

An improved approach to mate-paired library preparation for Illumina sequencing

Abstract

High quality data from mate-pair libraries provides long range sequence linkage across the genome, which is crucial for *de novo* assembly and structural-variant detection. Current commercial methods available for the construction of such libraries have differing limitations and are often linked to a single sequencing platform in a kit format, which may not be cost effective. We present an alternative mate-paired protocol, demonstrated using Illumina sequencing platforms, combining the specificity of hybridisation and ligation, to circularise fragments with high yield. An adapter sequence is incorporated between the junction site of the mate pairs, the length of which is evenly controlled by nick translation. We present a comparison of results from 3 Kb *E. coli* and *Plasmodium falciparum* 3D7 mate-pairs made with our protocol, alongside commercial mate-pair methods. Furthermore, we present the results of a set of 3 and 6 Kb mate-pair libraries from seven different mouse strains made with our mate-pair protocol to demonstrate its reliability and robustness.

Keywords

Circularisation • Next Generation Sequencing • Mate-Pair • Long Insert • Illumina • Nextera • Pippin Prep

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Introduction

Mate-pair libraries, also known as long-insert libraries, have been used successfully to aid *de novo* sequencing, structural-variant detection and genome finishing [1]. Distance information from mate-pair reads has particular value for joining contigs flanking repetitive sequences. The resolution of larger structural rearrangements such as insertions, deletions and inversions are aided by mapping mate-pair reads to a reference sequence [2].

However, the construction of mate-pair libraries is notoriously difficult [3], particularly for degraded samples or for samples with limited amounts of DNA. During mate-pair library preparation, distal sequences are brought together in a circularisation reaction after which the majority of the large fragment is removed, leaving the original ends as a juxtaposed mate-pair, insert sizes are typically between 2 and 40 Kb. Mate-pair libraries should map onto the reference sequence as 'outward-facing' paired reads, with a gap between the mapped reads that is approximately the same as the size of the original fragments that were selected. Low quality, damaged or degraded DNA samples often lead to an increase in undesirable 'inward-facing' reads, which align to the reference sequence pointing towards each other and tend to map close together. The desired insert size is directed by the specific project and this in turn directs selection of a suitable methodology. Techniques such as Illumina's (San Diego, CA, USA) Mate Pair Library Preparation Kit v2 and Nextera Mate Pair

Sample Prep Kit, as well as both of the SOLiD 4 and SOLiD 5500 methods (Life Technologies; Staley Road, Grand Island, NY, USA), utilise intramolecular circularisation to bring together the ends of smaller fragments (i.e. 3-10 Kb). Cre-Lox recombination has been used for both smaller and larger fragment libraries (i.e. 3-20 Kb) [3,4]. Even larger fragment sizes (i.e. 40 Kb) may be inserted into a fosmid or BAC vector as in the Lucigen (Middleton, WI, USA) NxSeq 40 Kb mate-pair cloning kit [5], or up to 300 Kb from the utilisation of existing BAC libraries previously prepared for Sanger capillary sequencing [6]. Each circularisation technique is suitable for sequencing on Illumina NGS platforms, or may be easily adapted to do so. As per the Illumina mate-pair methods, linear mate-pair fragments generated by other techniques can be captured on streptavidin beads for end-repair, A-tailing, adapter ligation and PCR amplification. However, in our experience, no single commercially available circularisation method for the generation of 3 Kb mate pair libraries is both reliable and optimal for all sample types.

Illumina mate-paired libraries

Illumina mate-paired libraries utilise blunt-ended circularisation of 3-5 Kb fragments, followed by a secondary fragmentation step. During Mate Pair Library Preparation Kit v2 (Illumina v2) library construction, biotinylated nucleotides are incorporated at the ends of the sheared fragments. Degraded samples may contain nicks into which the biotin can also insert which, after the

secondary fragmentation step, are bound by streptavidin beads alongside genuine mate-pairs. As ligation between blunt ends is generally more difficult to achieve than ligation between cohesive ends, [7] the circularisation yield may be poor, leading to a lower complexity final library. Ligation between two independent fragments can also generate an undesirable proportion of chimeric reads. Random secondary fragmentation of circularised fragments causes uneven genomic sequence length either side of the junction and, as the junction contains no adapter sequence, sequencing reads that pass through the junction of the two joined ends cannot be identified and pose problems during mapping and *de novo* assembly [4]. The Nextera Mate Pair Sample Prep Kit (Nextera) circumvents a number of these issues. Transposome mediated fragmentation and biotinylated adapter tagging of genomic DNA generates an identifiable mate-pair junction sequence. Whilst biotin will not incorporate into the nicks of degraded DNA, fragmentation of poor quality samples is likely to fragment such DNA to a size below the desired size range (Nextera Mate Pair Sample Preparation Guide). As with Illumina v2, circularisation proceeds via blunt ended ligation and random secondary fragmentation.

