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 We use this protocol and it's working

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## Workflow for SNP genotyping using the Hi-Plex method

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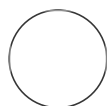
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### ABSTRACT

Many research questions in ecology and evolution require balancing sampling strategies between their spatial (how many populations? on which geographical, environmental gradients?), temporal (diachronic approaches), and genomic (how many and which loci?) dimensions. High-throughput molecular biology protocols often offer very good genomic coverage, but this is often only achievable at the expense of other sampling dimensions. This has led to the development of targeted genotyping strategies for SNP locus sets, in addition to whole or reduced genome sequencing strategies. We here present an adaptation of a protocol developed by the University of Melbourne for genotyping rare variants in human oncology to non-model species for use in ecology and evolution. Hi-Plex is an amplicon sequencing technique (sensu Meek & Larson 2019) in which all loci are co-amplified in a multiplex reaction before Illumina or Ion-Torrent sequencing (we used Illumina). Intermediate steps include dual indexing of individual samples used for demultiplexing.

**Keywords:** SNP genotyping, SNP panel, genotyping by sequencing, Illumina, Ion Torrent

## GUIDELINES

The Hi-Plex protocol is adapted for population genetic studies using SNPs or microhaplotypes. It is an amplicon sequencing technique (sensu Meek & Larson 2019) in which all loci are co-amplified in a multiplex reaction before Illumina or Ion-Torrent sequencing (we used Illumina). Depending on the number of loci and samples, and given actual reagent and sequencing costs, multilocus genotypes at up to 400 loci can be obtained for about 10 to 15€ per sample.

## MATERIALS

### Common supplies and reagents :

- Pipette : monochannel p10, p20, p200, p1000, multichannel p10 p100 and/or repeater pipette
- Filter tips corresponding on mono and multichannel pipettes
- 1.5 mL microcentrifuge tubes
- 2 mL microcentrifuge tubes
- 96-well PCR plates
- Adhesive sealing foil for PCR and storage plates
- DNase/RNase (nuclease) free water
- Strip tubes and caps

### Specific supplies and reagents for each step :

#### Gene Specific Primer (GSPs) and adaptator (TSITs) preparation steps

- 1x Low TE (10 mM Tris pH 8.0/0.1 mM EDTA)
- GSPs at 100 or 200 µM in TE
- TSITs A and TSITs P at 100 or 200 µM in TE

#### PCRs & Clean-up steps

- DNA
- Ice bath or cold cube
- 15 mL conical vials (e.g., Falcon tubes) for large PCR mixes
- Tube-strips and caps
- Pool of GSP at 50µM
- ThermoFisher Phusion<sup>TM</sup> Hot Start II High Fidelity (ref. F549L)
- ThermoFisher Phusion<sup>TM</sup> High-Fidelity (ref. F534L)
- TSIT prepared in plate at final concentration of 10µM
- EDTA 100mM predistributed in tube strip (MW 292.24 g/mol)
- dNTPs 20mM each (Promega ref U1330)
- Primers: P5 5'-AATGATACGGCGACCAACGA-3' and P7 5'-CAAGCAGAAGACGGCATAGCA-3'
- PCR machine
- Magnetic beads (Macherey-Nagel Nucleomag NGS cleanup ref. 744970.5)

- Magnetic tube rack
- 85% Ethanol

### Size selection step

#### *Option 1 : by electrophoresis in agarose gel*

- Agarose gel supplies and reagents : agarose powder, TAE 1x, Ethidium bromide, 50 bp ladder GeneRuler (ThermoFisher ref. SM0373), cuve and large electrophoresis comb
- Scalpel blades
- Nucleospin gel clean-up kit (Macherey-Nagel ref. 740609.5)

#### *Option 2: by Pippin™ prep*

- DNA Gel Cassettes 2% Agarose DF Marker L, dye free, w/ internal standards, Pippin™ Prep, 100-600 bp (SAGE SCIENCE- Ref : CDF2010)

### Quality control step

- Invitrogen™ Qubit™ Fluorometer or equivalent
- Agilent Fragment Analyzer System or Bioanalyser system or equivalent
- KAPA Library Quantification Kit Illumina® Platforms (Roche Sequencing Solutions)

### ILLUMINA sequencing

- Samplesheet
- Customs primers : Read1, i7\_read and Read2
- Illumina Miseq system or others and corresponding reagents

## SAFETY WARNINGS



- Reagents and samples should be stored at -20 °C and placed in the refrigerator at 4 °C until defrosted, ideally the day before any manipulation. To limit the risk of degradation of the primers and adaptors, avoid multiple freeze-thaw cycles by preparing multiple aliquots.
- Clean lab bench before work according to lab policy (example: Bleach/ethanol, DNAZap 1, DNAZap 2).
- Materials can be decontaminated in UV box.
- Working under a PCR workstation or in a sterile lab is recommended.
- Avoid potential contamination of PCR products by using filter tips.
- Lab coat and gloves required
- The use of ethidium bromide requires a specific waste treatment and implies to respect general rules to avoid contamination of benchwork, materials and lab technician.

- Select a set of SNP loci to include in your SNP panel. Prepare a fasta file with one 145bp sequence per locus. SNP loci can be selected from any genomic resource with information on polymorphism. If such resources do not exist, they can be generated from various protocols (see for instance Delord et al. 2018).
- The number of samples to be genotyped should ideally be a multiple of 96
- Choose a sequencing technology that allows reaching 100x read depth per locus per sample

## Summary

### 1 Foreword

The protocol that is described hereafter is adapted from Nguyen-Dumont et al. (2013) and Hammet et al. (2019). In this Summary section, potential alternatives to the protocol are mentioned *in italic*. These alternatives are not described later on (from section 2 onwards).

#### Protocol aim

The Hi-Plex protocol we describe allows the co-amplification and subsequent sequencing of targeted SNP panels (up to 456 SNPs in our trials), and runs through different steps: the synthesis and preparation of gene specific primers (GSPs) and adaptors (TSITs); a selective multiplex amplification of the SNP panel in a two-step PCR that allows building amplicons, the pooling of PCR products, a second PCR to increase the number of amplicon copies, interrupted with clean-up steps; quality control of the pooled libraries and their sequencing with custom sequencing primers. *The sequencing-specific sequences included in the library constructions allow for both Illumina or IonTorrent sequencing but we only tested and thereafter show the protocol for Illumina Miseq sequencing.*

#### Gene specific primers (GSPs)

GSPs are forward and reverse oligos designed from target fragments of 145bp for each SNP. Each resulting Gene Specific Primer (GSP) is built from a target specific sequence and a common heel used to add individual index and sequencing oligos (TSIT).

#### Adaptors (TSITs)

TSITs are adaptors that consist of a complementary sequence to the GSP common heel, a barcode, and a specific sequence for Illumina sequencing (P5/P7) for a total of about 60 bp for each TSIT A and TSIT P. The use of a combination of this 2 barcodes allows the specific identification of the DNA samples.

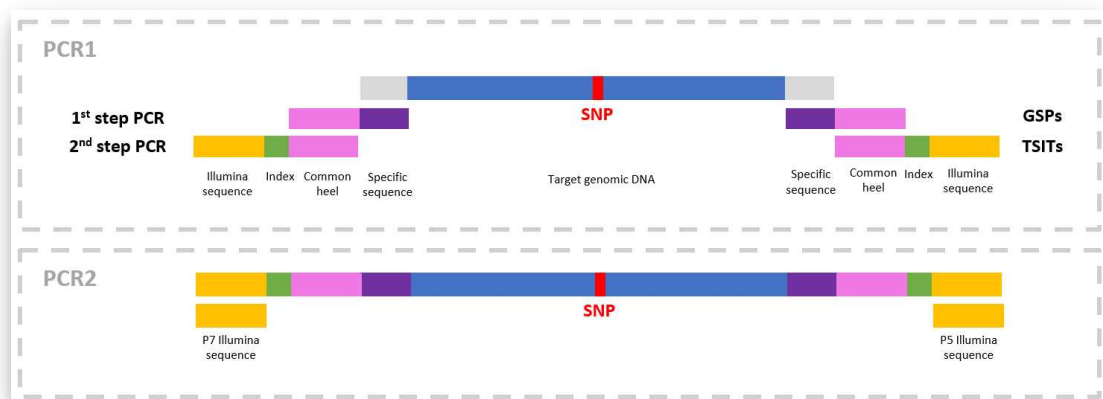
#### Library preparation

The first PCR uses a high fidelity hot start taq polymerase and is made of two phases. In the first phase, a pool of all GSPs (50 µM) is used, to which TSIT adaptors are added for the second phase. The first PCR phase starts with 8 cycles with 2 successive hybridization temperatures (TM) at 58°C and 60°C, followed by 4 cycles at 58°C. The second phase includes

4 cycles with a TM at 66 °C. The reaction is stopped by adding 100 mM EDTA at 72 °C and putting on ice.

The amplifications are pooled (the pooling unit is a 96-well plate) and purified using magnetic beads. Then, the second PCR is carried out using a high fidelity taq polymerase and the two Illumina's primers P5 and P7 at TM = 58 °C. The amplified fragments (250-300 bp) are size-selected by electrophoresis from an agarose gel colored with EtBr followed by extraction with a commercially available silica column or from an automatically size-selection system such as the Pipinn<sup>TM</sup> prep.

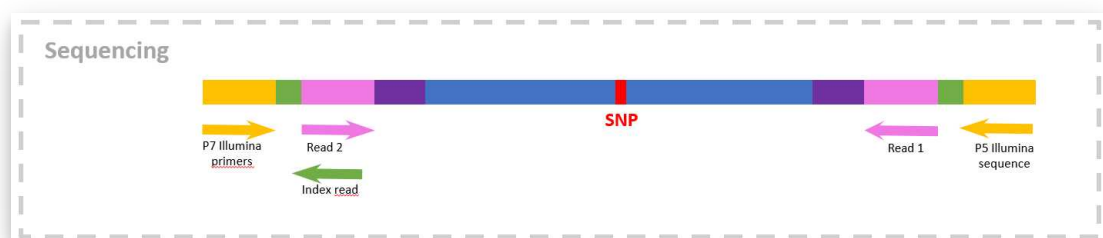
*The use of EtBr is essential if size selection from agarose gel is performed. SybrSafe reagent, for example, generated a lot of non-specific amplicons. This suggests that SybrSafe does not allow for clean separation of migrated fragments on agarose gels.*



Simplified schemes of PCR steps

### Quality controls and sequencing

Each library is checked on a Fragment Analyzer<sup>TM</sup> to estimate the purity of the amplification products. Library concentration is then measured by KAPA qPCR on a LightCycler according to the manufacturer protocol. All libraries are finally pooled together in equimolar quantities for sequencing performed on a Miseq platform (Illumina).



Simplified scheme of sequencing step

## 2 Gene Specific Primers (GSP) preparation

### GSP synthesis

- Prepare a fasta file with 145bp sequences that will be used to design GSPs. Sequence names should contain the position and polymorphism of the targeted SNP.

An example of one such sequence is:

>149126:289:-|73|[A/G]|LP\_LF\_hyb

```
CGGGCCGGCCCGTCCAAAAATAGCAAGCAGAGTGAAATCCTCCACATTCTAAATGGCCACATGCAC
AAACAAGCAGTGTTTTCTTAAAGAGACCACTGCTCCCAGCTCACCCAGCAGCCTCTCACAGACAGT
GGTGGGGGGGGGGG
```

- Send the file to Daniel Park (djp@unimelb.edu.au) and Bernie Pope (bjpope@unimelb.edu.au) for primer design.
- Add the following common heels to forward and reverse specific primers:  
Forward heel in 5'-3' : CTCTCTATGGGCAGTCGGTGATT  
Reverse heel in 5'-3' : CTGCGTGTCTCCGACTCAG

- Then, order GSPs in 96-well plates with a minimum yield of 10 nmol, purified using standard desalination with a purity of up to 70% to obtain 100 µL of 100-200 µM concentration in TE.

