the 8q24 prostate cancer risk locus. *Sci Rep.* 2016;6:22462.

Genome-wide association studies have identified > 100 independent susceptibility loci for prostate cancer, including a "hot spot" at 8q24. To identify genome-wide partners interacting with this hot spot, the authors coupled 4C-seq to an enhancer at 8q24 as "bait" in cell lines LNCaP and C4-2B. The 4C-identified regions were distributed in open nuclear compartments, featuring active histone marks H3K4me1, H3K4me2, and H3K27Ac.

Illumina Technology: HiSeq 2000 System

Loviglio M. N., Leleu M., Mannik K., et al. Chromosomal contacts connect loci associated with autism, BMI and head circumference phenotypes. *Mol Psychiatry*. 2016;.

The authors used 4C-seq to identify chromosomal regions that physically associate with the promoters of genes linked to autism-spectrum and other disorders, including MVP, KCTD13, ALDOA, TBX6 and MAPK3. They found that 2 CNV-prone regions at 16p11.2 were reciprocally engaged in complex chromatin looping, as successfully confirmed by 4C-seq, FISH, and Hi-C. Their results suggest that disruption of chromatin interplays at 16p11.2 could play a role in the observed phenotypes.

Illumina Technology: HiSeq System

Yang R., Kerschner J. L., Gosalia N., Neems D., Gorsic L. K., et al. Differential contribution of cis-regulatory elements to higher order chromatin structure and expression of the CFTR locus. *Nucleic Acids Res.* 2016;44:3082-3094.

The authors used CRISPR/Cas9 editing of cis-regulatory elements and small interfering RNA (siRNA)-mediated depletion of architectural proteins to determine the relative contribution of structural elements and enhancers to the higher order structure and expression of the cystic fibrosis transmembrane conductance regulator (CFTR) locus. They used CRISPR/Cas9-mediated deletion of a CTCF-binding insulator element 5' to the CFTR locus and a pivotal intronic enhancer, followed by 4C-seq. The results were consistent with a mechanism coordinating regulatory elements across the locus, which senses structural perturbations to maintain normal gene expression. However, they found that the loss of a key intronic enhancer on CFTR transcription could not be rescued by structural changes in the locus.

Illumina Technology: HiSeq System

312. Zhao Z., Tavoosidana G., Sjolinder M., et al. Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. *Nat Genet.* 2006;38:1341-1347.
313. Sajan S. A. and Hawkins R. D. Methods for identifying higher-order chromatin structure. *Annu Rev Genomics Hum Genet.* 2012;13:59-82.

Acemel R. D., Tena J. J., Irastorza-Azcarate I., Marletaz F., Gomez-Marin C., et al. A single three-dimensional chromatin compartment in amphioxus indicates a stepwise evolution of vertebrate Hox bimodal regulation. *Nat Genet.* 2016;48:336-341.

De S., Mitra A., Cheng Y., Pfeifer K. and Kassis J. A. Formation of a Polycomb-Domain in the Absence of Strong Polycomb Response Elements. *PLoS Genet.* 2016;12:e1006200.

Eckart N., Song Q., Yang R., et al. Functional Characterization of Schizophrenia-Associated Variation in CACNA1C. *PLoS One.* 2016;11:e0157086.

Kaaij L. J., Mokry M., Zhou M., Musheev M., Geeven G., et al. Enhancers reside in a unique epigenetic environment during early zebrafish development. *Genome Biol.* 2016;17:146.

Kandaswamy R., Sava G. P., Speedy H. E., et al. Genetic Predisposition to Chronic Lymphocytic Leukemia Is Mediated by a BMF Super-Enhancer Polymorphism. *Cell Rep*. 2016;16:2061-2067.

Lin C. Y., Erkek S., Tong Y., et al. Active medulloblastoma enhancers reveal subgroup-specific cellular origins. *Nature*. 2016;530:57-62.

Proudhon C., Snetkova V., Raviram R., Lobry C., Badri S., et al. Active and Inactive Enhancers Cooperate to Exert Localized and Long-Range Control of Gene Regulation. *Cell Rep.* 2016;15:2159-2169.

Rocha P. P., Raviram R., Fu Y., Kim J., Luo V. M., et al. A Damage-Independent Role for 53BP1 that Impacts Break Order and Igh Architecture during Class Switch Recombination. *Cell Rep.* 2016;16:48-55.

Vermunt M. W., Tan S. C., Castelijns B., et al. Epigenomic annotation of gene regulatory alterations during evolution of the primate brain. *Nat Neurosci.* 2016;19:494-503.

Wang Q., Sawyer I. A., Sung M. H., et al. Cajal bodies are linked to genome conformation. *Nat Commun*. 2016;7:10966.

Wani A. H., Boettiger A. N., Schorderet P., et al. Chromatin topology is coupled to Polycomb group protein subnuclear organization. *Nat Commun*. 2016;7:10291.

Associated Kits

TruSeq ChIP Library Prep Kit

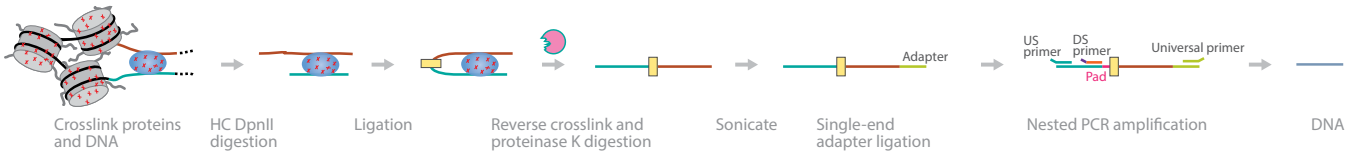
TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

UMI-4C: Circular Chromosome Conformation Capture with Unique Molecular Identifiers

This variation on the 4C approach uses UMIs to derive high-complexity quantitative chromosome contact profiles with controlled signal-to-noise ratios.[314] It is an efficient and accurate method for analyzing targeted loci. The method is paired with software to analyze the data (https://bitbucket.org/tanaylab/umi4cpackage).



