

Standard Operating Procedure (SOP)	
SOP Title	Preparation of PCR_2 tag plates.
SOP number	GbS01
SOP Version	2

1. SCOPE

This SOP describes the laboratory procedure to produce ‘tag’ plates for use in the PCR_2 step of the GbS process (Library preparation - GbS03).

A central feature of the GbS process is normalisation of library yields. These are achieved during the PCR itself; rather than requiring upfront or downstream processing.

Whilst PCR_1 involves a limited cycle PCR during which a low concentration of seeding primers specifically anneal to the genomic template, PCR_2 involves the addition of indexed tag primers annealing to and extending from the common 5'-tails incorporated during PCR_1. The normalisation effect relates to the number of PCR cycles and tag concentrations used in PCR_2 being sufficient to drive the reaction into plateau for a wide range of input DNA concentrations.

Due to the degree of miniaturisation of the PCR, it is impractical to add a solution of tag primer to PCR_1 prior to PCR_2. This is due to both the very small volume of liquid that would need to be added accurately (if adding concentrated tag primers), and the dilution effect on the PCR reagents (if adding more dilute primers). The only practical way of enabling PCR_2 involves the transfer of a volume of PCR_1 to another plate containing lyophilised tag primers. Thorough mixing of the reaction volume with these dried primers re-suspends them in solution.

Given that the samples destined for GbS typically are stored in 96 well plates, it is therefore sensible to use up to 96 i7 barcodes to indicate which well was the source for a given cluster. Each plate of samples destined to be pooled together is simultaneously barcoded with a single i5 tag, which therefore allows for up to 16x 96 well plates to be pooled together (for a maximum of 1536 samples simultaneously).

As we are drying the tag primers down into plates, the tags will necessarily have a fixed layout. We are running PCRs in 96 well plates, therefore the 96 i7 tags will be present in the same layout in each tag plate, and one different i5 tag will be present per plate.

2. MATERIALS REQUIRED:

2.1 REAGENTS AND MATERIALS		
Reagents	Supplier	Cat. No.
Buffer Elution Buffer (Buffer EB, 10 mM Tris-Cl, pH 8.5)	Qiagen	19086
i7 tag plates (at least 100µL @ 300µM, See appendix 1)		
i5 tag oligos in screwcap tubes (x16) (at least 130µL @ 100µM, See appendix 2)		
Triton X-100		
Tris EDTA (1x)		
Materials	Supplier	Cat. No.
96 well qPCR plate, skirted (AB2800)	Fisher Scientific	10032013
Qiagen seal	Qiagen	19570
Lo-bind Eppendorf tubes (1.5 mL)		
50mL Falcon centrifuge tubes (x10)		
15mL Falcon centrifuge tubes (x16)		
Centrifuge (requires a 96 well plate adaptor)		
Tube/plate vortexer		
Ice tray/cooling block/		
Tissue wipes (Azowipes)		
Repeater & tips (40µL)		
8 Multi-channel (200µL)		
12 Multi-channel (10µL)		
Pipettes (1000, 200, 100 and 20µL)		
Filtered tips of various volumes		
Stripette gun & Stripettes (25 & 50mL)		