### GSP pool preparation

- Prepare 40 µM plates from each 100 or 200 µM GSP plate using 1X low TE (10 mM Tris pH8.0/0.1mM EDTA):

A	B	C	D	E
Ci (µM)	Cf (µM)	Vi (µL)	Vf (µL)	V low TE (µL)
100	40	20	50	30
200	40	10	50	40

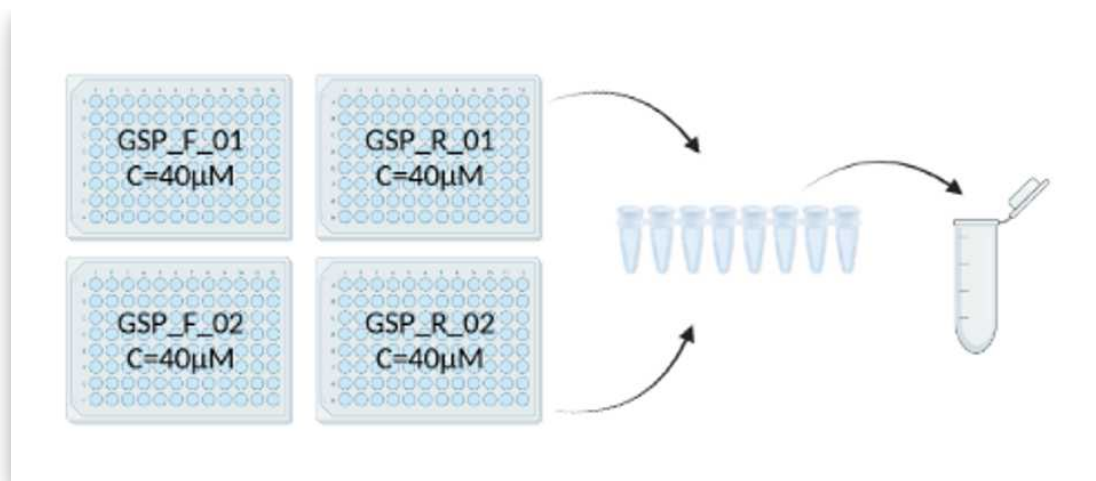
Ci: initial concentration; Cf: final concentration; Vi: initial volume; Vf: final volume

- Pool all forward and reverse GSPs.

First take 5 µL of each GSP-F and GSP-R at 40 µM and pool them by row in a 8-tube strip.

Then, pool the content of the strip into a 2 mL microcentrifuge tube. See diagram and table below.

Prepare as many aliquots as possible to avoid multiple freeze-thaw cycles.



A	B	C	D	E	F	G	H
SNP number in final pool	C <sub>i</sub> GSP (μM)	C <sub>f</sub> GSP (μM) =C <sub>i</sub> * V <sub>i</sub> /V <sub>f</sub> pool	V <sub>i</sub> GSP (μL)	Number of GSP =Nb F + Nb R	V <sub>f</sub> pool (μL) =V <sub>i</sub> GSP * Nb GSP	C <sub>f</sub> pool (μM) =C <sub>f</sub> GSP * Number of GSP	Nb aliquot 100 μL =V <sub>f</sub> pool/100 μL
For 96 SNP	40	0.208	5	192	960	40	9.6
For 192 SNP	40	0.104	5	384	1920	40	19.2
For 384 SNP	40	0.052	5	768	3840	40	38.4

- Prepare one tube of GSP-pool at 10 μM for PCR 1 in the Hi-Plex protocol.

A	B	C	D	E	F
Nb of 96-well PCR plates	C pool undiluted (μM)	C pool diluted (μM)	V <sub>i</sub> (μL)	V <sub>f</sub> (μL)	V low TE (μL)
1	40	10	12.5	50	37.5
2	40	10	25	100	75
3	40	10	37.5	150	112.5
4	40	10	50	200	150

- The tubes and plates should be stored at -20 °C.

### 3 Adaptator (TSITs) preparation

#### TSITs synthesis

- Order TSIT adaptors in two 96-well plates, one named "TSIT A" (these include the P5 sequence), and the other named "TSIT P" (these include the P7 sequence), with a purity of at least 80% in 100 μL at 200 μM concentration in TE. A minimum yield of 40 nmol is required.

The list of TSITs and the plate maps are given below.

TISIT_A	1	2	3	4	5	6	7	8	9	10	11	12	TISIT_P	1	2	3	4	5	6	7	8	9	10	11	12
A	N501	N509	N517										A	N701	N702	N703	N704	N705	N706	N707	N708	N709	N710	N711	N712
B	N502	N510	N518										B	N713	N714	N715	N716	N717	N718	N719	N720	N721	N722	N723	N724
C	N503	N511	N519										C	N725	N726	N727	N728	N729	N730	N731	N732	N733	N734	N735	N736
D	N504	N512	N520										D												
E	N505	N513	N521										E												
F	N506	N514	N522										F												
G	N507	N515	N523										G												
H	N508	N516	N524										H												

Schemes of the two TSITs plates

A	B	C
Plate well	TSITs P names	Sequences of TSITs P
A1	N701_TSIT_P	CAAGCAGAAGACGGCATACTGAGATTCGCCTTActccgctttcctctctatgggcagtcggtgat
A2	N702_TSIT_P	CAAGCAGAAGACGGCATACTGAGATCTAGTACGctccgctttcctctctatgggcagtcggtgat
A3	N703_TSIT_P	CAAGCAGAAGACGGCATACTGAGATTTCTGCCTctccgctttcctctctatgggcagtcggtgat
A4	N704_TSIT_P	CAAGCAGAAGACGGCATACTGAGATGCTCAGGActccgctttcctctctatgggcagtcggtgat
A5	N705_TSIT_P	CAAGCAGAAGACGGCATACTGAGATAGGAGTCCctccgctttcctctctatgggcagtcggtgat
A6	N706_TSIT_P	CAAGCAGAAGACGGCATACTGAGATCATGCCTActccgctttcctctctatgggcagtcggtgat
A7	N707_TSIT_P	CAAGCAGAAGACGGCATACTGAGATGTAGAGAGctccgctttcctctctatgggca gtcggtgat
A8	N708_TSIT_P	CAAGCAGAAGACGGCATACTGAGATCCTCTCTGctccgctttcctctctatgggcagtcggtgat
A9	N709_TSIT_P	CAAGCAGAAGACGGCATACTGAGATAGCGTAGCctccgctttcctctctatgggcagtcggtgat
A10	N710_TSIT_P	CAAGCAGAAGACGGCATACTGAGATCAGCCTCGctccgctttcctctctatgggcagtcggtgat
A11	N711_TSIT_P	CAAGCAGAAGACGGCATACTGAGATTGCCTCTTctccgctttcctctctatgggcagtcggtgat
A12	N712_TSIT_P	CAAGCAGAAGACGGCATACTGAGATTCCTCTACctccgctttcctctctatgggcagtcggtgat
B1	N713_TSIT_P	CAAGCAGAAGACGGCATACTGAGATCTTATCGCctccgctttcctctctatgggcagtcggtgat
B2	N714_TSIT_P	CAAGCAGAAGACGGCATACTGAGATTACGCTAGctccgctttcctctctatgggcagtcggtgat
B3	N715_TSIT_P	CAAGCAGAAGACGGCATACTGAGATGCCTTTCTctccgctttcctctctatgggcagtcggtgat
B4	N716_TSIT_P	CAAGCAGAAGACGGCATACTGAGATAGGAGCTCctccgctttcctctctatgggcagtcggtgat



A	B	C
B5	N717_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATGTCCAGGActccgctttcctctctatgggcag tcggtgat
B6	N718_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATCCTACATGctccgctttcctctctatgggca gtcggtgat
B7	N719_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATAGAGGTAGctccgctttcctctctatgggca gtcggtgat
B8	N720_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATTCTGCCTCctccgctttcctctctatgggcag tcggtgat
B9	N721_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATTAGCAGCGctccgctttcctctctatgggcag tcggtgat
B10	N722_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTCGCAGCctccgctttcctctctatgggcag tcggtgat
B11	N723_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATTCTTTGCCctccgctttcctctctatgggcag tcggtgat
B12	N724_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTACTCCTctccgctttcctctctatgggcag tcggtgat
C1	N725_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATGCCTTATCctccgctttcctctctatgggcag tcggtgat
C2	N726_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATAGTACGCTctccgctttcctctctatgggcag tcggtgat
C3	N727_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTGCCTTTctccgctttcctctctatgggcag tcggtgat
C4	N728_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATTCAGGAGCctccgctttcctctctatgggcag tcggtgat
C5	N729_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATGAGTCCAGctccgctttcctctctatgggcag tcggtgat
C6	N730_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATTGCCTACActccgctttcctctctatgggcag tcggtgat
C7	N731_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATAGAGAGGTctccgctttcctctctatgggca gtcggtgat
C8	N732_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATTCTCTGCCctccgctttcctctctatgggcag tcggtgat
C9	N733_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATCGTAGCAGctccgctttcctctctatgggcag tcggtgat
C10	N734_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATGCCTCGCActccgctttcctctctatgggcag tcggtgat
C11	N735_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATCCTCTTTGctccgctttcctctctatgggcag tcggtgat
C12	N736_ TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTCTACTCctccgctttcctctctatgggcag tcggtgat

List of TSITs P adaptors

A	B	C
<b>Plate well</b>	<b>TSITs A names</b>	<b>Sequences of TSITs A</b>

A	B	C
A1	N501_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTAGATCGCccatctcatccctg cgtgtctccgactcag
B1	N502_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTCTCTATccatctcatccctg cgtgtctccgactcag
C1	N503_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTATCCTCTccatctcatccctg cgtgtctccgactcag
D1	N504_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAGAGTAGAccatctcatccctg cgtgtctccgactcag
E1	N505_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGccatctcatccctg cgtgtctccgactcag
F1	N506_TSIT_A	AATGATACGGCGACCACCGAGATCTACACACTGCATAccatctcatccctg cgtgtctccgactcag
G1	N507_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAAGGAGTAccatctcatccctg cgtgtctccgactcag
H1	N508_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTccatctcatccctg cgtgtctccgactcag
A2	N509_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTCGCTAGAccatctcatccctg cgtgtctccgactcag
B2	N510_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTATCTCTccatctcatccctg cgtgtctccgactcag
C2	N511_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTCTTATCccatctcatccctg cgtgtctccgactcag
D2	N512_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTAGAAGAGccatctcatccctg cgtgtctccgactcag
E2	N513_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGGAGGTAAccatctcatccctg cgtgtctccgactcag
F2	N514_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCATAACTGccatctcatccctg cgtgtctccgactcag
G2	N515_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAGTAAAGGccatctcatccctg cgtgtctccgactcag
H2	N516_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGCCTCTAAccatctcatccctg cgtgtctccgactcag
A3	N517_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGATCGCTAccatctcatccctg cgtgtctccgactcag
B3	N518_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTCTATCTccatctcatccctg cgtgtctccgactcag
C3	N519_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTCCTCTTAccatctcatccctg cgtgtctccgactcag
D3	N520_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAGTAGAAGccatctcatccctg cgtgtctccgactcag
E3	N521_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAAGGAGGTccatctcatccctg cgtgtctccgactcag
F3	N522_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTGCATAACccatctcatccctg cgtgtctccgactcag
G3	N523_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGGAGTAAAccatctcatccctg cgtgtctccgactcag
H3	N524_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAAGCCTCTccatctcatccctg cgtgtctccgactcag

List of TSITs A adaptors

### TSITs preparation

- Make a new plate diluted to 50  $\mu\text{M}$  from each TSIT plate (TSIT\_A and TSIT\_P) at 100 or 200  $\mu\text{M}$  in 100  $\mu\text{L}$ /TSIT in TE: TSIT\_A\_50 $\mu\text{M}$  and TSIT\_P\_50 $\mu\text{M}$ .

A	B	C	D	E
Ci $\mu\text{M}$	Cf $\mu\text{M}$	Vi $\mu\text{L}$	Vf	V low TE
100	50	100	200	100
200	50	50	200	150

- Finally prepare 10  $\mu\text{M}$  plates by mixing 2 by 2 TSIT\_A with TSIT\_P to obtain 9 plates of different combinations (plates are named TSIT\_10 $\mu\text{M}$ \_1 to TSIT\_10 $\mu\text{M}$ \_9).