A schematic overview of UMI-4C.

Advantages	Disadvantages
• Improved sensitivity and specificity • Multiplexing allows robust comparison of contact distributions between loci and conditions	• None reported

Reviews

None available yet.

References

Schwartzman O., Mukamel Z., Oded-Elkayam N., Olivares-Chauvet P., Lubling Y., et al. UMI-4C for quantitative and targeted chromosomal contact profiling. *Nat Methods.* 2016;13:685-691.

The authors describe the 4C-UMI method and show that it requires modest sequencing depth (100,000 reads per bait). The method can be multiplexed easily, allowing the selection of multiple viewpoints.

Illumina Technology: MiSeq System, NextSeq System, HiSeq System

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

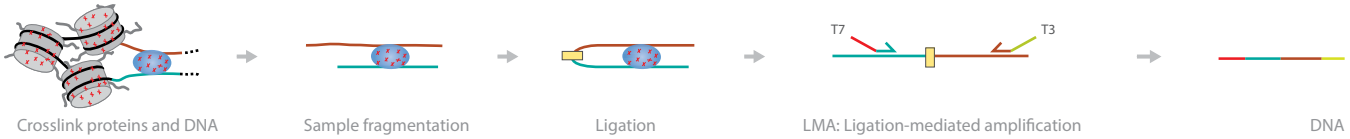
Nextera XT DNA Library Prep Kit

314. Schwartzman O., Mukamel Z., Oded-Elkayam N., et al. UMI-4C for quantitative and targeted chromosomal contact profiling. *Nat Methods*. 2016;13:685-691.

5C: Chromatin Conformation Capture Carbon Copy

5C[315] allows concurrent determination of interactions among multiple sequences and is a high-throughput version of 3C.[316]

In this method, DNA-protein complexes are crosslinked using formaldehyde. The sample is fragmented and the DNA ligated and digested with restriction enzymes. The resulting DNA fragments are amplified using ligation-mediated PCR and sequenced. Deep sequencing provides base-pair resolution of the ligated fragments.



A schematic overview of 5C.

Advantages	Disadvantages
• Different from 4C, 5C provides a matrix of interaction frequencies for many pairs of sites[317] • Can be used to reconstruct the (average) 3D conformation of larger genomic regions[120]	• Requires a priori information of the regulatory sites[318] • Detection may not necessarily mean an interaction, resulting from random chromosomal collisions • Cannot scale to genome-wide studies that would require a large amount of primers

Reviews

Sati S. and Cavalli G. Chromosome conformation capture technologies and their impact in understanding genome function. *Chromosoma*. 2016;.

Reuter J. A., Spacek D. V. and Snyder M. P. High-throughput sequencing technologies. *Mol Cell.* 2015;58:586-597.

References

Smith E. M., Lajoie B. R., Jain G. and Dekker J. Invariant TAD Boundaries Constrain Cell-Type-Specific Looping Interactions between Promoters and Distal Elements around the CFTR Locus. *Am J Hum Genet.* 2016;98:185-201.

Globally, chromosomes are organized into active and inactive compartments while, at the gene level, looping interactions connect promoters to regulatory elements. Topologically associating domains (TADs) represent an intermediate level of organization. The authors designed a 5C experiment to interrogate looping interactions between HindIII fragments containing TSSs and any other HindIII restriction fragments (distal fragments) in the target region. Their results showed that the same TAD boundaries were present in all cell types, and they suggest that TADs represent a universal chromosome architecture.

Illumina Technology: Genome Analyzer$_{IIx}$ System

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Prep Kit

TruSeq DNA PCR-Free Library Prep Kit

315. Dostie J. and Dekker J. Mapping networks of physical interactions between genomic elements using 5C technology. *Nat Protoc.* 2007;2:988-1002.
316. Sajan S. A. and Hawkins R. D. Methods for identifying higher-order chromatin structure. *Annu Rev Genomics Hum Genet*. 2012;13:59-82.
317. de Wit E. and de Laat W. A decade of 3C technologies: insights into nuclear organization. *Genes Dev.* 2012;26:11-24.
318. Sati S. and Cavalli G. Chromosome conformation capture technologies and their impact in understanding genome function. *Chromosoma*. 2016;

Pu-seq: Polymerase Usage Sequencing

Pu-seq provides direct replication-origin location and efficiency data, as well as indirect estimates of replication timing.[319]

Alkali treatment of duplex ribonucleotide-containing DNA results in phosphate-backbone cleavage 3' to the ribose, resulting in a 5'-hydroxyl end. If the denatured DNA is used as a template for random-hexamer primer extension, 5' to 3' synthesis results in a flush end adjacent to the initial ribose. By generating a library from single-stranded DNA and placing distinct index primers at each end, sequencing reads can be mapped to individual strands, to determine the original ribonucleotide position with single-base accuracy.[318]

A schematic overview of Pu-seq.

Advantages	Disadvantages
• Single-base accuracy	• Only applied to yeast

Reviews

None available yet.

References

Keszthelyi A., Daigaku Y., Ptasinska K., Miyabe I. and Carr A. M. Mapping ribonucleotides in genomic DNA and exploring replication dynamics by polymerase usage sequencing (Pu-seq). *Nat Protoc.* 2015;10:1786-1801.

The authors used Pu-seq to define replication dynamics in yeast with a high level of detail. Although other methods that examine replication dynamics provide direct measures of replication timing and indirect estimates of origin efficiency, Pu-seq directly ascertains origin efficiency.

