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Workflow for SNP genotyping using the Hi-Plex method V.2

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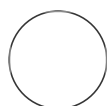
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We use this protocol and it's working

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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 adaptor (TSITs) preparation 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 50µM
- ThermoFisher Phusion™ Hot Start II High Fidelity (ref. F549L)
- ThermoFisher Phusion™ 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'-AATGATACGGCGACCACCGA-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 $-20\text{ }^{\circ}\text{C}$ and placed in the refrigerator at $4\text{ }^{\circ}\text{C}$ 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 INSTRUCTIONS

- 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 Ion Torrent 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)

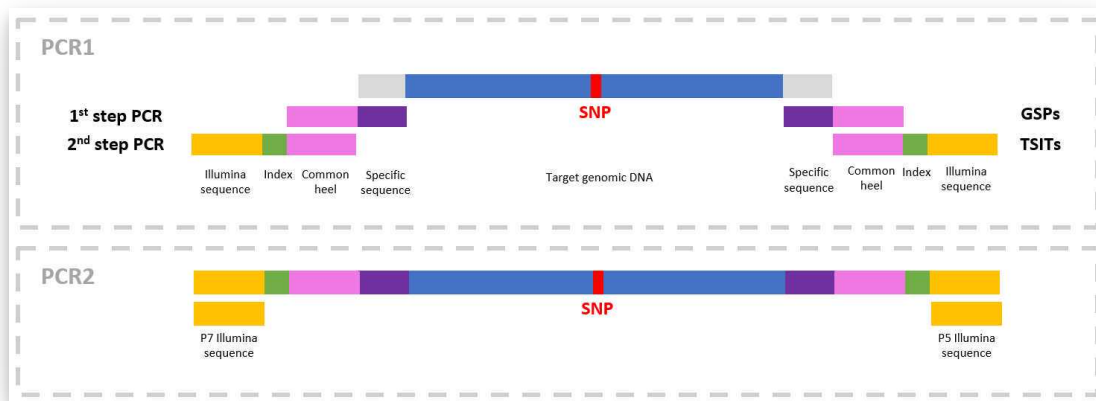
TSITs are adaptors that consist of a complementary sequence to the GSP common heel, a barcode, and a specific sequence for Illumina sequencing (P5/P7) for a total of about 60 bp for each TSIT A and TSIT P. The use of a combination of this 2 barcodes allows the specific identification of the DNA samples.

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 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 PipinnTM prep.

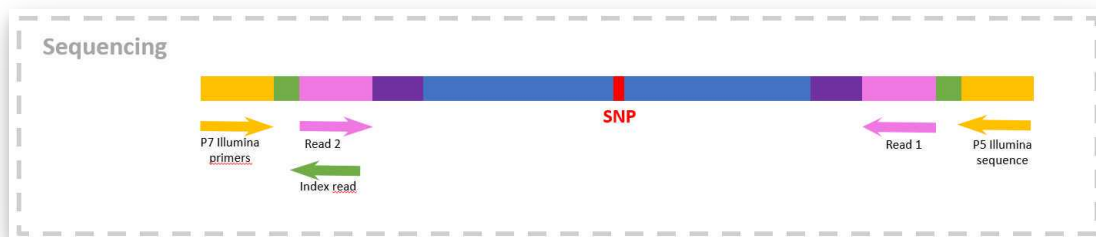
The use of EtBr is essential if size selection from agarose gel is performed. SybrSafe reagent, for example, generated a lot of non-specific amplicons. This suggests that SybrSafe does not allow for clean separation of migrated fragments on agarose gels.



Simplified schemes of PCR steps

Quality controls and sequencing

Each library is checked on a Fragment Analyzer™ 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 adaptor (TSITs) preparati...

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
```

```
CGGGCCGGCCCGTCCAAAATAGCAAGCAGAGTGAAATCCTCCACATTCTAAATGGCCACATGCACAAACA  
AGCAGTGTTTTCTTAAAGAGACCACTGCTCCCAGCTCACCCAGCAGCCTCTCACAGACAGTGGTGGGGGGG
```

GGG

- 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

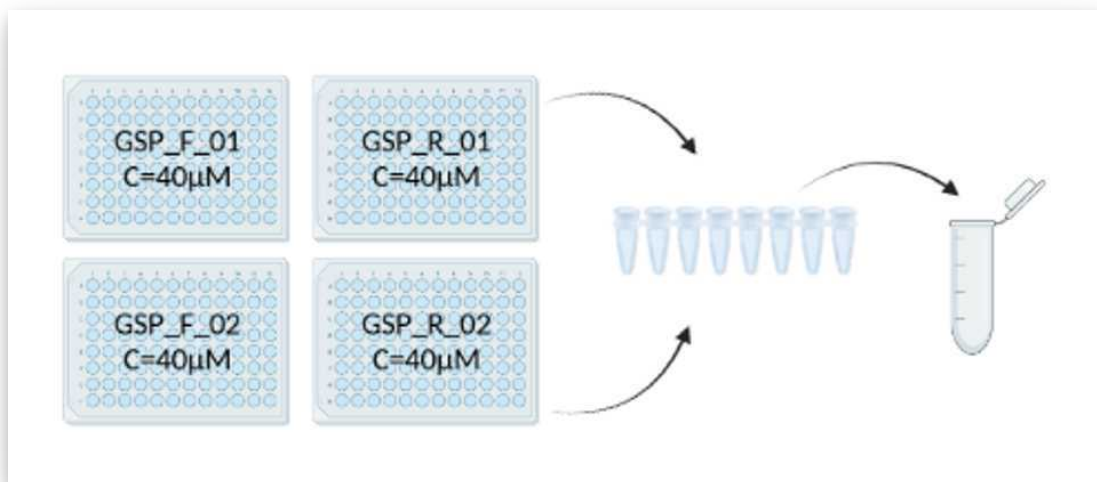
- Prepare 40 μM plates from each 100 or 200 μM GSP plate using 1X low TE (10 mM Tris pH8.0/0.1mM EDTA):

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	Ci GSP (μM)	Cf GSP (μM) =Ci * Vi / Vf pool	Vi GSP (μL)	Number of GSP =Nb F + Nb R	Vf pool (μL) =Vi GSP * Nb GSP	Cf pool (μM) =Cf GSP * Number of GSP	Nb aliquot 100 μL =Vf 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)	Vi (μL)	Vf (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 Adaptor (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
Plate well	TSITs P names	Sequences of TSITs P
A1	N701_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTCGCCTTActccgctttcctctctatgggcagtcggtg at
A2	N702_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTAGTACGctccgctttcctctctatgggcagtcggtg at
A3	N703_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTTCTGCCTctccgctttcctctctatgggcagtcggtg at
A4	N704_TSIT_P	CAAGCAGAAGACGGCATAACGAGATGCTCAGGActccgctttcctctctatgggcagtcggtg at
A5	N705_TSIT_P	CAAGCAGAAGACGGCATAACGAGATAGGAGTCCctccgctttcctctctatgggcagtcggtg at
A6	N706_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCATGCCTActccgctttcctctctatgggcagtcggtg at
A7	N707_TSIT_P	CAAGCAGAAGACGGCATAACGAGATGTAGAGAGctccgctttcctctctatgggcagtcggtg at
A8	N708_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCCTCTCTGctccgctttcctctctatgggcagtcggtg at
A9	N709_TSIT_P	CAAGCAGAAGACGGCATAACGAGATAGCGTAGCctccgctttcctctctatgggcagtcggtg at
A10	N710_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCAGCCTCGctccgctttcctctctatgggcagtcggtg at
A11	N711_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTGCCTCTTctccgctttcctctctatgggcagtcggtg at
A12	N712_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTCTCTACctccgctttcctctctatgggcagtcggtg at
B1	N713_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTTATCGCctccgctttcctctctatgggcagtcggtg at
B2	N714_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTACGCTAGctccgctttcctctctatgggcagtcggtg at
B3	N715_TSIT_P	CAAGCAGAAGACGGCATAACGAGATGCCTTTCTctccgctttcctctctatgggcagtcggtg at
B4	N716_TSIT_P	CAAGCAGAAGACGGCATAACGAGATAGGAGCTCctccgctttcctctctatgggcagtcggtg at
B5	N717_TSIT_P	CAAGCAGAAGACGGCATAACGAGATGTCCAGGActccgctttcctctctatgggcagtcggtg at
B6	N718_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCCTACATGctccgctttcctctctatgggcagtcggt gat
B7	N719_TSIT_P	CAAGCAGAAGACGGCATAACGAGATAGAGGTAGctccgctttcctctctatgggcagtcggtg at
