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Getting started with next-generation sequencing (NGS)

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Why is now the best time to consider next-generation sequencing (NGS)?

Game-changing discovery power using NGS

Taking an unbiased approach to scientific research can free your experimental design from the bounds of prior understanding and preconceived expectations, providing untapped insights into biological phenomena, pathways, and systems. NGS offers the power to sequence vast amounts of genetic material at a fraction of the time and cost of traditional methods.[1-4] This allows for a shift from profiling select markers with known significance to profiling many markers, even those with low abundance or unknown biological significance. Researchers are leveraging the unbiased lens of NGS to reveal a broader landscape of molecular entities, enabling the discovery of novel drug targets, signaling networks, and markers of disease.[2]

> *When you're studying something that's completely new or uncharacterized, you need a much bigger picture. In our project, there was a lot of uncertainty about what mechanisms are influencing our pathways. And given how little we knew about the mechanisms, we needed to use a broader approach like NGS to more fully understand the mechanism in its entirety.*
>
> **Amanda Touey, PhD candidate in Dr. Paula Cohen's lab at Cornell University**

Fortunately, you don't need to be an expert in NGS to get started. This resource aims to lay the foundation for understanding the research impact, experimental benefits, and overall workflow of NGS while providing actionable steps for getting started.

Unbiased discovery with NGS can:

- Expand the scope of your experimental studies
- Find the answers to your boldest research questions
- Drive your field of expertise forward

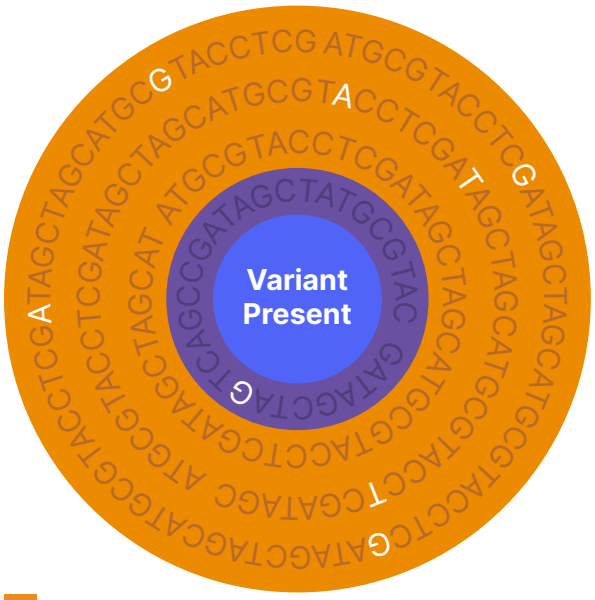
NGS powers unbiased discovery- *Expand the scope and impact of your research by starting with a broader view.*

NGS is more accessible than ever

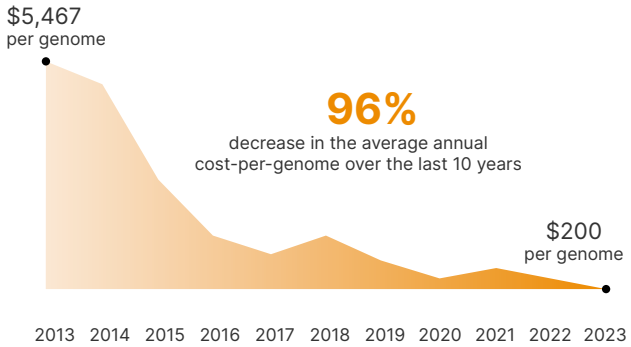
The development of Sanger sequencing in 1977 was the first step to unraveling the genetic code. While revolutionary, this method was **limited to sequencing one or a few gene regions at a time**.[3]

The dawn of the NGS era—powered by the advent of Illumina sequencing by synthesis (SBS) technology—has significantly expanded the rapid and efficient sequencing of large pools of genetic material.[3-5] In addition to enhanced speed and efficiency, NGS enables higher discovery power (including the ability to identify novel variants) and higher resolution when compared to both Sanger sequencing and traditional targeted methods, such as quantitative PCR (qPCR).[3-5]

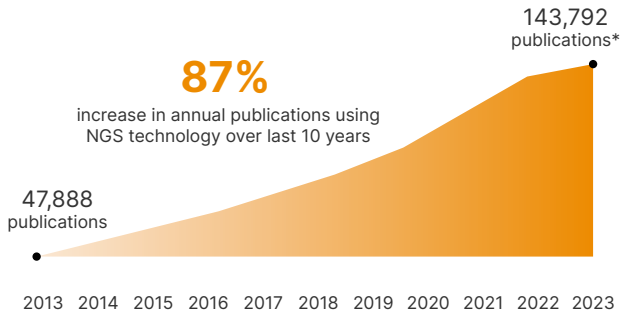
Despite these benefits, the barrier to NGS entry initially remained high due to expensive costs and lack of widespread expertise.[3] However, over the last twenty years, the cost of NGS has rapidly decreased and workflows have become well-defined, leading to an increase in approachability.[6,7,8] Data analysis of large NGS datasets has also become increasingly accessible and user-friendly, allowing for automated data interpretation even without prior knowledge of bioinformatics.[3,4] Consequently, the total adoption of NGS to answer research questions has boomed, exemplified by the striking increase in research publications employing NGS over the last decade.[9]



NGS supports enhanced discovery power compared to traditional methods – *Illuminate rare variants with single base resolution across hundreds to thousands of target regions.*[3]



NGS is more cost-effective than ever – *since 2013, there has been a 96% decrease in the average cost-per-genome.*[6,7]

