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A Rapid CRISPR/Cas-based Mutagenesis Assay in Zebrafish for Identification of Genes Involved in Thyroid Morphogenesis and Function

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Supplementary Tables 1 – 2

Supplementary Table 1: gRNA target sequences and primers used for genotyping

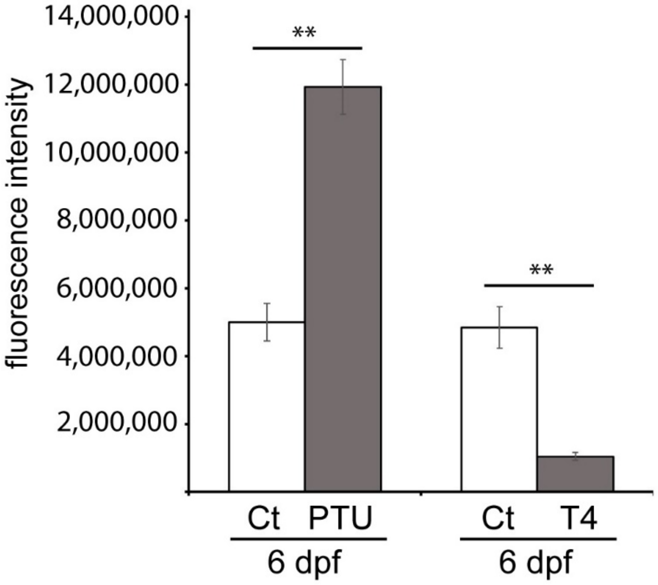
target	**gRNA target sequence (5′-3′)**	**strand**	**forward primer (5′-3′)**	**reverse primer (5′-3′)**	**product size**
adamtsl2 exon 1	GGCTGGAAGTGATCACATAC	+	GCTTCAGACAGTGTCTCTCCTG	GCTTCAGACAGTGTCTCTCCTG	206 bp
adamtsl2 exon 13	AGAGGTCAGATGCTCAGATG	+	CAGCTAAATGTGGTCGCAAG	GGTCCCCACTCGGATGCT	151 bp
duox exon 23	GTCATCTACGCCATCTCCGC	+	ACCGGAGTCAATTTTCCCTTG	ACTTACAGATGCATCTTTCC	210 bp
duoxa exon 2	GTATTCCCGGTAGAATGAAG	+	GCTTTAGTTCTCGCCTGTGG	TCACCGATCTGCCACGTATT	178 bp
nkx2.4b exon 1	GTTCGAGCCGTACCAAGCCG	-	GAGCCATAACGCGTACCACA	GTATCTCGGCTCCGGGTTC	169 bp
nkx2.4b exon 2	AGCACGCGCCGTTTCCTGCG	-	AAAGTCTCCAGGTTTATGGG	CGCTCCAGCTCGTATACCT	152 bp
pax2a exon 2	GGCGGTGTGAACCAGCTAGG	+	TGTGTCTGTGTTGTTGTATCCC	TGGCTGACCCGTAGTTGTCT	167 bp
tshr exon 4	GCTGACATTCATCCACCCAG	+	GGAACACACGAAAGCTGACAT	AACAGTGATTTTGCTCACAGATAC	85 bp
tshr exon 10	GATCATAGGTGTCTCTGAAG	-	GAACCTGGCATCGACTTGAG	GTTTCACCGAAACCCATGTT	128 bp

Supplementary Table 2: Primers used for PCR-based approach of RNA probe preparation

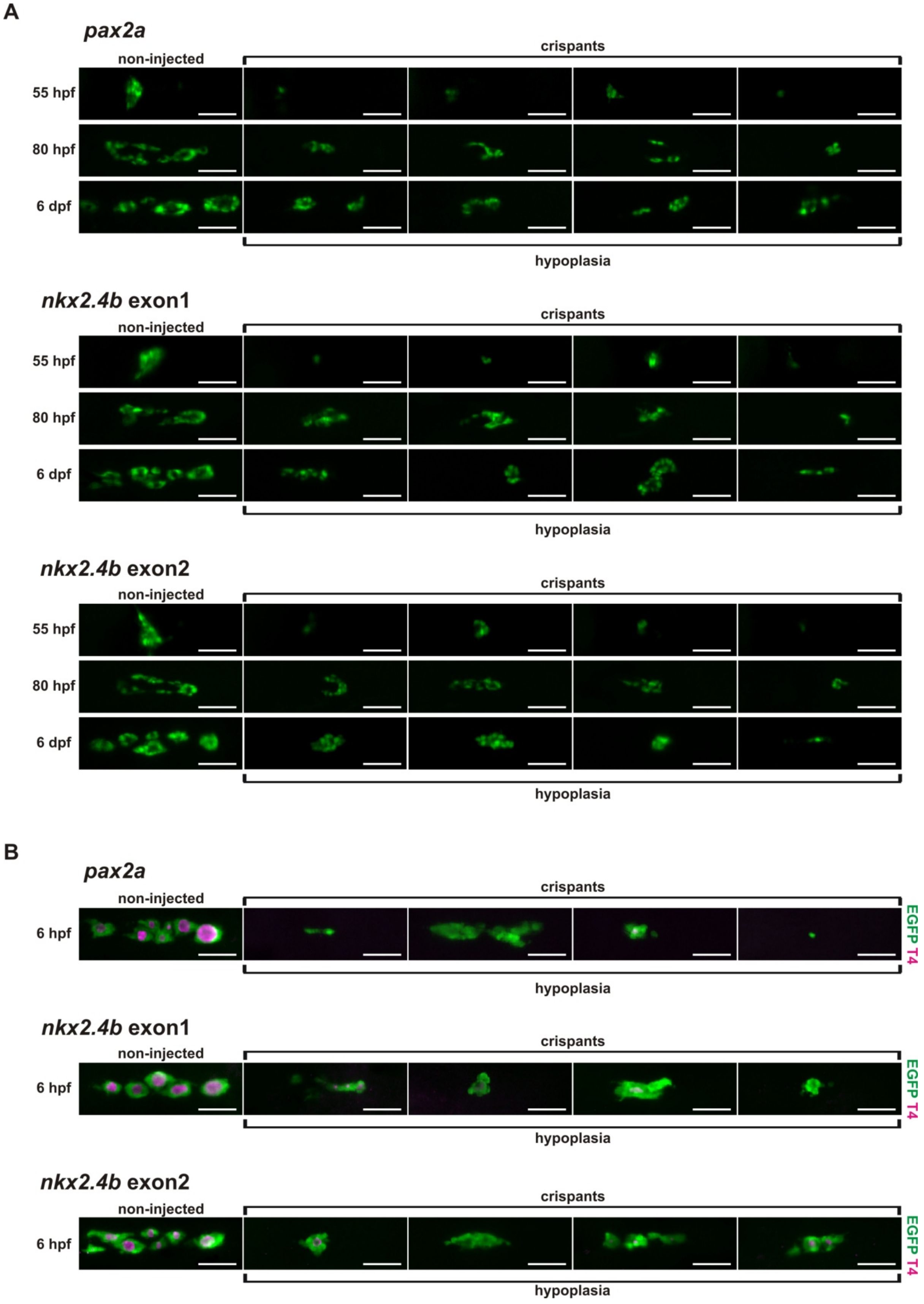
target	**GenBank Accession**	**forward primer (5′-3′)**	**reverse primer (5′-3′)**	**probe size**
adamtsl2	XM_021476679	AAGAAAACAACAACCGGTAAAGAC	CTGTAAAACACTCACAACCTCCAC	857 bp
nkx2.4b	NM_131589	TTTCTCAGTGAGCGATATTTTGAG	CGTATACAATTCACGTCAAAGAGG	1058 bp
slc5a5	NM_001089391	TTTGTATGGCTTCAAGTTCCTGTA	TGTTTTCTCTGTGAAATGGTGATT	906 bp
tg	NM_001329865	TAATTGTAGTGACGGCCAGTTTTA	TTTTCTCCTTGTAGCTGAAGAGGT	796 bp
tshb	NM_181494	TCCAGACAGACATCCTCATACACT	TGAACCCTATTAAAACCACACCTT	857 bp

Note: A sequence containing the T3 polymerase promoter was added to the 5’ end of each reverse primer sequence in order to obtain a PCR product that could be used to generate an antisense probe during *in vitro* transcription. Therefore, the final design of the reverse primers used in this study was as follows: 5’ GGATCCAATTAACCCTCACTAAAGGGAA(N)24 3’ with N representing the 24 nucleotides of the gene-specific reverse primer. The bases of the T3 polymerase promoter become double-stranded promoter sequence during the PCR reaction.