3. METHODOLOGY

3.1 i5 OLIGO PLATE PRODUCTION	
ELUTION BUFFER + TRITON X-100 (EB-TX100)	
3.1.01	<p>Add Triton X-100 to Buffer EB at a final concentration of 0.01%v/v.</p> <ul style="list-style-type: none"> • Add 40mL of Buffer EB to a 50mL Falcon tubes • Dispense 4µL of neat Triton X-100 - Onto the inner surface of the tube slowly, using a P10 pipette, <u>i.e. not into the buffer</u> • Vortex thoroughly until all the Triton X-100 is in solution • Each assay will require <u>seven</u> 50mL Falcon tubes
i5 OLIGO	
3.1.02	Defrost the sixteen i5 oligo tubes and thoroughly vortex
3.1.03	<p>From the information sheet add the suggested volume to produce a concentration of 100µM.</p> <p>N.B. The i5 oligos should be ordered in at a concentration of 100µM.</p>
3.1.04	Add 12,375µL of prepared buffer EB-TX100 to sixteen 15mL Falcon tubes.
3.1.05	Label each tube with the unique i5 oligo identifier.
3.1.06	Add 125µl of the corresponding i5 tag oligo (100µM) to the correct Falcon tube and vortex thoroughly. (N.B. 1 oligo per tube).
3.2 i7 OLIGO PLATE PRODUCTION	
3.2.01	Defrost the stock i7 oligo 96 well plate and thoroughly vortex.
3.2.02	Pulse centrifuge the stock i7 oligo 96 well plate.
3.2.03	<p>Dilute the 300µM to 50µM with TE (x1) into a new 96 well plate. (N.B. 1 in 6 dilution):</p> <ul style="list-style-type: none"> • Add 7.5µL of each i7 tag primer to a new 96 well plate. • Add the calculated volume of TE (x1) for each i7 primer.
3.2.04	Label 16 plates with the i7 oligos.
3.2.05	<p>Transfer 2.5µL of each specific diluted i7 tag oligo (50µM) to its corresponding well of the new 96 well plates (i.e. create a stamp).</p> <p>Plates can be centrifuged (Store @ 4°C until required).</p> <p>Plates can be frozen for medium and the long term.</p>
3.2.06	The remaining volume (~5µL) in the i7 oligo dilution plate can be disposed of.

3.3 COMBINED PLATES (i7 + i5)	
3.3.01	Retrieve sixteen pre-aliquoted i7 oligo plates and thoroughly vortex (from section 3.2).
3.3.02	Label each one of the i7 oligo plates with a unique i5 oligo identifier.
3.3.03	Add 122.5µL of the corresponding diluted i5 Tag Oligo (from section 3.1) to each well.
3.3.04	Seal the i7 + i5 oligo stock plate with a sticky lid and briefly vortex them using the plate vortexer to fully mix the primers in each well into homogenous solutions.
3.3.05	Repeat 3.3.02 to 3.3.04 for other plates making sure that each i7 oligo plate receives the allocated diluted i5 oligo.
The processing of the combined plates (1µM) can be paused at this point indefinitely. The combined 96 well plates are to be stored at -20°C.	

3.4 OLIGO STOCK PLATES (i7 + i5)	
3.4.01	Label sixteen 96 well plates with the i5 oligo identifier, which will be dispensed in each one (N.B. 1 oligo per plate).
3.4.02	Transfer 40µL of each specific diluted well from the “combined plates” (section 3.3, i7 + i5, 1µM) to its corresponding well of the new 96 well plates (i.e. create a stamp).
3.4.03	Add 40µL of EB-TX100 into each well of the sixteen 96 well plates.
The oligo stock plates (500nM) processing can be paused at this point indefinitely. The oligo stock plates (500nM) are to be stored at -20°C.	

3.5 PCR_2 PLATE PRODUCTION – 96 WELL PLATE (1 TAG).

Each oligo stock plate contains sufficient volume to allow the stamping of at least 20 PCR_2 plates

(80µL stock and 4µL per 96 well PCR plate = 20 plates)

Given likely GbS pipeline throughput and the unknown longevity of dried-down primers, it may be optimal to stamp out from three or four of each set of tag stock plates at any one time, retaining the others in the freezer until required.

3.5.01	Transfer 4µL of each well from the oligo stock plate into each of the twenty 96 well PCR plates and label accordingly.
3.5.02	Centrifuge all stamped plates in the plate centrifuge (1000g for 30sec is sufficient).
3.5.03	Lyophilise the primers by placing plates in the fan oven (set to 50°C) and leave for at least one hour or until all wells are all dry.

Drying time is entirely dependent on the heater output, fan speed, oven size and number/layout of plates present in the oven, so there is really no substitute for visually inspecting each plate to confirm that the liquid has evaporated. The dried down tag primer will be invisible to the eye, so a fully dried plate will look identical to an empty plate to the naked eye. Over-drying is not a concern in this process.

