Jun 21, 2023 Version 2

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

DOI
dx.doi.org/10.17504/protocols.io.8epv5jnnnl1b/v2

Anne-Laure Besnard[1], Daniel J. Park[2], Bernard J. Pope[3], Fleur Hammet[4], Sophie Michon-Coudouel[5], Marine Biget[6], Stacy A. Krueger-Hadfield[7], Stéphane Mauger[8], Eric J. Petit[1]

[1]DECOD (Ecosystem Dynamics and Sustainability), INRAE, L'Institut Agro, IFREMER, Rennes, France;
[2]Department of Biochemistry and Pharmacology, Melbourne Bioinformatics, The University of Melbourne, Melbourne, Australia;
[3]Melbourne Bioinformatics, The University of Melbourne, Melbourne, Australia;
[4]School of Clinical Sciences, Precision Medicine, Monash University, Melbourne, Australia;
[5]Université de Rennes, CNRS, UAR3343 OSUR, Plateforme EcogenO, Avenue du Général Leclerc , 35042 Rennes Cedex, France;
[6]IGEPP, INRAE, Institut Agro, Université de Rennes, 35000 Rennes, France;
[7]The University of Alabama at Birmingham, Birmingham, AL, 35294, USA;
[8]IRL 3614, Evolutionary Biology and Ecology of Algae, CNRS, Sorbonne Université, Université Catholique, Université Australe du Chili, Roscoff, France

Anne-Laure Besnard
INRAE

**DOI:** https://dx.doi.org/10.17504/protocols.io.8epv5jnnnl1b/v2

**Protocol Citation:** Anne-Laure Besnard, Daniel J. Park, Bernard J. Pope, Fleur Hammet, Sophie Michon-Coudouel, Marine Biget, Stacy A. Krueger-Hadfield, Stéphane Mauger, Eric J. Petit 2023. Workflow for SNP genotyping using the Hi-Plex method. **protocols.io** https://dx.doi.org/10.17504/protocols.io.8epv5jnnnl1b/v2Version created by **Eric J. Petit**



**Protocol status:** Working
**We use this protocol and it's working**

**Created:** June 13, 2023

**Last Modified:** June 21, 2023

**Protocol Integer ID:** 83344

**Keywords:** SNP genotyping, SNP panel, genotyping by sequencing, Illumina, Ion Torrent, genotyping rare variant, good genomic coverage, targeted genotyping strategy, sequencing strategy, sequencing technique, development of targeted genotyping strategy, rare variants in human oncology, sequencing, genomic, throughput molecular biology protocol, molecular biology protocol, snp locus set, plex method many research questions in ecology, sampling strategy, snp, balancing sampling strategy, dual indexing of individual sample, non model species for use, many population

## 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 multichanel 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 µM in TE
- TSITs A and TSITs P at 100 or 200 µM in TE

**PCRs & Clean-up steps**

- DNA
- Ice bath or cold cube
- 15 mL conical vials (e.g., Falcon tubes) for large PCR mixes
- Tube-strips and caps
- Pool of GSP at 50µM
- ThermoFisher Phusion$^{TM}$ Hot Start II High Fidelity (ref. F549L)
- ThermoFisher Phusion$^{TM}$ 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$^{TM}$ prep*

- DNA Gel Cassettes 2% Agarose DF Marker L, dye free, w/ internal standards, Pippin$^{TM}$ 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

## Troubleshooting

## 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 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 specifics 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)**

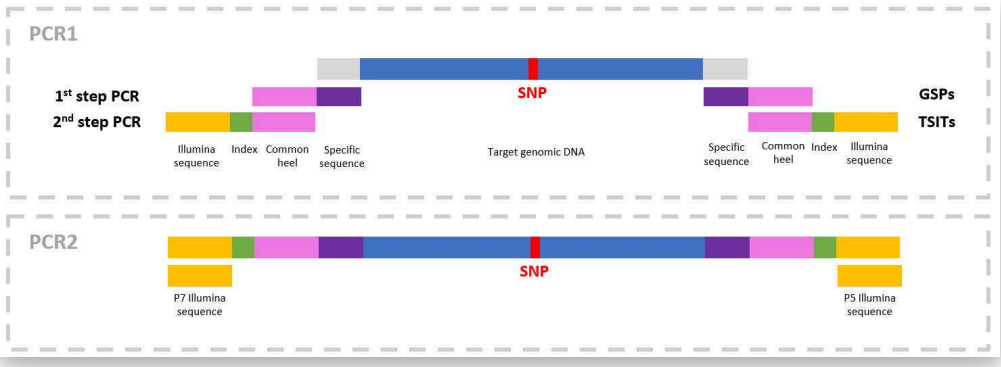
TSITs are adaptors that consist of a complementary sequence to the GSP common heel, a barcode, and a specific sequence for Illumina sequencing (P5/P7) for a total of about 60 bp for each TSIT A and TSIT P. The use of a combination of this 2 barcodes allows the specific identification of the DNA samples.

**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 µM) is used, to which TSIT adaptors are added for the second phase. The first PCR phase starts with 8 cycles with 2 successive hybridization temperatures (TM) at 58˚C and 60˚C, followed by 4 cycles at 58˚C. The second phase includes 4 cycles with a TM at 66˚C. The reaction is stopped by adding 100 mM EDTA at 72 ˚C and putting on ice.

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

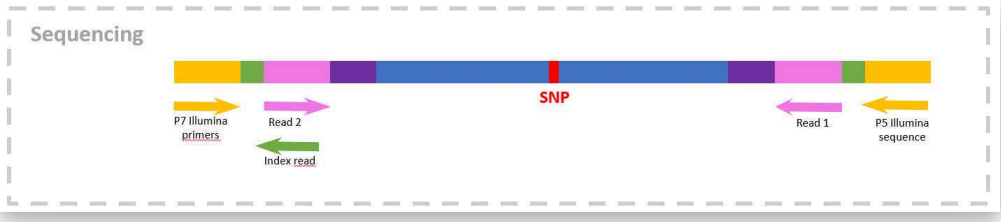
*The use of EtBr is essential if size selection from agarose gel is performed. SybrSafe reagent, for example, generated a lot of non-specific amplicons. This suggests that SybrSafe does not allow for clean separation of migrated fragments on agarose gels.*




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 manufacter 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
ATGCACAAACAAGCAGTGTTTTCTTAAAGAGACCACTGCTCCCAGCTCACCCAGCAGCCTC
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**

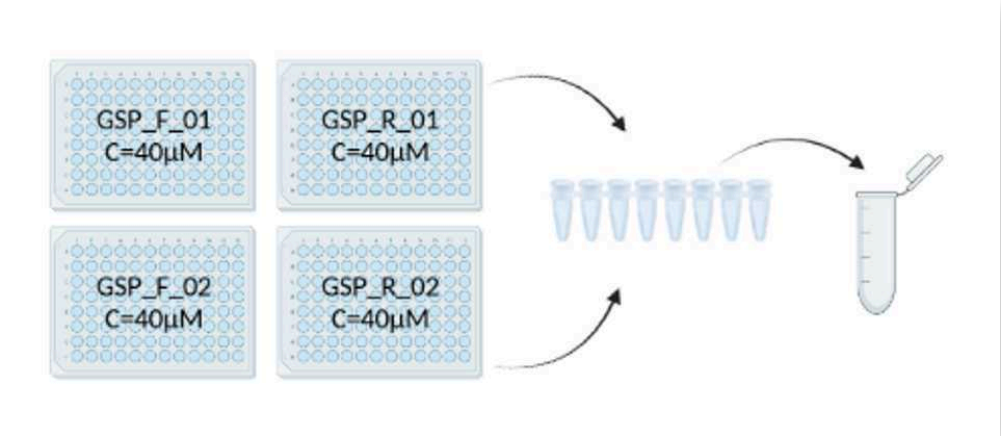
- Prepare 40 µM plates from each 100 or 200 µM GSP plate using 1X low TE (10 mM Tris pH8.0/0.1mM EDTA):

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | ***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.

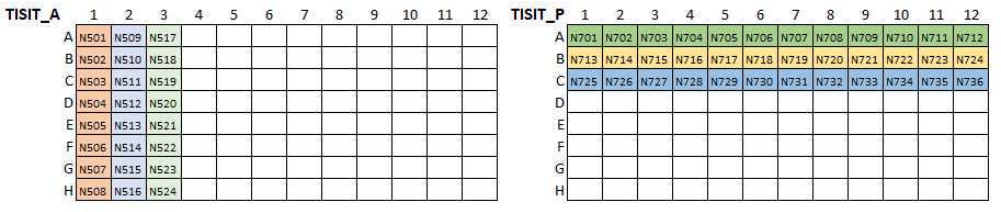
| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | **Nb of 96-well PCR plates** | **C pool undiluted (µM)** | **C pool diluted (µM)** | **Vi (µL)** | **Vf (µL)** | **V low TE (µL)** |
| | 1 | 40 | 10 | 12.5 | 50 | 37.5 |
| | 2 | 40 | 10 | 25 | 100 | 75 |
| | 3 | 40 | 10 | 37.5 | 150 | 112.5 |
| | 4 | 40 | 10 | 50 | 200 | 150 |