B8	N720_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTCTGCCTCctccgctttcctctctatgggcagtcggtg at
B9	N721_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTAGCAGCGctccgctttcctctctatgggcagtcggtg at
B10	N722_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTCGCAGCctccgctttcctctctatgggcagtcggtg at
B11	N723_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTCTTTGCCctccgctttcctctctatgggcagtcggtg at
B12	N724_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTACTCCTctccgctttcctctctatgggcagtcggtg at

A	B	C
C1	N725_TSIT_P	CAAGCAGAAGACGGCATAACGAGATGCCTTATCctccgctttcctctctatgggcagtcggtg at
C2	N726_TSIT_P	CAAGCAGAAGACGGCATAACGAGATAGTACGCTctccgctttcctctctatgggcagtcggtg at
C3	N727_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTGCCTTTctccgctttcctctctatgggcagtcggtg at
C4	N728_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTGAGGAGCctccgctttcctctctatgggcagtcggtg at
C5	N729_TSIT_P	CAAGCAGAAGACGGCATAACGAGATGAGTCCAGctccgctttcctctctatgggcagtcggtg at
C6	N730_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTGCCTACActccgctttcctctctatgggcagtcggtg at
C7	N731_TSIT_P	CAAGCAGAAGACGGCATAACGAGATAGAGAGGTctccgctttcctctctatgggcagtcggtg at
C8	N732_TSIT_P	CAAGCAGAAGACGGCATAACGAGATTCTCTGCCctccgctttcctctctatgggcagtcggtg at
C9	N733_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCGTAGCAGctccgctttcctctctatgggcagtcggtg at
C10	N734_TSIT_P	CAAGCAGAAGACGGCATAACGAGATGCCTCGCActccgctttcctctctatgggcagtcggtg at
C11	N735_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCCTCTTTGctccgctttcctctctatgggcagtcggtg at
C12	N736_TSIT_P	CAAGCAGAAGACGGCATAACGAGATCTCTACTCctccgctttcctctctatgggcagtcggtg at

List of TSITs P adaptors

A	B	C
Plate well	TSITs A names	Sequences of TSITs A
A1	N501_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTAGATCGCccatctcatccctgcgtgtc tccgactcag
B1	N502_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTCTCTATccatctcatccctgcgtgtc ccgactcag
C1	N503_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTATCCTCTccatctcatccctgcgtgtc ccgactcag
D1	N504_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAGAGTAGAccatctcatccctgcgtgtc tccgactcag
E1	N505_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGccatctcatccctgcgtgtc tccgactcag
F1	N506_TSIT_A	AATGATACGGCGACCACCGAGATCTACACACTGCATAccatctcatccctgcgtgtc tccgactcag
G1	N507_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAAGGAGTAccatctcatccctgcgtgtc tccgactcag
H1	N508_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTccatctcatccctgcgtgtc tccgactcag

A	B	C
A2	N509_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTCGCTAGAccatctcatccctgcgtgtc tccgactcag
B2	N510_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTATCTCTccatctcatccctgcgtgtct ccgactcag
C2	N511_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTCTTATCccatctcatccctgcgtgtct ccgactcag
D2	N512_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTAGAAGAGccatctcatccctgcgtgtc tccgactcag
E2	N513_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGGAGGTAAccatctcatccctgcgtgtc tccgactcag
F2	N514_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCATAACTGccatctcatccctgcgtgtc tccgactcag
G2	N515_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAGTAAAGGccatctcatccctgcgtgtc tccgactcag
H2	N516_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGCCTCTAAccatctcatccctgcgtgtc tccgactcag
A3	N517_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGATCGCTAccatctcatccctgcgtgtc tccgactcag
B3	N518_TSIT_A	AATGATACGGCGACCACCGAGATCTACACCTCTATCTccatctcatccctgcgtgtct ccgactcag
C3	N519_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTCCTCTTAccatctcatccctgcgtgtct ccgactcag
D3	N520_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAGTAGAAGccatctcatccctgcgtgtc tccgactcag
E3	N521_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAAGGAGGTccatctcatccctgcgtgtc tccgactcag
F3	N522_TSIT_A	AATGATACGGCGACCACCGAGATCTACACTGCATAACccatctcatccctgcgtgtc tccgactcag
G3	N523_TSIT_A	AATGATACGGCGACCACCGAGATCTACACGGAGTAAccatctcatccctgcgtgtc tccgactcag
H3	N524_TSIT_A	AATGATACGGCGACCACCGAGATCTACACAAGCCTCTccatctcatccctgcgtgtc tccgactcag

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
C_i μM	C_f μM	V_i μL	V_f	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_10µM_1 to TSIT_10µM_9). As the TSIT_A are arranged columnwise in plate TSIT_A_50µM and TSIT_P are sorted rowwise in plate TSIT_P_50µM, distribute 5µL 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 volmue
TSIT_10µM_1	1	5	A	5	15	25
TSIT_10µM_2	1	5	B	5	15	25
TSIT_10µM_3	1	5	C	5	15	25
TSIT_10µM_4	2	5	A	5	15	25
TSIT_10µM_5	2	5	B	5	15	25
TSIT_10µM_6	2	5	C	5	15	25
TSIT_10µM_7	3	5	A	5	15	25
TSIT_10µM_8	3	5	B	5	15	25
TSIT_10µM_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-P...

