Herpetology Evolution

Integrative Biology

Introduction to short read NGS:

Library construction, UCE capture and ddRADseq

The Natural History Museum, London

Autumn 2021

Instructor: Jeff Streicher

j.streicher@nhm.ac.uk

Litoria iris, Papua New Guinea

Unit 4: Double digest restriction-site associated DNA sequencing (ddRADseq)

GitHub https://github.com/nhm-herpetology/museum-NGS-training

Unit 3 Review

Lecture

- Reduced representation genome sequencing
- Targeted sequence capture and UCEs

Bioinformatics Lab

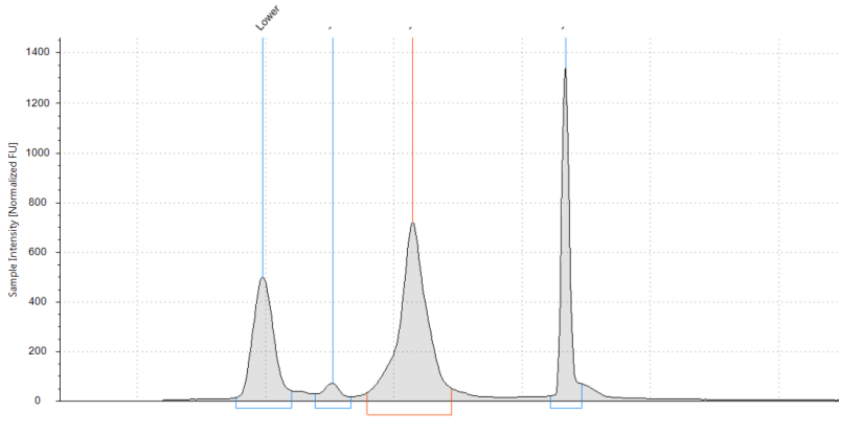
- How to download and process UCE data

Molecular Lab

- Targeted sequence capture of UCEs from shotgun libraries made in Unit 2

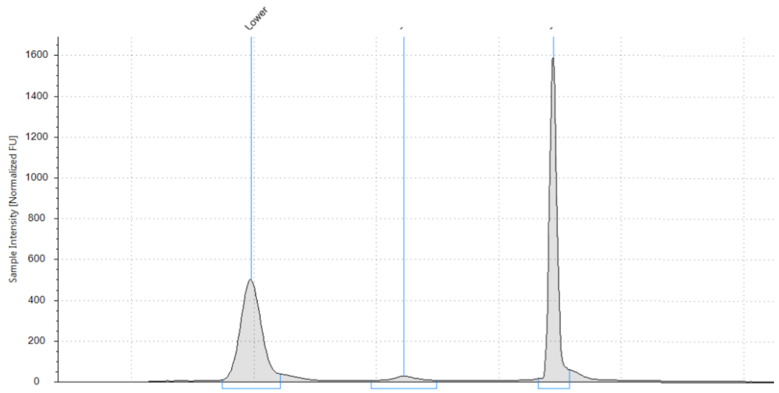
Tapestation results

Peak: B1: NGS_library_15_cycle



Unit 2

Peak: B1: Seq_cap-lib_squamates



Unit 3

Unit 4 Overview

Lecture

- Restriction Enzymes
- Restriction-site associated DNA sequencing (RADseq)
- ddRADseq

Bioinformatics Lab

- How to process ddRADseq data

Molecular Lab

- Restriction digestion and adapter design (Tomorrow)
- PCR and TapeStation (Monday)

Reduced-representation NGS sequencing

- Genomes can be large!
- We might want to compare multiple individuals/species
- Targeted Sequence Capture (TSC) [Unit 3]
- Restriction site associated DNA sequencing (RADseq)

Restriction endonucleases

Restriction enzymes cleave DNA into fragments at or near specific recognition sites within molecules known as restriction sites. These enzymes are found in bacteria and archaea and provide a defense mechanism against invading viruses.

Text mostly from Wikipedia ☺

EcoR1

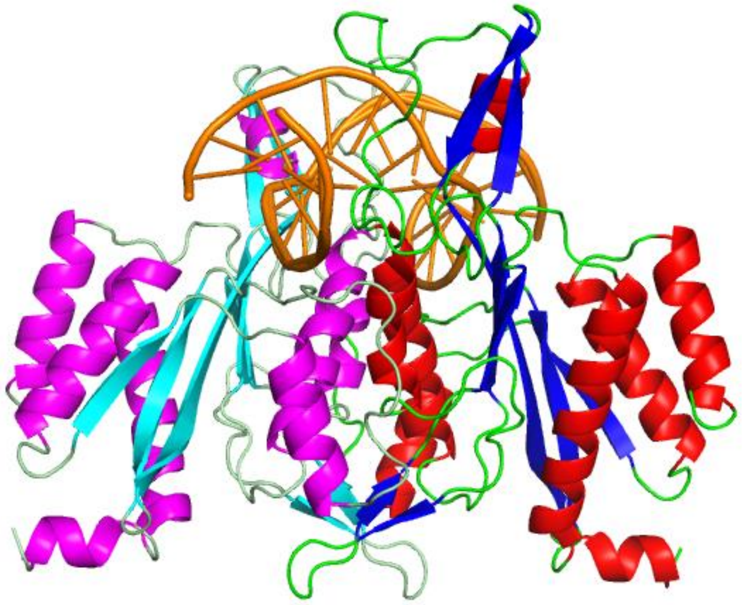


Image by A2-33 with CCL

Type II restriction enzymes

They form homodimers, with recognition sites that are usually undivided and palindromic and 4–8 nucleotides in length. They recognize and cleave DNA at the same site throughout the genome and can either cleave at the center of both strands to yield a **blunt end**, or at a staggered position leaving overhangs called **sticky (protruding) ends**.

Text mostly from Wikipedia ☺

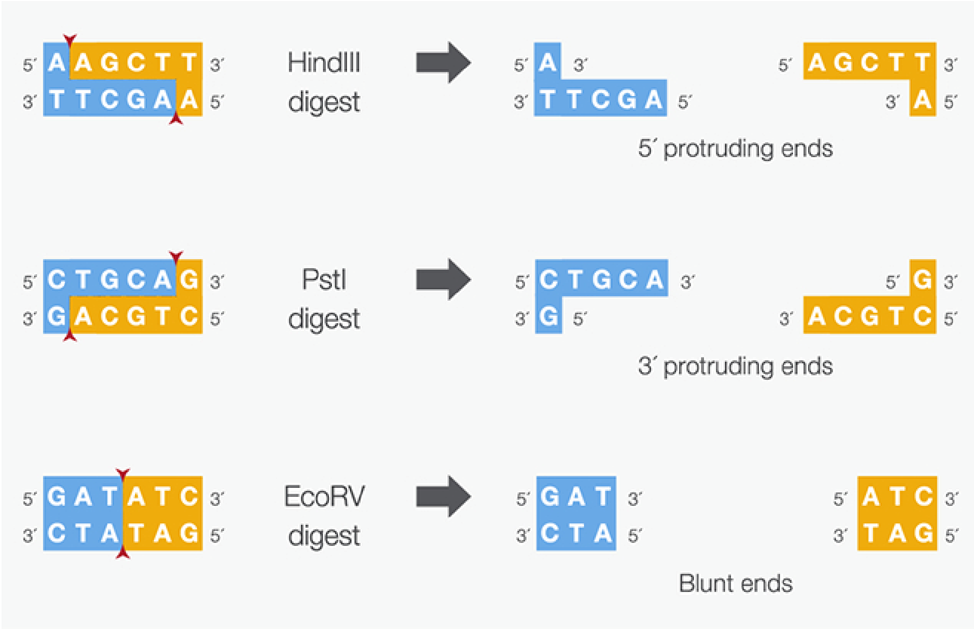
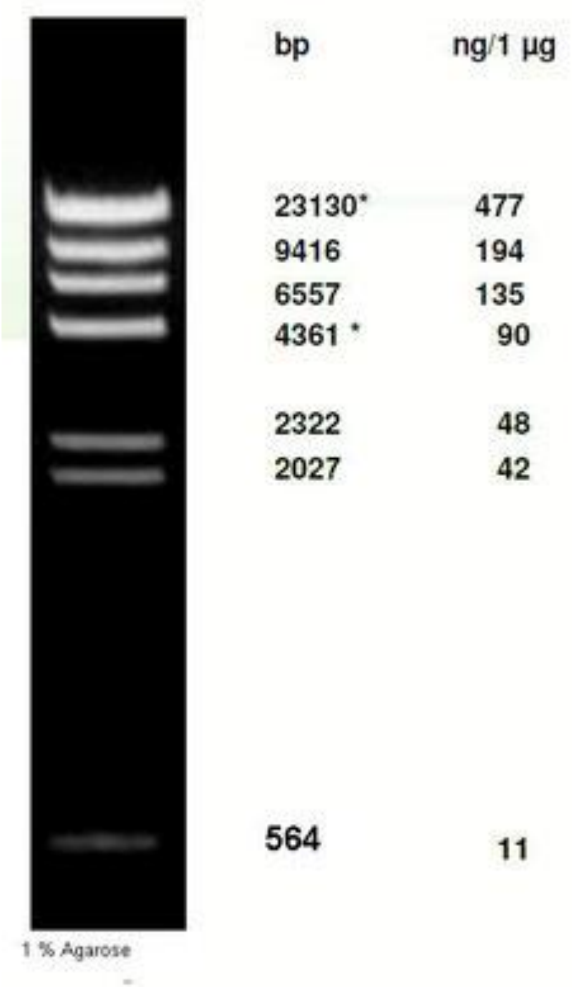


Image by Thermo Fisher Scientific

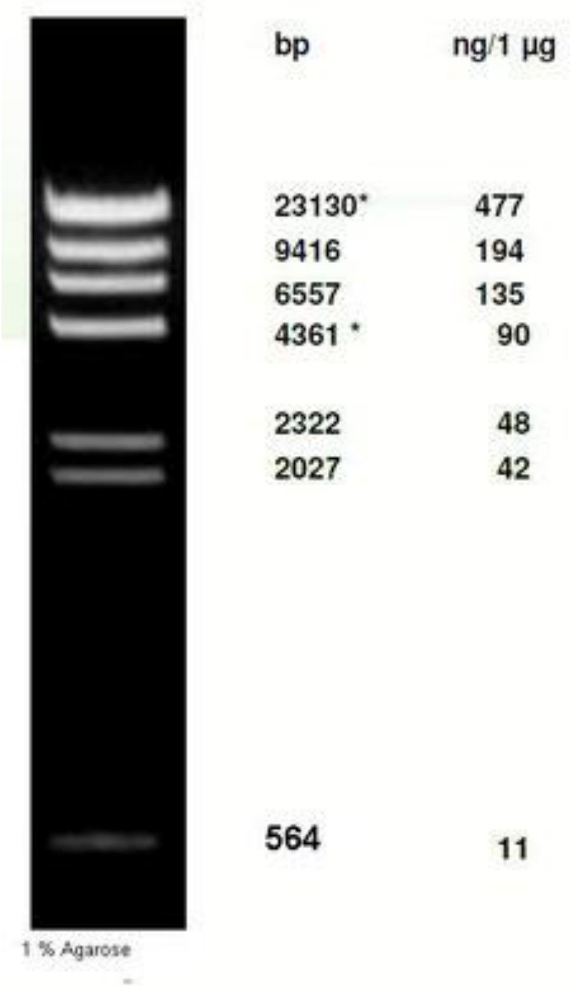
Restriction digestion

- DNA Ladders (size standards)
- Restriction fragment length polymorphism (RFLP)
- Amplified fragment length polymorphism (AFLP)



bp	ng/1 µg
23130*	477
9416	194
6557	135
4361 *	90
2322	48
2027	42
564	11

DNA Ladder (Lambda phage genome) cut with Hind III restriction enzyme



bp	ng/1 µg
23130*	477
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2322	48
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Hind III Recognition Site

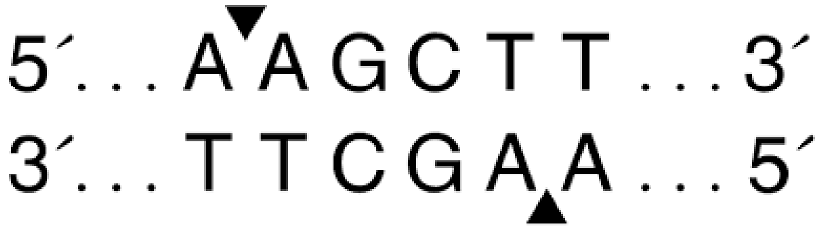


Image by New England BioLabs

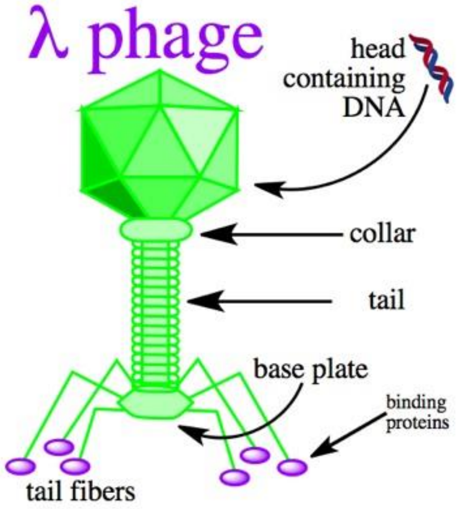
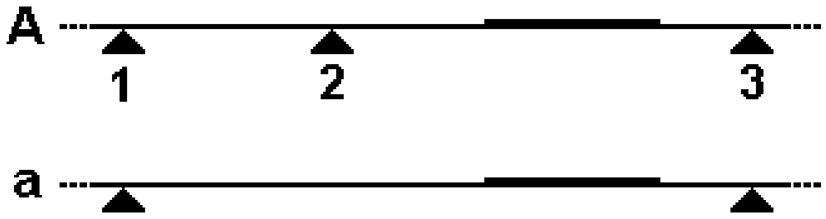


Image by Lizanne Koch/Public Domain

DNA Ladder (Lambda phage genome) cut with Hind III restriction enzyme

Restriction fragment length polymorphism (RFLP)

DNA in a diploid organism



- DNA is digested using a restriction enzyme.
- DNA fragments produced by the digest are then separated by length through agarose gel electrophoresis and transferred to a membrane via the Southern blot procedure.
- Hybridization of the membrane to a labeled DNA probe then determines the length of the fragments which are complementary to the probe.