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

## 3 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.




Schemes of the two TSITs plates

| | A | B | C |
|---|---|---|---|
| | **Plate well** | **TSITs P names** | **Sequences of TSITs P** |
| | A1 | N701_TSIT_P | CAAGCAGAAGACGGCATACGAGATTCGCCTTActccgctttcctctctatgggcagtcggtgat |
| | A2 | N702_TSIT_P | CAAGCAGAAGACGGCATACGAGATCTAGTACGctccgctttcctctctatgggcagtcggtgat |
| | A3 | N703_TSIT_P | CAAGCAGAAGACGGCATACGAGATTTCTGCCTctccgctttcctctctatgggcagtcggtgat |
| | A4 | N704_TSIT_P | CAAGCAGAAGACGGCATACGAGATGCTCAGGActccgctttcctctctatgggcagtcggtgat |
| | A5 | N705_TSIT_P | CAAGCAGAAGACGGCATACGAGATAGGAGTCCctccgctttcctctctatgggcagtcggtgat |
| | A6 | N706_TSIT_P | CAAGCAGAAGACGGCATACGAGATCATGCCTActccgctttcctctctatgggcagtcggtgat |
| | A7 | N707_TSIT_P | CAAGCAGAAGACGGCATACGAGATGTAGAGAGctccgctttcctctctatgggcagtcggtgat |
| | A8 | N708_TSIT_P | CAAGCAGAAGACGGCATACGAGATCCTCTCTGctccgctttcctctctatgggcagtcggtgat |
| | A9 | N709_TSIT_P | CAAGCAGAAGACGGCATACGAGATAGCGTAGCctccgctttcctctctatgggcagtcggtgat |

| | A | B | C |
|---|---|---|---|
| | A10 | N710_TSIT_P | CAAGCAGAAGACGGCATACGAGATCAGCCTCGctccgctttcctctctatgggcagtcggtgat |
| | A11 | N711_TSIT_P | CAAGCAGAAGACGGCATACGAGATTGCCTCTTctccgctttcctctctatgggcagtcggtgat |
| | A12 | N712_TSIT_P | CAAGCAGAAGACGGCATACGAGATTCCTCTACctccgctttcctctctatgggcagtcggtgat |
| | B1 | N713_TSIT_P | CAAGCAGAAGACGGCATACGAGATCTTATCGCctccgctttcctctctatgggcagtcggtgat |
| | B2 | N714_TSIT_P | CAAGCAGAAGACGGCATACGAGATTACGCTAGctccgctttcctctctatgggcagtcggtgat |
| | B3 | N715_TSIT_P | CAAGCAGAAGACGGCATACGAGATGCCTTTCTctccgctttcctctctatgggcagtcggtgat |
| | B4 | N716_TSIT_P | CAAGCAGAAGACGGCATACGAGATAGGAGCTCctccgctttcctctctatgggcagtcggtgat |
| | B5 | N717_TSIT_P | CAAGCAGAAGACGGCATACGAGATGTCCAGGActccgctttcctctctatgggcagtcggtgat |
| | B6 | N718_TSIT_P | CAAGCAGAAGACGGCATACGAGATCCTACATGctccgctttcctctctatgggcagtcggtgat |
| | B7 | N719_TSIT_P | CAAGCAGAAGACGGCATACGAGATAGAGGTAGctccgctttcctctctatgggcagtcggtgat |
| | B8 | N720_TSIT_P | CAAGCAGAAGACGGCATACGAGATTCTGCCTCctccgctttcctctctatgggcagtcggtgat |
| | B9 | N721_TSIT_P | CAAGCAGAAGACGGCATACGAGATTAGCAGCGctccgctttcctctctatgggcagtcggtgat |
| | B10 | N722_TSIT_P | CAAGCAGAAGACGGCATACGAGATCTCGCAGCctccgctttcctctctatgggcagtcggtgat |
| | B11 | N723_TSIT_P | CAAGCAGAAGACGGCATACGAGATTCTTTGCCctccgctttcctctctatgggcagtcggtgat |
| | B12 | N724_TSIT_P | CAAGCAGAAGACGGCATACGAGATCTACTCCTctccgctttcctctctatgggcagtcggtgat |
| | C1 | N725_TSIT_P | CAAGCAGAAGACGGCATACGAGATGCCTTATCctccgctttcctctctatgggcagtcggtgat |
| | C2 | N726_TSIT_P | CAAGCAGAAGACGGCATACGAGATAGTACGCTctccgctttcctctctatgggcagtcggtgat |
| | C3 | N727_TSIT_P | CAAGCAGAAGACGGCATACGAGATCTGCCTTTctccgctttcctctctatgggcagtcggtgat |

| | A | B | C |
|---|---|---|---|
| | C4 | N728_TSIT_P | CAAGCAGAAGACGGCATACGAGATTCAGGAGCctccgctttcctctctatgggcagtcggtgat |
| | C5 | N729_TSIT_P | CAAGCAGAAGACGGCATACGAGATGAGTCCAGctccgctttcctctctatgggcagtcggtgat |
| | C6 | N730_TSIT_P | CAAGCAGAAGACGGCATACGAGATTGCCTACActccgctttcctctctatgggcagtcggtgat |
| | C7 | N731_TSIT_P | CAAGCAGAAGACGGCATACGAGATAGAGAGGTctccgctttcctctctatgggcagtcggtgat |
| | C8 | N732_TSIT_P | CAAGCAGAAGACGGCATACGAGATTCTCTGCCctccgctttcctctctatgggcagtcggtgat |
| | C9 | N733_TSIT_P | CAAGCAGAAGACGGCATACGAGATCGTAGCAGctccgctttcctctctatgggcagtcggtgat |
| | C10 | N734_TSIT_P | CAAGCAGAAGACGGCATACGAGATGCCTCGCActccgctttcctctctatgggcagtcggtgat |
| | C11 | N735_TSIT_P | CAAGCAGAAGACGGCATACGAGATCCTCTTTGctccgctttcctctctatgggcagtcggtgat |
| | C12 | N736_TSIT_P | CAAGCAGAAGACGGCATACGAGATCTCTACTCctccgctttcctctctatgggcagtcggtgat |

List of TSITs P adaptors

| | A | B | C |
|---|---|---|---|
| | **Plate well** | **TSITs A names** | **Sequences of TSITs A** |
| | A1 | N501_TSIT_A | AATGATACGGCGACCACCGAGATCTACACTAGATCGCccatctcatccctgcgtgtctccgactcag |
| | B1 | N502_TSIT_A | AATGATACGGCGACCACCGAGATCTACACCTCTCTATccatctcatccctgcgtgtctccgactcag |
| | C1 | N503_TSIT_A | AATGATACGGCGACCACCGAGATCTACACTATCCTCTccatctcatccctgcgtgtctccgactcag |
| | D1 | N504_TSIT_A | AATGATACGGCGACCACCGAGATCTACACAGAGTAGAccatctcatccctgcgtgtctccgactcag |
| | E1 | N505_TSIT_A | AATGATACGGCGACCACCGAGATCTACACGTAAGGAGccatctcatccctgcgtgtctccgactcag |
| | F1 | N506_TSIT_A | AATGATACGGCGACCACCGAGATCTACACACTGCATAccatctcatccctgcgtgtctccgactcag |

| | A | B | C |
|---|---|---|---|
| | G1 | N507_TSIT_A | AATGATACGGCGACCACCGAGATCTACACAAGGAGTAccatctcatccctgcgtgtctccgactcag |
| | H1 | N508_TSIT_A | AATGATACGGCGACCACCGAGATCTACACCTAAGCCTccatctcatccctgcgtgtctccgactcag |
| | A2 | N509_TSIT_A | AATGATACGGCGACCACCGAGATCTACACTCGCTAGAccatctcatccctgcgtgtctccgactcag |
| | B2 | N510_TSIT_A | AATGATACGGCGACCACCGAGATCTACACCTATCTCTccatctcatccctgcgtgtctccgactcag |
| | C2 | N511_TSIT_A | AATGATACGGCGACCACCGAGATCTACACCTCTTATCccatctcatccctgcgtgtctccgactcag |
| | D2 | N512_TSIT_A | AATGATACGGCGACCACCGAGATCTACACTAGAAGAGccatctcatccctgcgtgtctccgactcag |
| | E2 | N513_TSIT_A | AATGATACGGCGACCACCGAGATCTACACGGAGGTAAccatctcatccctgcgtgtctccgactcag |
| | F2 | N514_TSIT_A | AATGATACGGCGACCACCGAGATCTACACCATAACTGccatctcatccctgcgtgtctccgactcag |
| | G2 | N515_TSIT_A | AATGATACGGCGACCACCGAGATCTACACAGTAAAGGccatctcatccctgcgtgtctccgactcag |
| | H2 | N516_TSIT_A | AATGATACGGCGACCACCGAGATCTACACGCCTCTAAccatctcatccctgcgtgtctccgactcag |
| | A3 | N517_TSIT_A | AATGATACGGCGACCACCGAGATCTACACGATCGCTAccatctcatccctgcgtgtctccgactcag |
| | B3 | N518_TSIT_A | AATGATACGGCGACCACCGAGATCTACACCTCTATCTccatctcatccctgcgtgtctccgactcag |
| | C3 | N519_TSIT_A | AATGATACGGCGACCACCGAGATCTACACTCCTCTTAccatctcatccctgcgtgtctccgactcag |
| | D3 | N520_TSIT_A | AATGATACGGCGACCACCGAGATCTACACAGTAGAAGccatctcatccctgcgtgtctccgactcag |
| | E3 | N521_TSIT_A | AATGATACGGCGACCACCGAGATCTACACAAGGAGGTccatctcatccctgcgtgtctccgactcag |
| | F3 | N522_TSIT_A | AATGATACGGCGACCACCGAGATCTACACTGCATAACccatctcatccctgcgtgtctccgactcag |
| | G3 | N523_TSIT_A | AATGATACGGCGACCACCGAGATCTACACGGAGTAAAccatctcatccctgcgtgtctccgactcag |
| | H3 | N524_TSIT_A | AATGATACGGCGACCACCGAGATCTACACAAGCCTCTccatctcatccctgcgtgtctccgactcag |

List of TSITs A adaptors

**TSITs preparation**

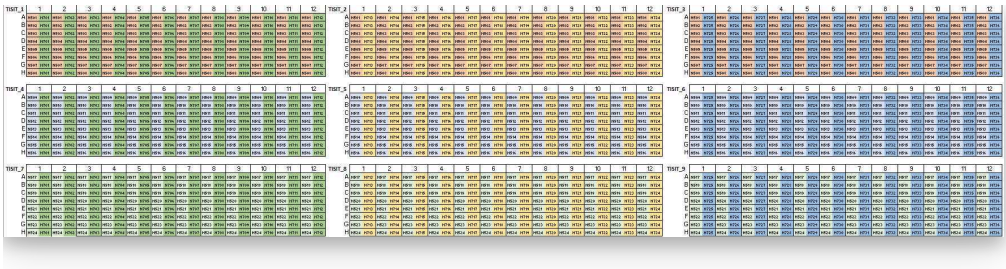
- Make a new plate diluted to 50 µM from each TSIT plate (TSIT_A and TSIT_P) at 100 or 200 µM in 100 µL/TSIT in TE: TSIT_A_50µM and TSIT_P_50µM.

| | 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_10µM_1 to TSIT_10µM_9).