Illumina Technology: NextSeq System

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

319. Daigaku Y., Keszthelyi A., Muller C. A., et al. A global profile of replicative polymerase usage. *Nat Struct Mol Biol.* 2015;22:192-198.

PB–seq: Protein/DNA Binding Followed by High-Throughput Sequencing

PB–seq is a DNA-binding assay that allows the DNA-protein binding energy landscape to be characterized genome-wide, in the absence of chromatin.[320] It belongs to the family of methods more commonly known as systematic evolution of ligands by exponential enrichment (SELEX). [321]

Genomic DNA is sonicated, size-selected, and purified. After hybridization to the DNA-binding protein the protein-bound DNA is isolated, extracted, and prepared for sequencing.

A schematic overview of PB–seq.

Advantages	Disadvantages
• Determines binding efficiencies independent of chromatin structure	• Not yet adopted widely by the scientific community

Reviews

None available yet.

References

Guertin M. J., Martins A. L., Siepel A. and Lis J. T. Accurate prediction of inducible transcription factor binding intensities in vivo. *PLoS Genet.* 2012;8:e1002610.

The authors used PB–seq to show that DNase I hypersensitivity and tetra-acetylation of H4 are the most influential covariates in predicting changes in Drosophila heat shock factor (HSF) binding affinity. They developed an unbiased model of HSF binding sequences, which revealed distinct biophysical properties of the HSF/HSE interaction and a previously unrecognized substructure within the DNA sequence element.

Illumina Technology: Genome Analyzer II System

Associated Kits

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

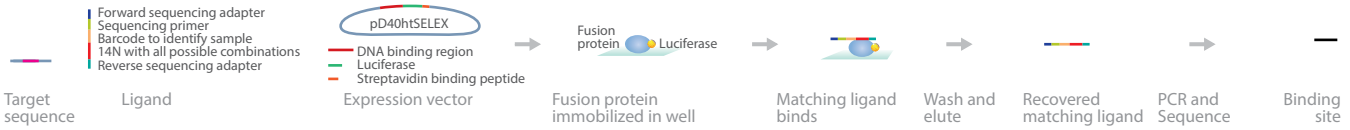
TruSeq DNA PCR-Free Library Prep Kit

320. Guertin M. J., Martins A. L., Siepel A. and Lis J. T. Accurate prediction of inducible transcription factor binding intensities in vivo. *PLoS Genet.* 2012;8:e1002610.
321. Ozer A., Pagano J. M. and Lis J. T. New Technologies Provide Quantum Changes in the Scale, Speed, and Success of SELEX Methods and Aptamer Characterization. *Mol Ther Nucleic Acids.* 2014;3:e183.

SELEX or SELEX-seq: Systematic Evolution of Ligands by Exponential Enrichment
HT-SELEX: High-Throughput Systematic Evolution of Ligands by Exponential Enrichment

From the time that the first SELEX experiments were described by 3 independent groups in 1990[322,323,324] the method has been adapted to a wide range of technologies.[325,326] A highly multiplexed, parallel HT-SELEX method was developed for NGS.[327] A variation of SELEX-seq[328] uses Nextera adapter sequences for efficient library preparation.[329]

In this method, proteins are expressed as fusions with streptavidin-binding peptide (SBP), conjugated to Gaussia luciferase, in the pD40htSELEX expression vector. Each DNA ligand contains a 14 bp randomized region (14N), and a 5 bp barcode that uniquely identifies the individual SELEX sample. Partially nested primers are used in successive SELEX rounds. A double-stranded DNA mixture containing all possible 14 bp sequences is incubated with a DNA-binding protein immobilized into a well of a 96-well plate, resulting in binding of DNA to the protein. After washing and elution, the resulting population of more specific sequences is amplified by PCR and sequenced.[326]



A schematic overview of HT-SELEX.

Advantages	Disadvantages
• High-throughput and efficient • Software pipelines are available[330,331]	• Could contain sequence bias[332]

Reviews

Anzalone A. V., Lin A. J., Zairis S., Rabadan R. and Cornish V. W. Reprogramming eukaryotic translation with ligand-responsive synthetic RNA switches. *Nat Methods.* 2016;13:453-458.

Jijakli K., Khraiwesh B., Fu W., et al. The *in vitro* selection world. *Methods*. 2016;106:3-13.

322. Ellington A. D. and Szostak J. W. In vitro selection of RNA molecules that bind specific ligands. *Nature*. 1990;346:818-822.
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326. Takahashi M., Wu X., Ho M., et al. High throughput sequencing analysis of RNA libraries reveals the influences of initial library and PCR methods on SELEX efficiency. *Sci Rep.* 2016;6:33697.
327. Jolma A., Kivioja T., Toivonen J., et al. Multiplexed massively parallel SELEX for characterization of human transcription factor binding specificities. *Genome Res.* 2010;20:861-873.
328. Slattery M., Riley T., Liu P., et al. Cofactor binding evokes latent differences in DNA binding specificity between Hox proteins. *Cell*. 2011;147:1270-1282.
329. Zhang Y., Lee J. K., Toso E. A., et al. DNA-binding sequence specificity of DUX4. *Skelet Muscle.* 2016;6:8.
330. Hoinka J. and Przytycka T. AptaPLEX - A dedicated, multithreaded demultiplexer for HT-SELEX data. *Methods*. 2016;106:82-85.
331. Caroli J., Taccioli C., De La Fuente A., Serafini P. and Bicciato S. APTANI: a computational tool to select aptamers through sequence-structure motif analysis of HT-SELEX data. *Bioinformatics*. 2016;32:161-164.
332. Takahashi M., Wu X., Ho M., et al. High throughput sequencing analysis of RNA libraries reveals the influences of initial library and PCR methods on SELEX efficiency. *Sci Rep.* 2016;6:33697.

