A COMPLEX REGULATORY LANDSCAPE INVOLVED IN THE DEVELOPMENT OF EXTERNAL GENITALS

Short title: *Hox* gene regulation during the development of genitals

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Supplementary material

Legends to figures supplements 1 to 5
Figures supplements 1 to 5
Tables supplements 1 to 5

LEGENDS TO FIGURES SUPPLEMENTS

Figure supplement 1: *Hox* genes expression profile during GT development. Bar plots show the quantification of *Hoxa, Hoxb* and *Hoxc* genes transcripts by RNA-seq (FPKM values) in GT cells at E12.5, E16.5 and E18.5. The gene cluster is indicated on top of each bar plot.

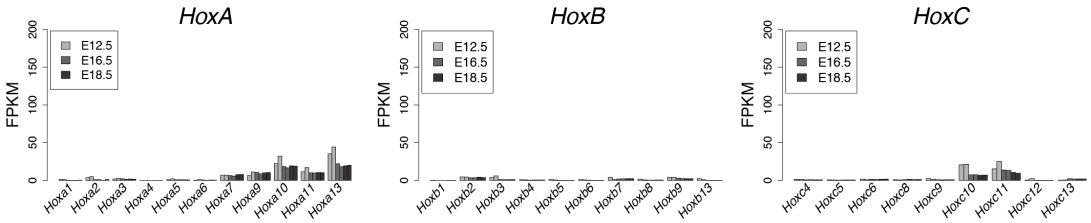
Figure supplement 2: Quantification of interactions in C-DOM during GT development. A) Bar plots show the quantification of the ratio of the number of normalized reads (+/- 5Mb around the viewpoint) in selected regulatory regions, using mouse ES cells as a reference. The regulatory element analyzed is indicated on top of each plot. **B)** 4C-seq profiles at the *HoxD* cluster and C-DOM, using GT cells at E12.5, E13.5, E15.5, E17.5 and forebrain cells. Coordinates (mm10): chr2:73815520-74792376. The GT2 (upper panel, blue line) and island V (lower panel, red line) were used as viewpoints.

Figure supplement 3: Interaction landscape in the *Del(GT2)* allele. A) ATAC-seq profiles of *HoxD* and C-DOM in wildtype and mutant *Del(GT2)* E13.5 GTs. Coordinates (mm10): chr2:73815520-74792376. The wildtype track is the average of three biological replicates and the *Del(GT2)* track the average of two biological replicates. Peaks called using MACS2 are displayed below, for each individual replicate (vertical black lines below). The red arrows delineate the deleted region and black arrows indicate a peak lost in *Del(GT2)*. **B)** Overlay of 4C-seq profiles of E13.5 GT cells using *Hoxd13* as viewpoint, wildtype in blue and *Del(GT2)* in red (average of two biological replicates). Coordinates (mm10): chr2:73815520-74792376. Viewpoint is highlighted by a gray line. The red arrow indicates the deleted region. **C)** ATAC-seq profile of E10.5 CR (average of two biological replicates). The black arrow points to the GT2 enhancer. Coordinates (mm10): chr2: 73815520-74792376. **D)** RT-qPCR of wildtype and mutant *Del(GT2)* E10.5 CR. *Hoxd13* mRNA levels were analyzed and the values plotted indicate the ratio of expression using wildtype as a reference (blue dots) (n=6 biologically independent WT or mutant GTs). A Welch's *t*-test was used to evaluate the statistical significance of changes in gene expression. Bars indicate mean with SD, * p=0.0125. We observed a 27% decrease in the mRNA levels of *Hoxd13.*

Figure supplement 4: Chromatin accessibility in the mutant *Del(V)* and *Inv(V)* alleles. A) ATAC-seq profiles of wildtype and mutant *Del(V)* and *Inv(V)* E13.5 GTs. Coordinates (mm10): chr2:73815520-74792376. The wildtype track is the average of three biological replicates and the *Del(V)* and *Inv(V)* tracks are the average of two biological replicates. Peaks called using MACS2 are displayed below for each individual replicate (vertical black lines below). The red arrows highlight the deleted or inverted region and the black arrow points to the loss of a peak in the *Inv(V)* allele. **B)** Graphical representation of the percentage of interactions centromeric (red) or telomeric (blue) to island V, for each biological replicate. Coordinates (mm10): centromeric: chr2:74015789-74276083; telomeric: chr2:74332870-74671433.

