

# Advancements in NGS Library Preparation for Challenging Samples

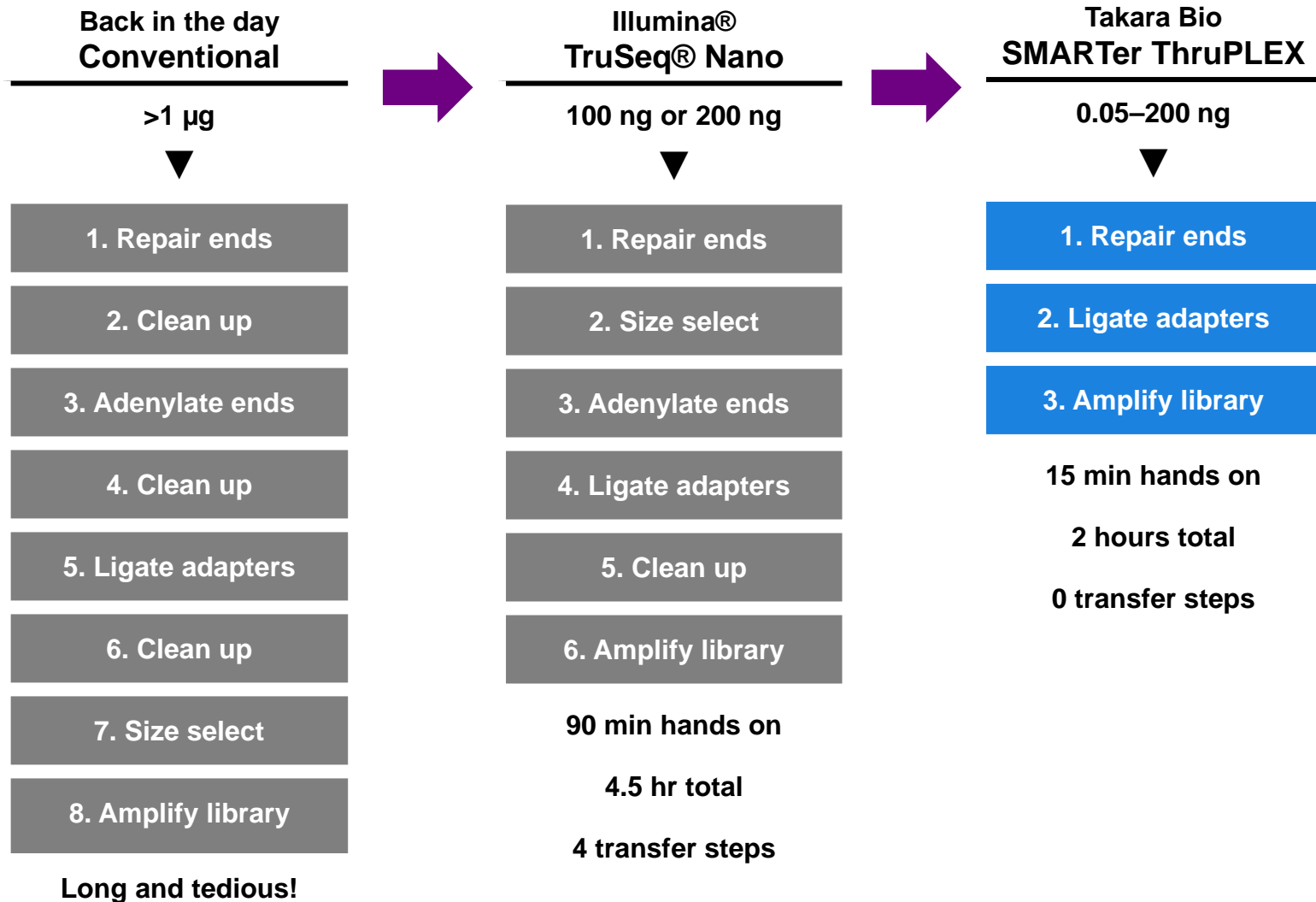
Suvarna Gandlur, Ph.D.  
Takara Bio USA, Inc.

# SMARTer® NGS portfolio

Encompasses three key technologies, all featuring **high sensitivity** and a **streamlined workflow**

	DNA SEQUENCING	TARGETED SEQUENCING	WHOLE TRANSCRIPTOME ANALYSIS
SMART® Technology	<ul style="list-style-type: none"><li>• ChIP-seq</li><li>• Meth-seq</li></ul>	<ul style="list-style-type: none"><li>• Targeted RNA-seq</li><li>• Small RNA-seq</li><li>• Immune profiling</li></ul>	<ul style="list-style-type: none"><li>• Single-cell/ultra-low-input RNA-seq</li><li>• Total RNA-seq</li></ul>
ThruPLEX® Technology	<ul style="list-style-type: none"><li>• Illumina library construction</li><li>• Targeted sequencing</li><li>• ChIP-seq</li></ul>	<ul style="list-style-type: none"><li>• Targeted DNA-seq with major enrichment platforms</li></ul>	
PicoPLEX® Technology	<ul style="list-style-type: none"><li>• Whole genome amplification</li><li>• Aneuploidy/CNV detection</li></ul>	New additions to the SMARTer NGS portfolio	

