Icahn School of Medicine at Mount Sinai LINCS Center for Drug Toxicity Signatures

Standard Operating Procedure:

High-Throughput mRNASeq Library Construction for 3'Digital Gene Expression (DGE)

DToxS SOP Index: A-6.0
Last Revision: 12/13/2015
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Ravi Iyengar (12/13/2015)

Quality Assurance/Control (QA/QC) steps are indicated with green highlight.

Metadata recording is highlighted with yellow highlight and superscript indices.

This protocol was developed at the Broad Institute by Magali Soumillon and Tarjei Mikkelsen and is performed there.

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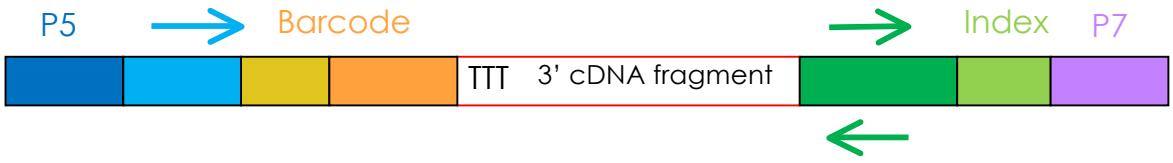
0. Final library construct for RNA-DGE, for reference purposes
    a. These libraries should be run as paired-end with a 50 bp cycle kit (see SOP A-7.0.



Barcode:	**Well/Cell index (N_6) + Unique Molecular Identifier (N_{10})**
Index:	**Plate indexing**
P5:	Flow Cell Sequence proceeding I5 (5'end Index)
P7:	Flow Cell Sequence following I7 (3'end Index)

1) Reverse Transcription (RT) / Template Switching
   a) Primers for this step:
      i) RT Primer: E3V6NEXT

      5'-/5Biosg/ACACTCTTTCCCTACACGACGCTCTTCCGATCT[BC6]N
      NNNNNNNNNTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'

      5Biosg: 5' biotin
      V: (AGC)
      N: (AGCT)

      [BC6]: 6bp sample-specific barcode**
      The barcodes were designed such that each barcode differs from the others by at least two nucleotides, so that a single sequencing error cannot lead to the misidentification of the barcode.
      ***See supplementary pages for the complete list of BC6 sequences*

      (N)10: Unique Molecular Identifier (UMI)—molecule-specific sequence tag

      ii) Template Switching Primer: E5V6NEXT

      5'-iCiGiCACACTCTTTCCCTACACGACGCrGrGrG-3'

      iC : iso-dC
      iG: iso-dG
      rG: RNA G

   b) Set up the following RT/template switching mix, for each sample (e.g., one sample per well of a 96 well plate):
      i) 10 ng of total RNA
      ii) 2 µL of 5x RT buffer (250 mMTris-HCl, pH 8.3 at 25°C)
      iii) 1 µL of dNTPs[1]
      iv) 1 µL of 10µM E5V6NEXT adapter[2]
      v) 1 µL of barcoded, 10µM, E3V6NEXT adapter[3]
      vi) 0.125 µL of Maxima H Minus Reverse Transcriptase[4]
      vii) complete to 10 µL with Nuclease-Free Water[5] (not DEPC-Treated)
      ***If total RNA is too dilute, either re-precipitate the sample and resuspend in the appropriate volume or scale up the reaction mix proportional to the volume given above.*

   c) Incubate the plate at 42°C for 1 hour 30 minutes, then at 80°C for 10 minutes

2) cDNA pooling and purification

   a) Pool all wells (samples) together
   b) Add 7x volume of DNA Binding Buffer[6] to the pooled cDNAs.
      i. For example, 96 samples from Step 1 each 10 µL in volume yields ~960 µL total volume; to this one adds 0.960 mL*7=6.72 mL.

c) Purify all pooled cDNAs through one single DNA Clean & Concentrator-5 column[7] according to the manufacturer's protocol.
d) Elute cDNAs in 18 µL of Nuclease-Free Water

3) Exonuclease I treatment

a) Add 2 µL of 10X Exonuclease I reaction buffer (from NEB) and 1µL of Exonuclease I[8] to the cDNAs
b) Incubate the reaction at 37°C for 30 minutes, then at 80°C for 20 minutes

4) Full length cDNA amplification

a) Full Length cDNA Amplification Primer-SINGV6

5'-/5Biosg/ACACTCTTTCCCTACACGACGC-3'