As the TSIT_A are arranged columnwise in plate TSIT_A_50µM and TSIT_P are sorted rowise 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-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 |
| | MgCl2 | 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 |
| | ***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 |

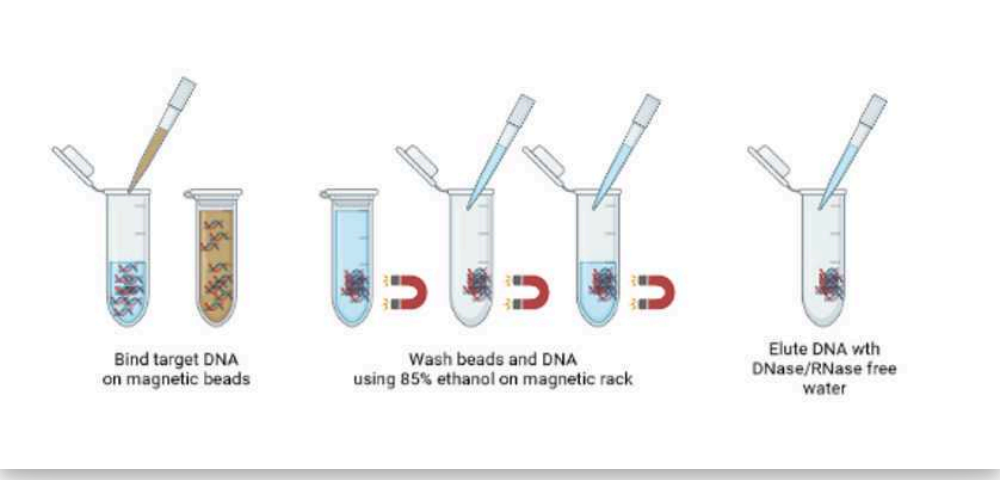
| | A | B | C |
|---|---|---|---|
| | 58°C | 02:30 | |
| | 72°C | 01:00 | |
| | 72°C | Needed to add TSITs | |
| | 98°C | 00:30 | x4 |
| | 66°C | 02:00 | |
| | 72°C | 01:00 | |
| | 72°C | Needed to add EDTA | |

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 ˚C until the end of the protocol or at -20 ˚C for longer term storage.
2. Add 864µL of presuspended magnetic beads (0.9×960µL=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 (0.9×70µL=63µL) to the previously eluted DNA.
2. Mix up and down 10 times.
3. Incubate at room temperature for 5 min.
4. Place the tube in a magnetic rack and wait until all the beads are against the side of the tube. This could take a few minutes.
5. Remove the supernatant without removing any bead.

- **Wash the beads with 85% ethanol**

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

- **Elute DNA**

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

8 **PCR2 - Fragment enrichment**

- **PCR mix**

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

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

| | A | B | C | D |
|---|---|---|---|---|
| | **Reagents** | **Initial conc.** | **Final conc.** | **Vol. for one reaction** |

| | A | B | C | D |
|---|---|---|---|---|
| | DNase/RNase free water | - | - | 22.5 |
| | 5 X Green buffer | 5X | 1X | 15 |
| | MgCl2 | 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*** |
| | ***Purifyed 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 µL loading volumes.

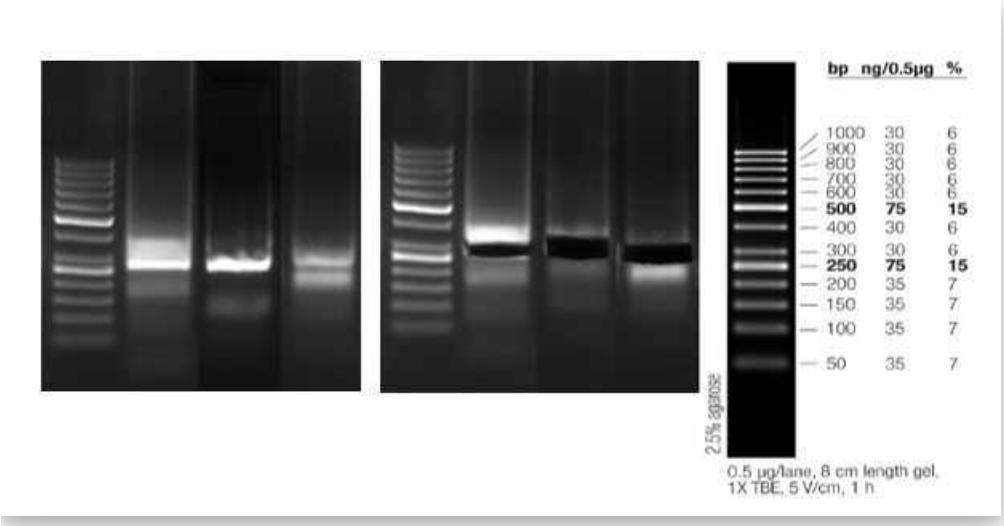
1. Prepare a 1.75% agarose gel using 1X TAE and ethidium bromide (EtBr).
2. Add directly 55µL of each sample to a well (possible due to the use of the green buffer in previous PCR2 step)
3. Add 10 µL of the 50 bp ladder to at least one well (adapat to the number of samples or your gel size).
4. Run the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

- **Size selection**

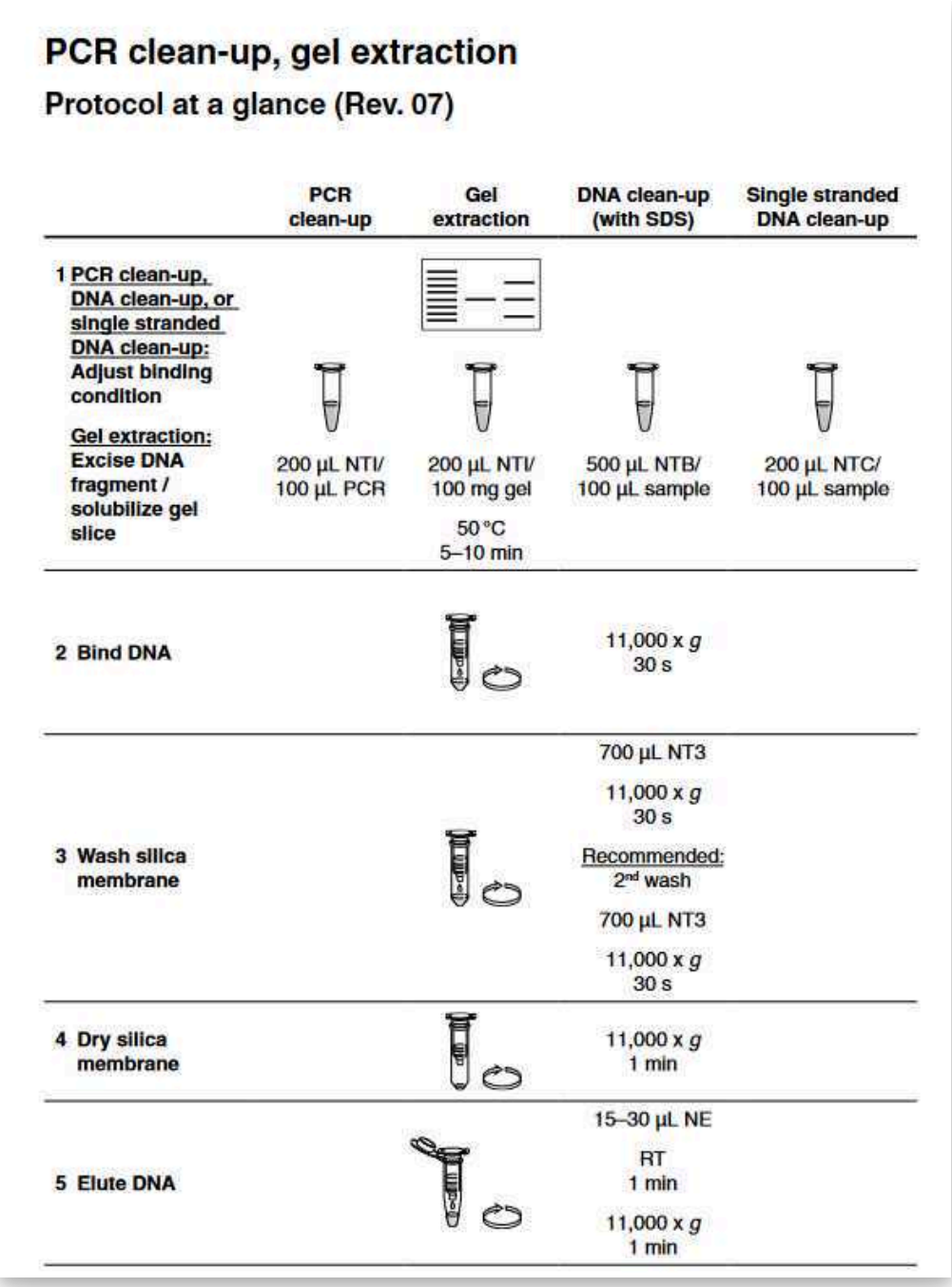
1. Cut out an agarose gel band between 250-300 bp with a scalpel for each sample.
2. Weigh an empty 2 mL microcentrifuge tube. Then, add the gel slice to the tube and re-weigh.
3. Note the weigh of the agarose gel slice to adapt the correct volume of NTI buffer in next step.

