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Workflow for SNP genotyping using the Hi-Plex method V.1

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We use this protocol and it's working

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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.



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) preparataion steps

- 1x Low TE (10 mM Tris pH 8.0/0.1 mM EDTA)
- GSPs at 100 or 200 μ M in TE
- TSITs A and TSITs P at 100 or 200 μ M 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 50 μ M
- ThermoFisher PhusionTM Hot Start II High Fidelity (ref. F549L)
- ThermoFisher PhusionTM High-Fidelity (ref. F534L)
- TSIT prepared in plate at final concentrion of 10 μ M
- EDTA 100mM predistributed in tube strip (MW 292.24 g/mol)
- dNTPs 20mM each (Promega ref U1330)
- Primers: P5 5'-AATGATACGGCGACCAACCGA-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 PippinTM 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.

Before start

- 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 Hammett 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)

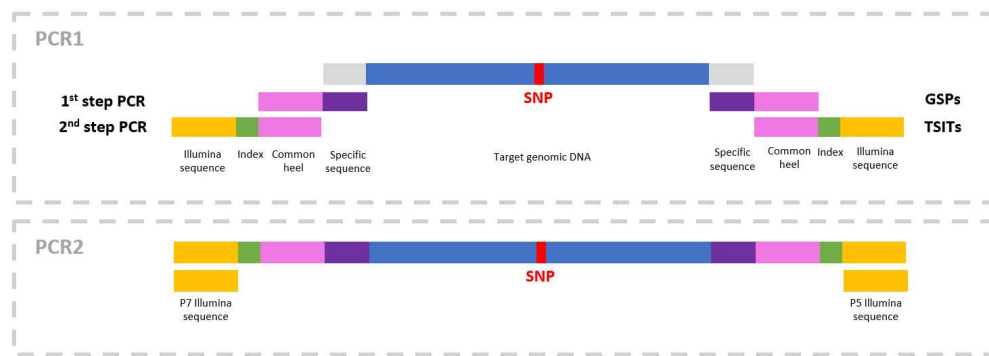
TSITs are adaptors that consist of a complementary sequence to the GSP common heel, a barcode, and a specific sequence for Illumina sequencing (P5/P7) for a total of about 60 bp for each TSIT A and TSIT P. The use of a combination of this 2 barcodes allows the specific identification of the DNA samples.

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 μ M) 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 $T_M = 58^\circ\text{C}$. 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 PipinnTM prep.

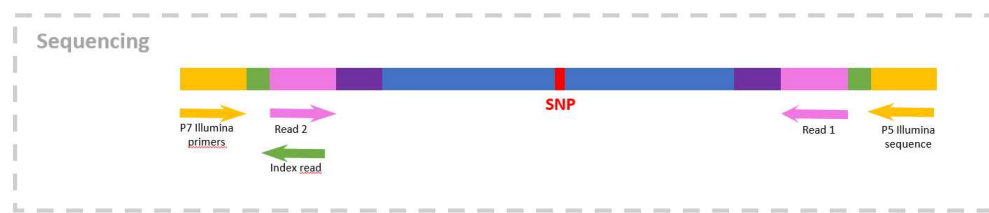
The use of EtBr is essential if size selection from agarose gel is performed. SybrSafe reagent, for example, generated a lot of non-specific amplicons. This suggests that SybrSafe does not allow for clean separation of migrated fragments on agarose gels.



Simplified schemes of PCR steps

Quality controls and sequencing

Each library is checked on a Fragment AnalyzerTM 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

Gene Specific Primers (GSPs) and adaptator (TSITs) preparation

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

```
CGGGCCGGCCCGTCCAAAAATAGCAAGCAGAGTGAAATCCTCCACATTCTAAATGGCCAC
ATGCACAAACAAGCAGTGT TTTCTTAAAGAGACCACTGCTCCCAGCTCACCCAGCAGCCTC
TCACAGACAGTGGTGGGGGGGGGG
```

- 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
	<i>C_i</i> (µM)	<i>C_f</i> (µM)	<i>V_i</i> (µL)	<i>V_f</i> (µL)	<i>V_{low TE}</i> (µL)
	100	40	20	50	30
	200	40	10	50	40

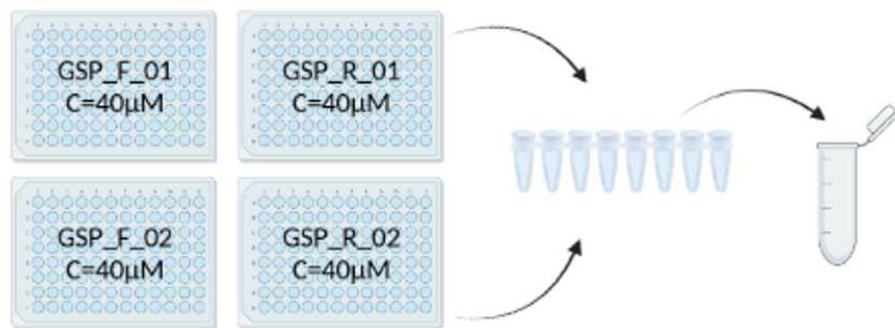
Ci: initial concentration; Cf: final concentration; Vi: initial volume; Vf: final volume

- 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_i GSP (μM)	C_f GSP (μM) =C _i * V _i / V _f pool	V_i GSP (μL)	Number of GSP =Nb F + Nb R	V_f pool (μL) =V _i GSP * Nb GSP	C_f pool (μM) =C _f GSP * Number of GSP	Nb aliquot 100 μL =V _f 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_i (μL)	V_f (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.

TSIT_A	1	2	3	4	5	6	7	8	9	10	11	12	TSIT_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
	Pla te wel l	TSITs P names	Sequences of TSITs P
	A1	N701_TSIT_P	CAAGCAGAAGACGGCATACTGAGATTCGCCTTActccgctttcctctcta tgggcagtcggtgat
	A2	N702_TSI T_P	CAAGCAGAAGACGGCATACTGAGATCTAGTACGctccgctttcctctctat gggcagtcggtgat
	A3	N703_TSI T_P	CAAGCAGAAGACGGCATACTGAGATTTCTGCCTctccgctttcctctctat gggcagtcggtgat
	A4	N704_TSI T_P	CAAGCAGAAGACGGCATACTGAGATGCTCAGGActccgctttcctctcta tgggcagtcggtgat
	A5	N705_TSIT _P	CAAGCAGAAGACGGCATACTGAGATAGGAGTCCctccgctttcctctcta tgggcagtcggtgat
	A6	N706_TSIT _P	CAAGCAGAAGACGGCATACTGAGATCATGCCTActccgctttcctctctat gggcagtcggtgat
	A7	N707_TSIT _P	CAAGCAGAAGACGGCATACTGAGATGTAGAGAGctccgctttcctctcta tgggcagtcggtgat
	A8	N708_TSI T_P	CAAGCAGAAGACGGCATACTGAGATCCTCTCTGctccgctttcctctcta tgggcagtcggtgat
	A9	N709_TSIT _P	CAAGCAGAAGACGGCATACTGAGATAGCGTAGCctccgctttcctctcta tgggcagtcggtgat



	A	B	C
	A10	N710_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCAGCCTCGctccgctttcctctcta tgggcagtcggtgat
	A11	N711_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTGCCTCTTctccgctttcctctctat gggcagtcggtgat
	A12	N712_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTCTCTACctccgctttcctctctat gggcagtcggtgat
	B1	N713_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTTATCGCctccgctttcctctctat gggcagtcggtgat
	B2	N714_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTACGCTAGctccgctttcctctctat gggcagtcggtgat
	B3	N715_TSIT_P	CAAGCAGAAGACGGCATAACGAGATGCCTTTCTctccgctttcctctctat gggcagtcggtgat
	B4	N716_TSIT_P	CAAGCAGAAGACGGCATAACGAGATAGGAGCTCctccgctttcctctcta tgggcagtcggtgat
	B5	N717_TSIT_P	CAAGCAGAAGACGGCATAACGAGATGTCCAGGActccgctttcctctcta tgggcagtcggtgat
	B6	N718_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCCTACATGctccgctttcctctctat gggcagtcggtgat
	B7	N719_TSIT_P	CAAGCAGAAGACGGCATAACGAGATAGAGGTAGctccgctttcctctctat gggcagtcggtgat
	B8	N720_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTCTGCCTCctccgctttcctctcta tgggcagtcggtgat
	B9	N721_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTAGCAGCGctccgctttcctctcta tgggcagtcggtgat
	B10	N722_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTCGCAGCctccgctttcctctcta tgggcagtcggtgat
	B11	N723_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTCTTTGCCctccgctttcctctcta tgggcagtcggtgat
	B12	N724_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTACTCCTctccgctttcctctctat gggcagtcggtgat
	C1	N725_TSIT_P	CAAGCAGAAGACGGCATAACGAGATGCCTTATCctccgctttcctctctat gggcagtcggtgat
	C2	N726_TSIT_P	CAAGCAGAAGACGGCATAACGAGATAGTACGCTctccgctttcctctctat gggcagtcggtgat
	C3	N727_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTGCCTTTctccgctttcctctctat gggcagtcggtgat