# Evolution of DNA library prep



# ThruPLEX enabled pioneers in cfDNA research to make groundbreaking findings

- Kitzman *et al.* Noninvasive Whole-Genome Sequencing of a Human Fetus. *Science Translational Medicine* (2012).
  - Jay Shendure lab: Howard Hughes Medical Institute and the Department of Genome Sciences at the University of Washington
- Murtaza *et al.* Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature* (2013).
  - Nitzan Rosenfeld lab: Cancer Research UK Cambridge Institute

RESEARCH ARTICLE

GENOMICS

## Noninvasive Whole-Genome Sequencing of a Human Fetus

Jacob O. Kitzman,<sup>1</sup> Matthew W. Snyder,<sup>1</sup> Mario Ventura,<sup>1,2</sup> Alexandra P. Lewis,<sup>1</sup> Ivuon Olu,<sup>1</sup> Latoya E. Simmons,<sup>1</sup> Hilary N. Gammal,<sup>1</sup> Craig E. Hubert,<sup>1</sup> Debra A. Santillan,<sup>1</sup> Jeffrey C. Murray,<sup>1</sup> Holly K. Tabor,<sup>3,4</sup> Michael J. Bamshad,<sup>5,6</sup> Evan E. Eichler,<sup>1,5</sup> Jay Shendure<sup>1\*</sup>

Analysis of cell-free fetal DNA in maternal plasma holds promise for the development of noninvasive prenatal genetic diagnostics. Previous studies have been restricted to detection of fetal trisomies, to specific paternally inherited mutations, or to genotyping common polymorphisms using material obtained invasively, for example, through chorionic villus sampling. Here, we combine genome sequencing of two parents, genome-wide maternal haplotyping, and deep sequencing of maternal plasma DNA to noninvasively determine the genome sequence of a human fetus at 16.5 weeks of gestation. Inheritance was predicted at 2.8 × 10<sup>6</sup> parental heterozygous sites with 98.1% accuracy. Furthermore, 39 of 44 de novo point mutations in the fetal genome were detected, albeit with limited specificity. Subsampling these data and analyzing a second family trio by the same approach indicate that parental haplotype blocks of ~300 kilobase pairs combined with shallow sequencing of maternal plasma DNA is sufficient to substantially determine the inherited complement of a fetal genome. However, ultradeep sequencing of maternal plasma DNA is necessary for the practical detection of fetal de novo mutations genome-wide. Although technical and analytical challenges remain, we anticipate that noninvasive analysis of inherited variation and de novo mutations in fetal genomes will facilitate prenatal diagnosis of both recessive and dominant Mendelian disorders.

**INTRODUCTION**  
On average, ~13% of cell-free DNA isolated from maternal plasma during pregnancy is fetal in origin (1). The concentration of cell-free fetal DNA in the maternal circulation rises between individuals, increases during gestation, and is rapidly cleared postpartum (2, 3). Despite this variability, cell-free fetal DNA has been successfully targeted for noninvasive prenatal diagnosis including for development of targeted assays for single-gene disorders (4). More recently, several groups have demonstrated that shotgun, massively parallel sequencing from maternal plasma is a robust approach to sequencing fetal aneuploidies such as trisomy 21 (5). Ideally, it should be possible to obtain a genome sequence of a fetus to high accuracy, thereby enabling the comprehensive prenatal diagnosis and creating the need for invasive procedures such as chorionic villus sampling with their ever-increasing technical obstacles that must be achieved using cell-free DNA. Here, we present representation of fetal-derived sequences from low-frequency alleles inherited from the plasma

... arising from de novo mutations in the fetal genome. Second, maternal DNA polymorphisms in the mother's plasma, making it difficult to assess maternally inherited variation at individual sites in the fetal genome. Recently, Lu *et al.* showed that fetal-derived DNA is distributed sufficiently evenly in maternal plasma to support the inference of fetal genome, and further, they demonstrated how knowledge of parental haplotypes could be leveraged to this end (7). However, their study was limited in several ways. First, the proposed method allowed

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## LETTER

### Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA

Muhammad Murtaza<sup>1</sup>, Sarah Jane Dawson<sup>1,2</sup>, Diana W. Y. Tang<sup>1</sup>, Emma Gilg<sup>1</sup>, Tim Forde<sup>1</sup>, Anna M. Pihlman<sup>1</sup>, Christine Parkman<sup>1</sup>, Scott Hong Chai<sup>1</sup>, Zoya Jagtap<sup>1</sup>, Alvin S. C. Wong<sup>1</sup>, Francisco Martin<sup>1</sup>, Scott Humphrey<sup>1</sup>, James Harford<sup>1</sup>, David Bentley<sup>1</sup>, Tim Chin<sup>1,3</sup>, James D. Brenton<sup>1,4</sup>, Carlos Cordon-Cardo<sup>1,5</sup> & Nitzan Rosenfeld<sup>1</sup>