5Biosg: 5' biotin

b) Full length cDNA is amplified by single primer PCR using the Advantage 2 PCR Enzyme System[9]
c) Set up the PCR reaction
i) 20 µL of cDNA from previous step (essentially the whole sample)
ii) 5 µL of 10X Advantage 2 PCR buffer (from kit)
iii) 1 µL of dNTPs (from kit)
iv) 1 µL of the10µM, SINGV6 primer[10]
v) 1 µL of the Advantage 2 Polymerase Mix (from kit)
vi) 22 µL of Nuclease-Free Water (PCR grade water, preferably not DEPC treated water)
d) Perform PCR using the following program:
i) 95°C 1 min
ii) x cycles of (see below for cycle #):
- 95°C 15 sec
- 65°C 30 sec
- 68°C 6 min
iii) 72°C 10 min
iv) 4°C storage

e) PCR cycle numbers: x=10 cycles for 48-96 samples pooled together
x=15 cycles for < 48 samples

5) Full length cDNA purification and quantification

a) Purify the full length cDNAs with 30 µL of Agencourt AMPure XP magnetic beads[11] according to the manufacturer's protocol.
b) Elute the full length cDNAs in 12µL of Nuclease-Free Water (preferably not DEPC-treated)
c) QA/QC1: quantify eluted cDNA on the Qubit 2.0 Flurometer[12] using the dsDNA HS Assay[13] according to the manufacturer's protocol. Optionally (QA/QC2), the eluted cDNA can also be checked using Bioanalyzer[20] DNA High Sensitivity Assay[21] according to the manufacturer's protocol.

6) Sequencing Library Preparation

a) Replacement of i5 Primer for 2nd PCR : P5NEXTPT5

5'-
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC
C*G*A*T*C*Ts-3'
*: phosphorothioate bond

General Structure of i7** Primer for 2nd PCR:

5' CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG
***See supplementary pages for the complete list of i7 sequences*

b) Proceed to the tagmentation using the Nextera DNA kit[14], suitable for 50 ng of input, using the manufacturer's protocol. For less than 50 ng of pooled, purified and pre-amplified cDNA, scale down all reagents and reaction volume by the input concentration, according to the manufacturer's protocol.
c) Purify the tagmented cDNA on a single DNA Clean & Concentrator-5 column[7] according to the manufacturer's protocol.
d) Set up the PCR reaction
   i) 25 µL of cDNA from previous step c
   ii) 15 µL of NPM reaction mix (from the Nextera kit)
   iii) 5 µL of the5 µM,Illumina i7 index primer (N70*)[16]
   iv) 5 µL of the5 µM, P5NEXTPT5[15]
e) Perform PCR:
   i) 72°C 3 min
   ii) 98°C 30 sec
   iii) x cycles of(see below for cycle #):
      - 98°C 10sec
      - 63°C 30 sec
      - 72°C 3min
   iv) 4°C storage

f) PCR cycle numbers: x=10 cycles if the input cDNAwas less than 20ng
x=5 cycles if the input cDNA was more than 20ng
***Depending on the input cDNA measured in QA/QC1step (prior to tagmentation), PCR cycle number is determined.*

7) Sequencing Library Purification and Size Selection

a) Purify the sequencing library with 30 µL of Agencourt AMPure XP[11] magnetic beads according to the manufacturer's protocol and elute it in 20 µLof nuclease-free water (preferably not DEPC-treated).
b) The entire library is run on an E-Gel EX Gel, 2%[17] and the band corresponding to a size range of 350 to 800 bp (QA/QC3) is excised, purified using the QIA quick Gel Extraction Kit[19] according to the manufacturer's protocol and eluted in 15 µL of nuclease-free water. Use E-gel 50bp Ladder[18]

8) Sequencing Library Quality Assessment

a) QA/QC4: The library is quantified on the Qubit 2.0 Flurometer[12] using the dsDNA HS Assay[13].
b) QA/QC5 (optional): The quality and average size of the library are assessed by BioAnalyzer[20] with the DNAHigh Sensitivity kit[21].