Text mostly from Wikipedia ☺

Restriction fragment length polymorphism (RFLP)

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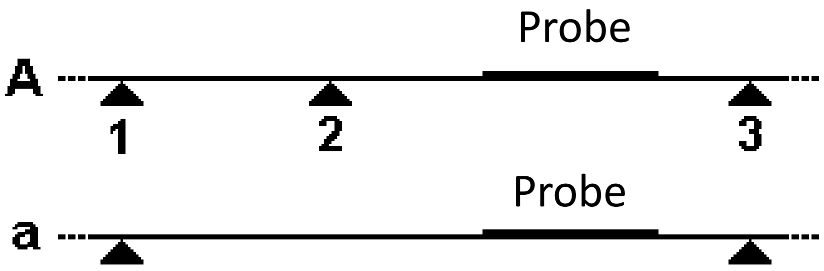
DNA in a diploid organism



Probe

Probe

Arrows = Recognition sites

Restriction fragment length polymorphism (RFLP)

- DNA is digested using a restriction enzyme.
- DNA fragments produced by the digest are then separated by length through agarose gel electrophoresis and transferred to a membrane via the Southern blot procedure.
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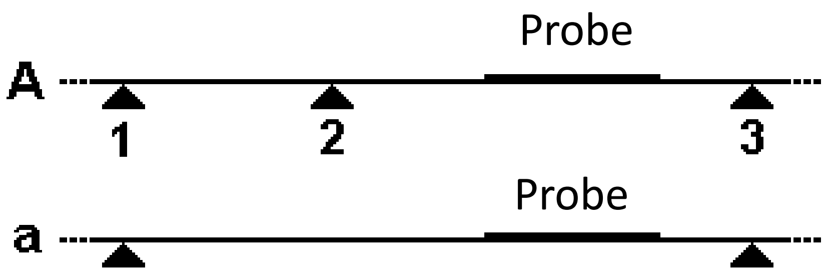
DNA in a diploid organism



Arrows = Recognition sites

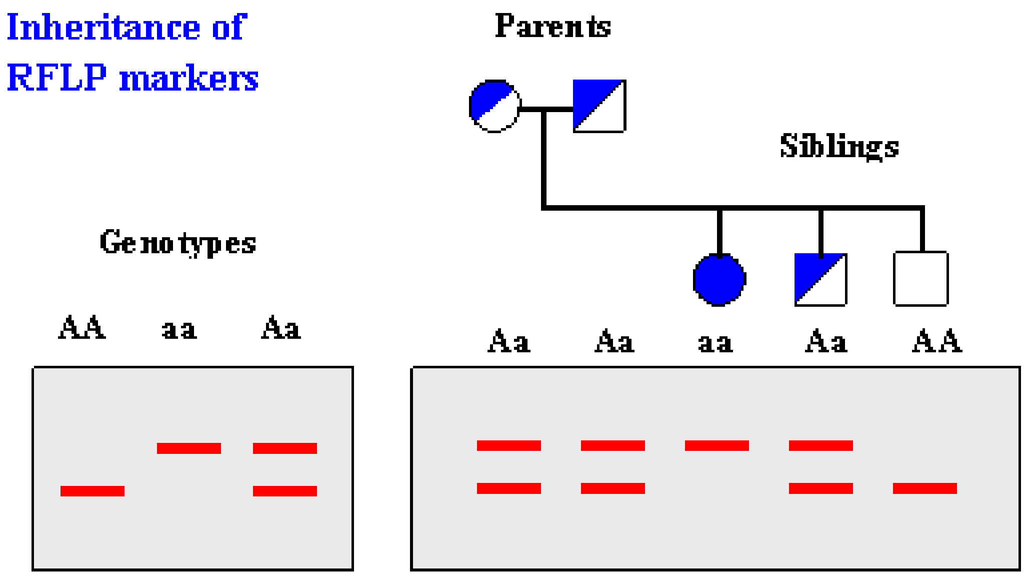
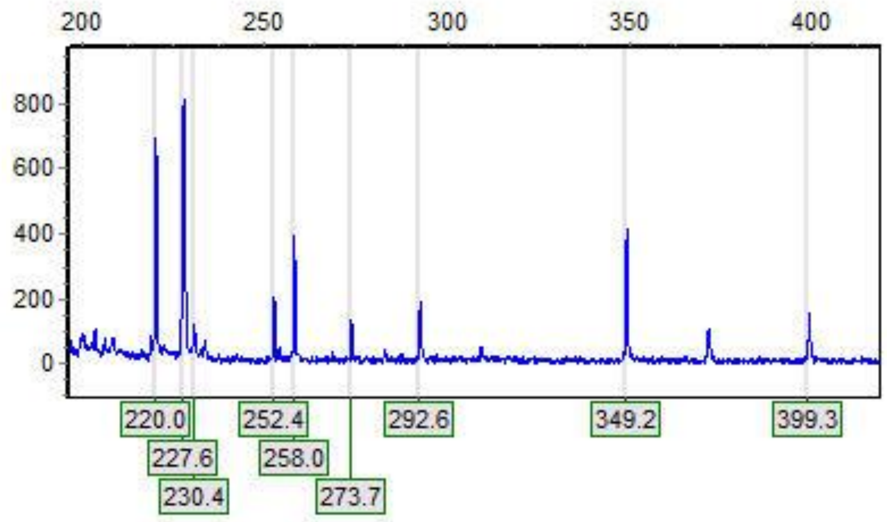


Image by National Institutes of Health

Amplified fragment length polymorphism (AFLP)

- AFLP uses restriction enzymes to digest genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments is then selected to be amplified.
- The amplified fragments are separated and visualized on denaturing on agarose gel electrophoresis , either through autoradiography or fluorescence methodologies, or via automated capillary sequencing instruments.

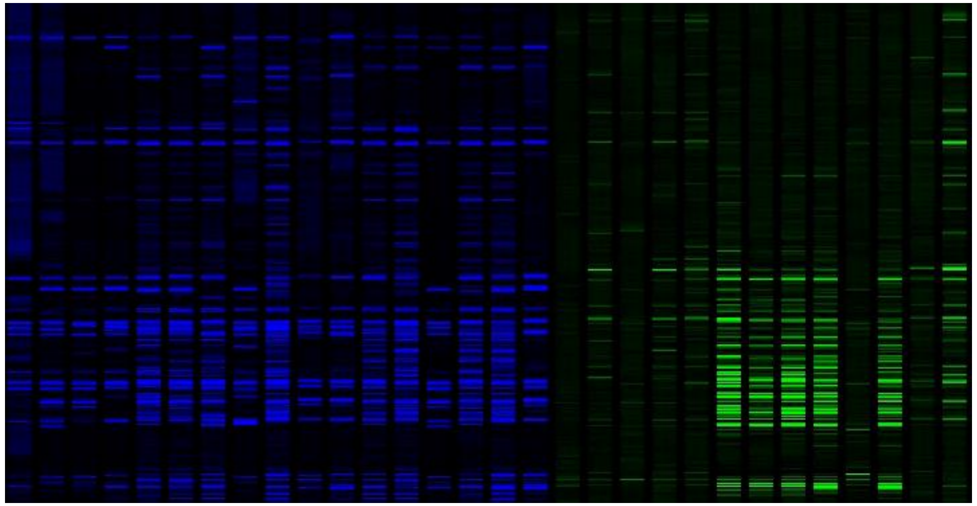
Text mostly from Wikipedia ☺



Public Domain

AFLP data, scoring different size digest fragments

Amplified fragment length polymorphism (AFLP)



Anthony Genova

Amplified Fragment Length Polymorphisms (*Anolis*)

Phylogenetic analysis

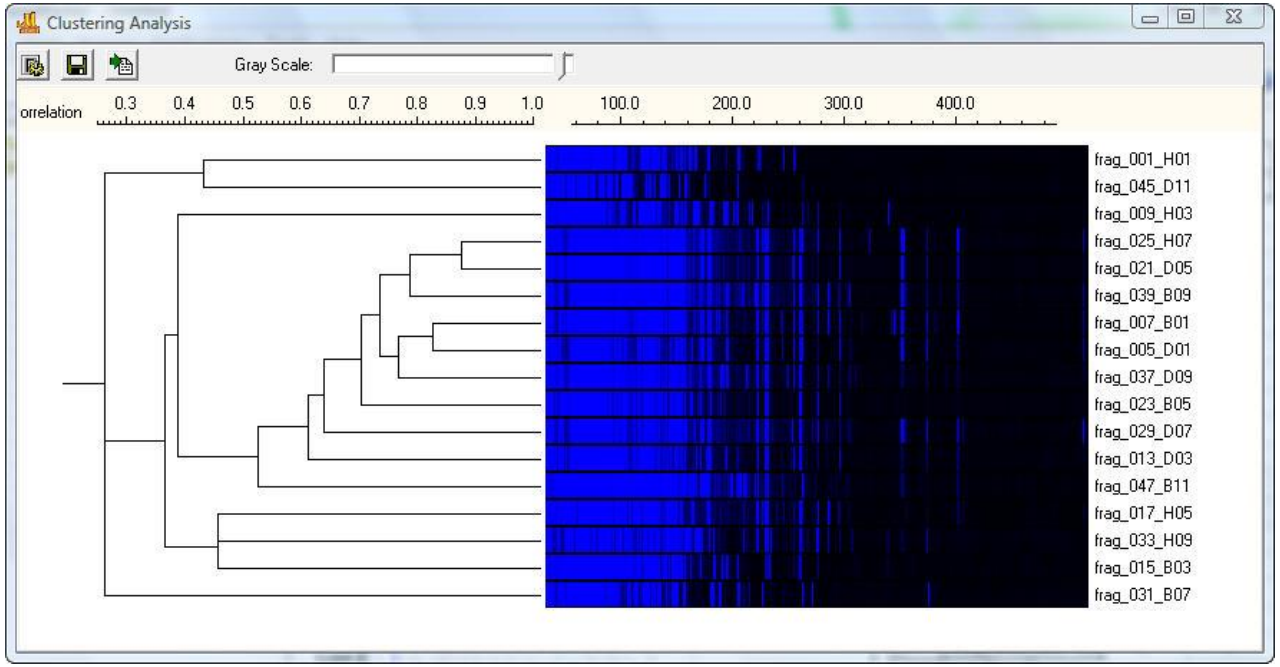


Image by Gene Marker

Restriction Enzymes
+
NGS short read sequencing

Reduced-representation NGS sequencing

- Restriction enzymes can be used to identify homologous blocks of the genome
- Valuable for comparative research in genomics, population genetics etc.
- ‘RADseq’

Restriction-site Associate DNA Sequencing (RADseq)

