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Chemistry & Molecular Biology

early 2015

Requirements

- Reversible termination
- Differentiation of nucleotides
- Ability to manipulate on solid support

Preliminary studies

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A new class of cleavable fluorescent nucleotides: synthesis and optimization as reversible terminators for DNA sequencing by synthesis†

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ABSTRACT

Fluorescent 2′-deoxynucleotides containing a protecting group at the 3′-*O*-position are reversible terminators enabling array-based DNA sequencing by synthesis (SBS) approaches. Herein, we describe the synthesis of a new family of 3′-OH unprotected cleavable fluorescent 2′-deoxynucleotides and their evaluation as reversible terminators for high-throughput DNA SBS strategies. In this first version, all four modified nucleotides bearing a cleavable disulfide Alexa Fluor® 594 dye were assayed for their ability to act as a reversible stop for the incorporation of the next labeled base. Their use in SBS leaded to a signal–no signal output after successive addition of each labeled nucleotide during the sequencing process (binary read-out). Solid-phase immobilized synthetic DNA target sequences were used to optimize the method that has been applied to DNA polymerized colonies or clusters obtained by *in situ* solid-phase amplification of fragments of genomic DNA templates.

DNA molecules (3) and clusters or polymerized colonies (4–6) generated by solid-phase *in situ* amplification of DNA. In SBS methods, a primer hybridized to its target sequence is extended after nucleotide incorporation into the growing DNA strand using a DNA polymerase. The detection of the incorporated nucleotide immediately after each incorporation reaction allows the sequence assignment along the DNA synthesis process. Furthermore, the removal of the reporter signal, such as a fluorophore, after each base identification is essential to ensure that the residual fluorescence from the previous nucleotide incorporation does not affect the identification of the next incorporated fluorescent nucleotide.

In the design of fluorescently labeled reversible chain terminators for SBS, the linker used as a chemically cleavable moiety to attach the fluorophore to 2′-deoxynucleotides, must satisfy several requirements: (1) stability during the polymerase-mediated extension step, (2) its structure (geometry and size) and its location within the 2′-deoxynucleotide moiety must not prevent the recognition of the resulting labeled nucleotide by standard DNA polymerases, (3) cleavage under mild conditions compatible with the stability of DNA biopolymers (single and double strands) and the functionalized surface of DNA biochips, (4) easy synthetic access and high bioconjugation

Nucleic Acids Research, 2006, Vol. 34, No. 3 ***e22***
doi:10.1093/nar/gnj023

BTA, a novel reagent for DNA attachment on glass and efficient generation of solid-phase amplified DNA colonies

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ABSTRACT

The tricarboxylate reagent benzene-1,3,5-triacetic acid (BTA) was used to attach 5′-aminated DNA primers and templates on an aminosilanized glass surface for subsequent generation of DNA colonies by *in situ* solid-phase amplification. We have characterized the derivatized surfaces for the chemical attachment of oligonucleotides and evaluate the properties relevant for the amplification process: surface density, thermal stability towards thermocycling, functionalization reproducibility and storage stability. The derivatization process, first developed for glass slides, was then adapted to microfabricated glass channels containing integrated fluidic connections. This implementation resulted in an important reduction of reaction times, consumption of reagents and process automation. Innovative analytical methods for the characterization of attached DNA were developed for assessing the surface immobilized DNA content after amplification. The results obtained showed that the BTA chemistry is compatible and suitable for forming highly dense arrays of DNA colonies with optimal surface coverage

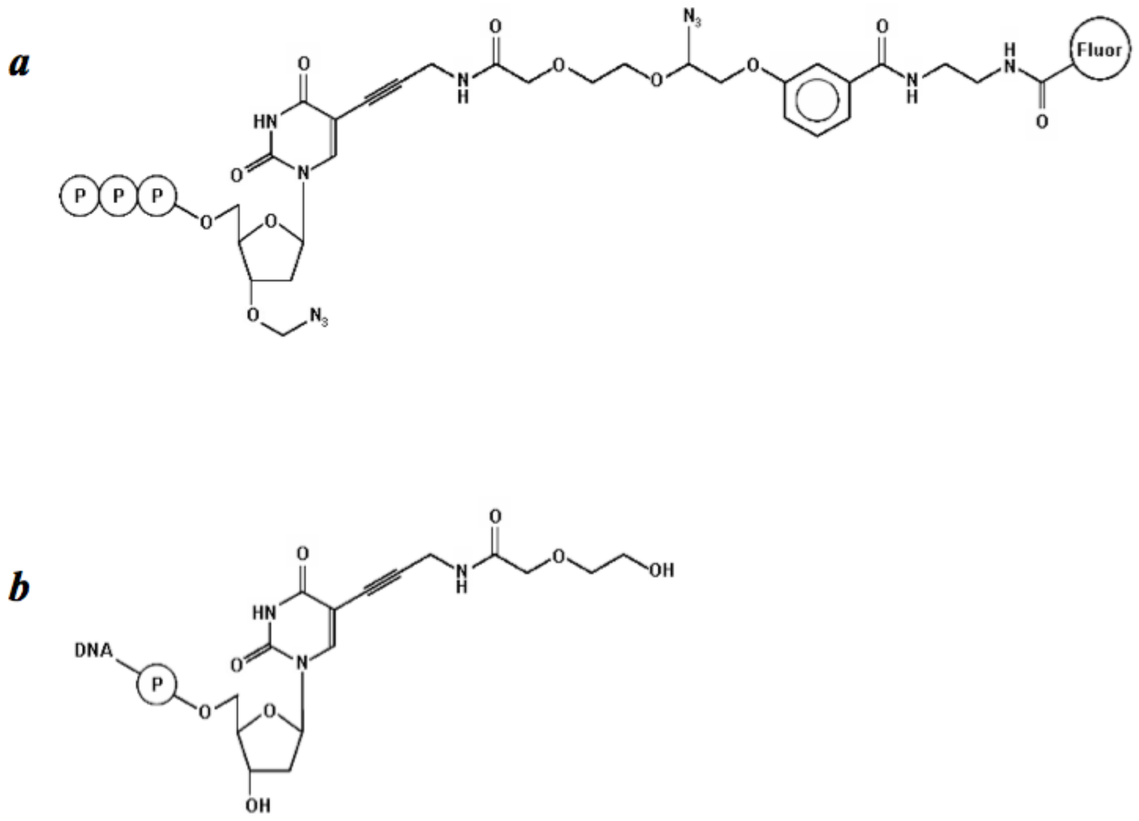
INTRODUCTION

The goal of a cost-effective approach to whole-genome resequencing is the impetus for current research efforts that are focused on the development of novel, highly efficient DNA sequencing methods (1). Next-generation technologies for low-cost DNA sequencing will be widely applicable, and will have a strong impact on biomedical research. An important example is the sequencing of individual genomes as a component of predictive and preventive medicine, and for hypothesis testing toward the discovery of genotype–phenotype associations (2–6).

A series of massively parallel DNA sequencing methods have been developed toward the goal of ultra low-cost sequencing (7–11). One of the most promising techniques make use of parallel sequencing through the synthesis of very dense DNA colony arrays, generated by solid-phase amplification of surface-attached single-template molecules (12–14).

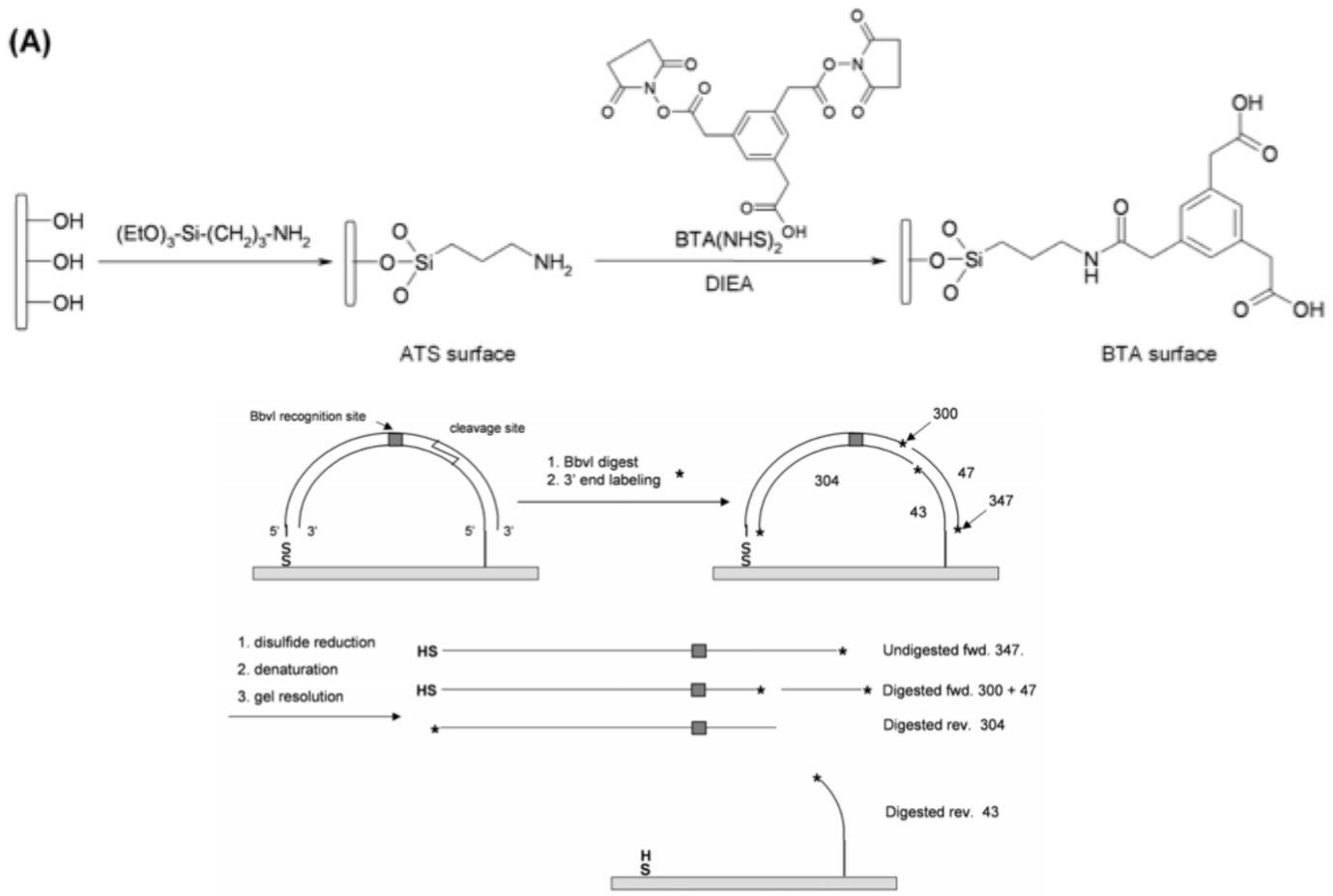
A suitable approach for performing the *in situ* amplification of target DNA templates (generation of DNA colonies) consists of the initial attachment of amplification primers by 5′ termini, which allows the free 3′ ends to prime DNA synthesis through DNA templates that hybridize to the surface-bound primers. With this method, DNA can be amplified by two mechanisms: (i) interfacial amplification (priming step) followed by surface amplification (12), or (ii) amplification of primers and target templates after simultaneous attachment