SOLiD mate-paired libraries

The Life Technologies 2x50 bp Mate-Paired Library kit for the SOLiD 4 system incorporates hybridisation and ligation in order to circularise fragments. As described in the manufacturer's protocol, an adapter is ligated to both ends of the 3 Kb fragment; the adapter has a 2-base overhang. A biotinylated internal adapter, complementary to the two overhangs is added and the ligation reaction is held at 20°C to complete circularisation. The efficiency of the circularisation is often low, probably due to the short 2 bp hybridisation region which may limit the efficiency of the ligation [8]. The mate-pair protocol for the SOLiD 5500 series improves the yield of circularisation in comparison to the SOLiD 4 method (Life Technologies press release). A different left and right adapter are ligated which contain longer base overhangs, the exact length of which is undisclosed, blocked by short oligonucleotides. The reaction is heated to 70°C and cooled, during which time the blocking groups denature, allowing the left and right complementary overhangs to anneal to each other, thus forming a circle. Because the left and right adapters are of different sequence composition, only ~50% of adapter-ligated fragments are amenable to circularisation. Additionally, the 70°C temperature of the circularisation reaction may be detrimental to AT-rich genomes and/or to degraded samples. All SOLiD circularised fragments contain two nicks on opposite strands either side of the biotinylated adapter, which are nick-translated into the inserted genomic sequence, and subsequently digested with T7 and S1 exonuclease at the translated nick site to give linear dsDNA for streptavidin-bead capture. This process of nick translation and digestion from an adapter sequence is favourable in comparison to random shearing, which causes uneven genomic sequence length either side of the junction. As the junction of the joined DNA ends is marked by a known adapter sequence, the reads can be trimmed or split easily.

Cre-Lox recombination

Roche (Penzberg, Germany) GS-FLX paired-end libraries generate 3-20 Kb mate-pairs with cre-recombinase mediated recombination. Adaptations of this method to generate Illumina sequencing-ready libraries have previously been reported in the literature [3,4]. Circularisation adapters which contain LoxP sites are ligated to both ends of the fragment. This product undergoes cre recombination to generate circularised DNA containing biotinylated adapter sequence at the junction site. Although this method of circularisation is highly efficient, random secondary fragmentation of circularised fragments generates uneven sequence length either side of the LoxP adapter sequence, which may cause sequencing reads to pass through the adapter and into the other side, resulting in mapping issues.

Improved (Sanger) mate-paired libraries

In order to generate unbiased and diverse Illumina mate-paired libraries containing even genomic sequence either side of a common adapter sequence, we altered the Illumina mate-pair protocol to use a modified SOLiD 4 hybridisation and ligation circularisation approach. A single double stranded adapter (coloured green in Figure 1) is ligated to each end of the sheared fragment, leaving a 9 base overhang. This increased length of sticky end, as shown in Figure 2, generates a stable structure for subsequent ligation to a biotinylated internal adapter (coloured red in Figure 1). Due to the absence of a phosphate at the 5' end of the "Adapter Bottom" oligonucleotide, the circularised fragments contain one nick on each strand, which are used for nick translation into the genomic sequence, as is done with both SOLiD methods. The nicked sites are extended outward from the mate-pair region by T7 exonuclease (New England Biosciences; Ipswich, MA, USA), generating a single stranded region. This single stranded region is digested by S1 nuclease (Life Technologies), releasing a linear biotinylated mate-paired fragment from the rest of the circle. Subsequently only the biotinylated mate-paired fragment is captured by streptavidin-beads for Illumina library preparation.

This new method (See Supplementary method) was compared to the Illumina v2, Nextera, SOLiD 4, SOLiD 5500, and the cre-lox methods of circularisation by making 3 Kb *E. coli* and *Plasmodium falciparum* 3D7 (*pf3D7*) mate-pair libraries. Each method was adapted for sequencing on Illumina platforms and sequenced in a multiplexed pool. The robustness and reproducibility of our mate-pair method was further demonstrated by 3 Kb and 6 Kb mate-paired libraries made from seven mouse strains. For each library, we present an analysis of the post-circularisation yield and the total library yield. We also show sequencing quality metrics for mapped read percentage, total mapped reads, proper-paired reads along with the number of singletons, duplicates and chimeras.