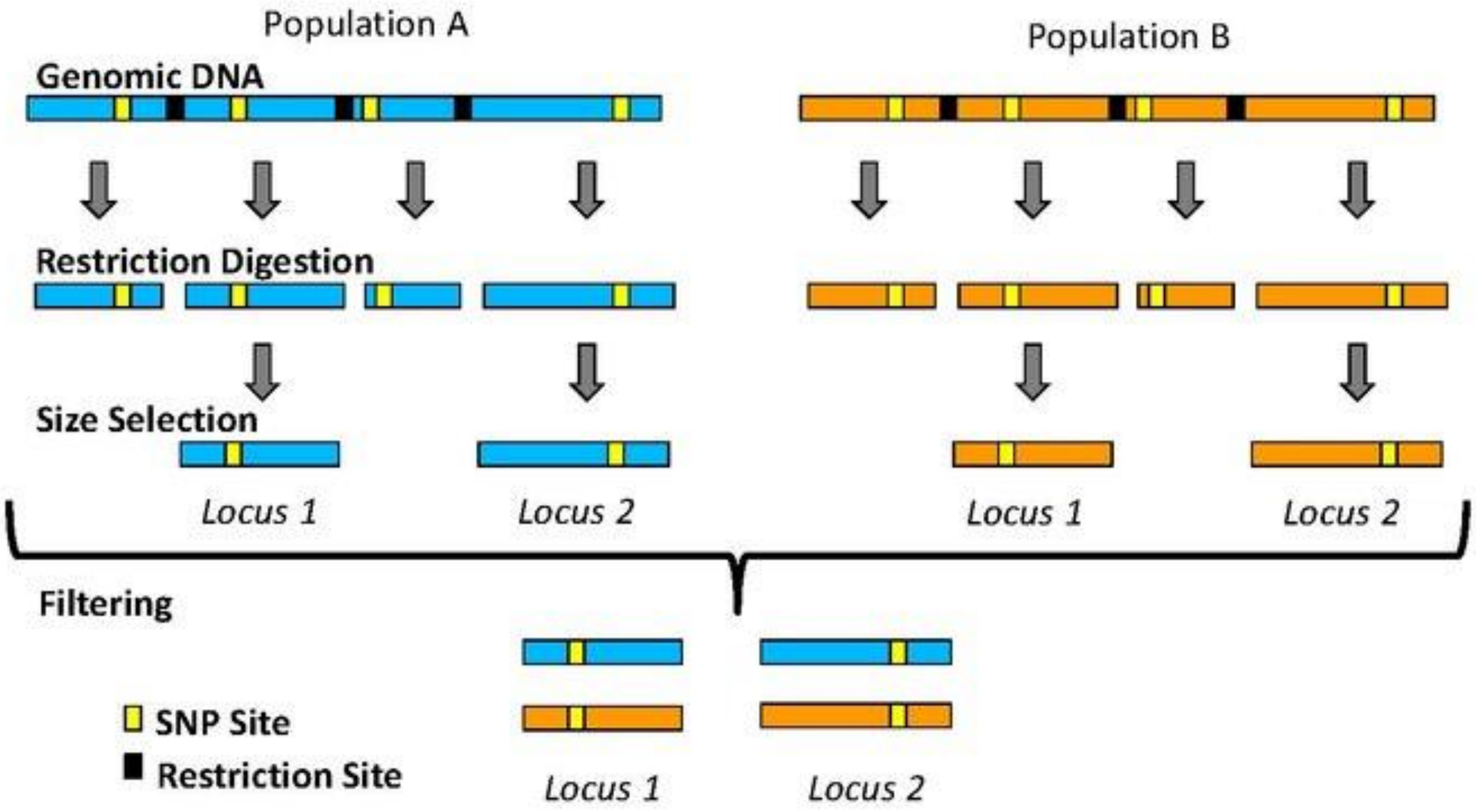
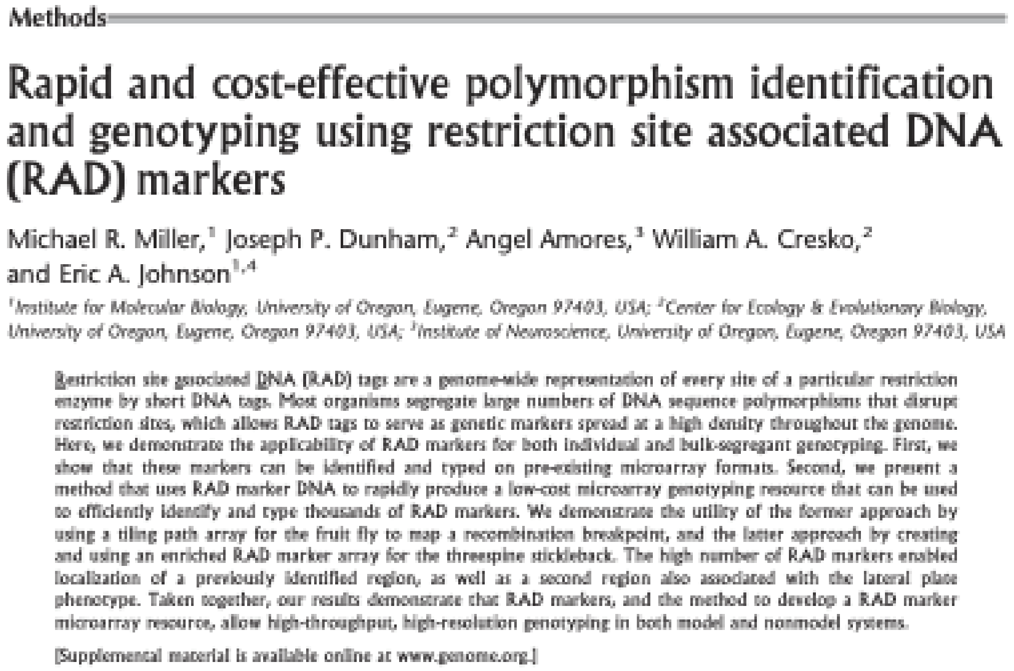


Image by Jonathan Clark CCAS 4.0



Methods

Rapid and cost-effective polymorphism identification and genotyping using restriction site associated DNA (RAD) markers

Michael R. Miller,[1] Joseph P. Dunham,[2] Angel Amores,[3] William A. Cresko,[2] and Eric A. Johnson[1,4]

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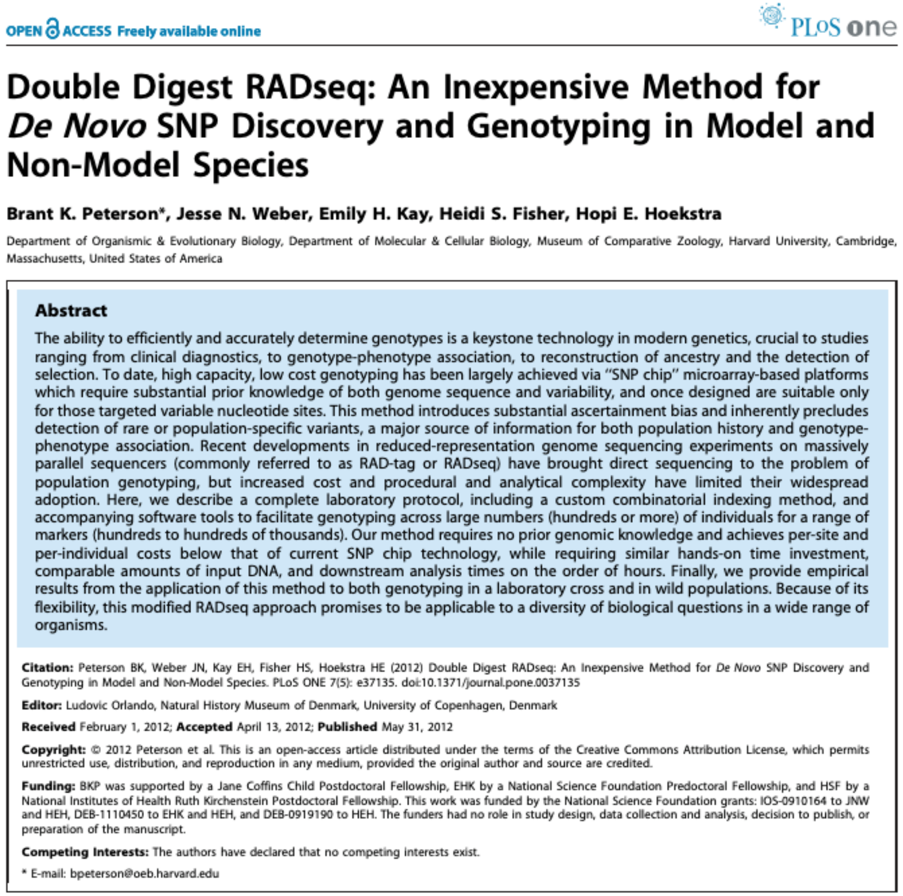
Restriction site associated DNA (RAD) tags are a genome-wide representation of every site of a particular restriction enzyme by short DNA tags. Most organisms segregate large numbers of DNA sequence polymorphisms that disrupt restriction sites, which allows RAD tags to serve as genetic markers spread at a high density throughout the genome. Here, we demonstrate the applicability of RAD markers for both individual and bulk-segregant genotyping. First, we show that these markers can be identified and typed on pre-existing microarray formats. Second, we present a method that uses RAD marker DNA to rapidly produce a low-cost microarray genotyping resource that can be used to efficiently identify and type thousands of RAD markers. We demonstrate the utility of the former approach by using a tiling path array for the fruit fly to map a recombination breakpoint, and the latter approach by creating and using an enriched RAD marker array for the threespine stickleback. The high number of RAD markers enabled localization of a previously identified region, as well as a second region also associated with the lateral plate phenotype. Taken together, our results demonstrate that RAD markers, and the method to develop a RAD marker microarray resource, allow high-throughput, high-resolution genotyping in both model and nonmodel systems.

[Supplemental material is available online at www.genome.org.]

Miller et al. 2007, Gen. Res.



OPEN ACCESS Freely available online

PLoS one

Double Digest RADseq: An Inexpensive Method for *De Novo* SNP Discovery and Genotyping in Model and Non-Model Species

Brant K. Peterson*, Jesse N. Weber, Emily H. Kay, Heidi S. Fisher, Hopi E. Hoekstra

Department of Organismic & Evolutionary Biology, Department of Molecular & Cellular Biology, Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts, United States of America

Abstract

The ability to efficiently and accurately determine genotypes is a keystone technology in modern genetics, crucial to studies ranging from clinical diagnostics, to genotype-phenotype association, to reconstruction of ancestry and the detection of selection. To date, high capacity, low cost genotyping has been largely achieved via "SNP chip" microarray-based platforms which require substantial prior knowledge of both genome sequence and variability, and once designed are suitable only for those targeted variable nucleotide sites. This method introduces substantial ascertainment bias and inherently precludes detection of rare or population-specific variants, a major source of information for both population history and genotype-phenotype association. Recent developments in reduced-representation genome sequencing experiments on massively parallel sequencers (commonly referred to as RAD-tag or RADseq) have brought direct sequencing to the problem of population genotyping, but increased cost and procedural and analytical complexity have limited their widespread adoption. Here, we describe a complete laboratory protocol, including a custom combinatorial indexing method, and accompanying software tools to facilitate genotyping across large numbers (hundreds or more) of individuals for a range of markers (hundreds to hundreds of thousands). Our method requires no prior genomic knowledge and achieves per-site and per-individual costs below that of current SNP chip technology, while requiring similar hands-on time investment, comparable amounts of input DNA, and downstream analysis times on the order of hours. Finally, we provide empirical results from the application of this method to both genotyping in a laboratory cross and in wild populations. Because of its flexibility, this modified RADseq approach promises to be applicable to a diversity of biological questions in a wide range of organisms.

Citation: Peterson BK, Weber JN, Kay EH, Fisher HS, Hoekstra HE (2012) Double Digest RADseq: An Inexpensive Method for *De Novo* SNP Discovery and Genotyping in Model and Non-Model Species. PLoS ONE 7(5): e37135. doi:10.1371/journal.pone.0037135

Editor: Ludovic Orlando, Natural History Museum of Denmark, University of Copenhagen, Denmark

Received February 1, 2012; **Accepted** April 13, 2012; **Published** May 31, 2012

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Funding: BKP was supported by a Jane Coffins Child Postdoctoral Fellowship, EHK by a National Science Foundation Predoctoral Fellowship, and HSF by a National Institutes of Health Ruth Kirchenstein Postdoctoral Fellowship. This work was funded by the National Science Foundation grants: IOS-0910164 to JNW and HEH, DEB-1110450 to EHK and HEH, and DEB-0919190 to HEH. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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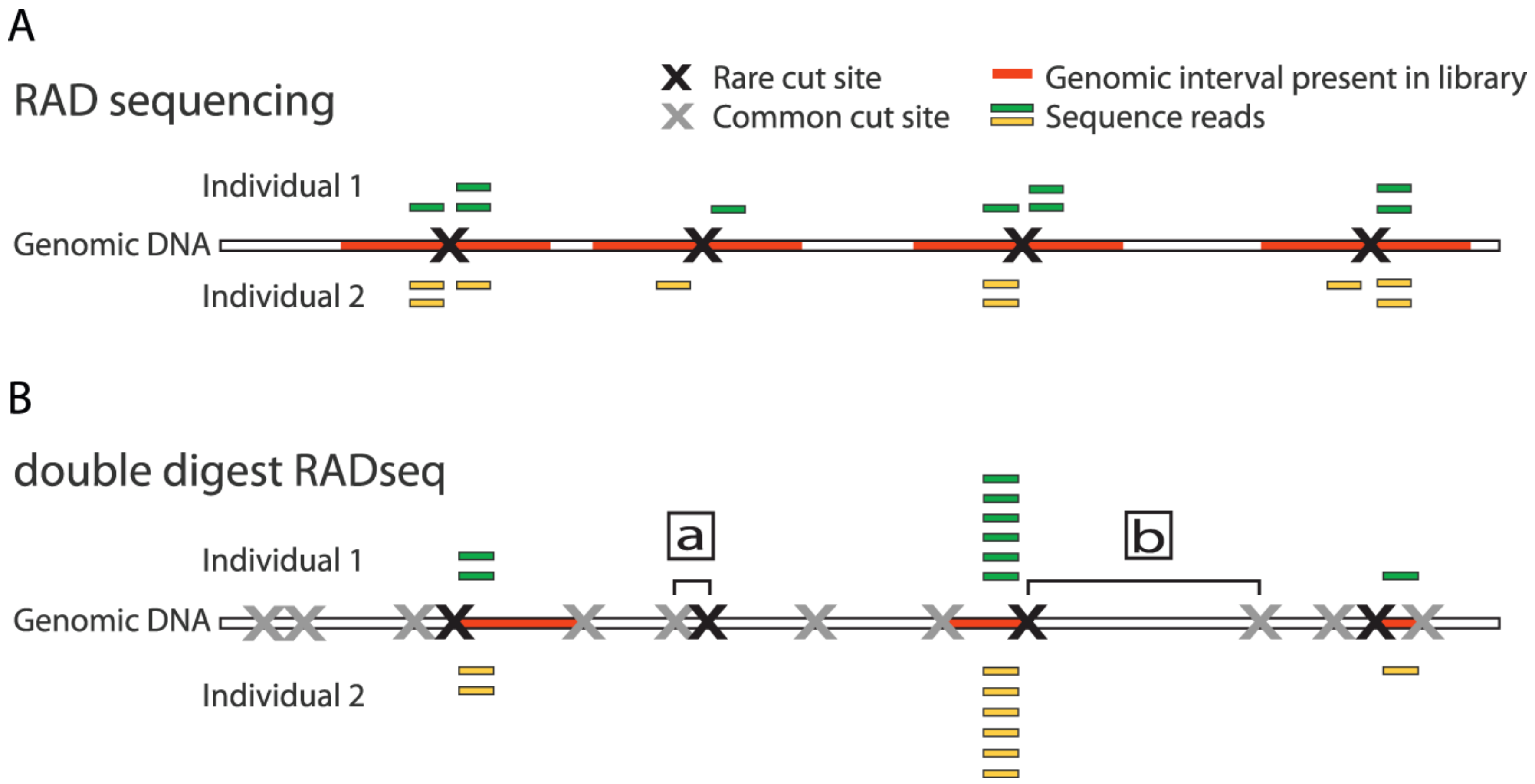
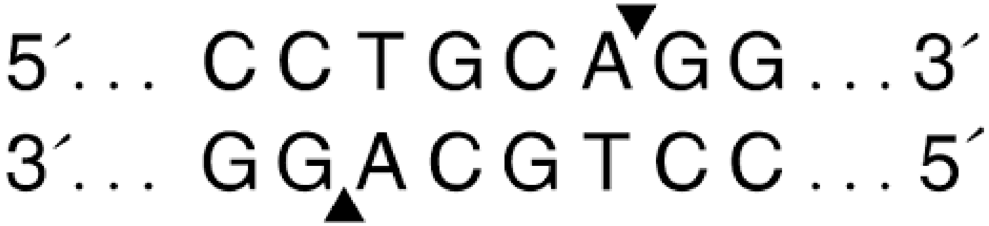
Peterson et al. 2012, PLoS ONE

