Test case: to test how -d and -x/-s work, test files were made that contained simulated overlapping reads generated on separate sequencing clusters (to test -d) and read pairs that overlap (generated from the same cluster; to test -x/-s). All bases in the simulated R1 and R2 fastq files were given a quality score of ‘E’.

The reference sequence was indexed:

```
[claire@gduserv Depth_test]$ bwa index test_ref.fa
[bwa_index] Pack FASTA... 0.00 sec
[bwa_index] Construct BWT for the packed sequence...
[bwa_index] 0.00 seconds elapse.
[bwa_index] Update BWT... 0.00 sec
[bwa_index] Pack forward-only FASTA... 0.00 sec
[bwa_index] Construct SA from BWT and Occ... 0.00 sec
[main] Version: 0.7.15-r1140
[main] CMD: bwa index test_ref.fa
[main] Real time: 0.003 sec; CPU: 0.003 sec
```

The reads were then aligned:

```
bwa mem -R @RG\\tID:test_seqs\\tSM:test_seqs -t 4 test_ref.fa test_seqs.R1_2.fq test_seqs.R2_2.fq |
samtools view -f 3 -F 2048 -u -b - | samtools sort -@ 4 -m 2G -o test_seqs.bam
[M::bwa_idx_load_from_disk] read 0 ALT contigs
[M::process] read 36 sequences (2592 bp)...
[M::mem_pestat] # candidate unique pairs for (FF, FR, RF, RR): (0, 18, 0, 0)
[M::mem_pestat] skip orientation FF as there are not enough pairs
[M::mem_pestat] analyzing insert size distribution for orientation FR...
[M::mem_pestat] (25, 50, 75) percentile: (137, 141, 149)
[M::mem_pestat] low and high boundaries for computing mean and std.dev: (113, 173)
[M::mem_pestat] mean and std.dev: (140.44, 8.77)
[M::mem_pestat] low and high boundaries for proper pairs: (101, 185)
[M::mem_pestat] skip orientation RF as there are not enough pairs
[M::mem_pestat] skip orientation RR as there are not enough pairs
[M::mem_process_seqs] Processed 36 reads in 0.003 CPU sec, 0.001 real sec
[main] Version: 0.7.15-r1140
[main] CMD: bwa mem -R @RG\tID:test_seqs\tSM:test_seqs -t 4 test_ref.fa test_seqs.R1_2.fq
test_seqs.R2_2.fq
[main] Real time: 0.002 sec; CPU: 0.004 sec
```

Checking the .bam file to make sure that the alignment was produced correctly:

```
samtools view test_seqs.bam -h
```

```
@HD        VN:1.5         SO:coordinate
@SQ        SN:test_ref    LN:154
@RG        ID:test_seqs   SM:test_seqs
@PG        ID:bwa         PN:bwa         VN:0.7.15-r1140   CL:bwa mem -R @RG\tID:test_seqs\tSM:test_seqs -t 4 test_ref.fa test_seqs.R1_2.fq test_seqs.R2_2.fq
@PG        ID:samtools    PN:samtools    PP:bwa            VN:1.12-26-ge77c7c6          CL:samtools view -h test_seqs.bam
```

Query name	FLAG	Reference name	1-based left-most position of alignment	Mapping quality	CIGAR string	Name of next read	1-based left-most position of read read	Total template length	Sequence	ASCII encoding of (base quality + 33)					
test1	99	test_ref	1	60	70M	=	83	154	(removed)	EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:1	MD:Z:51C18	AS:i:65	XS:i:0	RG:Z:test_seqs
test2	99	test_ref	1	60	72M	=	85	154		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:2	MD:Z:38A12C20	AS:i:62	XS:i:0	RG:Z:test_seqs
test3	99	test_ref	3	60	70M	=	81	150		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:2	MD:Z:36A12C20	AS:i:60	XS:i:0	RG:Z:test_seqs
test4	99	test_ref	3	60	72M	=	83	150		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:2	MD:Z:36A12C22	AS:i:62	XS:i:0	RG:Z:test_seqs
test5	99	test_ref	5	60	70M	=	79	146		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:1	MD:Z:47C22	AS:i:65	XS:i:0	RG:Z:test_seqs
test6	99	test_ref	5	60	72M	=	81	146		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:1	MD:Z:47C24	AS:i:67	XS:i:0	RG:Z:test_seqs
test14	99	test_ref	5	60	72M	=	83	150		EEEEEEEEE	NM:i:2	MD:Z:34A12	AS:i:62	XS:i:0	RG:Z:test_se

										EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE		C24			qs
test7	99	test_ref	7	60	70M	=	77	142		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:1	MD:Z:45C24	AS:i:65	XS:i:0	RG:Z:test_seqs
test8	99	test_ref	7	60	72M	=	79	142		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:2	MD:Z:45C25T0	AS:i:66	XS:i:0	RG:Z:test_seqs
test13	99	test_ref	7	60	72M	=	83	146		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:32A12C25T0	AS:i:61	XS:i:0	RG:Z:test_seqs
test9	99	test_ref	9	60	70M	=	75	138		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:30A12C25T0	AS:i:59	XS:i:0	RG:Z:test_seqs
test10	99	test_ref	9	60	72M	=	77	138		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:30A12C25T2	AS:i:59	XS:i:0	RG:Z:test_seqs
test11	99	test_ref	9	60	70M	=	79	138		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:30A12C25T0	AS:i:59	XS:i:0	RG:Z:test_seqs
test12	99	test_ref	9	60	72M	=	83	138		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:30A12C25T2	AS:i:59	XS:i:0	RG:Z:test_seqs
test15	99	test_ref	12	60	70M	=	71	135		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:27A12C25T3	AS:i:56	XS:i:0	RG:Z:test_seqs
test16	99	test_ref	12	60	76M	=	71	129		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:27A12C25T9	AS:i:61	XS:i:0	RG:Z:test_seqs
test17	99	test_ref	15	60	89M	=	64	126		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:24A12C25T25	AS:i:74	XS:i:0	RG:Z:test_seqs
test18	99	test_ref	17	60	87M	=	74	124		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:22A12C25T25	AS:i:72	XS:i:0	RG:Z:test_seqs
test17	147	test_ref	64	60	77M	=	15	-126		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:14T29A30A1	AS:i:65	XS:i:0	RG:Z:test_seqs
test15	147	test_ref	71	60	76M	=	12	-135		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:7T29A30A7	AS:i:61	XS:i:0	RG:Z:test_seqs
test16	147	test_ref	71	60	70M	=	12	-129		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:7T29A30A1	AS:i:58	XS:i:0	RG:Z:test_seqs
test18	147	test_ref	74	60	67M	=	17	-124		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:4T29A30A1	AS:i:55	XS:i:0	RG:Z:test_seqs
test9	147	test_ref	75	60	72M	=	9	-138		EEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:3T29A30A7	AS:i:58	XS:i:0	RG:Z:test_seqs

										EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE					
test7	147	test_ref	77	60	72M	=	7	-142		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:2	MD:Z:1T60A9	AS:i:65	XS:i:0	RG:Z:test_seqs
test10	147	test_ref	77	60	70M	=	9	-138		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:2	MD:Z:1T29A38	AS:i:63	XS:i:0	RG:Z:test_seqs
test5	147	test_ref	79	60	72M	=	5	-146		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:1	MD:Z:71G0	AS:i:71	XS:i:0	RG:Z:test_seqs
test8	147	test_ref	79	60	70M	=	7	-142		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:1	MD:Z:29A40	AS:i:65	XS:i:0	RG:Z:test_seqs
test11	147	test_ref	79	60	68M	=	9	-138		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:1	MD:Z:29A38	AS:i:63	XS:i:0	RG:Z:test_seqs
test3	147	test_ref	81	60	72M	=	3	-150		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:27A30A10G2	AS:i:59	XS:i:0	RG:Z:test_seqs
test6	147	test_ref	81	60	70M	=	5	-146		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:2	MD:Z:58A10G0	AS:i:64	XS:i:0	RG:Z:test_seqs
test1	147	test_ref	83	60	72M	=	1	-154		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:2	MD:Z:56A10G4	AS:i:62	XS:i:0	RG:Z:test_seqs
test4	147	test_ref	83	60	70M	=	3	-150		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:1	MD:Z:67G2	AS:i:67	XS:i:0	RG:Z:test_seqs
test12	147	test_ref	83	60	64M	=	9	-138		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:2	MD:Z:25A30A7	AS:i:54	XS:i:0	RG:Z:test_seqs
test13	147	test_ref	83	60	70M	=	7	-146		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:25A30A10G2	AS:i:57	XS:i:0	RG:Z:test_seqs
test14	147	test_ref	83	60	72M	=	5	-150		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:3	MD:Z:25A30A10G4	AS:i:57	XS:i:0	RG:Z:test_seqs
test2	147	test_ref	85	60	70M	=	1	-154		EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE	NM:i:1	MD:Z:65G4	AS:i:65	XS:i:0	RG:Z:test_seqs