As the TSIT\_A are arranged columnwise in plate TSIT\_A\_50 $\mu\text{M}$  and TSIT\_P are sorted rowwise in plate TSIT\_P\_50 $\mu\text{M}$ , distribute 5 $\mu\text{L}$  of each column of TSIT\_A and each row of TSIT\_P in the 9 plates according to the table below.

A	B	C	D	E	F	G
Final plate at 10 $\mu\text{M}$	TSIT_A column number	Vol. of TSIT_A at 50 $\mu\text{M}$	TSIT_P row number	Vol. of TSIT_P à 50 $\mu\text{M}$	Vol. of low TE	Final volmue
TSIT_10 $\mu\text{M}$ _1	1	5	A	5	15	25
TSIT_10 $\mu\text{M}$ _2	1	5	B	5	15	25
TSIT_10 $\mu\text{M}$ _3	1	5	C	5	15	25
TSIT_10 $\mu\text{M}$ _4	2	5	A	5	15	25
TSIT_10 $\mu\text{M}$ _5	2	5	B	5	15	25
TSIT_10 $\mu\text{M}$ _6	2	5	C	5	15	25
TSIT_10 $\mu\text{M}$ _7	3	5	A	5	15	25
TSIT_10 $\mu\text{M}$ _8	3	5	B	5	15	25
TSIT_10 $\mu\text{M}$ _9	3	5	C	5	15	25

TSIT_1	1	2	3	4	5	6	7	8	9	10	11	12	TSIT_2	1	2	3	4	5	6	7	8	9	10	11	12	TSIT_3	1	2	3	4	5	6	7	8	9	10	11	12
A	0001	0002	0003	0004	0005	0006	0007	0008	0009	0010	0011	0012	A	0013	0014	0015	0016	0017	0018	0019	0020	0021	0022	0023	A	0024	0025	0026	0027	0028	0029	0030	0031	0032	0033	0034		
B	0013	0014	0015	0016	0017	0018	0019	0020	0021	0022	0023	0024	B	0025	0026	0027	0028	0029	0030	0031	0032	0033	0034	0035	B	0036	0037	0038	0039	0040	0041	0042	0043	0044	0045	0046		
C	0025	0026	0027	0028	0029	0030	0031	0032	0033	0034	0035	0036	C	0037	0038	0039	0040	0041	0042	0043	0044	0045	0046	0047	C	0048	0049	0050	0051	0052	0053	0054	0055	0056	0057	0058		
D	0037	0038	0039	0040	0041	0042	0043	0044	0045	0046	0047	0048	D	0049	0050	0051	0052	0053	0054	0055	0056	0057	0058	0059	D	0060	0061	0062	0063	0064	0065	0066	0067	0068	0069	0070		
E	0049	0050	0051	0052	0053	0054	0055	0056	0057	0058	0059	0060	E	0061	0062	0063	0064	0065	0066	0067	0068	0069	0070	0071	E	0072	0073	0074	0075	0076	0077	0078	0079	0080	0081	0082		
F	0061	0062	0063	0064	0065	0066	0067	0068	0069	0070	0071	0072	F	0073	0074	0075	0076	0077	0078	0079	0080	0081	0082	0083	F	0084	0085	0086	0087	0088	0089	0090	0091	0092	0093	0094		
G	0073	0074	0075	0076	0077	0078	0079	0080	0081	0082	0083	0084	G	0085	0086	0087	0088	0089	0090	0091	0092	0093	0094	0095	G	0096	0097	0098	0099	0100	0101	0102	0103	0104	0105	0106		
H	0085	0086	0087	0088	0089	0090	0091	0092	0093	0094	0095	0096	H	0097	0098	0099	0100	0101	0102	0103	0104	0105	0106	0107	H	0108	0109	0110	0111	0112	0113	0114	0115	0116	0117	0118		
I	0097	0098	0099	0100	0101	0102	0103	0104	0105	0106	0107	0108	I	0109	0110	0111	0112	0113	0114	0115	0116	0117	0118	I	0119	0120	0121	0122	0123	0124	0125	0126	0127	0128	0129			

TSIT_1	1	2	3	4	5	6	7	8	9	10	11	12	TSIT_2	1	2	3	4	5	6	7	8	9	10	11	12	TSIT_3	1	2	3	4	5	6	7	8	9	10	11	12
A	0001	0002	0003	0004	0005	0006	0007	0008	0009	0010	0011	0012	A	0013	0014	0015	0016	0017	0018	0019	0020	0021	0022	0023	A	0024	0025	0026	0027	0028	0029	0030	0031	0032	0033	0034		
B	0013	0014	0015	0016	0017	0018	0019	0020	0021	0022	0023	0024	B	0025	0026	0027	0028	0029	0030	0031	0032	0033	0034	0035	B	0036	0037	0038	0039	0040	0041	0042	0043	0044	0045	0046		
C	0025	0026	0027	0028	0029	0030	0031	0032	0033	0034	0035	0036	C	0037	0038	0039	0040	0041	0042	0043	0044	0045	0046	0047	C	0048	0049	0050	0051	0052	0053	0054	0055	0056	0057	0058		
D	0037	0038	0039	0040	0041	0042	0043	0044	0045	0046	0047	0048	D	0049	0050	0051	0052	0053	0054	0055	0056	0057	0058	0059	D	0060	0061	0062	0063	0064	0065	0066	0067	0068	0069	0070		
E	0049	0050	0051	0052	0053	0054	0055	0056	0057	0058	0059	0060	E	0061	0062	0063	0064	0065	0066	0067	0068	0069	0070	0071	E	0072	0073	0074	0075	0076	0077	0078	0079	0080	0081	0082		
F	0061	0062	0063	0064	0065	0066	0067	0068	0069	0070	0071	0072	F	0073	0074	0075	0076	0077	0078	0079	0080	0081	0082	0083	F	0084	0085	0086	0087	0088	0089	0090	0091	0092	0093	0094		
G	0073	0074	0075	0076	0077	0078	0079	0080	0081	0082	0083	0084	G	0085	0086	0087	0088	0089	0090	0091	0092	0093	0094	0095	G	0096	0097	0098	0099	0100	0101	0102	0103	0104	0105	0106		
H	0085	0086	0087	0088	0089	0090	0091	0092	0093	0094	0095	0096	H	0097	0098	0099	0100	0101	0102	0103	0104	0105	0106	0107	H	0108	0109	0110	0111	0112	0113	0114	0115	0116	0117	0118		
I	0097	0098	0099	0100	0101	0102	0103	0104	0105	0106	0107	0108	I	0109	0110	0111	0112	0113	0114	0115	0116	0117	0118	I	0119	0120	0121	0122	0123	0124	0125	0126	0127	0128	0129			

TSIT_1	1	2	3	4	5	6	7	8	9	10	11	12	TSIT_2	1	2	3	4	5	6	7	8	9	10	11	12	TSIT_3	1	2	3	4	5	6	7	8	9	10	11	12
A	0001	0002	0003	0004	0005	0006	0007	0008	0009	0010	0011	0012	A	0013	0014	0015	0016	0017	0018	0019	0020	0021	0022	0023	A	0024	0025	0026	0027	0028	0029	0030	0031	0032	0033	0034		
B	0013	0014	0015	0016	0017	0018	0019	0020	0021	0022	0023	0024	B	0025	0026	0027	0028	0029	0030	0031	0032	0033	0034	0035	B	0036	0037	0038	0039	0040	0041	0042	0043	0044	0045	0046		
C	0025	0026	0027	0028	0029	0030	0031	0032	0033	0034	0035	0036	C	0037	0038	0039	0040	0041	0042	0043	0044	0045	0046	0047	C	0048	0049	0050	0051	0052	0053	0054	0055	0056	0057	0058		
D	0037	0038	0039	0040	0041	0042	0043	0044	0045	0046	0047	0048	D	0049	0050	0051	0052	0053	0054	0055	0056	0057	0058	0059	D	0060	0061	0062	0063	0064	0065	0066	0067	0068	0069	0070		
E	0049	0050	0051	0052	0053	0054	0055	0056	0057	0058	0059	0060	E	0061	0062	0063	0064	0065	0066	0067	0068	0069	0070	0071	E	0072	0073	0074	0075	0076	0077	0078	0079	0080	0081	0082		
F	0061	0062	0063	0064	0065	0066	0067	0068	0069	0070	0071	0072	F	0073	0074	0075	0076	0077	0078	0079	0080	0081	0082	0083	F	0084	0085	0086	0087	0088	0089	0090	0091	0092	0093	0094		
G	0073	0074	0075	0076	0077	0078	0079	0080	0081	0082	0083	0084	G	0085	0086	0087	0088	0089	0090	0091	0092	0093	0094	0095	G	0096	0097	0098	0099	0100	0101	0102	0103	0104	0105	0106		
H	0085	0086	0087	0088	0089	0090	0091	0092	0093	0094	0095	0096	H	0097	0098	0099	0100	0101	0102	0103	0104	0105	0106	0107	H	0108	0109	0110	0111	0112	0113	0114	0115	0116	0117	0118		
I	0097	0098	0099	0100	0101	0102	0103	0104	0105	0106	0107	0108	I	0109	0110	0111	0112	0113	0114	0115	0116	0117	0118	I	0119	0120	0121	0122	0123	0124	0125	0126	0127	0128	0129			

Schemes of the nine final TSIT plates at 10μM

## PCR Preparation and Clean-Up for SNP genotyping using Hi...

### 4 Important informations before starting PCRs

Please read the GUIDELINES & WARNINGS to set up appropriate genotyping experiments. Make sure your number of samples is a multiple of 96 and the sequencing strategy you have chosen allows to reach 100X read depth for each sample at each locus. Note that replicating some samples can provide interesting information about genotyping quality (we run at least one set of 96 samples in triplicate for each new species or SNP panel we analyze).

Then,

- prepare a PCR plate layout,
- and determine the TSIT combinations that will be used for each sample/DNA plate. Make sure not to use the same TSIT plate twice in the same sequencing run. Each barcode combination must be unique.

For each PCR, we advise to report those informations in tables, for example:

A	B	C	D
PCR name	DNA plate name	TSIT plate name	PCR dates
<i>PCR_01</i>	DNA_01_rep1	TSIT_10μM_01	2023/xx/xx
<i>PCR_02</i>	DNA_01_rep2	TSIT_10μM_02	2023/xx/xx

### 5 PCR1 - SNP amplification step

-  On ice **PCR mix for one 96-well PCR plate**

=> Keep all reagents, PCR plate on ice or cold cube during all process.

1. Prepare the PCR mix following the table below. The PCR mix can be used right away or stored on ice or at 4 °C for several hours.
2. Distribute 2 μL of DNA (or control) into each well – make sure to design a plate layout for the

samples.


3. Distribute 21  $\mu\text{L}$  of mix to each well.
4. Cover the PCR plate with foil sealing films and keep on ice until PCR running.