Nucleotides



Bentley et al. 2008

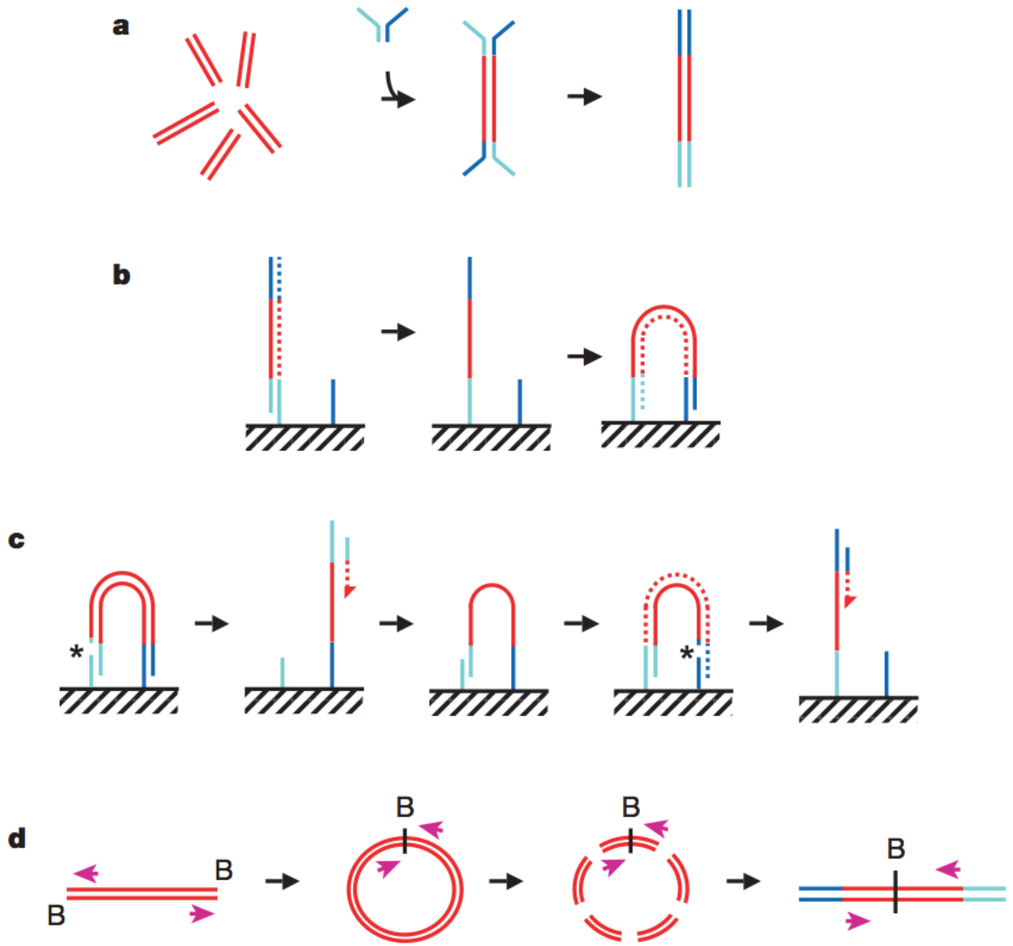
Solid support



Fedurco et al. 2006

How it works

The overall idea

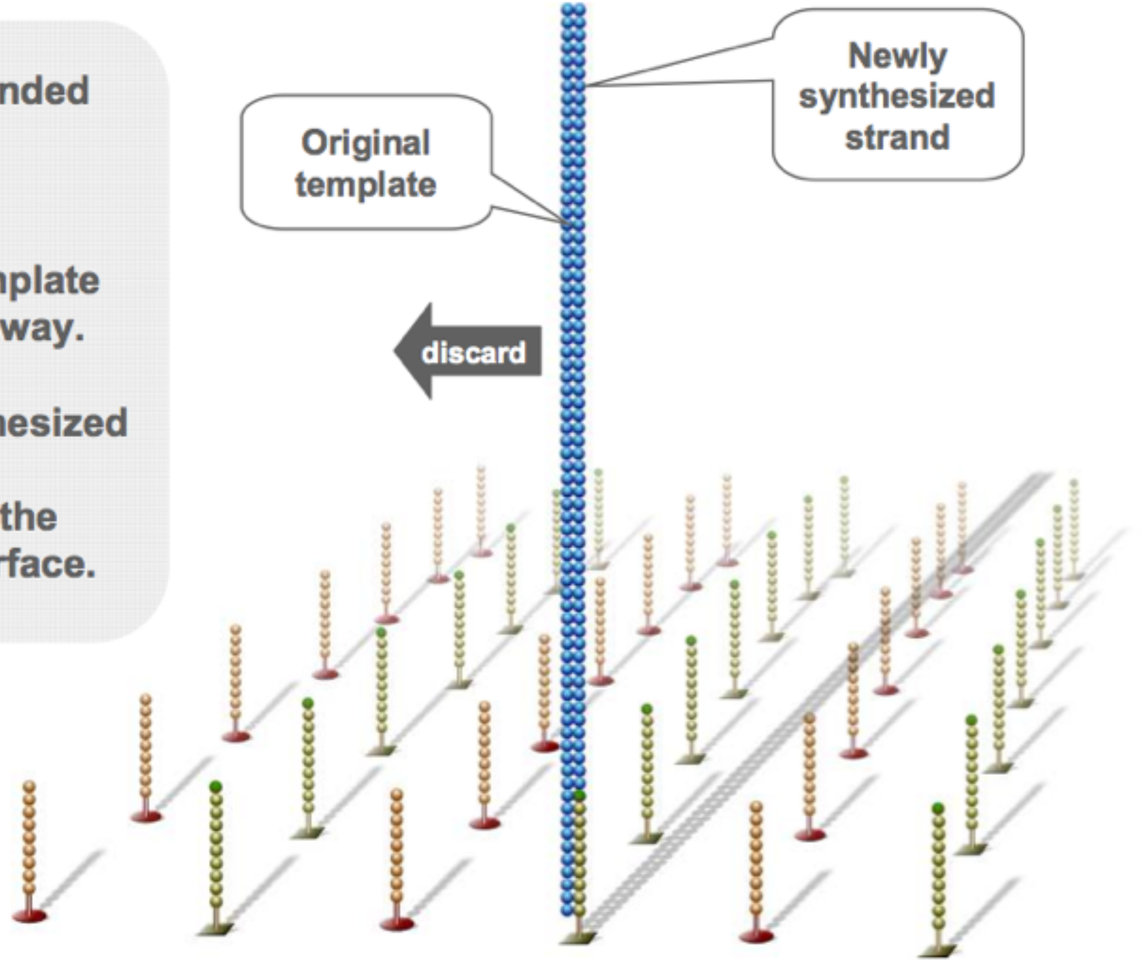


Cluster generation:
Denature double-stranded DNA

Double-stranded molecule is denatured.

Original template is washed away.

Newly synthesized covalently attached to the flow cell surface.