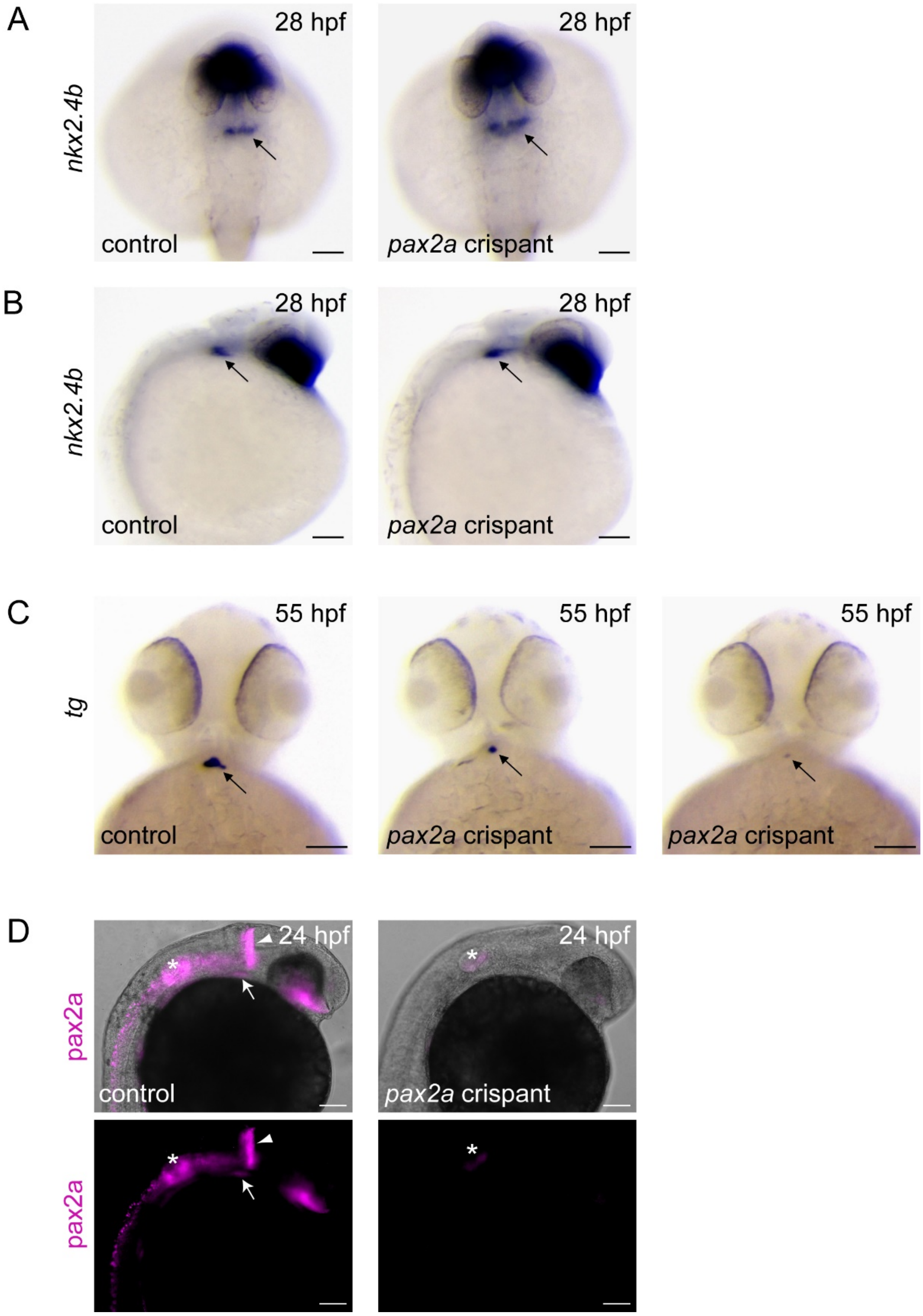
Supplementary Figures 1 – 10



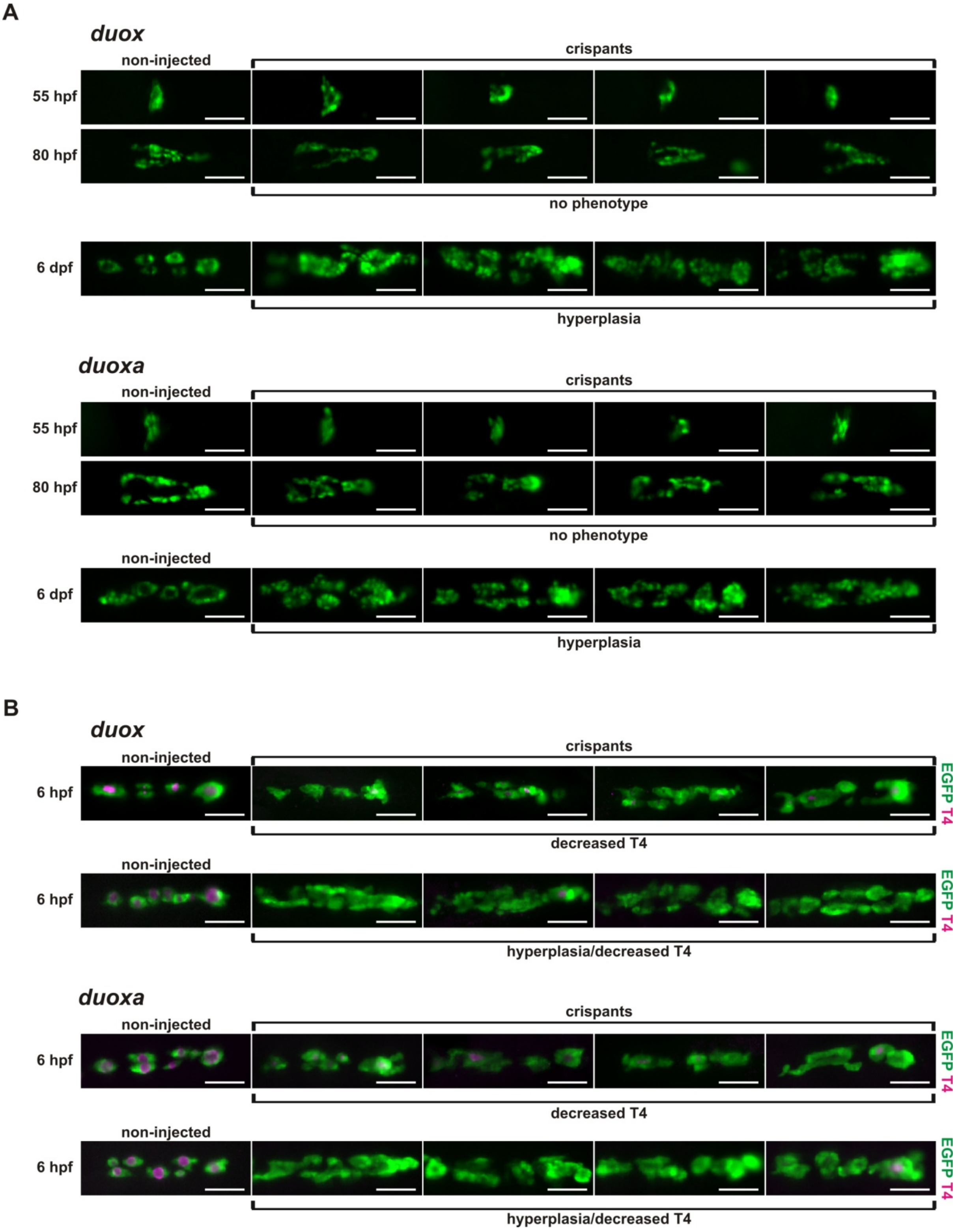
Supplementary Figure 1: Quantification of fluorescence signal intensity in live transgenic *Tg(tg:nlsEGFP)* zebrafish larvae at 6 dpf. Transgenic zebrafish were treated from 52 hpf onwards with 30 mg/L phenylthiourea (PTU) or 5.0 µg/L thyroxine (T4). Fluorescent reporter signal intensities were analysed in untreated controls (Ct) and treated fish by epifluorescence microscopy. Results are shown as means±S.E.M. (*N*=7-9). Asterisks denote significant differences between treatment means (** $P < 0.01$, Student *t*-test).