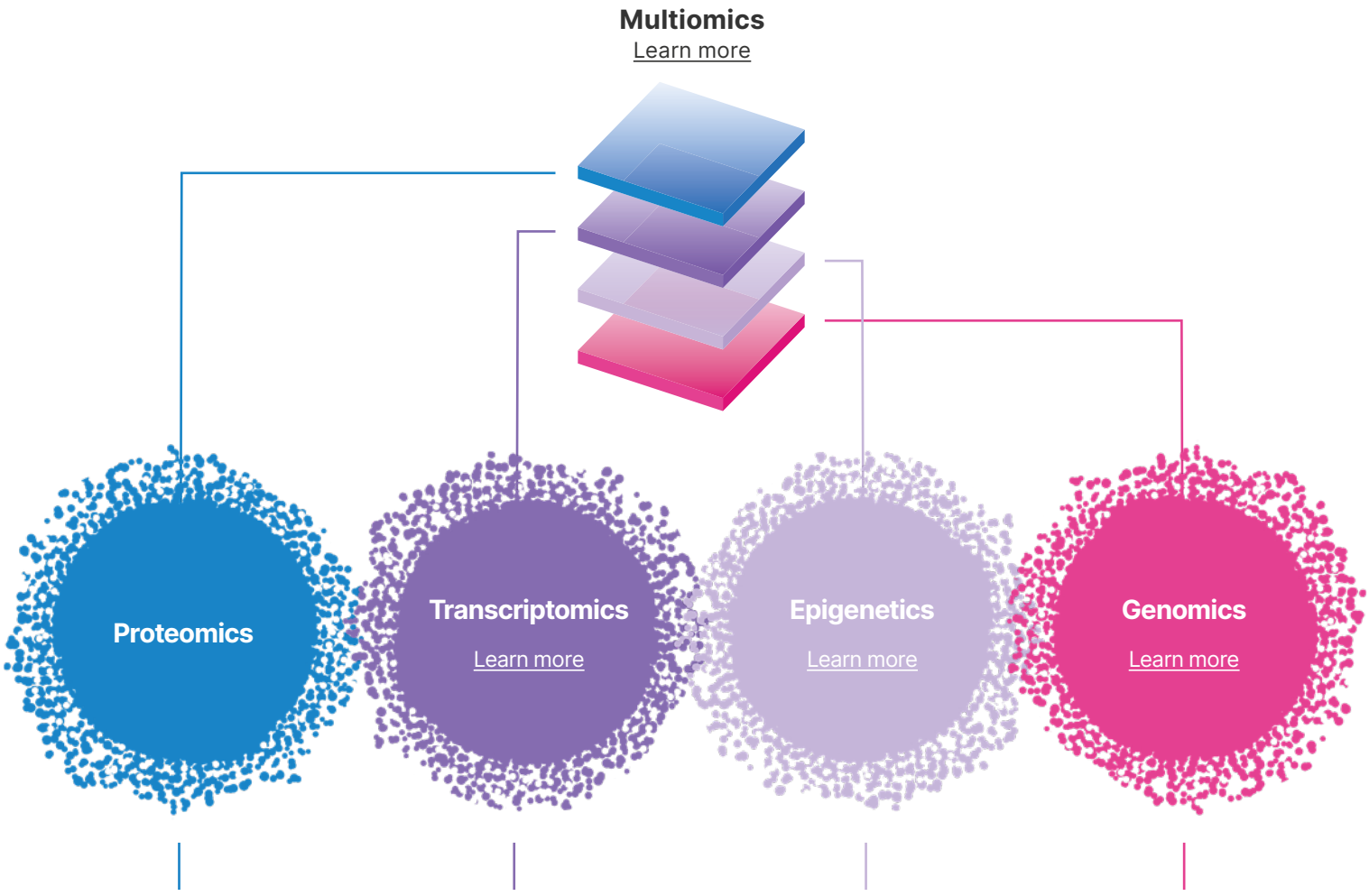


*predicted end-of-year total based on monthly publications in first 3 months of 2023

NGS publications are on the rise – *since 2013, there has been a predicted 87% increase in the number of publications featuring data obtained using NGS.*[9]

Applications of NGS

NGS can play an important role in pursuing the answer to a variety of biological questions using a wide array of published methods for diverse sample types. NGS enables the unbiased investigation of multiple biological "-omes", such as the proteome, transcriptome, epigenome, and genome. A combinatorial approach interrogating multiple -omes at once, called multiomics, can also be achieved with NGS.[10-14]



NGS delivers insights into various -omes – *Gain access to large-scale, unbiased proteomics, transcriptomics, epigenomics, genomics, and combinatorial multiomics approaches by adopting an NGS workflow into your lab.*[10-14]

NGS accelerates transcriptomics research

Transcriptomics, the characterization of the complete set or a designated subset of RNA transcripts produced by the genome, is an accessible entry point for using NGS, given its versatility across use cases and well-established workflows.[10,15] NGS-based RNA-Seq enables researchers to generate large-scale, high-throughput datasets that provide insights into gene expression and regulation across diverse samples.[10]

Depending on your research question, RNA-Seq can lay the groundwork for:

1. Understanding how differential gene expression is responsible for normal development and function[10]
2. Uncovering gene expression patterns that drive the development of disease[10]
3. Identifying new biomarkers and drug targets[10]
4. Elucidating the functional effects of gene variants[10]

RNA-seq can be applied to your research using several distinct methodologies that differ in the scope of detected transcripts.

Method	Description[10]
mRNA-Seq	Sensitive, accurate measurement of gene expression, after mRNA enrichment using poly-A capture
Enrichment-based RNA-Seq	Targeted probe-based enrichment of specific transcripts of interest from low-input or low-quality samples
Total RNA-Seq	Bulk, high-throughput analysis of the total coding and noncoding RNA landscape

NGS enables several RNA-Seq methodologies – *Profile mRNA transcripts only, a particular pre-defined subset of RNA transcripts, or the complete set of RNA transcripts with NGS-based RNA-Seq.*

How does RNA-Seq compare to qPCR?