References

Urak K. T., Shore S., Rockey W. M., et al. In vitro RNA SELEX for the generation of chemically-optimized therapeutic RNA drugs. *Methods*. 2016;103:167-174.

This publication describes the SELEX process for the rapid identification of RNA aptamers for *in vivo* applications. It contains a detailed protocol for the selection of chemically optimized RNA aptamers using the original *in vitro* SELEX methodology.

Illumina Technology: Various

Ahirwar R., Vellarikkal S. K., Sett A., et al. Aptamer-Assisted Detection of the Altered Expression of Estrogen Receptor Alpha in Human Breast Cancer. *PLoS One.* 2016;11:e0153001.

The authors used HT-SELEX to identify a DNA aptamer that selectively binds and facilitates the detection of estrogen receptor alpha (ERβ) in human breast cancer tissue sections. They validated the results of aptamer-assisted histochemical analysis of ERβ in tissue samples from breast cancer patients and performed further validation by immunohistochemistry on the same samples with an ERβ antibody. The results demonstrated a significant consistency in the aptamer-assisted detection of ERβ in strong-positive, moderate-positive, and negative breast cancer tissues.

Illumina Technology: HiSeq 2500 System

Iaboni M., Fontanella R., Rienzo A., et al. Targeting Insulin Receptor with a Novel Internalizing Aptamer. *Mol Ther Nucleic Acids.* 2016;5:e365.

The authors used a cell-based SELEX method to discover a nuclease-resistant RNA aptamer, named GL56, which specifically recognizes the insulin receptor (IR). It rapidly internalizes into target cells and is able to discriminate IR from the highly homologous insulin-like growth factor receptor 1. When applied to IR-expressing cancer cells, the aptamer inhibited IR-dependent signaling, suggesting its potential use as a delivery tool for IR-dependent cancers.

Illumina Technology: MiSeq System

Long Y., Qin Z., Duan M., et al. Screening and identification of DNA aptamers toward Schistosoma japonicum eggs via SELEX. *Sci Rep.* 2016;6:24986.

Janowski R., Heinz G. A., Schlundt A., et al. Roquin recognizes a non-canonical hexaloop structure in the 3'-UTR of Ox40. *Nat Commun*. 2016;7:11032.

Schneider T., Hung L. H., Schreiner S., et al. CircRNA-protein complexes: IMP3 protein component defines subfamily of circRNPs. *Sci Rep.* 2016;6:31313.

Stewart H., Bingham R. J., White S. J., et al. Identification of novel RNA secondary structures within the hepatitis C virus genome reveals a cooperative involvement in genome packaging. *Sci Rep.* 2016;6:22952.

Oakes B. L., Xia D. F., Rowland E. F., et al. Multi-reporter selection for the design of active and more specific zinc-finger nucleases for genome editing. *Nat Commun.* 2016;7:10194.

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

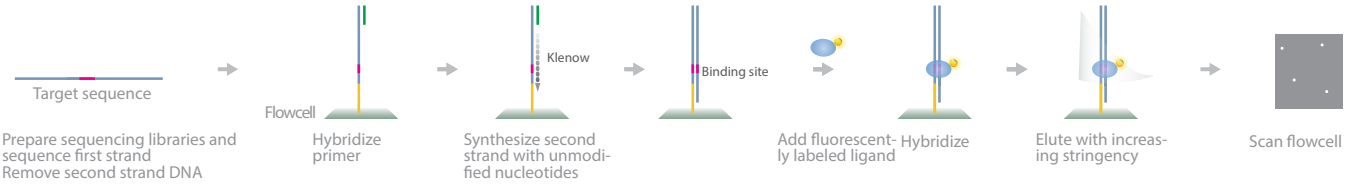
Nextera DNA Library Prep Kit

Nextera XT DNA Library Prep Kit

HiTS-FLIP: High-Throughput Sequencing With Fluorescent Ligand Interaction Profiling

HiTS-FLIP is a technique for measuring quantitative protein-DNA binding affinity at unprecedented depth. In this approach, the optics built into a high-throughput sequencer are used to visualize *in vitro* binding of a protein to sequenced DNA in a flow cell.[333]

A microfluidic flow cell with anchored single-stranded DNA is sequenced by synthesis. Second-strand DNA is stripped and rebuilt using Klenow polymerase and unmodified dNTPs to form double-stranded DNA clusters. Fluorescently labeled binding protein is introduced at different concentrations, and binding is imaged.



A schematic overview of HiTS-FLIP.

Advantages	Disadvantages
• Quantitative and comprehensive	• Requires specialized hardware • Not yet adopted widely by the scientific community

Reviews

None available yet.

References

Nutiu R., Friedman R. C., Luo S., Khrebtukova I., Silva D., et al. Direct measurement of DNA affinity landscapes on a high-throughput sequencing instrument. Nat Biotechnol. 2011;29:659-664.

The authors applied HiTS-FLIP to study the S. cerevisiae protein GCN4, the master regulator of the yeast amino-acid starvation response. The study yielded ~440 million binding measurements, which enabled the determination of dissociation constants for all 12-mer sequences that had a submicromolar affinity for GCN4.