9) Metadata

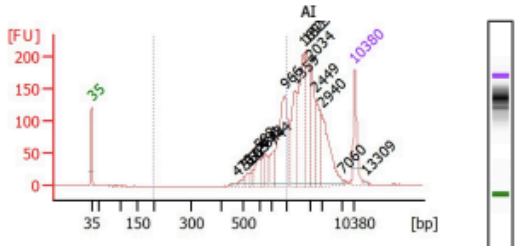
1. dNTPs (New England Biolabs, #N0447L)
2. E5V6NEXT adapter (Eurogentec/Integrated DNA Technologies, #custom, refer to the protocol for sequences/modification)
3. E3V6NEXT adapter(Integrated DNA Technologies, #custom, refer to the protocol sequences/modification)
4. Maxima H Minus Reverse Transcriptase(Thermo Scientific, #EP0753)
5. Nuclease-Free Water(LifeTechnologies, #AM9937)
6. DNA Binding Buffer(Zymo Research, #D4004-1-L)
7. DNA Clean & Concentrator-5 column (Zymo Research, #D4013)
8. Exonuclease I (New England Biolabs, #M0293L)
9. Advantage 2 PCR Enzyme System (Clontech, #639206)
10. SINGV6 primer (Integrated DNA Technologies, #custom, refer to the protocol for sequences/modification)
11. AgencourtAMPure XP magnetic beads (Beckman Coulter, #A63880)
12. Qubit 2.0 Fluorometer (Life Technologies, Q32866)
13. dsDNA HS Assay (Life Technologies. #Q32851)
14. Nextera DNA kit (FC-121-1031)
15. P5NEXTPT5 (Integrated DNA Technologies, #custom, refer to the protocol for sequences/modification)
16. i7Primers(Integrated DNA Technologies,TruGrade, #custom, refer to the protocol for sequences/modification
17. E-Gel EX Gel, 2% (Life Technologies, #G4010-02)
18. E-gel 50bp Ladder (Life Technologies, #10488-099)
19. QIAquick Gel Extraction Kit (Qiagen, #28704)
20. BioAnalyzer (Agilent, G2939AA)
21. High Sensitivitykit (Agilent, #5067- 4626)

Quality Assurance/Control Steps (QA/QC)

QA/QC1: Quantify eluted cDNA from the first PCR on the Qubit 2.0 Flurometer[12] using the dsDNA HS Assay[13]according to the manufacturer's protocol. Passing QC1 is defined by the total amount of cDNA> 30ng.

QA/QC2 (Optional): this QC step can be supplemented with Bioanalyzer High Sensitivity Assay[20,21] to check for the full length cDNA integrity.

A sample trace of the bioanalyzerhigh senstivityassay of the cDNA for a successful prep is shown below:
If the peak sizes corresponds to the range of mRNA transcript length, as shown below, it can be assumed that the cDNA step was successful.



Overall Results for sample 2 : AI

Number of peaks found:	17
Noise:	0.6
Corr. Area 1:	694.5

Region table for sample 2 : AI

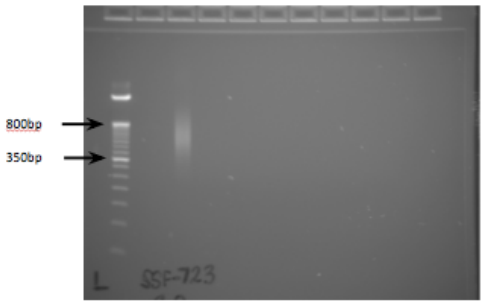
From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	694.5	28	759	20.3	372.08	783.4	

Peak table for sample 2 : AI

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations	Peak Height	Peak Width
1	35	125.00	5,411.3	Lower Marker	121.5	1.1
2	470	6.24	20.1		6.2	1.8
3	510	15.09	44.8		15.7	2.0
4	532	17.03	48.5		21.7	1.3
5	555	13.73	37.5		25.5	0.9
6	593	51.03	130.5		46.4	2.1
7	631	30.52	73.3		50.1	1.0
8	648	27.91	65.2		48.6	0.9
9	744	36.68	74.7		55.3	1.1
10	966	216.72	339.8		141.1	3.8
11	1,359	134.43	149.9		150.4	2.1
12	1,691	159.31	142.7		206.3	1.9
13	1,829	120.96	100.2		213.4	1.3
14	2,034	101.78	75.8		185.6	1.4
15	2,449	83.24	51.5		134.7	1.5
16	2,940	127.54	65.7		111.3	5.6
17	7,060	3.09	0.7		9.9	0.9
18	10,380	75.00	10.9	Upper Marker	179.5	3.3
19	13,309	0.00	0.0		7.1	1.1

QA/QC3: During the gel purification step after the 2nd PCR, the size of the library is visualized using the E-Gel EX Gel, 2%[16] (manufacturer's protocol). This step can be used to check if thelibrary cDNAlength distribution is primarily from ~350bp to ~800bp.