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 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	400μM	0.5	49 μL
Phusion HF Hot Start taq	2U/μL	1U	0.5	49 μL
GSP pool	10μM	0.2μM	0.5	49 μL
<i>TOTAL Volume mix</i>			<i>21 μL</i>	<i>2058 μL</i>
<i>DNA or control per well</i>			<i>2 μL</i>	

- **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	
72°C	01:00	
72°C	Needed to add EDTA	

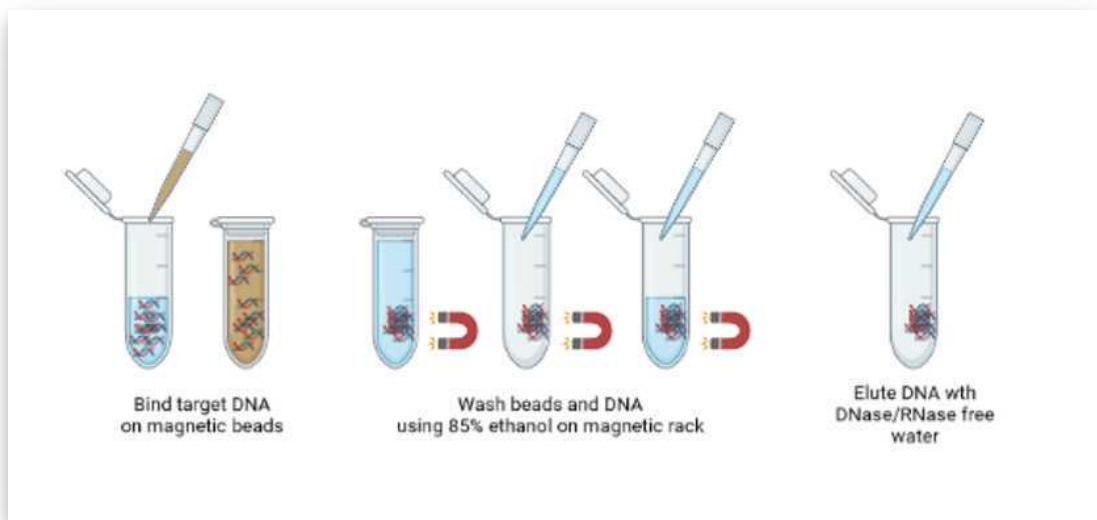
1. To limit aspecific amplification, the PCR program requires a pre-heating step at 98 °C. Start the PCR program without inserting the plate. Store the plate on ice until the heating block on the machine has reached 98 °C. Then, insert the plate.
2. At the 1st step at 72°C forever, stop the run and open 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 °C until the end of the protocol or at -20 °C for longer term storage.
2. Add 864µL of presuspended magnetic beads (0.9x960µL=864µL).
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 70µl 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 ($0.9 \times 70 \mu\text{L} = 63 \mu\text{L}$) 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 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 μ L 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 ₂	50 mM	2.5 mM	1.5
dNTPs	20 mM	400 μ M	1.5
Phusion HF Hot Start taq	2U/ μ L	1U	1.5
P5 primer : 5'-AATGATACGGCGACCACCGA-3'	50 μ M	1 μ M	1.5
P7 primer : 5'-CAAGCAGAAGACGGCATAGCA-3'	50 μ M	1 μ M	1.5
<i>TOTAL volume mix</i>			45
<i>Purified PCR1 product or control</i>			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.

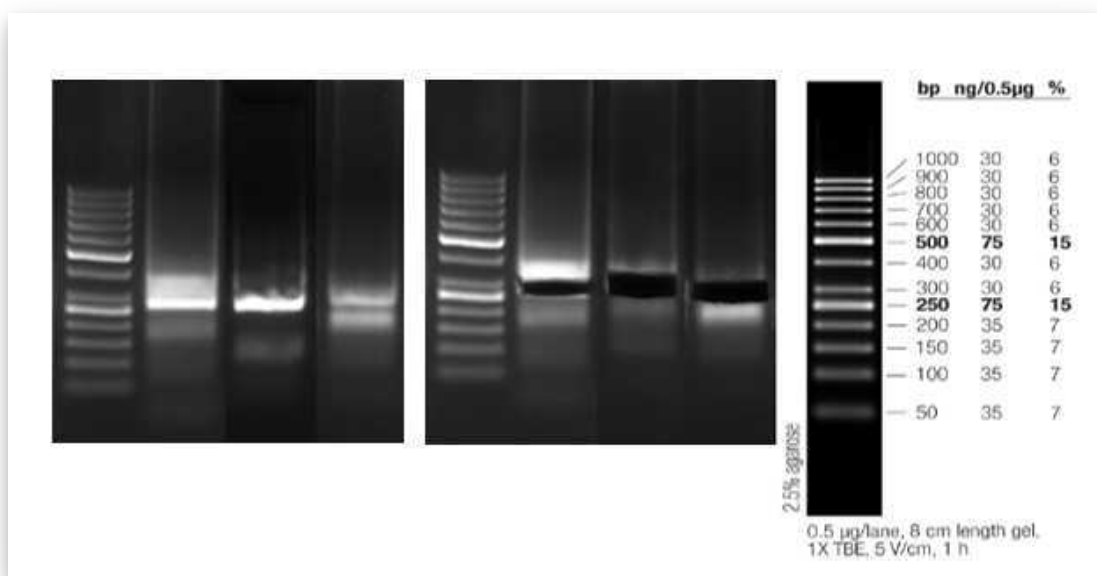
1. Prepare a 1.75% agarose gel using 1X TAE and ethidium bromide (EtBr).
2. Add directly 55 μ L of each sample to a well (possible due to the use of the green buffer in previous PCR2 step)
3. Add 10 μ L of the 50 bp ladder to at least one well (adapat to the number of samples or your gel size).
4. Run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



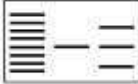






1. Use the MN Nucleospin gel clean-up kit using the gel extraction protocol supplied by the manufacturer.
2. Prepare the NT3 and add the appropriate volume of 96-100% ethanol.
3. Adapt the volume of NTI buffer for each sample following : Vol. NTI = 2x agarose weight.
4. Elute with 2 x 25 μ L of buffer NE (50 μ 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
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 <u>Recommended:</u> 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

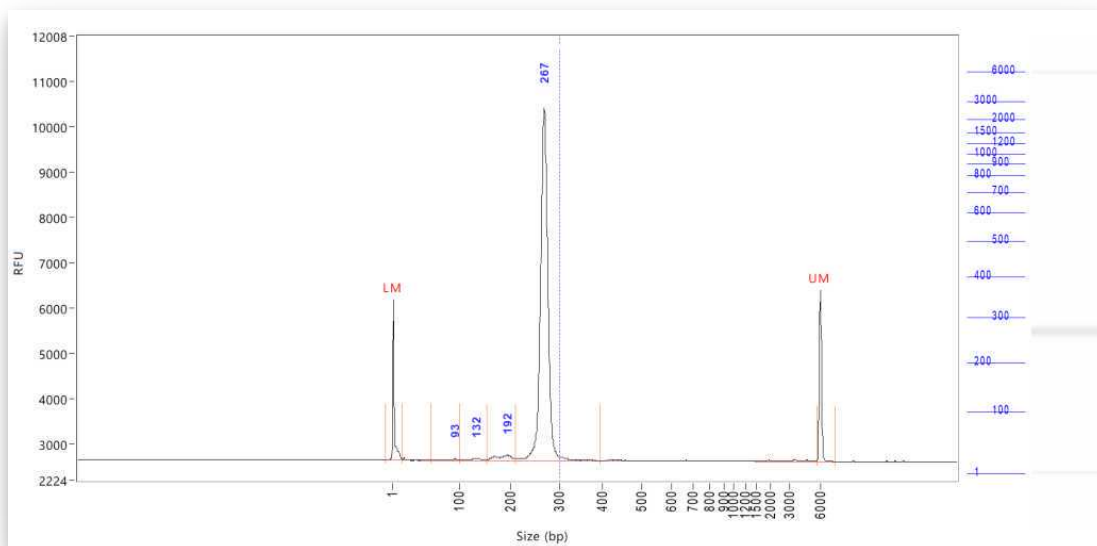
1. Bring each DNA sample from PCR2 up to 60µl with water.
2. Combine 60µl of DNA sample with 20µl of loading/marker mix (Marker L).

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.