Illumina Technology: Genome Analyzer System

Associated Kits

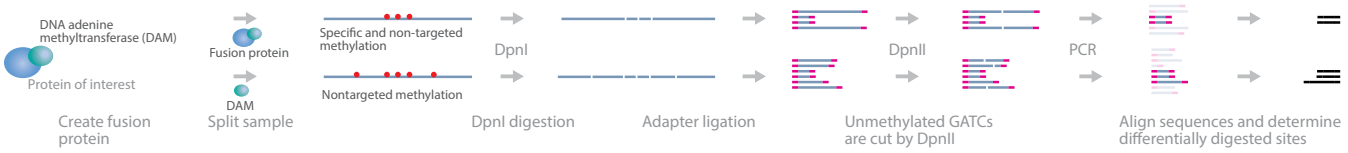
TruSeq ChIP Library Prep Kit

333. Nutiu R., Friedman R. C., Luo S., et al. Direct measurement of DNA affinity landscapes on a high-throughput sequencing instrument. *Nat Biotechnol*. 2011;29:659-664.

DamID: DNA Adenine Methyltransferase Interaction Detection

DamID allows the identification of protein-binding sites in living cells without the need for crosslinking or immunoprecipitation. It was developed in 2006 as a microarray method before it was adapted to NGS.[334]

DamID involves the low-level expression of a fusion protein consisting of DNA adenine methyltransferase (Dam) and a chromatin protein of interest. This fusion protein is targeted to the native binding sites of the chromatin protein, where Dam methylates adenines in the surrounding DNA. Subsequently, the methylated DNA fragments are isolated, amplified by selective PCR, and sequenced.[333]



A schematic overview of DamID.

Advantages	Disadvantages
• Allows the identification of protein-binding sites in living cells without the need for crosslinking or immunoprecipitation • Dedicated algorithms are available[335]	• Dam can be toxic • Limited to kilobase-sized regions

Reviews

None available yet.

References

Pindyurin A. V., Pagie L., Kozhevnikova E. N., van Arensbergen J. and van Steensel B. Inducible DamID systems for genomic mapping of chromatin proteins in Drosophila. *Nucleic Acids Res.* 2016;44:5646-5657.

The authors used an intein-based approach to tune the expression level of Dam and Dam-fusion proteins in Drosophila by addition of a ligand to fly food. With this method, they generated a glia-specific map of Polycomb binding sites in small samples of brain tissue.

Illumina Technology: HiSeq 2000 System

Mitchell A. C., Javidfar B., Bicks L. K., et al. Longitudinal assessment of neuronal 3D genomes in mouse prefrontal cortex. *Nat Commun.* 2016;7:12743.

The authors introduce "Neuro-Dam" for assessing epigenome status retrospectively. They showed that short-term expression of Dam, tethered to the Gad1 gene promoter in mouse prefrontal cortex neurons, resulted in stable GMeATC tags at Gad1-bound chromosomal contacts. The NeuroDam data revealed that mice with defective cognition 4 months after pharmacological blockade of the N-methyl-D-aspartate (NMDA) receptor already were affected by disrupted chromosomal conformations, shortly after drug exposure.

Illumina Technology: HiSeq 2000 System

Perovanovic J., Dell'Orso S., Gnochi V. F., et al. Laminopathies disrupt epigenomic developmental programs and cell fate. *Sci Transl Med.* 2016;8:335ra358.

The authors used DamID-seq with mutated forms of the lamin A protein fused to Dam, to define euchromatic-heterochromatin (epigenomic) transitions at the nuclear envelope during myogenesis. They found that lamin A missense mutations disrupted formation of lamin A–associated heterochromatin domains in an allele-specific manner.

Illumina Technology: HiScan System

334. Vogel M. J., Guelen L., de Wit E., et al. Human heterochromatin proteins form large domains containing KRAB-ZNF genes. *Genome Res.* 2006;16:1493-1504.
335. Li R., Hempel L. U. and Jiang T. A non-parametric peak calling algorithm for DamID-Seq. *PLoS One.* 2015;10:e0117415.

McCann T. S., Guo Y., McDonald W. H. and Tansey W. P. Antagonistic roles for the ubiquitin ligase Asr1 and the ubiquitin-specific protease Ubp3 in subtelomeric gene silencing. *Proc Natl Acad Sci U S A.* 2016;113:1309-1314.

Carl S. H. and Russell S. Common binding by redundant group B Sox proteins is evolutionarily conserved in Drosophila. *BMC Genomics*. 2015;16:292.

Kind J., Pagie L., de Vries S. S., et al. Genome-wide maps of nuclear lamina interactions in single human cells. *Cell*. 2015;163:134-147.

Klocko A. D., Rountree M. R., Grisafi P. L., et al. Neurospora importin alpha is required for normal heterochromatic formation and DNA methylation. *PLoS Genet.* 2015;11:e1005083.

Steglich B., Stralfors A., Khorosjutina O., et al. The Fun30 chromatin remodeler Fft3 controls nuclear organization and chromatin structure of insulators and subtelomeres in fission yeast. *PLoS Genet.* 2015;11:e1005101.

Associated Kits

TruSeq Nano DNA Library Prep Kit

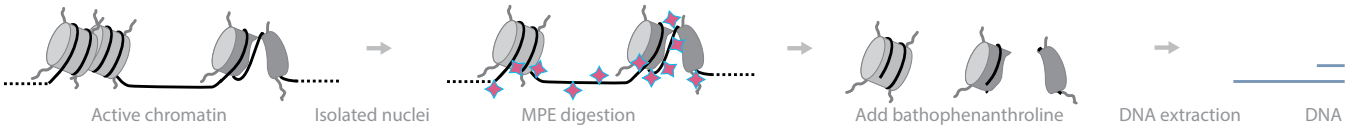
TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

MPE-seq: Methidiumpropyl Ethylenediaminetetraacetic Acid (EDTA) Sequencing

MPE-seq is a method for the genome-wide characterization of chromatin that involves the treatment of nuclei with a complex of methidiumpropyl-EDTA (MPE) and ferrous iron. The MPE-Fe(II) complex binds to DNA via intercalation of the methidium moiety and then generates single- and double-stranded DNA breaks in the presence of oxygen.