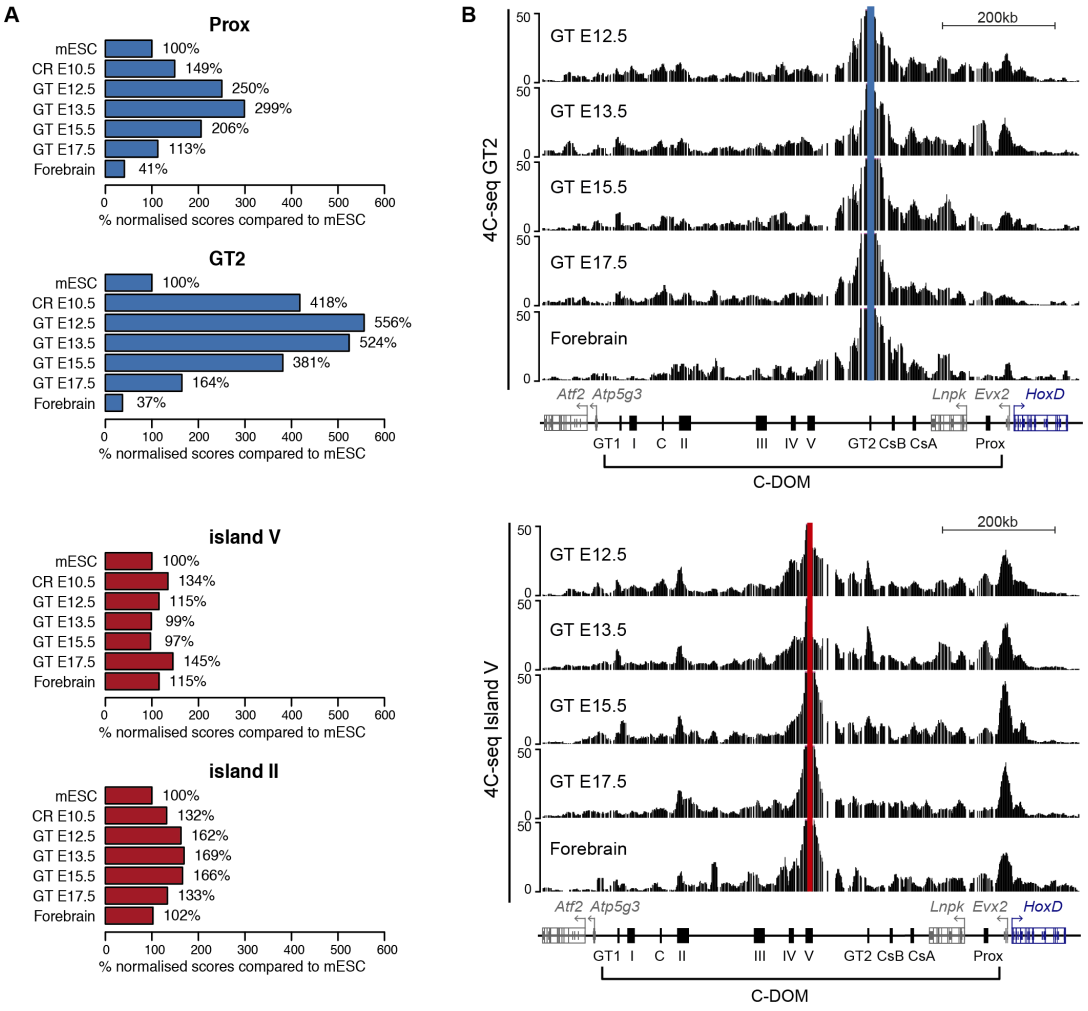
Figure supplement 5: Alleles generated by CRISPR-Cas9. Sanger sequencing results of F0 animals for all alleles generated. Scissors indicate CRISPR-Cas9 mediated breakpoints flanking each regulatory region. SgRNA sequences are marked in red or in green. PCR based genotyping was carried out with primers designed on both sides of sgRNAs targets, deletions were screened with primers F1/R2, inversions with primers F1/F2 and R2/R1, and WT were amplified with primers F1/R1 and F2/R2. See Table 1 for all primer sequences and related PCR product sizes.