- **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 × 25 µL of buffer NE (50 µL 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<br>Gel extraction: Excise DNA fragment / solubilize gel slice | 200 µL NTI/ 100 µL PCR | 200 µL NTI/ 100 mg gel<br>50 °C<br>5–10 min | 500 µL NTB/ 100 µL sample | 200 µL NTC/ 100 µL sample |
| 2 Bind DNA | | | 11,000 x *g*<br>30 s | |
| 3 Wash silica membrane | | | 700 µL NT3<br>11,000 x *g*<br>30 s<br>Recommended:<br>2nd wash<br>700 µL NT3<br>11,000 x *g*<br>30 s | |
| 4 Dry silica membrane | | | 11,000 x *g*<br>1 min | |
| 5 Elute DNA | | | 15–30 µL NE<br>RT<br>1 min<br>11,000 x *g*<br>1 min | |

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

**Option 2: Size selection by Pippin$^{TM}$ prep**

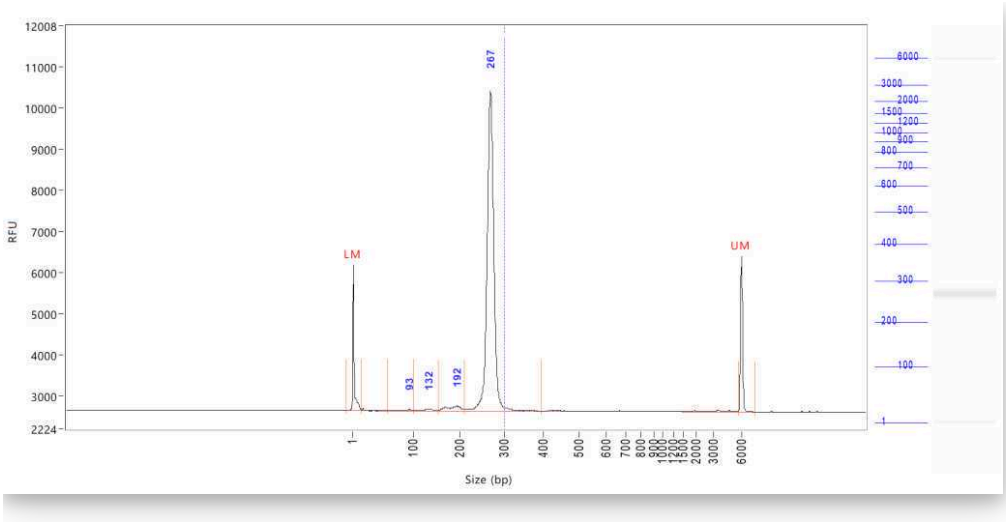
1. Bring each DNA sample from PCR2 up to 60µl with water.
2. Combine 60µl of DNA sample with 20µl of loading/marker mix (Marker L).
3. Mix samples thoroughly (vortex mixer). Briefly centrifuge to collect.
4. Remove 40µl of buffer from two "**Sample Well**" of the cassette, and load 40µl of sample into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

5. For each sample cut out band between 250-300 bp with “Range” option in Pippin$^{TM}$ Prep software.
6. After migration, remove 40µl of sample from the two "**Elution Module**" of the cassette.

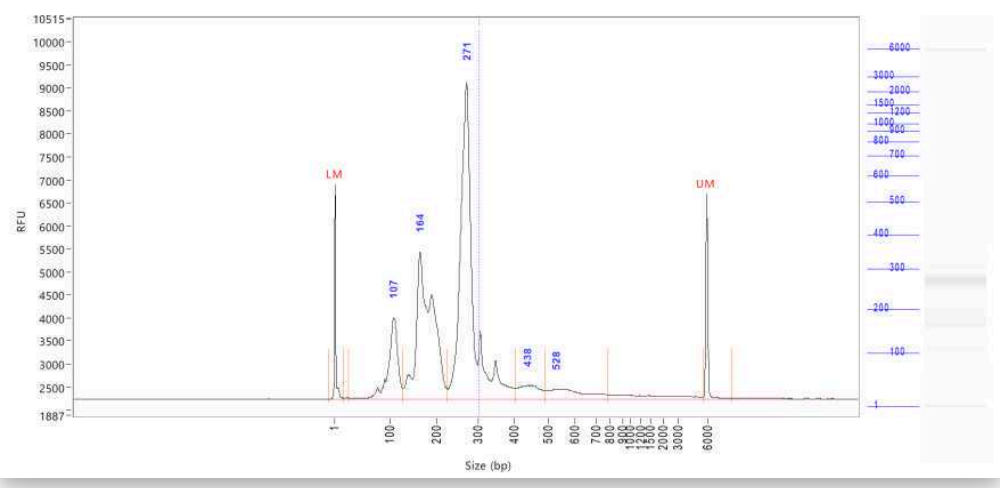
## Quality and quantity control of libraries

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

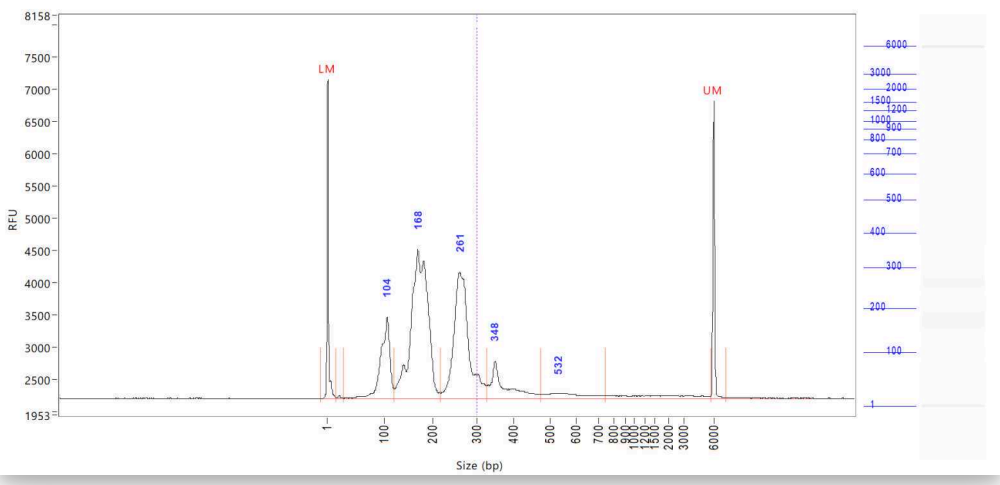
1. Use Invitrogen™ Qubit™ Fluorometer or equivalent to estimate DNA concentrations for each library
2. Prepare a 50pg/µL to 5ng/µL dilution of each library.
3. Then, follow the instructions from the manufacter.




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 ILLUMNA 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 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Platewell** | **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** |
| | | **Pcomplement** | **BarcodA** | **Pcomplement** | **BarcodA** | **Pcomplement** | **BarcodA** | **Pcomplement** | **BarcodA** | **Pcomplement** | **BarcodA** | **Pcomplement** | **BarcodA** | **Pcomplement** | **BarcodA** | **Pcomplement** | **BarcodA** | **Pcomplement** | **BarcodA** |
| | A1 | TAAGGGCGA | TAGATCGC | GCGATAAG | TAGATCGC | GATAAGGC | TAGATCGC | TAAGGGCGA | TCGCTAGA | GCGATAAG | TCGCTAGA | GATAAGGC | TCGCTAGA | TAAGGGCGA | GATCGCTA | GCGATAAG | GATCGCTA | GATAAGGC | GATCGCTA |
| | B1 | TAAGGGCGA | CTCTCTAT | GCGATAAG | CTCTCTAT | GATAAGGC | CTCTCTAT | TAAGGGCGA | CTATCTCT | GCGATAAG | CTATCTCT | GATAAGGC | CTATCTCT | TAAGGGCGA | CTCTATCT | GCGATAAG | CTCTATCT | GATAAGGC | CTCTATCT |
| | C1 | TAAGGGCGA | TATCCTCT | GCGATAAG | TATCCTCT | GATAAGGC | TATCCTCT | TAAGGGCGA | CTCTTATC | GCGATAAG | CTCTTATC | GATAAGGC | CTCTTATC | TAAGGGCGA | TCCTCTTA | GCGATAAG | TCCTCTTA | GATAAGGC | TCCTCTTA |
| | D1 | TAAGGGCGA | AGAGTAGA | GCGATAAG | AGAGTAGA | GATAAGGC | AGAGTAGA | TAAGGGCGA | TAGAAGAG | GCGATAAG | TAGAAGAG | GATAAGGC | TAGAAGAG | TAAGGGCGA | AGTAGAAG | GCGATAAG | AGTAGAAG | GATAAGGC | AGTAGAAG |

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E 1 | TAAGGGCGA | GTAAGGAG | GCGATAAG | GTAAGGAG | GATAAGGC | GTAAGGAG | TAAGGGCGA | GGAGGGTAA | GCGATAAG | GGAGGGTAA | GATAAGGC | GGAGGGTAA | TAAGGGCGA | AAGGAAGGT | GCGATAAG | AAGGAAGGT | GATAAGGC | AAGGAAGGT |
| | F 1 | TAAGGGCGA | ACTGCATA | GCGATAAG | ACTGCATA | GATAAGGC | ACTGCATA | TAAGGGCGA | CATAACTG | GCGATAAG | CATAACTG | GATAAGGC | CATAACTG | TAAGGGCGA | TGCATAAC | GCGATAAG | TGCATAAC | GATAAGGC | TGCATAAC |
| | G 1 | TAAGGGCGA | AAGGAAGTA | GCGATAAG | AAGGAAGTA | GATAAGGC | AAGGAAGTA | TAAGGGCGA | AGTAAAGG | GCGATAAG | AGTAAAGG | GATAAGGC | AGTAAAGG | TAAGGGCGA | GGAGTAAA | GCGATAAG | GGAGTAAA | GATAAGGC | GGAGTAAA |
| | H 1 | TAAGGGCGA | CTAAGCCT | GCGATAAG | CTAAGCCT | GATAAGGC | CTAAGCCT | TAAGGGCGA | GCCTCTAA | GCGATAAG | GCCTCTAA | GATAAGGC | GCCTCTAA | TAAGGGCGA | AAGCCTCT | GCGATAAG | AAGCCTCT | GATAAGGC | AAGCCTCT |
| | A 2 | CGTACTAG | TAGAATCGC | CTAGCGTA | TAGAATCGC | AGCGTACT | TAGAATCGC | CGTACTAG | TCGCTAGA | CTAGCGTA | TCGCTAGA | AGCGTACT | TCGCTAGA | CGTACTAG | GATCGCTA | CTAGCGTA | GATCGCTA | AGCGTACT | GATCGCTA |
| | B 2 | CGTACTAG | CTCTCTAT | CTAGCGTA | CTCTCTAT | AGCGTACT | CTCTCTAT | CGTACTAG | CTATCTCT | CTAGCGTA | CTATCTCT | AGCGTACT | CTATCTCT | CGTACTAG | CTCTATCT | CTAGCGTA | CTCTATCT | AGCGTACT | CTCTATCT |
| | C 2 | CGTAC | TATCC | CTAGC | TATCC | AGCGT | TATCC | CGTAC | CTCTT | CTAGC | CTCTT | AGCGT | CTCTT | CGTAC | TCCTC | CTAGC | TCCTC | AGCGT | TCCTC |