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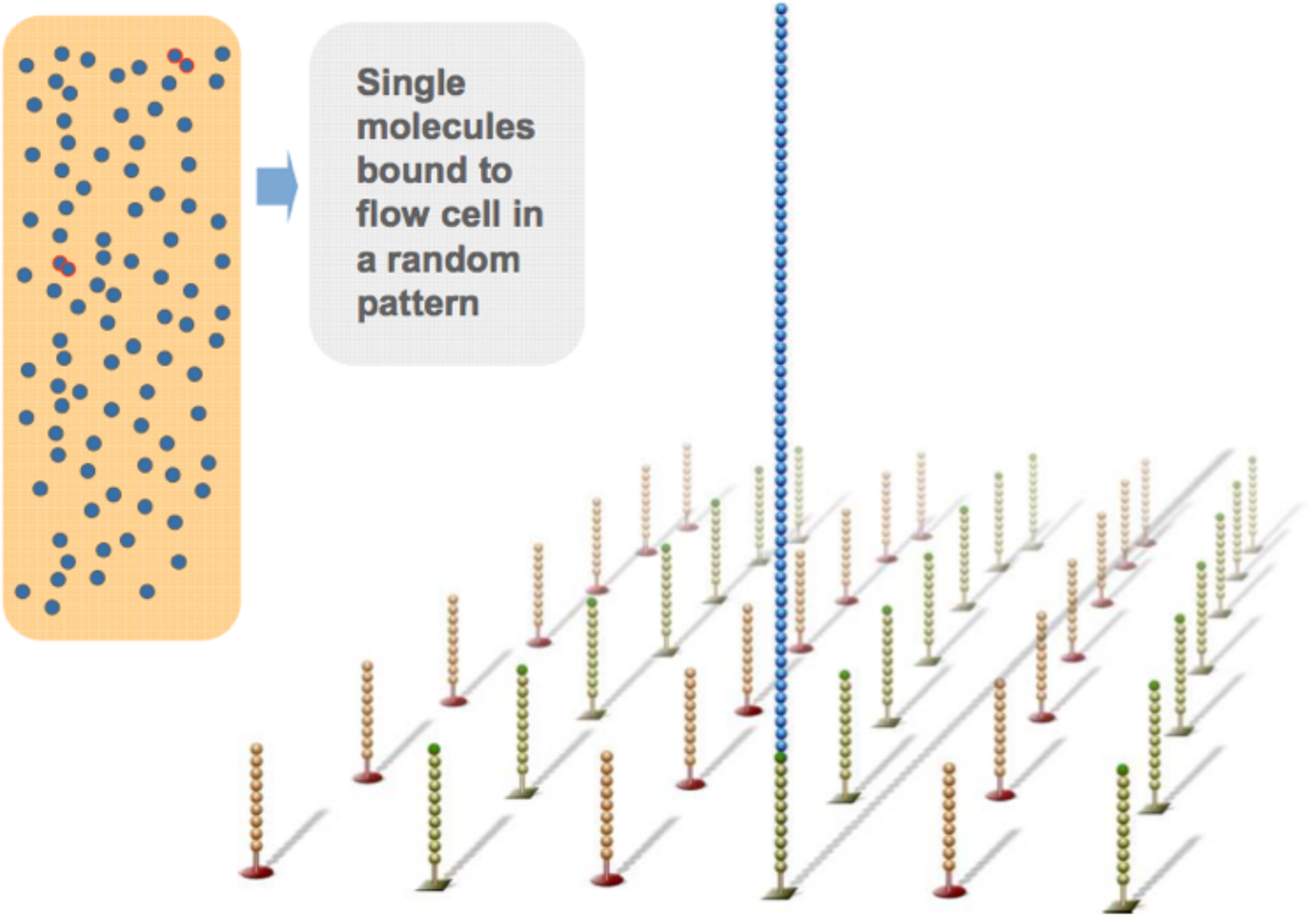
Cluster generation: Covalently bound spatially separated single molecules



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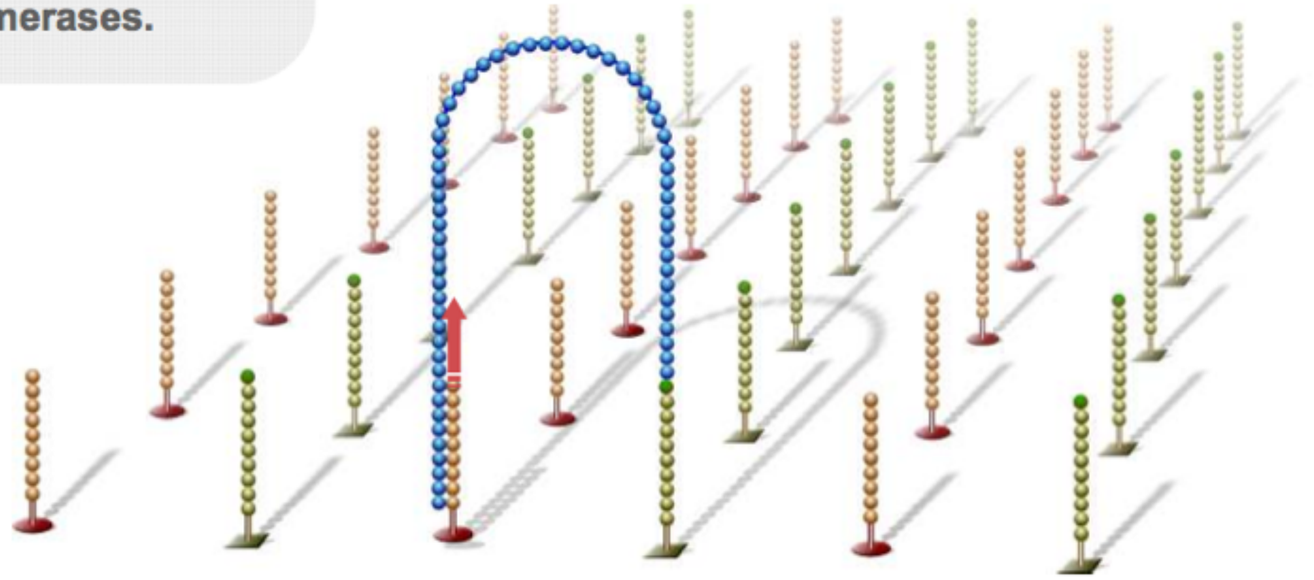
Cluster generation: Bridge amplification

Single-strand flips over to hybridize to adjacent primers to form a bridge.

Hybridized primer is extended by polymerases.



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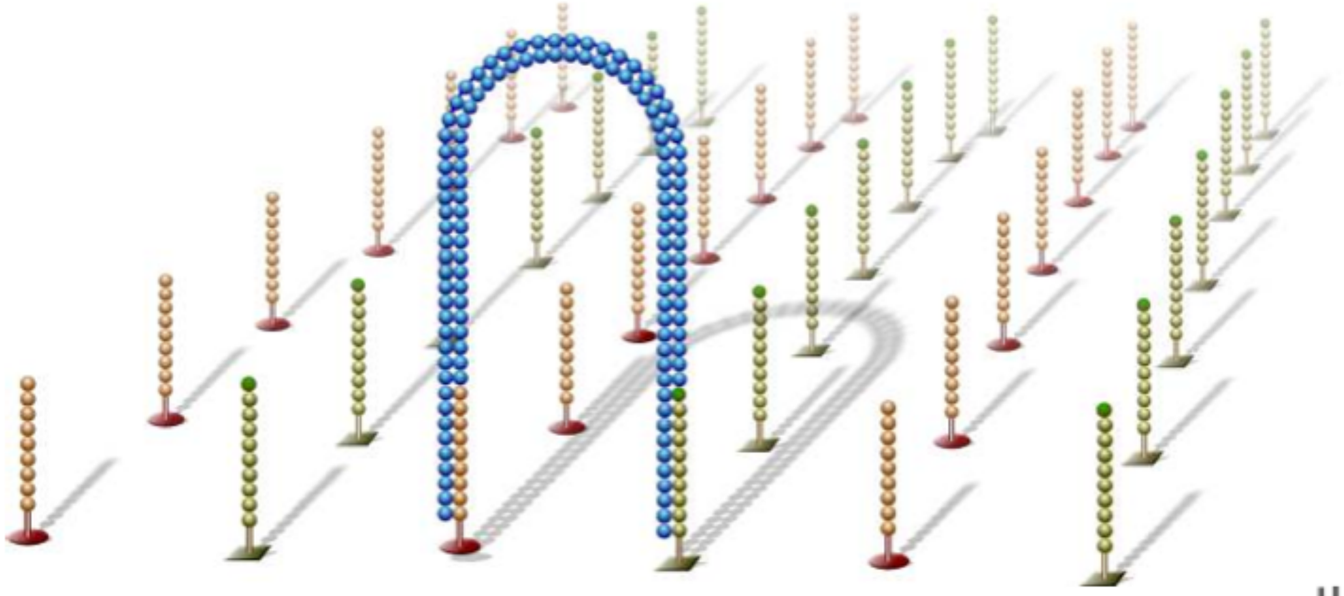
Cluster generation: Bridge amplification

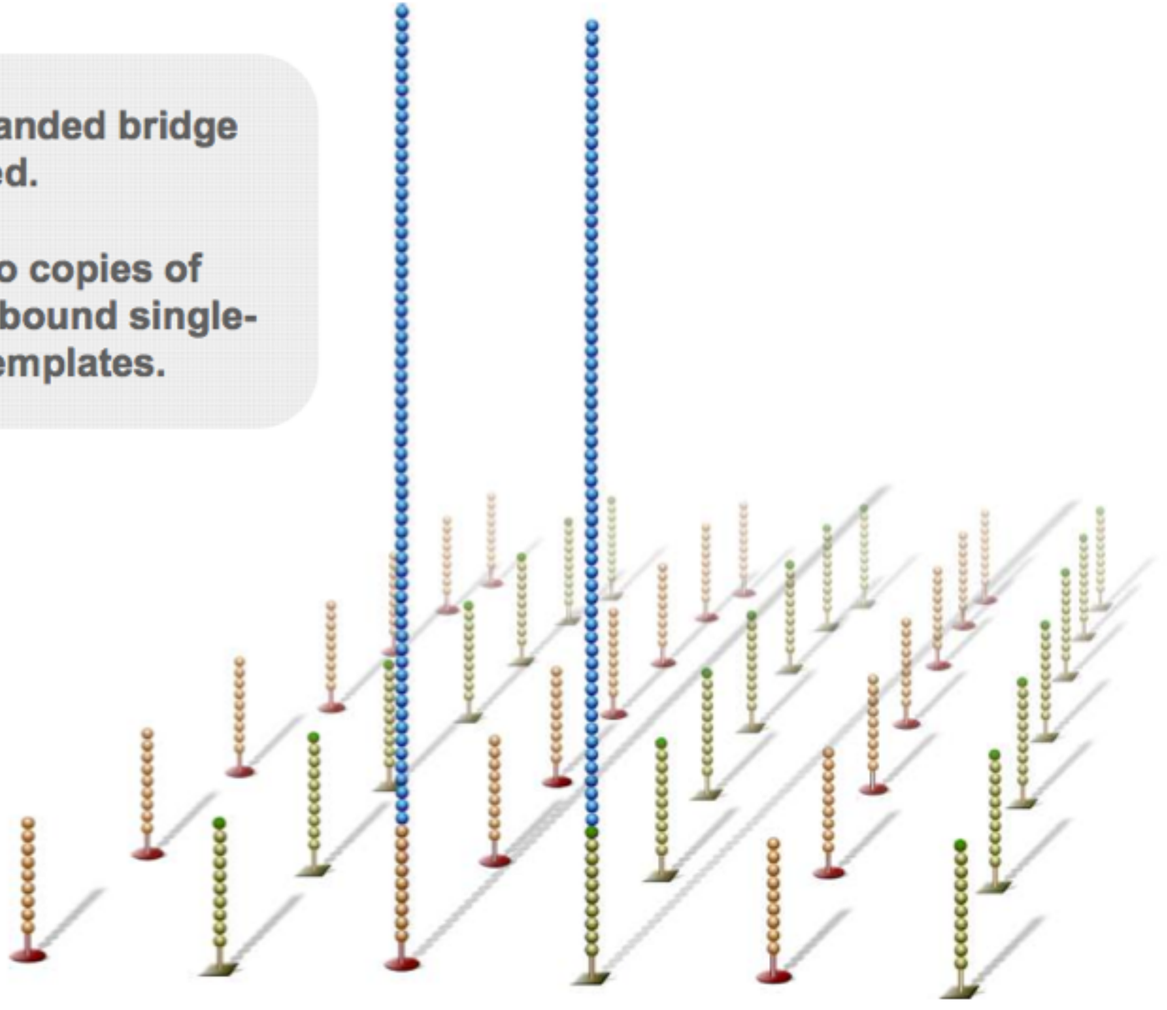
→ double-stranded bridge is formed.