A	B	C	D	E
Reagents	Initial conc.	Final conc.	Vol. for one reaction	Vol. for one 96-well plate
DNase/RNase free water	-	-	14	1372 $\mu\text{L}$
5X Buffer	5X	1X	5	490 $\mu\text{L}$
MgCl <sub>2</sub>	50mM	1mM	0.5	49 $\mu\text{L}$
dNTPs	20mM	400 $\mu\text{M}$	0.5	49 $\mu\text{L}$
Phusion HF Hot Start taq	2U/ $\mu\text{L}$	1U	0.5	49 $\mu\text{L}$
GSP pool	10 $\mu\text{M}$	0.2 $\mu\text{M}$	0.5	49 $\mu\text{L}$
<b>TOTAL Volume mix</b>			<b>21 <math>\mu\text{L}</math></b>	<b>2058 <math>\mu\text{L}</math></b>
<b>DNA or control per well</b>			<b>2 <math>\mu\text{L}</math></b>	


#### ■ PCR cycles

A	B	C
Temperature	Time	Steps information and Number of cycles
98°C	Needed to reach 98°C	Preheat step
98°C	01:00	x1
98°C	00:30	x8
58°C	02:30	
60°C	02:30	
72°C	01:00	
98°C	00:30	x4
58°C	02:30	
72°C	01:00	
72°C	Needed to add TSITs	
98°C	00:30	x4
66°C	02:00	

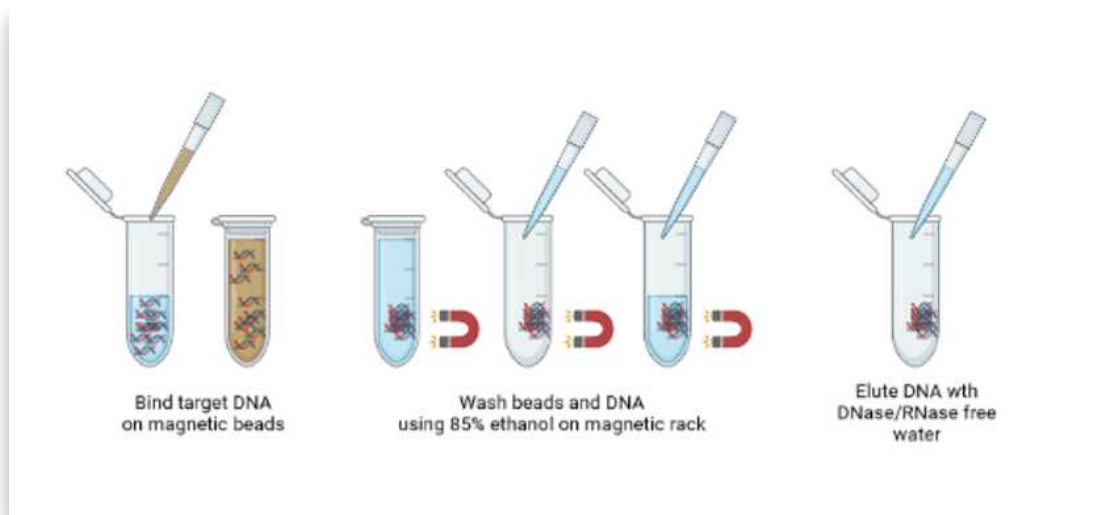
A	B	C
72°C	01:00	
72°C	Needed to add EDTA	

1. To limit aspecific amplification, the PCR program requires a pre-heating step at 98 °C. Start the PCR program without inserting the plate. Store the plate on ice until the heating block on the machine has reached 98 °C. Then, insert the plate.
2. At the 1st step at 72°C forever, stop the run and open the thermocycler. Keep the PCR plate in the PCR machine at 72 °C.
3. Take the appropriate TSIT\_10µM plates stored at 4 °C, centrifuge the plate, and carefully remove the sealing film.
4. Carefully remove the PCR plate sealing film.
5. Add 2.5 µL of each TSIT\_10µM to each column of the PCR plate with a multichannel pipette. Mix by carefully pipetting up and down. Change tips for each column.
6. Close the PCR and TSIT\_10µM plates with new sealing films. Restart the PCR program for the following steps.
7. At the final step at 72°C forever, stop the run and open the thermocycler. Keep the PCR plate in the PCR machine at 72 °C.
8. Take the EDTA 100mM in tube strips stored at 4°C, centrifuge the strip and carefully open up the caps.
9. Carefully remove the PCR plate sealing film.
10. Add 2.5 µL of EDTA 100mM to each column of the PCR plate with a multichannel pipette. Mix by carefully pipetting up and down. Change tips for each column.
11. Seal the plate with a new storage sealing foil film and put on ice immediately.
12.  On ice Plates are placed directly on ice and stored at 4°C until the next steps.

## 6 Pooling PCR products

1. Centrifuge each PCR plate before remove sealing foil film.
2.  On ice Prepare one 2mL tube per 96-well PCR plate. Use a multichannel pipette to transfer 20 µL of each PCR product to a tube strip. Combine all contents of the tube strip into a 2 mL microcentrifuge tube. For each 96-well plate, the final volume must reach 1,920 µL.
3. Store at 4 °C until the next step or at -20°C for longer storage.

## 7 Cleaning-up PCR pool on magnetic beads



Simplified scheme of clean-up PCR pool (Created with BioRender.com)

### Room temperature

Ensure magnetic beads have been placed at room temperature for at least 30 minutes before starting the clean-up step. All steps are run twice to ensure optimal library preparation.

#### ■ Bind target DNA fragment onto magnetic beads

To remove the smallest fragments and primer dimers, use a ratio 0.9/1 (beads/sample).

1. Transfer 960  $\mu\text{L}$  of PCR products to a new 2 mL tube. Leave the remaining 960  $\mu\text{L}$  at 4 °C until the end of the protocol or at -20 °C for longer term storage.
2. Add 864  $\mu\text{L}$  of presuspended magnetic beads ( $0.9 \times 960 \mu\text{L} = 864 \mu\text{L}$ ).
3. Mix up and down 10 times with the pipette.
4. Incubate at room temperature for 5 min.
5. Place the tube in a magnetic rack and wait until all the beads are against the side of the tube. This could take a few minutes.
6. Remove the supernatant without removing any bead.

#### ■ Wash the beads with 85% ethanol

1. Add 500  $\mu\text{L}$  of 85% ethanol to the beads and incubate at room temperature for 30 s.
2. Return the tube to the magnetic tube strip.
3. Remove the supernatant without removing any bead.
4. Repeat step 1 (second washing step).
5. Let beads dry on the magnetic rack for 5-15 min at room temperature.

#### ■ Elute DNA

1. To elute DNA, remove the tubes from the magnetic rack.
2. Add 70  $\mu\text{L}$  of DNase/RNase free water and mix by pipetting up and down 10 times.
3. Incubate at room temperature for 1-5 min.
4. Place the tube to the magnetic rack and wait for the beads to be against the magnetic strip.
5. Transfer the 70  $\mu\text{L}$  of water containing target DNA into a new 1.5 or 2 mL tube.

### ■ Bind eluted DNA onto magnetic beads

1. Add 63µL of presuspend magnetic beads (0.9x70µL=63µL) to the previously eluted DNA.
2. Mix up and down 10 times.
3. Incubate at room temperature for 5 min.
4. Place the tube in a magnetic rack and wait until all the beads are against the side of the tube.  
This could take a few minutes.
5. Remove the supernatant without removing any bead.

### ■ Wash the beads with 85% ethanol

1. Add 180 µL of 85% ethanol to the beads and incubate at room temperature for 30 s.
2. Return tube to the magnetic tube strip.
3. Remove the supernatant without removing any bead.
4. Let beads dry on magnetic rack for 5-15 min at room temperature.

### ■ Elute DNA

1. To elute DNA, remove the tubes from the magnetic rack.
2. Add 70 µL of DNase/RNase free water and mix by pipetting up and down 10 times.
3. Incubate at room temperature for 1-5 min.
4. Place the tube to the magnetic rack and wait for the beads to be against the magnetic strip.
5. Transfer the 70µl of water containing target DNA into a new 1.5 mL tube.

## 8 PCR2 - Fragment enrichment

### ■ PCR mix

=> *Keep all reagents, PCR plate on ice or cold cube during all process.*

1. Prepare the PCR mix followong indications described in the table below. Adjust volumes to the number of purified PCR1 product and controls needed. The PCR mix can be used right away or stored on ice or at 4 °C for several hours.
2. Distribute 10 µL of purified PCR1 product (or control) to each well in strip-tubes.
3. Distribute 45 µL of mix to each well.
4. Cap each tube.

A	B	C	D
Reagents	Initial conc.	Final conc.	Vol. for one reaction
DNase/RNase free water	-	-	22.5
5 X Green buffer	5X	1X	15
MgCl <sub>2</sub>	50 mM	2.5 mM	1.5
dNTPs	20 mM	400 µM	1.5
Phusion HF Hot Start taq	2U/µL	1U	1.5
P5 primer : 5'-AATGATACGGCGACCACCGA-3'	50 µM	1 µM	1.5



A	B	C	D
P7 primer : 5'-CAAGCAGAAGACGGCATAGCA-3'	50µM	1 µM	1.5
<b><i>TOTAL volume mix</i></b>			<b><i>45</i></b>
<b><i>Purified PCR1 product or control</i></b>			<b><i>10</i></b>

#### ■ PCR cycles

Program the PCR machine according to the following table :

A	B	C
Temperature	Time	Number of cycles
98°C	Needed to reach 98°C	Preheat step
98°C	05:00	
98°C	00:30	X30
58°C	01:00	
72°C	01:00	
72°C	07:00	
15°C	Forever	

## 9 Size selection

### Option 1: Size selection by electrophoresis in agarose gel

#### ■ Agarose gel electrophoresis

Important: the electrophoresis comb must afford 55 µL loading volumes.

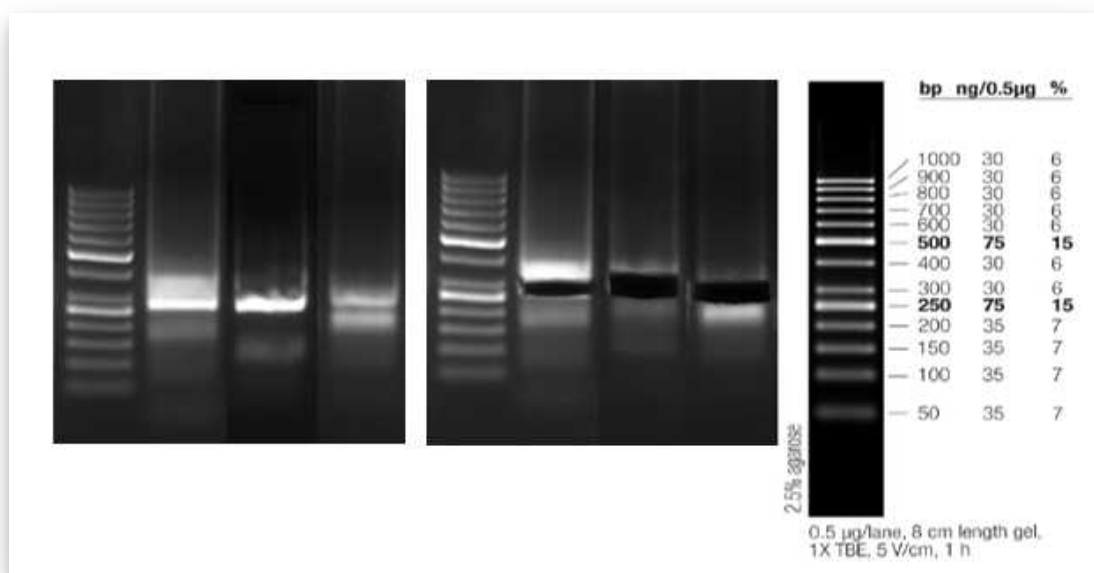
1. Prepare a 1.75% agarose gel using 1X TAE and ethidium bromide (EtBr).
2. Add directly 55µL of each sample to a well (possible due to the use of the green buffer in previous PCR2 step)
3. Add 10 µL of the 50 bp ladder to at least one well (adapt to the number of samples or your gel size).
4. Run the gel at 100V for about 50-75 min to get more than 5 cm migration.

#### ■ Size selection

1. Cut out an agarose gel band between 250-300 bp with a scalpel for each sample.
2. Weigh an empty 2 mL microcentrifuge tube. Then, add the gel slice to the tube and re-weigh.
3. Note the weigh of the agarose gel slice to adapt the correct volume of NTI buffer in next step.

#### ■ DNA purification on silica column


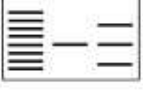







1. Use the MN Nucleospin gel clean-up kit using the gel extraction protocol supplied by the manufacturer.
2. Prepare the NT3 and add the appropriate volume of 96-100% ethanol.
3. Adapt the volume of NTI buffer for each sample following : Vol. NTI = 2x agarose weight.
4. Elute with 2 x 25  $\mu$ L of buffer NE (50  $\mu$ L total).
5. Store at 4 °C.
6. The samples are now ready for the sequencing platform.



Example of 1.75% agarose gel before and after cutting 250-300bp bands.