Cancer acquires resistance to systemic treatment as a result of clonal evolution and selection<sup>1</sup>. Repeat biopsies to study genomic evolution as a result of therapy are difficult, invasive and are confounded by tumor heterogeneity<sup>2,3</sup>. Recent studies have shown that genetic alterations in tumor samples can be tracked by massively parallel sequencing of circulating cell-free tumor DNA released from cancer cells into plasma, representing a non-invasive liquid biopsy<sup>4</sup>. Here, we report sequencing of cancer genome in cell-free plasma samples in 16 cancer patients with advanced breast, ovarian and lung cancer who were followed over 1–2 years. For each case, cancer sequencing was performed on 2–5 plasma samples (19 in total) spanning multiple courses of treatment. Individualized tumor-free allele frequencies of numerous mutations in plasma, high, allowing improved sensitivity. For two cases, synchronous biopsies were also analyzed, confirming genome-wide representation of the tumor genome in plasma. Quantification of allele fractions in plasma identified increased representation of alleles in association with emergence of therapy resistance. These included activating mutations in *PIK3CA* (phosphatidylinositol-3-OH kinase class IA), *cazotinib* (cazotinib alpha) following treatment with *pipecolif* a mTORC1 inhibitor, *ERK1* (extracellular signal-regulated kinase 1) following treatment with *lapatinib*, a tyrosine kinase inhibitor, *MEK1* (mitogen-activated protein kinase 1) following treatment with *trastuzumab*<sup>5,6</sup>, and following subsequent treatment with *lapatinib*<sup>7,8</sup>. A splicing mutation in *GAS2* (growth arrest specific 2) in the same patient, and a resistance-associated mutation in *EGFR* (epidermal growth factor receptor 2) following treatment with *gefitinib*<sup>9</sup>. These results establish proof of principle that cancer-wide analysis of circulating tumor DNA could complement current invasive biopsy approaches to identify mutations associated with acquired drug resistance in advanced cancer. Initial analysis of cancer genome in plasma indicates a new paradigm for the study of clonal evolution in human cancer.

Serial sampling of the tumor genome is required to identify the resistance mechanisms underlying drug resistance<sup>10</sup>. Serial tumor biopsies are invasive and often unfeasible. Tumor-free heterozygous and copy-neutral events and novel copy number alterations, these are tracked both spatially and temporally. Analysis of fetal cell-free circulating tumor cells (CTCs) has been proposed, but circulating tumor DNA (ctDNA) is more accessible and easier to process<sup>11</sup>. Previous studies of cancer evolution in plasma have analyzed wild-type loci genes or structural variants to quantify tumor burden and to detect previously unobserved resistance-conferring mutations<sup>12–14</sup>. Genome-wide sequencing of plasma samples is used in prenatal diagnosis, demonstrating comprehensive coverage of the genome<sup>15</sup>. More recently, genome-wide sequencing of plasma DNA has been

... demonstrated in a pilot study for the detection of disease or analysis of tumor burden in patients with advanced cancer<sup>16</sup>. This study established that plasma DNA contains representations of the entire human genome<sup>17</sup>, using together variants originating from multiple independent tumors<sup>18</sup>. This suggested deeper sequencing of plasma DNA, applied to serial samples with high tumor burden in blood, may allow assessment of clonal heterogeneity and evolution. In this study, we applied cancer sequencing of ctDNA in a pilot study for non-invasive analysis of tumor evolution during systemic cancer treatment (Fig. 1).

Figure 1 | Identification of treatment-associated clonal changes from cancer sequencing of cell-free plasma samples. Overview of the study design: serial sampling of plasma and tumor samples and analysis of mutations during treatment and follow-up of advanced cancer patients. Genome sequencing was performed on plasma and tumor samples at multiple time points during the course of treatment, and genome DNA. Mutations were identified across plasma samples, and their clonal evolution (allele fraction) at different time points was compared. Genomes of metastatic tumor samples were sequenced to identify mutations that were not detected in plasma samples. Allele fraction analysis of mutations in plasma samples was used to identify mutations that were not detected in plasma samples. Allele fraction analysis of mutations in plasma samples was used to identify mutations that were not detected in plasma samples.

\*Correspondence: Nitzan Rosenfeld (n.rosenfeld@cancer.gov.uk) or Jay Shendure (shendure@u.washington.edu) (both authors contributed equally and significantly to the work). Full list of author information is available at the end of the article. © 2013 Nature Publishing Group. All rights reserved.

# ThruPLEX: the gold standard for ChIP-seq

Widely cited in high-impact publications performing ChIP-seq to profile transcription factor binding sites:

- Liu, Y. *et al.* Transcriptional landscape of the human cell cycle. *PNAS* (2017).
- Warrick, J. I. *et al.* FOXA1, GATA3 and PPAR $\gamma$  cooperate to drive luminal subtype in bladder cancer: A molecular analysis of established human cell lines. *Sci. Reps.* (2016).
- Cejas, P. *et al.* Chromatin immunoprecipitation from fixed clinical tissues reveals tumor-specific enhancer profiles. *Nat. Med.* (2016).