Amândio et al.
Supplementary Figure 1



Amândio et al.

Supplementary Figure 2



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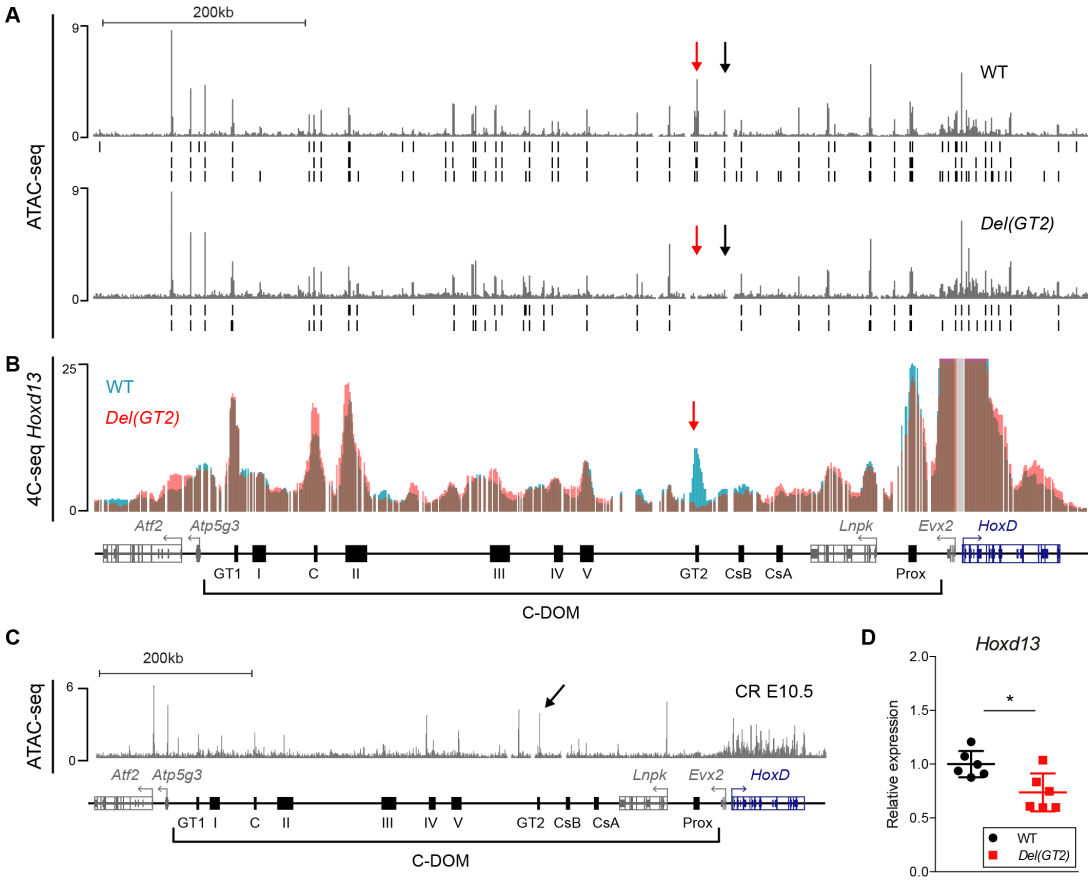
Supplementary Figure 3