	A	B	C
	C4	N728_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTCAGGAGCctccgctttcctctcta tgggcagtcggtgat
	C5	N729_TSIT_P	CAAGCAGAAGACGGCATAACGAGATGAGTCCAGctccgctttcctctcta tgggcagtcggtgat
	C6	N730_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTGCCTACActccgctttcctctcta tgggcagtcggtgat
	C7	N731_TSIT_P	CAAGCAGAAGACGGCATAACGAGATAGAGAGGTctccgctttcctctctat gggcagtcggtgat
	C8	N732_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTCTCTGCCctccgctttcctctcta tgggcagtcggtgat
	C9	N733_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCGTAGCAGctccgctttcctctcta tgggcagtcggtgat
	C10	N734_TSIT_P	CAAGCAGAAGACGGCATAACGAGATGCCTCGCActccgctttcctctcta tgggcagtcggtgat
	C11	N735_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCCTCTTTGctccgctttcctctcta tgggcagtcggtgat
	C12	N736_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTCTACTCctccgctttcctctctat gggcagtcggtgat

List of TSITs P adaptors

	A	B	C
	Plate well	TSITs A names	Sequences of TSITs A
	A1	N501_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTAGATCGCccatctc atccctgcgtgtctccgactcag
	B1	N502_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTCTCTATccatctca tccctgcgtgtctccgactcag
	C1	N503_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTATCCTCTccatctca tccctgcgtgtctccgactcag
	D1	N504_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAGAGTAGAccatctc atccctgcgtgtctccgactcag
	E1	N505_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGccatctc atccctgcgtgtctccgactcag
	F1	N506_TSIT_A	AATGATACGGCGACCACCGAGATCTACACACTGCATAccatctc atccctgcgtgtctccgactcag



	A	B	C
	G1	N507_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAAGGAGTAccatctc atccctgcggtgtctccgactcag
	H1	N508_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTccatctc atccctgcggtgtctccgactcag
	A2	N509_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTCGCTAGAccatctc atccctgcggtgtctccgactcag
	B2	N510_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTATCTCTccatctca tccctgcggtgtctccgactcag
	C2	N511_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTCTTATCccatctc atccctgcggtgtctccgactcag
	D2	N512_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTAGAAGAGccatctc atccctgcggtgtctccgactcag
	E2	N513_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGGAGGTAAccatctc atccctgcggtgtctccgactcag
	F2	N514_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCATAACTGccatctc atccctgcggtgtctccgactcag
	G2	N515_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAGTAAAGGccatctc atccctgcggtgtctccgactcag
	H2	N516_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGCCTCTAAccatctc atccctgcggtgtctccgactcag
	A3	N517_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGATCGCTAccatctc atccctgcggtgtctccgactcag
	B3	N518_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTCTATCTccatctca tccctgcggtgtctccgactcag
	C3	N519_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTCCTCTTAccatctc atccctgcggtgtctccgactcag
	D3	N520_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAGTAGAAGccatctc atccctgcggtgtctccgactcag
	E3	N521_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAAGGAGGTccatctc atccctgcggtgtctccgactcag
	F3	N522_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTGCATAACccatctc atccctgcggtgtctccgactcag
	G3	N523_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGGAGTAAAccatctc atccctgcggtgtctccgactcag
	H3	N524_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAAGCCTCTccatctc atccctgcggtgtctccgactcag

List of TSITs A adaptors

TSITs preparation

- Make a new plate diluted to 50 μM from each TSIT plate (TSIT_A and TSIT_P) at 100 or 200 μM in 100 μL /TSIT in TE: TSIT_A_50 μM and TSIT_P_50 μM .

	A	B	C	D	E
	Ci μM	Cf μM	Vi μL	Vf	V low TE
	100	50	100	200	100
	200	50	50	200	150

- Finally prepare 10 μM plates by mixing 2 by 2 TSIT_A with TSIT_P to obtain 9 plates of different combinations (plates are named TSIT_10 μM _1 to TSIT_10 μM _9).

As the TSIT_A are arranged columnwise in plate TSIT_A_50 μM and TSIT_P are sorted rowwise in plate TSIT_P_50 μM , distribute 5 μL 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 10μM	TSIT_A column number	Vol. of TSIT_A at 50μM	TSIT_P row number	Vol. of TSIT_P à 50μM	Vol. of low TE	Final volume
	TSIT_10 μM _1	1	5	A	5	15	25
	TSIT_10 μM _2	1	5	B	5	15	25
	TSIT_10 μM _3	1	5	C	5	15	25
	TSIT_10 μM _4	2	5	A	5	15	25
	TSIT_10 μM _5	2	5	B	5	15	25
	TSIT_10 μM _6	2	5	C	5	15	25
	TSIT_10 μM _7	3	5	A	5	15	25
	TSIT_10 μM _8	3	5	B	5	15	25
	TSIT_10 μM _9	3	5	C	5	15	25

Schemes of the nine final TSIT plates at 10µM

PCR Preparation and Clean-Up for SNP genotyping using Hi-Plex

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
PCR_01	DNA_01_rep1	TSIT_10µM_01	2023/xx/xx
PCR_02	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 μL 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 μL
5X Buffer	5X	1X	5	490 μL
MgCl ₂	50mM	1mM	0.5	49 μL
dNTPs	20mM	400 μM	0.5	49 μL
Phusion HF Hot Start taq	2U/ μL	1U	0.5	49 μL
GSP pool	10 μM	0.2 μM	0.5	49 μL
TOTAL Volume mix			21 μL	2058 μL
DNA or control per well			2 μL	

■ 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

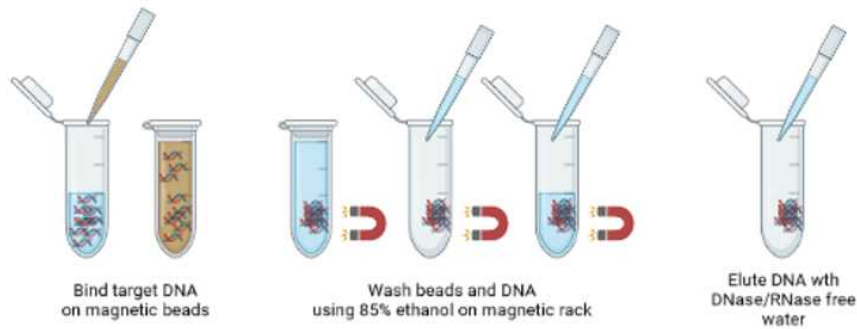
	A	B	C
	58°C	02:30	
	72°C	01:00	
	72°C	Needed to add TSITs	
	98°C	00:30	x4
	66°C	02:00	
	72°C	01:00	
	72°C	Needed to add EDTA	

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 μL of PCR products to a new 2 mL tube. Leave the remaining 960 μL at 4 $^{\circ}\text{C}$ until the end of the protocol or at -20 $^{\circ}\text{C}$ for longer term storage.
2. Add 864 μL 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 μL 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 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 70 µl of water containing target DNA into a new 1.5 or 2 mL tube.