## Recent ChIP-seq Publications using ThruPLEX Technology

Our collaborators and customers are constantly making scientific breakthroughs. Here are the latest published results obtained using ThruPLEX DNA-seq for chromatin immunoprecipitation sequencing (ChIP-seq).

1. Baejen, C. *et al.* Genome-wide analysis of RNA Polymerase II termination at protein-coding. *Genes. Mol. Cell* **66**, 1–12 (2017).

This paper used ChIP-seq, ChIP-qPCR, and other functional genomic methods to understand how RNA Pol II termination occurs in yeast. ThruPLEX DNA-seq kit was used to generate libraries for ChIP-seq. The author showed that the 3'-transition in budding yeast requires the Pol II elongation factor Spt5, and that polymerase II release from DNA requires the Rat1 exonuclease.

[Read now »](#)

2. Maatouk, D.M. *et al.* Genome-wide identification of regulatory elements in Sertoli cells. *Development* **144**, 720–30 (2017).

This study used ChIP-seq, DNase-seq, and RNA-seq to identify regulatory elements in mouse Sertoli cells during sex determination. ThruPLEX DNA-seq kit was used to prepare ChIP-seq libraries from FACS-sorted mouse Sertoli cells. By overlapping DNase-seq peaks with the chromatin landscape for H3K27ac, the authors were able to identify enhancers active only in Sertoli cells during the early stages of sex determination.

[Read now »](#)

3. Liu, Y. *et al.* Transcriptional landscape of the human cell cycle. *PNAS* **114**, 3473–78 (2017).

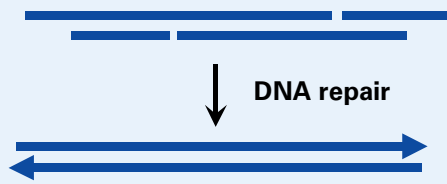
This paper investigated the transcriptional landscape across the cell cycles using a combination of ChIP-seq, DNase-seq, RNA-seq, and GRO-seq. ThruPLEX DNA-seq kit was used to prepare libraries for ChIP-seq and DNase-seq. Using the MCF-7 breast cancer cell line as a model, the authors revealed lag between transcription and steady-state RNA expression at the cell-cycle level. Other findings highlighted the importance of transcriptional and epigenetic dynamics during cell-cycle progression.

[Read now »](#)