Results and Discussion

An ideal mate-pair library will have the following features: a diverse population of reads which align to the reference

```

Adapter Top          5' pCTGCTTGTGGACGTTGTACATCGTGGTGC 3'
Adapter Bottom       5' TGTACAACGTCCACAAGCAG 3'
Internal Adapter Top  5' pGGAGCCTAGTGCGCACCACGA 3'
Internal Adapter Bottom 5' pGCACTAGGCCTCGCACCACGA 3'

Double stranded Adapter after annealing
5' pCTGCTTGTGGACGTTGTACATCGTGGTGC 3'
3' GACGAACACCTGCAACATGT 5'

Double stranded Internal Adapter after annealing
5' pGGAGCCTAGTGCGCACCACGA 3'
3' AGCACCACGCCCTCGGATCACGp 5'
    
```

Figure 1 Adapter and Internal Adapter Oligonucleotides. The double stranded adapter (green) has a 9-base overhang which is complementary to each 9-base overhang of the internal adapter (red). The biotinylated thymidine (T) is highlighted in yellow.

Post Adapter ligation:

```

5' TGTACAACGTCCACAAGCAG NNNNNNNN CTGCTTGTGGACGTTGTACATCGTGGTGC 3'
3' CGTGGTGCTACATGTTGCAGGTGTTTCGTC NNNNNNNN GACGAACACCTGCAACATGT 5'
    
```

Post Circularisation with the Internal Adapter:

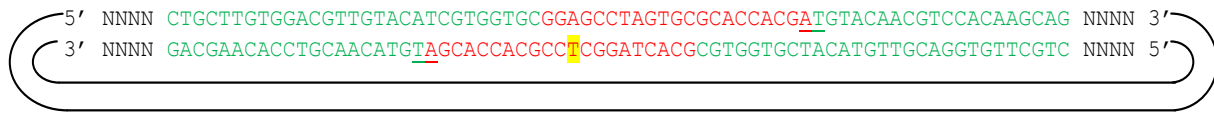


Figure 2. Schematic of the library preparation steps. NNNN denotes 3 Kb DNA fragments ligated to the adapter. The internal adapter hybridises and ligates to the adapter leaving a nick (underlined) to enable translation into the genomic region. The biotinylated thymidine (T) is highlighted in yellow.

genome in an outward-facing orientation, reads which do not reach adapter sequence, an average size between the outward-facing paired reads matching that desired, no chimeric reads, and no GC bias. Generation of fragments of the desired size is dependent upon the genomic starting material being sufficiently intact, the method employed for fragmentation being reliable and the method (if any) used to size select for the desired fragment range being accurate and selective. In the case of the hybridisation/ligation/circularisation approach used within this paper, inward-facing paired reads with a small insert size are caused by the non-specific capture of non-biotinylated library fragments. This is in contrast to the Illumina v2 mate-pair method, in which biotinylated bases can insert into nicked sites of damaged DNA; the resulting molecules can be captured on the streptavidin-coated beads, and yield inward-facing paired reads. The diversity of the final mate-pair library is dependent upon a number of factors, including the amount of adapter-ligated DNA going into the circularisation reaction, the circularisation yield (which can be determined after Plasmid Safe® digestion (Epicentre; Madison, WI, USA) of remaining linear DNA) and the number of PCR cycles.

Mate-Pair Method Comparison

We compared the method presented in this paper with the Illumina v2, Nextera, SOLiD 4, SOLiD 5500 and cre-lox methods

of circularisation. Both Nextera protocols, using 1 µg input with an AMPure (Beckman Coulter; Brea, CA, USA) bead size selection, and using a 4 µg input with a gel size selection were carried out. Each experiment was performed in duplicate. Where possible, variables such as fragmentation, size selection, circularisation input amount and Illumina sequencing preparation were standardised in order to aid a fair comparison. Each method was evaluated with the preparation and sequencing of both *E.Coli* (50.8% GC) and *Plasmodium falciparum 3D7* (19.3% GC) genomes.