■ Bind eluted DNA onto magnetic beads

1. Add 63 µL of presuspend magnetic beads ($0.9 \times 70 \mu\text{L} = 63 \mu\text{L}$) 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 70 µl 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 following 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

A	B	C	D
DNase/RNase free water	-	-	22.5
5 X Green buffer	5X	1X	15
MgCl ₂	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
P7 primer : 5'-CAAGCAGAAGACGGCATAGCA-3'	50μM	1 μM	1.5
TOTAL volume mix			45
Purified PCR1 product or control			10

■ 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 μ L loading volumes.

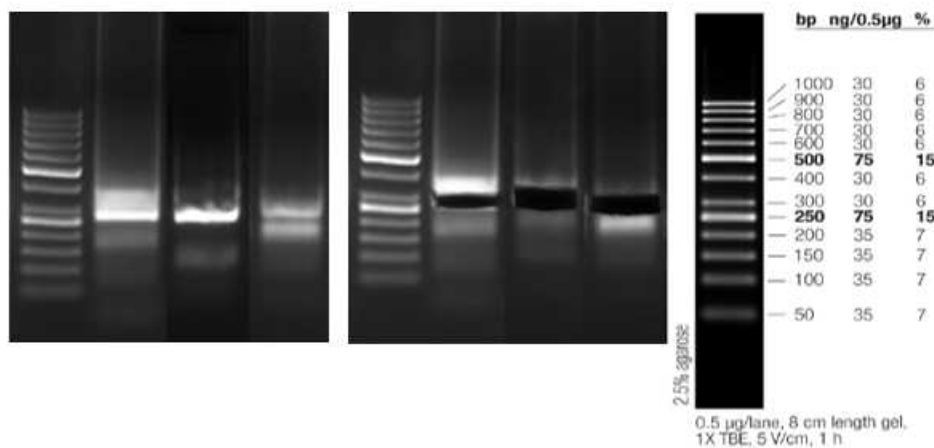
1. Prepare a 1.75% agarose gel using 1X TAE and ethidium bromide (EtBr).
2. Add directly 55 μ L of each sample to a well (possible due to the use of the green buffer in previous PCR2 step)
3. Add 10 μ L of the 50 bp ladder to at least one well (adapt to the number of samples or your gel size).
4. Run 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

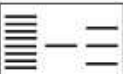
1. Use the MN Nucleospin gel clean-up kit using the gel extraction protocol supplied by the manufacturer.
2. Prepare the NT3 and add the appropriate volume of 96-100% ethanol.
3. Adapt the volume of NTI buffer for each sample following : Vol. NTI = 2x agarose weight.
4. Elute with 2 $\times$ 25 μ L of buffer NE (50 μ L total).
5. Store at 4 $^{\circ}$ 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
1 PCR clean-up, DNA clean-up, or single stranded DNA clean-up: Adjust binding condition Gel extraction: 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
2 Bind DNA		 11,000 x g 30 s		
3 Wash silica membrane		 700 µL NT3 11,000 x g 30 s Recommended: 2 nd wash 700 µL NT3 11,000 x g 30 s		
4 Dry silica membrane		 11,000 x g 1 min		
5 Elute DNA		 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

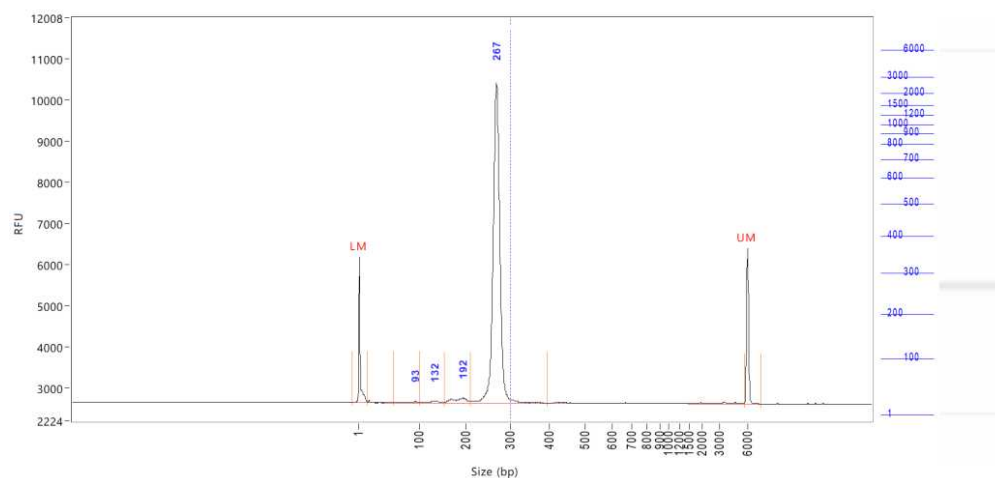
1. Bring each DNA sample from PCR2 up to 60µl with water.
2. Combine 60µl of DNA sample with 20µl of loading/marker mix (Marker L).
3. Mix samples thoroughly (vortex mixer). Briefly centrifuge to collect.
4. Remove 40µl of buffer from two "**Sample Well**" of the cassette, and load 40µl of sample into 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.

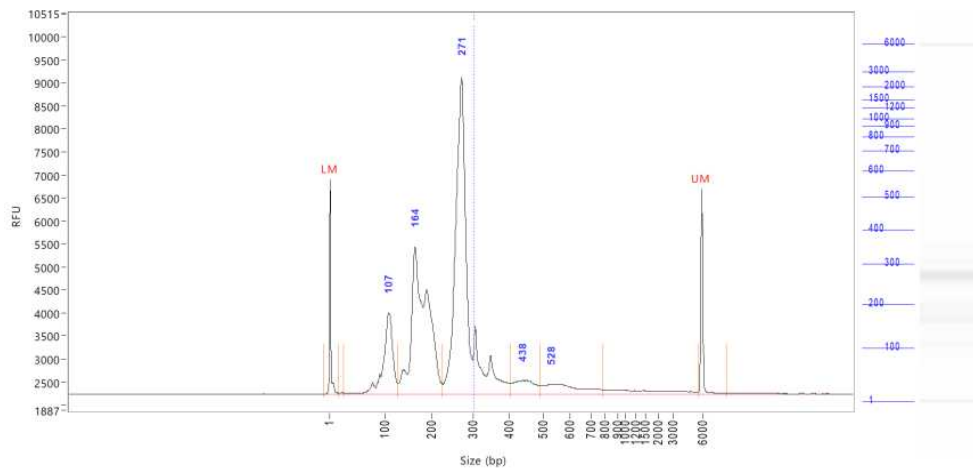
Quality and quantity control of libraries

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

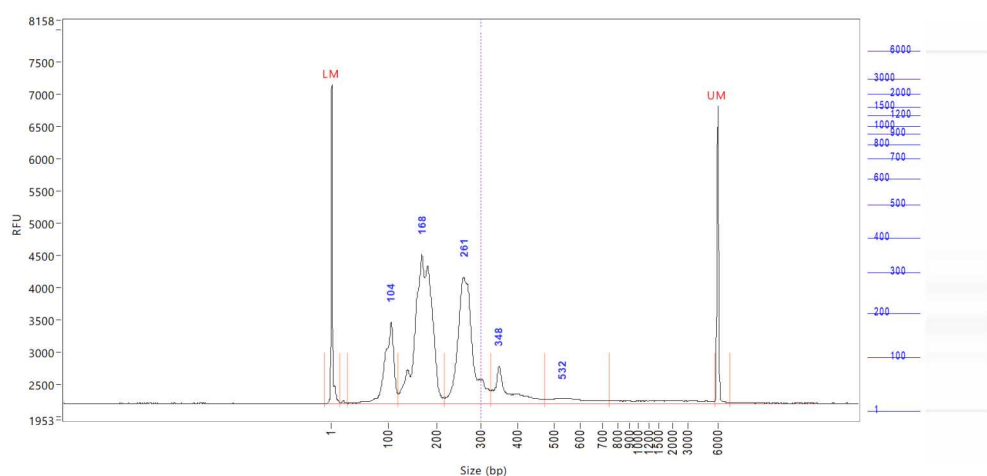
1. Use Invitrogen™ Qubit™ Fluorometer or equivalent to estimate DNA concentrations for each library
2. Prepare a 50pg/µL to 5ng/µL dilution of each library.
3. Then, follow the instructions from the manufacturer.



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.