MPE-Fe(II) preferentially cleaves the linker DNA between nucleosomes with little sequence bias, unlike MNase. For example, DNA sequences at RNA splice sites are hypersensitive to digestion by MNase but not by MPE-Fe(II). The combined use of MPE-seq and MNase-Seq should allow the identification of noncanonical chromatin structures.



A schematic overview of MPE-seq.

Advantages	Disadvantages
• Very little sequence bias	• Not replicated in other laboratories

Reviews

None available yet.

References

Ishii H., Kadonaga J. T. and Ren B. MPE-seq, a new method for the genome-wide analysis of chromatin structure. Proc Natl Acad Sci U S A. 2015;112:E3457-3465.

The authors treated mouse ESCs with MPE-Fe(II). For comparison, they also digested nuclei in parallel with MNase. Analysis of the nucleosome positioning analysis with MPE-seq revealed peaks in active promoter regions that were not seen with MNase-seq. The authors also observed that MPE-seq can be used to detect sequence-specific DNA binding by some transcription factors.

Illumina Technology: HiSeq System

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

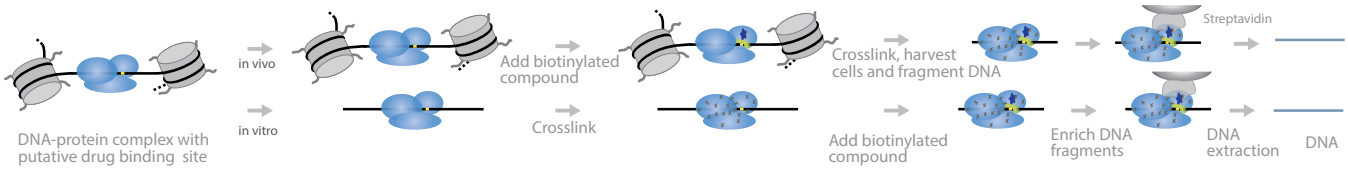
Nextera XT DNA Library Prep Kit

336. Hertzberg R. P. and Dervan P. B. Cleavage of double helical DNA by methidium-propyl-EDTA-iron(II). *Journal of the American Chemical Society.* 1982;104:313-315.
337. Ishii H., Kadonaga J. T. and Ren B. MPE-seq, a new method for the genome-wide analysis of chromatin structure. *Proc Natl Acad Sci U S A.* 2015;112:E3457-3465.

Chem-seq: Identify Sites Bound by Small Chemical Molecules

Chem-seq can be used to detect the binding of small-molecule ligands, such as therapeutic drugs, to proteins associated with the genome. This information may provide important insights into the perturbation of cellular function by small-molecule drugs.[338]

The Chem-seq method uses 2 approaches. In living cells, the biotinylated drug is added to allow drug-target binding. The complex is crosslinked with formaldehyde, the cells are lysed and sonicated, and the complex is captured on streptavidin beads. The enriched DNA fragments are purified and sequenced. For *in vitro* analysis, the biotinylated drug is added to a cell extract, and the remaining steps are performed as for the *in vivo* procedure.



A schematic overview of Chem-seq.

Advantages	Disadvantages
• Can be applied to living, human cells	• Creating biotin derivative may alter drug activity

Reviews

None available yet.

References

Anders L., Guenther M. G., Qi J., Fan Z. P., Marineau J. J., et al. Genome-wide localization of small molecules. *Nat Biotechnol.* 2014;32:92-96.

The authors used Chem-seq to investigate the genome-wide binding of the bromodomain inhibitor JQ1 to the BET bromodomain family members BRD2, BRD3, and BRD4 in MM1.S multiple myeloma cells. They synthesized a biotinylated JQ1 derivative, which had only slightly reduced activity in MM1.S cells. Both *in vivo* and *in vitro* Chem-seq showed that the genomic sites bound by biotinylated JQ1 are highly similar to the sites occupied by BRD2, BRD3 and BRD4. A functionally inactive enantiomer of biotinylated JQ1 did not produce significant Chem-seq signals.

Illumina Technology: Genome Analyzer System

Jin C., Yang L., Xie M., et al. Chem-seq permits identification of genomic targets of drugs against androgen receptor regulation selected by functional phenotypic screens. *Proc Natl Acad Sci U S A.* 2014;111:9235-9240.