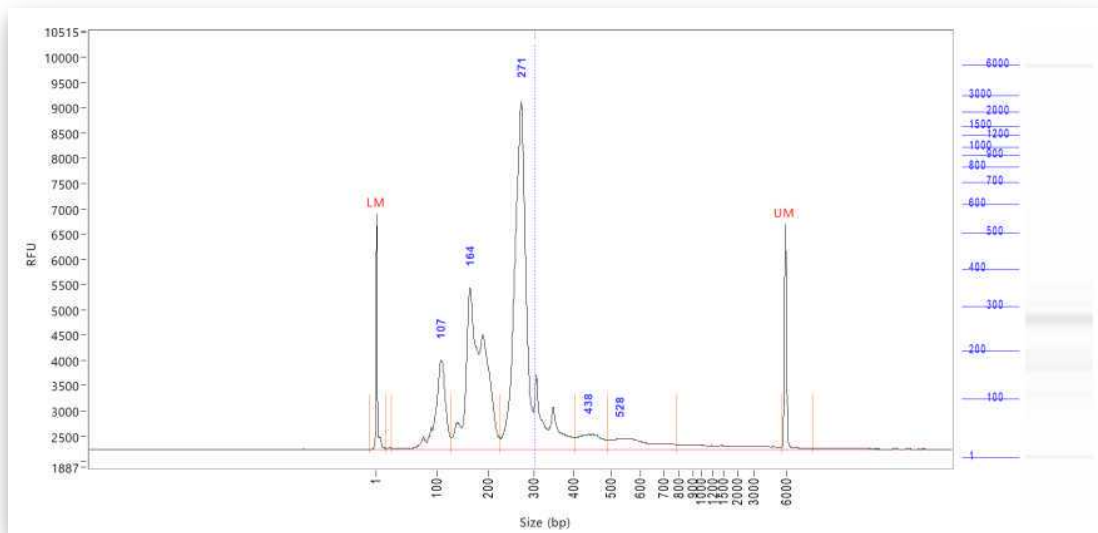
Quality and quantity control of libraries

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

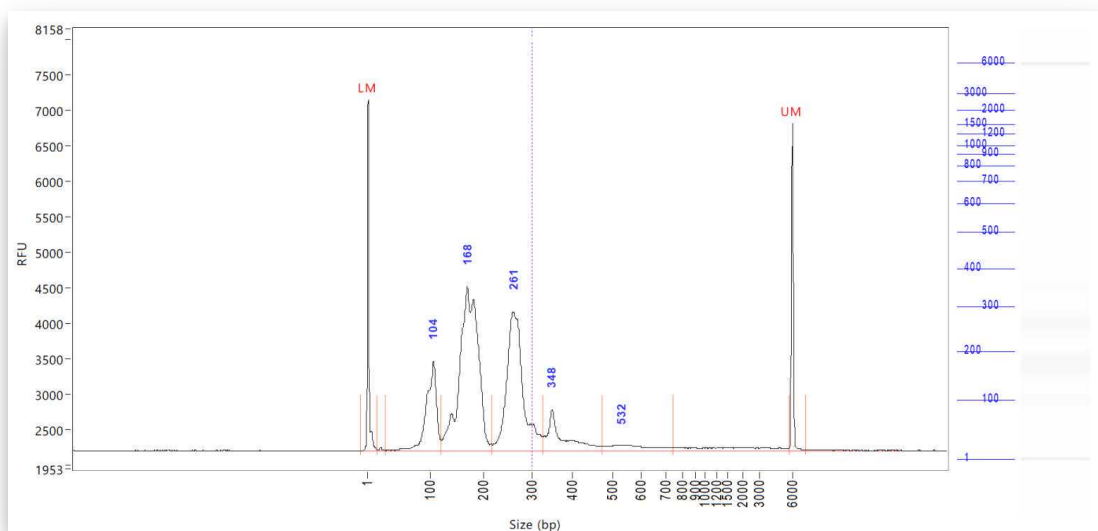
1. Use Invitrogen™ Qubit™ Fluorometer or equivalent to estimate DNA concentrations for each library
2. Prepare a 50pg/µL to 5ng/µL dilution of each library.
3. Then, follow the instructions from the manufacturer.



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 ILLU...

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
Plate well	TSIT_1	TSIT_1	TSIT_2	TSIT_2	TSIT_3	TSIT_3	TSIT_4	TSIT_4	TSIT_5	TSIT_5	TSIT_6	TSIT_6	TSIT_7	TSIT_7	TSIT_8	TSIT_8	TSIT_9	TSIT_9
	P complement	Barcod A	P complement	Barcod A	P complement	Barcod A	P complement	Barcod A	P complement	Barcod A	P complement	Barcod A	P complement	Barcod A	P complement	Barcod A	P complement	Barcod A
A1	TAA GG CG A	TA GA TC GC	GC GA TAA G	TA GA TC GC	GAT AA GG C	TA GA TC GC	TAA GGC GA	TC GC TA GA	GC GAT AA G	TC GC TA GA	GAT AA GG C	TC GC TA GA	TAA GGC GA	GA TC GC TA	GCG ATA AG	GA TC GC TA	GAT AA GG C	GA TC GC TA
B1	TAA GG CG A	CT CT CT AT	GC GA TAA G	CT CT CT AT	GAT AA GG C	CT CT CT AT	TAA GGC GA	CT AT CT CT	GC GAT AA G	CT AT CT CT	GAT AA GG C	CT AT CT CT	TAA GGC GA	CT CT AT CT	GCG ATA AG	CT CT AT CT	GAT AA GG C	CT CT AT CT
C1	TAA GG CG A	TA TC CT CT	GC GA TAA G	TA TC CT CT	GAT AA GG C	TA TC CT CT	TAA GGC GA	CT CT TA TC	GC GAT AA G	CT CT TA TC	GAT AA GG C	CT CT TA TC	TAA GGC GA	TC CT CT TA	GCG ATA AG	TC CT CT TA	GAT AA GG C	TC CT CT TA
D1	TAA GG CG A	AG AG TA GA	GC GA TAA G	AG AG TA GA	GAT AA GG C	AG AG TA GA	TAA GGC GA	TA GA AG AG	GC GAT AA G	TA GA AG AG	GAT AA GG C	TA GA AG AG	TAA GGC GA	AG TA GA AG	GCG ATA AG	AG TA GA AG	GAT AA GG C	AG TA GA AG
E1	TAA GG CG A	GT AA GG AG	GC GA TAA G	GT AA GG AG	GAT AA GG C	GT AA GG AG	TAA GGC GA	GG AG GT AA	GC GAT AA G	GG AG GT AA	GAT AA GG C	GG AG GT AA	TAA GGC GA	AA GG AG GT	GCG ATA AG	AA GG AG GT	GAT AA GG C	AA GG AG GT
F1	TAA GG CG A	AC TG CA TA	GC GA TAA G	AC TG CA TA	GAT AA GG C	AC TG CA TA	TAA GGC GA	CA TA AC TG	GC GAT AA G	CA TA AC TG	GAT AA GG C	CA TA AC TG	TAA GGC GA	TG CA TA AC	GCG ATA AG	TG CA TA AC	GAT AA GG C	TG CA TA AC
G1	TAA GG CG A	AA GG AG TA	GC GA TAA G	AA GG AG TA	GAT AA GG C	AA GG AG TA	TAA GGC GA	AG TA AA GG	GC GAT AA G	AG TA AA GG	GAT AA GG C	AG TA AA GG	TAA GGC GA	GG AG TA AA	GCG ATA AG	GG AG TA AA	GAT AA GG C	GG AG TA AA
H1	TAA GG CG A	CT AA GC CT	GC GA TAA G	CT AA GC CT	GAT AA GG C	CT AA GC CT	TAA GGC GA	GC CT CT AA	GC GAT AA G	GC CT CT AA	GAT AA GG C	GC CT CT AA	TAA GGC GA	AA GC CT CT	GCG ATA AG	AA GC CT CT	GAT AA GG C	AA GC CT CT
A2	CG TAC TA G	TA GA TC GC	CTA GC GT A	TA GA TC GC	AG CGT ACT	TA GA TC GC	CGT ACT AG	TC GC TA GA	CTA GC GTA	TC GC TA GA	AG CGT ACT	TC GC TA GA	CGT ACT AG	GA TC GC TA	CTA GCG TA	GA TC GC TA	AG CGT ACT	GA TC GC TA
B2	CG TAC TA G	CT CT CT AT	CTA GC GT A	CT CT CT AT	AG CGT ACT	CT CT CT AT	CGT ACT AG	CT AT CT CT	CTA GC GTA	CT AT CT CT	AG CGT ACT	CT AT CT CT	CGT ACT AG	CT CT AT CT	CTA GCG TA	CT CT AT CT	AG CGT ACT	CT CT AT CT

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
C2	CG TAC TA G	TA TC CT CT	CTA GC GT A	TA TC CT CT	AG CGT ACT	TA TC CT CT	CGT ACT AG	CT CT TA TC	CTA GC GTA	CT CT TA TC	AG CGT ACT	CT CT TA TC	CGT ACT AG	TC CT CT TA	CTA GCG TA	TC CT CT TA	AG CGT ACT	TC CT CT TA
D2	CG TAC TA G	AG AG TA GA	CTA GC GT A	AG AG TA GA	AG CGT ACT	AG AG TA GA	CGT ACT AG	TA GA AG AG	CTA GC GTA	TA GA AG AG	AG CGT ACT	TA GA AG AG	CGT ACT AG	AG TA GA AG	CTA GCG TA	AG TA GA AG	AG CGT ACT	AG TA GA AG
E2	CG TAC TA G	GT AA GG AG	CTA GC GT A	GT AA GG AG	AG CGT ACT	GT AA GG AG	CGT ACT AG	GG AG GT AA	CTA GC GTA	GG AG GT AA	AG CGT ACT	GG AG GT AA	CGT ACT AG	AA GG AG GT	CTA GCG TA	AA GG AG GT	AG CGT ACT	AA GG AG GT