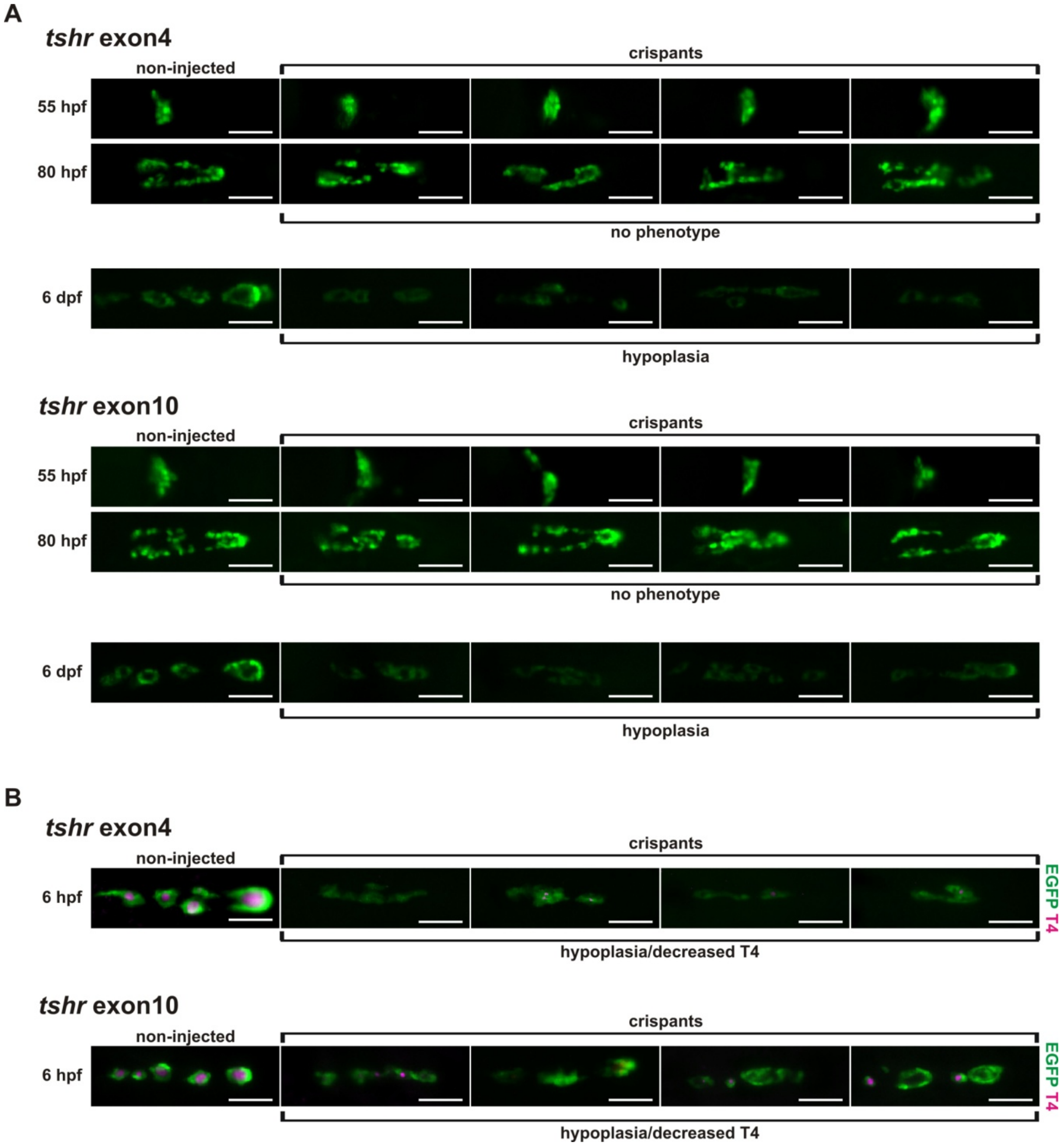
Supplementary Figure 2: Phenotypic spectrum of *pax2a* and *nkx2.4b* crispants. (A) Epifluorescence live imaging of *Tg*(*tg:nlsEGFP*) zebrafish larvae injected with Cas9 protein and sgRNAs targeting *pax2a* or *nkx2.4b* (exon 1 and exon 2), respectively. Ventral views of the thyroid region (anterior to the right) are shown for non-injected control fish and four different crispants displaying hypoplasia of variable severity. Images were aquired at 55 hpf, 80 hpf, and 6 dpf. (B) Whole-mount immunofluorescence staining of *Tg*(*tg:nlsEGFP*) zebrafish for EGFP and colloidal T4 at 6 dpf. Ventral views of the thyroid region (anterior to the right) are shown for non-injected control fish and four different crispants displaying hypoplasia of variable severity. Scale bars: 50 µM.



