# Brad’s Rapid Ravi for low starting mRNA amounts

Original: Brad Townsley, annotated by Kaisa Kajala, latest update 12/8/16.

## Purpose and Background

Preparing indexed non-strand specific RNA-seq libraries from low mRNA starting amounts. Great for multiplexing!

Note: this is **not** Brad-seq (which is strand-specific 3’ sequencing) but Brad’s rapid version of Ravi Kumar’s RNA-seq prep.

**References:**

<http://journal.frontiersin.org/article/10.3389/fpls.2015.00366/full> - Brad’s paper incl. the protocol for this prep and for Brad-seq

<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3428589/> - Original Ravi prep

## Materials

|  |  |  |
| --- | --- | --- |
| **Item** | **Cat number** | **Provider** |
|  |  | (VMCS = UCD stores) |
| **Consumables** |  |  |
| NEB Streptavadin Magnetic Beads, 5ml | S1420S | NEB / VMCS |
| RevertAid Reverse Transcriptase (200 U/μL), 10,000U | EP0441 | Thermo / VMCS |
| DNA polymerase I (10U/ul), 500 units | EP0041 | Fermentas / VMCS |
| Ampure XP beads | A63881 | Beckman |
| Phusion high-fidelity polymerase | M0530S | NEB |
| dNTP mix, 25 mM each | R1121 | Thermo / VMCS |
| Random Primers | 48190-011 | Invitrogen |
| RnaseH | M0297S | NEB / VMCS |
| NEBNext® End Repair Module | E6050L | NEB / VMCS |
| Taq | M0273L | NEB / VMCS |
| T4 DNA Ligase (Rapid) 600 units/μl | L603-HC-L | Enzymatics |
| Biotinylated oligos (Bio-linker-dT20) | Custom primer |  |
| Adapters (annealed primers PE1-lig and ILL-lig) | Custom primer |  |
| Primers (S1, S2, PE1, barcoded primers) | Custom primer |  |
|  |  |  |
| **For buffers & washes** |  |  |
| 1 M Tris-HCl pH 8 | E76004 | VMCS |
| Lithium chloride solution (8 M) | L7026-500ml | Sigma |
| 500 mM EDTA pH 8 | E76000 | VMCS |
| SDS | ABI01266 | VMCS |
| DTT | D0632-1G | Sigma |
| Antifoam A | A5633-100G | Sigma |
| 5 M NaCl | E76025 | VMCS |
| 2-Mercaptoethanol | WIMM2993 | VMCS |
| Rnase-free water | WI717861LT | VMCS |
| ethanol |  |  |
| PEG 8000 |  |  |
|  |  |  |
| **Plastics** |  |  |
| RNase-free PCR strips |  | USA scientific |
| Filter tips, 1-10ul |  | Denville |
| Filter tips, 2-20ul |  | Denville |
| Filter tips, 20-200ul |  | Denville |
|  |  |  |
| **Devices** |  |  |
| Magwell 96 well magnetic separator | #57624 | EdgeBio |
| Beadbeater |  |  |
| PCR machine |  |  |
| MicroPlate Genie™ multiple well plate mixer | SI-0400 | Scientific Industries |
| 8-multichannel pipettes (2-20ul, 20-200ul) |  |  |
| Chillette™ 20 Portable Tube Cooler | R6620 | Denville |

## Preparations:

**This protocol starts with mRNA extracted with streptavidin-coated magnetic beads** (see separate protocol).

**ABR buffer (Ampure XP Bead Resuspension)**

15% PEG 8000

2.5M NaCL

**Non-strand specific “Y” adapter annealing**

Non-strand specific libraries use a “Y” shaped adapter which is ligated to both ends of a double stranded DNA molecule,

The adapters should be prepared in advance and can be stored at -20 C indefinitely.