F2	CG TAC TA G	AC TG CA TA	CTA GC GT A	AC TG CA TA	AG CGT ACT	AC TG CA TA	CGT ACT AG	CA TA AC TG	CTA GC GTA	CA TA AC TG	AG CGT ACT	CA TA AC TG	CGT ACT AG	TG CA TA AC	CTA GCG TA	TG CA TA AC	AG CGT ACT	TG CA TA AC
G2	CG TAC TA G	AA GG AG TA	CTA GC GT A	AA GG AG TA	AG CGT ACT	AA GG AG TA	CGT ACT AG	AG TA AA GG	CTA GC GTA	AG TA AA GG	AG CGT ACT	AG TA AA GG	CGT ACT AG	GG AG TA AA	CTA GCG TA	GG AG TA AA	AG CGT ACT	GG AG TA AA
H2	CG TAC TA G	CT AA GC CT	CTA GC GT A	CT AA GC CT	AG CGT ACT	CT AA GC CT	CGT ACT AG	GC CT CT AA	CTA GC GTA	GC CT CT AA	AG CGT ACT	GC CT CT AA	CGT ACT AG	AA GC CT CT	CTA GCG TA	AA GC CT CT	AG CGT ACT	AA GC CT CT
A3	AG GC AG AA	TA GA TC GC	AG AA AG GC	TA GA TC GC	AAA GG CA G	TA GA TC GC	AGG CAG AA	TC GC TA GA	AG AAA GG C	TC GC TA GA	AAA GG CA G	TC GC TA GA	AGG CAG AA	GA TC GC TA	AGA AAG GC	GA TC GC TA	AAA GG CA G	GA TC GC TA
B3	AG GC AG AA	CT CT CT AT	AG AA AG GC	CT CT CT AT	AAA GG CA G	CT CT CT AT	AGG CAG AA	CT AT CT CT	AG AAA GG C	CT AT CT CT	AAA GG CA G	CT AT CT CT	AGG CAG AA	CT CT AT CT	AGA AAG GC	CT CT AT CT	AAA GG CA G	CT CT AT CT
C3	AG GC AG AA	TA TC CT CT	AG AA AG GC	TA TC CT CT	AAA GG CA G	TA TC CT CT	AGG CAG AA	CT CT TA TC	AG AAA GG C	CT CT TA TC	AAA GG CA G	CT CT TA TC	AGG CAG AA	TC CT CT TA	AGA AAG GC	TC CT CT TA	AAA GG CA G	TC CT CT TA
D3	AG GC AG AA	AG AG TA GA	AG AA AG GC	AG AG TA GA	AAA GG CA G	AG AG TA GA	AGG CAG AA	TA GA AG AG	AG AAA GG C	TA GA AG AG	AAA GG CA G	TA GA AG AG	AGG CAG AA	AG TA GA AG	AGA AAG GC	AG TA GA AG	AAA GG CA G	AG TA GA AG
E3	AG GC AG AA	GT AA GG AG	AG AA AG GC	GT AA GG AG	AAA GG CA G	GT AA GG AG	AGG CAG AA	GG AG GT AA	AG AAA GG C	GG AG GT AA	AAA GG CA G	GG AG GT AA	AGG CAG AA	AA GG AG GT	AGA AAG GC	AA GG AG GT	AAA GG CA G	AA GG AG GT
F3	AG GC AG AA	AC TG CA TA	AG AA AG GC	AC TG CA TA	AAA GG CA G	AC TG CA TA	AGG CAG AA	CA TA AC TG	AG AAA GG C	CA TA AC TG	AAA GG CA G	CA TA AC TG	AGG CAG AA	TG CA TA AC	AGA AAG GC	TG CA TA AC	AAA GG CA G	TG CA TA AC
G3	AG GC AG AA	AA GG AG TA	AG AA AG GC	AA GG AG TA	AAA GG CA G	AA GG AG TA	AGG CAG AA	AG TA AA GG	AG AAA GG C	AG TA AA GG	AAA GG CA G	AG TA AA GG	AGG CAG AA	GG AG TA AA	AGA AAG GC	GG AG TA AA	AAA GG CA G	GG AG TA AA
H3	AG GC AG AA	CT AA GC CT	AG AA AG GC	CT AA GC CT	AAA GG CA G	CT AA GC CT	AGG CAG AA	GC CT CT AA	AG AAA GG C	GC CT CT AA	AAA GG CA G	GC CT CT AA	AGG CAG AA	AA GC CT CT	AGA AAG GC	AA GC CT CT	AAA GG CA G	AA GC CT CT

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
A4	TCC TG AG C	TA GA TC GC	GA GC TCC T	TA GA TC GC	GCT CCT GA	TA GA TC GC	TCC TGA GC	TC GC TA GA	GA GCT CCT	TC GC TA GA	GCT CCT GA	TC GC TA GA	TCC TGA GC	GA TC GC TA	GAG CTC CT	GA TC GC TA	GCT CCT GA	GA TC GC TA
B4	TCC TG AG C	CT CT CT AT	GA GC TCC T	CT CT CT AT	GCT CCT GA	CT CT CT AT	TCC TGA GC	CT AT CT CT	GA GCT CCT	CT AT CT CT	GCT CCT GA	CT AT CT CT	TCC TGA GC	CT CT AT CT	GAG CTC CT	CT CT AT CT	GCT CCT GA	CT CT AT CT
C4	TCC TG AG C	TA TC CT CT	GA GC TCC T	TA TC CT CT	GCT CCT GA	TA TC CT CT	TCC TGA GC	CT CT TA TC	GA GCT CCT	CT CT TA TC	GCT CCT GA	CT CT TA TC	TCC TGA GC	TC CT CT TA	GAG CTC CT	TC CT CT TA	GCT CCT GA	TC CT CT TA
D4	TCC TG AG C	AG AG TA GA	GA GC TCC T	AG AG TA GA	GCT CCT GA	AG AG TA GA	TCC TGA GC	TA GA AG AG	GA GCT CCT	TA GA AG AG	GCT CCT GA	TA GA AG AG	TCC TGA GC	AG TA GA AG	GAG CTC CT	AG TA GA AG	GCT CCT GA	AG TA GA AG
E4	TCC TG AG C	GT AA GG AG	GA GC TCC T	GT AA GG AG	GCT CCT GA	GT AA GG AG	TCC TGA GC	GG AG GT AA	GA GCT CCT	GG AG GT AA	GCT CCT GA	GG AG GT AA	TCC TGA GC	AA GG AG GT	GAG CTC CT	AA GG AG GT	GCT CCT GA	AA GG AG GT
F4	TCC TG AG C	AC TG CA TA	GA GC TCC T	AC TG CA TA	GCT CCT GA	AC TG CA TA	TCC TGA GC	CA TA AC TG	GA GCT CCT	CA TA AC TG	GCT CCT GA	CA TA AC TG	TCC TGA GC	TG CA TA AC	GAG CTC CT	TG CA TA AC	GCT CCT GA	TG CA TA AC