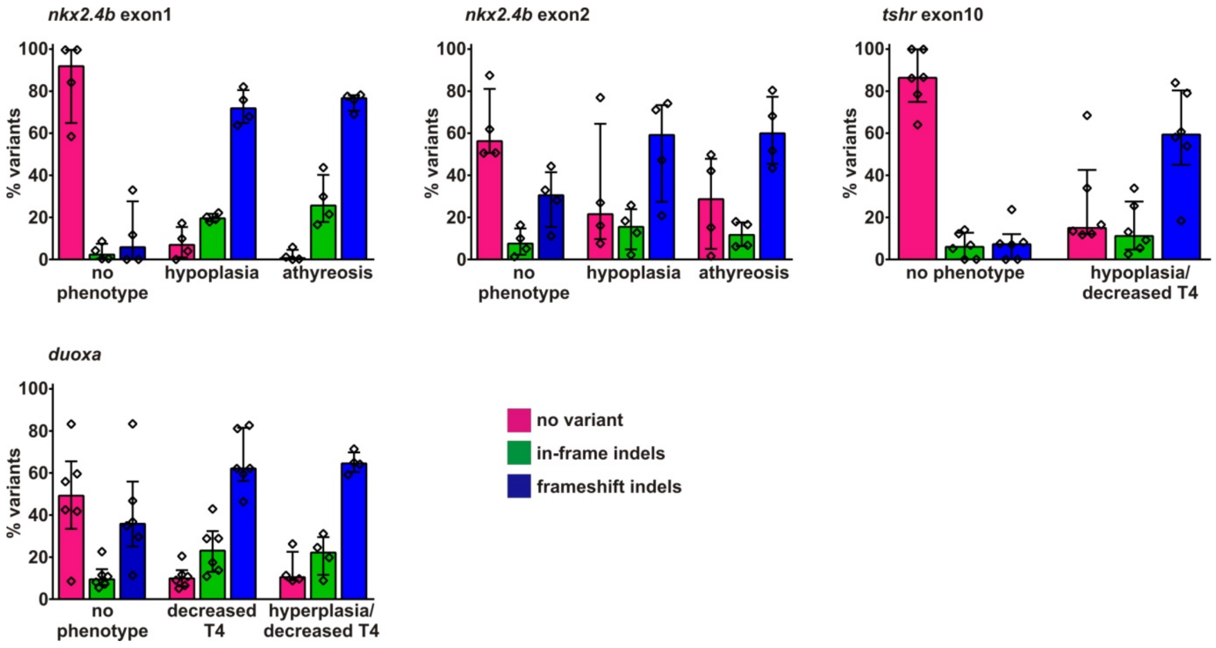
Supplementary Figure 3: Thyroid anlage specification is not perturbed in *pax2a* crispants. (A,B) Whole-mount *in situ* hybridization for the early thyroid marker *nkx2.4b* in non-injected control embryos and *pax2a* crispants. Thyroidal *nkx2.4b* expression (arrow) was not different between experimental groups at 28 hpf. Panel A shows dorsal views of stained specimen (anterior is to the top). Panel B shows lateral views (anterior is to the right). (C) Loss of thyroid marker expression in later stage *pax2a* crispants. Whole-mount *in situ* hybridization for the thyroid differentiation marker *tg* in non-injected control embryos and *pax2a* crispants at 55 hpf. In contrast to the strong *tg* staining in the compact and slightly ovoid thyroid primordium of control embryos, half of the *pax2a* crispants showed either a complete absence of detectable *tg* staining (data not shown) or presented thyroid primordia of greatly reduced size. Ventral views are shown (anterior is to the top). (D) *pax2a* crispants display strongly reduced immunoreactivity to a pax2a antibody. Whole-mount immunofluorescence staining of pax2a (magenta) was performed on non-injected control embryos and *pax2a* crispants at 24 hpf using a pax2a antibody directed against an epitope located C-terminal to the sgRNA target site. Approximately half of the *pax2a* crispants (53.7%; *N*=79/147) displayed a strongly reduced pax2a immunofluorescence signal. Lateral views are shown (anterior is to the right). Arrowheads: mid-hindbrain boundary; arrows: thyroid anlage; asterisks: otic vesicle. Scale bars: 100 µm.



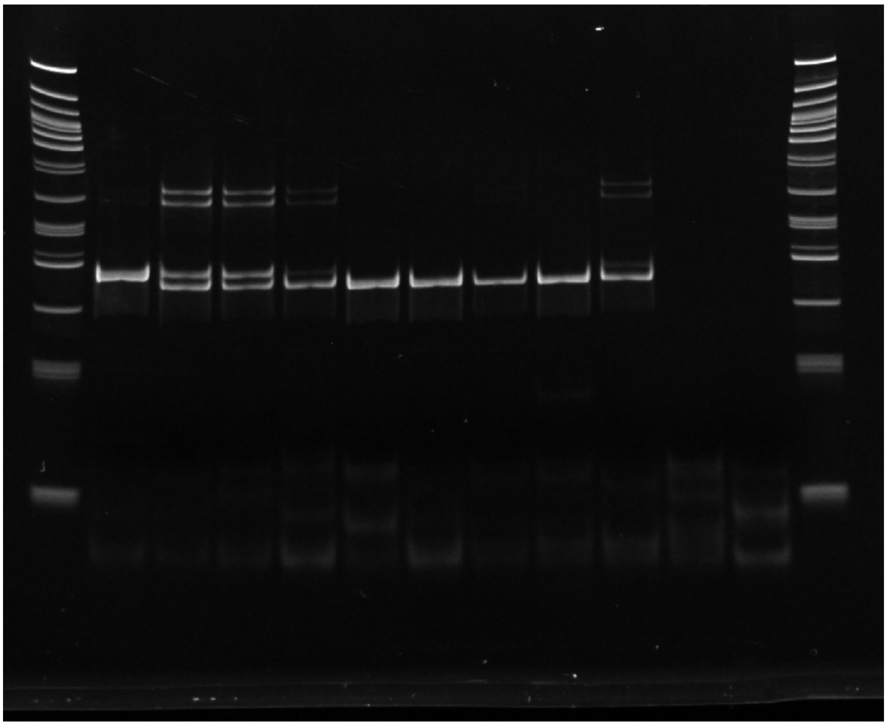
Supplementary Figure 4: Phenotypic spectrum of *duox* and *duoxa* crispants. (A) Epifluorescence live imaging of *Tg*(*tg:nlsEGFP*) zebrafish larvae injected with Cas9 protein and sgRNAs targeting *duox* or *duoxa*, respectively. Ventral views of the thyroid region (anterior to the right) are shown for non-injected control fish and four crispants. Images were aquired at 55 hpf, 80 hpf, and 6 dpf. No deviation from control thyroid morphology was evident in *duox* and *duoxa* crispants at 55 and 80 hpf. By 6 dpf, hyperplastic thyroid enlargement (goiter) was detectable in many *duox* and *duoxa* crispants. (B) Whole-mount immunofluorescence staining of *Tg*(*tg:nlsEGFP*) zebrafish for EGFP and colloidal T4 at 6 dpf. Ventral views of the thyroid region (anterior to the right) are shown for non-injected control fish and different crispants displaying hypothyroidism (decreased T4 staining) that was often but not always associated with a hyperplastic thyroid enlargement. Scale bars: 50 µm.