A sample gel picture of a passing test is shown below. If the library size distribution spans across 350-800bp as it is shown, QC result is pass. If not, QC result is fail.
*** Do not go lower than 300bp as it will risk enriching small insert libraries and concatemers of adapters (i.e. no insert). Final library size may appear smaller than what is shown below if tagmentation step was too long or an incorrect amount of input cDNA (too little) was used.*

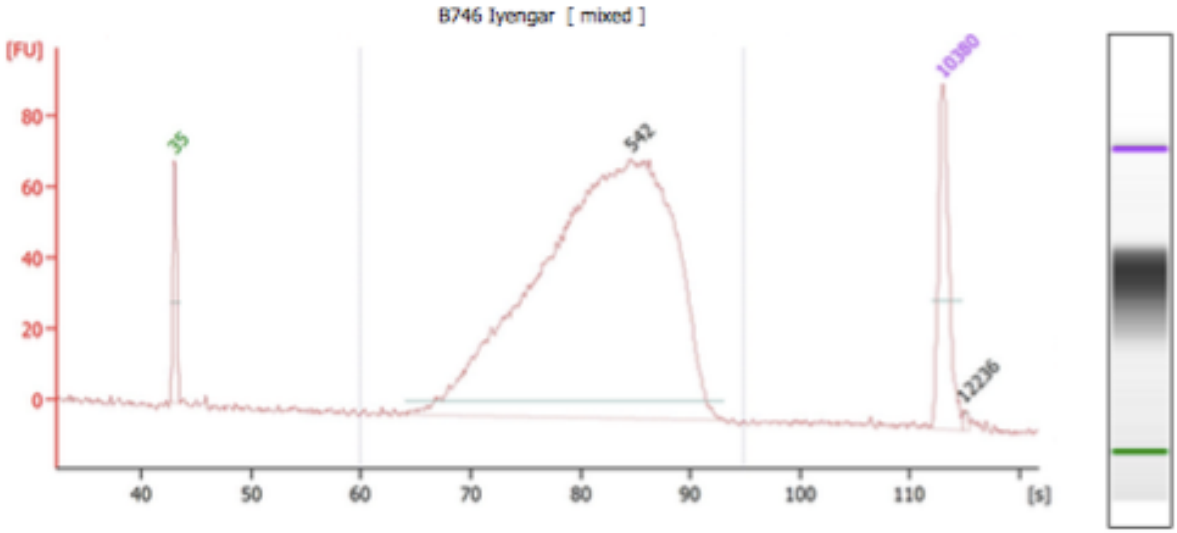


50bp ladder (Life technologies)

QA/QC4: The library is quantified on the Qubit 2.0 Flurometer[12] using the dsDNA HS Assay[13], as in QA/QC1

QA/QC5 (optional):The quality and library length distribution are assessed by BioAnalyzer[20]with the DNA High Sensitivitykit[21].

A sample bioanalyzer trace of the final library (after 2nd PCR) is shown below. The left peak labeled 35 and the right peak labeled 10380 are lower and upper markers used as length standards, and the broad distribution between is the library, whose mode is 542 bp. If the library size (middle bump in the trace below) corresponds to the size selection of 350pb-800bp, QC result is pass. Lower than 350bp peaks mean that the size selection might have not done properly and cut into the adapter dimers. If that happened, we recommend repeating gel purification and excise the gel at the proper library size again. QC fails if the library size is wrong (peaks < 350 bp) and the sample concentration is too low to repeat the gel purification step.