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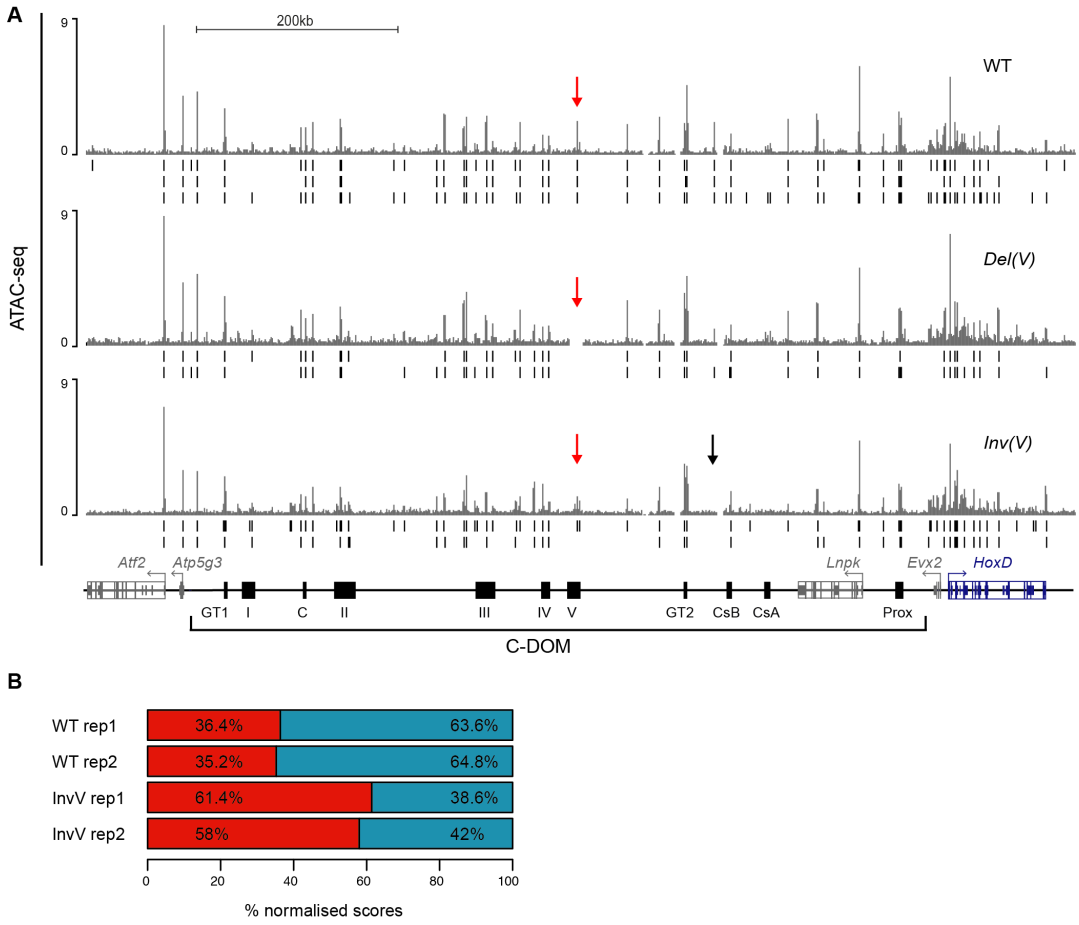
Supplementary Figure 4



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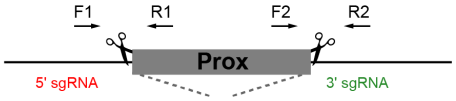
Supplementary Figure 5