* Hydrate the main stocks of sense and antisense 5-prime adapter oligos when they arrive to a concentration of 100 μM.
* Prepare adapters by adding 8μl of 100μM PE1-lig oligo and 8μl of 100μM 5’ phosphorylated ILL-lig oligo. Add 784 μl of H2O, vortex and spin down. Aliquot 100 μl in 8 strip flip-cap strip tubes and spin the strip so that all droplets are collected and run the following annealing program:

94C 1min, (94C 10sec) X 60 cycles -1C/cycle, 20C 1min, 4C hold)

* The final concentration should be 1 μM of the Y shaped ligation adapter.
* It is a good idea to use different colors of flip-cap 8 strip tubes for the different types of adapters to avoid confusion.
* OLIGO SEQUENCES:

PE1-lig CACTCTTTCCCTACACGACGCTCTTCCGATCT

ILL-lig **P**-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC

## General tips:

* Use multichannel pipettes, nuclease-free reservoirs and master mixes (always add +10%) to make the work faster. Filter tips can be used to ensure contamination-free samples. Master mixes can be first pipetted into one PCR strip and then into all the samples using a multi-channel pipette.
* Remember to resuspend all beads carefully before pipetting them. Ampure XP beads are expensive – pipette out the total amount you need for a day and then aliquot into individual tubes to prevent contaminating back-and-forth pipetting.

## Protocol:

## 4. RNA fragmentation and cDNA priming

Here magnesium ions in the first strand buffer are used to fragment the mRNA at high temperature, followed by priming of the 1st strand cDNA by random primers.

* For each sample, add following into a fresh tube:

1.5 μl 5X Thermo Scientific RT buffer

0.5 μl Invitrogen random primers

8 μl RNA

* (Place remaining RNA into -80C for troubleshooting or future library preps.)
* Spin down samples to ensure all of sample is at the bottom of the tube.
* Place in thermocycler for fragmentation and 1st strand priming.

25°C 1 second,

94°C 1.5 min,

4°C 5 min,

4°C hold

## 5. First strand cDNA synthesis

* Prepare 1st strand master mix.

Reagent vol/rxn

5X Thermo Scientific RT buffer 1.5 μl

0.1M DTT 1.5 μl

H2O 1 μl

25mM dNTPs .5 μl

RevertAid RT enzyme .5 μl

Add 5 μl of mix to each fragmented RNA sample and mix well.

Total reaction volume 15 μl

* Incubate in thermocycler for reverse transcription step with the following program:

25C 10min

42C 50min

50C 10min

70C 10min

4C hold

## X2. Rapid Ravi steps: Second strand synthesis, end repair, A-tailing, adapter ligation

**1: Second strand synthesis, end preparation and A-tailing**

* Prepare master mix.

Reagent vol/rxn

H2O 1.5 uL

25mM dNTPs 0.4 uL

PolI 1 uL

RNaseH 0.1 uL

T4 Pol+PNK mix (End Repair Module) 0.4 uL

Taq 0.2 uL

End Repair Buffer 1.4 uL

Total 5 uL

* Add 5 ul of mix to each sample on ice. Mix and spin down strip.
* Place in thermocycler and run following program:

16C 20m,

20C 20m,

72C 20m,

4C Hold

* Add 30 ul Ampure XP beads, mix and let stand at RT for 5 minutes.
* Magnetize and remove all but 5 uL of the supernatant.
* Wash 2x with 200 ul 80% EtOH – both times add the ethanol and leave it on for 30 sec. No need to resuspend beads. Remove all the supernatant.
* Leave strip on magnetic rack and allow beads to dry (until look dry & start to crack)

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**2: Adapter ligation**

* Add 3 ul of annealed 1 uM universal adapters to each dry bead pellet. (It is possible that PEG with the DNA ligase affects elution, so you might pipette up and down to elute already here – very hard with such low volume though, and KK does not do it.)
* Prepare following master mix:

Reagent volume per reaction

H2O 1.75 ul

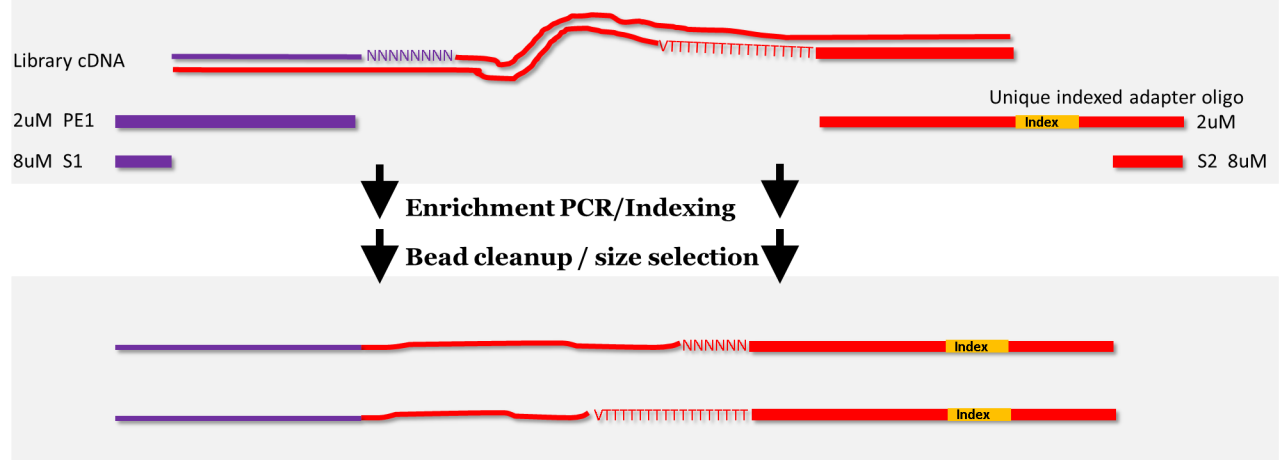
**2X Rapid** T4 ligase buffer 5.0 ul (note: if not rapid – will not work)

DNA ligase 0.25 ul

Total 7 ul

* Add 7 ul of the ligase master mix to each sample and mix by pipetting up and down making sure to resuspend Ampure beads.
* Place strip lid on to avoid evaporation and allow to stand at Room Temperature for 15 minutes.
* Add 10 ul of 50 mM EDTA to each sample.
* Add 25 ul of ABR to each sample and mix by pipetting up and down.
* Allow to stand 5 min at RT.
* Magnetize and remove all but 5 uL of the supernatant.
* Wash 2x with 200 ul 80% EtOH – both times add the ethanol and leave it on for 30 sec. No need to resuspend beads. Remove all the supernatant.
* Leave strip on magnetic rack and allow beads to dry (until look dry & start to crack).
* When beads are dry add 20-22 ul 10mM Tris to each sample and re-suspend beads by pipetting up and down 10 times. Incubate at RT for 2min.
* Magnetize and transfer the supernatant into fresh tubes.

## 10. Enrichment, adapter extension and final cleanup



**10.1: Enrichment and adapter extension**

The enrichment PCR uses four primers concurrently, two long primers to complete the adapter sequence at low concentration, and two short primers consisting of the terminal most sequence of the adapters at higher concentration. This is to selectively amplify molecules with complete adapter sequences.

* Make enrichment master mix (at RT, hot start is not necessary).

Reagent volume per reaction

5X Phusion HF Buffer 4 μl

H2O 2.6 μl

2 μM PE1 primer 1 μl

8 μM each EnrichS1 + S2 primers 1 μl

25mM dNTPs 0.2 μl

Phusion Polymerase 0.2 μl

* Add **9 μl** of master mix to a set of new tubes.
* Transfer **1 μl** of appropriate unique indexed enrichment oligo to each well (2 μM ILL-BC primer).
* Transfer **10 μl** of adapterized cDNA (that has been eluted off the Ampure beads) to each well.
* Primer sequences:

EnrichS1 AATGATACGGCGACCACCGA

EnrichS2 CAAGCAGAAGACGGCATACGA

PE1 AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

Indexed enrichment oligo sequences at the end of this document

* Mix well by pipetting up and down. The total volume of the reaction is 20ul.
* [**Optional but HIGHLY recommended**, especially if doing libraries for these samples for the first time]

Without changing tips from previous step, transfer 10 ul of enrichment PCR mix to second 8 strip PCR tubes

! Hedging your bets: Particularly with small samples or any time you don’t have a reasonable idea how many amplification cycles will provide sufficient amplification without over-amplifying, transfer 10 μl of the pre-amplification reaction volume and freeze at -20 C, then amplify the remainder of the reaction volume and run 2 ul on an agarose gel. This will allow you to adjust the number of cycles for the remainder of the reaction volume. The remaining 8 ul of enriched libraries should provide enough sample for the final cleanup and pooling.