Short information for sequencing Hi-Plex libraries on the ILLUMINA Miseq system

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
	P l a t e w e l l	T S I T _1	T S I T _1	T S I T _2	T S I T _2	T S I T _3	T S I T _3	T S I T _4	T S I T _4	T S I T _5	T S I T _5	T S I T _6	T S I T _6	T S I T _7	T S I T _7	T S I T _8	T S I T _8	T S I T _9	T S I T _9
		P c o m p l e m e n t	B a r c o d A	P c o m p l e m e n t	B a r c o d A	P c o m p l e m e n t	B a r c o d A	P c o m p l e m e n t	B a r c o d A	P c o m p l e m e n t	B a r c o d A	P c o m p l e m e n t	B a r c o d A	P c o m p l e m e n t	B a r c o d A	P c o m p l e m e n t	B a r c o d A	P c o m p l e m e n t	B a r c o d A
	A 1	T A A G G C G A	T A G A T C G C	G C G A T A A G	T A G A T C G C	G A T A A G G C	T A G A T C G C	T A A G G C G A	T C G C T A G A	G C G A T A A G	T C G C T A G A	G A T A A G G C	T C G C T A G A	T A A G G C G A	G A T C G C T A	G C G A T A A G	G A T C G C T A	G A T A A G G C	G A T C G C T A
	B 1	T A A G G C G A	C T C T C T A T	G C G A T A A G	C T C T C T A T	G A T A A G G C	C T C T C T A T	T A A G G C G A	C T A T C T C T	G C G A T A A G	C T A T C T C T	G A T A A G G C	C T A T C T C T	T A A G G C G A	C T C T A T C T	G C G A T A A G	C T C T A T C T	G A T A A G G C	C T C T A T C T
	C 1	T A A G G C G A	T A T C C T C T	G C G A T A A G	T A T C C T C T	G A T A A G G C	T A T C C T C T	T A A G G C G A	C T C T T A T C	G C G A T A A G	C T C T T A T C	G A T A A G G C	C T C T T A T C	T A A G G C G A	T C C T C T T A	G C G A T A A G	T C C T C T T A	G A T A A G G C	T C C T C T T A
	D 1	T A A G G C G A	A G A G T A G A	G C G A T A A G	A G A G T A G A	G A T A A G G C	A G A G T A G A	T A A G G C G A	T A G A A G A G	G C G A T A A G	T A G A A G A G	G A T A A G G C	T A G A A G A G	T A A G G C G A	A G T A G A A G	G C G A T A A G	A G T A G A A G	G A T A A G G C	A G T A G A A G



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
	E 1	T A A G G C G A	G T A A G G A G	G C G A T A A G	G T A A G G A G	G A T A A G G C	G T A A G G A G	T A A G G C G A	G G A G G T A A	G C G A T A A G	G G A G G T A A	G A T A A G G C	G G A G G T A A	T A A G G C G A	A A G G A G G T	G C G A T A A G	A A G G A G G T	G A T A A G G C	A A G G A G G T
	F 1	T A A G G C G A	A C T G C A T A	G C G A T A A G	A C T G C A T A	G A T A A G G C	A C T G C A T A	T A A G G C G A	C A T A A C T G	G C G A T A A G	C A T A A C T G	G A T A A G G C	C A T A A C T G	T A A G G C G A	T G C A T A A C	G C G A T A A G	T G C A T A A C	G A T A A G G C	T G C A T A A C
	G 1	T A A G G C G A	A A G G A G T A	G C G A T A A G	A A G G A G T A	G A T A A G G C	A A G G A G T A	T A A G G C G A	A G T A A A G G	G C G A T A A G	A G T A A A G G	G A T A A G G C	A G T A A A G G	T A A G G C G A	G G A G T A A A	G C G A T A A G	G G A G T A A A	G A T A A G G C	G G A G T A A A
	H 1	T A A G G C G A	C T A A G C C T	G C G A T A A G	C T A A G C C T	G A T A A G G C	C T A A G C C T	T A A G G C G A	G C C T C T A A	G C G A T A A G	G C C T C T A A	G A T A A G G C	G C C T C T A A	T A A G G C G A	A A G C C T C T	G C G A T A A G	A A G C C T C T	G A T A A G G C	A A G C C T C T
	A 2	C G T A C T A G	T A G A T T C G C	C T A G C G T A	T A G A T T C G C	A G C G T A C T	T A G A T T C G C	C G T A C T A G	T C G C T A G A	C T A G C G T A	T C G C T A G A	A G C G T A C T	T C G C T A G A	C G T A C T A G	G A T C G C T A	C T A G C G T A	G A T C G C T A	A G C G T A C T	G A T C G C T A
	B 2	C G T A C T A G	C T C T C T A T	C T A G C G T A	C T C T C T A T	A G C G T A C T	C T C T C T A T	C G T A C T A G	C T A T C T C T	C T A G C G T A	C T A T C T C T	A G C G T A C T	C T A T C T C T	C G T A C T A G	C T C T A T C T	C T A G C G T A	C T C T A T C T	A G C G T A C T	C T C T A T C T
	C 2	C G T A C	T A T C C	C T A G C	T A T C C	A G C G T	T A T C C	C G T A C	C T C T T	C T A G C	C T C T T	A G C G T	C T C T T	C G T A C	T C C T C	C T A G C	T C C T C	A G C G T	T C C T C



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
		T A G	T C T	G T A	T C T	A C T	T C T	T A G	A T C	G T A	A T C	A C T	A T C	T A G	T T A	G T A	T T A	A C T	T T A
D 2		C G T A C T A G	A G A G T A G A	C T A G C G T A	A G A G T A G A	A G C G T A C T	A G A G T A G A	C G T A C T A G	T A G A A G A G	C T A G C G T A	T A G A A G A G	A G C G T A C T	T A G A A G A G	C G T A C T A G	A G T A G A A G	C T A G C G T A	A G T A G A A G	A G C G T A C T	A G T A G A A G
E 2		C G T A C T A G	G T A A G G A G	C T A G C G T A	G T A A G G A G	A G C G T A C T	G T A A G G A G	C G T A C T A G	G G A G G T A A	C T A G C G T A	G G A G G T A A	A G C G T A C T	G G A G G T A A	C G T A C T A G	A A G G A G G T	C T A G C G T A	A A G G A G G T	A G C G T A C T	A A G G A G G T
F 2		C G T A C T A G	A C T G C A T A	C T A G C G T A	A C T G C A T A	A G C G T A C T	A C T G C A T A	C G T A C T A G	C A T A A C T G	C T A G C G T A	C A T A A C T G	A G C G T A C T	C A T A A C T G	C G T A C T A G	T G C A T A A C	C T A G C G T A	T G C A T A A C	A G C G T A C T	T G C A T A A C
G 2		C G T A C T A G	A A G G A G T A	C T A G C G T A	A A G G A G T A	A G C G T A C T	A A G G A G T A	C G T A C T A G	A G T A A A G G	C T A G C G T A	A G T A A A G G	A G C G T A C T	A G T A A A G G	C G T A C T A G	G G A G T A A A	C T A G C G T A	G G A G T A A A	A G C G T A C T	G G A G T A A A
H 2		C G T A C T A G	C T A A G C C T	C T A G C G T A	C T A A G C C T	A G C G T A C T	C T A A G C C T	C G T A C T A G	G C C T C T A A	C T A G C G T A	G C C T C T A A	A G C G T A C T	G C C T C T A A	C G T A C T A G	A A G C C T C T	C T A G C G T A	A A G C C T C T	A G C G T A C T	A A G C C T C T
A 3		A G G C A G A A	T A G A T C G C	A G A A A G G C	T A G A T C G C	A A A G G C A G	T A G A T C G C	A G G C A G A A	T C G C T A G A	A G A A A G G C	T C G C T A G A	A A A G G C A G	T C G C T A G A	A G G C A G A A	G A T C G C T A	A G A A A G G C	G A T C G C T A	A A A G G C A G	G A T C G C T A