Cluster generation: Bridge amplification

Double-stranded bridge is denatured.

Result: Two copies of covalently bound single-stranded templates.



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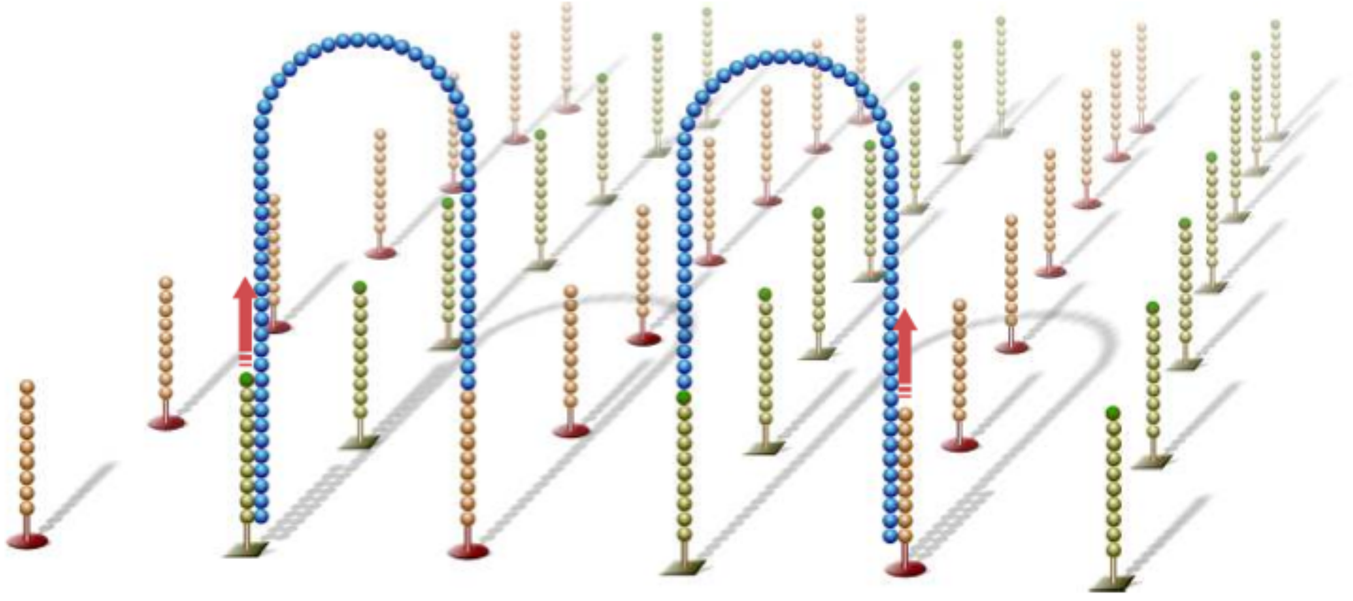
Cluster generation: Bridge amplification

Single-strands flip over to hybridize to adjacent primers to form bridges.

Hybridized primer is extended by polymerase.



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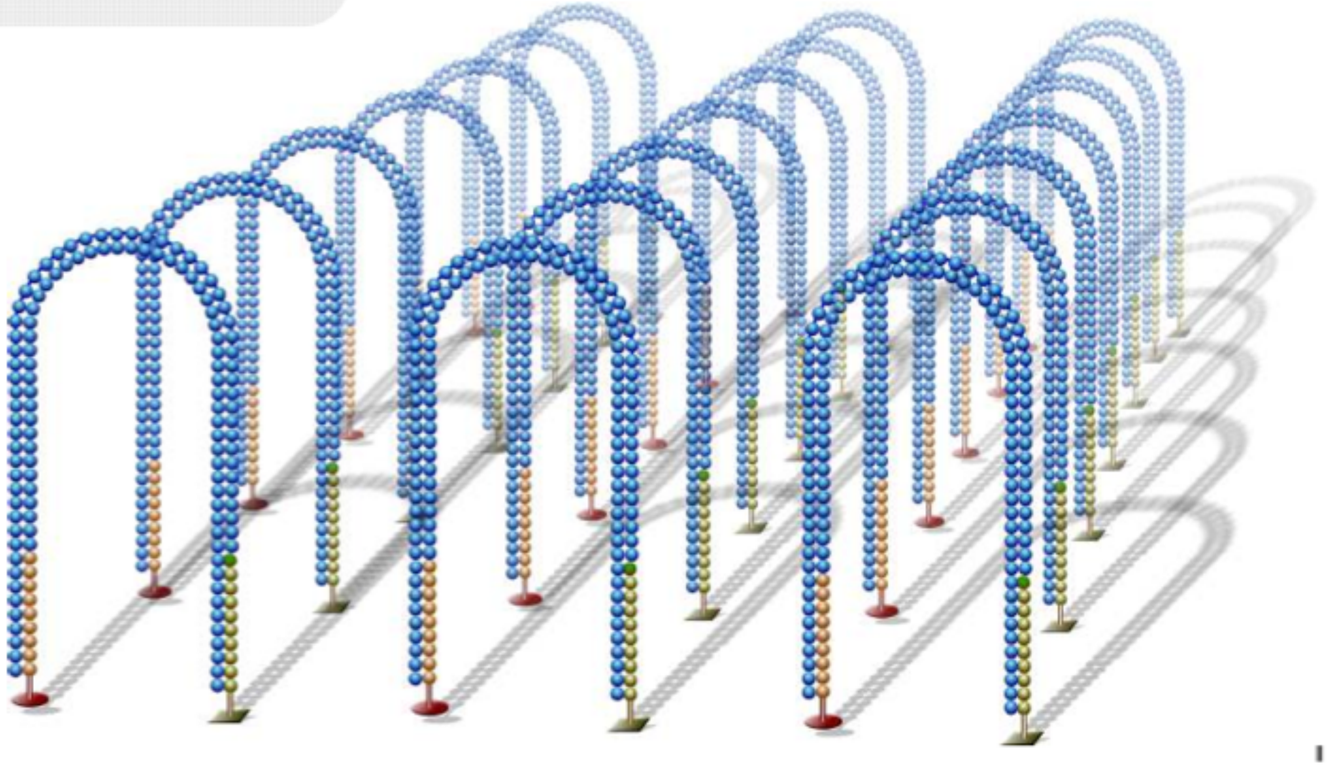
Cluster generation: Bridge amplification