Associated Kits

TruSeq ChIP Library Prep Kit

TruSeq Nano DNA Library Prep Kit

TruSeq DNA Sample Preparation Kit

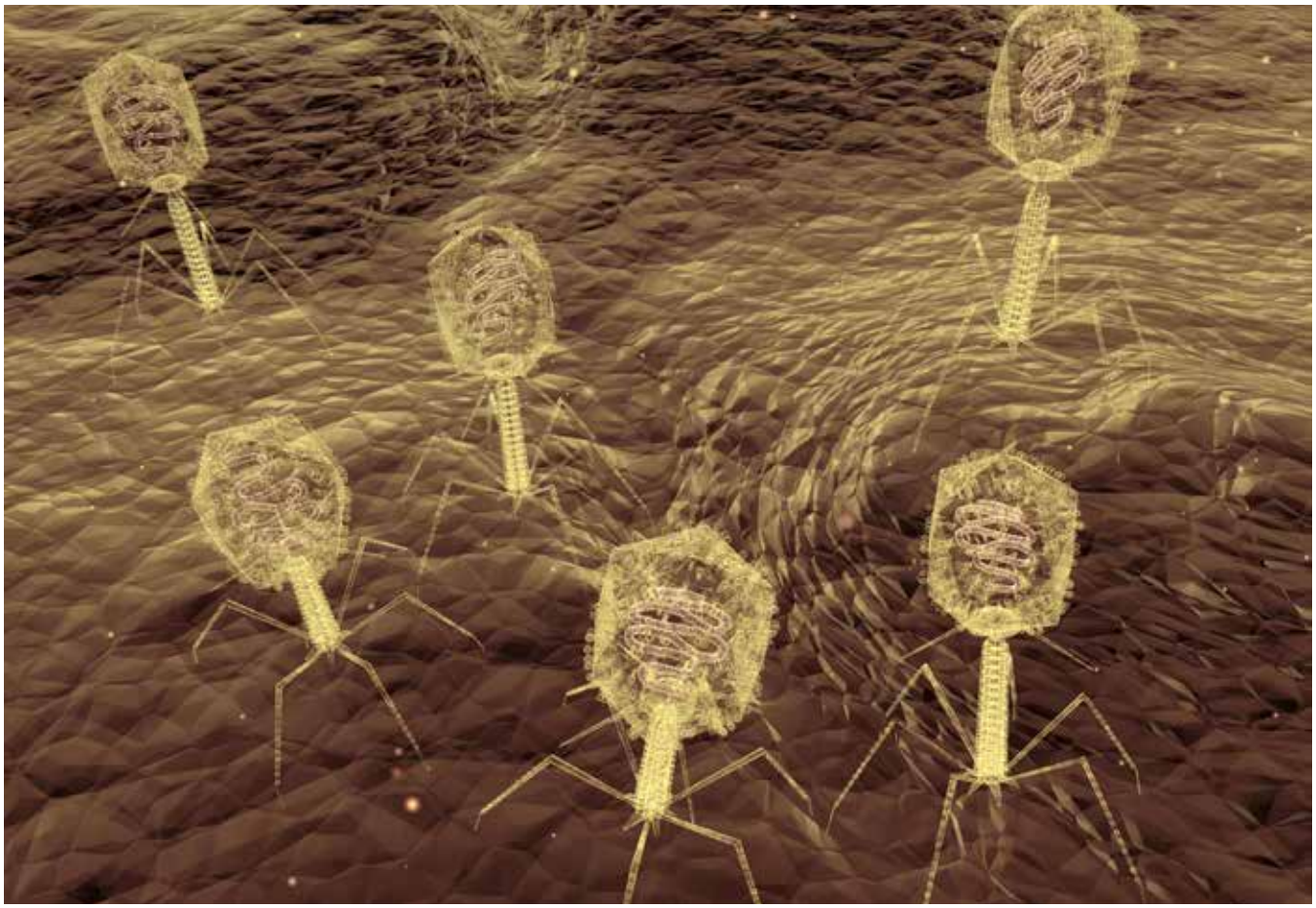
TruSeq DNA PCR-Free Library Prep Kit

Nextera DNA Library Prep Kit

338. Anders L., Guenther M. G., Qi J., et al. Genome-wide localization of small molecules. *Nat Biotechnol.* 2014;32:92-96.

PROTEIN-PROTEIN INTERACTION

The use of NGS to study protein-protein interactions may seem an unlikely application, but phage display of recombinant proteins is a well-established method for developing specific affinity reagents for various applications.[339] High-throughput sequencing is poised to change all aspects of the discovery and engineering of antibodies and other protein-binding molecules. Millions of available sequence reads provide an unprecedented sampling depth able to guide the design and construction of effective, high quality naïve libraries containing tens of billions of unique molecules. [340]



Phage display technology is widely used for high-throughput screening of protein-protein interactions. The gene encoding the protein of interest is inserted into phages and displayed by the phage. The displayed proteins are screened against target proteins to check for binding.

Reviews

Caricciо V. L., Domina M., Benfatto S., et al. Phage display revisited: Epitope mapping of a monoclonal antibody directed against Neisseria meningitidis adhesin A using the PROFILER technology. *MAbs*. 2016;8:741-750.

Jijakli K., Khraiwesh B., Fu W., Luo L., Alzahmi A., et al. The *in vitro* selection world. *Methods*. 2016;106:3-13.

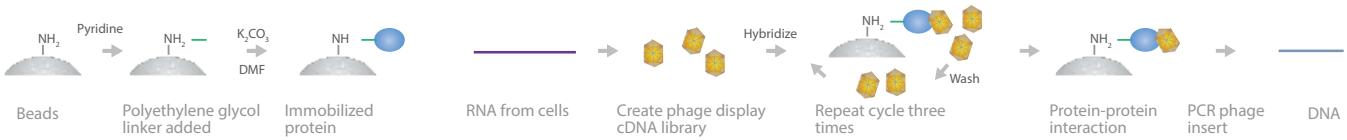
Glanville J., D'Angelo S., Khan T. A., Reddy S. T., Naranjo L., et al. Deep sequencing in library selection projects: what insight does it bring? *Curr Opin Struct Biol.* 2015;33:146-160.

339. Bradbury A. R., Sidhu S., Dubel S. and McCafferty J. Beyond natural antibodies: the power of in vitro display technologies. *Nat Biotechnol.* 2011;29:245-254.
340. Glanville J., D'Angelo S., Khan T. A., et al. Deep sequencing in library selection projects: what insight does it bring? *Curr Opin Struct Biol.* 2015;33:146-160.

PD-Seq: Candidate Cellular Protein Target Identification

PD-Seq is a protocol to identify cellular targets for proteins through protein-protein interactions.[341]

In this method, researchers identified cellular targets for apigenin. They coupled apigenin to amino polyethyleneglycol-polyacrylamide copolymer (PEGA) beads after activation with 4-nitrophenyl bromoacetate. The apigenin-loaded beads were used for screening phage display cDNA library generated using mRNA from human breast cancer tumor cells. After 3 rounds, the fractions were used to make libraries and sequenced on the Illumina platform.