G4	TCC TG AG C	AA GG AG TA	GA GC TCC T	AA GG AG TA	GCT CCT GA	AA GG AG TA	TCC TGA GC	AG TA AA GG	GA GCT CCT	AG TA AA GG	GCT CCT GA	AG TA AA GG	TCC TGA GC	GG AG TA AA	GAG CTC CT	GG AG TA AA	GCT CCT GA	GG AG TA AA
H4	TCC TG AG C	CT AA GC CT	GA GC TCC T	CT AA GC CT	GCT CCT GA	CT AA GC CT	TCC TGA GC	GC CT CT AA	GA GCT CCT	GC CT CT AA	GCT CCT GA	GC CT CT AA	TCC TGA GC	AA GC CT CT	GAG CTC CT	AA GC CT CT	GCT CCT GA	AA GC CT CT
A5	GG ACT CCT	TA GA TC GC	TCC TG GA C	TA GA TC GC	CTG GA CTC	TA GA TC GC	GGA CTC CT	TC GC TA GA	TCC TGG AC	TC GC TA GA	CTG GA CTC	TC GC TA GA	GGA CTC CT	GA TC GC TA	TCC TGG AC	GA TC GC TA	CTG GA CTC	GA TC GC TA
B5	GG ACT CCT	CT CT CT AT	TCC TG GA C	CT CT CT AT	CTG GA CTC	CT CT CT AT	GGA CTC CT	CT AT CT CT	TCC TGG AC	CT AT CT CT	CTG GA CTC	CT AT CT CT	GGA CTC CT	CT CT AT CT	TCC TGG AC	CT CT AT CT	CTG GA CTC	CT CT AT CT
C5	GG ACT CCT	TA TC CT CT	TCC TG GA C	TA TC CT CT	CTG GA CTC	TA TC CT CT	GGA CTC CT	CT CT TA TC	TCC TGG AC	CT CT TA TC	CTG GA CTC	CT CT TA TC	GGA CTC CT	TC CT CT TA	TCC TGG AC	TC CT CT TA	CTG GA CTC	TC CT CT TA
D5	GG ACT CCT	AG AG TA GA	TCC TG GA C	AG AG TA GA	CTG GA CTC	AG AG TA GA	GGA CTC CT	TA GA AG AG	TCC TGG AC	TA GA AG AG	CTG GA CTC	TA GA AG AG	GGA CTC CT	AG TA GA AG	TCC TGG AC	AG TA GA AG	CTG GA CTC	AG TA GA AG
E5	GG ACT CCT	GT AA GG AG	TCC TG GA C	GT AA GG AG	CTG GA CTC	GT AA GG AG	GGA CTC CT	GG AG GT AA	TCC TGG AC	GG AG GT AA	CTG GA CTC	GG AG GT AA	GGA CTC CT	AA GG AG GT	TCC TGG AC	AA GG AG GT	CTG GA CTC	AA GG AG GT
F5	GG ACT CCT	AC TG CA TA	TCC TG GA C	AC TG CA TA	CTG GA CTC	AC TG CA TA	GGA CTC CT	CA TA AC TG	TCC TGG AC	CA TA AC TG	CTG GA CTC	CA TA AC TG	GGA CTC CT	TG CA TA AC	TCC TGG AC	TG CA TA AC	CTG GA CTC	TG CA TA AC

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
G5	GG ACT CCT	AA GG AG TA	TCC TG GA C	AA GG AG TA	CTG GA CTC	AA GG AG TA	GGA CTC CT	AG TA AA GG	TCC TGG AC	AG TA AA GG	CTG GA CTC	AG TA AA GG	GGA CTC CT	GG AG TA AA	TCC TGG AC	GG AG TA AA	CTG GA CTC	GG AG TA AA
H5	GG ACT CCT	CT AA GC CT	TCC TG GA C	CT AA GC CT	CTG GA CTC	CT AA GC CT	GGA CTC CT	GC CT CT AA	TCC TGG AC	GC CT CT AA	CTG GA CTC	GC CT CT AA	GGA CTC CT	AA GC CT CT	TCC TGG AC	AA GC CT CT	CTG GA CTC	AA GC CT CT
A6	TA GG CAT G	TA GA TC GC	CAT GT AG G	TA GA TC GC	TGT AG GC A	TA GA TC GC	TAG GCA TG	TC GC TA GA	CAT GTA GG	TC GC TA GA	TGT AG GC A	TC GC TA GA	TAG GCA TG	GA TC GC TA	CAT GTA GG	GA TC GC TA	TGT AG GC A	GA TC GC TA
B6	TA GG CAT G	CT CT CT AT	CAT GT AG G	CT CT CT AT	TGT AG GC A	CT CT CT AT	TAG GCA TG	CT AT CT CT	CAT GTA GG	CT AT CT CT	TGT AG GC A	CT AT CT CT	TAG GCA TG	CT CT AT CT	CAT GTA GG	CT CT AT CT	TGT AG GC A	CT CT AT CT
C6	TA GG CAT G	TA TC CT CT	CAT GT AG G	TA TC CT CT	TGT AG GC A	TA TC CT CT	TAG GCA TG	CT CT TA TC	CAT GTA GG	CT CT TA TC	TGT AG GC A	CT CT TA TC	TAG GCA TG	TC CT CT TA	CAT GTA GG	TC CT CT TA	TGT AG GC A	TC CT CT TA
D6	TA GG CAT G	AG AG TA GA	CAT GT AG G	AG AG TA GA	TGT AG GC A	AG AG TA GA	TAG GCA TG	TA GA AG AG	CAT GTA GG	TA GA AG AG	TGT AG GC A	TA GA AG AG	TAG GCA TG	AG TA GA AG	CAT GTA GG	AG TA GA AG	TGT AG GC A	AG TA GA AG
E6	TA GG CAT G	GT AA GG AG	CAT GT AG G	GT AA GG AG	TGT AG GC A	GT AA GG AG	TAG GCA TG	GG AG GT AA	CAT GTA GG	GG AG GT AA	TGT AG GC A	GG AG GT AA	TAG GCA TG	AA GG AG GT	CAT GTA GG	AA GG AG GT	TGT AG GC A	AA GG AG GT
F6	TA GG CAT G	AC TG CA TA	CAT GT AG G	AC TG CA TA	TGT AG GC A	AC TG CA TA	TAG GCA TG	CA TA AC TG	CAT GTA GG	CA TA AC TG	TGT AG GC A	CA TA AC TG	TAG GCA TG	TG CA TA AC	CAT GTA GG	TG CA TA AC	TGT AG GC A	TG CA TA AC
G6	TA GG CAT G	AA GG AG TA	CAT GT AG G	AA GG AG TA	TGT AG GC A	AA GG AG TA	TAG GCA TG	AG TA AA GG	CAT GTA GG	AG TA AA GG	TGT AG GC A	AG TA AA GG	TAG GCA TG	GG AG TA AA	CAT GTA GG	GG AG TA AA	TGT AG GC A	GG AG TA AA