Bridge amplification cycle repeated till multiple bridges are formed



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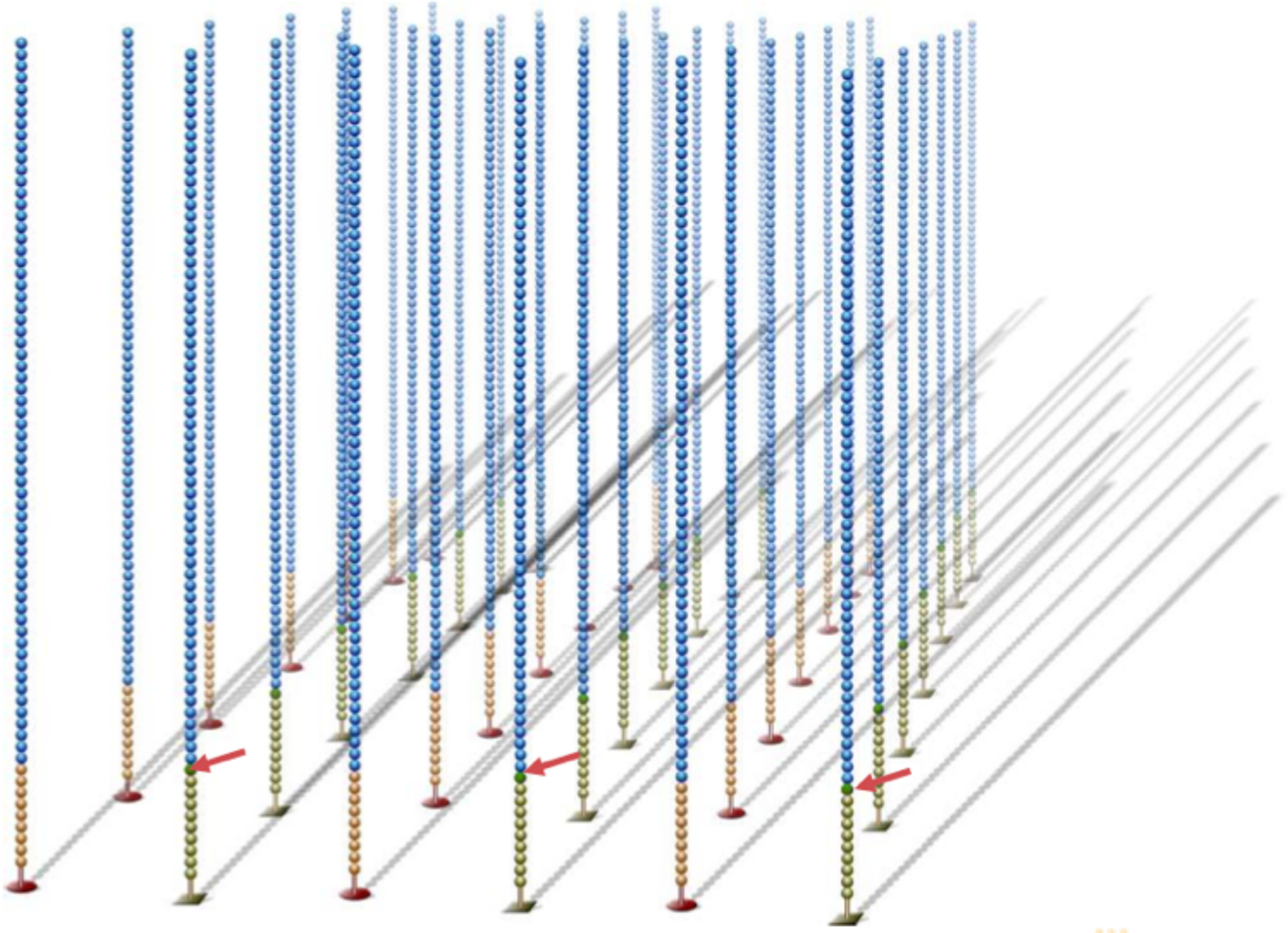
Cluster generation

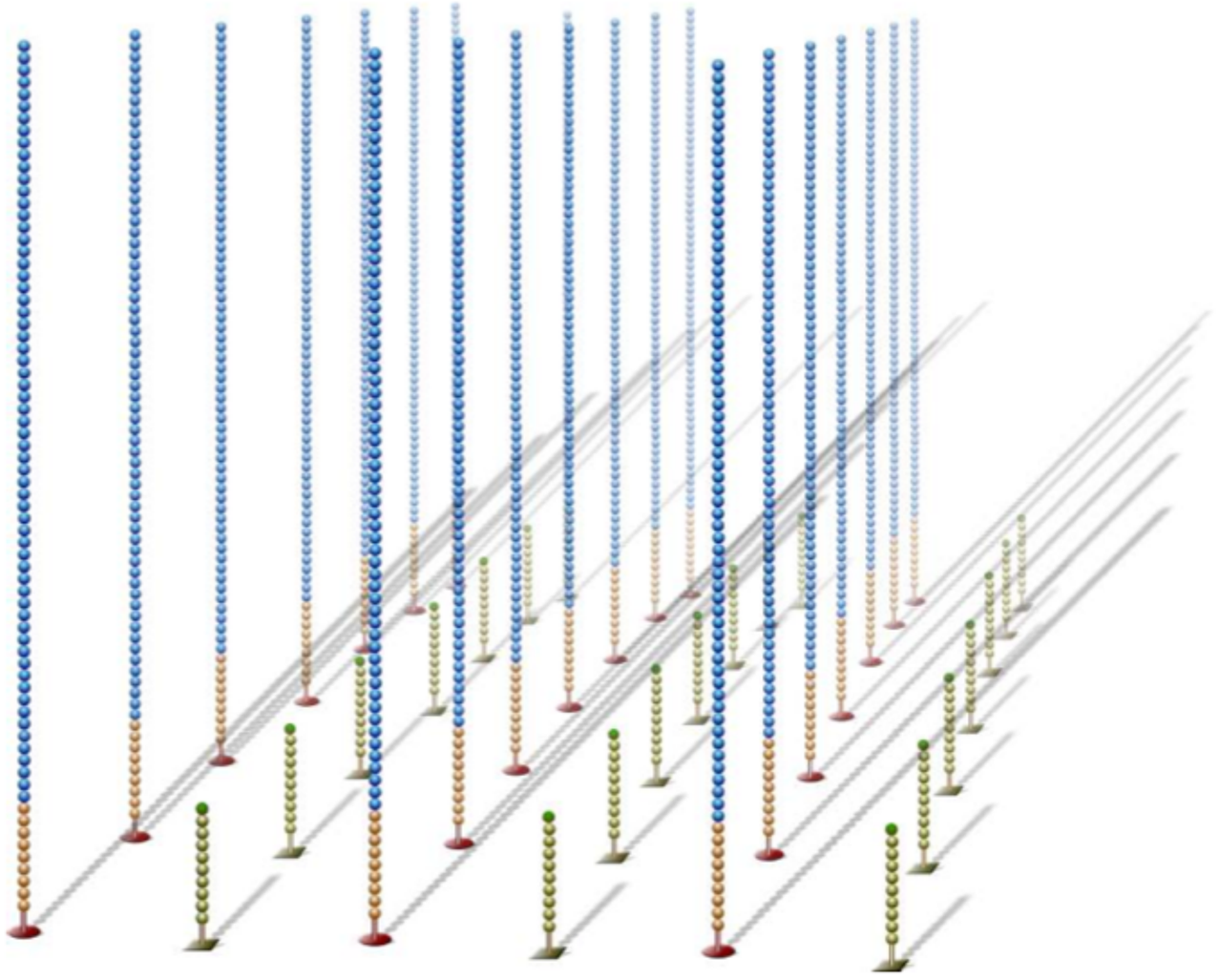
dsDNA bridges denatured.

Reverse strands cleaved and washed away.



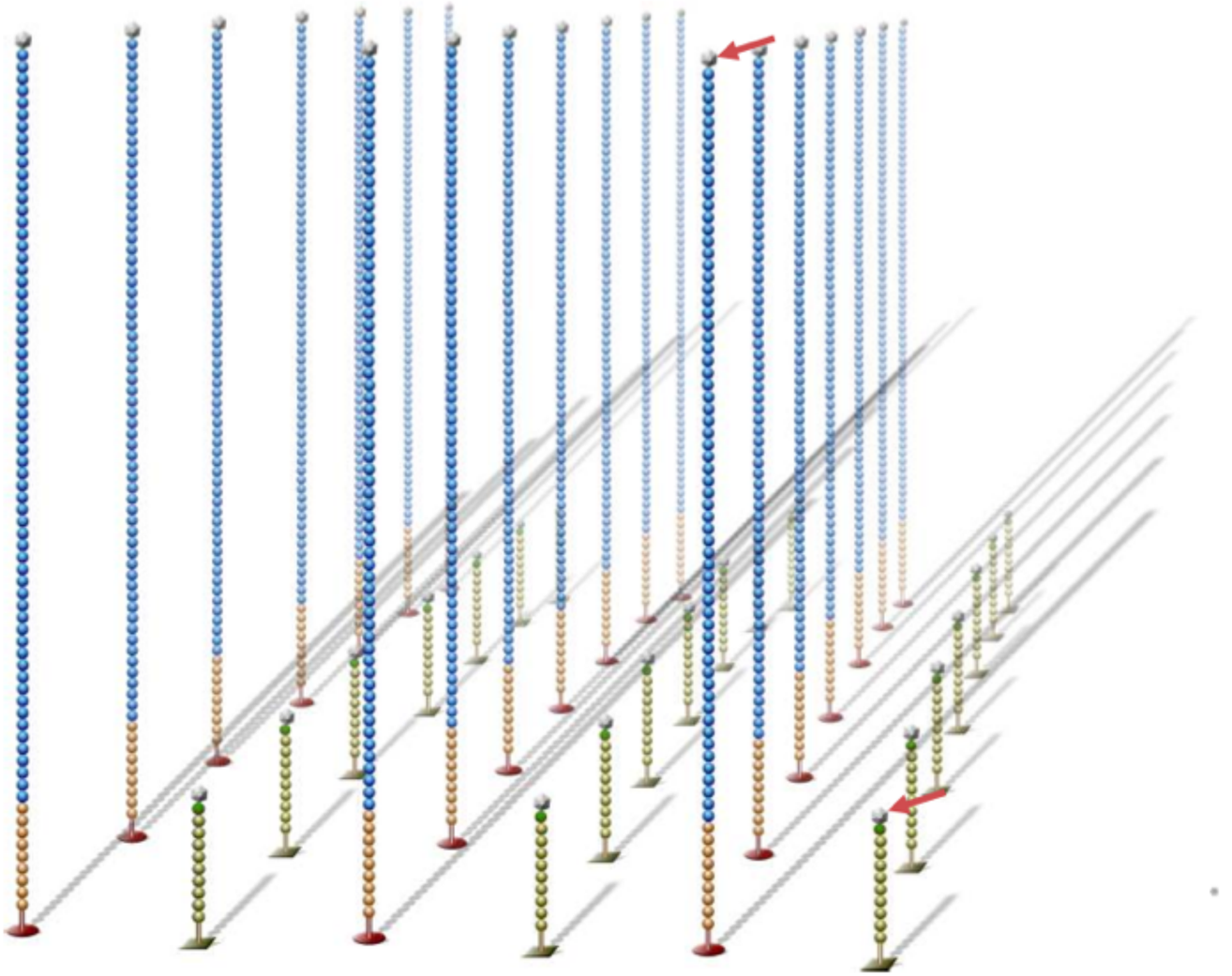
Cluster generation

… leaving a cluster with forward strands only.