A schematic overview of PD-Seq.

Advantages	Disadvantages
• Can detect a large number of cellular targets	• Not yet adopted widely by the scientific community

Reviews

Arango D., Morohashi K., Yilmaz A., Kuramochi K., Parihar A., et al. Molecular basis for the action of a dietary flavonoid revealed by the comprehensive identification of apigenin human targets. *Proc Natl Acad Sci U S A.* 2013;110:E2153-2162.

References

None available yet.

Associated Kits

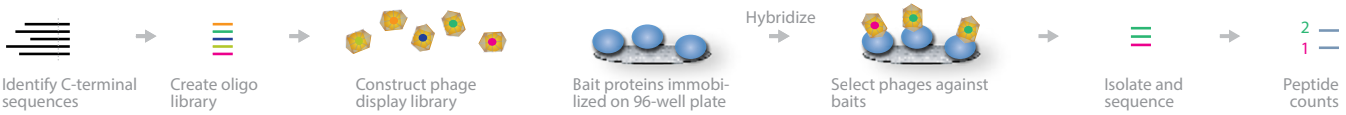
TruSeq DNA PCR-Free Library Prep Kit

341. Arango D., Morohashi K., Yilmaz A., et al. Molecular basis for the action of a dietary flavonoid revealed by the comprehensive identification of apigenin human targets. *Proc Natl Acad Sci U S A.* 2013;110:E2153-2162.

ProP-PD: Proteomic Peptide-Phage Display
PDZ-Seq: PDZ Domains Sequencing

ProP-PD is a protocol that identifies short linear motif (SLiM) interactions or PDZ domains (PDZ-Seq).[342,343] ProP-PD can be used for the direct identification of ligands with potential biological relevance.[344] This method contrasts with the commonly used combinatorial phage display, where highly diverse libraries are used to establish preferred binding motifs.

In this method, C-terminal sequences are first identified and an oligonucleotide library is created. Next, a phage display library is constructed, which is hybridized to immobilized bait proteins. After multiple rounds of selection, the selected pools that bind to the PDZ domains are sequenced and counted. Deep sequencing provides detailed information pertaining to the motifs that interact with specific proteins.



A schematic overview of ProP-PD.

Advantages	Disadvantages
• Direct identification of the ligands	• Construction of the oligonucleotide library may be biased

Reviews

None available yet.

References

Garrido-Urbani S., Garg P., Ghossoub R., et al. Proteomic peptide phage display uncovers novel interactions of the PDZ1-2 supramodule of syntenin. *FEBS Lett.* 2016;590:3-12.

The authors used ProP-PD to profile syntenin PDZ1-2 by using a library that displays C-terminal regions of the human proteome. The protein recognized a broad range of peptides, with a preference for hydrophobic motifs and internal cryptic ligands.

Illumina Technology: Unspecified Illumina system

Ivarsson Y., Arnold R., McLaughlin M., Nim S., Joshi R., et al. Large-scale interaction profiling of PDZ domains through proteomic peptide-phage display using human and viral phage peptidomes. *Proc Natl Acad Sci U S A.* 2014;111:2542-2547.

Associated Kits

TruSeq DNA PCR-Free Library Prep Kit

TruSeq Nano DNA Library Prep Kit

342. Ivarsson Y., Arnold R., McLaughlin M., et al. Large-scale interaction profiling of PDZ domains through proteomic peptide-phage display using human and viral phage peptidomes. *Proc Natl Acad Sci U S A.* 2014;111:2542-2547.
343. Ernst A., Gfeller D., Kan Z., et al. Coevolution of PDZ domain-ligand interactions analyzed by high-throughput phage display and deep sequencing. *Mol Biosyst.* 2010;6:1782-1790.
344. Sundell G. N. and Ivarsson Y. Interaction analysis through proteomic phage display. *Biomed Res Int.* 2014;2014:176172.

Scientific Publication Reviews can be accessed at
www.illumina.com/pubreviews

RNA Sequencing Methods Collection

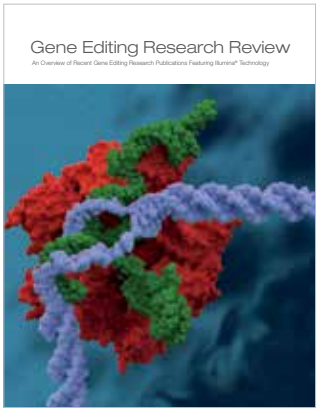
This publication is a collection of next-generation sequencing (NGS) methods for RNA sequencing, compiled from the scientific literature. It is both a tribute to the creativity of the users and the versatility of the technology. We hope it will inspire researchers to use these methods or to develop new ones to address new scientific challenges.

A method refers to the processing steps between extracting the nucleic acids (sample preparation) and the addition of oligonucleotide adapters for sequencing (library preparation). With a few extra processing steps, a wide range of scientific questions can be addressed by this technology.

Single-Cell Research Review

Most of the impetus for single-cell tissue sequencing has come from cancer research, where cell lineage and the detection of residual disease are of paramount concern. The same approaches are being used to improve our understanding of massively complex biological systems, such as neural development and immunology.

This document highlights recent publications that demonstrate the use of Illumina technology for single-cell sequencing and very low input applications and techniques.



Gene Editing Research Review

CRISPR-Cas9 is a recently developed genome editing technique that allows scientists to perform precise genomic manipulation quickly and conveniently. This technology has a vast spectrum of applications. As any molecular biology technique, it is crucial that the obtained results have high levels of specificity. This review highlights recent publications that demonstrate the use of genomic technologies and high-throughput sequencing in CRISPR-Cas9 experiments for checking specificity and genome-wide off target effects.

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