# PCR clean-up, gel extraction

Protocol at a glance (Rev. 07)

	PCR clean-up	Gel extraction	DNA clean-up (with SDS)	Single stranded DNA clean-up
<b>1 PCR clean-up, DNA clean-up, or single stranded DNA clean-up:</b> Adjust binding condition  <b>Gel extraction:</b> Excise DNA fragment / solubilize gel slice		 		
	200 µL NT1/ 100 µL PCR	200 µL NT1/ 100 mg gel  50 °C 5–10 min	500 µL NTB/ 100 µL sample	200 µL NTC/ 100 µL sample
<b>2 Bind DNA</b>			11,000 x g 30 s	
<b>3 Wash silica membrane</b>			700 µL NT3 11,000 x g 30 s <u>Recommended:</u> 2 <sup>nd</sup> wash 700 µL NT3 11,000 x g 30 s	
<b>4 Dry silica membrane</b>			11,000 x g 1 min	
<b>5 Elute DNA</b>			15–30 µL NE RT 1 min 11,000 x g 1 min	

Extract from NucleoSpin® Gel and PCR Clean-up manual, Version of April 2022 / Rev. 07

## Option 2: Size selection by Pippin™ prep

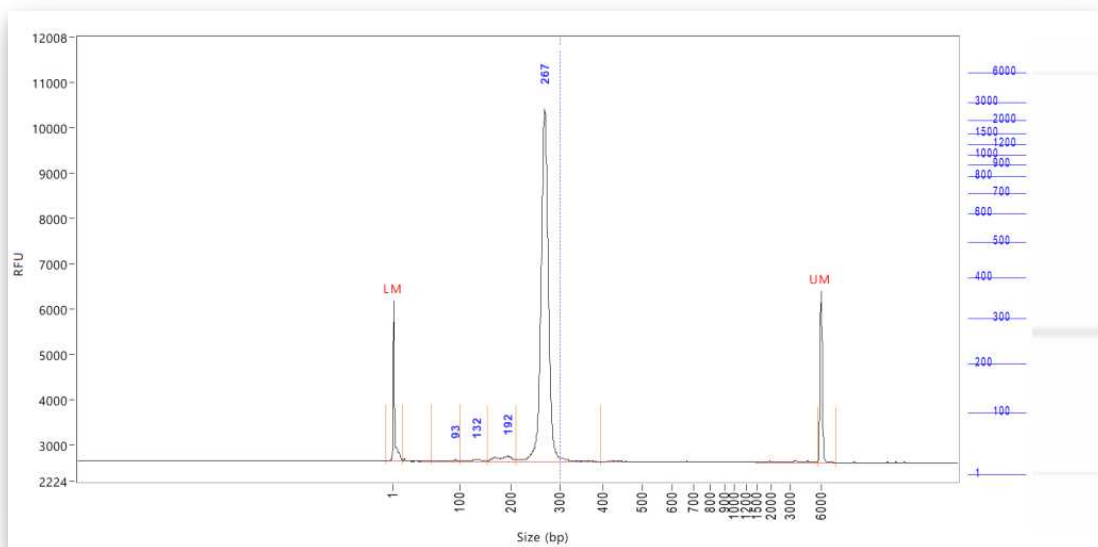
1. Bring each DNA sample from PCR2 up to 60µl with water.
2. Combine 60µl of DNA sample with 20µl of loading/marker mix (Marker L).

3. Mix samples thoroughly (vortex mixer). Briefly centrifuge to collect.
4. Remove 40µl of buffer from two "**Sample Well**" of the cassette, and load 40µl of sample into each well (2 wells per sample).
5. For each sample cut out band between 250-300 bp with "Range" option in Pippin™ Prep software.
6. After migration, remove 40µl of sample from the two "**Elution Module**" of the cassette.

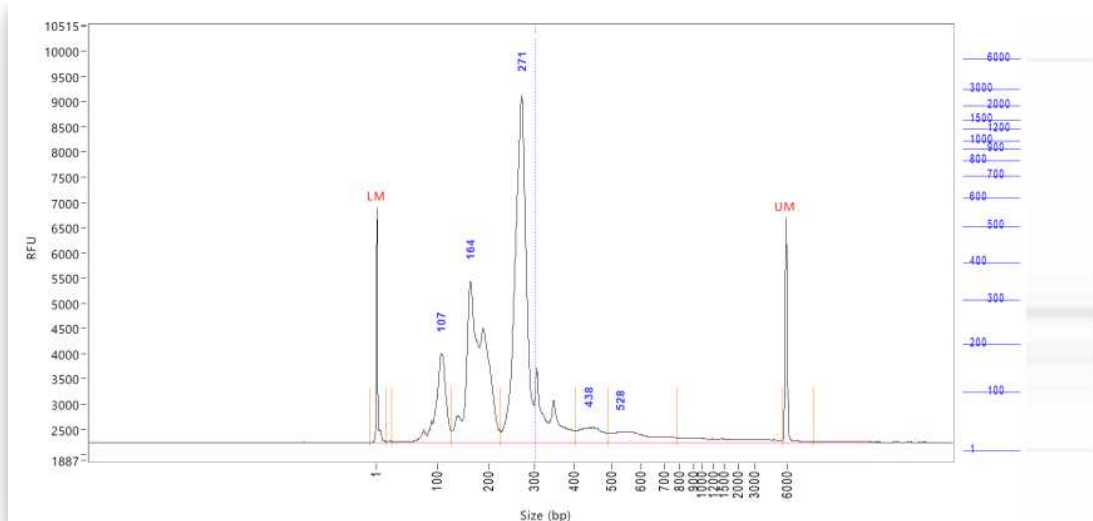
## Quality and quantity control of libraries

### 10 Check the purity of each library on a Fragment Analyzer™

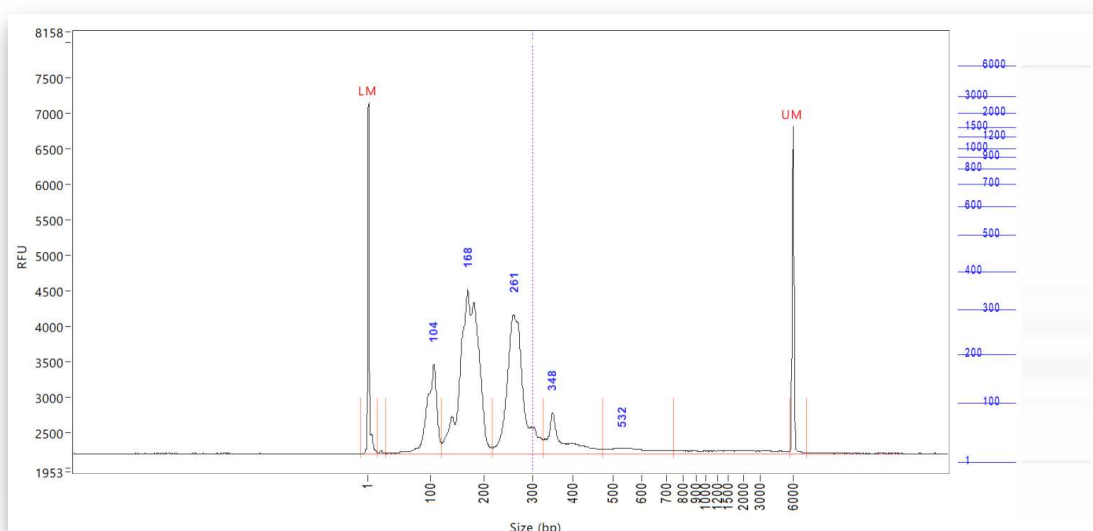
1. Use Invitrogen™ Qubit™ Fluorometer or equivalent to estimate DNA concentrations for each library
2. Prepare a 50pg/µL to 5ng/µL dilution of each library.
3. Then, follow the instructions from the manufacturer.



Example of conform library (267bp) presenting a very low contamination by primer dimers (<200bp).



Example of library (274bp) presenting a large amount of primer dimers (<200bp).



Example of non conform library presenting more primer dimers (<200bp) than target DNA (250-300bp).

## 11 Determine the quantity of DNA available for each library

1. Determine the DNA concentration of each library following the instruction of the KAPA Library Quantification Kit Illumina® Platforms (Roche Sequencing Solutions).
2. Based on concentrations measured for each library, make a final pool of equimolar concentrations.

**This final pool is ready for sequencing following Illumina instructions.**

## 12 Informations for samplesheets

Report the corresponding "barcod P complement" and "barcod A" according to TSIT plate added at PCR1 step.

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
Plat e we ll	TSI T_1	TS IT_1	TSI T_2	TS IT_2	TSI T_3	TS IT_3	TSI T_4	TS IT_4	TSI T_5	TS IT_5	TSI T_6	TS IT_6	TSI T_7	TS IT_7	TSI T_8	TS IT_8	TSI T_9	TS IT_9
	P co mpl em ent	Bar cod A	P co mpl em ent	Bar cod A	P co mpl em ent	Bar cod A	P co mpl em ent	Bar cod A	P co mpl em ent	Bar cod A	P co mpl em ent	Bar cod A	P co mpl em ent	Bar cod A	P co mpl em ent	Bar cod A	P co mpl em ent	Bar cod A
A1	TA AG GC GA	TA GA TC GC	GC GA TA AG	TA GA TC GC	GA TA AG GC	TA GA TC GC	TA AG GC GA	TC GC TA GA	GC GA TA AG	TC GC TA GA	GA TA AG GC	TC GC TA GA	TA AG GC GA	GA TC GC TA	GC GA TA AG	GA TC GC TA	GA TA AG GC	GA TC GC TA
B1	TA AG GC GA	CT CT CT AT	GC GA TA AG	CT CT CT AT	GA TA AG GC	CT CT CT AT	TA AG GC GA	CT AT CT CT	GC GA TA AG	CT AT CT CT	GA TA AG GC	CT AT CT CT	TA AG GC GA	CT CT AT CT	GC GA TA AG	CT CT AT CT	GA TA AG GC	CT CT AT CT
C1	TA AG GC GA	TA TC CT CT	GC GA TA AG	TA TC CT CT	GA TA AG GC	TA TC CT CT	TA AG GC GA	CT CT TA TC	GC GA TA AG	CT CT TA TC	GA TA AG GC	CT CT TA TC	TA AG GC GA	TC CT CT TA	GC GA TA AG	TC CT CT TA	GA TA AG GC	TC CT CT TA
D1	TA AG GC GA	AG AG TA GA	GC GA TA AG	AG AG TA GA	GA TA AG GC	AG AG TA GA	TA AG GC GA	TA GA AG AG	GC GA TA AG	TA GA AG AG	GA TA AG GC	TA GA AG AG	TA AG GC GA	AG TA GA AG	GC GA TA AG	AG TA GA AG	GA TA AG GC	AG TA GA AG
E1	TA AG GC GA	GT AA GG AG	GC GA TA AG	GT AA GG AG	GA TA AG GC	GT AA GG AG	TA AG GC GA	GG AG GT AA	GC GA TA AG	GG AG GT AA	GA TA AG GC	GG AG GT AA	TA AG GC GA	AA GG AG GT	GC GA TA AG	AA GG AG GT	GA TA AG GC	AA GG AG GT
F1	TA AG GC GA	AC TG CA TA	GC GA TA AG	AC TG CA TA	GA TA AG GC	AC TG CA TA	TA AG GC GA	CA TA AC TG	GC GA TA AG	CA TA AC TG	GA TA AG GC	CA TA AC TG	TA AG GC GA	TG CA TA AC	GC GA TA AG	TG CA TA AC	GA TA AG GC	TG CA TA AC
G1	TA AG GC GA	AA GG AG TA	GC GA TA AG	AA GG AG TA	GA TA AG GC	AA GG AG TA	TA AG GC GA	AG TA AA GG	GC GA TA AG	AG TA AA GG	GA TA AG GC	AG TA AA GG	TA AG GC GA	GG AG TA AA	GC GA TA AG	GG AG TA AA	GA TA AG GC	GG AG TA AA
H1	TA AG GC GA	CT AA GC CT	GC GA TA AG	CT AA GC CT	GA TA AG GC	CT AA GC CT	TA AG GC GA	GC CT CT AA	GC GA TA AG	GC CT CT AA	GA TA AG GC	GC CT CT AA	TA AG GC GA	AA GC CT CT	GC GA TA AG	AA GC CT CT	GA TA AG GC	AA GC CT CT
A2	CG TA CT AG	TA GA TC GC	CT AG CG TA	TA GA TC GC	AG CG TA CT	TA GA TC GC	CG TA CT AG	TC GC TA GA	CT AG CG TA	TC GC TA GA	AG CG TA CT	TC GC TA GA	CG TA CT AG	GA TC GC TA	CT AG CG TA	GA TC GC TA	AG CG TA CT	GA TC GC TA