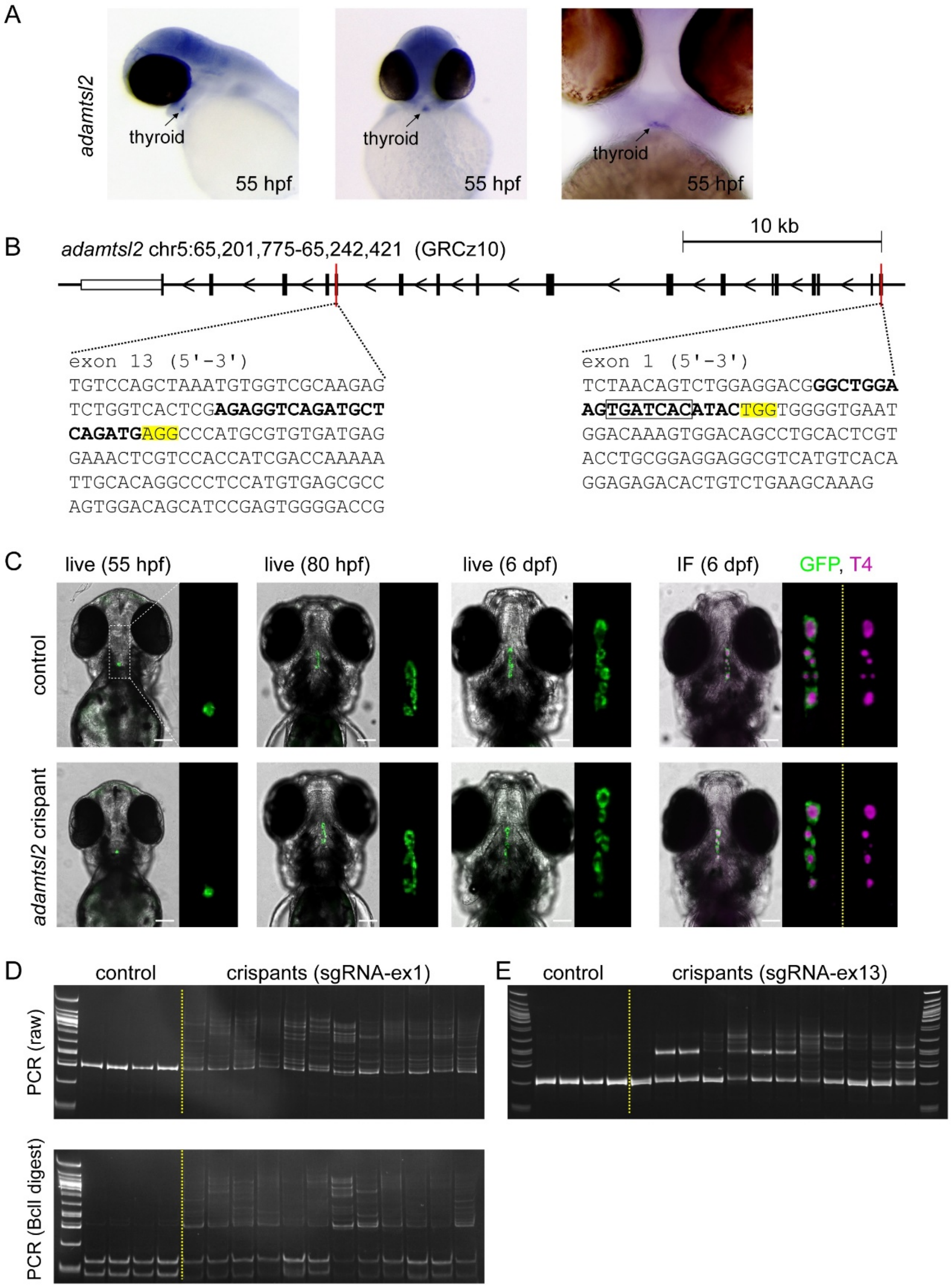
Supplementary Figure 5: Phenotypic spectrum of *tshr* crispants. (A) Epifluorescence live imaging of *Tg*(*tg:nlsEGFP*) zebrafish larvae injected with Cas9 protein and sgRNAs targeting exon 4 or exon 10 of *tshr*, respectively. Ventral views of the thyroid region (anterior to the right) are shown for non-injected control fish and four crispants. Images were aquired at 55 hpf, 80 hpf, and 6 dpf. No deviation from control thyroid morphology was evident in *tshr* crispants at 55 and 80 hpf. By 6 dpf, thyroids of *tshr* crispants had an atrophic/hypoplastic appearance and displayed a greatly reduced GFP reporter signal. (B) Whole-mount immunofluorescence staining of *Tg*(*tg:nlsEGFP*) zebrafish for EGFP and colloidal T4 at 6 dpf. Ventral views of the thyroid region (anterior to the right) are shown for non-injected control fish and different *tshr* crispants displaying hypothyroidism (decreased T4 staining), variable thyroid hypoplasia and reduced GFP expression. Scale bars: 50 µm.



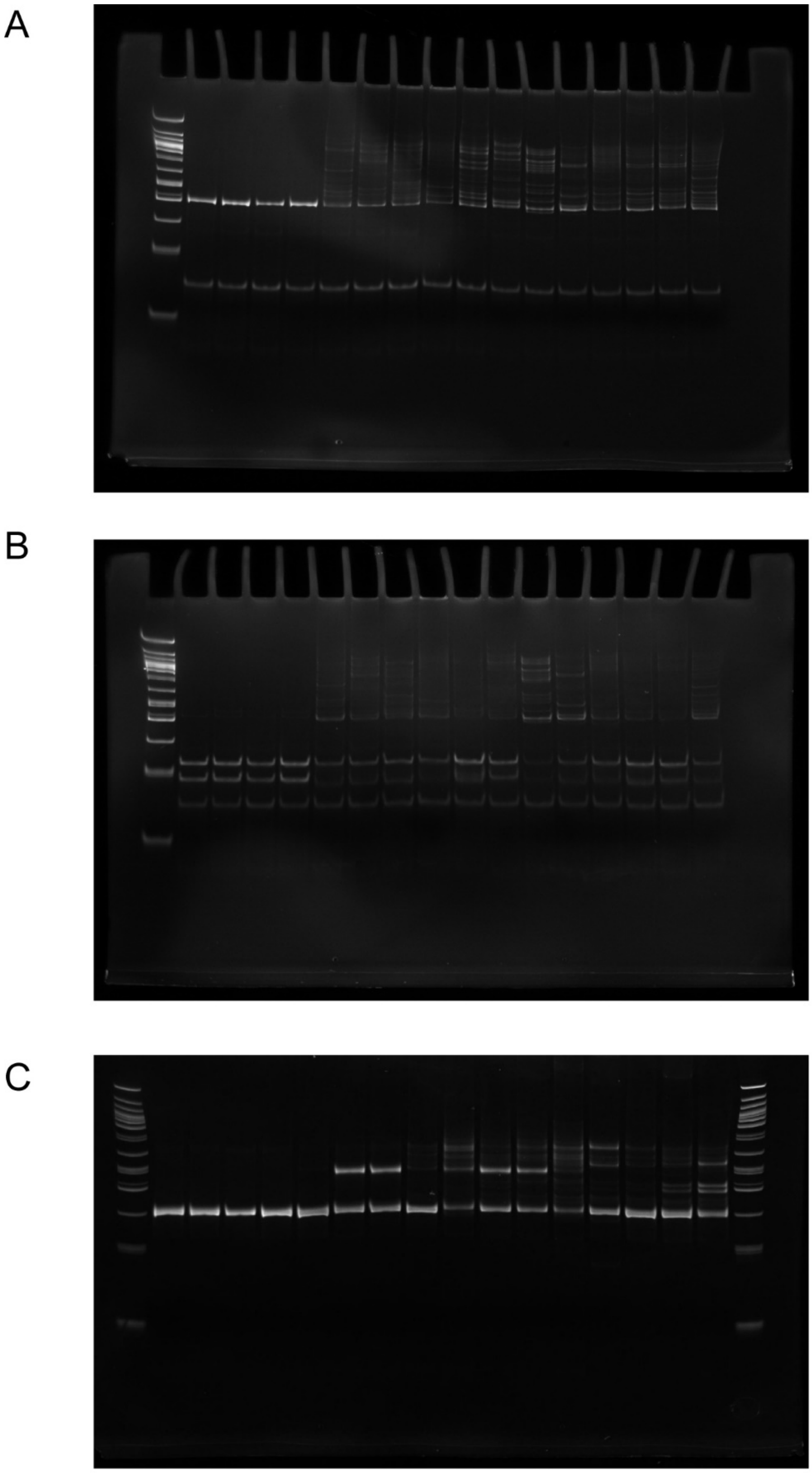
Supplementary Figure 6: Illumina HiSeq analyses of *nkx2.4b*, *duoxa* and *tshr* crispants. Distribution of allelic variants as determined by Illumina HiSeq analysis of individual crispants revealed high mutagenesis efficiency in larvae presenting thyroid dysgenesis (target: exon 1 of *nkx2.4b*, exon 2 of *nkx2.4b*), thyroid hypoplasia and decreased T4 content (target: exon 10 of *tshr*) and hypothyroidism/hyperplastic thyroid enlargement (target: *duoxa*). The percentage of WT alleles (no variant call), in-frame indels, or frameshift indels is shown for *N* = 4-6 larvae per phenotypic category (median values with interquartile range).