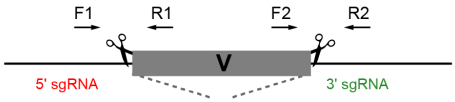


REF TTCCAGTCCATCTATCACTATTTGCTCCC....NNN....ACGATACTTAAGACCTAGTCTGGAAAGCT

Del(Prox) TTCCAGTCCATCTATCACTATTTGCT.....GTCTGGAAAGCT

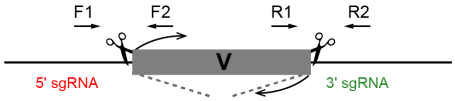
REF ACCCCAGAACAGAGATCTGCTTGGCACGT....NNN....GCAGTGCGTGCTGGTTCGCCCGGCCACAT

Del(GT2) ACCCCAGAACAGAGATC.....GGCCACAT



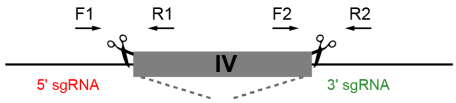
REF CAGTGACGCTCAAATCGTACAGCGCTGCC....NNN.....GTAGATAACTCCACCCCGTGCACGTACCA

Del(V) CAGTGACGCTCAAATCGTACAGCGCTG.....ATAACTCCACCCCGTGCACGTACCA



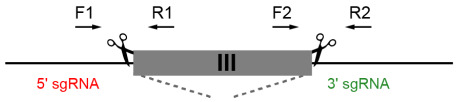
REF CAGTGACGCTCAAATCGTACAGCGCTGCC....NNN.....GTAGATAACTCCACCCCGTGCACGTACCA

Inv(V) CAGTGACGCTCAAATCGT.....CCACCCCGTGCACGTACCA



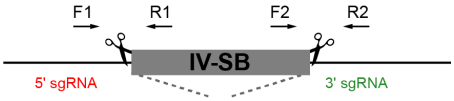
REF TCTCCCCCCAAGTCGCATCTGCCTTTGCG....NNN....CTTGCCGCTTGACCTGCTAATGGTCCTGT

Del(IV) TCTCCCCCC.....TAATGGTCCTGT



REF AGTGGTCCTCCGTACTACCAATGATTCTA....NNN....TTACGGAAATCTTAGAACCAACAGTTCTC

Del(IIIE) AGTGGTCCTCCG.....CGGAAATCTTAGAACCAACAGTTCTC



REF CCTGATGCTCTCTTGTTCTTGATCGACAT....NNN....CTTGCCGCTTGACCTGCTAATGGTCCTGT

Del(IV-SB) CCTGATGCTCTCTTGTTCTTGATCGAC.....TAATGGTCCTGT

Table 1: List of genotyping primers

Allele	Primer name	Sequence	Product Size (bp)
Del(Prox)	590	GACTGTGTTTTGAGGAGGACAGTG	PCR 590-591: WT: 488bp PCR 594-597: Del:369bp
	591	CTGTGGCAGTAAGAGTGTGCTGAG	
	594	CCCAGCTGCAGCAGAAGACC	
	597	GTTTCAAAAGGCAAGTCCATGACTCTCTG	
Del(GT2)	435	TGGGTACAGTTTGGCTCCAT	PCR 435-436: WT: 575bp PCR 433-436: Del: 500bp
	436	GCCCTTGGTGGCATGTTTAG	
	433	CTGCCACCTACCTTCTCCTC	
Del(V) and Inv(V)	RA7	GCTTGTGTCTTGCTGTGTCA	PCR RA7-RA8: WT: 419bp PCR RA7-RA10: Del:440bp PCR RA7-RA9: Inv: approx 300bp
	RA8	AAGGAAAGTGTGTGTGCTGG	
	RA9	CCGAATCCCTAGCTGTCGAG	
	RA10	CATCTGTAGGTTCTGTGCTTATG	
Del(IV)	502	CAGTTCTTTTCCACGGTGAGGAAGC	PCR 502-503: WT: 504bp PCR 501-507: Del: approx 450bp
	503	CACAAAGATGTGTCAAAGTTGGGGCTG	
	501	CCTCTTGAACCACGTGCATCGC	
	507	GCCAGCAGGAATAGCTATACTAACAGG	
Del(III)	545	GGAGAGCTCTGGGTGTGATTGC	PCR 545-542: Wt: 397bp PCR 537-544: Del: 600bp
	542	TGGGAAGTGTGGAGTCTTCTGCC	
	537	CCCTTCCCCCTATCACTGTATCTCC	
	544	CAGACCTTTGCAGTAGGGTCATGG	
Del(IV-SB)	RA21	TCTGCCTCCGTTCTCACAAT	PCR RA21-RA22: Wt: 480bp PCR RA21-RA24: Del:300bp
	RA22	GGACCATCAAGAAGCATCCG	
	RA24	GCACTAATCCAAAGCCAGCA	

Table 2: List of sgRNAs

Allele	**5' sgRNA sequence**	**3' sgRNA sequence**
Del(Prox)	ATCTATCACTATTTGCTCCC	ACGATACTTAAGACCTAGTC
Del(GT2)	CAGAGATCTGCTTGGCACGT	GCAGTGCGTGCTGGTTCGCC
Del(V)/Inv(V)	TCAAATCGTACAGCGCTGCC	CACGGGGTGGAGTTATCTAC
Del(IV)	CGCAAAGGCAGATGCGACTT	CTTGCCGCTTGACCTGCTAA
Del(III)	TAGAATCATTGGTAGTACGG	TGGTTCTAAGATTTCCGTAA
Del(IV-SB)	CTCTTGTTCTTGATCGACAT	CTTGCCGCTTGACCTGCTAA

Table 3: List of fosmids

Clone Name	Coordinates	Length
WI1-1879J12 (island IV)	chr2:74235922-74281101	45180 bps
WI1-2556D5 (island III)	chr2:74180517-74223072	42556 bps
WI1-1741G6 (island V)	chr2:74270669-74309212	38544 bps
WI1-1129A16 (between is-III/SB)	chr2:74141402-74180933	39532 bps
WI1-109B16 (between is-V/GT2)	chr2:74346499-74384278	37780 bps