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
	B2	CG TA CT AG	CT CT CT AT	CT AG CG TA	CT CT CT AT	AG CG TA CT	CT CT CT AT	CG TA CT AG	CT AT CT CT	CT AG CG TA	CT AT CT CT	AG CG TA CT	CT AT CT CT	CG TA CT AG	CT CT AT CT	CT AG CG TA	CT CT AT CT	AG CG TA CT	CT AT CT
	C2	CG TA CT AG	TA TC CT CT	CT AG CG TA	TA TC CT CT	AG CG TA CT	TA TC CT CT	CG TA CT AG	CT CT TA TC	CT AG CG TA	CT CT TA TC	AG CG TA CT	CT CT TA TC	CG TA CT AG	TC CT CT TA	CT AG CG TA	TC CT CT TA	AG CG TA CT	TC CT CT TA
	D2	CG TA CT AG	AG AG TA GA	CT AG CG TA	AG AG TA GA	AG CG TA CT	AG AG TA GA	CG TA CT AG	TA GA AG AG	CT AG CG TA	TA GA AG AG	AG CG TA CT	TA GA AG AG	CG TA CT AG	AG TA GA AG	CT AG CG TA	AG TA GA AG	AG CG TA CT	AG TA GA AG
	E2	CG TA CT AG	GT AA GG AG	CT AG CG TA	GT AA GG AG	AG CG TA CT	GT AA GG AG	CG TA CT AG	GG AG GT AA	CT AG CG TA	GG AG GT AA	AG CG TA CT	GG AG GT AA	CG TA CT AG	AA GG AG GT	CT AG CG TA	AA GG AG GT	AG CG TA CT	AA GG AG GT
	F2	CG TA CT AG	AC TG CA TA	CT AG CG TA	AC TG CA TA	AG CG TA CT	AC TG CA TA	CG TA CT AG	CA TA AC TG	CT AG CG TA	CA TA AC TG	AG CG TA CT	CA TA AC TG	CG TA CT AG	TG CA TA AC	CT AG CG TA	TG CA TA AC	AG CG TA CT	TG CA TA AC
	G2	CG TA CT AG	AA GG AG TA	CT AG CG TA	AA GG AG TA	AG CG TA CT	AA GG AG TA	CG TA CT AG	AG TA AA GG	CT AG CG TA	AG TA AA GG	AG CG TA CT	AG TA AA GG	CG TA CT AG	GG AG TA AA	CT AG CG TA	GG AG TA AA	AG CG TA CT	GG AG TA AA
	H2	CG TA CT AG	CT AA GC CT	CT AG CG TA	CT AA GC CT	AG CG TA CT	CT AA GC CT	CG TA CT AG	GC CT CT AA	CT AG CG TA	GC CT CT AA	AG CG TA CT	GC CT CT AA	CG TA CT AG	AA GC CT CT	CT AG CG TA	AA GC CT CT	AG CG TA CT	AA GC CT CT
	A3	AG GC AG AA	TA GA TC GC	AG AA AG GC	TA GA TC GC	AA AG GC AG	TA GA TC GC	AG GC AG AA	TC GC TA GA	AG AA AG GC	TC GC TA GA	AA AG GC AG	TC GC TA GA	AG GC AG AA	GA TC GC TA	AG AA AG GC	GA TC GC TA	AA AG GC AG	GA TC GC TA
	B3	AG GC AG AA	CT CT CT AT	AG AA AG GC	CT CT CT AT	AA AG GC AG	CT CT CT AT	AG GC AG AA	CT AT CT CT	AG AA AG GC	CT AT CT CT	AA AG GC AG	CT AT CT CT	AG GC AG AA	CT CT AT CT	AG AA AG GC	CT CT AT CT	AA AG GC AG	CT CT AT CT
	C3	AG GC AG AA	TA TC CT CT	AG AA AG GC	TA TC CT CT	AA AG GC AG	TA TC CT CT	AG GC AG AA	CT CT TA TC	AG AA AG GC	CT CT TA TC	AA AG GC AG	CT CT TA TC	AG GC AG AA	TC CT CT TA	AG AA AG GC	TC CT CT TA	AA AG GC AG	TC CT CT TA
	D3	AG GC AG AA	AG AG TA GA	AG AA AG GC	AG AG TA GA	AA AG GC AG	AG AG TA GA	AG GC AG AA	TA GA AG AG	AG AA AG GC	TA GA AG AG	AA AG GC AG	TA GA AG AG	AG GC AG AA	AG TA GA AG	AG AA AG GC	AG TA GA AG	AA AG GC AG	AG TA GA AG
	E3	AG GC AG AA	GT AA GG AG	AG AA AG GC	GT AA GG AG	AA AG GC AG	GT AA GG AG	AG GC AG AA	GG AG GT AA	AG AA AG GC	GG AG GT AA	AA AG GC AG	GG AG GT AA	AG GC AG AA	AA GG AG GT	AG AA AG GC	AA GG AG GT	AA AG GC AG	AA GG AG GT
	F3	AG GC AG AA	AC TG CA TA	AG AA AG GC	AC TG CA TA	AA AG GC AG	AC TG CA TA	AG GC AG AA	CA TA AC TG	AG AA AG GC	CA TA AC TG	AA AG GC AG	CA TA AC TG	AG GC AG AA	TG CA TA AC	AG AA AG GC	TG CA TA AC	AA AG GC AG	TG CA TA AC
	G3	AG GC AG AA	AA GG AG TA	AG AA AG GC	AA GG AG TA	AA AG GC AG	AA GG AG TA	AG GC AG AA	AG TA AA GG	AG AA AG GC	AG TA AA GG	AA AG GC AG	AG TA AA GG	AG GC AG AA	GG AG TA AA	AG AA AG GC	GG AG TA AA	AA AG GC AG	GG AG TA AA

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
H3	AG GC AG AA	CT AA GC CT	AG AA AG GC	CT AA GC CT	AA AG GC AG	CT AA GC CT	AG GC AG AA	GC CT CT AA	AG AA AG GC	GC CT CT AA	AA AG GC AG	GC CT CT AA	AG GC AG AA	AA GC CT CT	AG AA AG GC	AA GC CT CT	AA AG GC AG	AA GC CT CT
A4	TC CT GA GC	TA GA TC GC	GA GC TC CT	TA GA TC GC	GC TC CT GA	TA GA TC GC	TC CT GA GC	TC GC TA GA	GA GC TC CT	TC GC TA GA	GC TC CT GA	TC GC TA GA	TC CT GA GC	GA TC GC TA	GA GC TC CT	GA TC GC TA	GC TC CT GA	GA TC GC TA
B4	TC CT GA GC	CT CT CT AT	GA GC TC CT	CT CT CT AT	GC TC CT GA	CT CT CT AT	TC CT GA GC	CT AT CT CT	GA GC TC CT	CT AT CT CT	GC TC CT GA	CT AT CT CT	TC CT GA GC	CT CT AT CT	GA GC TC CT	CT CT AT CT	GC TC CT GA	CT CT AT CT
C4	TC CT GA GC	TA TC CT CT	GA GC TC CT	TA TC CT CT	GC TC CT GA	TA TC CT CT	TC CT GA GC	CT CT TA TC	GA GC TC CT	CT CT TA TC	GC TC CT GA	CT CT TA TC	TC CT GA GC	TC CT CT TA	GA GC TC CT	TC CT CT TA	GC TC CT GA	TC CT CT TA
D4	TC CT GA GC	AG AG TA GA	GA GC TC CT	AG AG TA GA	GC TC CT GA	AG AG TA GA	TC CT GA GC	TA GA AG AG	GA GC TC CT	TA GA AG AG	GC TC CT GA	TA GA AG AG	TC CT GA GC	AG TA GA AG	GA GC TC CT	AG TA GA AG	GC TC CT GA	AG TA GA AG
E4	TC CT GA GC	GT AA GG AG	GA GC TC CT	GT AA GG AG	GC TC CT GA	GT AA GG AG	TC CT GA GC	GG AG GT AA	GA GC TC CT	GG AG GT AA	GC TC CT GA	GG AG GT AA	TC CT GA GC	AA GG AG GT	GA GC TC CT	AA GG AG GT	GC TC CT GA	AA GG AG GT
F4	TC CT GA GC	AC TG CA TA	GA GC TC CT	AC TG CA TA	GC TC CT GA	AC TG CA TA	TC CT GA GC	CA TA AC TG	GA GC TC CT	CA TA AC TG	GC TC CT GA	CA TA AC TG	TC CT GA GC	TG CA TA AC	GA GC TC CT	TG CA TA AC	GC TC CT GA	TG CA TA AC
G4	TC CT GA GC	AA GG AG TA	GA GC TC CT	AA GG AG TA	GC TC CT GA	AA GG AG TA	TC CT GA GC	AG TA AA GG	GA GC TC CT	AG TA AA GG	GC TC CT GA	AG TA AA GG	TC CT GA GC	GG AG TA AA	GA GC TC CT	GG AG TA AA	GC TC CT GA	GG AG TA AA
H4	TC CT GA GC	CT AA GC CT	GA GC TC CT	CT AA GC CT	GC TC CT GA	CT AA GC CT	TC CT GA GC	GC CT CT AA	GA GC TC CT	GC CT CT AA	GC TC CT GA	GC CT CT AA	TC CT GA GC	AA GC CT CT	GA GC TC CT	AA GC CT CT	GC TC CT GA	AA GC CT CT
A5	GG AC TC CT	TA GA TC GC	TC CT GG AC	TA GA TC GC	CT GG AC TC	TA GA TC GC	GG AC TC CT	TC GC TA GA	TC CT GG AC	TC GC TA GA	CT GG AC TC	TC GC TA GA	GG AC TC CT	GA TC GC TA	TC CT GG AC	GA TC GC TA	CT GG AC TC	GA TC GC TA
B5	GG AC TC CT	CT CT CT AT	TC CT GG AC	CT CT CT AT	CT GG AC TC	CT CT CT AT	GG AC TC CT	CT AT CT CT	TC CT GG AC	CT AT CT CT	CT GG AC TC	CT AT CT CT	GG AC TC CT	CT AT CT CT	TC CT GG AC	CT CT AT CT	CT GG AC TC	CT CT AT CT
C5	GG AC TC CT	TA TC CT CT	TC CT GG AC	TA TC CT CT	CT GG AC TC	TA TC CT CT	GG AC TC CT	CT CT TA TC	TC CT GG AC	CT CT TA TC	CT GG AC TC	CT CT TA TC	GG AC TC CT	TC CT CT TA	TC CT GG AC	TC CT CT TA	CT GG AC TC	TC CT CT TA
D5	GG AC TC CT	AG AG TA GA	TC CT GG AC	AG AG TA GA	CT GG AC TC	AG AG TA GA	GG AC TC CT	TA GA AG AG	TC CT GG AC	TA GA AG AG	CT GG AC TC	TA GA AG AG	GG AC TC CT	AG TA GA AG	TC CT GG AC	AG TA GA AG	CT GG AC TC	AG TA GA AG
E5	GG AC TC CT	GT AA GG AG	TC CT GG AC	GT AA GG AG	CT GG AC TC	GT AA GG AG	GG AC TC CT	GG AG GT AA	TC CT GG AC	GG AG GT AA	CT GG AC TC	GG AG GT AA	GG AC TC CT	AA GG AG GT	TC CT GG AC	AA GG AG GT	CT GG AC TC	AA GG AG GT