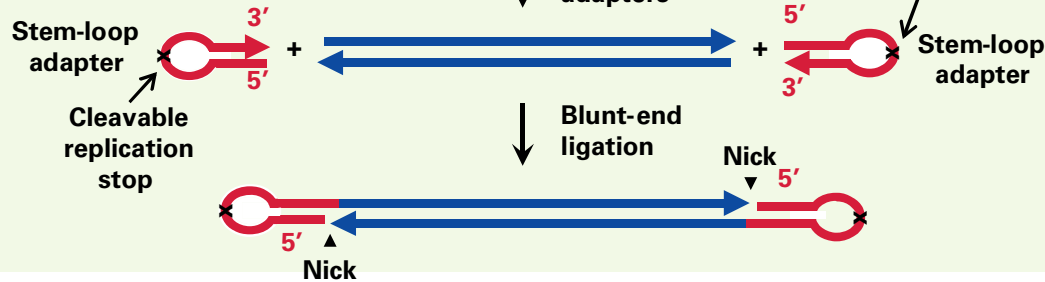
# ThruPLEX technology

## 1. Repair

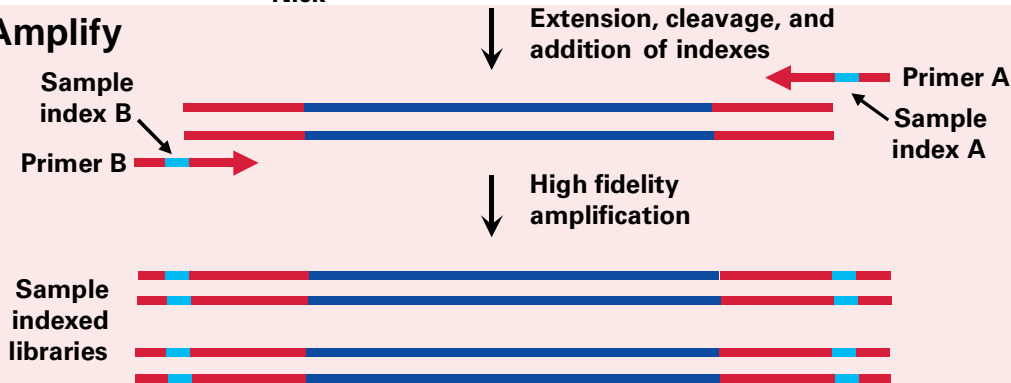
Fragmented dsDNA  
or cell-free DNA



## 2. Ligate



## 3. Amplify



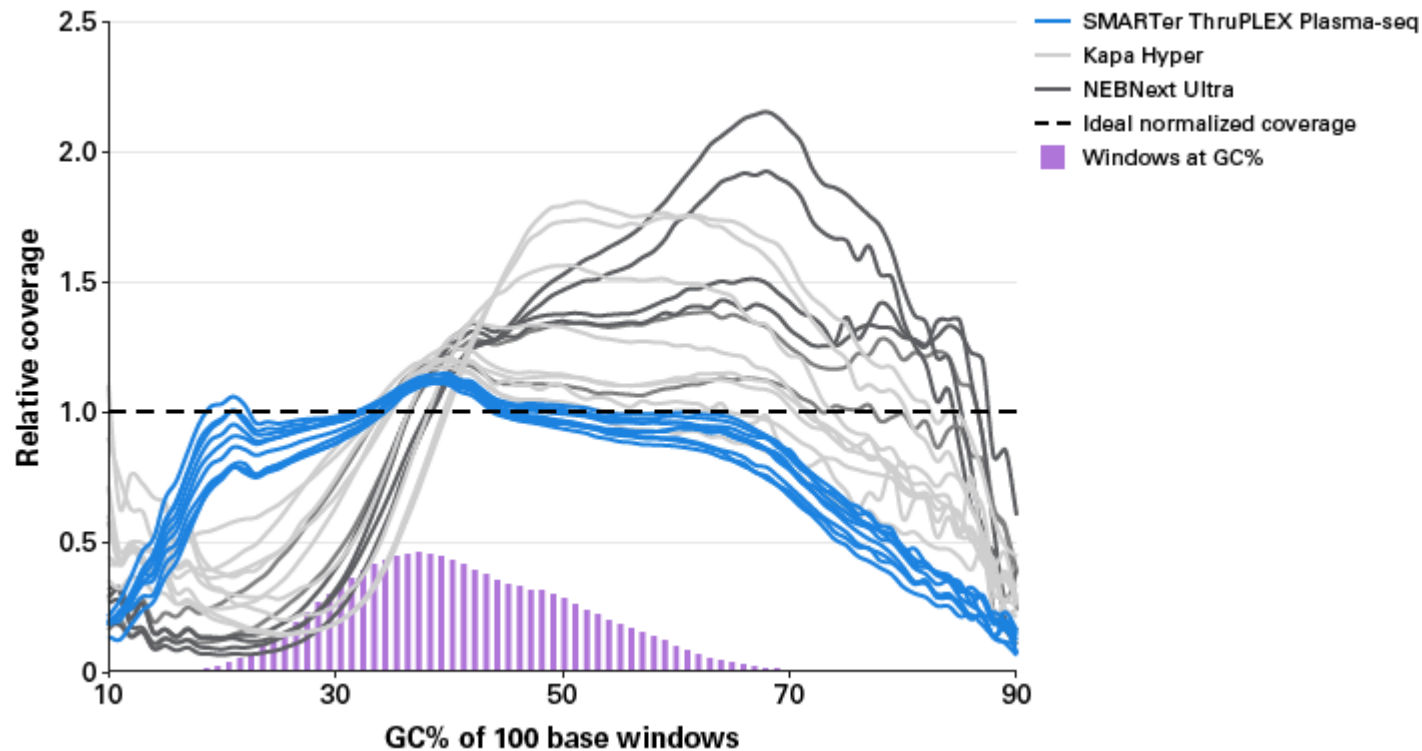
Superior performance and single-tube workflow resulting from innovative adapter design and highly efficient ligation reaction

- Innovative degradable stem-loop adapters
- Highly efficient single-stranded blunt-end ligation
- Excess adapter molecules degraded
- Post-ligation purification step eliminated