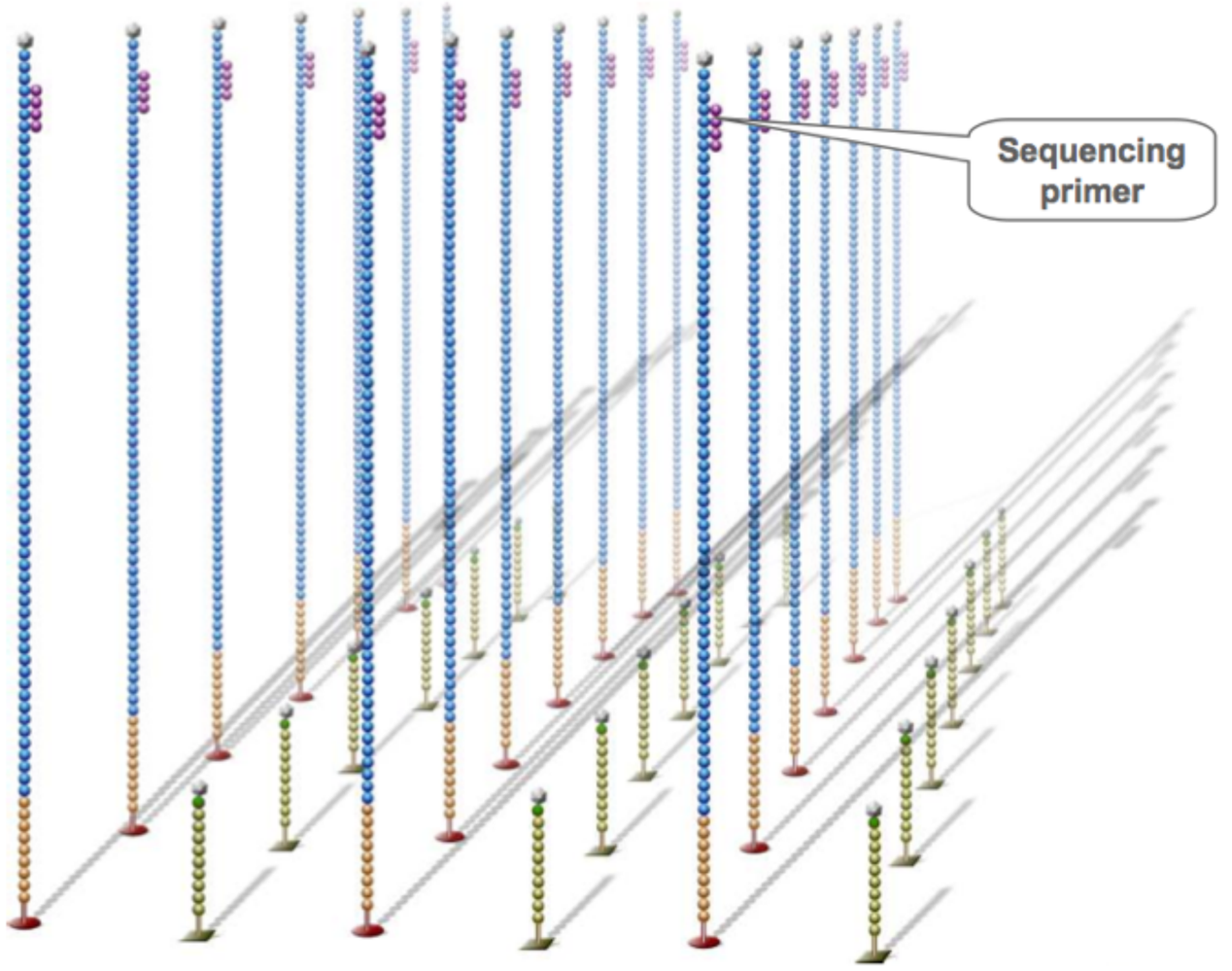
Cluster generation

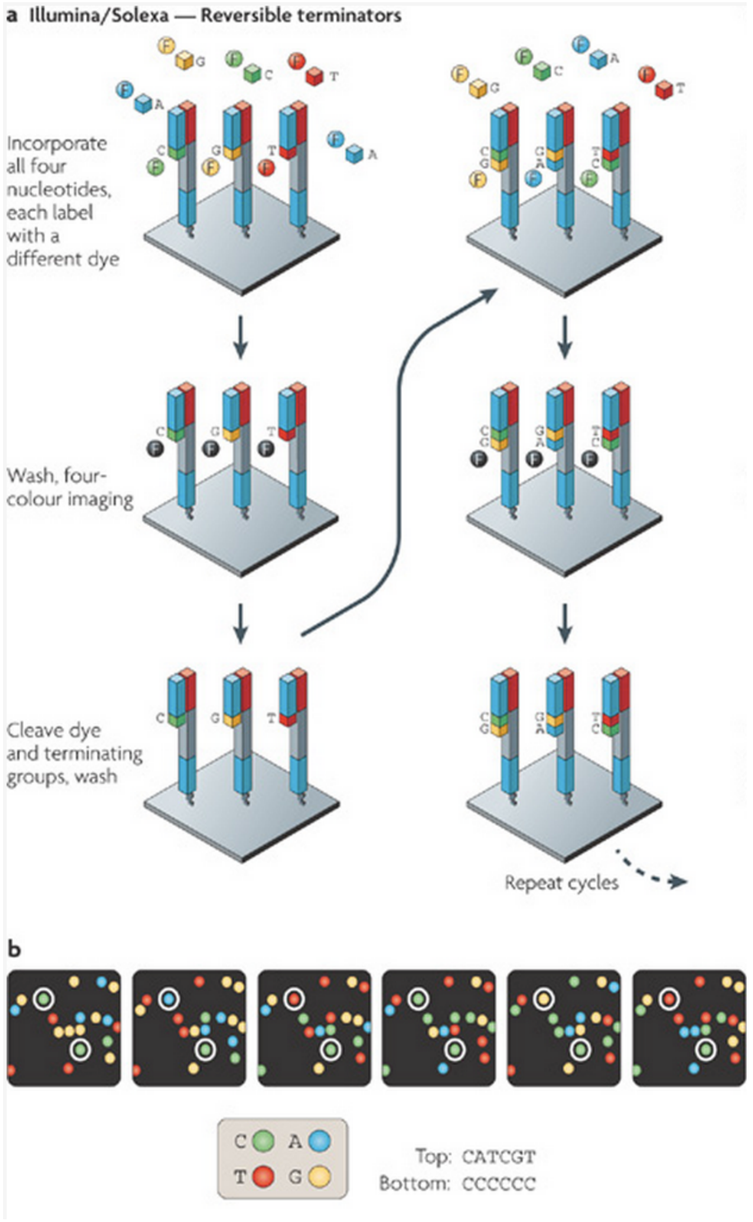
Free 3’ ends are blocked to prevent unwanted DNA priming.



Sequencing

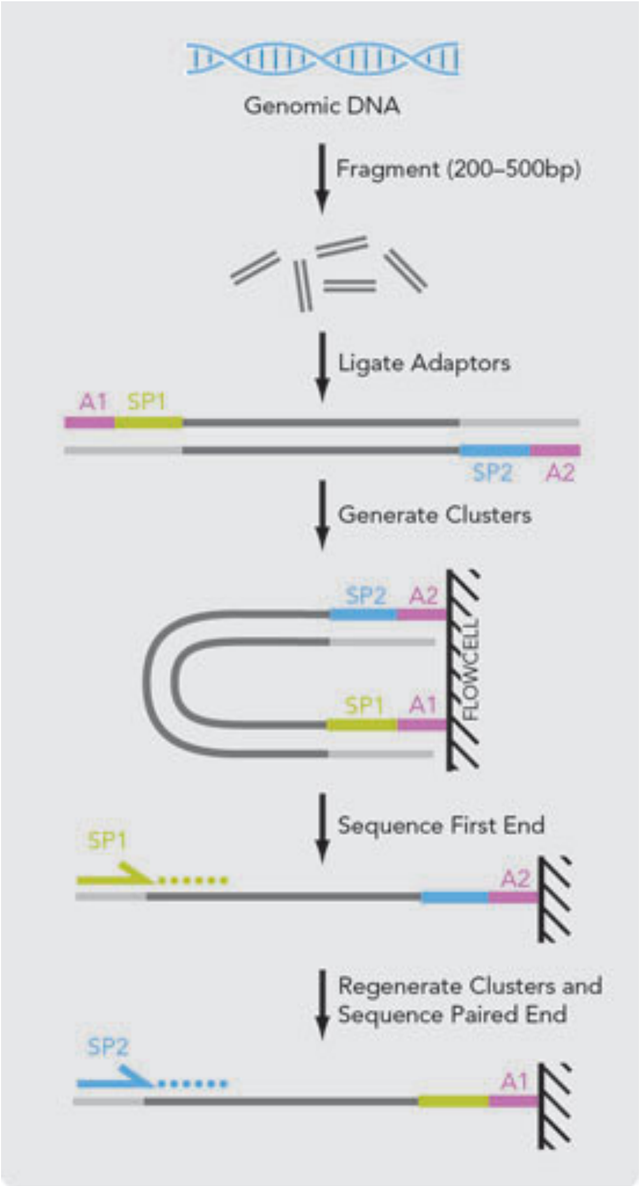
Sequencing primer is hybridized to adapter sequence.