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
	B 3	A G G C A G A A	C T C T C T A T	A G A A A G G C	C T C T C T A T	A A A G G C A G	C T C T C T A T	A G G C A G A A	C T A T C T C T	A G A A A G G C	C T A T C T C T	A A A G G C A G	C T A T C T C T	A G G C A G A A	C T C T A T C T	A G A A A G G C	C T C T A T C T	A A A G G C A G	C T C T A T C T
	C 3	A G G C A G A A	T A T C C T C T	A G A A A G G C	T A T C C T C T	A A A G G C A G	T A T C C T C T	A G G C A G A A	C T C T T A T C	A G A A A G G C	C T C T T A T C	A A A G G C A G	C T C T T A T C	A G G C A G A A	T C C T C T T A	A G A A A G G C	T C C T C T T A	A A A G G C A G	T C C T C T T A
	D 3	A G G C A G A A	A G A G T A G A	A G A A A G G C	A G A G T A G A	A A A G G C A G	A G A G T A G A	A G G C A G A A	T A G A A G A G	A G A A A G G C	T A G A A G A G	A A A G G C A G	T A G A A G A G	A G G C A G A A	A G T A G A A G	A G A A A G G C	A G T A G A A G	A A A G G C A G	A G T A G A A G
	E 3	A G G C A G A A	G T A A A G G A G	A G A A A G G C	G T A A A G G A G	A A A G G C A G	G T A A A G G A G	A G G C A G A A	G G A G G T A A	A G A A A G G C	G G A G G T A A	A A A G G C A G	G G A G G T A A	A G G C A G A A	A A G G A G G T	A G A A A G G C	A A G G A G G T	A A A G G C A G	A A G G A G G T
	F 3	A G G C A G A A	A C T G C A T A	A G A A A G G C	A C T G C A T A	A A A G G C A G	A C T G C A T A	A G G C A G A A	C A T A A C T G	A G A A A G G C	C A T A A C T G	A A A G G C A G	C A T A A C T G	A G G C A G A A	T G C A T A A C	A G A A A G G C	T G C A T A A C	A A A G G C A G	T G C A T A A C
	G 3	A G G C A G A A	A A G G A G T A	A G A A A G G C	A A G G A G T A	A A A G G C A G	A A G G A G T A	A G G C A G A A	A G T A A A G G	A G A A A G G C	A G T A A A G G	A A A G G C A G	A G T A A A G G	A G G C A G A A	G G A G T A A A	A G A A A G G C	G G A G T A A A	A A A G G C A G	G G A G T A A A
	H 3	A G G C A	C T A A G	A G A A A	C T A A G	A A A G G	C T A A G	A G G C A	G C C T C	A G A A A	G C C T C	A A A G G	G C C T C	A G G C A	A A G C C	A G A A A	A A G C C	A A A G G	A A G C C



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
		G A A	C C T	G G C	C C T	C A G	C C T	G A A	T A A	G G C	T A A	C A G	T A A	G A A	T C T	G G C	T C T	C A G	T C T
A 4		T C C T G A G C	T A G A T C G C	G A G C T C C T	T A G A T C G C	G C T C C T G A	T A G A T C G C	T C C T G A G C	T C G C T A G A	G A G C T C C T	T C G C T A G A	G C T C C T G A	T C G C T A G A	T C C T G A G C	G A T C G C T A	G A G C T C C T	G A T C G C T A	G C T C C T G A	G A T C G C T A
B 4		T C C T G A G C	C T C T C T A T	G A G C T C C T	C T C T C T A T	G C T C C C T G A	C T C T C T A T	T C C T G A G C	C T A T C T C T	G A G C T C C T	C T A T C T C T	G C T C C C T G A	C T A T C T C T	T C C T G A G C	C T C T A T C T	G A G C T C C T	C T C T A T C T	G C T C C T G A	C T C T A T C T
C 4		T C C T G A G C	T A T C C T C T	G A G C T C C T	T A T C C T C T	G C T C C C T G A	T A T C C T C T	T C C T G A G C	C T C T T A T C	G A G C T C C T	C T C T T A T C	G C T C C C T G A	C T C T T A T C	T C C T G A G C	T C C T C T T A	G A G C T C C T	T C C T C T T A	G C T C C T G A	T C C T C T T A
D 4		T C C T G A G C	A G A G T A G A	G A G C T C C T	A G A G T A G A	G C T C C C T G A	A G A G T A G A	T C C T G A G C	T A G A A G A G	G A G C T C C T	T A G A A G A G	G C T C C C T G A	T A G A A G A G	T C C T G A G C	A G T A G A A G	G A G C T C C T	A G T A G A A G	G C T C C T G A	A G T A G A A G
E 4		T C C T G A G C	G T A A G G A G	G A G C T C C T	G T A A G G A G	G C T C C C T G A	G T A A G G A G	T C C T G A G C	G G A G G T A A	G A G C T C C T	G G A G G T A A	G C T C C C T G A	G G A G G T A A	T C C T G A G C	A A G G A G G T	G A G C T C C T	A A G G A G G T	G C T C C T G A	A A G G A G G T
F 4		T C C T G A G C	A C T G C A T A	G A G C T C C T	A C T G C A T A	G C T C C C T G A	A C T G C A T A	T C C T G A G C	C A T A A C T G	G A G C T C C T	C A T A A C T G	G C T C C C T G A	C A T A A C T G	T C C T G A G C	T G C A T A A C	G A G C T C C T	T G C A T A A C	G C T C C T G A	T G C A T A A C

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
	G 4	T C C T G A G C	A A G G A G T A	G A G C T C C T	A A G G A G T A	G C T C C T G A	A A G G A G T A	T C C T G A G C	A G T A A A G G	G A G C T C C T	A G T A A A G G	G C T C C T G A	A G T A A A G G	T C C T G A G C	G G A G T A A A	G A G C T C C T	G G A G T A A A	G C T C C T G A	G G A G T A A A
	H 4	T C C T G A G C	C T A A G C C T	G A G C T C C T	C T A A G C C T	G C T C C T G A	C T A A G C C T	T C C T G A G C	G C C T C T A A	G A G C T C C T	G C C T C T A A	G C T C C T G A	G C C T C T A A	T C C T G A G C	A A G C C T C T	G A G C T C C T	A A G C C T C T	G C T C C T G A	A A G C C T C T
	A 5	G G A C T C C T	T A G A T C G C	T C C T G G A C	T A G A T C G C	C T G G A C T C	T A G A T C G C	G G A C T C C T	T C G C T A G A	T C C T G G A C	T C G C T A G A	C T G G A C T C	T C G C T A G A	G G A C T C C T	G A T C G C T A	T C C T G G A C	G A T C G C T A	C T G G A C T C	G A T C G C T A
	B 5	G G A C T C C T	C T C T C T A T	T C C T G G A C	C T C T C T A T	C T G G A C T C	C T C T C T A T	G G A C T C C T	C T A T C T C T	T C C T G G A C	C T A T C T C T	C T G G A C T C	C T A T C T C T	G G A C T C C T	C T C T A T C T	T C C T G G A C	C T C T A T C T	C T G G A C T C	C T C T A T C T
	C 5	G G A C T C C T	T A T C C T C T	T C C T G G A C	T A T C C T C T	C T G G A C T C	T A T C C T C T	G G A C T C C T	C T C T T A T C	T C C T G G A C	C T C T T A T C	C T G G A C T C	C T C T T A T C	G G A C T C C T	T C C T C T T A	T C C T G G A C	T C C T C T T A	C T G G A C T C	T C C T C T T A
	D 5	G G A C T C C T	A G A G T A G A	T C C T G G A C	A G A G T A G A	C T G G A C T C	A G A G T A G A	G G A C T C C T	T A G A A G A G	T C C T G G A C	T A G A A G A G	C T G G A C T C	T A G A A G A G	G G A C T C C T	A G T A G A A G	T C C T G G A C	A G T A G A A G	C T G G A C T C	A G T A G A A G
	E 5	G G A C T	G T A A G	T C C T G	G T A A G	C T G G A	G T A A G	G G A C T	G G A G G	T C C T G	G G A G G	C T G G A	G G A G G	G G A C T	A A G G A	T C C T G	A A G G A	C T G G A	A A G G A