While qPCR is a reliable, widely established method for analyzing gene expression, RNA-Seq can increase the quality and scale of a given research project by providing visibility into previously undetectable changes in gene expression.[10,16] While qPCR relies on pre-defined targets, total RNA-Seq and mRNA-Seq do not always require prior knowledge of relevant transcripts for detection, enabling an unbiased view of the transcriptome that empowers researchers to better understand novel molecular mechanisms underlying biological phenomena.[10,16]

- Increased discovery power for detecting novel transcripts
- Enhanced sensitivity for detecting rare variants and lowly expressed genes
- Higher throughput for simultaneous sequencing of multiple genes across multiple samples
- Wider dynamic range for quantifying expression of genes without background noise or signal saturation

RNA-Seq offers more than qPCR – *Experience unparalleled, unbiased discovery power to interrogate the transcriptome with NGS.*[10,16]

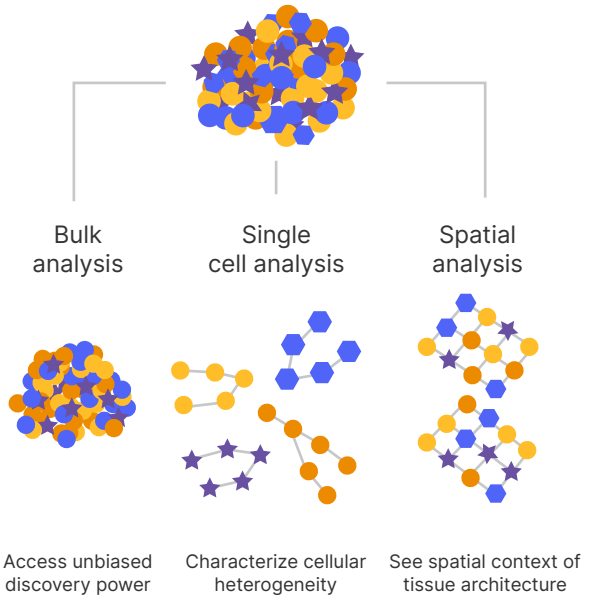
NGS-based transcriptomics at any biological resolution

The process of analyzing RNA from homogenized whole tissue samples using NGS-based RNA-Seq is referred to as "bulk RNA-Seq".[10] Bulk RNA-Seq has exceptionally well-defined workflows and has been implemented in thousands of scientific publications.[10,17] The ease of bulk RNA-Seq execution, combined with its comparatively low cost, make it an ideal method for transcriptomics researchers who are new to NGS. Since tissues are comprised of multiple different cell types, bulk RNA-Seq of whole tissues yields averaged results of gene expression profiles across these various cell types.[10]

Single-cell RNA-Seq (scRNA-Seq) examines gene expression from a population of single cells isolated from a tissue sample.[10] Implementation of scRNA-Seq facilitates the identification of novel biomarkers and rare cell types that would otherwise be missed with bulk RNA-Seq.[22,23] This single-cell resolution is a valuable tool in the study of multiple biological phenomena, including the tumor microenvironment and cellular differentiation.[18,19]

Additionally, spatially resolved transcriptomics, also called “spatial RNA-Seq”, is a ground-breaking molecular profiling method that enables RNA-Seq analysis of genes within the context of a tissue sample.[10, 20] This allows for insights into how the tissue microenvironment can influence the gene expression profiles of the cells that comprise it, since cells can be influenced by neighboring cells, local signaling events, cell–cell interactions, and more. Revealing a tissue’s complex mixture of cell types with spatial techniques has already enabled profound new discoveries within the fields of neuroscience, developmental biology, cancer, and more.[21]

While bulk RNA-Seq lacks the resolution of single-cell and spatial RNA-Seq, this method can be used to compare gene expression patterns between distinct cell populations by first using flow cytometry to sort cells into distinct populations, followed by bulk RNA-Seq to identify transcriptomic differences between those populations.[24]



Emerging applications of RNA-Seq technologies– *Be at the forefront of rapidly advancing single-cell and spatial methodologies with NGS.*[10]

How can NGS revolutionize your research?

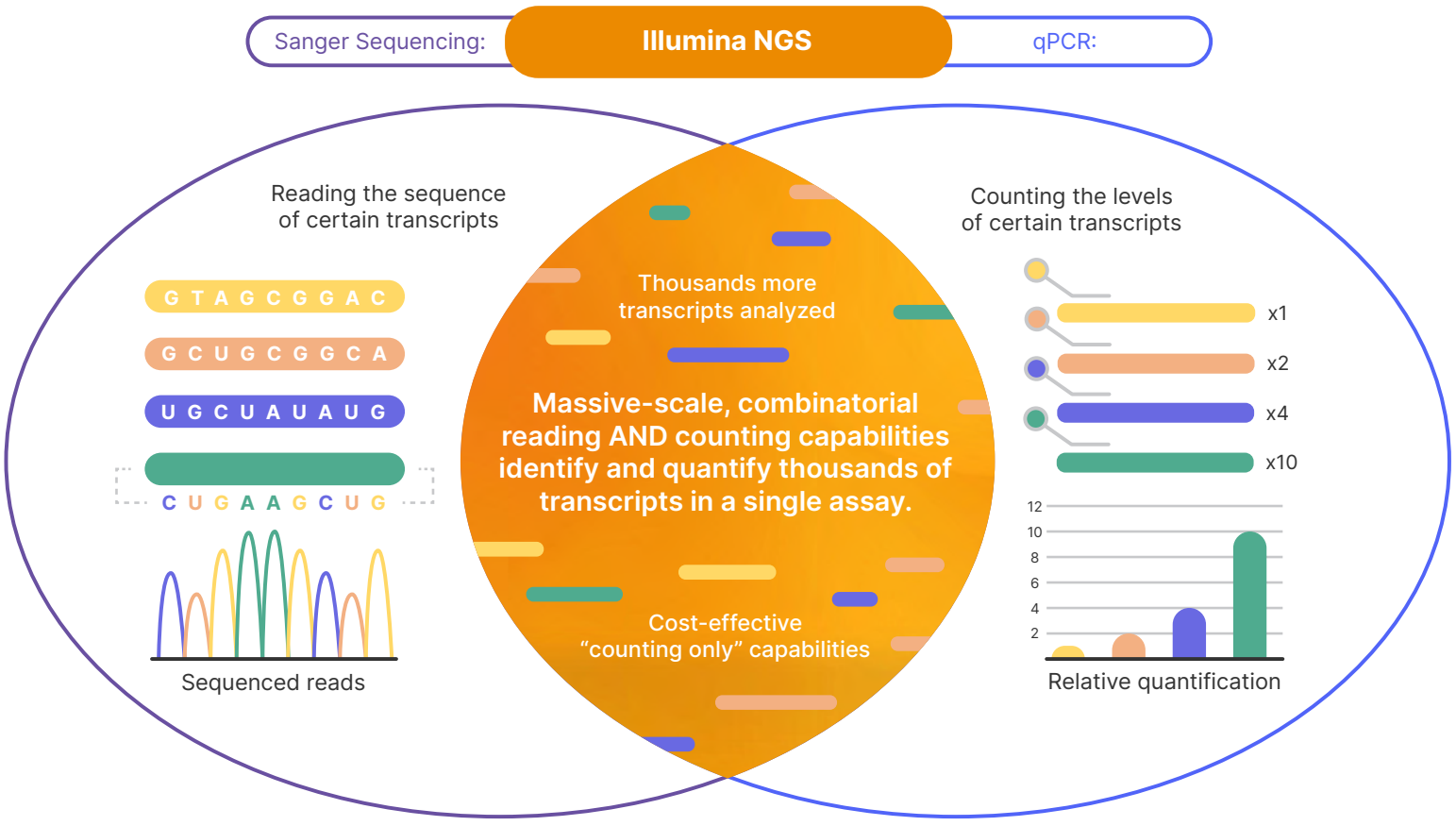
Benefits of NGS over traditional molecular methods