3.6 PCR_2 PLATE PRODUCTION –384 WELL PLATE (3 TAGS).

Each oligo stock plate contains sufficient volume to allow the stamping of at least 20 x 384-well PCR_2 plates

(80µL stock and 4µL per 384 well PCR plate = 20 plates)

Given likely GbS pipeline throughput and the unknown longevity of dried-down primers, it may be optimal to stamp out from three or four of each set of tag stock plates at any one time, retaining the others in the freezer until required.

Transfer 4µL of each well/quadrant in interweaved format (see below) from the oligo stock plate into each of the twenty 384 well PCR plates and label accordingly. Leave quadrant 4 empty.

384 well plate – i5 = 1, 2, & 3

384 well plate – i5 = 4, 5, & 6

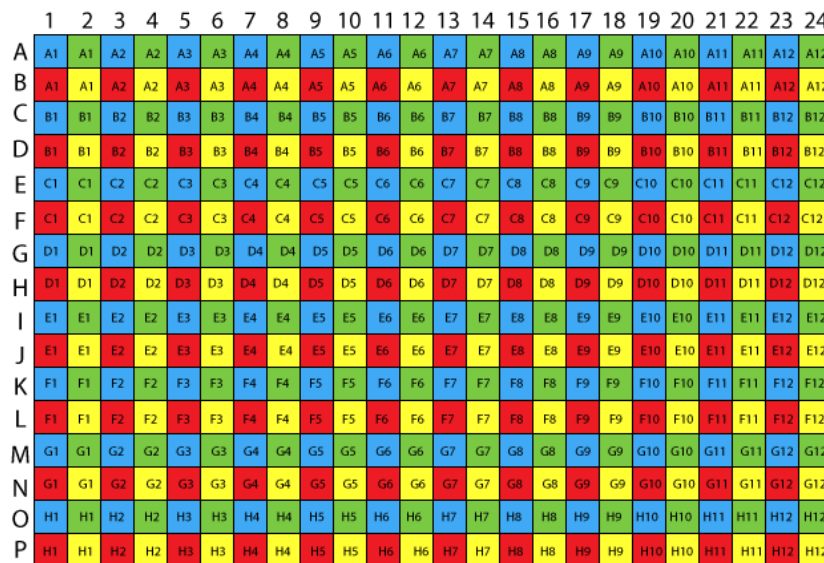
384 well plate – i5 = 7, 8, & 9

384 well plate – i5 = 10, 11, & 12

384 well plate – i5 = 13, 14, 15 & 16

The diagram below shows the 96-well plate locations in each of the corresponding quadrants

3.6.01



3.6.02

Centrifuge all stamped plates in the plate centrifuge (1000g for 30sec is sufficient).

3.6.03

Lyophilise the primers by placing plates in the fan oven (set to 50°C) and leave for at least one hour or until all wells are dry.

3.7 PCR_2 PLATE PRODUCTION –384 WELL PLATE (4 TAGS).

Each oligo stock plate contains sufficient volume to allow the stamping of at least 20 x 384-well PCR_2 plates

(80µL stock and 4µL per 384 well PCR plate = 20 plates)

Given likely GbS pipeline throughput and the unknown longevity of dried-down primers, it may be optimal to stamp out from three or four of each set of tag stock plates at any one time, retaining the others in the freezer until required.

Transfer 4µL of each well/quadrant in interweaved format (see below) from the oligo stock plate into each of the twenty 384 well PCR plates and label accordingly.