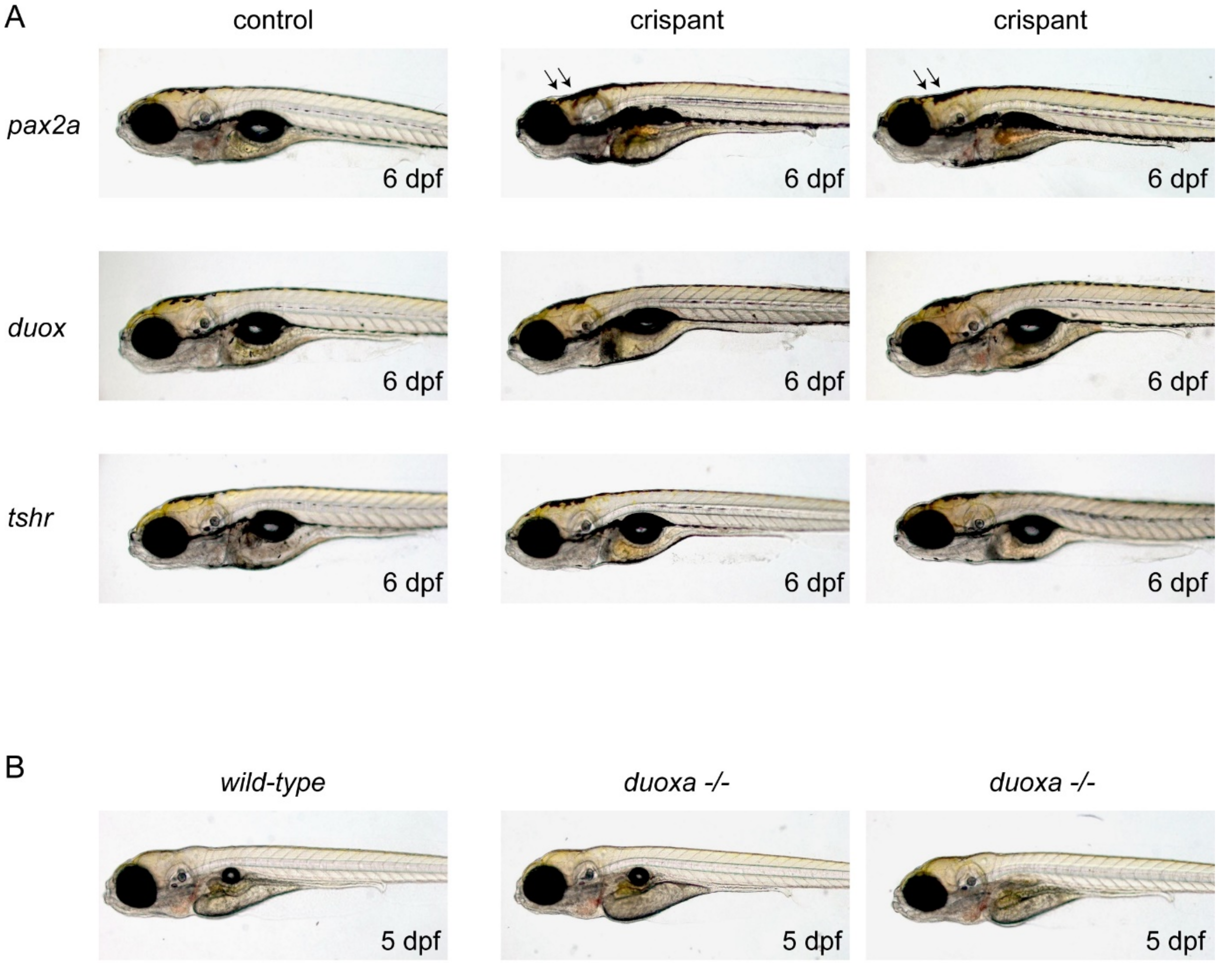
Supplementary Figure 7: Full length gel for the cropped gel image shown in Figure 7B. Polyacrylamide gel for PCR amplicons of wild-type fish (single PCR product of 178 bp), heterozygous carriers of the *duoxa* Δ11 allele (main PCR products of 178 and 167 bp) and homozygous carriers of the *duoxa* Δ11 allele (PCR product of 167 bp). DNA molecular size markers are shown in lane 1 and 13, wild-type sample in lane 2, heterozygous *duoxa* mutants in lane 3, 4, 5 and 10, homozygous *duoxa* mutants in lanes 6-9 and negative controls (H_2O) in lane 11 and 12. Additional slow-migrating heteroduplex bands can be seen in PCR samples of heterozygous *duoxa* mutant fish (lanes 3,4,5,10).



Supplementary Figure 8: Characterization of *adamtsl2* crispants. (A) *adamtsl2* mRNA is expressed at high levels in the zebrafish thyroid primordium (arrow) as demonstrated by whole-mount *in situ* hybridization. Lateral and frontal views of 55 hpf embryos stained with an *adamtsl2*-specific riboprobe are shown. (B) Zebrafish *adamtsl2* genomic locus on chromosome 5 with sequences for exon 1 and exon 13. Underlined sequences are target sites for sgRNA-ex1 and sgRNA-ex13, respectively. PAM sequences are highlighted in yellow and the boxed sequence in exon 1 is the BclI restriction enzyme recognition site. (C) Thyroid phenotyping of *adamtsl2* crispants did not reveal alteration in thyroid morphogenesis or thyroid function. Results shown are from an injection experiment with sgRNA-ex13 and similar results were obtained with sgRNA-ex1. Epifluorescence live imaging of *Tg*(*tg:nlsEGFP*) zebrafish at 55 hpf, 80 hpf, and 6 dpf. Ventral views of the head region and 3-fold magnified views of the thyroid region (GFP channel only) are shown for non-injected controls and *adamtsl2* crispants. Whole-mount immunofluorescence (IF) staining of 6 dpf *Tg*(*tg:nlsEGFP*) zebrafish for EGFP (thyroid cells) and thyroxine (colloidal T4). Ventral views of the head region (anterior is to the top) and 3-fold magnified views of the thyroid region (GFP, T4) are shown. Scale bars: 100 µm. (D) Polyacrylamide gel electrophoresis (PAGE) of PCR amplicons generated with primers spanning the target site of sgRNA-ex1. Upper panel shows a gel for PCR amplicons of *WT* larvae (single PCR product of 206 bp) and *adamtsl2* crispants (multiple slow-migrating heteroduplex bands). Lower panel shows a gel for PCR amplicons digested with BclI. Note complete digestion of PCR amplicons from *WT* larvae resulting in two restriction fragments of 111bp and 95 bp, respectively. Images of full-length gels are shown in Supplementary Figure 9A,B. (E) PAGE analysis of PCR amplicons generated with primers spanning the target site of sgRNA-ex13. Gel showing PCR amplicons of non-injected *WT* larvae (single PCR product of 151 bp) and *adamtsl2* crispants (multiple slow-migrating heteroduplex bands in most samples). Full-length gel is shown in Supplementary Figure 9C.



Supplementary Figure 9: Full length gels for the cropped gels shown in Supplementary Figure 8. (A) Full length gel for the cropped gel shown in the upper panel of Supplementary Figure 8D. (B) Full length gel for the cropped gel shown in the lower panel of Supplementary Figure 8D. (C) Full length gel for the cropped gel shown in Supplementary Figure 8E.



Supplementary Figure 10: Brightfield micrographs of crispants and germline mutant fish (A) Brightfield microscopy of control larvae and crispants displaying a thyroid phenotype after injection of gRNAs targetting *pax2a*, *duox* and *tshr*. Lateral views (anterior to the left) of 6 dpf old fish are shown. Arrows highlight defective brain development in *pax2a* crispants. (B) Brightfield microscopy of *WT* larvae and homozygous carriers of the *duoxa* Δ11 allele. Lateral views (anterior to the left) of 5 dpf old larvae (pigment-less *casper* fish) are shown.