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | TAG | TCT | GTA | TCT | ACT | TCT | TAG | ATC | GTA | ATC | ACT | ATC | TAG | TTA | GTA | TTA | ACT | TTA |
| | D2 | CGTACTAG | AGAAGTAGA | CTAGCCGTA | AGAAGTAGA | AGCCGTACT | AGAAGTAGA | CGTACTAG | TAGAAGAG | CTAGCCGTA | TAGAAGAG | AGCCGTACT | TAGAAGAG | CGTACTAG | AGTAGAAG | CTAGCCGTA | AGTAGAAG | AGCCGTACT | AGTAGAAG |
| | E2 | CGTACTAG | GTAAGGAG | CTAGCCGTA | GTAAGGAG | AGCCGTACT | GTAAGGAG | CGTACTAG | GGAGGTAA | CTAGCCGTA | GGAGGTAA | AGCCGTACT | GGAGGTAA | CGTACTAG | AAGGAGGT | CTAGCCGTA | AAGGAGGT | AGCCGTACT | AAGGAGGT |
| | F2 | CGTACTAG | ACTGCATA | CTAGCCGTA | ACTGCATA | AGCGTACT | ACTGCATA | CGTACTAG | CATAACTG | CTAGCCGTA | CATAACTG | AGCGTACT | CATAACTG | CGTACTAG | TGCATAAC | CTAGCCGTA | TGCATAAC | AGCGTACT | TGCATAAC |
| | G2 | CGTACTAG | AAGGAGTA | CTAGCCGTA | AAGGAGTA | AGCGTACT | AAGGAGTA | CGTACTAG | AGTAAAGG | CTAGCCGTA | AGTAAAGG | AGCGTACT | AGTAAAGG | CGTACTAG | GGAGTAAA | CTAGCCGTA | GGAGTAAA | AGCGTACT | GGAGTAAA |
| | H2 | CGTACTAG | CTAAGCCT | CTAGCCGTA | CTAAGCCT | AGCGTACT | CTAAGCCT | CGTACTAG | GCCTCTAA | CTAGCCGTA | GCCTCTAA | AGCGTACT | GCCTCTAA | CGTACTAG | AAGCCTCT | CTAGCCGTA | AAGCCTCT | AGCGTACT | AAGCCTCT |
| | A3 | AGGCAGAA | TAGATCGC | AGAAAGGC | TAGATCGC | AAAGGCAG | TAGATCGC | AGGCAGAA | TCGCTAGA | AGAAAGGC | TCGCTAGA | AAAGGCAG | TCGCTAGA | AGGCAGAA | GATCGCTA | AGAAAGGC | GATCGCTA | AAAGGCAG | GATCGCTA |

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | B 3 | AGGCAGAAA | CTCCTCTAT | AGAAAAGGC | CTCCTCTAT | AAAAGGCAG | CTCCTCTAT | AGGCAGAAA | CTATCTCT | AGAAAAGGC | CTATCTCT | AAAAGGCAG | CTATCTCT | AGGCAGAAA | CTCCTATCT | AGAAAAGGC | CTCCTATCT | AAAAGGCAG | CTCCTATCT |
| | C 3 | AGGCAGAAA | TATCCTCCT | AGAAAAGGC | TATCCTCCT | AAAAGGCAG | TATCCTCCT | AGGCAGAAA | CTCCTTATC | AGAAAAGGC | CTCCTTATC | AAAAGGCAG | CTCCTTATC | AGGCAGAAA | TCCCTCTTA | AGAAAAGGC | TCCCTCTTA | AAAAGGCAG | TCCCTCTTA |
| | D 3 | AGGCAGAAA | AGAAGTAGA | AGAAAAGGC | AGAAGTAGA | AAAAGGCAG | AGAAGTAGA | AGGCAGAAA | TAGAAAAGAG | AGAAAAGGC | TAGAAAAGAG | AAAAGGCAG | TAGAAAAGAG | AGGCAGAAA | AGTAGAAAG | AGAAAAGGC | AGTAGAAAG | AAAAGGCAG | AGTAGAAAG |
| | E 3 | AGGCAGAAA | GTAAGGAAG | AGAAAAGGC | GTAAGGAAG | AAAAGGCAG | GTAAGGAAG | AGGCAGAAA | GGAGGGTAA | AGAAAAGGC | GGAGGGTAA | AAAAGGCAG | GGAGGGTAA | AGGCAGAAA | AAGGAAGGT | AGAAAAGGC | AAGGAAGGT | AAAAGGCAG | AAGGAAGGT |
| | F 3 | AGGCAGAAA | ACTGCATA | AGAAAAGGC | ACTGCATA | AAAAGGCAG | ACTGCATA | AGGCAGAAA | CATAACTG | AGAAAAGGC | CATAACTG | AAAAGGCAG | CATAACTG | AGGCAGAAA | TGCATAAC | AGAAAAGGC | TGCATAAC | AAAAGGCAG | TGCATAAC |
| | G 3 | AGGCAGAAA | AAGGAGTA | AGAAAAGGC | AAGGAGTA | AAAAGGCAG | AAGGAGTA | AGGCAGAAA | AGTAAAGG | AGAAAAGGC | AGTAAAGG | AAAAGGCAG | AGTAAAGG | AGGCAGAAA | GGAGTAAA | AGAAAAGGC | GGAGTAAA | AAAAGGCAG | GGAGTAAA |
| | H 3 | AGGCA | CTAAG | AGAAA | CTAAG | AAAGG | CTAAG | AGGCA | GCCTC | AGAAA | GCCTC | AAAGG | GCCTC | AGGCA | AAGCC | AGAAA | AAGCC | AAAGG | AAGCC |

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | GAA | CCT | GGC | CCT | CAG | CCT | GAA | TAA | GGC | TAA | CAG | TAA | GAA | TCT | GGC | TCT | CAG | TCT |
| | A4 | TCCCTTGAGC | TAGAATCGGC | GAGGCTTCCCT | TAGAATCGGC | GCTTCCCTTGA | TAGAATCGGC | TCCCTTGAGC | TCGGCTAGAA | GAGGCTTCCCT | TCGGCTAGAA | GCTTCCCTTGA | TCGGCTAGAA | TCCCTTGAGC | GATCGGCTA | GAGGCTTCCCT | GATCGGCTA | GCTTCCCTTGA | GATCGGCTA |
| | B4 | TCCCTTGAGC | CTCTTCTAT | GAGGCTTCCCT | CTCTTCTAT | GCTTCCCTTGA | CTCTTCTAT | TCCCTTGAGC | CTATCTTCT | GAGGCTTCCCT | CTATCTTCT | GCTTCCCTTGA | CTATCTTCT | TCCCTTGAGC | CTCTTATCT | GAGGCTTCCCT | CTCTTATCT | GCTTCCCTTGA | CTCTTATCT |
| | C4 | TCCCTTGAGC | TATCCTTCT | GAGGCTTCCCT | TATCCTTCT | GCTTCCCTTGA | TATCCTTCT | TCCCTTGAGC | CTCTTTATC | GAGGCTTCCCT | CTCTTTATC | GCTTCCCTTGA | CTCTTTATC | TCCCTTGAGC | TCCTCTTTA | GAGGCTTCCCT | TCCTCTTTA | GCTTCCCTTGA | TCCTCTTTA |
| | D4 | TCCCTTGAGC | AGAAGTAGA | GAGGCTTCCCT | AGAAGTAGA | GCTTCCCTTGA | AGAAGTAGA | TCCCTTGAGC | TAGAAGAAG | GAGGCTTCCCT | TAGAAGAAG | GCTTCCCTTGA | TAGAAGAAG | TCCCTTGAGC | AGTAGAAAG | GAGGCTTCCCT | AGTAGAAAG | GCTTCCCTTGA | AGTAGAAAG |
| | E4 | TCCCTTGAGC | GTAAGGAAG | GAGGCTTCCCT | GTAAGGAAG | GCTTCCCTTGA | GTAAGGAAG | TCCCTTGAGC | GGAGGGTAA | GAGGCTTCCCT | GGAGGGTAA | GCTTCCCTTGA | GGAGGGTAA | TCCCTTGAGC | AAGGAAGGT | GAGGCTTCCCT | AAGGAAGGT | GCTTCCCTTGA | AAGGAAGGT |
| | F4 | TCCCTTGAGC | ACTTGCATA | GAGGCTTCCCT | ACTTGCATA | GCTTCCCTTGA | ACTTGCATA | TCCCTTGAGC | CATAACTTG | GAGGCTTCCCT | CATAACTTG | GCTTCCCTTGA | CATAACTTG | TCCCTTGAGC | TGCATTAAC | GAGGCTTCCCT | TGCATTAAC | GCTTCCCTTGA | TGCATTAAC |