Overall Results for sample 3 : B746 Iyengar

Number of peaks found:	2	Corr. Area 1:	1,387.6
Noise:	0.7		

Peak table for sample 3 : B746 Iyengar

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125.00	5,411.3	Lower Marker
2		542	1,346.46	3,763.4	
3	▶	10,380	75.00	10.9	Upper Marker
4		12,236	0.00	0.0	

Supplementary Information for Oligo Sequences:

i7 Index Sequences

This index sequence can be used for high throughput setting (i.e. prepping library for more than 384 samples at a time)

i7 bases in adapter	i7 index name	i7 bases for entry on sample sheet
TCGCCTTA	N701	TAAGGCGA
CTAGTACG	N702	CGTACTAG
TTCTGCCT	N703	AGGCAGAA
GCTCAGGA	N704	TCCTGAGC
AGGAGTCC	N705	GGACTCCT
CATGCCTA	N706	TAGGCATG
GTAGAGAG	N707	CTCTCTAC
CCTCTCTG	N708	CAGAGAGG
AGCGTAGC	N709	GCTACGCT
CAGCCTCG	N710	CGAGGCTG
TGCCTCTT	N711	AAGAGGCA
TCCTCTAC	N712	GTAGAGGA

http://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/experiment-design/illumina-customer-sequence-letter.pdf

Barcode Sequences:

BC1	**AAAACT**
BC2	**AAAATC**
BC3	**AAAGTT**
BC4	**AAATAC**
BC5	**AAATTG**
BC6	**AACAAT**
BC7	**AAGATT**
BC8	**AAGTAT**
BC9	**AATACA**
BC10	**AATAGT**
BC11	**AATCTT**
BC12	**AATGAT**
BC13	**AATTCT**
BC14	**AATTGA**
BC15	**ACAATA**
BC16	**ACATAA**

BC17	**ACTTAT**
BC18	**ACTTTA**
BC19	**AGATTA**
BC20	**AGTAAT**
BC21	**ATAAAC**
BC22	**ATAACA**
BC23	**ATACAA**
BC24	**ATACTT**
BC25	**AAACAT**
BC26	**AAACTA**
BC27	**AAATCA**
BC28	**AAATGT**
BC29	**AACATA**
BC30	**AACTAA**
BC31	**AAGTTA**
BC32	**AATAAC**
BC33	**AATATG**
BC34	**AATCAA**
BC35	**AATGTA**
BC36	**AATTAG**
BC37	**AATTTC**
BC38	**ACAAAT**
BC39	**ACTAAA**
BC40	**ACTATT**
BC41	**AGAATT**
BC42	**AGATAT**
BC43	**AGTATA**
BC44	**AGTTAA**
BC45	**ATAAGT**
BC46	**ATAATG**
BC47	**ATAGAT**
BC48	**ATAGTA**
BC49	**ATATAG**
BC50	**ATATCT**
BC51	**ATCAAA**
BC52	**ATCATT**
BC53	**ATGAAT**
BC54	**ATGATA**
BC55	**ATTACT**

BC56	**ATTAGA**
BC57	**ATTCTA**
BC58	**ATTGAA**
BC59	**ATTTCA**
BC60	**ATTTGT**
BC61	**CAAATA**
BC62	**CAATAA**
BC63	**CATTAT**
BC64	**CATTTA**
BC65	**CTATAT**
BC66	**CTATTA**
BC67	**CTTTAA**
BC68	**GAAATT**
BC69	**GATAAT**
BC70	**GATATA**
BC71	**GTAATA**
BC72	**GTATAA**
BC73	**ATATGA**
BC74	**ATATTC**
BC75	**ATCTAT**
BC76	**ATCTTA**
BC77	**ATGTAA**
BC78	**ATTAAG**
BC79	**ATTATC**
BC80	**ATTCAT**
BC81	**ATTGTT**
BC82	**ATTTAC**
BC83	**ATTTTG**
BC84	**CAAAAT**
BC85	**CATAAA**
BC86	**CATATT**
BC87	**CTAAAA**
BC88	**CTAATT**
BC89	**CTTAAT**
BC90	**CTTATA**
BC91	**GAATAT**
BC92	**GAATTA**
BC93	**GATTAA**
BC94	**GTAAAT**