Supplementary Information 2: NGS data

A Rapid CRISPR/Cas-based Mutagenesis Assay in Zebrafish for Identification of Genes Involved in Thyroid Morphogenesis and Function

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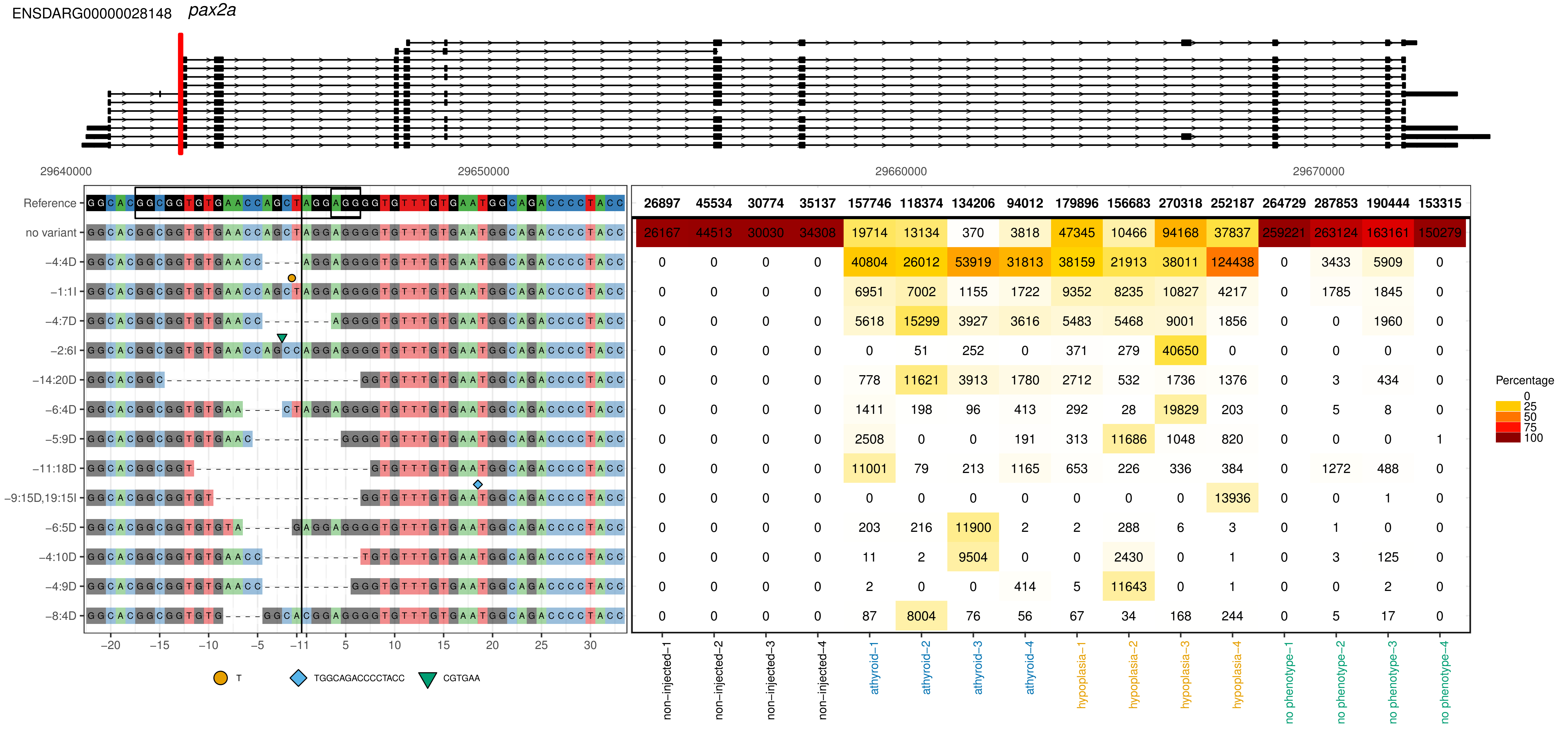
Allele frequencies of individual F0 crispants as analysed by CrispRVariants.

Data of HiSeq sequencing of zebrafish mutagenesis experiments were analysed by CrispRVariants[26,45]. For each target locus, variant allele plots and barplots are presented for individual zebrafish categorized by phenotype. Only variants with a frequency of ≥ 5% in at least one zebrafish were considered.

Variant allele plots show on the left the alignment of each variant to the reference genome (top line) with the sgRNA sequence and the 3' PAM indicated by boxes. The cut site is indicated by a vertical line. The gene scheme on the top shows the binding site of the sgRNA (in red) in relation to known Ensemble transcripts. Within the alignment, deletions and insertions are indicated by ´-` and by symbols, respectively, with the corresponding insert sequence explained below the plot. Deletions, insertions, single nucleotide variants and chimeric reads are indicated by D, I, SNV, or 'other' respectively. Each variant is further characterized by its size and by the position of the most upstream base in relation to the cut site. For example, a 4 bp deletion starting at -4 bases upstream of the cut site would be -4:4D. The right panel of the variant allele plots shows the frequency (in read counts) of each variant for individual zebrafish with the top line indicating the total number of derived sequences. Color codes on the right represent the relative frequencies in percentages of each variant.

Barplots show the relative frequencies (x-axis) of no variant alleles, frameshift indels, inframe indels, single nucleotide variants, and chimeric reads for individual zebrafish (Y-axis) as indicated by color codes. The number of different alleles (variants) and the total number of sequences for each individual zebrafish are shown to the right of the barplots.