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
		CCT	GAG	GAC	GAG	CTC	GAG	CTC	TAA	GAC	TAA	CTC	TAA	CTC	GGT	GAC	GGT	CTC	GGT
F 5		GGACCTCCT	ACTGCATA	TCCCTGGAC	ACTTGCAATA	CTGGACTC	ACTGCAATA	GGACTCCT	CATAACTTG	TCCCTGGAC	CATAACTTG	CTGGACTC	CATAACTTG	GGACTCCT	TGCATAAAC	TCCCTGGAC	TGCATAAAC	CTGGACTC	TGCATAAAC
G 5		GGACCTCCT	AAGGAGTA	TCCCTGGAC	AAGGAGTA	CTGGACTC	AAGGAGTA	GGACTCCT	AGTAAGAGG	TCCCTGGAC	AGTAAGAGG	CTGGACTC	AGTAAGAGG	GGACTCCT	GGAGTAACA	TCCCTGGAC	GGAGTAACA	CTGGACTC	GGAGTAACA
H 5		GGACCTCCT	CTAAGCCT	TCCCTGGAC	CTAAGCCT	CTGGACTC	CTAAGCCT	GGACTCCT	GCCTCTAA	TCCCTGGAC	GCCTCTAA	CTGGACTC	GCCTCTAA	GGACTCCT	AAGCCTCT	TCCCTGGAC	AAGCCTCT	CTGGACTC	AAGCCTCT
A 6		TAGGCATTG	TAGATTCGC	CATTGTAGG	TAGATTCGC	TGTAGGCA	TAGATTCGC	TAGGCATTG	TCGCTAGGA	CATTGTAGG	TCGCTAGGA	TGTAGGCA	TCGCTAGGA	TAGGCATTG	GATTCGCTA	CATTGTAGG	GATTCGCTA	TGTAGGCA	GATTCGCTA
B 6		TAGGCATTG	CTCTCTTAT	CATTGTAGG	CTCTCTTAT	TGTAGGCA	CTCTCTTAT	TAGGCATTG	CTATCTCT	CATTGTAGG	CTATCTCT	TGTAGGCA	CTATCTCT	TAGGCATTG	CTCTATCT	CATTGTAGG	CTCTATCT	TGTAGGCA	CTCTATCT
C 6		TAGGCATTG	TATCCTCT	CATTGTAGG	TATCCTCT	TGTAGGCA	TATCCTCT	TAGGCATTG	CTCTTATC	CATTGTAGG	CTCTTATC	TGTAGGCA	CTCTTATC	TAGGCATTG	TCCCTCTTA	CATTGTAGG	TCCCTCTTA	TGTAGGCA	TCCCTCTTA



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
	D 6	T A G G C A T G	A G A G T A G A	C A T G T A G G	A G A G T A G A	T G T A G G C A	A G A G T A G A	T A G G C A T G	T A G A A G A G	C A T G T A G G	T A G A A G A G	T G T A G G C A	T A G A A G A G	T A G G C A T G	A G T A G A A G	C A T G T A G G	A G T A G A A G	T G T A G G C A	A G T A G A A G
	E 6	T A G G C A T G	G T A A G G A G	C A T G T A G G	G T A A G G A G	T G T A G G C A	G T A A G G A G	T A G G C A T G	G G A G G T A A	C A T G T A G G	G G A G G T A A	T G T A G G C A	G G A G G T A A	T A G G C A T G	A A G G A G G T	C A T G T A G G	A A G G A G G T	T G T A G G C A	A A G G A G G T
	F 6	T A G G C A T G	A C T G C A T A	C A T G T A G G	A C T G C A T A	T G T A G G C A	A C T G C A T A	T A G G C A T G	C A T A A C T G	C A T G T A G G	C A T A A C T G	T G T A G G C A	C A T A A C T G	T A G G C A T G	T G C A T A A C	C A T G T A G G	T G C A T A A C	T G T A G G C A	T G C A T A A C
	G 6	T A G G C A T G	A A G G A G T A	C A T G T A G G	A A G G A G T A	T G T A G G C A	A A G G A G T A	T A G G C A T G	A G T A A A G G	C A T G T A G G	A G T A A A G G	T G T A G G C A	A G T A A A G G	T A G G C A T G	G G A G T A A A	C A T G T A G G	G G A G T A A A	T G T A G G C A	G G A G T A A A
	H 6	T A G G C A T G	C T A A G C C T	C A T G T A G G	C T A A G C C T	T G T A G G C A	C T A A G C C T	T A G G C A T G	G C C T C T A A	C A T G T A G G	G C C T C T A A	T G T A G G C A	G C C T C T A A	T A G G C A T G	A A G C C T C T	C A T G T A G G	A A G C C T C T	T G T A G G C A	A A G C C T C T
	A 7	C T C T C T A C	T A G A T C G C	C T A C C T C T	T A G A T C G C	A C C T C T C T	T A G A T C G C	C T C T C T A C	T C G C T A G A	C T A C C T C T	T C G C T A G A	A C C T C T C T	T C G C T A G A	C T C T C T A C	G A T C G C T A	C T A C C T C T	G A T C G C T A	A C C T C T C T	G A T C G C T A
	B 7	C T C T C	C T C T C	C T A C C	C T C T C	A C C T C	C T C T C	C T C T C	C T A T C	C T A C C	C T A T C	A C C T C	C T A T C	C T C T C	C T C T A	C T A C C	C T C T A	A C C T C	C T C T A



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
		TAC	TAT	TCT	TAT	TCT	TAT	TAC	TCT	TCT	TCT	TCT	TCT	TAC	TCT	TCT	TCT	TCT	TCT
	C7	CCTCTTAC	TATCCTCT	CTACCCTCT	TATCCTCT	ACCTCTCT	TATCCTCT	CTCTCTAC	CTCTTATC	CTACCCTCT	CTCTTATC	ACCTCTCT	CTCTTATC	CTCTCTAC	TCCTCTTA	CTACCCTCT	TCCTCTTA	ACCTCTCT	TCCTCTTA
	D7	CTCTCTAC	AGAGTAGA	CTACCCTCT	AGAGTAGA	ACCTCTCT	AGAGTAGA	CTCTCTAC	TAGAGAGA	CTACCCTCT	TAGAGAGA	ACCTCTCT	TAGAGAGA	CTCTCTAC	AGTAGAGA	CTACCCTCT	AGTAGAGA	ACCTCTCT	AGTAGAGA
	E7	CTCTCTAC	GTAAGGAG	CTACCCTCT	GTAAGGAG	ACCTCTCT	GTAAGGAG	CTCTCTAC	GGAAGTAA	CTACCCTCT	GGAAGTAA	ACCTCTCT	GGAAGTAA	CTCTCTAC	AAGGAGGT	CTACCCTCT	AAGGAGGT	ACCTCTCT	AAGGAGGT
	F7	CTCTCTAC	ACTGCATA	CTACCCTCT	ACTGCATA	ACCTCTCT	ACTGCATA	CTCTCTAC	CATAACTG	CTACCCTCT	CATAACTG	ACCTCTCT	CATAACTG	CTCTCTAC	TGCATAAC	CTACCCTCT	TGCATAAC	ACCTCTCT	TGCATAAC
	G7	CTCTCTAC	AAGGAGTA	CTACCCTCT	AAGGAGTA	ACCTCTCT	AAGGAGTA	CTCTCTAC	AGTAAGAG	CTACCCTCT	AGTAAGAG	ACCTCTCT	AGTAAGAG	CTCTCTAC	GGAGTAAA	CTACCCTCT	GGAGTAAA	ACCTCTCT	GGAGTAAA
	H7	CTCTCTAC	CTAAGCCT	CTACCCTCT	CTAAGCCT	ACCTCTCT	CTAAGCCT	CTCTCTAC	GCTCTTAA	CTACCCTCT	GCTCTTAA	ACCTCTCT	GCTCTTAA	CTCTCTAC	AAGCCTCT	CTACCCTCT	AAGCCTCT	ACCTCTCT	AAGCCTCT