* Incubate in thermocycler with following program:

98C 30 sec,

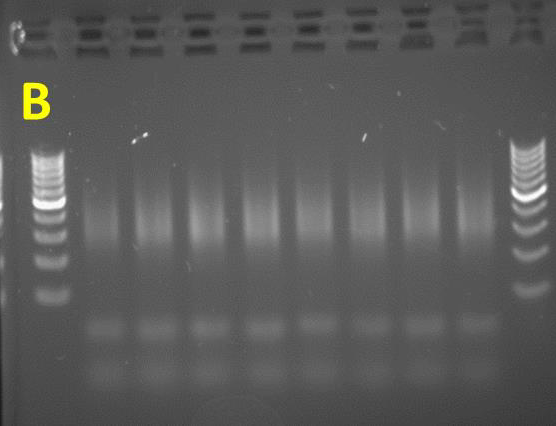
(98 C 10 sec, 65 C 30 sec, 72 C 30 sec) \*N cycles,

72 C 5 min,

10 C hold.

N for our TRAP work has been 15 cycles.

* If you have not optimized your library cycle number for the sample type yet, check PCR results on a gel. **“If you can see it, you can sequence it”.** If desired amplification is obtained proceed to cleanup.
  + Run 2 μl on 1% Agarose gel for 20 minutes at 100 Volts to check results (KK) **or**
  + **Or** run 2ul on 8% acrylamide gel and stain with SYBR Gold to increase the detection (JBS lab).
  + Sometimes KK runs up to 5ul of the first enrichment test to see the sample, enriches the rest (10-15ul), cleans it up, and does spot checks for libraries for adapter/primer contamination by running 1ul on gel.
* If desired amplification is not obtained for all samples, use remaining adapterized cDNA for enrichment with increased or decreased number of cycles.



***Non-strand specific RNA-seq libraries run at 9 cycles.***

2 ul of enrichment reaction loaded on to 1% SB gel run 20 minutes at 100 volts.

Ladder used is 1 ul of Thermo Scientific O’GeneRuler 100bp DNA ladder.

**10.2: Final library cleanup**

This step gets rid of primers and adapters and selects for specific size of library molecules. Use fresh Ampure beads. Two alternative approaches (depending how good your Ampure beads are).

Brady lab uses homemade beads and following cleanup:

* Add 1.5 volumes (i.e. 12 ul for 8 ul of enrichment product) resuspended Ampure beads to sample, mix well and let sit at room temperature for 5 minutes.
* Place on magnetic tray and remove supernatant
* Wash 2X with 200 ul of 80% EtOH. Allow pellet to dry.
* Re-suspend pellet in 10uL 10mM Tris pH 8.0 and re-suspend beads by pipetting up and down 10 times. Incubate at RT for 2min.
* Magnetize and transfer the supernatant into fresh tubes.
* **Repeat the wash.**

Bailey-Serres lab uses AmpureXP beads and following cleanup:

* Add 1.1 volumes (i.e. 8.8 ul for 8 ul of enrichment product) resuspended Ampure beads to sample, mix well and let sit at room temperature for 5 minutes.
* Place on magnetic tray and remove supernatant
* Wash 2X with 200 ul of 80% EtOH. Allow pellet to dry.
* Re-suspend pellet in 10uL 10mM Tris pH 8.0 and re-suspend beads by pipetting up and down 10 times. Incubate at RT for 2min.
* Magnetize and transfer the supernatant into fresh tubes.
* 1-2 ul can be run on a gel to make sure of (sufficient recovery and) complete lack of adapter contamination.
* Proceed to quantification (SYBR green with plate reader – see separate protocol) and pooling.
* Clean up pooled libraries with 0.8 volumes of Ampure beads to bring down the volume and to remove any residual adapters and empty library molecules.