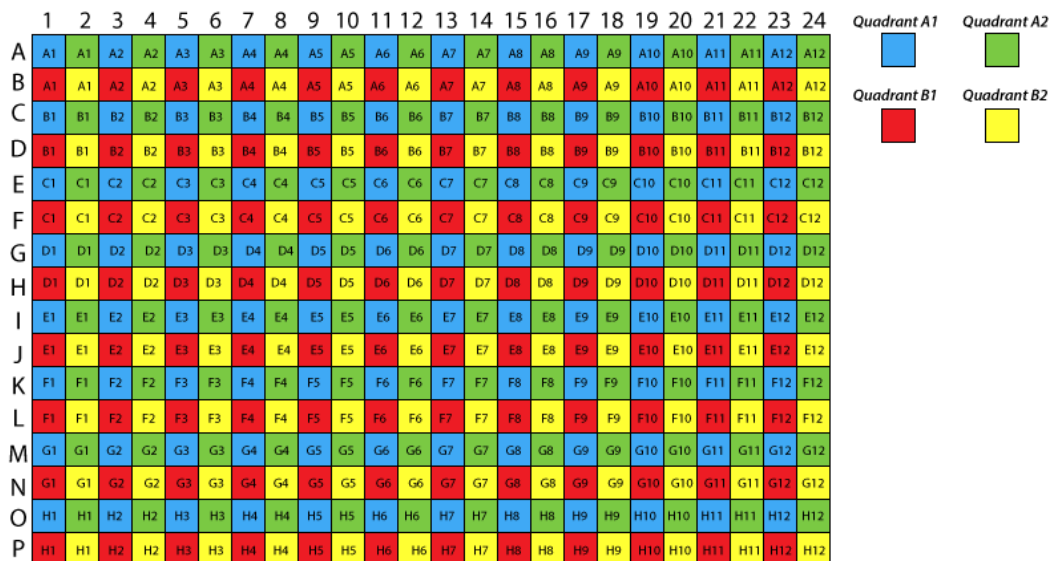
384 well plate – i5 = 1, 2, 3 & 4

384 well plate – i5 = 5, 6, 7 & 8

384 well plate – i5 = 9, 10, 11 & 12

384 well plate – i5 = 13, 14, 15 & 16

The diagram below shows the 96-well plate locations in each of the corresponding quadrants



3.7.01

3.7.02

Centrifuge all stamped plates in the plate centrifuge (1000g for 30sec is sufficient).

3.7.03

Lyophilise the primers by placing plates in the fan oven (set to 50°C) and leave for at least one hour or until all wells are dry.

4. STORAGE OF PREPARED OLIGO PLATES

STORAGE

Seal each plate using a tape lid, and collect together in batches of 10 of each tag group and place into a Ziploc bag and seal.

Store the completed oligo PCR plates in a cool, dark, dry place, e.g. a cupboard in an air-conditioned room. Under these conditions, the oligo plates should last at least 6-12 months.

5. QUALITY CONTROL OF PREPARED OLIGO PLATES

QC

Given the use of the tag plates as part of the input to the process the i7 tags can be regarded as having been QC'd already. Furthermore, we will only be using i5 tags which have also historically passed a separate QC. Therefore, both tag primers in any well can be regarded as having been tested already. However, it is advisable on each occasion that a new batch of tag plates is prepared from a new synthesis batch of i5s or i7s to perform a comparison GbS run (ideally using every well of the tag plate) against a previous batch to serve as confirmation that the tags are performing correctly.

PCR_2 REACTION CONCENTRATION

Tag primers are at 200nM in the final ~10ul PCR_2 reactions.

The stock plates contain the tag primers (i5 & i7 combined) at 500nM. The 4µl stamp is created before drying down the tag plates. There is therefore 2pmol of each tag primer present, which we rehydrate with the 10µl PCR_1 volume when transferred.

$$2 \text{ pmol in } 10\mu\text{l} = 200\text{nM}$$

APPENDIX 1 – 17 PRIMERS (300µM)

Index sequencing is performed as a separate read using the iPCRtagseq sequencing oligo (see below for sequence).
LARGE YIELD – AT LEAST 100µL @ 300µM