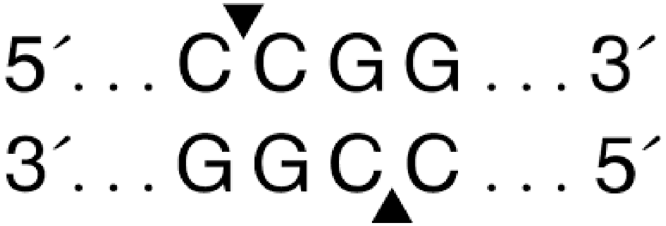


Peterson et al. 2012, PLoS ONE 7: e37135

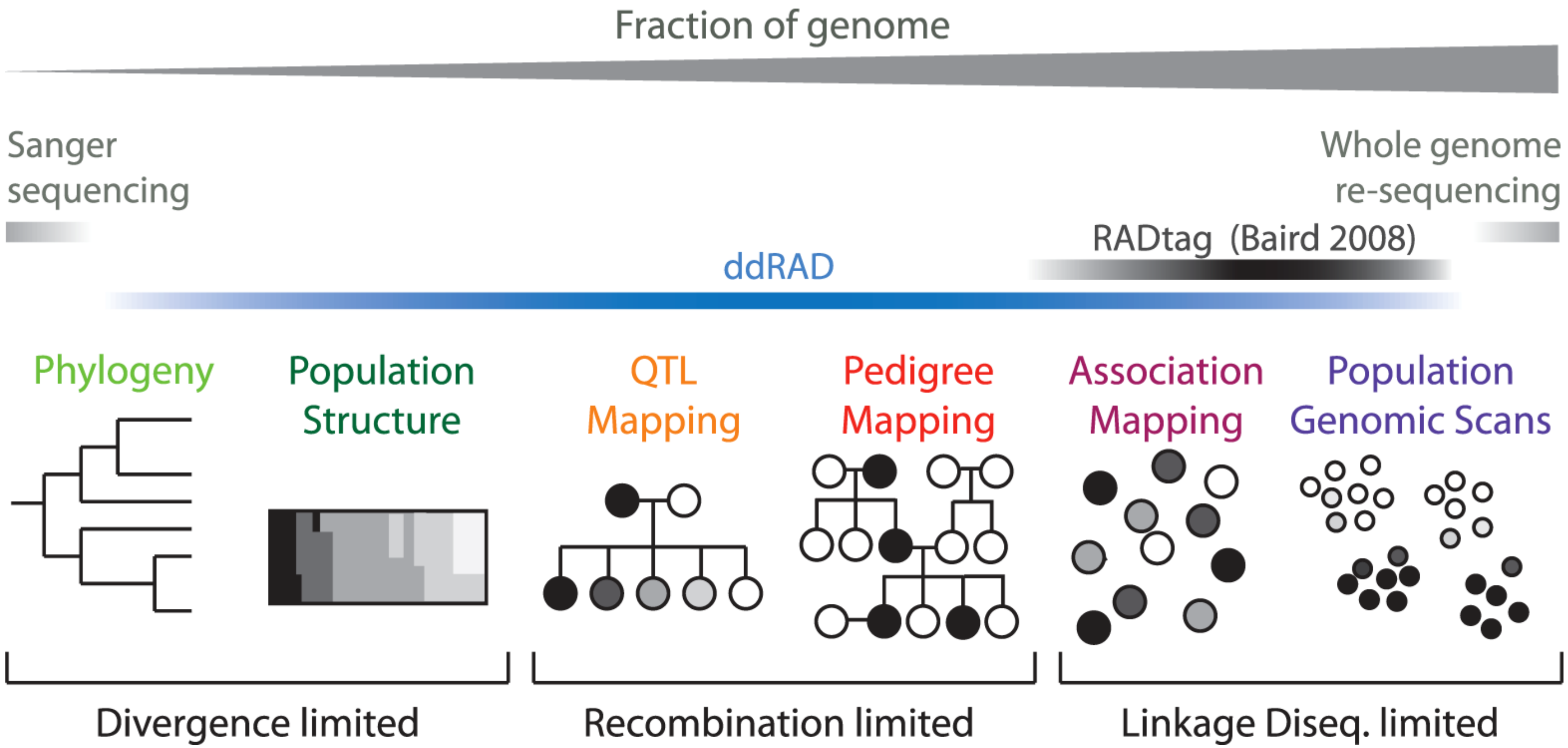
Examples of cut-sites


5´. . . C C T G C A G G . . . 3´
3´. . . G G A C G T C C . . . 5´

SbfI – ‘rare cutter’ 6 nucleotide recognition sequence



MspI – ‘common cutter’ 3 nucleotide recognition sequence



Fraction of genome

Sanger sequencing

Whole genome re-sequencing

RADtag (Baird 2008)

ddRAD

Phylogeny

Population Structure

QTL Mapping

Pedigree Mapping

Association Mapping

Population Genomic Scans

Divergence limited

Recombination limited

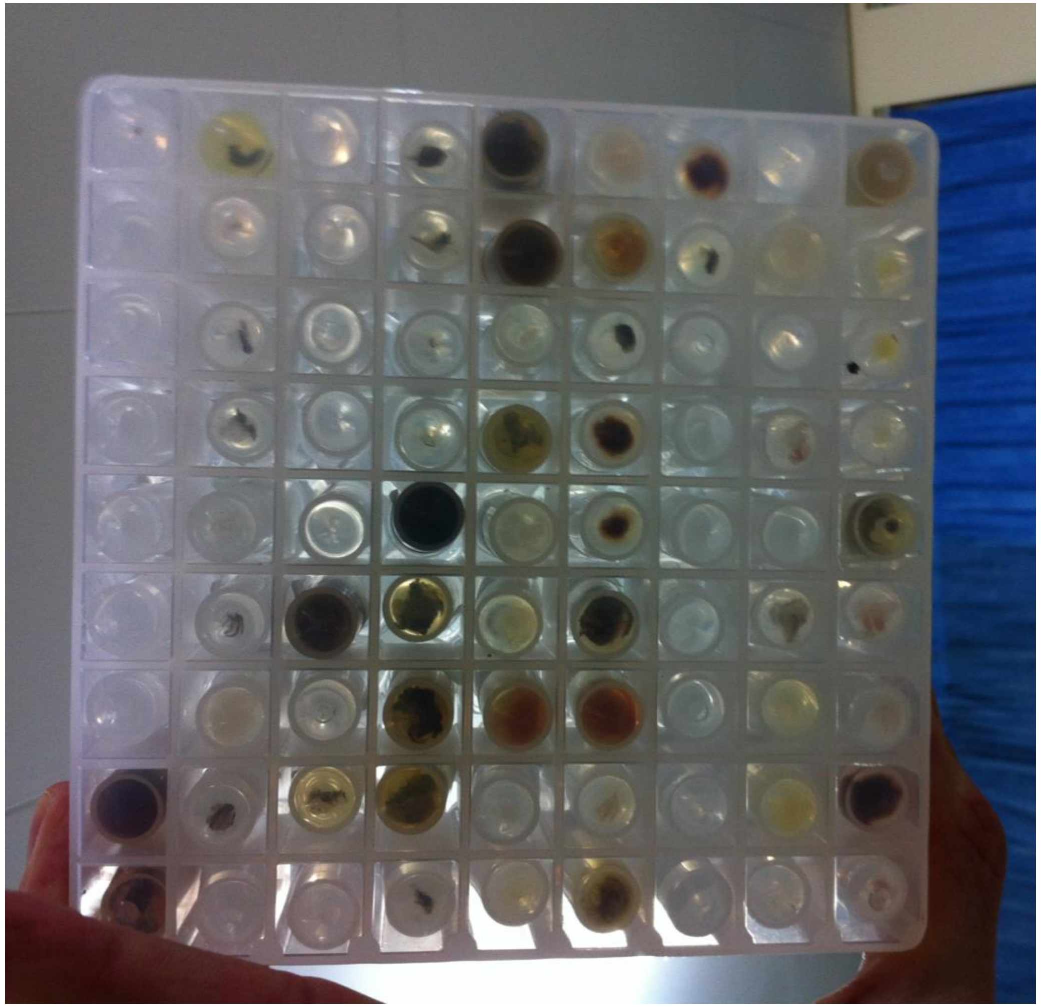
Linkage Diseq. limited

Peterson et al. 2012, PLoS ONE 7: e37135

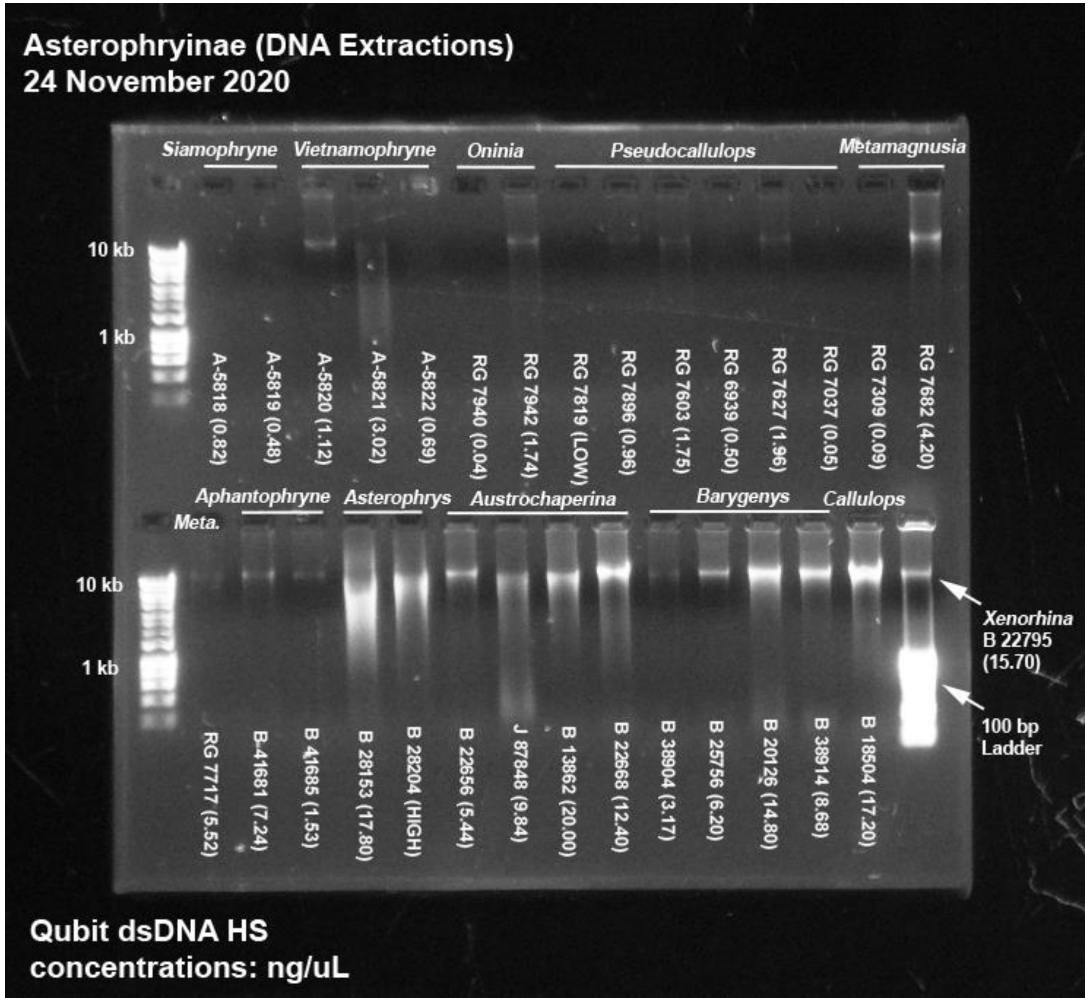
ddRADseq Laboratory methods

- DNA extraction
- Restriction digestion with two Type II enzymes
- Ligation of adapters
- Size-selection
- PCR amplification
- Pooling and Illumina sequencing

DNA extraction



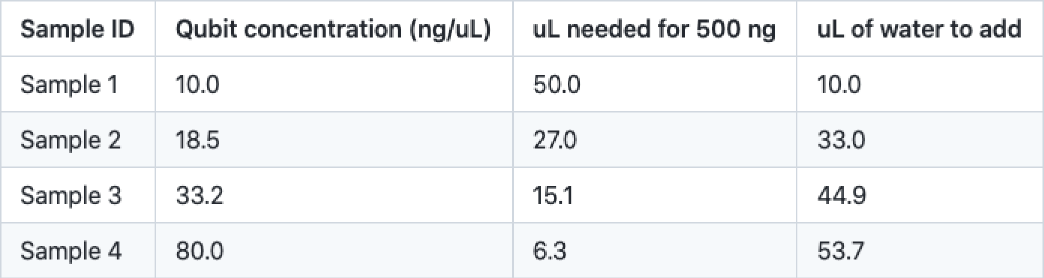
Tissue sampling
(Muscle, liver, etc.)