BC95	**GTTAAA**
BC96	**GTTATT**
BC97	**GTTTAT**
BC98	**GTTTTA**
BC99	**TAAAGT**
BC100	**TAAATG**
BC101	**TAAGAT**
BC102	**TAAGTA**
BC103	**TAATGA**
BC104	**TAATTC**
BC105	**TACTAT**
BC106	**TACTTA**
BC107	**TAGTAA**
BC108	**TAGTTT**
BC109	**TATAGA**
BC110	**TATATC**
BC111	**TATGAA**
BC112	**TATGTT**
BC113	**TATTGT**
BC114	**TATTTG**
BC115	**TCATAT**
BC116	**TCATTA**
BC117	**TCTTAA**
BC118	**TGAAAT**
BC119	**TGATTT**
BC120	**TGTAAA**
BC121	**TAAAAC**
BC122	**TAAACA**
BC123	**TAACAA**
BC124	**TAACTT**
BC125	**TAATAG**
BC126	**TAATCT**
BC127	**TACAAA**
BC128	**TACATT**
BC129	**TAGAAT**
BC130	**TAGATA**
BC131	**TATAAG**
BC132	**TATACT**
BC133	**TATCAT**

BC134	**TATCTA**
BC135	**TATTAC**
BC136	**TATTCA**
BC137	**TCAAAA**
BC138	**TCAATT**
BC139	**TCTAAT**
BC140	**TCTATA**
BC141	**TGAATA**
BC142	**TGATAA**
BC143	**TGTATT**
BC144	**TGTTAT**
BC145	**TGTTTA**
BC146	**TTAAAG**
BC147	**TTAATC**
BC148	**TTACAT**
BC149	**TTAGTT**
BC150	**TTATAC**
BC151	**TTATTG**
BC152	**TTCAAT**
BC153	**TTGAAA**
BC154	**TTGATT**
BC155	**TTTACA**
BC156	**TTTAGT**
BC157	**TTTCTT**
BC158	**TTTGTA**
BC159	**TTTTGA**
BC160	**TCTTTC**
BC161	**AGACCT**
BC162	**AGGGAT**
BC163	**CACCAA**
BC164	**CAGTCA**
BC165	**CCACAT**
BC166	**CCGATT**
BC167	**CTAGTG**
BC168	**CTTCTG**
BC169	**TTAACT**
BC170	**TTAAGA**
BC171	**TTACTA**
BC172	**TTAGAA**

BC173	**TTATCA**
BC174	**TTATGT**
BC175	**TTCATA**
BC176	**TTCTAA**
BC177	**TTGTTA**
BC178	**TTTAAC**
BC179	**TTTATG**
BC180	**TTTCAA**
BC181	**TTTTAG**
BC182	**TTTTCT**
BC183	**TTGGAT**
BC184	**ACCGTA**
BC185	**ATCGAG**
BC186	**CAAGCT**
BC187	**CATCAG**
BC188	**CATGGT**
BC189	**CGACTT**
BC190	**CGATTG**
BC191	**GAAGAC**
BC192	**GATCGT**
BC193	**GCTAGA**
BC194	**GCTTAC**
BC195	**GGGATT**
BC196	**GTACAC**
BC197	**GTTCGA**
BC198	**TAGTGG**
BC199	**TCTGCA**
BC200	**TTCCTC**
BC201	**CCAACC**
BC202	**CCTTCC**
BC203	**GTACCG**
BC204	**ACCCCC**
BC205	**ACCGGC**
BC206	**ACGCCG**
BC207	**ACGGGG**
BC208	**AGCCCG**
BC209	**AGCGGG**
BC210	**AGGCCC**
BC211	**AGGGGC**

BC212	**CACCCC**
BC213	**CACGGC**
BC214	**CAGCCG**
BC215	**CAGGGG**
BC216	**CCACCG**
BC217	**GGACAT**
BC218	**GGCAAT**
BC219	**GTCAAG**
BC220	**GTGACT**
BC221	**TCCAAC**
BC222	**TCGAAG**
BC223	**TTGTCC**
BC224	**TTTGGC**
BC225	**CTCTCC**
BC226	**GGACCA**
BC227	**ACCCGG**
BC228	**ACCGCG**
BC229	**ACGCGC**
BC230	**ACGGCC**
BC231	**AGCCGC**
BC232	**AGCGCC**
BC233	**AGGCGG**
BC234	**AGGGCG**
BC235	**CACCGG**
BC236	**CACGCG**
BC237	**CAGCGC**
BC238	**CAGGCC**
BC239	**CCACGC**
BC240	**CCAGGG**
BC241	**CCCACG**
BC242	**CCCAGC**
BC243	**CCCCGT**
BC244	**CCCCTG**
BC245	**CCCTGG**
BC246	**CCGAGG**
BC247	**CCGGAC**
BC248	**CCGGCA**
BC249	**CCGTCG**
BC250	**CCGTGC**