Oligo name	Index	Sequence
iPCRtagT1	AACGTGAT	CAAGCAGAAGACGGCATAACGAGAT AACGTGAT GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT2	AAACATCG	CAAGCAGAAGACGGCATAACGAGAT AAACATCG GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT3	ATGCCTAA	CAAGCAGAAGACGGCATAACGAGAT ATGCCTAA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT4	AGTGGTCA	CAAGCAGAAGACGGCATAACGAGAT AGTGGTCA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT5	ACCACTGT	CAAGCAGAAGACGGCATAACGAGAT ACCACTGT GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT6	ACATTGGC	CAAGCAGAAGACGGCATAACGAGAT ACATTGGC GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT7	CAGATCTG	CAAGCAGAAGACGGCATAACGAGAT CAGATCTG GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT8	CATCAAGT	CAAGCAGAAGACGGCATAACGAGAT CATCAAGT GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT9	CGCTGATC	CAAGCAGAAGACGGCATAACGAGAT CGCTGATC GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT10	ACAAGCTA	CAAGCAGAAGACGGCATAACGAGAT ACAAGCTA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT11	CTGTAGCC	CAAGCAGAAGACGGCATAACGAGAT CTGTAGCC GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT12	AGTACAAG	CAAGCAGAAGACGGCATAACGAGAT AGTACAAG GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT13	AACAACCA	CAAGCAGAAGACGGCATAACGAGAT AACAACCA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT14	AACCGAGA	CAAGCAGAAGACGGCATAACGAGAT AACCGAGA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT15	AACGCTTA	CAAGCAGAAGACGGCATAACGAGAT AACGCTTA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT16	AAGACGGA	CAAGCAGAAGACGGCATAACGAGAT AAGACGGA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT17	AAGGTACA	CAAGCAGAAGACGGCATAACGAGAT AAGGTACA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT18	ACACAGAA	CAAGCAGAAGACGGCATAACGAGAT ACACAGAA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT19	ACAGCAGA	CAAGCAGAAGACGGCATAACGAGAT ACAGCAGA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT20	ACCTCCAA	CAAGCAGAAGACGGCATAACGAGAT ACCTCCAA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT21	ACGCTCGA	CAAGCAGAAGACGGCATAACGAGAT ACGCTCGA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT22	ACGTATCA	CAAGCAGAAGACGGCATAACGAGAT ACGTATCA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT23	ACTATGCA	CAAGCAGAAGACGGCATAACGAGAT ACTATGCA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT24	AGAGTCAA	CAAGCAGAAGACGGCATAACGAGAT AGAGTCAA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT25	AGATCGCA	CAAGCAGAAGACGGCATAACGAGAT AGATCGCA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT26	AGCAGGAA	CAAGCAGAAGACGGCATAACGAGAT AGCAGGAA GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T