# SMARTer ThruPLEX Plasma-seq Kit

Optimized specifically for cell-free DNA

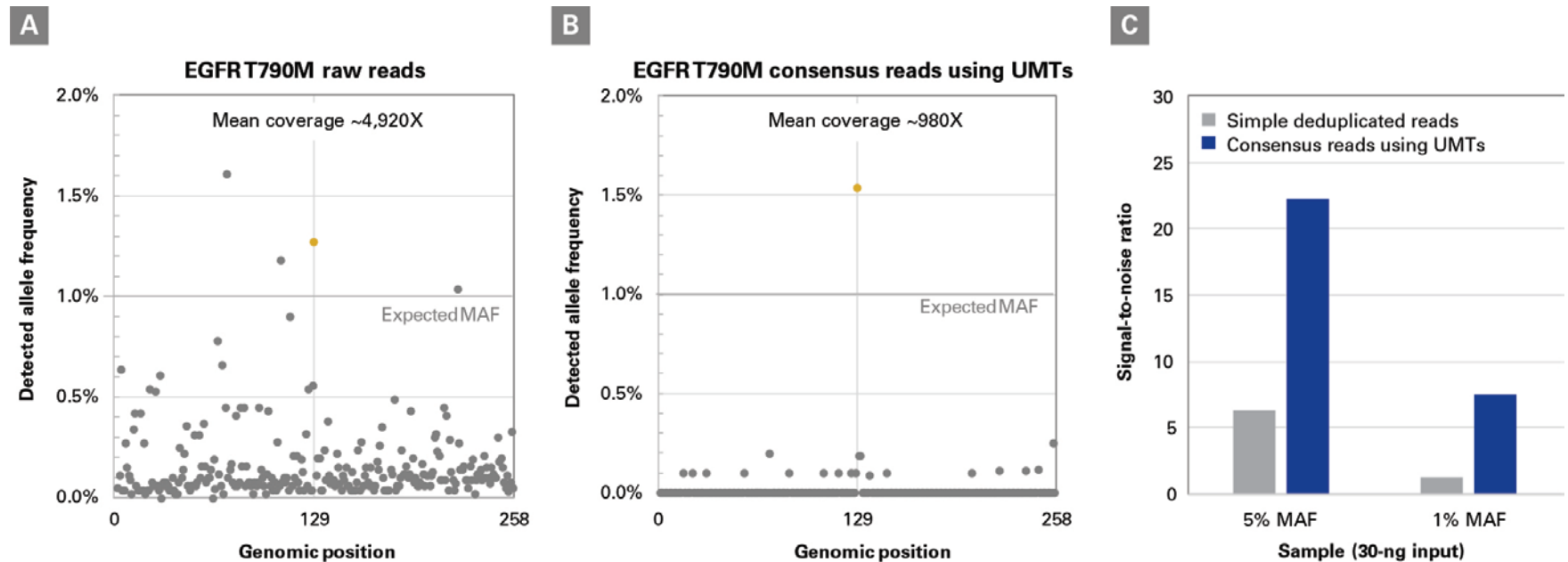
Single-tube workflow and minimal pipetting operations translate into highly reproducible sequencing results



# SMARTer ThruPLEX Tag-seq Kit

## Confident rare variant detection with unique molecular tags

Unique molecular tags incorporated during ligation to remove PCR and sequencing errors, resulting in confident detection of rare variants





# MicroRNA-seq challenges

- Most state-of-the-art ligation-based technologies for miRNA-seq suffer from ligation-induced bias
- This results in inaccurate representation of the biological state of the sample



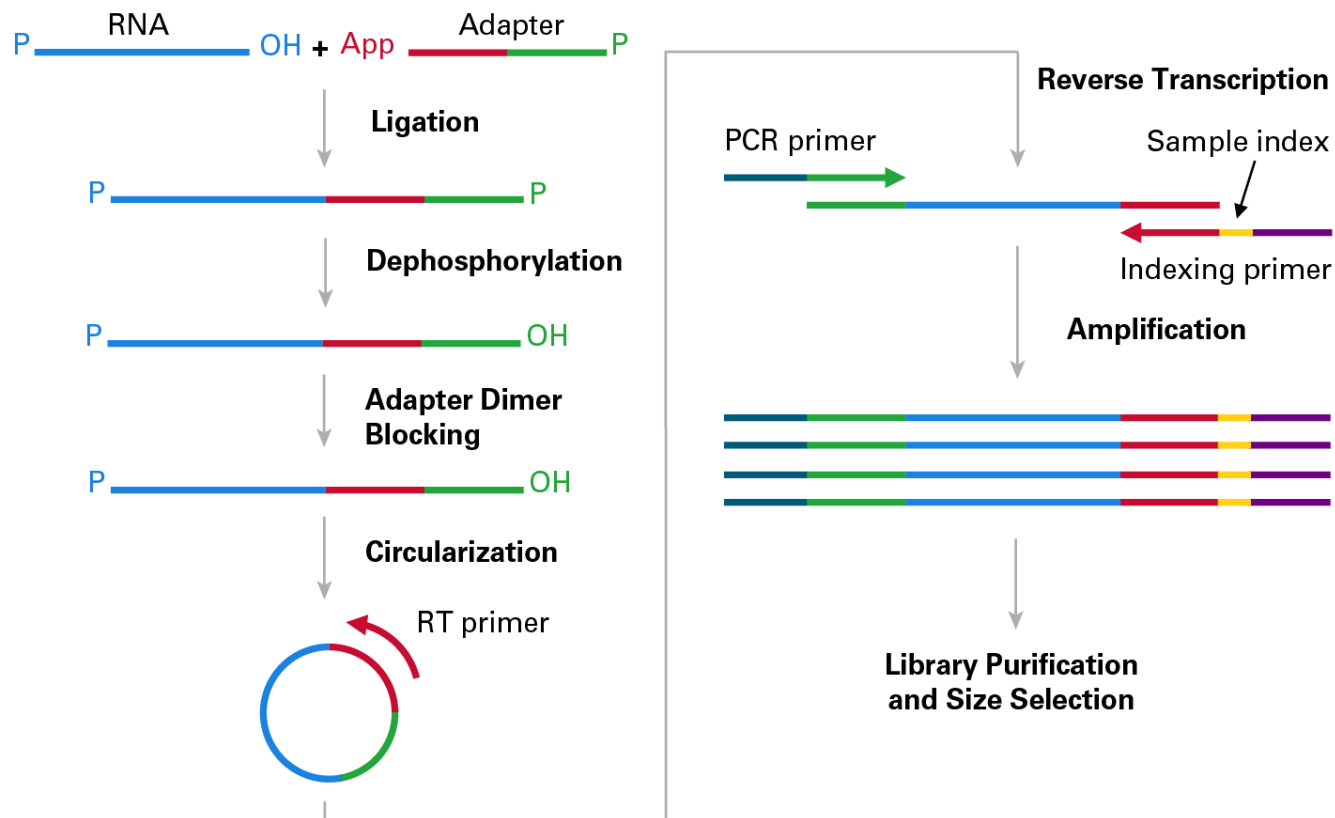
Jackson, T. J. *et al.* Evaluating bias-reducing protocols for RNA sequencing library preparation. *BMC Genomics* (2014).