Circularisation

In theory, any improvement in the yield of circularisation should directly increase the complexity of the final mate-pair library. The yield of each circularisation reaction from a normalised input of 400 ng material is presented in Table 1. For both genomes, the Sanger method demonstrated a ~1.7-fold improvement in yield above the SOLiD 5500 method and a ~4-fold improvement in yield above the SOLiD 4 adapter protocol. The estimated library size (defined as the total number of unique fragments (Table 2)) for the SOLiD 4 *E.Coli* libraries, reflects this positive correlation between circularisation yield and final library complexity. This correlation is also demonstrated with the SOLiD 5500 *pf3D7* library; however, the estimated library size of the *E.Coli* SOLiD 5500 library is twice that of the Sanger

library, indicating the relationship between circularisation yield and final library diversity may be influenced by other factors. In the case of the SOLiD 4 method, extremely poor circularisation of *pf3D7* (Table 1) led to an inability to produce a successful library. The Illumina v2 protocol demonstrated a ~1.3-fold improvement in circularisation yield above the Sanger method. However, sequencing metrics (Table 2) indicates this is at least partially due to the formation of chimeric circles by two unrelated fragments (~15% of mapped reads) and not genuine mate-pairs. Conversely, the Nextera protocol demonstrated an improvement of 1.7-2.9-fold in circularisation yield which is not as markedly related to an increase in chimeric reads (although this is still elevated at ~2.3%). Circularisation yields of the cre-lox libraries were undetectable by high sensitivity qubit and generated a ~5-fold lower estimated library size than the Sanger libraries.

Mate-Pair Size

Genomic DNA was mechanically sheared for each 3 Kb library with the exception of the Nextera libraries. These libraries underwent transposome mediated fragmentation and adapter tagging of genomic DNA. Transposome mediated fragmentation is dependent upon high quality and accurately quantified starting material. In our experience, the accurate quantification of “real life” samples is often difficult and inaccurate due to impurities, even with the use of fluorometric based methods specific for duplex DNA such as the Qubit dsDNA BR kit. Additionally, the GC content of the genome alters the fragmentation pattern and, despite the proportional scaling up of all reaction components, the 4 µg fragmentation of *pf3D7* generated a ~2.7-fold larger mean fragment size than that of the 1 µg fragmentation. With the exception of the 1 µg Nextera libraries, all libraries were size selected using the Blue Pippin (Sage Science; Beverly, MA, USA) using conditions shown in supplementary Table 1 and 2,

to target the peak size maximum as determined by the Agilent Bioanalyzer (Agilent; Santa Clara, CA, USA).

Library quality

Library quality statistics of reads mapped to the *E.Coli* and *pf3D7* genomes are given in Table 2. Despite multiple attempts *pf3D7* SOLiD 4 libraries failed to yield a final library. All methods yielded a high proportion (>75%) of mappable reads, with the exception of one *E.Coli* SOLiD 4 library (38%) of which only 34% were proper pairs and the cre-lox libraries (59-67%), of which only 36-55% were proper pairs. All other libraries were 70-87% proper pairs, the lower end being the Nextera libraries. Singleton reads ranged from 2 to 5%, except for *E.coli* Nextera libraries (~11%) and cre-lox libraries (8-24%). Inward facing reads were low for all libraries, the highest being the Sanger (0.8-1.4%) and Illumina v2 (0.9-1.8%) libraries. Although still low, the inward facing reads are likely due to insufficient removal of non-biotinylated material during the washing steps of these particular libraries. Duplicate rates ranged between 0.2-12%, Nextera libraries generating the lowest values and cre-lox libraries the highest.

Intermolecular circularisation may occur if two different fragments concatamerise or, in the case of the Sanger, SOLiD 4, SOLiD 5500 and cre-lox libraries, it is possible for two fragments to incorrectly ligate to each other in addition to ligating to the circularisation adapter. Either of these scenarios will result in an artefact of structural variation, chimeric reads. The presence of chimeric reads poses a major problem in the generation of mate-pair libraries and the elimination of these is highly desirable. For both genomes, the Sanger and cre-lox methods of circularisation generated the lowest number of chimeric reads (0.06/0.1% respectively for *E.Coli* and 0.7/0.3% respectively for *pf3D7*). Due to the poor performance of the cre-lox libraries and the high performance

Table 1. *E.coli/pf3D7* circularisation and final library yields. 400 ng material was used as input into each circularisation reaction; all libraries went through 13 cycles of PCR.