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | G4 | TCCTTGAGC | AAGGAAGTA | GAGCTTCCT | AAGGAAGTA | GCTTCCTTGA | AAGGAAGTA | TCCTTGAGC | AGTAAAAGG | GAGCTTCCT | AGTAAAAGG | GCTTCCTTGA | AGTAAAAGG | TCCTTGAGC | GGAGTAAAA | GAGCTTCCT | GGAGTAAAA | GCTTCCTTGA | GGAGTAAAA |
| | H4 | TCCTTGAGC | CTAAAGCCT | GAGCTTCCT | CTAAAGCCT | GCTTCCTTGA | CTAAAGCCT | TCCTTGAGC | GCCTTCTAA | GAGCTTCCT | GCCTTCTAA | GCTTCCTTGA | GCCTTCTAA | TCCTTGAGC | AAGCCTCT | GAGCTTCCT | AAGCCTCT | GCTTCCTTGA | AAGCCTCT |
| | A5 | GGACTTCCT | TAGAATCGC | TCCTTGGAC | TAGAATCGC | CTGGGACTC | TAGAATCGC | GGACTTCCT | TCGCTAGAA | TCCTTGGAC | TCGCTAGAA | CTGGGACTC | TCGCTAGAA | GGACTTCCT | GATCGCTA | TCCTTGGAC | GATCGCTA | CTGGGACTC | GATCGCTA |
| | B5 | GGACTTCCT | CTCTTCTAT | TCCTTGGAC | CTCTTCTAT | CTGGGACTC | CTCTTCTAT | GGACTTCCT | CTATCTCT | TCCTTGGAC | CTATCTCT | CTGGGACTC | CTATCTCT | GGACTTCCT | CTCTATCT | TCCTTGGAC | CTCTATCT | CTGGGACTC | CTCTATCT |
| | C5 | GGACTTCCT | TATCCTCT | TCCTTGGAC | TATCCTCT | CTGGGACTC | TATCCTCT | GGACTTCCT | CTCTTATC | TCCTTGGAC | CTCTTATC | CTGGGACTC | CTCTTATC | GGACTTCCT | TCCTCTTA | TCCTTGGAC | TCCTCTTA | CTGGGACTC | TCCTCTTA |
| | D5 | GGACTTCCT | AGAAGTAGA | TCCTTGGAC | AGAAGTAGA | CTGGGACTC | AGAAGTAGA | GGACTTCCT | TAGAAAGAG | TCCTTGGAC | TAGAAAGAG | CTGGGACTC | TAGAAAGAG | GGACTTCCT | AGTAGAAAG | TCCTTGGAC | AGTAGAAAG | CTGGGACTC | AGTAGAAAG |
| | E5 | GGACT | GTAAG | TCCTG | GTAAG | CTGGA | GTAAG | GGACT | GGAGG | TCCTG | GGAGG | CTGGA | GGAGG | GGACT | AAGGA | TCCTG | AAGGA | CTGGA | AAGGA |

| | 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 | CCT | TAA | GAC | TAA | CTC | TAA | CCT | GGT | GAC | GGT | CTC | GGT |
| | F5 | GGACTCCCT | ACTTGCCATA | TCCCTTGGAC | ACTTGCCATA | CTTGGACTCC | ACTTGCCATA | GGACTCCCT | CATAACTTG | TCCCTTGGAC | CATAACTTG | CTTGGACTCC | CATAACTTG | GGACTCCCT | TGCCATTAAC | TCCCTTGGAC | TGCCATTAAC | CTTGGACTCC | TGCCATTAAC |
| | G5 | GGACTCCCT | AAGGAAGTA | TCCCTTGGAC | AAGGAAGTA | CTTGGACTCC | AAGGAAGTA | GGACTCCCT | AGTAAAAGG | TCCCTTGGAC | AGTAAAAGG | CTTGGACTCC | AGTAAAAGG | GGACTCCCT | GGAGTAAAA | TCCCTTGGAC | GGAGTAAAA | CTTGGACTCC | GGAGTAAAA |
| | H5 | GGACTCCCT | CTAAGCCCT | TCCCTTGGAC | CTAAGCCCT | CTTGGACTCC | CTAAGCCCT | GGACTCCCT | GCCCTCTAA | TCCCTTGGAC | GCCCTCTAA | CTTGGACTCC | GCCCTCTAA | GGACTCCCT | AAGCCCTCT | TCCCTTGGAC | AAGCCCTCT | CTTGGACTCC | AAGCCCTCT |
| | A6 | TAGGGCATG | TAGAATCGC | CATGTAGGG | TAGAATCGC | TGTAGGGCA | TAGAATCGC | TAGGGCATG | TCGCCTAGA | CATGTAGGG | TCGCCTAGA | TGTAGGGCA | TCGCCTAGA | TAGGGCATG | GATCGGCTA | CATGTAGGG | GATCGGCTA | TGTAGGGCA | GATCGGCTA |
| | B6 | TAGGGCATG | CTCTCTAT | CATGTAGGG | CTCTCTAT | TGTAGGGCA | CTCTCTAT | TAGGGCATG | CTATCTCT | CATGTAGGG | CTATCTCT | TGTAGGGCA | CTATCTCT | TAGGGCATG | CTCTATCT | CATGTAGGG | CTCTATCT | TGTAGGGCA | CTCTATCT |
| | C6 | TAGGGCATG | TATCCTCT | CATGTAGGG | TATCCTCT | TGTAGGGCA | TATCCTCT | TAGGGCATG | CTCTTATC | CATGTAGGG | CTCTTATC | TGTAGGGCA | CTCTTATC | TAGGGCATG | TCCTCTTA | CATGTAGGG | TCCTCTTA | TGTAGGGCA | TCCTCTTA |

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | D6 | TAGGCATG | AGAGTAGA | CATGTAGG | AGAGTAGA | TGTAGGCA | AGAGTAGA | TAGGCATG | TAGAAGAG | CATGTAGG | TAGAAGAG | TGTAGGCA | TAGAAGAG | TAGGCATG | AGTAGAAG | CATGTAGG | AGTAGAAG | TGTAGGCA | AGTAGAAG |
| | E6 | TAGGCATG | GTAAGGAG | CATGTAGG | GTAAGGAG | TGTAGGCA | GTAAGGAG | TAGGCATG | GGAGGTAA | CATGTAGG | GGAGGTAA | TGTAGGCA | GGAGGTAA | TAGGCATG | AAGGAGGT | CATGTAGG | AAGGAGGT | TGTAGGCA | AAGGAGGT |
| | F6 | TAGGCATG | ACTGCATA | CATGTAGG | ACTGCATA | TGTAGGCA | ACTGCATA | TAGGCATG | CATAACTG | CATGTAGG | CATAACTG | TGTAGGCA | CATAACTG | TAGGCATG | TGCATAAC | CATGTAGG | TGCATAAC | TGTAGGCA | TGCATAAC |
| | G6 | TAGGCATG | AAGGAGTA | CATGTAGG | AAGGAGTA | TGTAGGCA | AAGGAGTA | TAGGCATG | AGTAAAGG | CATGTAGG | AGTAAAGG | TGTAGGCA | AGTAAAGG | TAGGCATG | GGAGTAAA | CATGTAGG | GGAGTAAA | TGTAGGCA | GGAGTAAA |
| | H6 | TAGGCATG | CTAAGCCT | CATGTAGG | CTAAGCCT | TGTAGGCA | CTAAGCCT | TAGGCATG | GCCTCTAA | CATGTAGG | GCCTCTAA | TGTAGGCA | GCCTCTAA | TAGGCATG | AAGCCTCT | CATGTAGG | AAGCCTCT | TGTAGGCA | AAGCCTCT |
| | A7 | CTCTCTAC | TAGATCGC | CTACCTCT | TAGATCGC | ACCTCTCT | TAGATCGC | CTCTCTAC | TCGCTAGA | CTACCTCT | TCGCTAGA | ACCTCTCT | TCGCTAGA | CTCTCTAC | GATCGGCTA | CTACCTCT | GATCGGCTA | ACCTCTCT | GATCGGCTA |
| | B7 | CTCTC | CTCTC | CTACC | CTCTC | ACCTC | CTCTC | CTCTC | CTATC | CTACC | CTATC | ACCTC | CTATC | CTCTC | CTCTA | CTACC | CTCTA | ACCTC | CTCTA |

| | 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 | CTCCTCCTAC | TATCCCTCCT | CTACCCTCCT | TATCCCTCCT | ACCCTCCTCT | TATCCCTCCT | CTCCTCCTAC | CTCCTTATC | CTACCCTCCT | CTCCTTATC | ACCCTCCTCT | CTCCTTATC | CTCCTCCTAC | TCCCTCCTTA | CTACCCTCCT | TCCCTCCTTA | ACCCTCCTCT | TCCCTCCTTA |
| | D7 | CTCCTCCTAC | AGAAGTAGAA | CTACCCTCCT | AGAAGTAGAA | ACCCTCCTCT | AGAAGTAGAA | CTCCTCCTAC | TAGAAGAAG | CTACCCTCCT | TAGAAGAAG | ACCCTCCTCT | TAGAAGAAG | CTCCTCCTAC | AGTAGAAAG | CTACCCTCCT | AGTAGAAAG | ACCCTCCTCT | AGTAGAAAG |
| | E7 | CTCCTCCTAC | GTAAGGAAG | CTACCCTCCT | GTAAGGAAG | ACCCTCCTCT | GTAAGGAAG | CTCCTCCTAC | GGAGGGTAA | CTACCCTCCT | GGAGGGTAA | ACCCTCCTCT | GGAGGGTAA | CTCCTCCTAC | AAGGAGGGT | CTACCCTCCT | AAGGAGGGT | ACCCTCCTCT | AAGGAGGGT |
| | F7 | CTCCTCCTAC | ACTGGCATA | CTACCCTCCT | ACTGGCATA | ACCCTCCTCT | ACTGGCATA | CTCCTCCTAC | CATAACTG | CTACCCTCCT | CATAACTG | ACCCTCCTCT | CATAACTG | CTCCTCCTAC | TGCATAAC | CTACCCTCCT | TGCATAAC | ACCCTCCTCT | TGCATAAC |
| | G7 | CTCCTCCTAC | AAGGAGTA | CTACCCTCCT | AAGGAGTA | ACCCTCCTCT | AAGGAGTA | CTCCTCCTAC | AGTAAAGG | CTACCCTCCT | AGTAAAGG | ACCCTCCTCT | AGTAAAGG | CTCCTCCTAC | GGAGTAAA | CTACCCTCCT | GGAGTAAA | ACCCTCCTCT | GGAGTAAA |
| | H7 | CTCCTCCTAC | CTAAGGCCT | CTACCCTCCT | CTAAGGCCT | ACCCTCCTCT | CTAAGGCCT | CTCCTCCTAC | GCCCTCCTAA | CTACCCTCCT | GCCCTCCTAA | ACCCTCCTCT | GCCCTCCTAA | CTCCTCCTAC | AAGCCCTCT | CTACCCTCCT | AAGCCCTCT | ACCCTCCTCT | AAGCCCTCT |