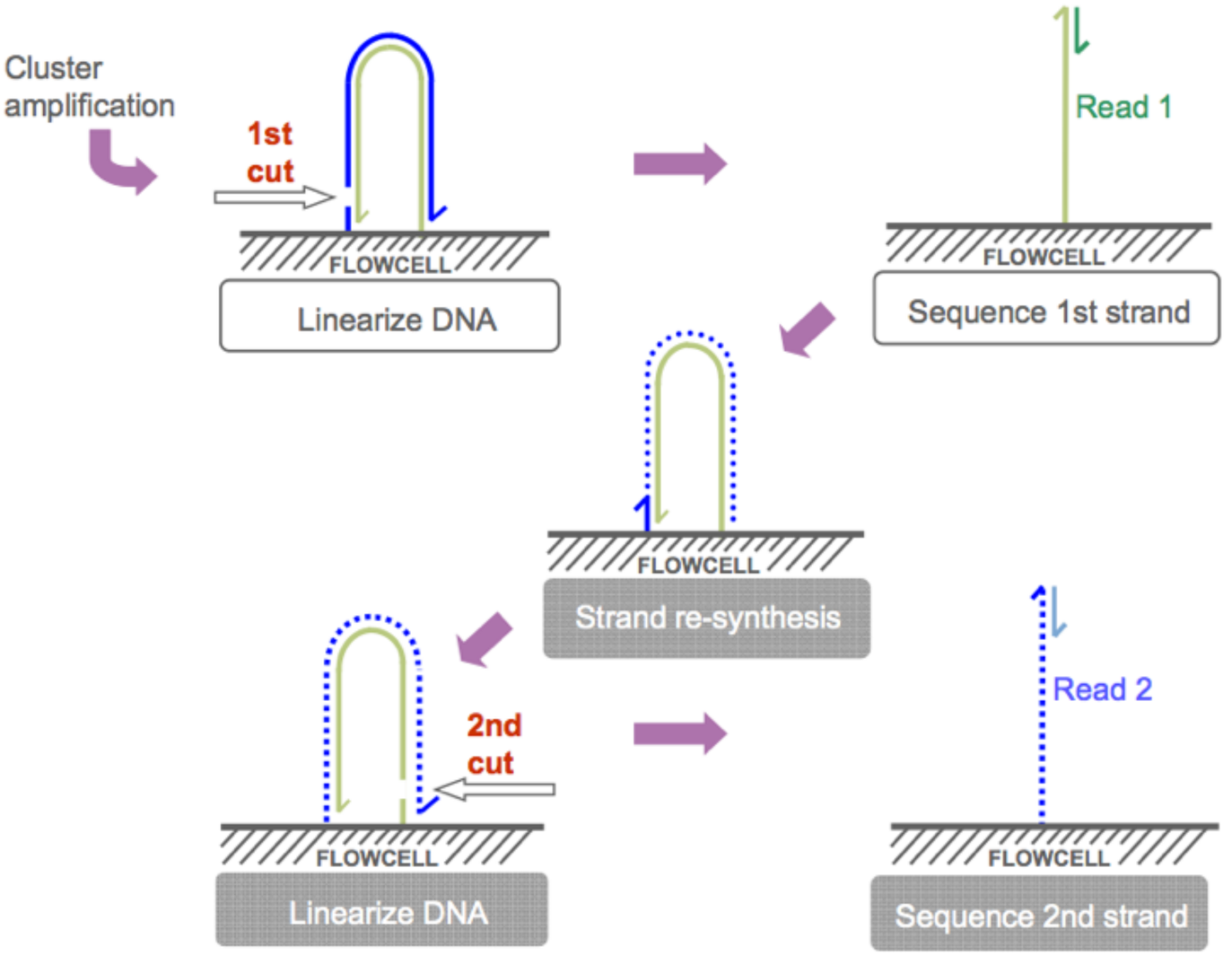
Metzker et al. 2010

Extending to paired ends



Paired-End Sequencing

Paired 36-50+ bp Reads Extends the Power of the Technology



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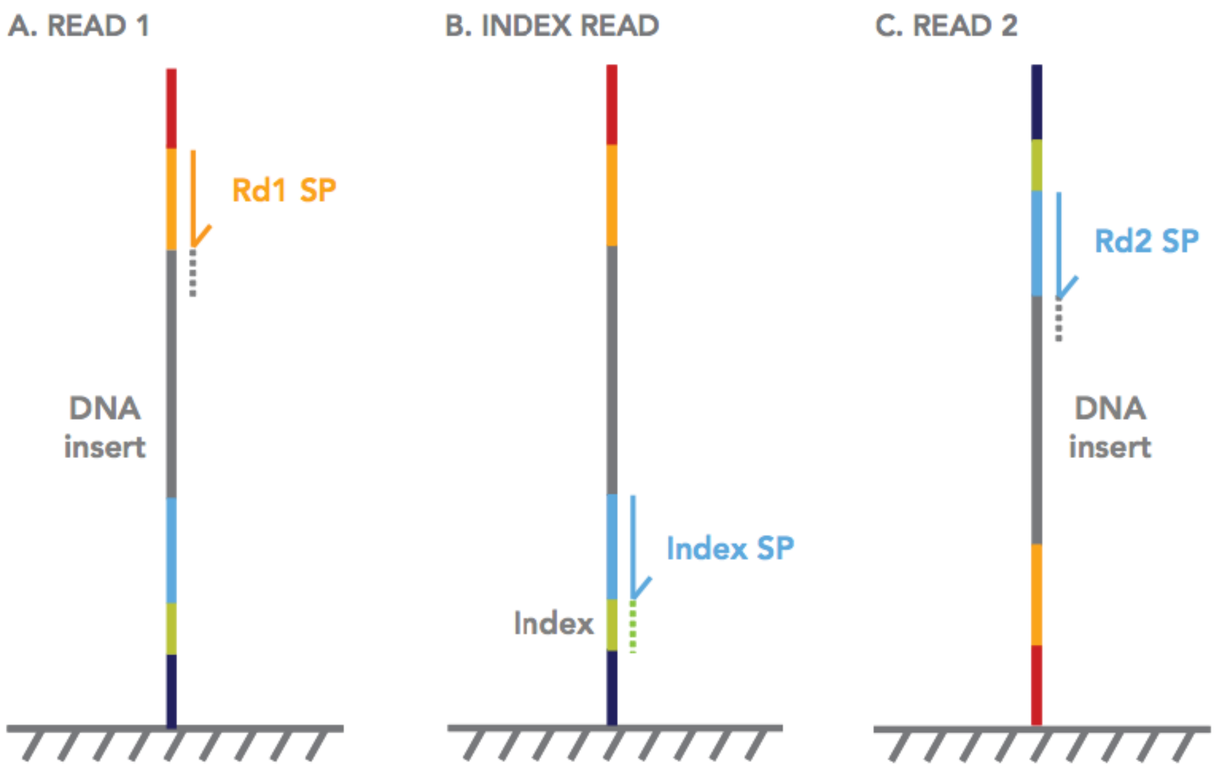
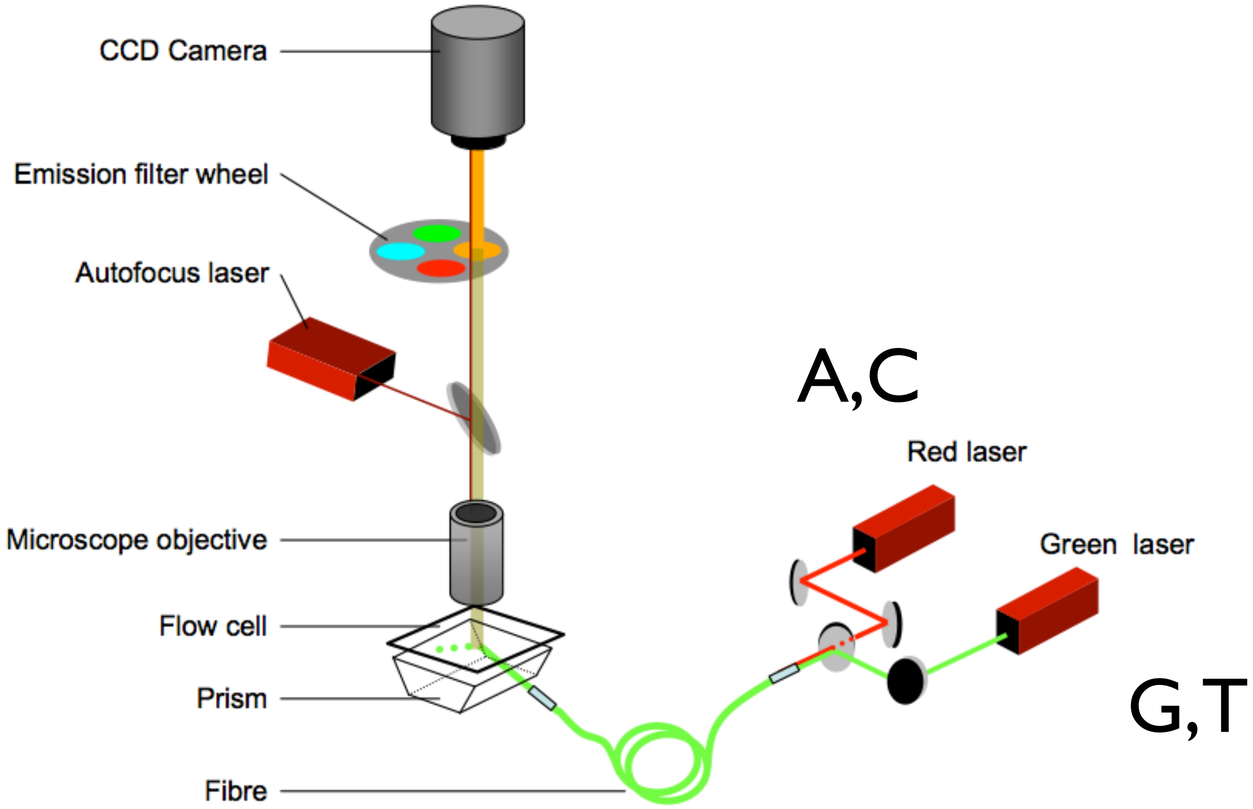
Multiplexing

FIGURE 1: MULTIPLEXED SEQUENCING PROCESS



Sample multiplexing involves a total of three sequencing reads, including a separate index read, which is generated automatically on the Genome Analyzer equipped with the Paired-End Module. A: Application read 1 (dotted line) is generated using the Read 1 Sequencing Primer (Rd1 SP). B: The read 1 product is removed and the Index Sequencing Primer (Index SP) is annealed to the same strand to produce the 6-bp index read (dotted line). C: If a paired-end read is required, the original template strand is used to regenerate the complementary strand. Then, the original strand is removed and the complementary strand acts as a template for application read 2 (dotted line), primed by the Read 2 Sequencing Primer (Rd2 SP). Pipeline Analysis software identifies the index sequence from each cluster so that the application reads can be assigned to a single sample. Hatch marks represent the flow cell surface.