DNA extraction
(Qiagen kit, Phenol-chloroform, salt extraction etc.)

Quantification of dsDNA

- Like other genomic library construction protocols (e.g. Unit 2) ddRADseq requires specific starting concentrations of DNA.
- We need to determine the concentration of double-stranded DNA (dsDNA) before or after the fragmentation.
- One of the most effective ways to do this (IMO) is with fluorometry.
- We will cover this during the molecular labs tomorrow and next week.



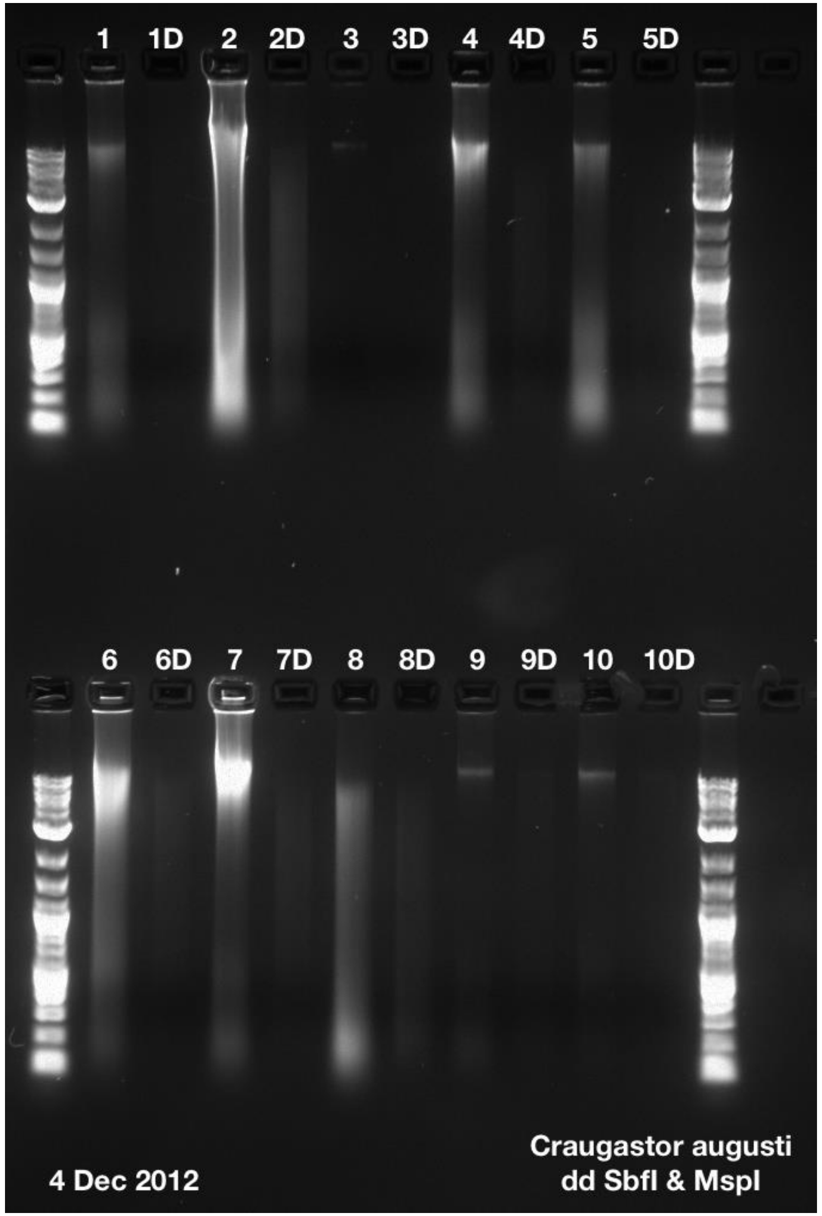
Sample ID	Qubit concentration (ng/uL)	uL needed for 500 ng	uL of water to add
Sample 1	10.0	50.0	10.0
Sample 2	18.5	27.0	33.0
Sample 3	33.2	15.1	44.9
Sample 4	80.0	6.3	53.7

Table from Unit 2 Molecular Lab Protocol
https://github.com/nhm-herpetology/museum-NGS-training/tree/main/Unit_02/Molecular_Lab

Qubit 2.0 Fluorometer

Digestion

Digest at optimal temperature
e.g. 37 C for 6 hours



Streicher et al. 2014, Mol. Ecol.

Ligation of ddRADseq adapters

- Designed specifically for RADseq protocol to match ‘sticky’ cut sites
- Similar in some ways to the adapters we talked about in Unit 1
- Let’s review…

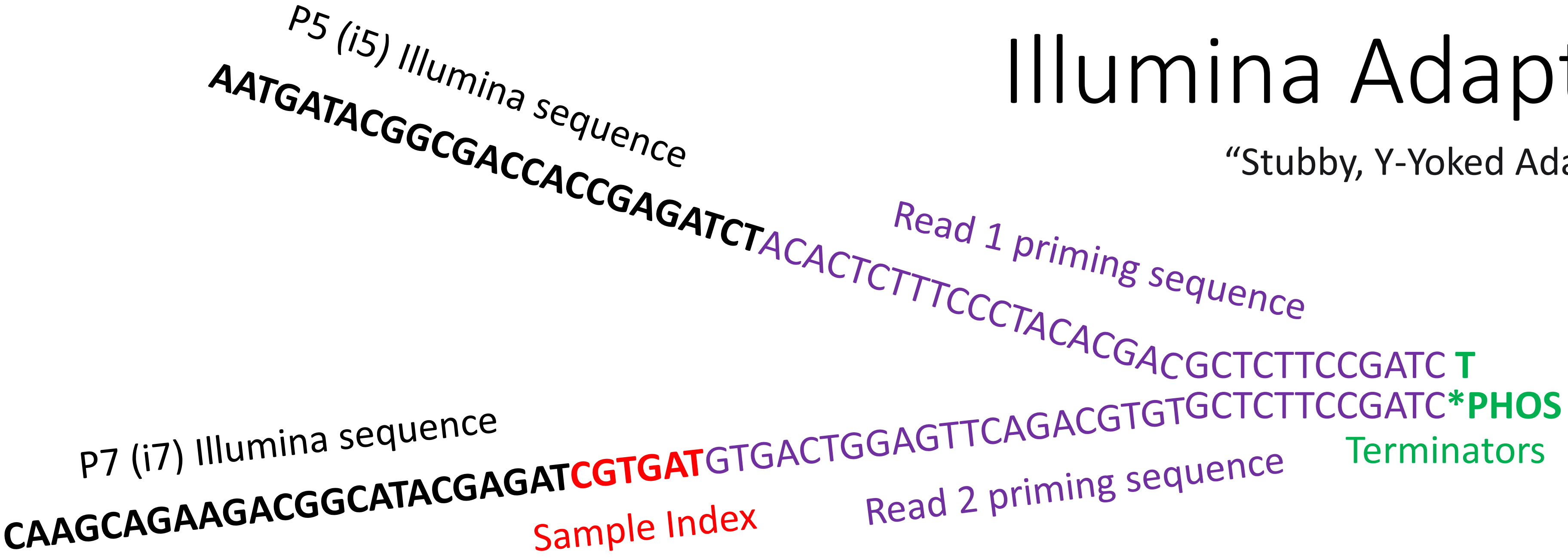
Illumina Adapter Design

“Stubby, Y-Yoked Adapters”

- One oligo with terminal thymine (Required)
- One oligo with phosphorylated terminal nucleotide (Required)
- Illumina P5 and P7 recognition sequences (Required)
- Read 1 and Read 2 priming sequences (Required)
- Unique Index (for multiplexing; Required)
- Second Index (for multiplexing; Optional)
- Unique Molecular Identifier (UMI; Optional)

Illumina Adapter Design

"Stubby, Y-Yoked Adapters"



P5 (i5) Illumina sequence

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC **T**

Read 1 priming sequence

P7 (i7) Illumina sequence

CAAGCAGAAGACGGCATACGAGAT **CGTGAT** GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC***PHOS**

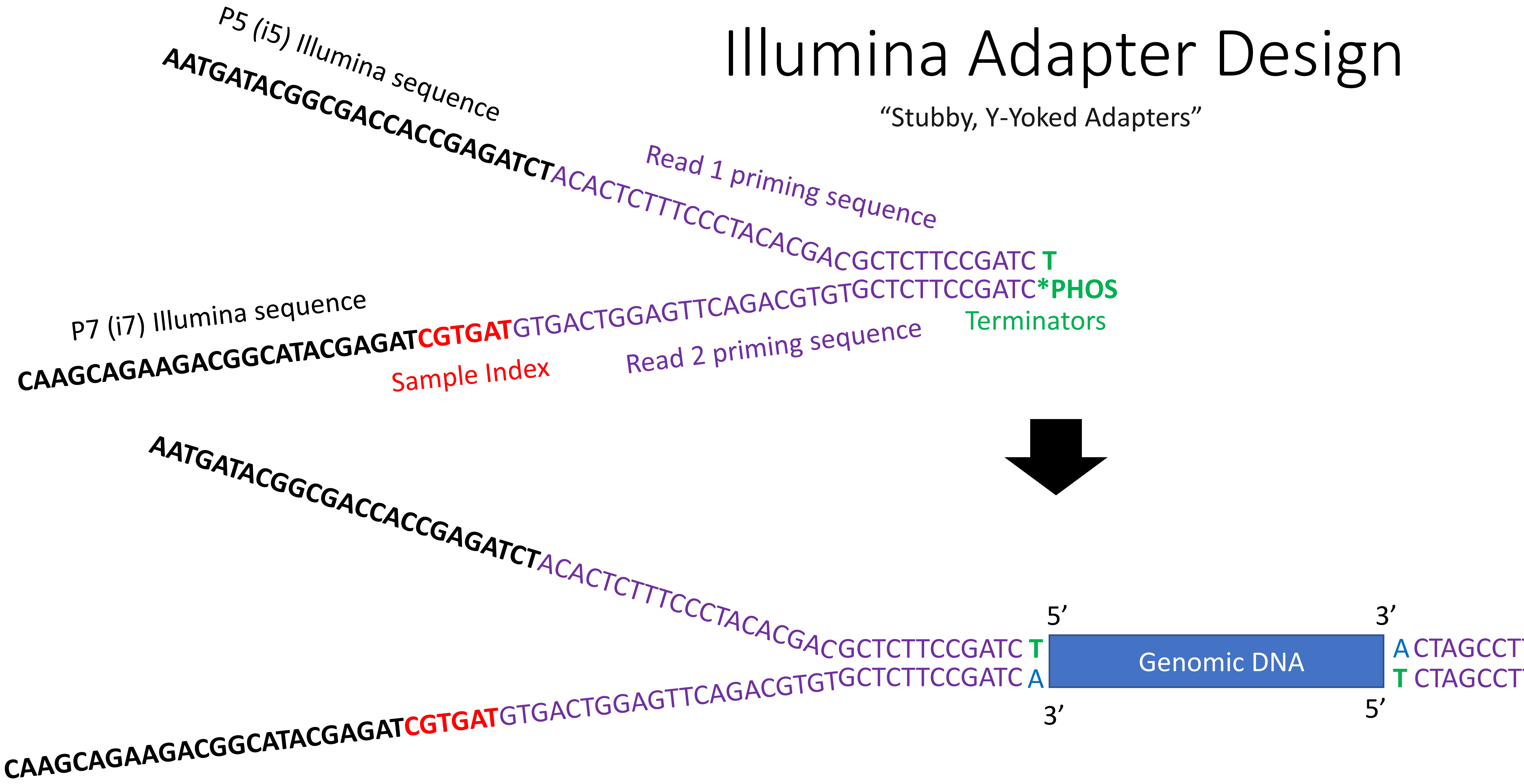
Sample Index

Read 2 priming sequence

Terminators

Illumina Adapter Design

“Stubby, Y-Yoked Adapters”



P5 (i5) Illumina sequence

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC **T**

Read 1 priming sequence

P7 (i7) Illumina sequence

CAAGCAGAAGACGGCATACGAGAT**CGTGAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC***PHOS**