H6	TA GG CAT G	CT AA GC CT	CAT GT AG G	CT AA GC CT	TGT AG GC A	CT AA GC CT	TAG GCA TG	GC CT CT AA	CAT GTA GG	GC CT CT AA	TGT AG GC A	GC CT CT AA	TAG GCA TG	AA GC CT CT	CAT GTA GG	AA GC CT CT	TGT AG GC A	AA GC CT CT
A7	CTC TCT AC	TA GA TC GC	CTA CCT CT	TA GA TC GC	ACC TCT CT	TA GA TC GC	CTC TCT AC	TC GC TA GA	CTA CCT CT	TC GC TA GA	ACC TCT CT	TC GC TA GA	CTC TCT AC	GA TC GC TA	CTA CCT CT	GA TC GC TA	ACC TCT CT	GA TC GC TA
B7	CTC TCT AC	CT CT CT AT	CTA CCT CT	CT CT CT AT	ACC TCT CT	CT CT CT AT	CTC TCT AC	CT AT CT CT	CTA CCT CT	CT AT CT CT	ACC TCT CT	CT AT CT CT	CTC TCT AC	CT CT AT CT	CTA CCT CT	CT CT AT CT	ACC TCT CT	CT CT AT CT
C7	CTC TCT AC	TA TC CT CT	CTA CCT CT	TA TC CT CT	ACC TCT CT	TA TC CT CT	CTC TCT AC	CT CT TA TC	CTA CCT CT	CT CT TA TC	ACC TCT CT	CT CT TA TC	CTC TCT AC	TC CT CT TA	CTA CCT CT	TC CT CT TA	ACC TCT CT	TC CT CT TA
D7	CTC TCT AC	AG AG TA GA	CTA CCT CT	AG AG TA GA	ACC TCT CT	AG AG TA GA	CTC TCT AC	TA GA AG AG	CTA CCT CT	TA GA AG AG	ACC TCT CT	TA GA AG AG	CTC TCT AC	AG TA GA AG	CTA CCT CT	AG TA GA AG	ACC TCT CT	AG TA GA AG

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
E7	CTC TCT AC	GT AA GG AG	CTA CCT CT	GT AA GG AG	ACC TCT CT	GT AA GG AG	CTC TCT AC	GG AG GT AA	CTA CCT CT	GG AG GT AA	ACC TCT CT	GG AG GT AA	CTC TCT AC	AA GG AG GT	CTA CCT CT	AA GG AG GT	ACC TCT CT	AA GG AG GT
F7	CTC TCT AC	AC TG CA TA	CTA CCT CT	AC TG CA TA	ACC TCT CT	AC TG CA TA	CTC TCT AC	CA TA AC TG	CTA CCT CT	CA TA AC TG	ACC TCT CT	CA TA AC TG	CTC TCT AC	TG CA TA AC	CTA CCT CT	TG CA TA AC	ACC TCT CT	TG CA TA AC
G7	CTC TCT AC	AA GG AG TA	CTA CCT CT	AA GG AG TA	ACC TCT CT	AA GG AG TA	CTC TCT AC	AG TA AA GG	CTA CCT CT	AG TA AA GG	ACC TCT CT	AG TA AA GG	CTC TCT AC	GG AG TA AA	CTA CCT CT	GG AG TA AA	ACC TCT CT	GG AG TA AA
H7	CTC TCT AC	CT AA GC CT	CTA CCT CT	CT AA GC CT	ACC TCT CT	CT AA GC CT	CTC TCT AC	GC CT CT AA	CTA CCT CT	GC CT CT AA	ACC TCT CT	GC CT CT AA	CTC TCT AC	AA GC CT CT	CTA CCT CT	AA GC CT CT	ACC TCT 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	CAG AGA GG	TC GC TA GA	GA GG CA GA	TC GC TA GA	GG CA GA GA	TC GC TA GA	CAG AGA GG	GA TC GC TA	GAG GCA 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	CAG AGA GG	CT AT CT CT	GA GG CA GA	CT AT CT CT	GG CA GA GA	CT AT CT CT	CAG AGA GG	CT CT AT CT	GAG GCA GA	CT CT AT CT	GG CA GA GA	CT CT AT 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	CAG AGA GG	CT CT TA TC	GA GG CA GA	CT CT TA TC	GG CA GA GA	CT CT TA TC	CAG AGA GG	TC CT CT TA	GAG GCA 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	CAG AGA GG	TA GA AG AG	GA GG CA GA	TA GA AG AG	GG CA GA GA	TA GA AG AG	CAG AGA GG	AG TA GA AG	GAG GCA 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	CAG AGA GG	GG AG GT AA	GA GG CA GA	GG AG GT AA	GG CA GA GA	GG AG GT AA	CAG AGA GG	AA GG AG GT	GAG GCA 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	CAG AGA GG	CA TA AC TG	GA GG CA GA	CA TA AC TG	GG CA GA GA	CA TA AC TG	CAG AGA GG	TG CA TA AC	GAG GCA 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	CAG AGA GG	AG TA AA GG	GA GG CA GA	AG TA AA GG	GG CA GA GA	AG TA AA GG	CAG AGA GG	GG AG TA AA	GAG GCA 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	CAG AGA GG	GC CT CT AA	GA GG CA GA	GC CT CT AA	GG CA GA GA	GC CT CT AA	CAG AGA GG	AA GC CT CT	GAG GCA GA	AA GC CT CT	GG CA GA GA	AA GC CT CT
A9	GC TAC GC T	TA GA TC GC	CG CT GC TA	TA GA TC GC	CTG CTA CG	TA GA TC GC	GCT ACG CT	TC GC TA GA	CG CTG CTA	TC GC TA GA	CTG CTA CG	TC GC TA GA	GCT ACG CT	GA TC GC TA	CGC TGC TA	GA TC GC TA	CTG CTA CG	GA TC GC TA
B9	GC TAC GC T	CT CT CT AT	CG CT GC TA	CT CT CT AT	CTG CTA CG	CT CT CT AT	GCT ACG CT	CT AT CT CT	CG CTG CTA	CT AT CT CT	CTG CTA CG	CT AT CT CT	GCT ACG CT	CT CT AT CT	CGC TGC TA	CT CT AT CT	CTG CTA CG	CT CT AT CT