- All forward sequences have a flag of 99 and all reverse sequences have a flag of 147, indicating proper mapping of read pairs
- Mapping quality is 60 for all reads, so this is well above any filters used in bcftools mpileup
- The CIGAR strings indicate that all bases align (no insertions or deletions)
- The start positions are all correct
- The total template lengths are all correct

Based on the .bam file above, the samtools/bcftools utilities will read the sequences in the following order:

```
          000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000000111111111111111111111111111111111111111111111111111111
          000000000111111111122222222223333333333444444444455555555556666666666777777777788888888889999999999000000000011111111112222222222333333333344444444445555
          1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234
          NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Test1-1   NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test2-1   NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test3-1     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test4-1     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test5-1       NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test6-1       NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test14-1      NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test7-1         NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test8-1         NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test13-1        NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test9-1           NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test10-1          NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test11-1          NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test12-1          NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test15-1             NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test16-1             NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test17-1                NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test18-1                  NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test17-2                                                                 NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test15-2                                                                        NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test16-2                                                                        NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test18-2                                                                           NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test9-2                                                                             NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test7-2                                                                               NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test10-2                                                                              NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test5-2                                                                                 NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test8-2                                                                                 NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test11-2                                                                                NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test3-2                                                                                   NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test6-2                                                                                   NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test1-2                                                                                     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test4-2                                                                                     NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test12-2                                                                                    NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test13-2                                                                                    NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test14-2                                                                                    NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Test2-2                                                                                       NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
```

Note: actual bases have been replaced by n’s .

Sequence depth was then determined using different settings and different versions of samtools depth and bcftools mpileup:

- samtools depth Version: 1.8 (using htslib 1.8) - cluster
- bcftools mpileup Version: 1.8 (using htslib 1.8) - cluster
- samtools depth Version: 1.12-26-ge77c7c6 (using htslib 1.12-25-g5b684ce) – local
- bcftools mpileup Version: 1.12-21-ga865a16 (using htslib 1.12-25-g5b684ce) – local

Commands were used as follows:

```
samtools depth test_seqs.bam -d [n] | head -n [n] | tail -n 1 (V1.8)

bcftools mpileup test_seqs.bam -f test_ref.fa -Q 20 -q 20 -a format/ad,format/dp,info/ad -d [n] [-x]
| head -n [n] | tail -n 1 (V1.8)

samtools depth test_seqs.bam -d [n] [-s] | head -n 10 | tail -n 1 (V1.12)

bcftools mpileup test_seqs.bam -f test_ref.fa -Q 20 -q 20 -a format/ad,format/dp,info/ad -d [n] [-x]
| head -n [n] | tail -n 1 (V1.12)
```

Note that samtools depth (V1.8) does not have the -s option (which, if used, does not count overlapping reads within a template).

Five sites were selected for detailed study:

Position	d	Overlap detection	depth -s	mpileup -x	Expected	samtools depth Version: 1.8 (using htslib 1.8)	bcftools mpileup Version: 1.8 (using htslib 1.8)	samtools depth Version: 1.12-26-ge77c7c6 (using htslib 1.12-25-g5b684ce)	bcftools mpileup Version: 1.12-21-ga865a16 (using htslib 1.12-25-g5b684ce)
10	1	no	no	yes	5	5	5	14	5
10	1	yes	yes	no	5	n/a	5	14	5
10	2	no	no	yes	10	6	6	14	6
10	2	yes	yes	no	10	n/a	6	14	6
10	3	no	no	yes	13	6	6	14	6
10	3	yes	yes	no	13	n/a	6	14	6
10	10	no	no	yes	14	11	11	14	11
10	10	yes	yes	no	14	n/a	11	14	11
10	20	no	no	yes	14	14	14	14	14
10	20	yes	yes	no	14	n/a	14	14	14
10	200	no	no	yes	14	14	14	14	14