Oligo name	Index	Sequence
iPCRtagT27	AGTCACTA	CAAGCAGAAGACGGCATAACGAGAT AGTCACT AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT28	ATCCTGTA	CAAGCAGAAGACGGCATAACGAGAT ATCCTGT AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT29	ATTGAGGA	CAAGCAGAAGACGGCATAACGAGAT ATTGAGG AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT30	CAACCACA	CAAGCAGAAGACGGCATAACGAGAT CAACCAC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT31	CAAGACTA	CAAGCAGAAGACGGCATAACGAGAT CAAGACT AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT32	CAATGGAA	CAAGCAGAAGACGGCATAACGAGAT CAATGGA AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT33	CACTTCGA	CAAGCAGAAGACGGCATAACGAGAT CACTTCG AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT34	CAGCGTTA	CAAGCAGAAGACGGCATAACGAGAT CAGCGTT AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT35	CATACCAA	CAAGCAGAAGACGGCATAACGAGAT CATACCA AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT36	CCAGTTCA	CAAGCAGAAGACGGCATAACGAGAT CCAGTTC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT37	CCGAAGTA	CAAGCAGAAGACGGCATAACGAGAT CCGAAGT AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT38	CCGTGAGA	CAAGCAGAAGACGGCATAACGAGAT CCGTGAG AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT39	CCTCCTGA	CAAGCAGAAGACGGCATAACGAGAT CCTCCTG AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT40	CGAACTTA	CAAGCAGAAGACGGCATAACGAGAT CGAACTT AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT41	CGACTGGA	CAAGCAGAAGACGGCATAACGAGAT CGACTGG AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT42	CGCATACA	CAAGCAGAAGACGGCATAACGAGAT CGCATAC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT43	CTCAATGA	CAAGCAGAAGACGGCATAACGAGAT CTCAATG AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT44	CTGAGCCA	CAAGCAGAAGACGGCATAACGAGAT CTGAGCC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT45	CTGGCATA	CAAGCAGAAGACGGCATAACGAGAT CTGGCAT AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT46	GAATCTGA	CAAGCAGAAGACGGCATAACGAGAT GAATCTG AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT47	GACTAGTA	CAAGCAGAAGACGGCATAACGAGAT GACTAGT AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT48	GAGCTGAA	CAAGCAGAAGACGGCATAACGAGAT GAGCTGA AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT49	GATAGACA	CAAGCAGAAGACGGCATAACGAGAT GATAGAC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT50	GCCACATA	CAAGCAGAAGACGGCATAACGAGAT GCCACAT AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT51	GCGAGTAA	CAAGCAGAAGACGGCATAACGAGAT GCGAGTA AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT52	GCTAACGA	CAAGCAGAAGACGGCATAACGAGAT GCTAACG AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT53	GCTCGGTA	CAAGCAGAAGACGGCATAACGAGAT GCTCGGT AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT54	GGAGAACA	CAAGCAGAAGACGGCATAACGAGAT GGAGAAC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT55	GGTGCGAA	CAAGCAGAAGACGGCATAACGAGAT GGTGCGA AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT56	GTACGCAA	CAAGCAGAAGACGGCATAACGAGAT GTACGCA AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T

Oligo name	Index	Sequence
iPCRtagT57	GTCGTAGA	CAAGCAGAAGACGGCATAACGAGAT GTCGTAG AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT58	GTCTGTCA	CAAGCAGAAGACGGCATAACGAGAT GTCGTGTC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT59	GTGTTCTA	CAAGCAGAAGACGGCATAACGAGAT GTGTTCT AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT60	TAGGATGA	CAAGCAGAAGACGGCATAACGAGAT TAGGATG AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT61	TATCAGCA	CAAGCAGAAGACGGCATAACGAGAT TATCAGC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT62	TCCGTCTA	CAAGCAGAAGACGGCATAACGAGAT TCCGTCT AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT63	TCTTCACA	CAAGCAGAAGACGGCATAACGAGAT TCTTCAC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT64	TGAAGAGA	CAAGCAGAAGACGGCATAACGAGAT TGAAGAG AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT65	TGGAACAA	CAAGCAGAAGACGGCATAACGAGAT TGGAACA AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT66	TGGCTTCA	CAAGCAGAAGACGGCATAACGAGAT TGGCTTC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT67	TGGTGGTA	CAAGCAGAAGACGGCATAACGAGAT TGGTGGT AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT68	TTCACGCA	CAAGCAGAAGACGGCATAACGAGAT TTCACGC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT69	AACTCACC	CAAGCAGAAGACGGCATAACGAGAT AACTCAC CGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT70	AAGAGATC	CAAGCAGAAGACGGCATAACGAGAT AAGAGAT CGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT71	AAGGACAC	CAAGCAGAAGACGGCATAACGAGAT AAGGACAC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT72	AATCCGTC	CAAGCAGAAGACGGCATAACGAGAT AATCCGT CGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT73	AATGTTGC	CAAGCAGAAGACGGCATAACGAGAT AATGTTGC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT74	ACACGACC	CAAGCAGAAGACGGCATAACGAGAT ACACGAC CGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT75	ACAGATTC	CAAGCAGAAGACGGCATAACGAGAT ACAGATTC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT76	AGATGTAC	CAAGCAGAAGACGGCATAACGAGAT AGATGTAC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT77	AGCACCTC	CAAGCAGAAGACGGCATAACGAGAT AGCACCT CGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT78	AGCCATGC	CAAGCAGAAGACGGCATAACGAGAT AGCCATGC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT79	AGGCTAAC	CAAGCAGAAGACGGCATAACGAGAT AGGCTAAC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT80	ATAGCGAC	CAAGCAGAAGACGGCATAACGAGAT ATAGCGAC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT81	ATCATTCC	CAAGCAGAAGACGGCATAACGAGAT ATCATTCC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT82	ATTGGCTC	CAAGCAGAAGACGGCATAACGAGAT ATTGGCT CGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT83	CAAGGAGC	CAAGCAGAAGACGGCATAACGAGAT CAAGGAG CGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT84	CACCTTAC	CAAGCAGAAGACGGCATAACGAGAT CACCTTAC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT85	CCATCCTC	CAAGCAGAAGACGGCATAACGAGAT CCATCCT CGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT86	CCGACAAC	CAAGCAGAAGACGGCATAACGAGAT CCGACAAC AGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T