When analyzing transcripts within a sample, researchers can choose between reading or counting applications.[25] "Reading" means sequencing transcripts of interest and aligning them to a reference database ("read mapping") to determine the nucleic acid sequence of transcripts within a sample.[26] Sanger sequencing was an early methodology used to read transcripts of interest.[27]

On the other hand, "counting" refers to the relative quantification of transcripts of interest within a sample, which provides insights into transcript abundances and gene expression changes under differential experimental conditions.[25,28] Traditionally, qPCR has been the go-to methodology used to count transcripts of interest.[10] Like these qPCR experiments, some NGS methods (such as 3' RNA-Seq) do not provide the full sequence information for a set of expressed genes, but rather, provide just enough information to determine whether their expression levels have changed.[28]

Illumina transcriptomics methods offer reading, counting, and combinatorial reading and counting applications, depending on the NGS method used.[10,28] NGS–based reading methods, which sequence full RNA transcripts, allow researchers to identify rare RNA isoforms, splicing variants, and single-nucleotide variants (SNVs).[10] On the other hand, NGS–based counting methods, which sequence only a part of RNA (or a barcode readout for another analyte), allow researchers to quantify gene expression at a low cost.[10,29] Finally, NGS methods that combine both reading and counting allow researchers to gain a full view of both sequence identity and abundance of transcripts in a single, high-throughput assay.[10]

For variant screening studies with a high number of samples, NGS is the most efficient and cost-effective approach for the sequencing of tens to thousands of genes compared to conventional methods.



Illumina NGS combines the power of traditional reading and counting applications – *Identify full sequences and quantify expression changes in thousands of transcripts in a single assay with NGS.*[10, 25-28]

NGS excels beyond familiar workflows

In contrast to qPCR and Sanger sequencing, NGS sequencing can identify variants across thousands of target regions (down to single-base resolution) in a single experiment.[10,16,30]

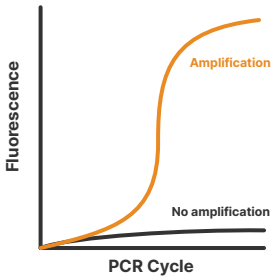
When compared to Sanger sequencing, certain NGS methods can:[10,30]

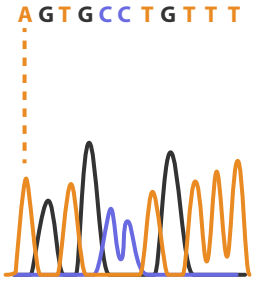
- Screen more samples cost-effectively
- Detect multiple variants across regions of the transcriptome
- Increase sequencing depth, mutation resolution, and discovery power

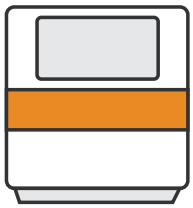
When compared to qPCR, certain NGS methods can:[10,16]

- Detect both known and novel transcripts
- Quantify individual sequence reads to produce absolute, not just relative, expression values
- Detect subtle changes in gene expression, down to 10%
- Identify novel transcripts, alternatively spliced isoforms, splice sites, and small and noncoding RNA species

When selecting the best method for answering a research question, it's important to consider not only the benefits of available methods, but also their associated challenges and limitations.







qPCR

Benefits[10,16,30]

- Familiar workflow
- Accessible equipment available in most labs

Challenges[10,16,30]

- Only accesses a predefined set of transcripts
- Detects only known transcripts, limiting discovery power
- Limited throughput and mutation resolution

Sanger Sequencing

Benefits[10,16,30]

- Familiar workflow
- Cost-effective when sequencing 1-20 targets

Challenges[10,16,30]

- Low sensitivity, throughput, and discovery power
- Not cost-effective for >20 targets

NGS

Benefits[10,16,30]

- High sequencing depth enables high sensitivity (down to 1%)
- High discovery power
- High mutation resolution
- Massively parallel sequencing enables high-throughput workflows and large datasets
- Detects gene expression changes down to 10%

Challenges[10,16,30]

- Not always as efficient for simple detection of a low number of targets

NGS enables large-scale, high-throughput, unbiased discovery – *Incorporate NGS into your research to enhance your discovery power over traditional molecular methods.*

NGS in action

NGS is revolutionizing a variety of basic and translational research areas by providing a deeper view into molecular mechanisms underlying cellular functions and disease states in a variety of biological systems (e.g., human samples, animal models, and cell culture).