Sample Index

Read 2 priming sequence

Terminators

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC **T**

CAAGCAGAAGACGGCATACGAGAT**CGTGAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC A

5’ 3’

Genomic DNA

3’ 5’

A CTAGCCT

T CTAGCCT

Illumina Adapter Design

“Stubby, Y-Yoked Adapters”

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- One oligo with phosphorylated terminal nucleotide (Required)
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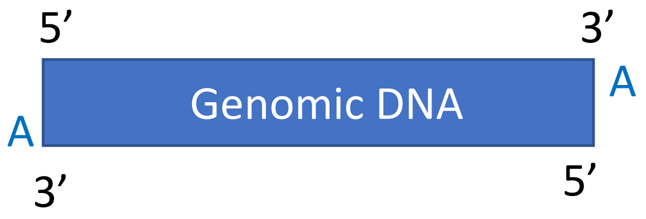
ddRADseq

Illumina Adapter Design

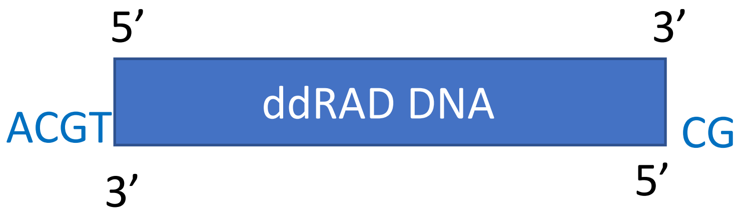
~~“Stubby, Y-Yoked Adapters”~~

- ~~One oligo with terminal thymine (Required)~~
- One oligo with phosphorylated terminal nucleotide (Required)
- Illumina P5 and P7 recognition sequences (Required)
- Read 1 and Read 2 priming sequences (Required)
- Unique Index (for multiplexing; Required)
- Second Index (for multiplexing; Required)
- Unique Molecular Identifier (UMI; Optional)
- One oligo that matches the rare cutter site
- One oligo that matches the common cutter site

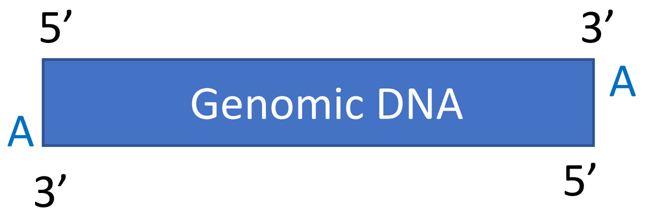
Standard Illumina Library Prep



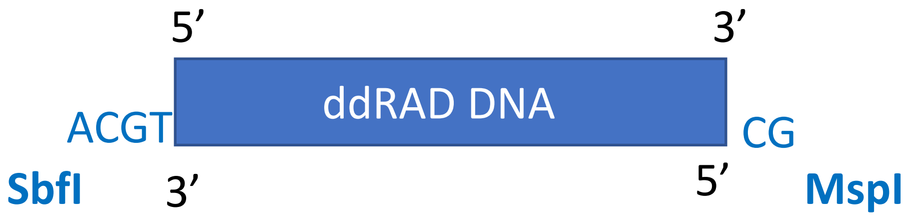
ddRADseq Illumina Library Prep



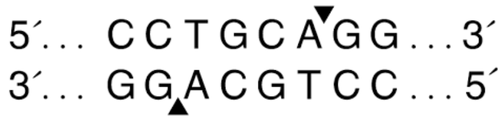
Standard Illumina Library Prep



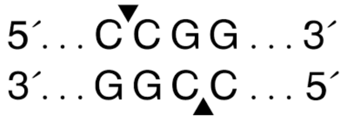
ddRADseq Illumina Library Prep



SbfI


5´. . . C C T G C A G G . . . 3´
3´. . . G G A C G T C C . . . 5´

MspI



P1
Adapter

```
ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNACTAGGTGCA
TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGANNNNNNNNTGATCC -5’PHOS
```

Unique Molecular Identifier

Read 1 priming sequence

Sample index

SbfI cutsite remnant

P1 Adapter

```
ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNACTAGGTGCA
TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGANNNNNNNNTGATCC -5’PHOS
```

Unique Molecular Identifier

Read 1 priming sequence

Sample index

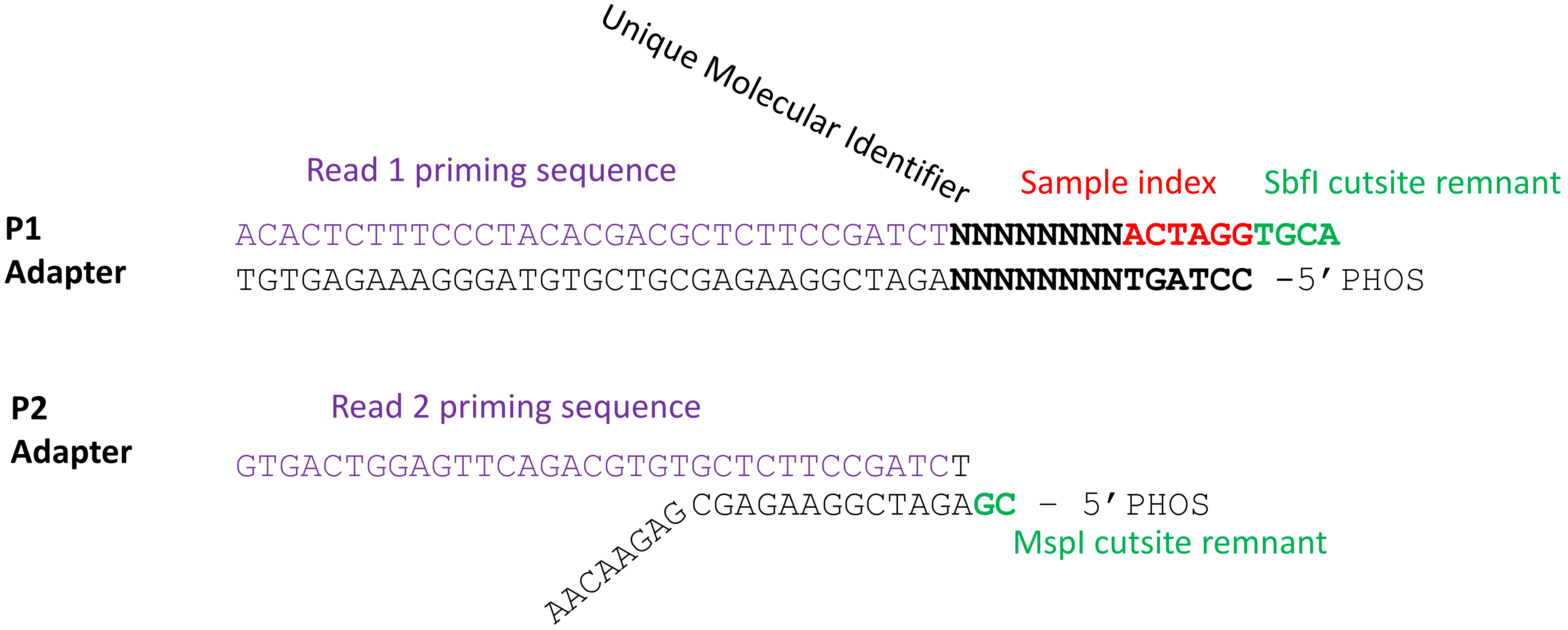
SbfI cutsite remnant

P1 Adapter

```
ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNACTAGGTGCA
TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGANNNNNNNNTGATCC -5’PHOS
```

P2 Adapter

```
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
            AACAAGAGCGAGAAGGCTAGAGC - 5’PHOS
```



Unique Molecular Identifier

Read 1 priming sequence

Sample index

SbfI cutsite remnant

P1 Adapter

```
ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNACTAGGTGCA
TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGANNNNNNNNTGATCC -5’PHOS
```

P2 Adapter

Read 2 priming sequence

```
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
                  AACAAGAG CGAGAAGGCTAGAGC – 5’PHOS
```

MspI cutsite remnant

Forked adapter permits PCR only from P1 side in first cycle

Unique Molecular Identifier

Read 1 priming sequence

Sample index

SbfI cutsite remnant

P1 Adapter

ACACTCTTTCCCTACACGACGCTCTTCCGATCT**NNNNNNNNACTAGGTGCA**

TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA**NNNNNNNNTGATCC** –5’PHOS

P2 Adapter

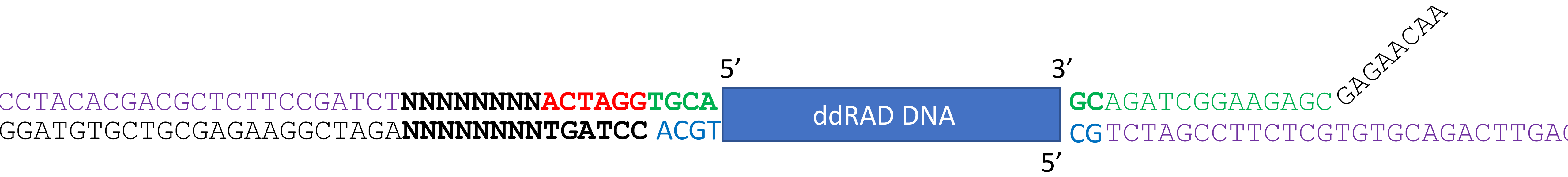
Read 2 priming sequence

GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

AACAAGAG CGAGAAGGCTAGA**GC** – 5’PHOS

MspI cutsite remnant

Forked adapter permits PCR only from P1 side in first cycle



CCTACACGACGCTCTTCCGATCT**NNNNNNNNACTAGGTGCA**

GGATGTGCTGCGAGAAGGCTAGA**NNNNNNNNTGATCC** ACGT

GCAGATCGGAAGAGC GAGAACAA

CGTCTAGCCTTCTCGTGTGCAGACTTGA

Size Selection

Illumina sequencers can only sequence DNA fragments >600 nucleotides in size, so making sure that the mean size of fragments in your libraries are smaller is critical.

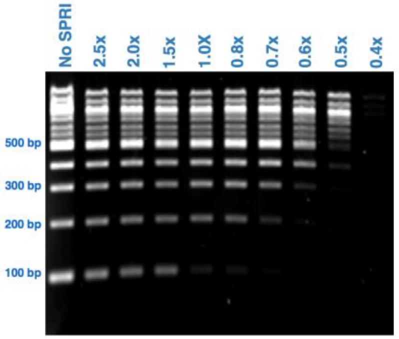


Image from Enseqlopedia

Bead-based size selection

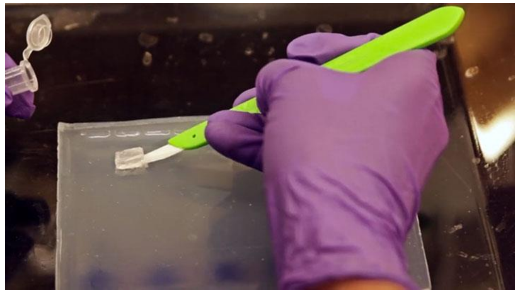


Image from NEB

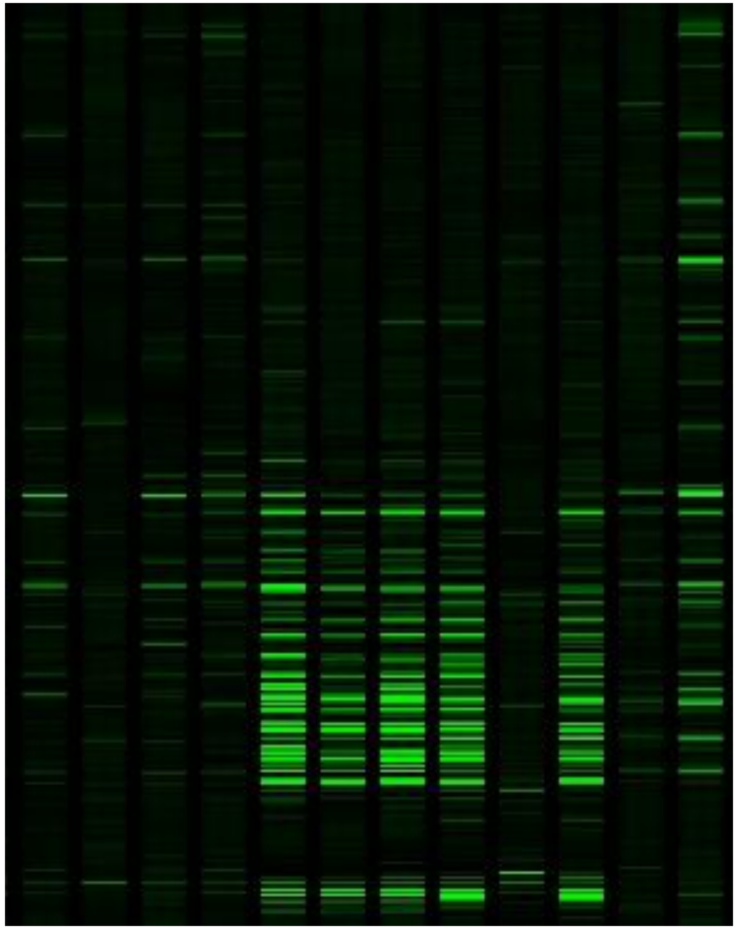
Gel-extraction size selection

Blue Pippin (Sage Science)

Automated Size Selection

ddRADseq – size selection determines which loci you will sequence

Amplified Fragment Length Polymorphisms (*Anolis*)