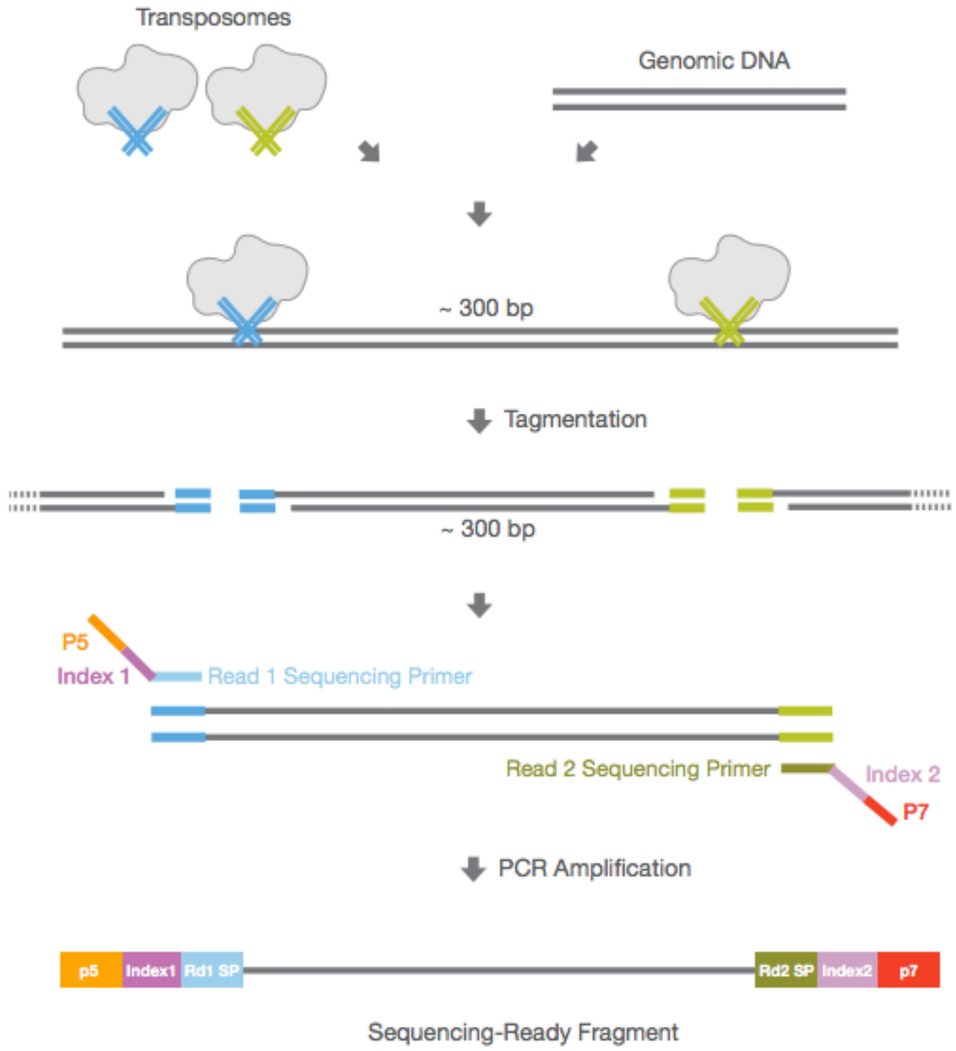
Detection system



Library prep strategies

- Nextera
- True-seq
- Moleculo

Figure 2: Nextera Library Preparation Biochemistry



Nextera chemistry simultaneously fragments and tags DNA in a single step. A simple PCR amplification then appends sequencing adapters and sample indexes to each fragment.

Table 1: TruSeq DNA Sample Preparation Kits

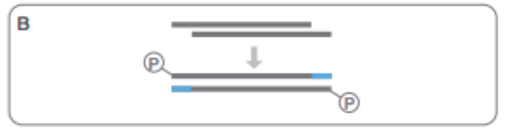
Specification	TruSeq Nano DNA	TruSeq DNA PCR-Free	TruSeq DNA
Description	Based upon widely adopted TruSeq sample prep, with lower input and improved data quality	Superior genomic coverage with radically reduced library bias and gaps	Original TruSeq next-generation sequencing sample preparation method
Input quantity	100–200 ng	1–2 µg	1 µg
Includes PCR	Yes	No	Yes
Assay time	~6 hours	~5 hours	1–2 days
Hands-on time	~5 hours	~4 hours	~8 hours
Target insert size	350 bp or 550 bp	350 bp or 550 bp	300 bp
Gel-Free	Yes	Yes	No
Number of samples supported	24 (LT) or 96 (HT) samples	24 (LT) or 96 (HT) samples	48 (LT) or 96 (HT) samples
Supports enrichment	No*	No*	Yes
Size-selection beads	Included	Included	Not included
Applications	Whole-genome sequencing applications, including whole-genome resequencing, *de novo* assembly, and metagenomics studies		
Sample multiplexing	24 single indices or 96 dual-index combinations		
Compatible Illumina sequencers	HiSeq®, HiScanSQ™, Genome Analyzer™, and MiSeq® systems		

*Nextera Rapid Capture products support a variety of enrichment applications. For more information, visit www.illumina.com/NRC.

True seq PCR free

Figure 2: Adapter Ligation Results in Sequence-Ready Constructs without PCR

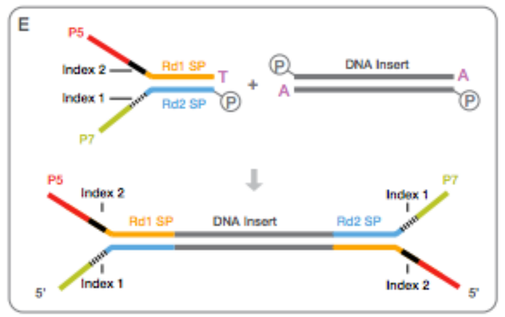
Library construction begins with genomic DNA that is subsequently fragmented.



Blunt-end fragments are created.

Fragments are narrowly size selected with sample purification beads.

A-base is added.

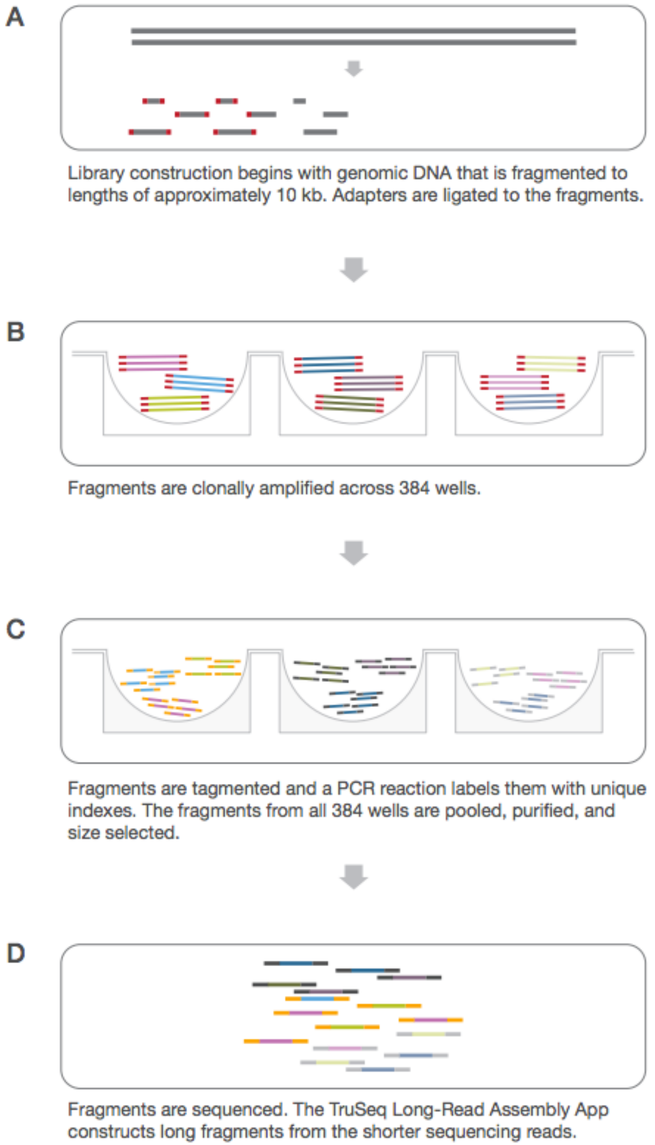


Dual-index adapters are ligated to the fragments* and final product is ready for cluster generation.

*The TruSeq DNA PCR-Free LT indexing solution features a single-index adapter at this step.

Moleculo

Figure 2: TruSeq Synthetic Long-Read DNA Library Preparation Workflow



The TruSeq Synthetic Long-Read DNA Library Prep Kit prepares DNA for sequencing. The TruSeq Long-Read Assembly App assembles the sequencing reads into long fragments.