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A8 | CAGAAGAAGG | TAGAATCGC | GAGGCAGAA | TAGAATCGC | GGCAGAAGA | TAGAATCGC | CAGAAGAGG | TCGCTAGAA | GAGGCAGAA | TCGCTAGAA | GGCAGAAGA | TCGCTAGAA | CAGAAGAGG | GATCGCTA | GAGGCAGAA | GATCGCTA | GGCAGAAGA | GATCGCTA |
| | B8 | CAGAAGAAGG | CTCTCTAT | GAGGCAGAA | CTCTCTAT | GGCAGAAGA | CTCTCTAT | CAGAAGAGG | CTATCTCT | GAGGCAGAA | CTATCTCT | GGCAGAAGA | CTATCTCT | CAGAAGAGG | CTCTATCT | GAGGCAGAA | CTCTATCT | GGCAGAAGA | CTCTATCT |
| | C8 | CAGAAGAAGG | TATCCTCT | GAGGCAGAA | TATCCTCT | GGCAGAAGA | TATCCTCT | CAGAAGAGG | CTCTTATC | GAGGCAGAA | CTCTTATC | GGCAGAAGA | CTCTTATC | CAGAAGAGG | TCCTCTTA | GAGGCAGAA | TCCTCTTA | GGCAGAAGA | TCCTCTTA |
| | D8 | CAGAAGAAGG | AGAAGTAGA | GAGGCAGAA | AGAAGTAGA | GGCAGAAGA | AGAAGTAGA | CAGAAGAGG | TAGAAGAAG | GAGGCAGAA | TAGAAGAAG | GGCAGAAGA | TAGAAGAAG | CAGAAGAGG | AGTAGAAGAAG | GAGGCAGAA | AGTAGAAGAAG | GGCAGAAGA | AGTAGAAGAAG |
| | E8 | CAGAAGAAGG | GTAAGGAG | GAGGCAGAA | GTAAGGAG | GGCAGAAGA | GTAAGGAG | CAGAAGAGG | GGAGGGTAA | GAGGCAGAA | GGAGGGTAA | GGCAGAAGA | GGAGGGTAA | CAGAAGAGG | AAGGAGGGT | GAGGCAGAA | AAGGAGGGT | GGCAGAAGA | AAGGAGGGT |
| | F8 | CAGAAGAGG | ACTGCATA | GAGGCAGAA | ACTGCATA | GGCAGAAGA | ACTGCATA | CAGAAGAGG | CATAACTG | GAGGCAGAA | CATAACTG | GGCAGAAGA | CATAACTG | CAGAAGAGG | TGCATAAC | GAGGCAGAA | TGCATAAC | GGCAGAAGA | TGCATAAC |
| | G8 | CAGAG | AAGGA | GAGGC | AAGGA | GGCAG | AAGGA | CAGAG | AGTAA | GAGGC | AGTAA | GGCAG | AGTAA | CAGAG | GGAGT | GAGGC | GGAGT | GGCAG | GGAGT |

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AGG | GTA | AGA | GTA | AGA | GTA | AGG | AGG | AGA | AGG | AGA | AGG | AGG | AAA | AGA | AAA | AGA | AAA |
| | H8 | CAGAGAGAGG | CTAAGCCCT | GAGGCAGAA | CTAAGCCCT | GGCAGAGAA | CTAAGCCCT | CAGAGAGAGG | GCCTCCTAA | GAGGCAGAA | GCCTCCTAA | GGCAGAGAA | GCCTCCTAA | CAGAGAGAGG | AAGCCCTCT | GAGGCAGAA | AAGCCCTCT | GGCAGAGAA | AAGCCCTCT |
| | A9 | GCTACGGCT | TAGAATCGC | CGCCTGGCTA | TAGAATCGC | CTGGCTACG | TAGAATCGC | GCTACGGCT | TCGGCTAGA | CGGCTGGCTA | TCGGCTAGA | CTGGCTACG | TCGGCTAGA | GCTACGGCT | GATCGGCTA | CGGCTGGCTA | GATCGGCTA | CTGGCTACG | GATCGGCTA |
| | B9 | GCTACGGCT | CTCTCTAT | CGCTGGCTA | CTCTCTAT | CTGGCTACG | CTCTCTAT | GCTACGGCT | CTATCTCT | CGCTGGCTA | CTATCTCT | CTGGCTACG | CTATCTCT | GCTACGGCT | CTCTATCT | CGCTGGCTA | CTCTATCT | CTGGCTACG | CTCTATCT |
| | C9 | GCTACGGCT | TATCCTCT | CGCTGGCTA | TATCCTCT | CTGGCTACG | TATCCTCT | GCTACGGCT | CTCTTATC | CGCTGGCTA | CTCTTATC | CTGGCTACG | CTCTTATC | GCTACGGCT | TCCTCTTA | CGCTGGCTA | TCCTCTTA | CTGGCTACG | TCCTCTTA |
| | D9 | GCTACGGCT | AGAGTAGA | CGCTGGCTA | AGAGTAGA | CTGGCTACG | AGAGTAGA | GCTACGGCT | TAGAAGAG | CGCTGGCTA | TAGAAGAG | CTGGCTACG | TAGAAGAG | GCTACGGCT | AGTAGAAG | CGCTGGCTA | AGTAGAAG | CTGGCTACG | AGTAGAAG |
| | E9 | GCTACGGCT | GTAAGGAG | CGCTGGCTA | GTAAGGAG | CTGGCTACG | GTAAGGAG | GCTACGGCT | GGAGGGTAA | CGCTGGCTA | GGAGGGTAA | CTGGCTACG | GGAGGGTAA | GCTACGGCT | AAGGAGGT | CGCTGGCTA | AAGGAGGT | CTGGCTACG | AAGGAGGT |

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F9 | GCTACGCCT | ACTGCATA | CGCTGCTA | ACTGCATA | CTGCTACG | ACTGCATA | GCTACGCT | CATAACTG | CGCTGCTA | CATAACTG | CTGCTACG | CATAACTG | GCTACGCT | TGCATAAC | CGCTGCTA | TGCATAAC | CTGCTACG | TGCATAAC |
| | G9 | GCTACGCCT | AAGGAGTA | CGCTGCTA | AAGGAGTA | CTGCTACG | AAGGAGTA | GCTACGCT | AGTAAAGG | CGCTGCTA | AGTAAAGG | CTGCTACG | AGTAAAGG | GCTACGCT | GGAGTAAA | CGCTGCTA | GGAGTAAA | CTGCTACG | GGAGTAAA |
| | H9 | GCTACGCCT | CTAAGCCT | CGCTGCTA | CTAAGCCT | CTGCTACG | CTAAGCCT | GCTACGCT | GCCTCTAA | CGCTGCTA | GCCTCTAA | CTGCTACG | GCCTCTAA | GCTACGCT | AAGCCTCT | CGCTGCTA | AAGCCTCT | CTGCTACG | AAGCCTCT |
| | A10 | CGAGGCTG | TAGATCGC | GCTGCGAG | TAGATCGC | TGCGAGGC | TAGATCGC | CGAGGCTG | TCGCTAGA | GCTGCGAG | TCGCTAGA | TGCGAGGC | TCGCTAGA | CGAGGCTG | GATCGCTA | GCTGCGAG | GATCGCTA | TGCGAGGC | GATCGCTA |
| | B10 | CGAGGCTG | CTCTCTAT | GCTGCGAG | CTCTCTAT | TGCGAGGC | CTCTCTAT | CGAGGCTG | CTATCTCT | GCTGCGAG | CTATCTCT | TGCGAGGC | CTATCTCT | CGAGGCTG | CTCTATCT | GCTGCGAG | CTCTATCT | TGCGAGGC | CTCTATCT |
| | C10 | CGAGGCTG | TATCCTCT | GCTGCGAG | TATCCTCT | TGCGAGGC | TATCCTCT | CGAGGCTG | CTCTTATC | GCTGCGAG | CTCTTATC | TGCGAGGC | CTCTTATC | CGAGGCTG | TCCTCTTA | GCTGCGAG | TCCTCTTA | TGCGAGGC | TCCTCTTA |
| | D10 | 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 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CTG | AGA | GAG | AGA | GGC | AGA | CTG | GAG | GAG | GAG | GGC | GAG | CTG | AAG | GAG | AAG | GGC | AAG |
| | E10 | CGAGGGCCTG | GTAAGGAAG | GCTTGCCGAG | GTAAGGAAG | TGCCGAGGGC | GTAAGGAAG | CGAGGGCCTG | GGAGGGTAA | GCTTGCCGAG | GGAGGGTAA | TGCCGAGGGC | GGAGGGTAA | CGAGGGCCTG | AAGGAAGGT | GCTTGCCGAG | AAGGAAGGT | TGCCGAGGGC | AAGGAAGGT |
| | F10 | CGAGGGCCTG | ACTTGCCATA | GCTTGCCGAG | ACTTGCCATA | TGCCGAGGGC | ACTTGCCATA | CGAGGGCCTG | CATAACTTG | GCTTGCCGAG | CATAACTTG | TGCCGAGGGC | CATAACTTG | CGAGGGCCTG | TGCATAACC | GCTTGCCGAG | TGCATAACC | TGCCGAGGGC | TGCATAACC |
| | G10 | CGAGGGCCTG | AAGGAAGTA | GCTTGCCGAG | AAGGAAGTA | TGCCGAGGGC | AAGGAAGTA | CGAGGGCCTG | AGTAAAGG | GCTTGCCGAG | AGTAAAGG | TGCCGAGGGC | AGTAAAGG | CGAGGGCCTG | GGAGTAAA | GCTTGCCGAG | GGAGTAAA | TGCCGAGGGC | GGAGTAAA |
| | H10 | CGAGGGCCTG | CTAAGGCCT | GCTTGCCGAG | CTAAGGCCT | TGCCGAGGGC | CTAAGGCCT | CGAGGGCCTG | GCCCTCTAA | GCTTGCCGAG | GCCCTCTAA | TGCCGAGGGC | GCCCTCTAA | CGAGGGCCTG | AAGGCCTCT | GCTTGCCGAG | AAGGCCTCT | TGCCGAGGGC | AAGGCCTCT |
| | A11 | AAGAGGGCA | TAGAATCGC | GGCAAAGAA | TAGAATCGC | CAAAGAGG | TAGAATCGC | AAGAGGGCA | TCGCCTAGA | GGCAAAGAA | TCGCCTAGA | CAAAGAGG | TCGCCTAGA | AAGAGGGCA | GATCGCCTA | GGCAAAGAA | GATCGCCTA | CAAAGAGG | GATCGCCTA |
| | B11 | AAGAGGGCA | CTCTCTAT | GGCAAAGAA | CTCTCTAT | CAAAGAGG | CTCTCTAT | AAGAGGGCA | CTATCTCT | GGCAAAGAA | CTATCTCT | CAAAGAGG | CTATCTCT | AAGAGGGCA | CTCTATCT | GGCAAAGAA | CTCTATCT | CAAAGAGG | CTCTATCT |