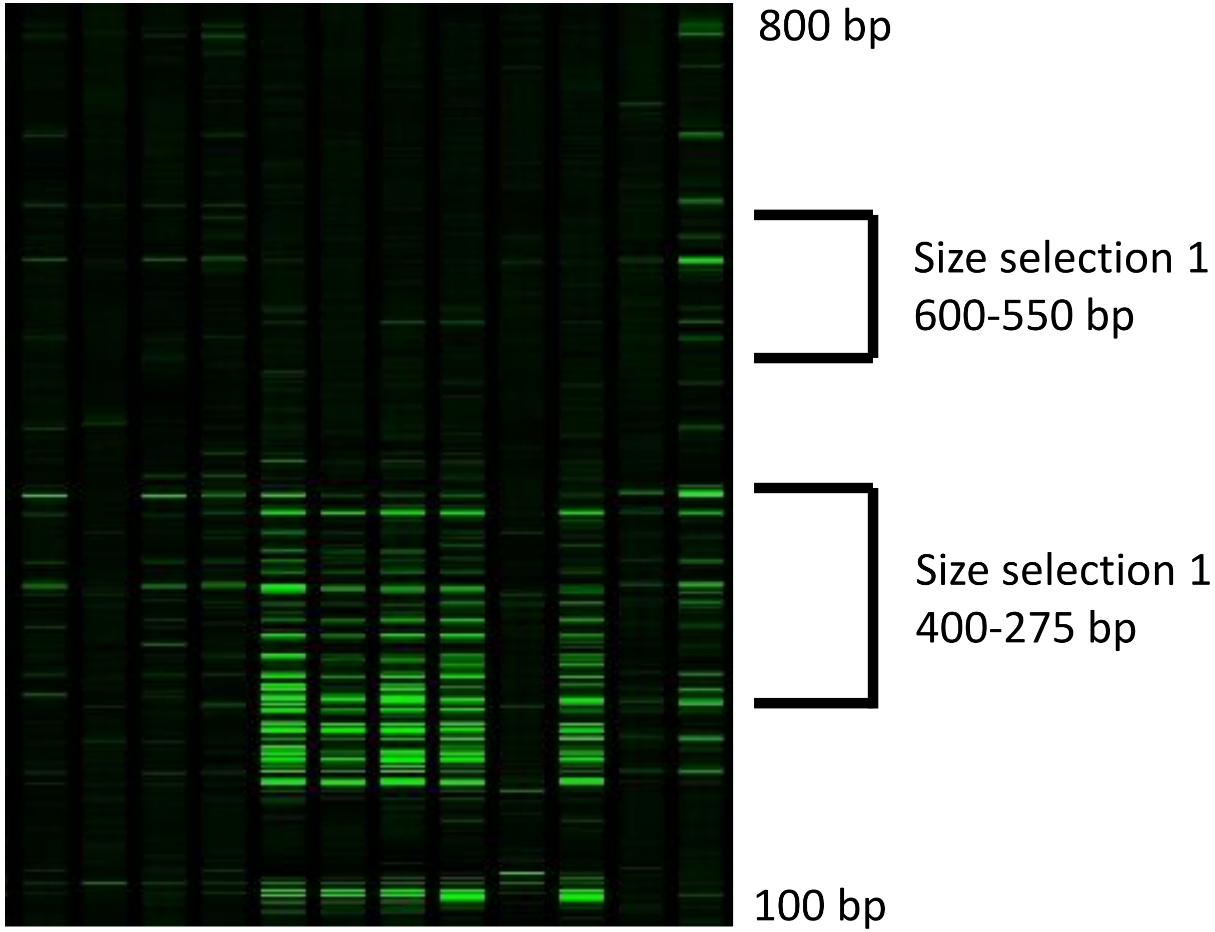


Anthony Genova

DISCLAIMER: I made up the AFLP gel sizes for the purpose of demonstration

ddRADseq – size selection determines which loci you will sequence

Amplified Fragment Length Polymorphisms (*Anolis*)



Anthony Genova

DISCLAIMER: I made up the AFLP gel sizes for the purpose of demonstration

Limited PCR Amplification

PCR usually of 8-12 cycles

PCR Primers Standard Illumina Library Prep

TruSeq P5: AATGATACGGCGACCACCGAGA

TruSeq P7: CAAGCAGAAGACGGCATACGAG

Hi-Fidelity Polymerase

Limited PCR Amplification

PCR usually of 8-12 cycles

PCR Primers Standard Illumina Library Prep

TruSeq P5: AATGATACGGCGACCACCGAGA

TruSeq P7: CAAGCAGAAGACGGCATACGAG

PCR Primers ddRADseq Illumina Library Prep

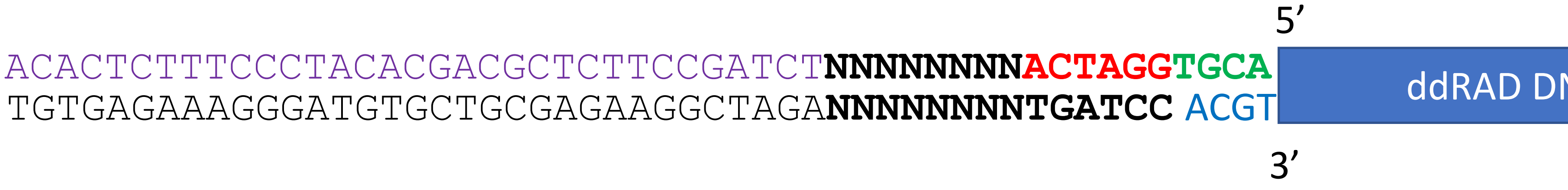
PCR 1: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG

PCR 2: CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGACGTGTGC

Hi-Fidelity Polymerase

Why the long primers?

Why the long primers?



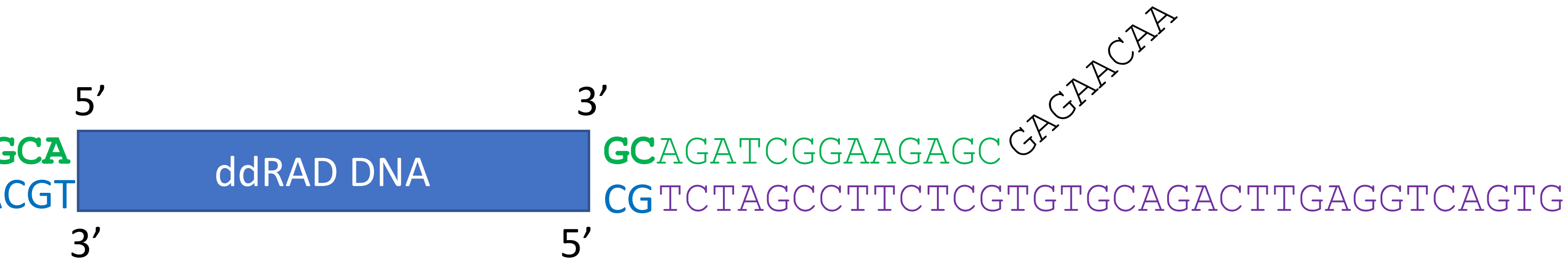
5'

ACACTCTTTCCCTACACGACGCTCTTCCGATCT**NNNNNNNNACTAGGTGCA**

TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA**NNNNNNNNTGATCC** ACGT

3'

ddRAD DN



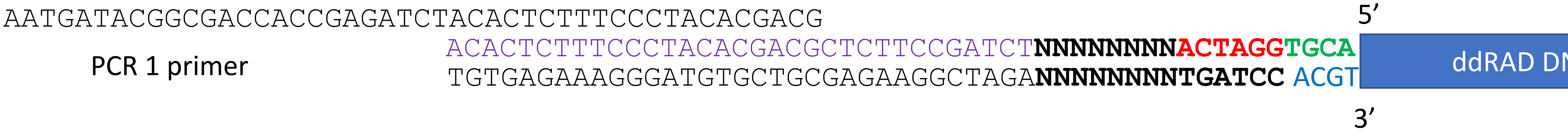
5' 3'

GCA ddRAD DNA **GC**AGATCGGAAGAGC GAGAACAA

CGT CGTCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG

3' 5'

Why the long primers?



AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG

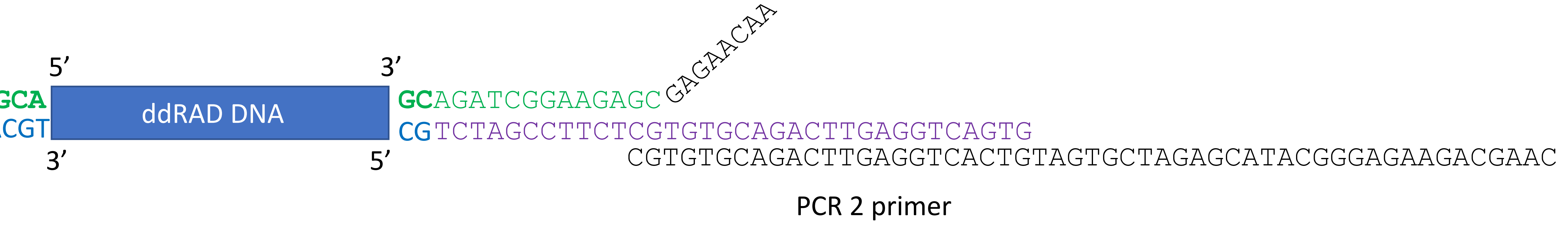
PCR 1 primer

5’

ACACTCTTTCCCTACACGACGCTCTTCCGATCT**NNNNNNNNACTAGGTGCA**

TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA**NNNNNNNNTGATCC** ACGT

3’



5’ 3’

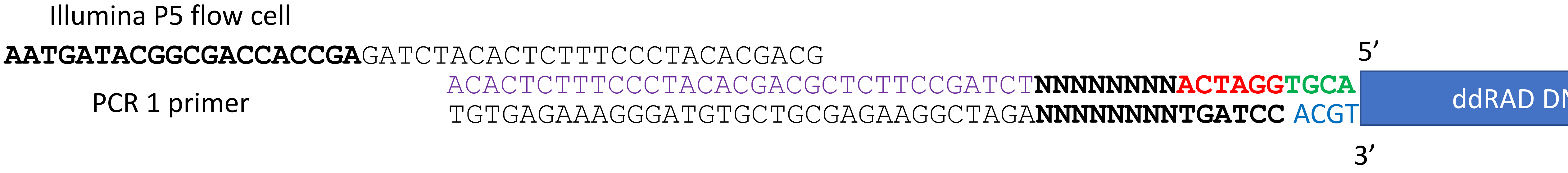
GCA **GC**AGATCGGAAGAGC GAGAACAA

CGT CGTCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG

3’ 5’ CGTGTGCAGACTTGAGGTCACTGTAGTGCTAGAGCATACGGAGAAGACGAAC

PCR 2 primer

Why the long primers?



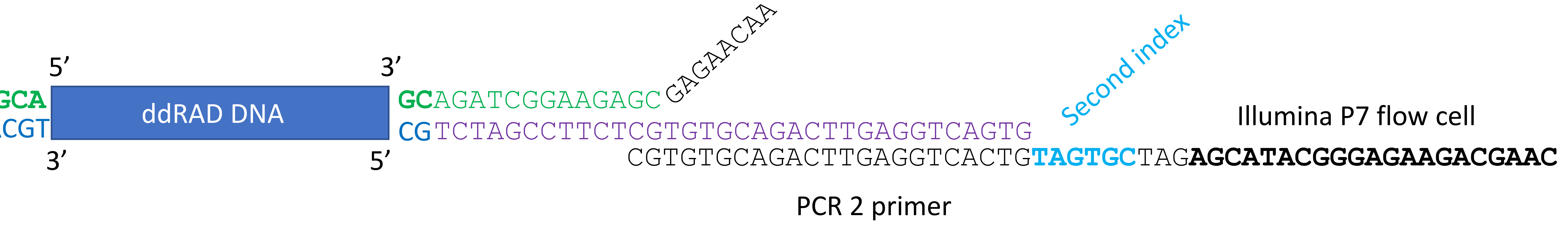
Illumina P5 flow cell

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG

PCR 1 primer

ACACTCTTTCCCTACACGACGCTCTTCCGATCT**NNNNNNNNACTAGGTGCA**

TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA**NNNNNNNNTGATCC** ACGT



GCAGATCGGAAGAGC GAGAACAA

CGTCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG

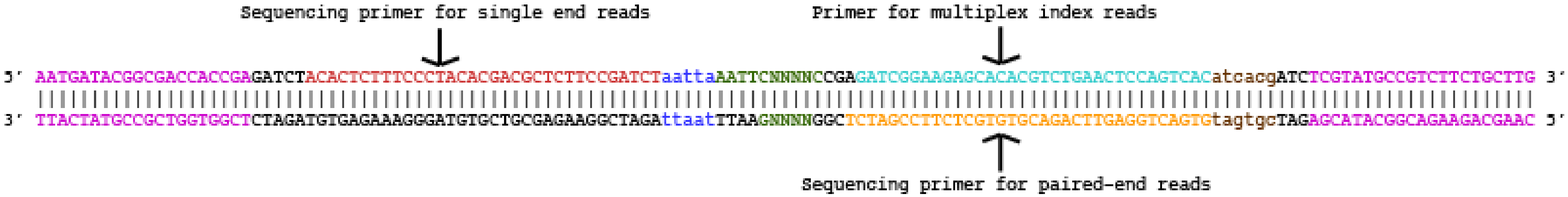
CGTGTGCAGACTTGAGGTCACTG**TAGTGC**TAG**AGCATACGGAGAAGACGAAC**

Second index

Illumina P7 flow cell

PCR 2 primer

Final sequencing library



Sequencing primer for single end reads

Primer for multiplex index reads

```
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTaattaAATTCNNNNCCGAGATCGGAAGAGCACACGTCTGAACTCCAGTCACatcacgATCTCGTATGCCGTCTTCTGCTTG 3'
   |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3' TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGAttaatTTAAGNNNNGGCTCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTGtagtgcTAGAGCATACGGCAGAAGACGAAC 5'
```