| Method | E.Coli | | | | P. falciparum 3D7 | | | |
|-------------|--|--|-----------------------------------|-------------------------------------|--|--|-----------------------------------|-------------------------------------|
| | Mean ± Stdev Output Post Plasmid Safe Digestion (ng) | Mean ± Stdev Circularisation Yield (%) | Mean ± Stdev Library Yield (pMol) | Mean ± Stdev Library Yield (nmol/l) | Mean ± Stdev Output Post Plasmid Safe Digestion (ng) | Mean ± Stdev Circularisation Yield (%) | Mean ± Stdev Library Yield (pMol) | Mean ± Stdev Library Yield (nmol/l) |
| Sanger | 37.2 ±3 | 9.3 ±0.7 | 0.028 ±0.001 | 1.4 ±0.07 | 41.7 ±10 | 10.4 ±2 | 0.0248 ±0.01 | 1.2 ±0.5 |
| Nextera 1ug | 110 ±12 | 27.6 ±3 | 2.921 ±0.2 | 146 ±10.4 | 74.0 ±6 | 18.5 ±1 | 1.941 ±1 | 97.1 ±55.0 |
| Nextera 4ug | 107.4 ±14 | 26.9 ±3 | 2.200 ±0.4 | 110 ±18.8 | 69.4 ±1 | 17.3 ±0.2 | 0.504 ±0.008 | 25.2 ±0.4 |
| SOLiD5500 | 22.5 ±0.6 | 5.6 ±0.2 | 0.052 ±0.005 | 2.6 ±0.2 | 23.0 ±0.1 | 5.7 ±0.02 | 0.012 ±0.0002 | 0.59 ±0.008 |
| SOLiD4 | 9.6 ±2 | 2.4 ±0.4 | 0.008 ±0.003 | 0.4 ±0.2 | 9.9 ±3 | 2.5 ±0.7 | Fail | Fail |
| Illuminav2 | 51.3 ±17 | 12.8 ±4 | 0.112 ±0.001 | 5.6 ±0.06 | 48.0 ±0 | 12.0 ±0 | 0.202 ±0.04 | 10.1 ±2.1 |
| 454 | ND | NA | 0.010 ±0.003 | 0.5 ±0.2 | ND | NA | 0.003 ±0.00001 | 0.15 ±0.0007 |

Table 2. Post sequencing analysis metrics for 3kb E.Coli and pf3D7 mate-paired libraries prepared using commercial methods alongside the Sanger method. Libraries were indexed, multiplexed and sequenced on the Illumina MiSeq. Singletons are defined as an individual read which does not have a corresponding mate-pair. Chimeras are defined as incorrect mate-pairs formed during circularisation when ligation occurs between unrelated DNA molecules.