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C 11 | AAGAGGCA | TATCCTCT | GGCAAAGA | TATCCTCT | CAAAAGAGG | TATCCTCT | AAGAGGCA | CTCTTATC | GGCAAAGA | CTCTTATC | CAAAAGAGG | CTCTTATC | AAGAGGCA | TCCTCTTA | GGCAAAGA | TCCTCTTA | CAAAAGAGG | TCCTCTTA |
| | D 11 | AAGAGGCA | AGAGTAGA | GGCAAAGA | AGAGTAGA | CAAAAGAGG | AGAGTAGA | AAGAGGCA | TAGAAGAG | GGCAAAGA | TAGAAGAG | CAAAAGAGG | TAGAAGAG | AAGAGGCA | AGTAGAAG | GGCAAAGA | AGTAGAAG | CAAAAGAGG | AGTAGAAG |
| | E 11 | AAGAGGCA | GTAAGGAG | GGCAAAGA | GTAAGGAG | CAAAAGAGG | GTAAGGAG | AAGAGGCA | GGAGGGTAA | GGCAAAGA | GGAGGGTAA | CAAAAGAGG | GGAGGGTAA | AAGAGGCA | AAGGAGGGT | GGCAAAGA | AAGGAGGGT | CAAAAGAGG | AAGGAGGGT |
| | F 11 | AAGAGGCA | ACTTGCATA | GGCAAAGA | ACTTGCATA | CAAAAGAGG | ACTTGCATA | AAGAGGCA | CATTAACTG | GGCAAAGA | CATTAACTG | CAAAAGAGG | CATTAACTG | AAGAGGCA | TGCATTAAC | GGCAAAGA | TGCATTAAC | CAAAAGAGG | TGCATTAAC |
| | G 11 | AAGAGGCA | AAGGAGTA | GGCAAAGA | AAGGAGTA | CAAAAGAGG | AAGGAGTA | AAGAGGCA | AGTAAAGG | GGCAAAGA | AGTAAAGG | CAAAAGAGG | AGTAAAGG | AAGAGGCA | GGAGTAAA | GGCAAAGA | GGAGTAAA | CAAAAGAGG | GGAGTAAA |
| | H 11 | AAGAGGCA | CTAAGCCT | GGCAAAGA | CTAAGCCT | CAAAAGAGG | CTAAGCCT | AAGAGGCA | GCCTCTAA | GGCAAAGA | GCCTCTAA | CAAAAGAGG | GCCTCTAA | AAGAGGCA | AAGCCTCT | GGCAAAGA | AAGCCTCT | CAAAAGAGG | AAGCCTCT |
| | A 12 | GTAGA | TAGAT | AGGAG | TAGAT | GAGTA | TAGAT | GTAGA | TCGCT | AGGAG | TCGCT | GAGTA | TCGCT | GTAGA | GATCG | AGGAG | GATCG | GAGTA | GATCG |

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | GGA | CGC | TAG | CGC | GAG | CGC | GGA | AGA | TAG | AGA | GAG | AGA | GGA | CTA | TAG | CTA | GAG | CTA |
| | B12 | GTAGAAGGGA | CTCCTCTTAT | AGGGAAGTTAG | CTCCTCTTAT | GAGTTAGAAG | CTCCTCTTAT | GTAGAAGGGA | CTATCTCTCT | AGGGAAGTTAG | CTATCTCTCT | GAGTTAGAAG | CTATCTCTCT | GTAGAAGGGA | CTCCTATCTCT | AGGGAAGTTAG | CTCCTATCTCT | GAGTTAGAAG | CTCCTATCTCT |
| | C12 | GTAGAAGGGA | TATCCCTCCT | AGGGAAGTTAG | TATCCCTCCT | GAGTTAGAAG | TATCCCTCCT | GTAGAAGGGA | CTCCTTATATC | AGGGAAGTTAG | CTCCTTATATC | GAGTTAGAAG | CTCCTTATATC | GTAGAAGGGA | TCCCTCCTTTA | AGGGAAGTTAG | TCCCTCCTTTA | GAGTTAGAAG | TCCCTCCTTTA |
| | D12 | GTAGAAGGGA | AGAGGAGTTAGA | AGGGAAGTTAG | AGAAGTTAGA | GAGTTAGAAG | AGAAGTTAGA | GTAGAAGGGA | TAGAAAGAAG | AGGGAAGTTAG | TAGAAAGAAG | GAGTTAGAAG | TAGAAAGAAG | GTAGAAGGGA | AGTTAGAAAG | AGGGAAGTTAG | AGTTAGAAAG | GAGTTAGAAG | AGTTAGAAAG |
| | E12 | GTAGAAGGGA | GTAAGGGAG | AGGGAAGTTAG | GTAAGGGAG | GAGTTAGAAG | GTAAGGGAG | GTAGAGGGA | GGAGGGTTAA | AGGGAAGTTAG | GGAGGGTTAA | GAGTTAGAAG | GGAGGGTTAA | GTAGAGGGA | AAGGAGAGGT | AGGGAAGTTAG | AAGGAGAGGT | GAGTTAGAAG | AAGGAGAGGT |
| | F12 | GTAGAAGGGA | ACTTGCATA | AGGGAAGTTAG | ACTTGCATA | GAGTTAGAAG | ACTTGCATA | GTAGAAGGGA | CATAAACTG | AGGGAAGTTAG | CATAAACTG | GAGTTAGAAG | CATAAACTG | GTAGAAGGGA | TGCATAAAC | AGGGAAGTTAG | TGCATAAAC | GAGTTAGAAG | TGCATAAAC |
| | G12 | GTAGAAGGGA | AAGGGAAGTTA | AGGGAAGTTAG | AAGGGAAGTTA | GAGTTAGAAG | AAGGGAAGTTA | GTAGAAGGGA | AGTAAAAGG | AGGGAAGTTAG | AGTAAAAGG | GAGTTAGAAG | AGTAAAAGG | GTAGAAGGGA | GGAGTAAAA | AGGGAAGTTAG | GGAGTAAAA | GAGTTAGAAG | GGAGTAAAA |
| | H1 | GT | CT | AG | CT | GA | CT | GT | GC | AG | GC | GA | GC | GT | AA | AG | AA | GA | AA |

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | AGAGGGA | AAGCCCT | GAGGTAG | AAGCCCT | GTAGAAG | AAGCCCT | AGAGGGA | CTCCTAAA | GAGGTAG | CTCCTAAA | GTAGAAG | CTCCTAAA | AGAGGGA | GCCCTCT | GAGGTAG | GCCCTCT | GTAGAAG | GCCCTCT |

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 |

## Protocol references

Delord, C., Lassalle, G., Oger, A., Barloy, D., Coutellec, M.-A., Delcamp, A., Evanno, G., Genthon, C., Guichoux, E., Bail, P.-Y.L., et al. (2018). A cost-and-time effective procedure to develop SNP markers for multiple species: A support for community genetics. Methods in Ecology and Evolution *9*, 1959–1974. https://doi.org/10.1111/2041-210X.13034

Hammet, F., Mahmood, K., Green, T.R., Nguyen-Dumont, T., Southey, M.C., Buchanan, D.D., Lonie, A., Nathanson, K.L., Couch, F.J., Pope, B.J., et al. (2019). Hi-Plex2: a simple and robust approach to targeted sequencing-based genetic screening. BioTechniques *67*, 118–122. https://doi.org/10.2144/btn-2019-0026

Meek, M.H., and Larson, W.A. (2019). The future is now: Amplicon sequencing and sequence capture usher in the conservation genomics era. Molecular Ecology Resources 19, 795–803. https://doi.org/10.1111/1755-0998.12998

Nguyen-Dumont, T., Pope, B.J., Hammet, F., Southey, M.C., and Park, D.J. (2013a). A high-plex PCR approach for massively parallel sequencing. BioTechniques *55*, 69–74. https://doi.org/10.2144/000114052

Nguyen-Dumont, T., Pope, B.J., Hammet, F., Mahmoodi, M., Tsimiklis, H., Southey, M.C., and Park, D.J. (2013b). Cross-platform compatibility of Hi-Plex, a streamlined approach for targeted massively parallel sequencing. Analytical Biochemistry *442*, 127–129. https://doi.org/10.1016/j.ab.2013.07.046

Nguyen-Dumont, T., Hammet, F., Mahmoodi, M., Pope, B.J., Giles, G.G., Hopper, J.L., Southey, M.C., and Park, D.J. (2015a). Abridged adapter primers increase the target scope of Hi-Plex. BioTechniques *58*, 33–36. https://doi.org/10.2144/000114247

Nguyen-Dumont, T., Mahmoodi, M., Hammet, F., Tran, T., Tsimiklis, H., Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer (kConFab), Giles, G.G., Hopper, J.L., Australian Breast Cancer Family Registry, Southey, M.C., et al. (2015b). Hi-Plex targeted sequencing is effective using DNA derived from archival dried blood spots. Analytical Biochemistry *470*, 48–51. https://doi.org/10.1016/j.ab.2014.10.010