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
	A 8	C A G A G A G G	T A G A T C G C	G A G G C A G A	T A G A T C G C	G G C A G A G A	T A G A T C G C	C A G A G A G G	T C G C T A G A	G A G G C A G A	T C G C T A G A	G G C A G A G A	T C G C T A G A	C A G A G A G G	G A T C G C T A	G A G G C A G A	G A T C G C T A	G G C A G A G A	G A T C G C T A
	B 8	C A G A G A G G	C T C T C T A T	G A G G C A G A	C T C T C T A T	G G C A G A G A	C T C T C T A T	C A G A G A G G	C T A T C T C T	G A G G C A G A	C T A T C T C T	G G C A G A G A	C T A T C T C T	C A G A G A G G	C T C T A T C T	G A G G C A G A	C T C T A T C T	G G C A G A G A	C T C T A T C T
	C 8	C A G A G A G G	T A T C C T C T	G A G G C A G A	T A T C C T C T	G G C A G A G A	T A T C C T C T	C A G A G A G G	C T C T T A T C	G A G G C A G A	C T C T T A T C	G G C A G A G A	C T C T T A T C	C A G A G A G G	T C C T C T T A	G A G G C A G A	T C C T C T T A	G G C A G A G A	T C C T C T T A
	D 8	C A G A G A G G	A G A G T A G A	G A G G C A G A	A G A G T A G A	G G C A G A G A	A G A G T A G A	C A G A G A G G	T A G A A G A G	G A G G C A G A	T A G A A G A G	G G C A G A G A	T A G A A G A G	C A G A G A G G	A G T A G A G A	G A G G C A G A	A G T A G A G A	G G C A G A G A	A G T A G A G A
	E 8	C A G A G A G G	G T A A G G A G	G A G G C A G A	G T A A G G A G	G G C A G A G A	G T A A G G A G	C A G A G A G G	G G A G G T A A	G A G G C A G A	G G A G G T A A	G G C A G A G A	G G A G G T A A	C A G A G A G G	A A G G A G G T	G A G G C A G A	A A G G A G G T	G G C A G A G A	A A G G A G G T
	F 8	C A G A G A G G	A C T G C A T A	G A G G C A G A	A C T G C A T A	G G C A G A G A	A C T G C A T A	C A G A G A G G	C A T A A C T G	G A G G C A G A	C A T A A C T G	G G C A G A G A	C A T A A C T G	C A G A G A G G	T G C A T A A C	G A G G C A G A	T G C A T A A C	G G C A G A G A	T G C A T A A C
	G 8	C A G A G	A A G G A	G A G G C	A A G G A	G G C A G	A A G G A	C A G A G	A G T A A	G A G G C	A G T A A	G G C A G	A G T A A	C A G A G	G G A G T	G A G G C	G G A G T	G G C A G	G G A G T



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
		A G G	G T A	A G A	G T A	A G A	G T A	A G G	A G G	A G A	A G G	A G A	A G G	A G G	A A A	A G A	A A A	A G A	A A A
	H 8	C A G A G A G G	C T A A G C C T	G A G G C A G A	C T A A G C C T	G G C A G A G A	C T A A G C C T	C A G A G A G G	G C C T C T A A	G A G G C A G A	G C C T C T A A	G G C A G A G A	G C C T C T A A	C A G A G A G G	A A G C C T C T	G A G G C A G A	A A G C C T C T	G G C A G A G A	A A G C C T C T
	A 9	G C T A C G C T	T A G A T C G C	C G C T G C T A	T A G A T C G C	C T G C T A C G	T A G A T C G C	G C T A C G C T	T C G C T A G A	C G C T G C T A	T C G C T A G A	C T G C T A C G	T C G C T A G A	G C T A C G C T	G A T C G C T A	C G C T G C T A	G A T C G C T A	C T G C T A C G	G A T C G C T A
	B 9	G C T A C G C T	C T C T C T A T	C G C T G C T A	C T C T C T A T	C T G C T A C G	C T C T C T A T	G C T A C G C T	C T A T C T C T	C G C T G C T A	C T A T C T C T	C T G C T A C G	C T A T C T C T	G C T A C G C T	C T C T A T C T	C G C T G C T A	C T C T A T C T	C T G C T A C G	C T C T A T C T
	C 9	G C T A C G C T	T A T C C T C T	C G C T G C T A	T A T C C T C T	C T G C T A C G	T A T C C T C T	G C T A C G C T	C T C T T A T C	C G C T G C T A	C T C T T A T C	C T G C T A C G	C T C T T A T C	G C T A C G C T	T C C T C T T A	C G C T G C T A	T C C T C T T A	C T G C T A C G	T C C T C T T A
	D 9	G C T A C G C T	A G A G T A G A	C G C T G C T A	A G A G T A G A	C T G C T A C G	A G A G T A G A	G C T A C G C T	T A G A A G A G	C G C T G C T A	T A G A A G A G	C T G C T A C G	T A G A A G A G	G C T A C G C T	A G T A G A A G	C G C T G C T A	A G T A G A A G	C T G C T A C G	A G T A G A A G
	E 9	G C T A C G C T	G T A A G G A G	C G C T G C T A	G T A A G G A G	C T G C T A C G	G T A A G G A G	G C T A C G C T	G G A G G T A A	C G C T G C T A	G G A G G T A A	C T G C T A C G	G G A G G T A A	G C T A C G C T	A A G G A G G T	C G C T G C T A	A A G G A G G T	C T G C T A C G	A A G G A G G T



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
	F 9	GCTACGCT	ACTGCAATA	CGCTGCTA	ACTGCAATA	CTGCTACG	ACTGCAATA	GCTACGCT	CATAACTG	CGCTGCTA	CATAACTG	CTGCTACG	CATAACTG	GCTACGCT	TGCATAAC	CGCTGCTA	TGCATAAC	CTGCTACG	TGCATAAC
	G 9	GCTACGCT	AAGGAGTA	CGCTGCTA	AAGGAGTA	CTGCTACG	AAGGAGTA	GCTACGCT	AGTAAGAAG	CGCTGCTA	AGTAAGAAG	CTGCTACG	AGTAAGAAG	GCTACGCT	GAGTAATA	CGCTGCTA	GAGTAATA	CTGCTACG	GAGTAATA
	H 9	GCTACGCT	CTAAGCCT	CGCTGCTA	CTAAGCCT	CTGCTACG	CTAAGCCT	GCTACGCT	GCCCTCTAA	CGCTGCTA	GCCCTCTAA	CTGCTACG	GCCCTCTAA	GCTACGCT	AAGCCTCT	CGCTGCTA	AAGCCTCT	CTGCTACG	AAGCCTCT
	A 1 0	CGAGGCTG	TAGATTCGC	GCTGCGAG	TAGATTCGC	TGCGAGGC	TAGATTCGC	CGAGGCTG	TCGCTAGAA	GCTGCGAG	TCGCTAGAA	TGCGAGGC	TCGCTAGAA	CGAGGCTG	GATCGCTA	GCTGCGAG	GATCGCTA	TGCGAGGC	GATCGCTA
	B 1 0	CGAGGCTG	CTCTCTAT	GCTGCGAG	CTCTCTAT	TGCGAGGC	CTCTCTAT	CGAGGCTG	CTATCTCT	GCTGCGAG	CTATCTCT	TGCGAGGC	CTATCTCT	CGAGGCTG	CTCTATCT	GCTGCGAG	CTCTATCT	TGCGAGGC	CTCTATCT
	C 1 0	CGAGGCTG	TATCCTCT	GCTGCGAG	TATCCTCT	TGCGAGGC	TATCCTCT	CGAGGCTG	CTCTTATC	GCTGCGAG	CTCTTATC	TGCGAGGC	CTCTTATC	CGAGGCTG	TCTCTTA	GCTGCGAG	TCTCTTA	TGCGAGGC	TCTCTTA
	D 1 0	CGAGG	AGAGT	GCTGC	AGAGT	TGCGA	AGAGT	CGAGG	TAGAA	GCTGC	TAGAA	TGCGA	TAGAA	CGAGG	AGTAG	GCTGC	AGTAG	TGCGA	AGTAG