Research/ Disease Area	NGS method	Application	Research Example
Cancer	Total RNA-Seq	Bulk sequencing of tumors to predict the likelihood of metastasis and associated clinical outcomes	Researchers assessed the gene expression profiles of primary ovarian cancer tumors and those that had metastasized. Bulk RNA-Seq analysis revealed a signature of 100 differentially expressed genes between the two tumor types, enabling researchers to predict the likelihood of tumor metastasis, which was suggestive of poor survival.[31]
Cancer	scRNA-Seq	Stratification of cell types within a tumor to predict a patient's clinical response to therapy	Using scRNA-Seq, researchers analyzed cancer-associated fibroblasts (CAFs) in tumor biopsies from patients with non-small cell lung cancer. This analysis found three distinct subtypes of CAFs among tumors, each differing in their gene expression profiles and each correlated with a unique clinical response therapy.[32]
Cancer	Total RNA-Seq and WGS	Unbiased discovery of cancer-associated genes and transcripts	A study analyzing tumor biopsies from 253 pediatric cancer patients using WGS and RNA-Seq revealed that 86% of patients had at least one finding that was diagnostic, prognostic, targetable, or indicative of germline predisposition.[33] Interestingly, RNA-Seq analysis of a tumor from one such patient identified a gene fusion in a targetable pathway known to drive cancer progression (the MEK pathway). This discovery led clinicians to change their therapeutic strategy (incorporation of MEK inhibitors), leading to effective cancer treatment.[34]
Microbial sequencing	Total RNA-Seq	Identification of differential viral microbiota in healthy vs. diseased patients	In-depth RNA-Seq analysis of the gut mucosa of patients with early Crohn's disease or ulcerative colitis found a differential abundance of certain viral transcripts compared to healthy controls, suggesting that specific gut virome signatures may influence intestinal inflammation and contribute to the pathogenesis of these diseases.[35]
Microbial sequencing	Metatranscriptomics[a]	Longitudinal analysis of microbiota-associated functional pathways that influence disease	Analysis of the metatranscriptome of human fecal samples isolated from healthy patients and patients with inflammatory bowel disease (IBD) revealed certain bacterial species-specific functional metabolic transcripts that were important for maintaining gut health.[36]
Complex disease research	Total RNA-Seq	Identification of previously undetected SNPs underlying disease	Researchers used RNA-Seq to screen four healthy patients and four patients with the lung disease silicosis and identified a functional SNP in a single immunomodulatory gene that was associated with increased susceptibility to silicosis.[37]
Complex disease research	Spatial and single-cell RNA-Seq	Discovery of distinct cellular niches that comprise a larger tissue	Researchers profiled the human heart and found eight distinct cellular regions with differential expression of ion channels and G-protein coupled receptors that influenced a myriad of cellular interactions.[38]

a. An NGS technique used to study the gene expression profiles of all microbes present within a given complex sample.

NGS powers discoveries across many therapeutic areas– *Unlock the unlimited potential of NGS to push your field of research forward.*

What does an NGS workflow look like?

NGS at a glance

The overall workflow for an NGS experiment starts with the isolation of genetic material. Once you have your sample, you don't need to be an expert in NGS to get started: send your samples out to a local service provider, such as an academic or commercial core lab.

Many service providers provide end-to-end support, and collaborating early in the experimental design process is the best way to get expert assistance at each step of your NGS workflow, from sample preparation to data analysis

Using a core lab was crucial for my ability to start NGS. Between graduate studies and many other methods that I needed to master, using the core helped me get started without being an expert.

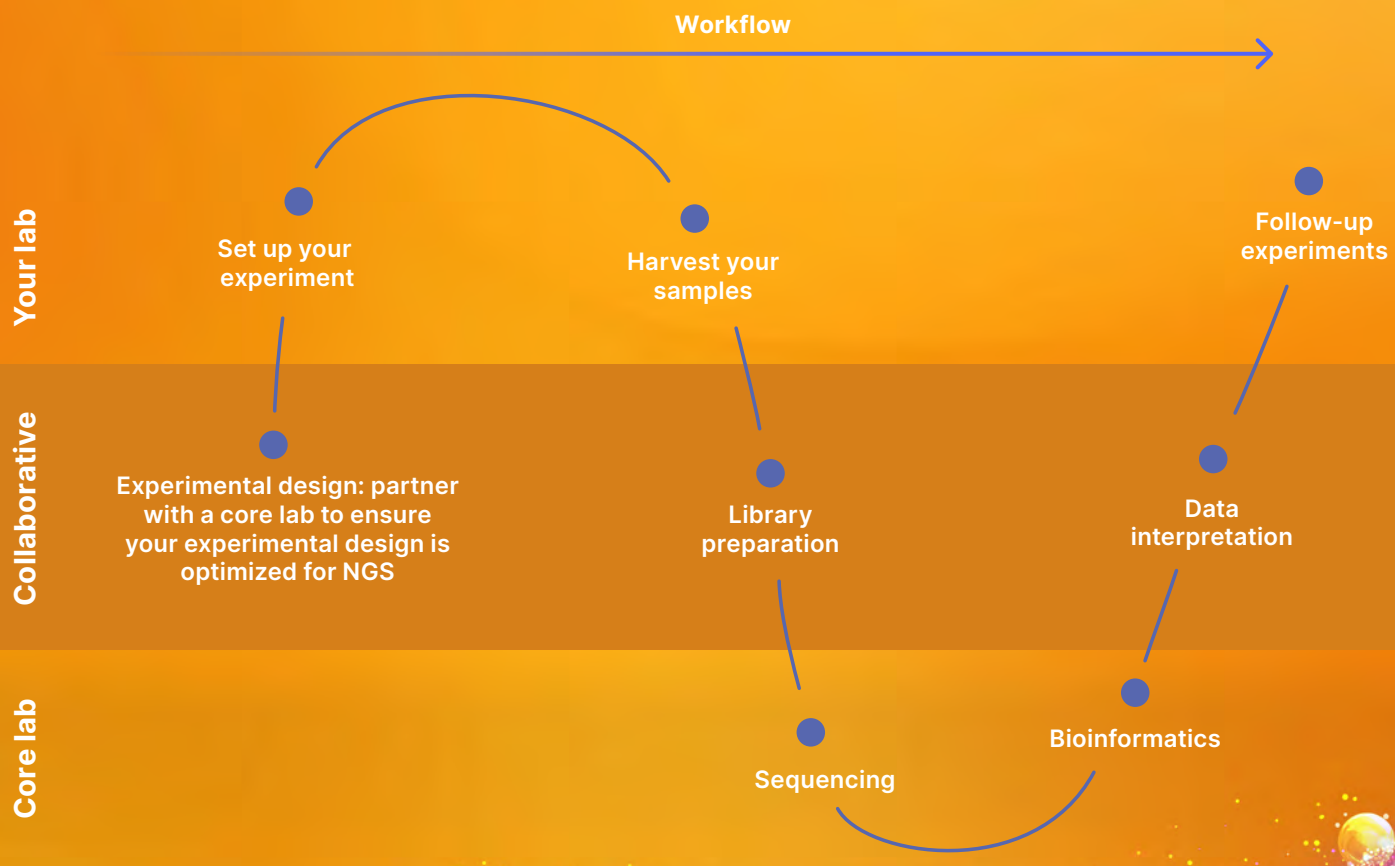
Amanda Touey, PhD candidate in Dr. Paula Cohen's lab at Cornell University