| Method | All reads | Mapped reads (%) | Proper pairs (%) | Singleton reads (%) | Inward reads (%) | Duplicates (%) | Chimeras (%) | Estimated Library Size | Insert Size Quartiles |
|--------------------------|-----------|------------------|------------------|---------------------|------------------|----------------|----------------|------------------------|-----------------------|
| E.Coli | | | | | | | | | |
| Sanger_1 | 1738250 | 1585574(91.22) | 1452332(83.55) | 83148(4.78) | 17220(0.99) | 90133(5.68) | 844(0.05) | 6,721,919 | 2496,2884,3414 |
| Sanger_2 | 1844424 | 1149143(85.18) | 1051838(77.97) | 75641(5.61) | 10770(0.8) | 43832(3.81) | 762(0.06) | 8,446,293 | 3148,3407,3818 |
| Nextera 1µg_1 | 1586134 | 1462202(92.28) | 1224990(66.42) | 213900(11.60) | 6060(0.33) | 3333(2.28) | 41127(2.25) | 532,093,793 | 1418,2023,2925 |
| Nextera 1µg_2 | 1349072 | 1558622(84.70) | 1318192(71.63) | 205162(11.15) | 8822(0.48) | 34952(2.24) | 37013(2.03) | 637,651,262 | 1862,2521,3570 |
| Nextera 4µg_1 | 1348984 | 1305714(82.32) | 1124720(70.91) | 169252(10.67) | 3478(0.22) | 23894(1.83) | 34923(2.22) | 231,344,363 | 2570,3222,4323 |
| Nextera 4µg_2 | 1188802 | 1379340(84.36) | 1169260(71.51) | 186088(11.38) | 5704(0.35) | 29072(2.11) | 36445(2.25) | 408,363,590 | 4094,4740,5336 |
| SOLID5500_1 | 1439736 | 1008034(74.73) | 957798(71.00) | 45338(3.36) | 1242(0.09) | 20201(2) | 13334(0.99) | 13,748,108 | 3249,3582,4137 |
| SOLID5500_2 | 1374586 | 1058409(89.03) | 1015338(85.41) | 34991(2.94) | 2024(0.17) | 19212(1.82) | 8379(0.71) | 17,608,752 | 3198,3531,4063 |
| SOLID4_1 | 1679048 | 1277513(88.73) | 1225494(85.12) | 41563(2.89) | 2686(0.19) | 74499(5.83) | 10824(0.76) | 4,697,911 | 3128,3386,3808 |
| SOLID4_2 | 1093040 | 522669(38.02) | 481644(35.04) | 38687(2.81) | 1362(0.1) | 40488(7.75) | 16788(1.24) | 748,054 | 3167,3409,3808 |
| Illumina2_1 | 1840202 | 1534005(91.36) | 1416994(84.39) | 97663(5.82) | 14522(0.86) | 27408(1.79) | 260410(15.56) | 46,305,416 | 2527,3050,3889 |
| Illumina2_2 | 1635128 | 998143(91.32) | 921338(84.29) | 62561(5.72) | 9832(0.9) | 11938(1.2) | 167799(15.39) | 45,559,230 | 2506,3017,3893 |
| 454_1 | 1735912 | 1035672(69.66) | 621090(35.78) | 399508(23.01) | 6486(0.37) | 124106(11.98) | 1695(0.12) | 1,022,158 | 3062,3415,4000 |
| 454_2 | 1613924 | 1080721(66.96) | 686376(42.53) | 381225(23.62) | 4722(0.29) | 102108(9.45) | 1421(0.1) | 1,578,265 | 3072,3406,3985 |
| P. falciparum 3D7 | | | | | | | | | |
| Sanger_1 | 682164 | 588694(86.30) | 536014(78.58) | 31129(4.56) | 10290(1.51) | 12540(2.13) | 4242(0.67) | 7,092,996 | 2614,2907,3332 |
| Sanger_2 | 1135602 | 960130(84.55) | 878902(77.40) | 44706(3.94) | 16178(1.42) | 33446(3.48) | 6919(0.67) | 6,662,099 | 2593,2867,3293 |
| Nextera 1µg_1 | 1817908 | 1545406(85.01) | 1434318(78.90) | 89371(4.92) | 2386(0.13) | 5040(0.33) | 40831(2.3) | 596,244,158 | 2073,3086,4689 |
| Nextera 1µg_2 | 944780 | 811459(85.89) | 753156(79.72) | 47012(4.98) | 1310(0.14) | 1624(0.2) | 21267(2.3) | 474,453,473 | 2079,3010,4549 |
| Nextera 4µg_1 | 1046822 | 920122(87.90) | 866396(82.76) | 43751(4.18) | 1286(0.12) | 2025(0.22) | 25111(2.45) | 217,591,242 | 6777,8032,10,223 |
| Nextera 4µg_2 | 1576742 | 1389665(88.14) | 1310604(83.12) | 64545(4.09) | 1994(0.13) | 44140(3.2) | 38810(2.51) | 221,719,888 | 6695,7976,10,109 |
| SOLID5500_1 | 1088608 | 895871(83.84) | 865328(80.98) | 18714(1.75) | 3722(0.35) | 5413(6.04) | 11413(1.09) | 3,119,007 | 3216,3592,4188 |
| SOLID5500_2 | 1006516 | 791843(78.67) | 763222(75.83) | 18314(1.82) | 3202(0.32) | 41250(5.21) | 11066(1.17) | 3,181,087 | 3199,3589,4189 |
| SOLID4_1 | - | - | - | - | - | - | - | - | - |
| SOLID4_2 | - | - | - | - | - | - | - | - | - |
| Illumina2_1 | 12186512 | 11251194(92.32) | 10652296(87.41) | 304631(2.50) | 217956(1.79) | 34891(3.1) | 1920467(15.91) | 96,778,532 | 2874,3176,3647 |
| Illumina2_2 | 9395990 | 8637064(91.92) | 8175824(87.01) | 233605(2.49) | 167792(1.79) | 194408(2.25) | 1477532(15.89) | 106,831,975 | 2872,3191,3962 |
| 454_1 | 1326766 | 776177(58.50) | 657592(49.56) | 111472(8.40) | 1606(0.12) | 76244(9.82) | 3455(0.35) | 1,436,903 | 2501,2906,3489 |
| 454_2 | 1546478 | 1005179(65.00) | 857836(55.47) | 137129(8.87) | 2226(0.14) | 109748(10.92) | 4281(0.34) | 1,666,050 | 2522,2913,3456 |

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