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
C9	GC TAC GC T	TA TC CT CT	CG CT GC TA	TA TC CT CT	CTG CTA CG	TA TC CT CT	GCT ACG CT	CT CT TA TC	CG CTG CTA	CT CT TA TC	CTG CTA CG	CT CT TA TC	GCT ACG CT	TC CT CT TA	CGC TGC TA	TC CT CT TA	CTG CTA CG	TC CT CT TA
D9	GC TAC GC T	AG AG TA GA	CG CT GC TA	AG AG TA GA	CTG CTA CG	AG AG TA GA	GCT ACG CT	TA GA AG AG	CG CTG CTA	TA GA AG AG	CTG CTA CG	TA GA AG AG	GCT ACG CT	AG TA GA AG	CGC TGC TA	AG TA GA AG	CTG CTA CG	AG TA GA AG
E9	GC TAC GC T	GT AA GG AG	CG CT GC TA	GT AA GG AG	CTG CTA CG	GT AA GG AG	GCT ACG CT	GG AG GT AA	CG CTG CTA	GG AG GT AA	CTG CTA CG	GG AG GT AA	GCT ACG CT	AA GG AG GT	CGC TGC TA	AA GG AG GT	CTG CTA CG	AA GG AG GT
F9	GC TAC GC T	AC TG CA TA	CG CT GC TA	AC TG CA TA	CTG CTA CG	AC TG CA TA	GCT ACG CT	CA TA AC TG	CG CTG CTA	CA TA AC TG	CTG CTA CG	CA TA AC TG	GCT ACG CT	TG CA TA AC	CGC TGC TA	TG CA TA AC	CTG CTA CG	TG CA TA AC
G9	GC TAC GC T	AA GG AG TA	CG CT GC TA	AA GG AG TA	CTG CTA CG	AA GG AG TA	GCT ACG CT	AG TA AA GG	CG CTG CTA	AG TA AA GG	CTG CTA CG	AG TA AA GG	GCT ACG CT	GG AG TA AA	CGC TGC TA	GG AG TA AA	CTG CTA CG	GG AG TA AA
H9	GC TAC GC T	CT AA GC CT	CG CT GC TA	CT AA GC CT	CTG CTA CG	CT AA GC CT	GCT ACG CT	GC CT CT AA	CG CTG CTA	GC CT CT AA	CTG CTA CG	GC CT CT AA	GCT ACG CT	AA GC CT CT	CGC TGC TA	AA GC CT CT	CTG CTA CG	AA GC CT CT
A10	CG AG GC TG	TA GA TC GC	GC TG CG AG	TA GA TC GC	TGC GA GG C	TA GA TC GC	CGA GGC TG	TC GC TA GA	GCT GC GA G	TC GC TA GA	TGC GA GG C	TC GC TA GA	CGA GGC TG	GA TC GC TA	GCT GCG AG	GA TC GC TA	TGC GA GG C	GA TC GC TA
B10	CG AG GC TG	CT CT CT AT	GC TG CG AG	CT CT CT AT	TGC GA GG C	CT CT CT AT	CGA GGC TG	CT AT CT CT	GCT GC GA G	CT AT CT CT	TGC GA GG C	CT AT CT CT	CGA GGC TG	CT CT AT CT	GCT GCG AG	CT CT AT CT	TGC GA GG C	CT CT AT CT
C10	CG AG GC TG	TA TC CT CT	GC TG CG AG	TA TC CT CT	TGC GA GG C	TA TC CT CT	CGA GGC TG	CT CT TA TC	GCT GC GA G	CT CT TA TC	TGC GA GG C	CT CT TA TC	CGA GGC TG	TC CT CT TA	GCT GCG AG	TC CT CT TA	TGC GA GG C	TC CT CT TA
D10	CG AG GC TG	AG AG TA GA	GC TG CG AG	AG AG TA GA	TGC GA GG C	AG AG TA GA	CGA GGC TG	TA GA AG AG	GCT GC GA G	TA GA AG AG	TGC GA GG C	TA GA AG AG	CGA GGC TG	AG TA GA AG	GCT GCG AG	AG TA GA AG	TGC GA GG C	AG TA GA AG
E10	CG AG GC TG	GT AA GG AG	GC TG CG AG	GT AA GG AG	TGC GA GG C	GT AA GG AG	CGA GGC TG	GG AG GT AA	GCT GC GA G	GG AG GT AA	TGC GA GG C	GG AG GT AA	CGA GGC TG	AA GG AG GT	GCT GCG AG	AA GG AG GT	TGC GA GG C	AA GG AG GT
F10	CG AG GC TG	AC TG CA TA	GC TG CG AG	AC TG CA TA	TGC GA GG C	AC TG CA TA	CGA GGC TG	CA TA AC TG	GCT GC GA G	CA TA AC TG	TGC GA GG C	CA TA AC TG	CGA GGC TG	TG CA TA AC	GCT GCG AG	TG CA TA AC	TGC GA GG C	TG CA TA AC
G10	CG AG GC TG	AA GG AG TA	GC TG CG AG	AA GG AG TA	TGC GA GG C	AA GG AG TA	CGA GGC TG	AG TA AA GG	GCT GC GA G	AG TA AA GG	TGC GA GG C	AG TA AA GG	CGA GGC TG	GG AG TA AA	GCT GCG AG	GG AG TA AA	TGC GA GG C	GG AG TA AA
H10	CG AG GC TG	CT AA GC CT	GC TG CG AG	CT AA GC CT	TGC GA GG C	CT AA GC CT	CGA GGC TG	GC CT CT AA	GCT GC GA G	GC CT CT AA	TGC GA GG C	GC CT CT AA	CGA GGC TG	AA GC CT CT	GCT GCG AG	AA GC CT CT	TGC GA GG C	AA GC CT CT

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

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
G1 2	GT AG AG GA	AA GG AG TA	AG GA GT AG	AA GG AG TA	GA GTA GA G	AA GG AG TA	GTA GAG GA	AG TA AA GG	AG GA GTA G	AG TA AA GG	GA GTA GA G	AG TA AA GG	GTA GAG GA	GG AG TA AA	AGG AGT AG	GG AG TA AA	GA GTA GA G	GG AG TA AA
H1 2	GT AG AG GA	CT AA GC CT	AG GA GT AG	CT AA GC CT	GA GTA GA G	CT AA GC CT	GTA GAG GA	GC CT CT AA	AG GA GTA G	GC CT CT AA	GA GTA GA G	GC CT CT AA	GTA GAG GA	AA GC CT CT	AGG AGT AG	AA GC CT CT	GA GTA GA G	AA GC 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	ctccgctttcctctctatgggcagtcggtgatt	14	4 µL