Sequencing primer for paired-end reads

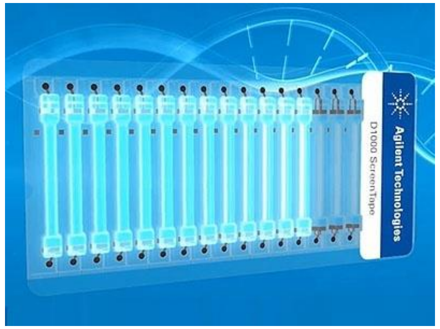
DNA Sequence Legend

READ 1 primer
READ 2 primer
MULTIPLEX READ primer
genomic DNA
barcode (aatta) - inline
index (atcacg) - multiplex
flowcell annealing

Peterson et al. 2012, PLoS ONE 7: e37135

Quantification of genomic DNA libraries

- Reasonably precise estimates of DNA concentration are needed for Illumina sequencer input



D1000 Screentape (Agilent)

TapeStation 2200 (Agilent)

Lower size standard

Upper size standard

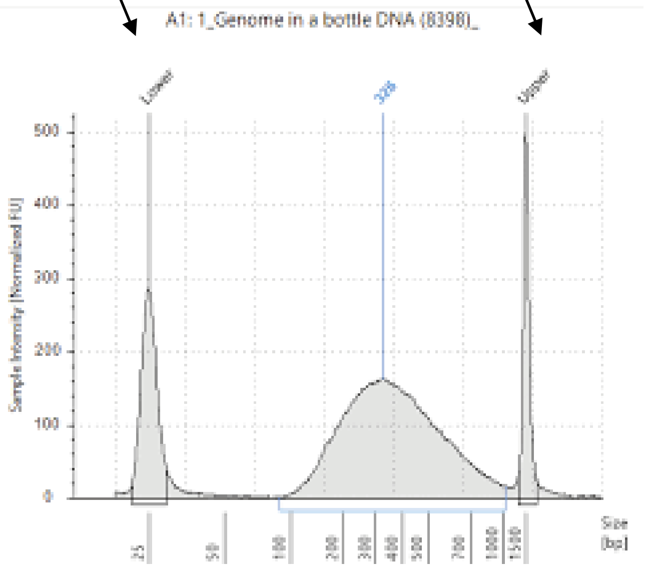
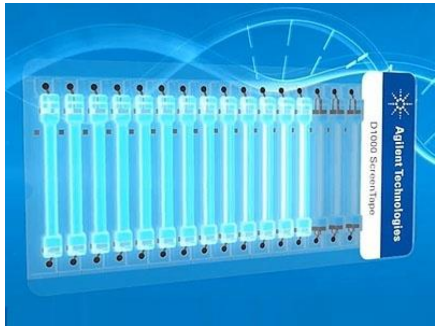


Image from Agilent

Illumina ddRADseq library

Quantification of ddRADseq libraries

- Reasonably precise estimates of DNA concentration are needed for Illumina sequencer input



D1000 Screentape (Agilent)

TapeStation 2200 (Agilent)

Lower size standard

Upper size standard

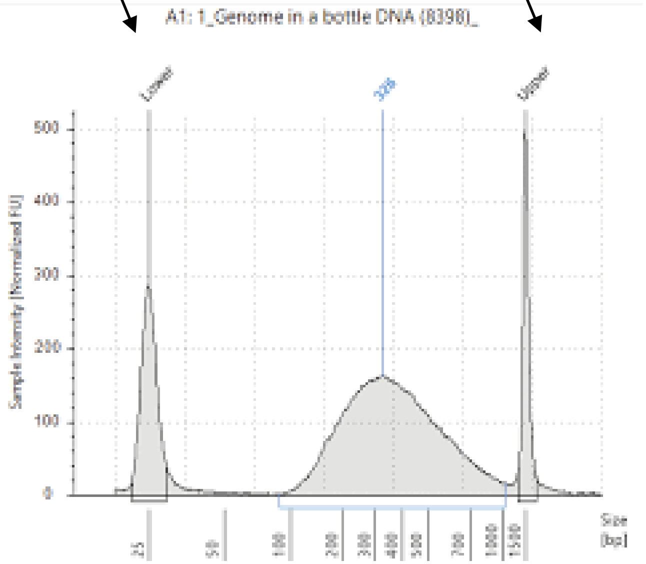


Image from Agilent

Illumina ddRADseq library

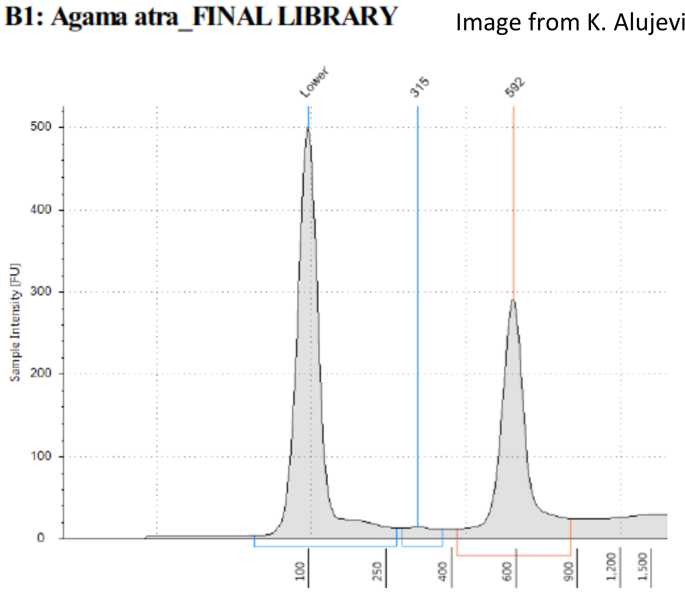


Image from K. Alujevic

Tighter size selection for smaller number of RAD loci

Analyzing ddRADseq data

- Stacks (Catchen et al.) provides a convenient way to demultiplex and sort data
- We will use this software in the bioinformatics lab today
- A brief overview…

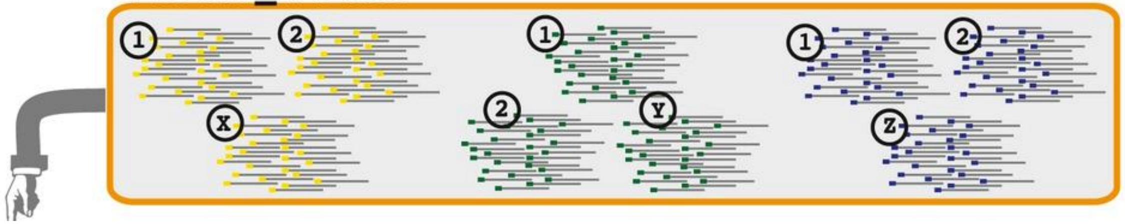
MOLECULAR ECOLOGY

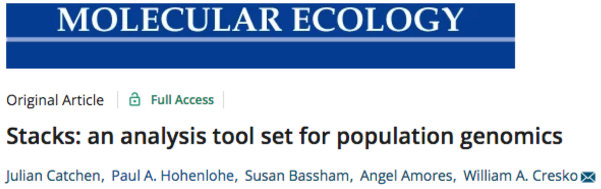
Original Article | Full Access

Stacks: an analysis tool set for population genomics

Julian Catchen, Paul A. Hohenlohe, Susan Bassham, Angel Amores, William A. Cresko

Process_Radtags





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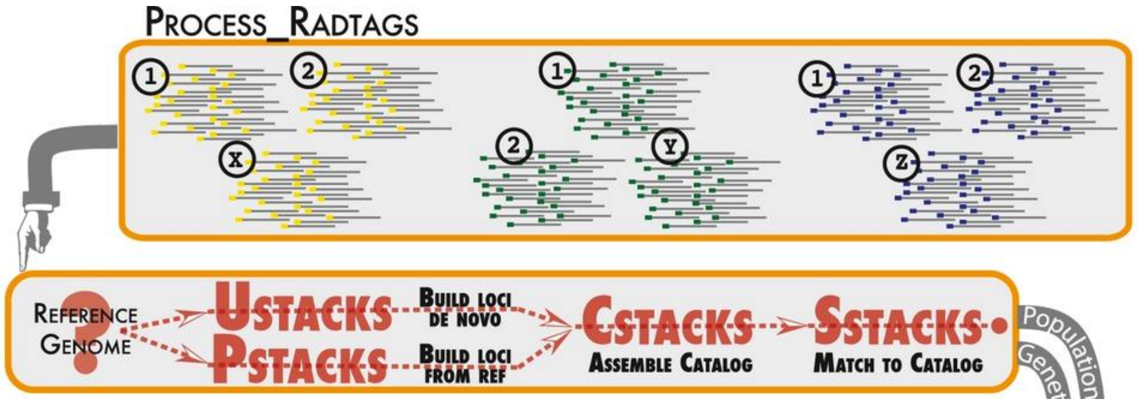

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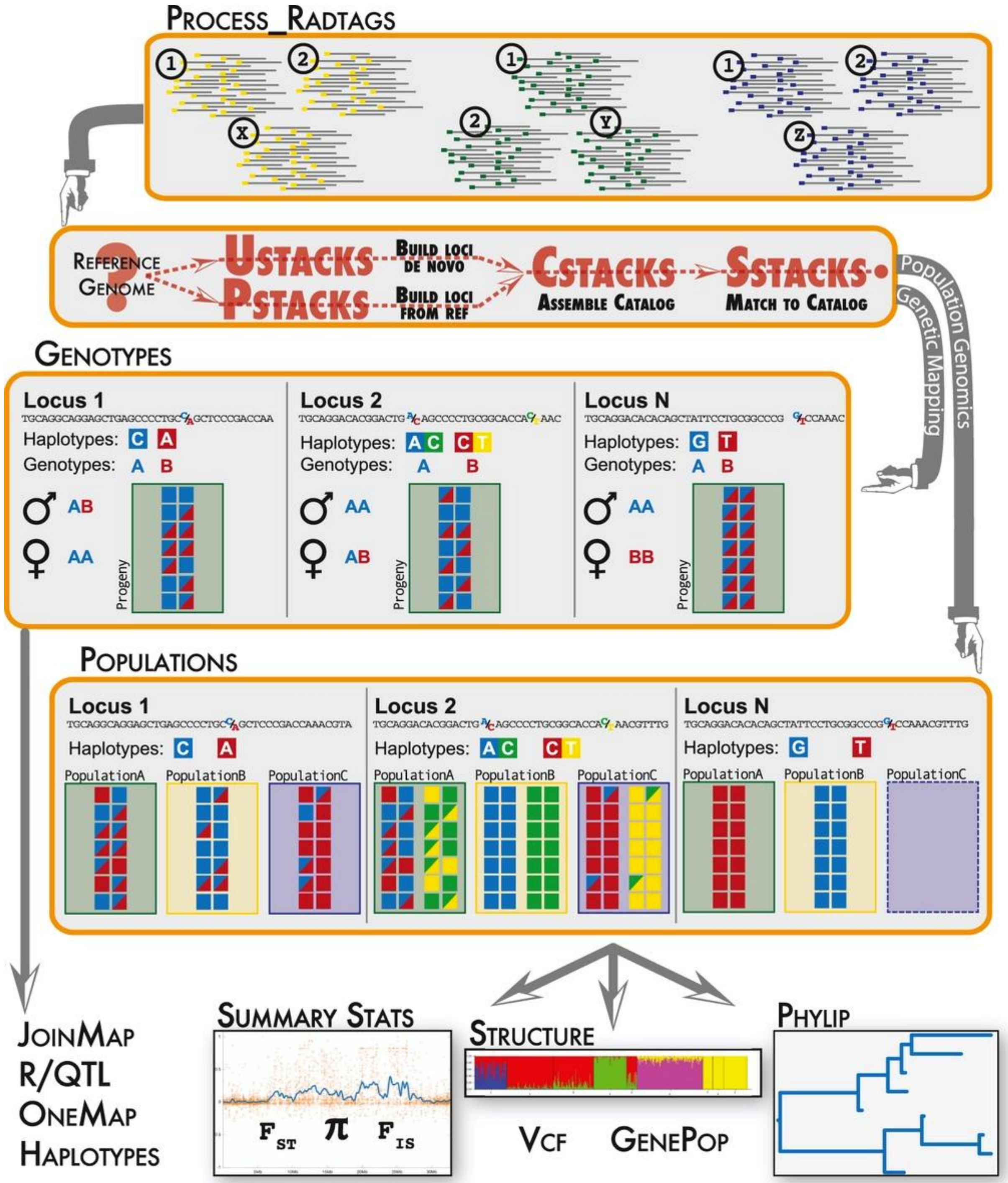

Unit 4: Double digest restriction-site associated DNA sequencing (ddRADseq)

Bioinformatics Lab

GitHub https://github.com/nhm-herpetology/museum-NGS-training