[Core labs] like to get involved with the researcher early on so that we can help direct experimental design, understand their biological question, and then make determinations from there in terms of what is the best method

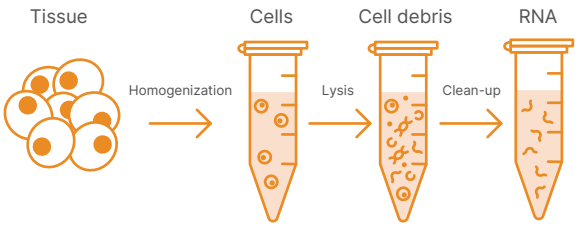
Dr. Adrian McNairn, Lead Biologist at the Genomics Innovation Hub

Illumina RNA Sequencing Workflow
Watch here

Collaboration with core labs simplifies the NGS workflow

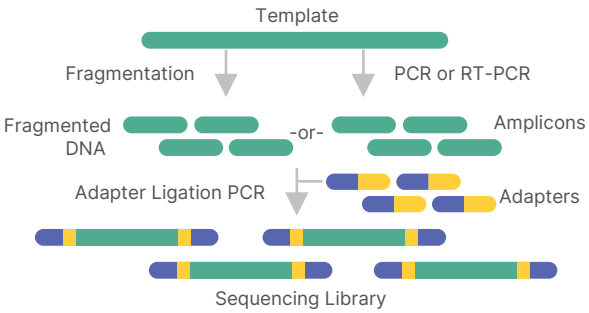


Step 1
Extraction



RNA isolation
Purification of total RNA or specific RNA species from samples via tissue homogenization, cell lysis, and debris clean-up.

Step 2
Library preparation

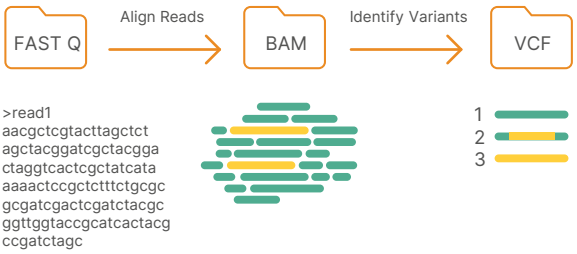


Fragmentation and adapter ligation
Fragmentation of your sample and addition of adapters that both identify your sample and make your sample compatible with Illumina sequencing systems.

Step 3
Sequencing

Sequencing by synthesis
Illumina sequencing technology employs fluorophore-conjugated nucleotides to enable easy identification of each base as it is incorporated during DNA synthesis.

Step 4
Data analysis



Bioinformatics and interpretation
Integrated data analysis of sequencing information, provides you with tangible results that are relevant to your research questions.

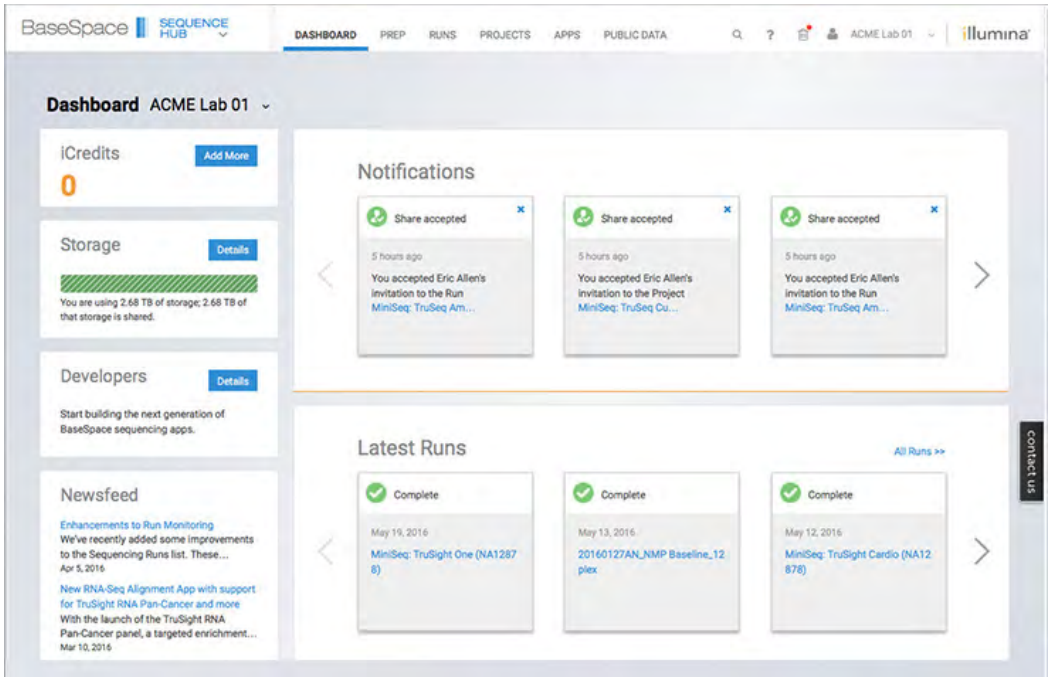
Illumina NGS workflow– *State-of-the-art sequencing capabilities that power your discoveries.*

The NGS workflow for other applications: Access our Genomics, Epigenomics, Transcriptomics, and Multiomics ebooks.