Table 4: List of primers used for recombineering

Primer name	**Sequence 5' > 3'**
WI1-2556D5 (island III) Fw	**CAGTTCTCAGTCTTCAACTTGCTGAGTCAAAAATCTGTTGTTCCTGTATT**GCTCGAGGTCGACGGTATCG
WI1-2556D5 (island III) Rev	**CTTTTCTGTGCTATTCTGAGGAGTGTTGGTGTTGTTACTTCTGGGAAGTG**ATCTATGTCGGGTGCGGAGAAAGAGGTAATGAAATGG CGTCCGCCATCTCCAGCAGC
WI1-1879J12 (island IV) Fw	**AATAAATGAACAGCGCTGCTCAGCTGCCCCTTCCCCCGTGGCTAGGCATT**GCTCGAGGTCGACGGTATCG
WI1-1879J12 (island IV) Rev	**TCTTAACAGAGTAGCTTCCTATGTGGAATGTCTGTGGTGGAAAGCGAGGA**ATCTATGTCGGGTGCGGAGAAAGAGGTAATGAAATGG CGTCCGCCATCTCCAGCAGC
WI1-1741G6 (island V) Fw	**ACTGTTCCCTAATTAATACTAAGCCATATGCAAAAACATTCTTGAATACC**ATCTATGTCGGGTGCGGAGAAAGAGGTAATGAAATGGGCTCGAGGTCGACGGTATCG
WI1-1741G6 (island V) Rev	**TCTTAACAGAGTAGCTTCCTATGTGGAATGTCTGTGGTGGAAAGCGAGGA**CGTCCGCCATCTCCAGCAGC
WI1-1129A16 (between is-III/SB) Fw	TTTTGATCCTGAGGCAGCGGAAGACTGTGCCACACTAGATGTAACTTGAGGCTCGAGGTCGACGGTATCG
WI1-1129A16 (between is-III/SB) Rev	TGATTCTTACCAGACTCTAGTTGTCTGCATCCCAAGTTCCCTACTAGGACATCTATGTCGGGTGCGGAGAAAGAGGTAATGAAATGG CGTCCGCCATCTCCAGCAGC
WI1-109B16 (between is-V/GT2) Fw	**CACCCAGTTTTTTTAATCCTGCCGGATCTTTGCATTGACGGTGGCTGTAA**GCTCGAGGTCGACGGTATCG
WI1-109B16 (between is-V/GT2) Rev	**CAAGCATCTCCTCTTTGGTGTCTCAGAAGCAGTTGGCTAAATGAGCTCCT**ATCTATGTCGGGTGCGGAGAAAGAGGTAATGAAATGG CGTCCGCCATCTCCAGCAGC

Homology Arm - restriction site (PISceI) - Primer

Table 5: List of 4C-seq primers

Viewpoint		**Sequence (5' > 3')**
Hoxd13	iF	AATGATACGGCGACCACCGAACACTCTTTCCCTACACGACGCTCTTCCGATCT XXXXAAAATCCTAGACCTGGTCATG
	iR	CAAGCAGAAGACGGCATACGAGGCCGATGGTGCTGTATAGG
GT2	iF	AATGATACGGCGACCACCGAACACTCTTTCCCTACACGACGCTCTTCCGATCT XXXXTTCTCTCTTTTAGTGACCTTGGAACA
	iR	CAAGCAGAAGACGGCATACGAAGAAATATCCAAAGGTAAAAATCAAGAA
island V	iF	AATGATACGGCGACCACCGAACACTCTTTCCCTACACGACGCTCTTCCGATCT XXXXGCTACAAGACTCATTCGTTTAA
	iR	CAAGCAGAAGACGGCATACGAAACTAACTTAAGTCCCCTCG
island IV	iF	AATGATACGGCGACCACCGAACACTCTTTCCCTACACGACGCTCTTCCGATCT XXXXTACAGCCTAGTCTTTTCTCATCACAT
	iR	CAAGCAGAAGACGGCATACGATGTAATTATTTCAGGGTTGGAGTAGAATCA

XXXX – Corresponds to possible barcode sequences