10	200	yes	yes	no	14	n/a	14	14	14

Position	d	Overlap detection	depth -s	mpileup -x	Expected	samtools depth Version: 1.8 (using htslib 1.8)	bcftools mpileup Version: 1.8 (using htslib 1.8)	samtools depth Version: 1.12-26-ge77c7c6 (using htslib 1.12-25-g5b684ce)	bcftools mpileup Version: 1.12-21-ga865a16 (using htslib 1.12-25-g5b684ce)
74	1	no	no	yes	9	9	9	19	9
74	1	yes	yes	no	6	n/a	9	15	9
74	2	no	no	yes	15	9	9	19	9
74	2	yes	yes	no	11	n/a	9	15	9
74	3	no	no	yes	18	9	9	19	9
74	3	yes	yes	no	14	n/a	9	15	9
74	10	no	no	yes	19	14	14	19	14
74	10	yes	yes	no	15	n/a	14	15	14
74	20	no	no	yes	19	18	18	19	18
74	20	yes	yes	no	15	n/a	18	15	18
74	200	no	no	yes	19	19	19	19	19
74	200	yes	yes	no	15	n/a	19	15	19

Position	d	Overlap detection	depth -s	mpileup -x	Expected	samtools depth Version: 1.8 (using htslib 1.8)	bcftools mpileup Version: 1.8 (using htslib 1.8)	samtools depth Version: 1.12-26-ge77c7c6 (using htslib 1.12-25-g5b684ce)	bcftools mpileup Version: 1.12-21-ga865a16 (using htslib 1.12-25-g5b684ce)
78	1	no	no	yes	9	9	9	17	9
78	1	yes	yes	no	5	n/a	9	11	9
78	2	no	no	yes	14	9	9	17	9
78	2	yes	yes	no	8	n/a	9	11	9
78	3	no	no	yes	16	9	9	17	9
78	3	yes	yes	no	10	n/a	9	11	9
78	10	no	no	yes	17	11	11	17	11
78	10	yes	yes	no	11	n/a	11	11	11
78	20	no	no	yes	17	16	16	17	16
78	20	yes	yes	no	11	n/a	16	11	16
78	200	no	no	yes	17	17	17	17	17
78	200	yes	yes	no	11	n/a	17	11	17

Position	d	Overlap detection	depth -s	mpileup -x	Expected	samtools depth Version: 1.8 (using htslib 1.8)	bcftools mpileup Version: 1.8 (using htslib 1.8)	samtools depth Version: 1.12-26-ge77c7c6 (using htslib 1.12-25-g5b684ce)	bcftools mpileup Version: 1.12-21-ga865a16 (using htslib 1.12-25-g5b684ce)
100	1	no	no	yes	11	11	11	20	11
100	1	yes	yes	no	9	n/a	11	18	11
100	2	no	no	yes	16	11	11	20	11
100	2	yes	yes	no	14	n/a	11	18	11
100	3	no	no	yes	18	11	11	20	11
100	3	yes	yes	no	16	n/a	11	18	11
100	10	no	no	yes	20	11	11	20	11
100	10	yes	yes	no	18	n/a	11	18	11
100	20	no	no	yes	20	19	19	20	19
100	20	yes	yes	no	18	n/a	19	18	19

100	200	no	no	yes	20	20	20	20	20
100	200	yes	yes	no	18	n/a	20	18	20

Position	d	Overlap detection	depth -s	mpileup -x	Expected	samtools depth Version: 1.8 (using htslib 1.8)	bcftools mpileup Version: 1.8 (using htslib 1.8)	samtools depth Version: 1.12-26-ge77c7c6 (using htslib 1.12-25-g5b684ce)	bcftools mpileup Version: 1.12-21-ga865a16 (using htslib 1.12-25-g5b684ce)
142	1	no	no	yes	7	7	7	15	7
142	1	yes	yes	no	7	n/a	7	15	7
142	2	no	no	yes	11	7	7	15	7
142	2	yes	yes	no	11	n/a	7	15	7
142	3	no	no	yes	13	7	7	15	7
142	3	yes	yes	no	13	n/a	7	15	7
142	10	no	no	yes	15	7	7	15	7
142	10	yes	yes	no	15	n/a	7	15	7
142	20	no	no	yes	15	15	15	15	15
142	20	yes	yes	no	15	n/a	15	15	15
142	200	no	no	yes	15	15	15	15	15
142	200	yes	yes	no	15	n/a	15	15	15

Observations:

- neither bcftools mpileup version 1.8 nor 1.12 have detected any overlapping sequences
- samtools depth version 1.18 and bcftools mpileup versions 1.8 and 1.12 all reported the same (often incorrect) depth
- when using samtools depth version 1.18 or bcftools mpileup versions 1.8 and 1.12, changing -d from 10 to 20 or from 20 to 200 can change the reported sequence depth at a given position, despite the fact that there are a maximum of five reads starting at any one site
- samtools depth version 1.12 is the only utility that is correctly identifying overlapping paired reads
- samtools depth version 1.12 does not generate an error message when -d [int] is passed, yet this option does not appear to do anything as this version of samtools depth reported the same depth irrespective of the value passed to -d

Given this final observation, does the newest version of samtools depth still have -d as an option?

The samtools depth manual page on the website (http://www.htslib.org/doc/samtools-depth.html) shows that -d is still an option:

```
“NAME
samtools depth – computes the read depth at each position or region
SYNOPSIS
samtools depth [options] [in1.sam|in1.bam|in1.cram [in2.sam|in2.bam|in2.cram] [...]]

DESCRIPTION
Computes the depth at each position or region.

OPTIONS
-a
Output all positions (including those with zero depth)

-a -a, -aa
Output absolutely all positions, including unused reference sequences. Note that when used in
conjunction with a BED file the -a option may sometimes operate as if -aa was specified if the
reference sequence has coverage outside of the region specified in the BED file.

-b FILE
Compute depth at list of positions or regions in specified BED FILE. []

-f FILE
Use the BAM files specified in the FILE (a file of filenames, one file per line) []

-H
```

Write a comment line showing column names at the beginning of the output. The names are CHROM, POS, and then the input file name for each depth column. If one of the inputs came from stdin, the name “-” will be used for the corresponding column.

-l INT
Ignore reads shorter than INT

-m, -d INT
At a position, read at most INT reads per input file. This means figures greater than INT may be reported in the output.

Setting this limit reduces the amount of memory and time needed to process regions with very high coverage. Passing zero for this option sets it to the highest possible value, effectively removing the depth limit. [8000]

Note that up to release 1.8, samtools would enforce a minimum value for this option. This no longer happens and the limit is set exactly as specified.

-o FILE
Write output to FILE. Using “-” for FILE will send the output to stdout (also the default if this option is not used).

-q INT
Only count reads with base quality greater than or equal to INT

-Q INT
Only count reads with mapping quality greater than or equal to INT

-r CHR:FROM-TO
Only report depth in specified region.

-X
If this option is set, it will allows user to specify customized index file location(s) if the data folder does not contain any index file. Example usage: samtools depth [options] -X /data_folder/in1.bam [/data_folder/in2.bam [...]] /index_folder/index1.bai [/index_folder/index2.bai [...]]

-g FLAGS
By default, reads that have any of the flags UNMAP, SECONDARY, QCFAIL, or DUP set are skipped. To include these reads back in the analysis, use this option together with the desired flag or flag combination. FLAGS can be specified in hex by beginning with `0x' (i.e. /^0x[0-9A-F]+/), in octal by beginning with `0' (i.e. /^0[0-7]+/), as a decimal number not beginning with '0' or as a comma-separated list of flag names. [0]

For a list of flag names see samtools-flags(1).

-G FLAGS
Discard any read that has any of the flags specified by FLAGS set. FLAGS are specified as for the -g option. [UNMAP,SECONDARY,QCFAIL,DUP]

-J
Include reads with deletions in depth computation.

-s
For the overlapping section of a read pair, count only the bases of a single read. This is accomplished by lowering to 0 the quality values of one read for the span of the overlapping section. As a consequence, the algorithm will consider only bases with a quality above 0.”

However, the manual page shown on the command line for my installation does not show -d as an option:

```
samtools depth
Usage: samtools depth [options] in.bam [in.bam ...]

Options:
  -a            Output all positions (including zero depth)
  -a -a, -aa    Output absolutely all positions, including unused ref seqs
  -r REG        Specify a region in chr or chr:from-to syntax
  -b FILE       Use bed FILE for list of regions
  -f FILE       Specify list of input BAM/SAM/CRAM filenames
  -X            Use custom index files (in -X *.bam *.bam.bai order)
  -g INT        Remove specified flags from default flag filter
  -G INT        Add specified flags to the default flag filter
  -H            Print a file header line
  -l INT        Minimum read length [0]
  -o FILE       Write output to FILE [stdout]
  -q INT        Minimum base quality [0]
  -Q INT        Minimum mapping quality [0]
  -H            Print a file header
  -J            Include reads with deletions in depth computation
  -s            Do not count overlapping reads within a template
      --input-fmt-option OPT[=VAL]
                Specify a single input file format option in the form
                of OPTION or OPTION=VALUE
  -@, --threads INT
                Number of additional threads to use [0]
      --verbosity INT
                Set level of verbosity
```