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
		C T G	A G A	G A G	A G A	G G C	A G A	C T G	G A G	G A G	G A G	G G C	G A G	C T G	A A G	G A G	A A G	G G C	A A G
E 1 0		C G A G G C T G	G T A A G G A G	G C T G C G A G	G T A A G G A G	T G C G A G G C	G T A A G G A G	C G A G G C T G	G G A G G T A A	G C T G C G A G	G G A G G T A A	T G C G A G G C	G G A G G T A A	C G A G G C T G	A A G G A G G T	G C T G C G A G	A A G G A G G T	T G C G A G G C	A A G G A G G T
F 1 0		C G A G G C T G	A C T G C A T A	G C T G C G A G	A C T G C A T A	T G C G A G G C	A C T G C A T A	C G A G G C T G	C A T A A C T G	G C T G C G A G	C A T A A C T G	T G C G A G G C	C A T A A C T G	C G A G G C T G	T G C A T A A C	G C T G C G A G	T G C A T A A C	T G C G A G G C	T G C A T A A C
G 1 0		C G A G G C T G	A A G G A G T A	G C T G C G A G	A A G G A G T A	T G C G A G G C	A A G G A G T A	C G A G G C T G	A G T A A A G G	G C T G C G A G	A G T A A A G G	T G C G A G G C	A G T A A A G G	C G A G G C T G	G G A G T A A A	G C T G C G A G	G G A G T A A A	T G C G A G G C	G G A G T A A A
H 1 0		C G A G G C T G	C T A A G C C T	G C T G C G A G	C T A A G C C T	T G C G A G G C	C T A A G C C T	C G A G G C T G	G C C T C T A A	G C T G C G A G	G C C T C T A A	T G C G A G G C	G C C T C T A A	C G A G G C T G	A A G C C T C T	G C T G C G A G	A A G C C T C T	T G C G A G G C	A A G C C T C T
A 1 1		A A G A G G C A	T A G A T C G C	G G C A A A G A	T A G A T C G C	C A A A G A G G	T A G A T C G C	A A G A G G C A	T C G C T A G A	G G C A A A G A	T C G C T A G A	C A A A G A G G	T C G C T A G A	A A G A G G C A	G A T C G C T A	G G C A A A G A	G A T C G C T A	C A A A G A G G	G A T C G C T A
B 1 1		A A G A G G C A	C T C T C T A T	G G C A A A G A	C T C T C T A T	C A A A G A G G	C T C T C T A T	A A G A G G C A	C T A T C T C T	G G C A A A G A	C T A T C T C T	C A A A G A G G	C T A T C T C T	A A G A G G C A	C T C T A T C T	G G C A A A G A	C T C T A T C T	C A A A G A G G	C T C T A T C T



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
	C 11	A A G A G G C A	T A T C C T C T	G G C A A A G A	T A T C C T C T	C A A A G A G G	T A T C C T C T	A A G A G G C A	C T C T T A T C	G G C A A A G A	C T C T T A T C	C A A A G A G G	C T C T T A T C	A A G A G G C A	T C C T C T T A	G G C A A A G A	T C C T C T T A	C A A A G A G G	T C C T C T T A
	D 11	A A G A G G C A	A G A G T A G A	G G C A A A G A	A G A G T A G A	C A A A G A G G	A G A G T A G A	A A G A G G C A	T A G A A G A G	G G C A A A G A	T A G A A G A G	C A A A G A G G	T A G A A G A G	A A G A G G C A	A G T A G A A G	G G C A A A G A	A G T A G A A G	C A A A G A G G	A G T A G A A G
	E 11	A A G A G G C A	G T A A G G A G	G G C A A A G A	G T A A G G A G	C A A A G A G G	G T A A G G A G	A A G A G G C A	G G A G G T A A	G G C A A A G A	G G A G G T A A	C A A A G A G G	G G A G G T A A	A A G A G G C A	A A G G A G G T	G G C A A A G A	A A G G A G G T	C A A A G A G G	A A G G A G G T
	F 11	A A G A G G C A	A C T G C A T A	G G C A A A G A	A C T G C A T A	C A A A G A G G	A C T G C A T A	A A G A G G C A	C A T A A C T G	G G C A A A G A	C A T A A C T G	C A A A G A G G	C A T A A C T G	A A G A G G C A	T G C A T A A C	G G C A A A G A	T G C A T A A C	C A A A G A G G	T G C A T A A C
	G 11	A A G A G G C A	A A G G A G T A	G G C A A A G A	A A G G A G T A	C A A A G A G G	A A G G A G T A	A A G A G G C A	A G T A A A G G	G G C A A A G A	A G T A A A G G	C A A A G A G G	A G T A A A G G	A A G A G G C A	G G A G T A A A	G G C A A A G A	G G A G T A A A	C A A A G A G G	G G A G T A A A
	H 11	A A G A G G C A	C T A A G C C T	G G C A A A G A	C T A A G C C T	C A A A G A G G	C T A A G C C T	A A G A G G C A	G C C T C T A A	G G C A A A G A	G C C T C T A A	C A A A G A G G	G C C T C T A A	A A G A G G C A	A A G C C T C T	G G C A A A G A	A A G C C T C T	C A A A G A G G	A A G C C T C T
	A 1 2	G T A G A	T A G A T	A G G A G	T A G A T	G A G T A	T A G A T	G T A G A	T C G C T	A G G A G	T C G C T	G A G T A	T C G C T	G T A G A	G A T C G	A G G A G	G A T C G	G A G T A	G A T C G

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
		G G A	C G C	T A G	C G C	G A G	C G C	G G A	A G A	T A G	A G A	G A G	A G A	G G A	C T A	T A G	C T A	G A G	C T A
B 1 2		G T A G A G G A	C T C T C T A T	A G G A G T A G	C T C T C T A T	G A G T A G A G	C T C T C T A T	G T A G A G G A	C T A T C T C T	A G G A G T A G	C T A T C T C T	G A G T A G A G	C T A T C T C T	G T A G A G G A	C T C T A T C T	A G G A G T A G	C T C T A T C T	G A G T A G A G	C T C T A T C T
C 1 2		G T A G A G G A	T A T C C T C T	A G G A G T A G	T A T C C T C T	G A G T A G A G	T A T C C T C T	G T A G A G G A	C T C T T A T C	A G G A G T A G	C T C T T A T C	G A G T A G A G	C T C T T A T C	G T A G A G G A	T C C T C T T A	A G G A G T A G	T C C T C T T A	G A G T A G A G	T C C T C T T A
D 1 2		G T A G A G G A	A G A G T A G A	A G G A G T A G	A G A G T A G A	G A G T A G A G	A G A G T A G A	G T A G A G G A	T A G A A G A G	A G G A G T A G	T A G A A G A G	G A G T A G A G	T A G A A G A G	G T A G A G G A	A G T A G A A G	A G G A G T A G	A G T A G A A G	G A G T A G A G	A G T A G A A G
E 1 2		G T A G A G G A	G T A A G G A G	A G G A G T A G	G T A A G G A G	G A G T A G A G	G T A A G G A G	G T A G A G G A	G G A G G T A A	A G G A G T A G	G G A G G T A A	G A G T A G A G	G G A G G T A A	G T A G A G G A	A A G G A G G T	A G G A G T A G	A A G G A G G T	G A G T A G A G	A A G G A G G T
F 1 2		G T A G A G G A	A C T G C A T A	A G G A G T A G	A C T G C A T A	G A G T A G A G	A C T G C A T A	G T A G A G G A	C A T A A C T G	A G G A G T A G	C A T A A C T G	G A G T A G A G	C A T A A C T G	G T A G A G G A	T G C A T A A C	A G G A G T A G	T G C A T A A C	G A G T A G A G	T G C A T A A C
G 1 2		G T A G A G G A	A A G G A G T A	A G G A G T A G	A A G G A G T A	G A G T A G A G	A A G G A G T A	G T A G A G G A	A G T A A A G G	A G G A G T A G	A G T A A A G G	G A G T A G A G	A G T A A A G G	G T A G A G G A	G G A G T A A A	A G G A G T A G	G G A G T A A A	G A G T A G A G	G G A G T A A A
H 1		G T	C T	A G	C T	G A	C T	G T	G C	A G	G C	G A	G C	G T	A A	A G	A A	G A	A A



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
2	A	A	G	A	A	G	A	A	C	G	C	G	C	A	G	G	G	G	G
	G	A	A	A	A	T	A	A	T	A	T	A	T	A	C	A	C	T	A
	A	G	G	G	G	A	G	G	C	G	C	A	C	A	C	T	C	A	G
	G	A	C	T	C	A	C	A	A	A	A	A	A	A	T	A	T	G	T

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	aatcaccgactgcccataagagaggaaagcggag	13	4 µL
TSIT_Read2	ctccgctttcctctctatgggcagtcggtgatt	14	4 µL

Protocol references

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