Zhuang F. *et al.* Small RNA expression profiling by High-Throughput Sequencing: Implications of Enzymatic Manipulation. *J. Nucleic Acids* (2012).

# MAGIC: minimize ligation-induced bias

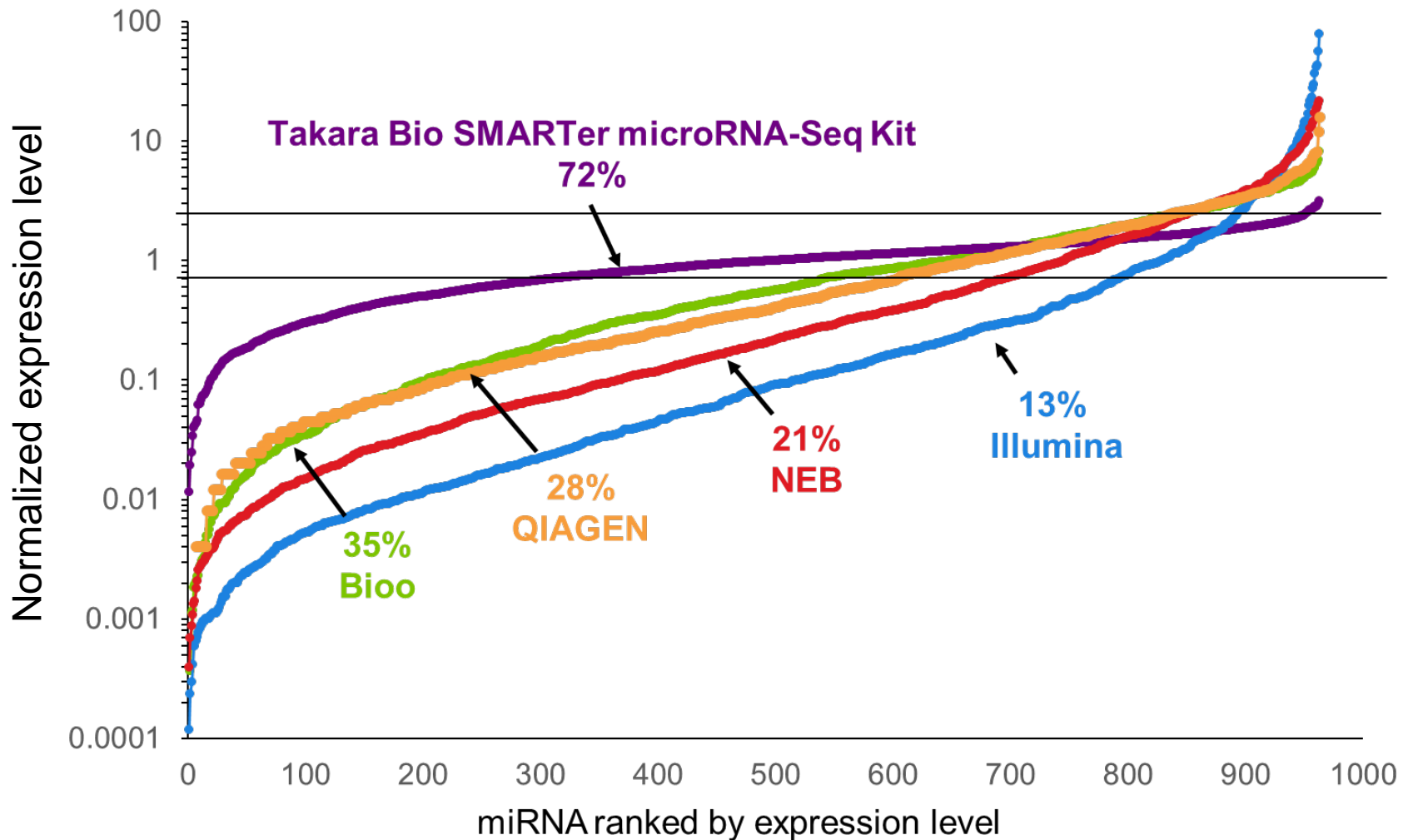
MAGIC technology:

## Mono-Adapter liGation and Intramolecular Circularization

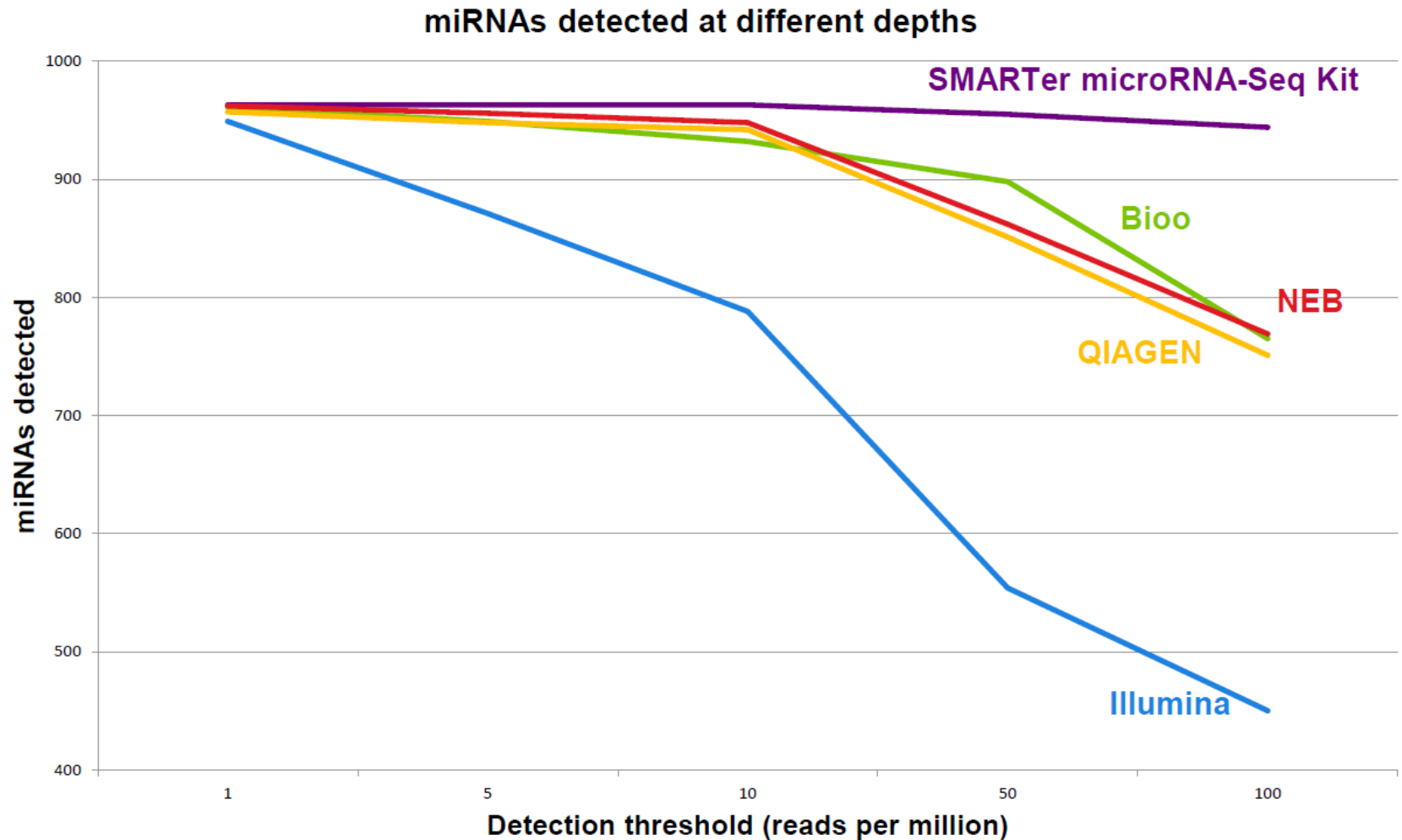


# SMARTer microRNA-Seq Kit

## Accurate expression profile of microRNAs



# SMARTer microRNA-Seq Kit Detects more microRNAs



# What else is new from Takara Bio?

- **SMARTer PicoPLEX Gold Single Cell DNA-seq Kit**—accurate and simultaneous detection of SNVs and CNVs from single cells
- **SMART-Seq® Stranded Kit**—random-primer-based single-cell RNA-seq kit for detecting coding and noncoding RNA and providing strand information of transcripts
- **SMARTer™ Apollo™ Library Prep System**—low to medium throughput benchtop automation system for NGS library preparation
- **SMARTer ICELL8® Single-Cell System**—open platform single-cell automation system for SMART-Seq library prep, TCR profiling, and more

# Thank you!

- Visit us at **Booth #300**
- Poster presentations
  - Confident detection of low-frequency mutations in cell-free DNA using SMARTer ThruPLEX technology with unique molecular tags
  - An unbiased and highly reproducible method for constructing microRNA NGS libraries for accurate expression profiling
  - A SMARTer solution to stranded single-cell RNA-seq
  - High-throughput single-cell transcriptomics with SMART-Seq technology





that's  
**GOOD**  
science!®

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