What can you do with your NGS data?

Once your NGS samples are sequenced, core lab technicians can help you with every step of your data analysis and interpretation. Data is also shared and can be viewed on the Illumina BaseSpace Sequence Hub, which enables easy access to and management of your NGS data. In addition, the BaseSpace Sequence Hub offers a variety of app-based programs with accessible user interfaces to simplify bioinformatics, allowing for automated visualization and interpretation of your sequencing data.

Example BaseSpace Sequence Hub dashboard



User-friendly data analysis apps

RNA-Seq Alignment
Illumina, Inc.

The RNA-Seq alignment app aligns raw RNA-Seq reads to reference genomes, quantifies gene expression, and calls small variants and gene fusions.

Cancer Variant Caller
Samsung SDS

The Cancer Variant Caller app detects SNVs and INDELs given a BAM file with high sensitivity.

DRAGEN RNA Pathogen Detection
Illumina Inc.

DRAGEN RNA Pathogen Detection uses a combined human + virus reference to analyze pathogen data and create consensus FASTAs.

DRAGEN Differential Expression
BaseSpace Labs

The DRAGEN Differential Expression Application performs secondary analysis of RNA transcripts.

How to get started with NGS

A step-by-step guide

With its growing accessibility and profound research applications, there's no better time than now to start using NGS. Illumina is committed to supporting your NGS journey from start to finish.

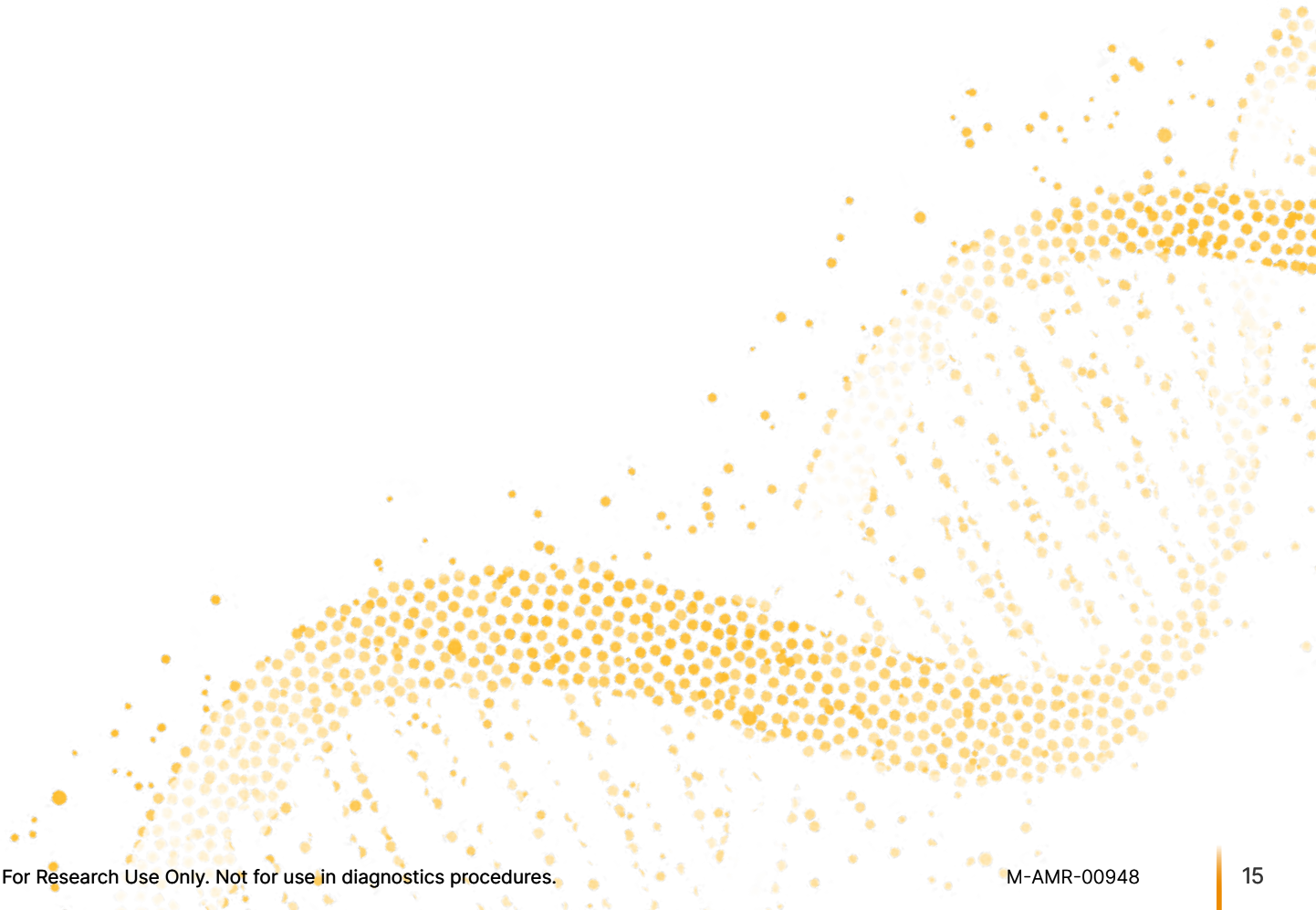
Do you need help identifying a local NGS service provider?	Do you need help with your experimental design?	Do you need more information on NGS?
Yes	Yes	Yes
Fill out this form	Access our beginner's guide to NGS	Access our wide range of online courses and webinars
Once submitted, an Illumina representative will contact you to direct you to service providers in your area	Reach out to our talented team of subject matter expoerts here	Explore all of our New to NGS resources here

Now is the time to get started because the cost is right and because we have so much experience under our belt now that we can really facilitate any genomic experiment you want to do or can think of.