BC251	**CCTGGC**
BC252	**CGACCC**
BC253	**CGAGGC**
BC254	**CGCACC**
BC255	**CGCCCT**
BC256	**CGCCGA**
BC257	**CGCGCA**
BC258	**CGCGGT**
BC259	**CGCTGC**
BC260	**CGGACG**
BC261	**CGGCCA**
BC262	**CGGCGT**
BC263	**CGGGCT**
BC264	**CGGGGA**
BC265	**CCCCAC**
BC266	**CCCCCA**
BC267	**CCCGAG**
BC268	**CCCGGA**
BC269	**CCGCAG**
BC270	**CCGCGA**
BC271	**CCGGGT**
BC272	**CCGGTG**
BC273	**CCTCGG**
BC274	**CCTGCG**
BC275	**CGACGG**
BC276	**CGAGCG**
BC277	**CGCAGG**
BC278	**CGCCAG**
BC279	**CGCCTC**
BC280	**CGCGAC**
BC281	**CGCGTG**
BC282	**CGCTCG**
BC283	**CGGAGC**
BC284	**CGGCAC**
BC285	**CGGCTG**
BC286	**CGGGAG**
BC287	**CGGGTC**
BC288	**CGGTCC**
BC289	**CGGTGG**

BC290	**CGTCCG**
BC291	**CGTGGG**
BC292	**CTCCCG**
BC293	**CTGCGG**
BC294	**CTGGCG**
BC295	**GACCGC**
BC296	**GACGCC**
BC297	**GAGCGG**
BC298	**GAGGCG**
BC299	**GCACGG**
BC300	**GCAGCG**
BC301	**GCCAGG**
BC302	**GCCCAG**
BC303	**GCCCTC**
BC304	**GCCGAC**
BC305	**GCCGTG**
BC306	**GCCTCG**
BC307	**GCGAGC**
BC308	**GCGCAC**
BC309	**GCGCTG**
BC310	**GCGGAG**
BC311	**GCGGTC**
BC312	**GCGTCC**
BC313	**CGTCGC**
BC314	**CGTGCC**
BC315	**CTCCGC**
BC316	**CTCGGG**
BC317	**CTGGGC**
BC318	**GACCCG**
BC319	**GACGGG**
BC320	**GAGCCC**
BC321	**GAGGGC**
BC322	**GCACCC**
BC323	**GCAGGC**
BC324	**GCCACC**
BC325	**GCCCCT**
BC326	**GCCCGA**
BC327	**GCCGCA**
BC328	**GCCGGT**

BC329	**GCCTGC**
BC330	**GCGACG**
BC331	**GCGCCA**
BC332	**GCGCGT**
BC333	**GCGGCT**
BC334	**GCGGGA**
BC335	**GCGTGG**
BC336	**GCTCCG**
BC337	**GCTCGC**
BC338	**GCTGCC**
BC339	**GGAGCC**
BC340	**GGAGGG**
BC341	**GGCCAC**
BC342	**GGCGAG**
BC343	**GGCGTC**
BC344	**GGCTCC**
BC345	**GGGCAG**
BC346	**GGGCCT**
BC347	**GGGGAC**
BC348	**GGGGCA**
BC349	**GGGTCG**
BC350	**GGGTGC**
BC351	**GGTGGC**
BC352	**GTCCCC**
BC353	**GTGCGC**
BC354	**GTGGCC**
BC355	**TCCCGC**
BC356	**TCCGGG**
BC357	**TCGGGC**
BC358	**TGCCCC**
BC359	**TGGCCG**
BC360	**TGGCGC**
BC361	**GCTGGG**
BC362	**GGACGC**
BC363	**GGCACG**
BC364	**GGCAGC**
BC365	**GGCGCT**
BC366	**GGCGGA**
BC367	**GGGACC**

BC368	**GGGAGG**
BC369	**GGGCGA**
BC370	**GGGCTC**
BC371	**GGGGGT**
BC372	**GGGGTG**
BC373	**GGTCCC**
BC374	**GGTGCG**
BC375	**GTCGCG**
BC376	**GTCGGC**
BC377	**GTGGGG**
BC378	**TCCCCG**
BC379	**TCGCGG**
BC380	**TCGGCG**
BC381	**TGCGCG**
BC382	**TGCGGC**
BC383	**TGGGCC**
BC384	**TGGGGG**