A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
F5	GG AC TC CT	AC TG CA TA	TC CT GG AC	AC TG CA TA	CT GG AC TC	AC TG CA TA	GG AC TC CT	CA TA AC TG	TC CT GG AC	CA TA AC TG	CT GG AC TC	CA TA AC TG	GG AC TC CT	TG CA TA AC	TC CT GG AC	TG CA TA AC	CT GG AC TC	TG CA TA AC
G5	GG AC TC CT	AA GG AG TA	TC CT GG AC	AA GG AG TA	CT GG AC TC	AA GG AG TA	GG AC TC CT	AG TA AA GG	TC CT GG AC	AG TA AA GG	CT GG AC TC	AG TA AA GG	GG AC TC CT	GG AG TA AA	TC CT GG AC	GG AG TA AA	CT GG AC TC	GG AG TA AA
H5	GG AC TC CT	CT AA GC CT	TC CT GG AC	CT AA GC CT	CT GG AC TC	CT AA GC CT	GG AC TC CT	GC CT CT AA	TC CT GG AC	GC CT CT AA	CT GG AC TC	GC CT CT AA	GG AC TC CT	AA GC CT CT	TC CT GG AC	AA GC CT CT	CT GG AC TC	AA GC CT CT
A6	TA GG CA TG	TA GA TC GC	CA TG TA GG	TA GA TC GC	TG TA GG CA	TA GA TC GC	TA GG CA TG	TC GC TA GA	CA TG TA GG	TC GC TA GA	TG TA GG CA	TC GC TA GA	TA GG CA TG	GA TC GC TA	CA TG TA GG	GA TC GC TA	TG TA GG CA	GA TC GC TA
B6	TA GG CA TG	CT CT CT AT	CA TG TA GG	CT CT CT AT	TG TA GG CA	CT CT CT AT	TA GG CA TG	CT AT CT CT	CA TG TA GG	CT AT CT CT	TG TA GG CA	CT AT CT CT	TA GG CA TG	CT CT AT CT	CA TG TA GG	CT CT AT CT	TG TA GG CA	CT CT AT CT
C6	TA GG CA TG	TA TC CT CT	CA TG TA GG	TA TC CT CT	TG TA GG CA	TA TC CT CT	TA GG CA TG	CT CT TA TC	CA TG TA GG	CT CT TA TC	TG TA GG CA	CT CT TA TC	TA GG CA TG	TC CT CT TA	CA TG TA GG	TC CT CT TA	TG TA GG CA	TC CT CT TA
D6	TA GG CA TG	AG AG TA GA	CA TG TA GG	AG AG TA GA	TG TA GG CA	AG AG TA GA	TA GG CA TG	TA GA AG AG	CA TG TA GG	TA GA AG AG	TG TA GG CA	TA GA AG AG	TA GG CA TG	AG TA GA AG	CA TG TA GG	AG TA GA AG	TG TA GG CA	AG TA GA AG
E6	TA GG CA TG	GT AA GG AG	CA TG TA GG	GT AA GG AG	TG TA GG CA	GT AA GG AG	TA GG CA TG	GG AG GT AA	CA TG TA GG	GG AG GT AA	TG TA GG CA	GG AG GT AA	TA GG CA TG	AA GG AG GT	CA TG TA GG	AA GG AG GT	TG TA GG CA	AA GG AG GT
F6	TA GG CA TG	AC TG CA TA	CA TG TA GG	AC TG CA TA	TG TA GG CA	AC TG CA TA	TA GG CA TG	CA TA AC TG	CA TG TA GG	CA TA AC TG	TG TA GG CA	CA TA AC TG	TA GG CA TG	TG CA TA AC	CA TG TA GG	TG CA TA AC	TG TA GG CA	TG CA TA AC
G6	TA GG CA TG	AA GG AG TA	CA TG TA GG	AA GG AG TA	TG TA GG CA	AA GG AG TA	TA GG CA TG	AG TA AA GG	CA TG TA GG	AG TA AA GG	TG TA GG CA	AG TA AA GG	TA GG CA TG	GG AG TA AA	CA TG TA GG	GG AG TA AA	TG TA GG CA	GG AG TA AA
H6	TA GG CA TG	CT AA GC CT	CA TG TA GG	CT AA GC CT	TG TA GG CA	CT AA GC CT	TA GG CA TG	GC CT CT AA	CA TG TA GG	GC CT CT AA	TG TA GG CA	GC CT CT AA	TA GG CA TG	AA GC CT CT	CA TG TA GG	AA GC CT CT	TG TA GG CA	AA GC CT CT
A7	CT CT CT AC	TA GA TC GC	CT AC CT CT	TA GA TC GC	AC CT CT CT	TA GA TC GC	CT CT CT AC	TC GC TA GA	CT AC CT CT	TC GC TA GA	AC CT CT CT	TC GC TA GA	CT CT CT AC	GA TC GC TA	CT AC CT CT	GA TC GC TA	AC CT CT CT	GA TC GC TA
B7	CT CT CT AC	CT CT CT AT	CT AC CT CT	CT CT CT AT	AC CT CT CT	CT CT CT AT	CT CT CT AC	CT AT CT CT	CT AC CT CT	CT AT CT CT	AC CT CT CT	CT AT CT CT	CT CT CT AC	CT CT AT CT	CT AC CT CT	CT CT AT CT	AC CT CT CT	CT CT AT CT
C7	CT CT CT AC	TA TC CT CT	CT AC CT CT	TA TC CT CT	AC CT CT CT	TA TC CT CT	CT CT CT AC	CT CT TA TC	CT AC CT CT	CT CT TA TC	AC CT CT CT	CT CT TA TC	CT CT CT AC	TC CT CT TA	CT AC CT CT	TC CT CT TA	AC CT CT CT	TC CT CT TA

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
D7	CT CT CT AC	AG AG TA GA	CT AC CT CT	AG AG TA GA	AC CT CT CT	AG AG TA GA	CT CT CT AC	TA GA AG AG	CT AC CT CT	TA GA AG AG	AC CT CT CT	TA GA AG AG	CT CT CT AC	AG TA GA AG	CT AC CT CT	AG TA GA AG	AC CT CT CT	AG TA GA AG
E7	CT CT CT AC	GT AA GG AG	CT AC CT CT	GT AA GG AG	AC CT CT CT	GT AA GG AG	CT CT CT AC	GG AG GT AA	CT AC CT CT	GG AG GT AA	AC CT CT CT	GG AG GT AA	CT CT CT AC	AA GG AG GT	CT AC CT CT	AA GG AG GT	AC CT CT CT	AA GG AG GT
F7	CT CT CT AC	AC TG CA TA	CT AC CT CT	AC TG CA TA	AC CT CT CT	AC TG CA TA	CT CT CT AC	CA TA AC TG	CT AC CT CT	CA TA AC TG	AC CT CT CT	CA TA AC TG	CT CT CT AC	TG CA TA AC	CT AC CT CT	TG CA TA AC	AC CT CT CT	TG CA TA AC
G7	CT CT CT AC	AA GG AG TA	CT AC CT CT	AA GG AG TA	AC CT CT CT	AA GG AG TA	CT CT CT AC	AG TA AA GG	CT AC CT CT	AG TA AA GG	AC CT CT CT	AG TA AA GG	CT CT CT AC	GG AG TA AA	CT AC CT CT	GG AG TA AA	AC CT CT CT	GG AG TA AA
H7	CT CT CT AC	CT AA GC CT	CT AC CT CT	CT AA GC CT	AC CT CT CT	CT AA GC CT	CT CT CT AC	GC CT CT AA	CT AC CT CT	GC CT CT AA	AC CT CT CT	GC CT CT AA	CT CT CT AC	AA GC CT CT	CT AC CT CT	AA GC CT CT	AC CT CT CT	AA GC CT CT
A8	CA GA GA GG	TA GA TC GC	GA GG CA GA	TA GA TC GC	GG CA GA GA	TA GA TC GC	CA GA GA GG	TC GC TA GA	GA GG CA GA	TC GC TA GA	GG CA GA GA	TC GC TA GA	CA GA GA GG	GA TC GC TA	GA GG CA GA	GA TC GC TA	GG CA GA GA	GA TC GC TA
B8	CA GA GA GG	CT CT CT AT	GA GG CA GA	CT CT CT AT	GG CA GA GA	CT CT CT AT	CA GA GA GG	CT AT CT CT	GA GG CA GA	CT AT CT CT	GG CA GA GA	CT AT CT CT	CA GA GA GG	CT CT AT CT	GA GG CA GA	CT AT CT CT	GG CA GA GA	CT AT CT CT
C8	CA GA GA GG	TA TC CT CT	GA GG CA GA	TA TC CT CT	GG CA GA GA	TA TC CT CT	CA GA GA GG	CT CT TA TC	GA GG CA GA	CT CT TA TC	GG CA GA GA	CT CT TA TC	CA GA GA GG	TC CT CT TA	GA GG CA GA	TC CT CT TA	GG CA GA GA	TC CT CT TA
D8	CA GA GA GG	AG AG TA GA	GA GG CA GA	AG AG TA GA	GG CA GA GA	AG AG TA GA	CA GA GA GG	TA GA AG AG	GA GG CA GA	TA GA AG AG	GG CA GA GA	TA GA AG AG	CA GA GA GG	AG TA GA AG	GA GG CA GA	AG TA GA AG	GG CA GA GA	AG TA GA AG
E8	CA GA GA GG	GT AA GG AG	GA GG CA GA	GT AA GG AG	GG CA GA GA	GT AA GG AG	CA GA GA GG	GG AG GT AA	GA GG CA GA	GG AG GT AA	GG CA GA GA	GG AG GT AA	CA GA GA GG	AA GG AG GT	GA GG CA GA	AA GG AG GT	GG CA GA GA	AA GG AG GT
F8	CA GA GA GG	AC TG CA TA	GA GG CA GA	AC TG CA TA	GG CA GA GA	AC TG CA TA	CA GA GA GG	CA TA AC TG	GA GG CA GA	CA TA AC TG	GG CA GA GA	CA TA AC TG	CA GA GA GG	TG CA TA AC	GA GG CA GA	TG CA TA AC	GG CA GA GA	TG CA TA AC
G8	CA GA GA GG	AA GG AG TA	GA GG CA GA	AA GG AG TA	GG CA GA GA	AA GG AG TA	CA GA GA GG	AG TA AA GG	GA GG CA GA	AG TA AA GG	GG CA GA GA	AG TA AA GG	CA GA GA GG	GG AG TA AA	GA GG CA GA	GG AG TA AA	GG CA GA GA	GG AG TA AA
H8	CA GA GA GG	CT AA GC CT	GA GG CA GA	CT AA GC CT	GG CA GA GA	CT AA GC CT	CA GA GA GG	GC CT CT AA	GA GG CA GA	GC CT CT AA	GG CA GA GA	GC CT CT AA	CA GA GA GG	AA GC CT CT	GA GG CA GA	AA GC CT CT	GG CA GA GA	AA GC CT CT
A9	GC TA CG CT	TA GA TC GC	CG CT GC TA	TA GA TC GC	CT GC TA CG	TA GA TC GC	GC TA CG CT	TC GC TA GA	CG CT GC TA	TC GC TA GA	CT GC TA CG	TC GC TA GA	GC TA CG CT	GA TC GC TA	CG CT GC TA	GA TC GC TA	CT GC TA CG	GA TC GC TA

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
B9	GC TA CG CT	CT CT CT AT	CG CT GC TA	CT CT CT AT	CT GC TA CG	CT CT CT AT	GC TA CG CT	CT AT CT CT	CG CT GC TA	CT AT CT CT	CT GC TA CG	CT AT CT CT	GC TA CG CT	CT CT AT CT	CG CT GC TA	CT CT AT CT	CT GC TA CG	CT AT CT
C9	GC TA CG CT	TA TC CT CT	CG CT GC TA	TA TC CT CT	CT GC TA CG	TA TC CT CT	GC TA CG CT	CT CT TA TC	CG CT GC TA	CT CT TA TC	CT GC TA CG	CT TA TC	GC TA CG CT	TC CT CT TA	CG CT GC TA	TC CT CT TA	CT GC TA CG	TC CT CT TA
D9	GC TA CG CT	AG AG TA GA	CG CT GC TA	AG AG TA GA	CT GC TA CG	AG AG TA GA	GC TA CG CT	TA GA AG AG	CG CT GC TA	TA GA AG AG	CT GC TA CG	TA GA AG AG	GC TA CG CT	AG TA GA AG	CG CT GC TA	AG TA GA AG	CT GC TA CG	AG TA GA AG
E9	GC TA CG CT	GT AA GG AG	CG CT GC TA	GT AA GG AG	CT GC TA CG	GT AA GG AG	GC TA CG CT	GG AG GT AA	CG CT GC TA	GG AG GT AA	CT GC TA CG	GG AG GT AA	GC TA CG CT	AA GG AG GT	CG CT GC TA	AA GG AG GT	CT GC TA CG	AA GG AG GT
F9	GC TA CG CT	AC TG CA TA	CG CT GC TA	AC TG CA TA	CT GC TA CG	AC TG CA TA	GC TA CG CT	CA TA AC TG	CG CT GC TA	CA TA AC TG	CT GC TA CG	CA TA AC TG	GC TA CG CT	TG CA TA AC	CG CT GC TA	TG CA TA AC	CT GC TA CG	TG CA TA AC
G9	GC TA CG CT	AA GG AG TA	CG CT GC TA	AA GG AG TA	CT GC TA CG	AA GG AG TA	GC TA CG CT	AG TA AA GG	CG CT GC TA	AG TA AA GG	CT GC TA CG	AG TA AA GG	GC TA CG CT	GG AG TA AA	CG CT GC TA	GG AG TA AA	CT GC TA CG	GG AG TA AA
H9	GC TA CG CT	CT AA GC CT	CG CT GC TA	CT AA GC CT	CT GC TA CG	CT AA GC CT	GC TA CG CT	GC CT CT AA	CG CT GC TA	GC CT CT AA	CT GC TA CG	GC CT CT AA	GC TA CG CT	AA GC CT CT	CG CT GC TA	AA GC CT CT	CT GC TA CG	AA GC CT CT
A1 0	CG AG GC TG	TA GA TC GC	GC TG CG AG	TA GA TC GC	TG CG AG GC	TA GA TC GC	CG AG GC TG	TC GC TA GA	GC TG CG AG	TC GC TA GA	TG CG AG GC	TC GC TA GA	CG AG GC TG	GA TC GC TA	GC TG CG AG	GA TC GC TA	TG CG AG GC	GA TC GC TA
B1 0	CG AG GC TG	CT CT CT AT	GC TG CG AG	CT CT CT AT	TG CG AG GC	CT CT CT AT	CG AG GC TG	CT AT CT CT	GC TG CG AG	CT AT CT CT	TG CG AG GC	CT AT CT CT	CG AG GC TG	CT AT CT CT	GC TG CG AG	CT AT CT CT	TG CG AG GC	CT AT CT CT
C1 0	CG AG GC TG	TA TC CT CT	GC TG CG AG	TA TC CT CT	TG CG AG GC	TA TC CT CT	CG AG GC TG	CT CT TA TC	GC TG CG AG	CT CT TA TC	TG CG AG GC	CT CT TA TC	CG AG GC TG	TC CT CT TA	GC TG CG AG	TC CT CT TA	TG CG AG GC	TC CT CT TA
D1 0	CG AG GC TG	AG AG TA GA	GC TG CG AG	AG AG TA GA	TG CG AG GC	AG AG TA GA	CG AG GC TG	TA GA AG AG	GC TG CG AG	TA GA AG AG	TG CG AG GC	TA GA AG AG	CG AG GC TG	AG TA GA AG	GC TG CG AG	AG TA GA AG	TG CG AG GC	AG TA GA AG
E1 0	CG AG GC TG	GT AA GG AG	GC TG CG AG	GT AA GG AG	TG CG AG GC	GT AA GG AG	CG AG GC TG	GG AG GT AA	GC TG CG AG	GG AG GT AA	TG CG AG GC	GG AG GT AA	CG AG GC TG	AA GG AG GT	GC TG CG AG	AA GG AG GT	TG CG AG GC	AA GG AG GT
F1 0	CG AG GC TG	AC TG CA TA	GC TG CG AG	AC TG CA TA	TG CG AG GC	AC TG CA TA	CG AG GC TG	CA TA AC TG	GC TG CG AG	CA TA AC TG	TG CG AG GC	CA TA AC TG	CG AG GC TG	TG CA TA AC	GC TG CG AG	TG CA TA AC	TG CG AG GC	TG CA TA AC
G1 0	CG AG GC TG	AA GG AG TA	GC TG CG AG	AA GG AG TA	TG CG AG GC	AA GG AG TA	CG AG GC TG	AG TA AA GG	GC TG CG AG	AG TA AA GG	TG CG AG GC	AG TA AA GG	CG AG GC TG	GG AG TA AA	GC TG CG AG	GG AG TA AA	TG CG AG GC	GG AG TA AA

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
	H1 0	CG AG GC TG	CT AA GC CT	GC TG CG AG	CT AA GC CT	TG CG AG GC	CT AA GC CT	CG AG GC TG	GC CT CT AA	GC TG CG AG	GC CT CT AA	TG CG AG GC	GC CT CT AA	CG AG GC TG	AA GC CT CT	GC TG CG AG	AA GC CT CT	TG CG AG GC	AA GC CT CT
	A1 1	AA GA GG CA	TA GA TC GC	GG CA AA GA	TA GA TC GC	CA AA GA GG	TA GA TC GC	AA GA GG CA	TC GC TA GA	GG CA AA GA	TC GC TA GA	CA AA GA GG	TC GC TA GA	AA GA GG CA	GA TC GC TA	GG CA AA GA	GA TC GC TA	CA AA GA GG	GA TC GC TA
	B1 1	AA GA GG CA	CT CT CT AT	GG CA AA GA	CT CT CT AT	CA AA GA GG	CT CT CT AT	AA GA GG CA	CT AT CT CT	GG CA AA GA	CT AT CT CT	CA AA GA GG	CT AT CT CT	AA GA GG CA	CT CT AT CT	GG CA AA GA	CT CT AT CT	CA AA GA GG	CT CT AT CT
	C1 1	AA GA GG CA	TA TC CT CT	GG CA AA GA	TA TC CT CT	CA AA GA GG	TA TC CT CT	AA GA GG CA	CT CT TA TC	GG CA AA GA	CT CT TA TC	CA AA GA GG	CT CT TA TC	AA GA GG CA	TC CT CT TA	GG CA AA GA	TC CT CT TA	CA AA GA GG	TC CT CT TA
	D1 1	AA GA GG CA	AG AG TA GA	GG CA AA GA	AG AG TA GA	CA AA GA GG	AG AG TA GA	AA GA GG CA	TA GA AG AG	GG CA AA GA	TA GA AG AG	CA AA GA GG	TA GA AG AG	AA GA GG CA	AG TA GA AG	GG CA AA GA	AG TA GA AG	CA AA GA GG	AG TA GA AG
	E1 1	AA GA GG CA	GT AA GG AG	GG CA AA GA	GT AA GG AG	CA AA GA GG	GT AA GG AG	AA GA GG CA	GG AG GT AA	GG CA AA GA	GG AG GT AA	CA AA GA GG	GG AG GT AA	AA GA GG CA	AA GG AG GT	GG CA AA GA	AA GG AG GT	CA AA GA GG	AA GG AG GT
	F1 1	AA GA GG CA	AC TG CA TA	GG CA AA GA	AC TG CA TA	CA AA GA GG	AC TG CA TA	AA GA GG CA	CA TA AC TG	GG CA AA GA	CA TA AC TG	CA AA GA GG	CA TA AC TG	AA GA GG CA	TG CA TA AC	GG CA AA GA	TG CA TA AC	CA AA GA GG	TG CA TA AC
	G1 1	AA GA GG CA	AA GG AG TA	GG CA AA GA	AA GG AG TA	CA AA GA GG	AA GG AG TA	AA GA GG CA	AG TA AA GG	GG CA AA GA	AG TA AA GG	CA AA GA GG	AG TA AA GG	AA GA GG CA	GG AG TA AA	GG CA AA GA	GG AG TA AA	CA AA GA GG	GG AG TA AA
	H1 1	AA GA GG CA	CT AA GC CT	GG CA AA GA	CT AA GC CT	CA AA GA GG	CT AA GC CT	AA GA GG CA	GC CT CT AA	GG CA AA GA	GC CT CT AA	CA AA GA GG	GC CT CT AA	AA GA GG CA	AA GC CT CT	GG CA AA GA	AA GC CT CT	CA AA GA GG	AA GC CT CT
	A1 2	GT AG AG GA	TA GA TC GC	AG GA GT AG	TA GA TC GC	GA GT AG AG	TA GA TC GC	GT AG AG GA	TC GC TA GA	AG GA GT AG	TC GC TA GA	GA GT AG AG	TC GC TA GA	GT AG AG GA	GA TC GC TA	AG GA GT AG	GA TC GC TA	GA GT AG AG	GA TC GC TA
	B1 2	GT AG AG GA	CT CT CT AT	AG GA GT AG	CT CT CT AT	GA GT AG AG	CT CT CT AT	GT AG AG GA	CT AT CT CT	AG GA GT AG	CT AT CT CT	GA GT AG AG	CT AT CT CT	GT AG AG GA	CT CT AT CT	AG GA GT AG	CT CT AT CT	GA GT AG AG	CT CT AT CT
	C1 2	GT AG AG GA	TA TC CT CT	AG GA GT AG	TA TC CT CT	GA GT AG AG	TA TC CT CT	GT AG AG GA	CT CT TA TC	AG GA GT AG	CT CT TA TC	GA GT AG AG	CT CT TA TC	GT AG AG GA	TC CT CT TA	AG GA GT AG	TC CT CT TA	GA GT AG AG	TC CT CT TA
	D1 2	GT AG AG GA	AG AG TA GA	AG GA GT AG	AG AG TA GA	GA GT AG AG	AG AG TA GA	GT AG AG GA	TA GA AG AG	AG GA GT AG	TA GA AG AG	GA GT AG AG	TA GA AG AG	GT AG AG GA	AG TA GA AG	AG GA GT AG	AG TA GA AG	GA GT AG AG	AG TA GA AG
	E1 2	GT AG AG GA	GT AA GG AG	AG GA GT AG	GT AA GG AG	GA GT AG AG	GT AA GG AG	GT AG AG GA	GG AG GT AA	AG GA GT AG	GG AG GT AA	GA GT AG AG	GG AG GT AA	GT AG AG GA	AA GG AG GT	AG GA GT AG	AA GG AG GT	GA GT AG AG	AA GG AG GT

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
	F1 2	GT	AC	AG	AC	GA	AC	GT	CA	AG	CA	GA	CA	GT	TG	AG	TG	GA	TG
AG		TG	GA	TG	GT	TG	AG	TA	GA	TA	GT	TA	AG	CA	GA	CA	GT	CA	
AG		CA	GT	CA	AG	CA	AG	AC	GT	AC	AG	AC	AG	TA	GT	TA	AG	TA	
GA		TA	AG	TA	AG	TA	GA	TG	AG	TG	AG	TG	GA	AC	AG	AC	AG	AC	
	G1 2	GT	AA	AG	AA	GA	AA	GT	AG	AG	AG	GA	AG	GT	GG	AG	GG	GA	GG
AG		GG	GA	GG	GT	GG	AG	TA	GA	TA	GT	TA	AG	AG	GA	AG	GT	AG	
AG		AG	GT	AG	AG	AG	AG	AA	GT	AA	AG	AA	AG	TA	GT	TA	AG	TA	
GA		TA	AG	TA	AG	TA	GA	GG	AG	GG	AG	GG	GA	AA	AG	AA	AG	AA	
	H1 2	GT	CT	AG	CT	GA	CT	GT	GC	AG	GC	GA	GC	GT	AA	AG	AA	GA	AA
AG		AA	GA	AA	GT	AA	AG	CT	GA	CT	GT	CT	CT	AG	GC	GA	GC	GT	GC
AG		GC	GT	GC	AG	GC	AG	AG	CT	GT	CT	AG	CT	AG	CT	GT	CT	AG	CT
GA		CT	AG	CT	AG	CT	GA	AA	AG	AA	AG	AA	GA	CT	AG	CT	AG	CT	CT

## 13 Custom primers

This method uses custom primers which are in positions 12, 13, and 14 of the MiSeq reagent cartridge. For more information or for using other ILLUMINA System, refer to "Custom Primers Guide" on ILLUMiNA website.

A	B	C	D
Primers	Séquence 5'-3'	Reservoir number	Vol. per run at 100µM
TSIT_Read1	ccatctcatccctgcgtgtctccgactcag	12	4 µL
TSIT_i7_read	aatcaccgactgcccatagagaggaaagcggag	13	4 µL
TSIT_Read2	ctccgcttctctctatgggcagtcggtgatt	14	4 µL