Ann Tate, Technical Services Supervisor at Cornell University

Summary

- NGS is easy to access and implement, and there's no better time to start than today.
- RNA-Seq powered by NGS unlocks deeper insights into the transcriptome for more impactful answers to your research questions.
- You don't need to be an expert to get started: the NGS workflow is accessible to everyone.
- Illumina is a leading expert in NGS and a trusted partner in helping you achieve research success.



Glossary of terms

ATAC-seq: a method in which genomic DNA is exposed to Tn5, a highly active transposase that preferentially inserts into open chromatin sites and adds sequencing primers. Subsequent NGS analysis provides insights into chromatin accessibility across the genome.

Antibody derived tags (ADTs): the use of DNA-barcoded oligonucleotides conjugated to protein-specific antibodies to enable NGS-based quantification of proteins.

Bulk RNA-Seq: a sequencing approach that assesses the average gene expression profile from a population of cells.

Chromatin immunoprecipitation sequencing (ChIP-Seq): the combination of using protein-specific antibodies to immunoprecipitate chromatin-associated proteins, followed by NGS to identify their DNA binding sites.

Counting: the relative quantification of a given gene within a sample.

Discovery power: the ability to identify novel variants or differentially regulated nucleic acids

Genomics: the study of the genome, which is the complete set of DNA within a biological sample.

Library preparation: A molecular biology protocol that converts a genomic DNA sample or a cDNA sample from a transcript into a sequencing library, which can then be sequenced on an NGS instrument. The first step in library preparation is random fragmentation of the DNA sample, followed by ligation of 5' and 3' adapters to each DNA fragment. Alternatively, “tagmentation” combines the fragmentation and ligation reactions into a single step and greatly increases the efficiency of the library preparation process.

Metatranscriptomics: An NGS technique to study the gene expression profiles of all microbes present within a given complex sample.

Multiomics: the combined study of multiple –omics, which provides a multidimensional, holistic view of a biological sample.

Mutation resolution: The size of mutation, in base pairs, a technology is able to detect. For example, karyotyping provides a mutation resolution of 5-10 Mb, while array comparative genomic hybridization provides “higher resolution” by detecting mutations down to 50 kb. NGS techniques provide the highest possible mutation resolution because they can provide single-base pair variant detection (detect the presence of a mutation) and nucleotide identification (detect the identity of a mutation).

Next-generation sequencing (NGS): a massively parallel sequencing technology that offers ultra-high throughput, scalability, and speed.

Proteomics: the study of the proteome, which is the complete set of proteins encoded by a biological system.

RNA sequencing (RNA-Seq): a highly sensitive and accurate method for gene expression analysis, which can assess the full transcriptome, rather than just a few selected transcripts. Read: the inferred sequence of base pairs output from a sequencing run.

Read: The process of next-generation DNA or cDNA sequencing involves using sophisticated instruments to determine the sequence of a DNA or RNA sample. In general terms, a sequence “read” refers to the data string of A,T, C, and G bases corresponding to the sample DNA or cDNA. With Illumina technology, millions of reads are generated in a single sequencing run.

Reading: the process of analyzing the full sequence of a DNA/RNA molecule and mapping it onto a reference genome for gene identification.

Sanger sequencing: the sequencing method, also known as capillary electrophoresis sequencing, developed in 1977 by Frederick Sanger. It involves a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication.

Sensitivity: In sequencing, the ability to detect low-frequency, rare variants.

Sequencing by synthesis (SBS): SBS technology uses fluorescently labeled nucleotides to sequence tens of millions of sequence templates in parallel. During each sequencing cycle, a single labeled dNTP is added to the nucleic acid chain. The nucleotide label serves as a "reversible terminator" for polymerization: after dNTP incorporation, the label is identified through laser excitation and imaging, then enzymatically cleaved to allow the next round of incorporation. As all four reversible terminator-bound dNTPs (A, C, T, G) are present, natural competition minimizes incorporation bias. Base calls are made directly from signal intensity measurements during each cycle, which greatly reduces raw error rates compared to other technologies. The result is highly accurate base-by-base sequencing that eliminates sequence-context-specific errors, enabling robust base calling across the genome, including repetitive sequence regions and within homopolymers.

Sequencing panel: A subset of genes or genomic regions of interest included in a targeted NGS assay. The sequencing panel (target regions) can be amplified or enriched using sequence-specific probe sets. The sequencing panel can be sequenced for a fraction of the time and cost compared to broader sequencing approaches.

Shotgun metagenomics: a next-generation sequencing approach that allows researchers to comprehensively sample all genes of all organisms present within a complex sample.

Single-cell RNA-Seq (scRNA-Seq): a sequencing approach that isolates single cells to assess the gene expression profiles of individual cells that make up a larger population.

Single-nucleotide polymorphism (SNP): a genomic variant that arises from a single base change in the genetic code.

Spatial RNA-Seq: a sequencing approach that uses intact tissues to capture the gene expression profile of cells within their native tissue environment.

Targeted resequencing: a next-generation sequencing approach in which a subset of genes or target regions are amplified or enriched before sequencing.

Transcriptomics: the study of the transcriptome, which is the complete set of RNA within a biological sample.

Unbiased discovery: the implementation of an experimental approach that does not rely on a targeted hypothesis, but rather, assesses a large scope of biological insights using high-throughput screening.

Whole-genome sequencing: a comprehensive next-generation sequencing method for analyzing entire genomes of experimental samples.

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