Oligo name	Index	Sequence
iPCRtagT87	CCTAATCC	CAAGCAGAAGACGGCATAACGAGAT CCTAATCC GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT88	CCTCTATC	CAAGCAGAAGACGGCATAACGAGAT CCTCTATC GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT89	CGACACAC	CAAGCAGAAGACGGCATAACGAGAT CGACACAC GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT90	CGGATTGC	CAAGCAGAAGACGGCATAACGAGAT CGGATTGC GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT91	CTAAGGTC	CAAGCAGAAGACGGCATAACGAGAT CTAAGGTC GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT92	GAACAGGC	CAAGCAGAAGACGGCATAACGAGAT GAACAGGC GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT93	GACAGTGC	CAAGCAGAAGACGGCATAACGAGAT GACAGTGC GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT94	GAGTTAGC	CAAGCAGAAGACGGCATAACGAGAT GAGTTAGC GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT95	GATGAATC	CAAGCAGAAGACGGCATAACGAGAT GATGAATC GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T
iPCRtagT96	GCCAAGAC	CAAGCAGAAGACGGCATAACGAGAT GCCAAGAC GAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATC*T

APPENDIX 2 – i5 PRIMERS - LYOPHILISED

Oligo name	Index	Sequence
GbS_i5_1	TATAGCCT	AATGATACGGCGACCACCGAGATCTACAC TATAGCCT ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_2	ATAGAGGC	AATGATACGGCGACCACCGAGATCTACAC ATAGAGGC ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_3	CCTATCCT	AATGATACGGCGACCACCGAGATCTACAC CCTATCCT ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_4	GGCTCTGA	AATGATACGGCGACCACCGAGATCTACAC GGCTCTGA ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_5	AGGCGAAG	AATGATACGGCGACCACCGAGATCTACAC AGGCGAAG ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_6	TAATCTTA	AATGATACGGCGACCACCGAGATCTACAC TAATCTTA ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_7	CAGGACGT	AATGATACGGCGACCACCGAGATCTACAC CAGGACGT ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_8	GTACTGAC	AATGATACGGCGACCACCGAGATCTACAC GTACTGAC ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_9	CTCTCTAT	AATGATACGGCGACCACCGAGATCTACAC CTCTCTAT ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_10	TATCCTCT	AATGATACGGCGACCACCGAGATCTACAC TATCCTCT ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_11	AGAGTAGA	AATGATACGGCGACCACCGAGATCTACAC AGAGTAGA ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_12	GTAAGGAG	AATGATACGGCGACCACCGAGATCTACAC GTAAGGAG ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_13	CTATTAAG	AATGATACGGCGACCACCGAGATCTACAC CTATTAAG ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_14	AAGGCTAT	AATGATACGGCGACCACCGAGATCTACAC AAGGCTAT ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_15	GAGCCTTA	AATGATACGGCGACCACCGAGATCTACAC GAGCCTTA ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T
GbS_i5_16	TTATGCGA	AATGATACGGCGACCACCGAGATCTACAC TTATGCGA ACTACTCTTTCCCTACACGACGCTCTTCCGATC*T

APPENDIX 3 – PLATE PRODUCTION:

