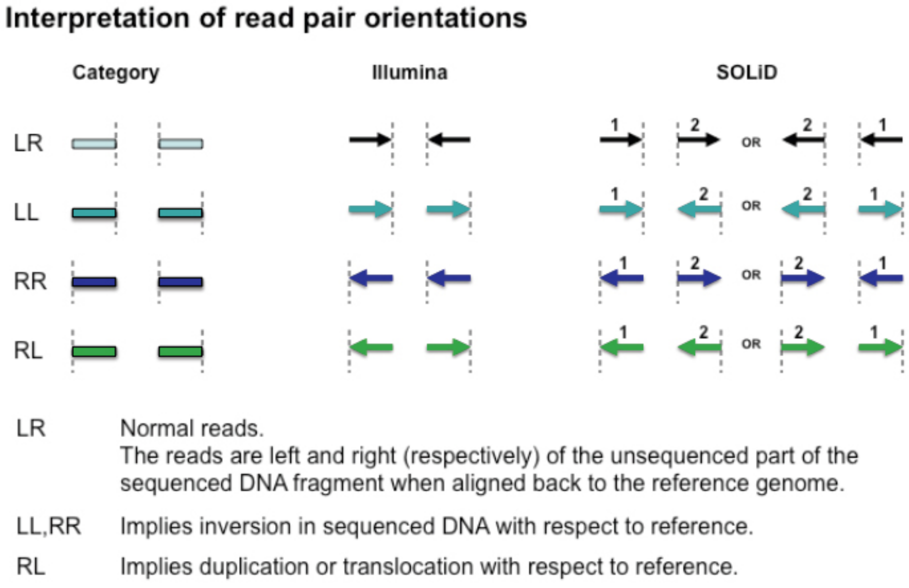
SAM FLAG field
Strictly speaking, 0x10 (16 in decimal) in the FLAG field means “SEQ being reverse complemented” [Based on SAM spec v 1.4]. However, Picard’s “SAM FLAG explainer” describes 0x10 as meaning, “read reverse strand”. Similarly, 0x20 strictly means “SEQ of next segment in the template being reversed”, but Picard describes this as “mate reverse strand”.

Illumina paired-end reads
Read1 and read2 are from opposite strands of the same DNA molecule (http://seqanswers.com/forums/showthread.php?t=18158).

Read orientation in Novoalign DNA-seq SAM files
I believe this also holds for Bowtie and BWA SAM files.



Interpretation of read pair orientations

LR Normal reads.
The reads are left and right (respectively) of the unsequenced part of the sequenced DNA fragment when aligned back to the reference genome.

LL,RR Implies inversion in sequenced DNA with respect to reference.

RL Implies duplication or translocation with respect to reference.

(From http://www.broadinstitute.org/software/igv/interpreting_pair_orientations)

IGV

```
<table><tr><td valign="top"><b>Left
alignment</b><br/>Sample = ASD2-41
Read group = 1
----------------------
Read name = HWI-ST0798_0086:3:2105:9728:41512#TAGCTT
Alignment start = 19558864 (+)
Cigar = 109M1H
Mapped = yes
Mapping quality = 12
----------------------
----------------------
Pair start = chr14:19558989 (-)
Pair is mapped = yes
Insert size = 234
Pair orientation = F2R1
----------------------
Second in pair
-------------------
LB = unknown
MD = 109
PG = novoalign
RG = 1
AM = 150
NM = 0
SM = 150
PQ = 1
UQ = 0
AS = 0
PU = HWI-ST0798_0062
-------------------</td><td valign="top"><b>Right
alignment</b><br/>Sample = ASD2-41
Read group = 1
----------------------
Read name = HWI-ST0798_0086:3:2105:9728:41512#TAGCTT
Alignment start = 19558989 (-)
Cigar = 110M
Mapped = yes
Mapping quality = 12
----------------------
Base = G
Base phred quality = 38
----------------------
Pair start = chr14:19558864 (+)
Pair is mapped = yes
Insert size = -234
Pair orientation = F2R1
----------------------
First in pair
```

```
-------------------
LB = unknown
MD = 110
PG = novoalign
RG = 1
AM = 150
NM = 0
SM = 150
PQ = 1
UQ = 1
AS = 1
PU = HWI-ST0798_0062
-------------------</td></tr></table>
Alignment start position = chr14:19558864
Null
```

BAM

```
HWI-ST0798_0086:3:2105:9728:41512#TAGCTT        163
chr14   19558864        12      109M1H  =       19558989
234
GGGTTTGATTTCAATACATAGCATAAAAATGAGTTTTCTCCTTTAAATATAACTAGTTG
GTGAAAGCTGTGGAATGTTATTTTGAAATCCTAGGATTTGTAATTTGTTT
AAAAABDBCCCFCCDCGCDCFGCDCDDDDDGDGDDDDGDGGDDDDDDEDEDDGEDFEDF
FDFDDDGGEG@=FADEGEECEEEEG?ABDFFEB>AADDDBBCCDCCBCB>
LB:Z:unknown    MD:Z:109        PG:Z:novoalign  RG:Z:1
AM:i:150        NM:i:0  SM:i:150        PQ:i:1  UQ:i:0
AS:i:0  PU:Z:HWI-ST0798_0062

HWI-ST0798_0086:3:2105:9728:41512#TAGCTT        83
chr14   19558989        12      110M    =       19558864
-234
AGGCCGTACAATGCCGGGAAGATGAATGTGCGTTAATGTTGCTGGAACATGGCACTGAT
CCGAATATTCCAGATGAGTATGGAAATACCGCTCTACACTATGCTATCTAC
@A@>@ECCCCCAEADEEFCCFCCEBBDFDGFGEEDDEGEEGGEGGDDGDEGGGDGEGDE
GGGDDEDEEGGDGDEGDGECEGGCCCB8FFGGDFDCGCFDCDEFCBBCA@@
LB:Z:unknown    MD:Z:110        PG:Z:novoalign  RG:Z:1
AM:i:150        NM:i:0  SM:i:150        PQ:i:1  UQ:i:1
AS:i:1  PU:Z:HWI-ST0798_0062
```

FASTQ

```
@HWI-ST0798_0086:3:2105:9728:41512#TAGCTT/1
GTAGATAGCATAGTGTAGAGCGGTATTTCCATACTCATCTGGAATATTCGGATCAGTGC
CATGTTCCAGCAACATTAACGCACATTCATCTTCCCGGCATTGTACGGCCT
+HWI-ST0798_0086:3:2105:9728:41512#TAGCTT/1
aaaeeeeeggggfhhgfhhiifiRbfhiiiiiiiiiiiiiiihiiiiiiiiiiiiihii
hihifffghhhiigggdgeeeecccbbb`ccccdcccac_cccdcda__aa
```

Peter Hickey 16/3/12 2:08 PM
Comment [1]: Read 2

Peter Hickey 16/3/12 2:08 PM
Comment [2]: Read 1

Peter Hickey 16/3/12 2:10 PM
Comment [3]: Reverse complent of FASTQ sequence of Read 1

```
@HWI-ST0798_0086:3:2105:9728:41512#TAGCTT/2
GGGTTTGATTTCAATACATAGCATAAAAATGAGTTTTCTCCTTTAAATATAACTAGTTG
GTGAAAGCTGTGGAATGTTATTTTGAAATCCTAGGATTTGTAATTTGTTTT
+HWI-ST0798_0086:3:2105:9728:41512#TAGCTT/2
bbbeeeeeggggiiiiiiihiiiiihhhiihiehffhiiiihihiiiiiihhhhhghi
ieghhhhhh_]e`gfhiidhgfgf\`adddeb_b_ddcb`cdecccdc^B
```

Read orientation in Bismark BS-seq SAM files

IGV

```
<b>Left alignment</b><br/>----------------------
Read name = SRR097428.6814839_HWI-
BRUNOP20X:0637:1:5:9089:67292_length=75/1
Alignment start = 19011703 (+)
Cigar = 75M
Mapped = yes
Mapping quality = 255
----------------------
Base = A
Base phred quality = 39
----------------------
Pair start = chr14:19011768 (+)
Pair is mapped = yes
Insert size = 140
Pair orientation = F1F2
----------------------
First in pair
-------------------
XG = CT
NM = 14
XM =
.x..h.h................h..h.x.............h...h....h.......
...x.......
.hh.x

XR = CT
XX = 1C2C1C16C2C1C13C3C3AC10C8CC1C
-------------------
Alignment start position = chr14:19011703
null

<b>Left alignment</b><br/>----------------------
Read name = SRR097428.6814839_HWI-
BRUNOP20X:0637:1:5:9089:67292_length=75/2
Alignment start = 19011768 (+)
Cigar = 75M
Mapped = yes
Mapping quality = 255
```

```
----------------------
Base = A
Base phred quality = 31
----------------------
Pair start = chr14:19011703 (+)
Pair is mapped = yes
Insert size = -140
Pair orientation = F1F2
----------------------
Second in pair
-------------------
XG = CT
NM = 16
XM =
......hh.x...h......................x.....hh..Z......h.h...
.h...hh...h
..hx.

XR = GA
XX = 6CC1C3C22C2T2CC9C1C4C3CC3C2CC1
-------------------
Alignment start position = chr14:19011768
Null
```

BAM

```
SRR097428.6814839_HWI-
BRUNOP20X:0637:1:5:9089:67292_length=75/1 67       chr14
19011703        255     75M     =       19011768        140

ATTGTTTTATGAAAAGGAATGTTTAATTTTGTGAGTTGAATGTAAGTATGGTAAAAAAG
TTTTTGAGAATGTTTT
HHHHHHHGGHHHHHHHHIHHHHHHHHHHHHHHHGHEHHFHHHIHHHDHHHHEEEHF9FF
<D@CDFGHGGDHAEHF     NM:i:14
XX:Z:1C2C1C16C2C1C13C3C3AC10C8CC1C
XM:Z:.x..h.h................h..h.x.............h...h....h..
........x........hh.x        XR:Z:CT XG:Z:CT

SRR097428.6814839_HWI-
BRUNOP20X:0637:1:5:9089:67292_length=75/2 131     chr14
19011768        255     75M     =       19011703        -
140
AGAATGTTTTTGTTTAGTTTTTATTTGAATATAATATTGNTTTTAACGAAAGGTTTAAA
GTTTTTTAAATATTTA
DGF@908/;(EGFDGDGGB@FDFFEHHFHGDADADDADD!A@A@?HBHHHHHHEHHHHH
GHHHHHHHHHEHHHHH     NM:i:16
XX:Z:6CC1C3C22C2T2CC9C1C4C3CC3C2CC1
XM:Z:......hh.x...h......................x.....hh..Z......h
.h....h...hh...h..hx.        XR:Z:GA XG:Z:CT
```

Peter Hickey 16/3/12 4:49 PM
Comment [4]: Neither read has the 0x10 flag set, i.e. it's an OT read in Bismark's terminology.

Peter Hickey 16/3/12 4:49 PM
Comment [5]: Neither read has the 0x10 flag set, i.e. it's an OT read in Bismark's terminology. HOWEVER, this conflicts with the SAM spec since the SEQ has been reverse complemented

Peter Hickey 16/3/12 12:42 PM
Comment [6]: Reverse complement of FASTQ sequence

FASTQ

```
@SRR097428.6814839 HWI-BRUNOP20X:0637:1:5:9089:67292
length=75
ATTGTTTTATGAAAAGGAATGTTTAATTTTGTGAGTTGAATGTAAGTATGGTAAAAAAG
TTTTTGAGAATGTTTT
+SRR097428.6814839 HWI-BRUNOP20X:0637:1:5:9089:67292
length=75
HHHHHHGGHHHHHHHHIHHHHHHHHHHHHHHHHGHEHHFHHHIHHHDHHHHEEEHF9FF
<D@CDFGHGGDHAEHF

@SRR097428.6814839 HWI-BRUNOP20X:0637:1:5:9089:67292
length=75
TAAATATTTAAAAAACTTTAAACCTTTCGTTAAAANCAATATTATATTCAAATAAAAAC
TAAACAAAAACATTCT
+SRR097428.6814839 HWI-BRUNOP20X:0637:1:5:9089:67292
length=75
HHHHEHHHHHHHHHHGHHHHHEHHHHHBH?@A@A!DDADDADADGHFHHEFFFDF@BGG
DGDFGE(;/809@FGD
```

Bismark translation

If we know that the CTOB and CTOT are merely theoretical, then we can use the "correct" strand information in the FLAG for each read and still uniquely determine whether a paired-read is informative for the OT or OB by checking the FLAG of the first read in the pair. If the strand of read1 is "+" then the paired-read is informative for the OT; if the strand of read1 is "-" then the paired-read is informative for the OB.

My proposal to resolve conflicts around the use of the FLAG field between the official SAM spec and Bismark's usage is as follows:

- Encode the "correct" strand information in the FLAG, i.e. +/- (0x20, 0x10) or -/+ (0x10, 0x20).
- To resolve ambiguities as to whether a read pair with +/- is informative for the OT or CTOB and whether a read pair with -/+ is informative for the OB or CTOT, encode this as a TAG XS:Z:OT, XS:Z:CTOB, XS:Z:CTOT, XS:Z:OB.

In this way the Bismark SAM files will conform to the SAM specifications (v1.4) while incorporating information about which of the four possible bisulfite strands of DNA the read originated from.

Table 1: Translation of Bismark strand flags for paired-end reads (read1/read2) for each of the four possible bisulfite strands of DNA. OT=original top; CTOB=complementary to original bottom; CTOT=complementary to original top; OB=original bottom. Only reads from the OT and OB strands are theoretically possible when using Illumina's non-directional BS-seq library. "Correct" strand refers to

the orientation of normal paired-end Illumina reads – IGV expects a paired to have this orientation in order to treat the read as a "correct" pair. Table partially based on information posted by Felix Krueger on seqanswers.com (http://seqanswers.com/forums/showthread.php?t=18422)

Read informative for	**Read conversion**	**Genome conversion**	**"Correct" strand**	**Bismark strand**	**Correct FLAG values**	**XS tag**
OT	CT	CT	+/-	+/+	0x20/0x10	XS:Z:OT
CTOB	GA	GA	+/-	-/-	0x20/0x10	XS:Z:CTOB
CTOT	GA	CT	-/+	+/+	0x10/0x20	XS:Z:CTOT
OB	CT	GA	-/+	-/-	0x10/0x20	XS:Z:OB

This information can be inferred from the XR and XG tags (encoding "read conversion" and "genome conversion" information) of Bismark's current SAM files.

I will write a pysam script to correct the strand information and add the XS tag to my files.