**Sequences of indexed adapter enrichment primers**

ILLSINHA01 CAAGCAGAAGACGGCATACGAGATTCAATCATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA02 CAAGCAGAAGACGGCATACGAGATAGGCAGTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA03 CAAGCAGAAGACGGCATACGAGATAGAATAGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA04 CAAGCAGAAGACGGCATACGAGATGTAACGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA05 CAAGCAGAAGACGGCATACGAGATTGTAGCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA06 CAAGCAGAAGACGGCATACGAGATGCACCACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA07 CAAGCAGAAGACGGCATACGAGATGACTCGTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA08 CAAGCAGAAGACGGCATACGAGATCATACTATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA09 CAAGCAGAAGACGGCATACGAGATCCAACCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA10 CAAGCAGAAGACGGCATACGAGATCGCAACATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA11 CAAGCAGAAGACGGCATACGAGATAACACACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA12 CAAGCAGAAGACGGCATACGAGATCGAGATACGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA13 CAAGCAGAAGACGGCATACGAGATAGTTCAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA14 CAAGCAGAAGACGGCATACGAGATAGGTGATTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA15 CAAGCAGAAGACGGCATACGAGATCATCGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA16 CAAGCAGAAGACGGCATACGAGATGGCACATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA17 CAAGCAGAAGACGGCATACGAGATCATGAGCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA18 CAAGCAGAAGACGGCATACGAGATCTGGCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA19 CAAGCAGAAGACGGCATACGAGATCTTCCAGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA20 CAAGCAGAAGACGGCATACGAGATCCTGATGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA21 CAAGCAGAAGACGGCATACGAGATATCTGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA22 CAAGCAGAAGACGGCATACGAGATGATATCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA23 CAAGCAGAAGACGGCATACGAGATCAGCATGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA24 CAAGCAGAAGACGGCATACGAGATTGGCATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA25 CAAGCAGAAGACGGCATACGAGATGTTCAGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA26 CAAGCAGAAGACGGCATACGAGATTAAGATGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA27 CAAGCAGAAGACGGCATACGAGATTCAGCCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA28 CAAGCAGAAGACGGCATACGAGATATAAGCAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA29 CAAGCAGAAGACGGCATACGAGATCGCCTGTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA30 CAAGCAGAAGACGGCATACGAGATCGGTTGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA31 CAAGCAGAAGACGGCATACGAGATCTCGGCGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA32 CAAGCAGAAGACGGCATACGAGATGGTAACCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA33 CAAGCAGAAGACGGCATACGAGATAATTGACCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA34 CAAGCAGAAGACGGCATACGAGATATGTACGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA35 CAAGCAGAAGACGGCATACGAGATGCATGTAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA36 CAAGCAGAAGACGGCATACGAGATCTCCTCAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA37 CAAGCAGAAGACGGCATACGAGATATTGATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA38 CAAGCAGAAGACGGCATACGAGATACCGAGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA39 CAAGCAGAAGACGGCATACGAGATGAATGCGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA40 CAAGCAGAAGACGGCATACGAGATGTTCTTATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA41 CAAGCAGAAGACGGCATACGAGATCAATGGTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA42 CAAGCAGAAGACGGCATACGAGATCGATTCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA43 CAAGCAGAAGACGGCATACGAGATTGAGCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA44 CAAGCAGAAGACGGCATACGAGATTATCCAACGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA45 CAAGCAGAAGACGGCATACGAGATAGCACCGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA46 CAAGCAGAAGACGGCATACGAGATCCGACAACGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA47 CAAGCAGAAGACGGCATACGAGATGGTTAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA48 CAAGCAGAAGACGGCATACGAGATTCCGGACAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA49 CAAGCAGAAGACGGCATACGAGATCGTCAGAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA50 CAAGCAGAAGACGGCATACGAGATCCGTGTCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA51 CAAGCAGAAGACGGCATACGAGATACTAGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA52 CAAGCAGAAGACGGCATACGAGATCAGCCGTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA53 CAAGCAGAAGACGGCATACGAGATACCTTCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA54 CAAGCAGAAGACGGCATACGAGATTAGTGTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA55 CAAGCAGAAGACGGCATACGAGATAAGTTAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA56 CAAGCAGAAGACGGCATACGAGATGATGCACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA57 CAAGCAGAAGACGGCATACGAGATTGCCTTGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA58 CAAGCAGAAGACGGCATACGAGATAATCTATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA59 CAAGCAGAAGACGGCATACGAGATGCGCTGGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA60 CAAGCAGAAGACGGCATACGAGATCAAGGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA61 CAAGCAGAAGACGGCATACGAGATCGCCGTAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA62 CAAGCAGAAGACGGCATACGAGATTGATAACAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA63 CAAGCAGAAGACGGCATACGAGATCTACCGTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA64 CAAGCAGAAGACGGCATACGAGATAGAGGTGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

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ILLSINHA89 CAAGCAGAAGACGGCATACGAGATAGCGAACCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

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ILLSINHA91 CAAGCAGAAGACGGCATACGAGATGTGGAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA92 CAAGCAGAAGACGGCATACGAGATCATTCGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA93 CAAGCAGAAGACGGCATACGAGATTAATCGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

ILLSINHA94 CAAGCAGAAGACGGCATACGAGATATGCTCTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT

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