ENSDARG00000028148 pax2a
29640000
29650000
29660000
29670000
Reference GGCACGGCGGTGTGAACCAGCTAGGAGGGGTGTTTGTGAATGGCAGACCCCTACC
no variant
−4:4D
−1:1I
−4:7D
−2:6I
−14:20D
−6:4D
−5:9D
−11:18D
−9:15D,19:15I
−6:5D
−4:10D
−4:9D
−8:4D
−20 −15 −10 −5 −1 1 5 10 15 20 25 30
● T
◆ TGGCAGACCCCTACC
▼ CGTGAA
26897 45534 30774 35137 157746 118374 134206 94012 179896 156683 270318 252187 264729 287853 190444 153315
26167 44513 30030 34308 19714 13134 370 3818 47345 10466 94168 37837 259221 263124 163161 150279
0 0 0 0 40804 26012 53919 31813 38159 21913 38011 124438 0 3433 5909 0
0 0 0 0 6951 7002 1155 1722 9352 8235 10827 4217 0 1785 1845 0
0 0 0 0 5618 15299 3927 3616 5483 5468 9001 1856 0 0 1960 0
0 0 0 0 0 51 252 0 371 279 40650 0 0 0 0 0
0 0 0 0 778 11621 3913 1780 2712 532 1736 1376 0 3 434 0
0 0 0 0 1411 198 96 413 292 28 19829 203 0 5 8 0
0 0 0 0 2508 0 0 191 313 11686 1048 820 0 0 0 1
0 0 0 0 11001 79 213 1165 653 226 336 384 0 1272 488 0
0 0 0 0 0 0 0 0 0 0 0 13936 0 0 1 0
0 0 0 0 203 216 11900 2 2 288 6 3 0 1 0 0
0 0 0 0 11 2 9504 0 0 2430 0 1 0 3 125 0
0 0 0 0 2 0 0 414 5 11643 0 1 0 0 2 0
0 0 0 0 87 8004 76 56 67 34 168 244 0 5 17 0
non−injected−1
non−injected−2
non−injected−3
non−injected−4
athyroid−1
athyroid−2
athyroid−3
athyroid−4
hypoplasia−1
hypoplasia−2
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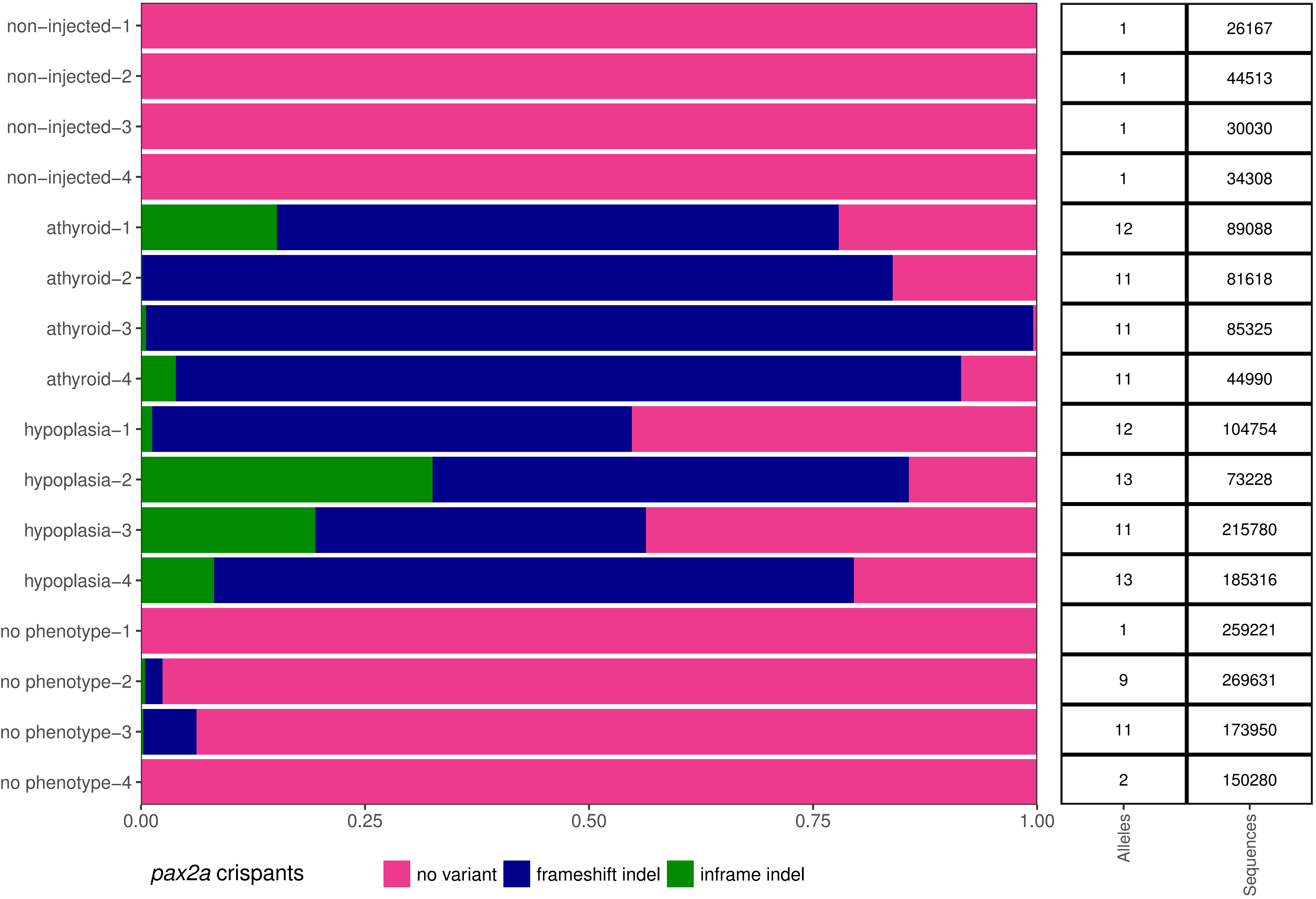
no phenotype−2
no phenotype−3
no phenotype−4
Percentage
0
25
50
75
100


non−injected−1
non−injected−2
non−injected−3
non−injected−4
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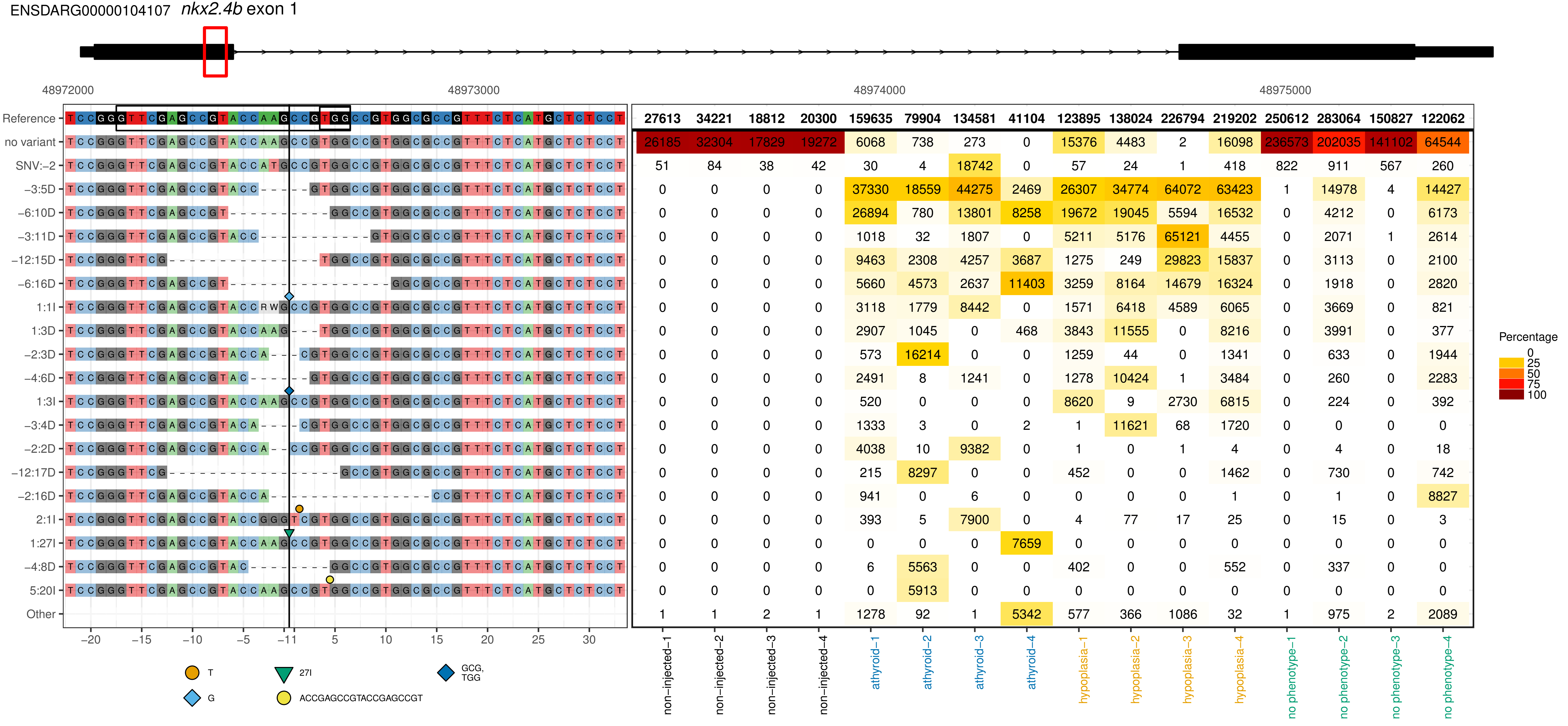
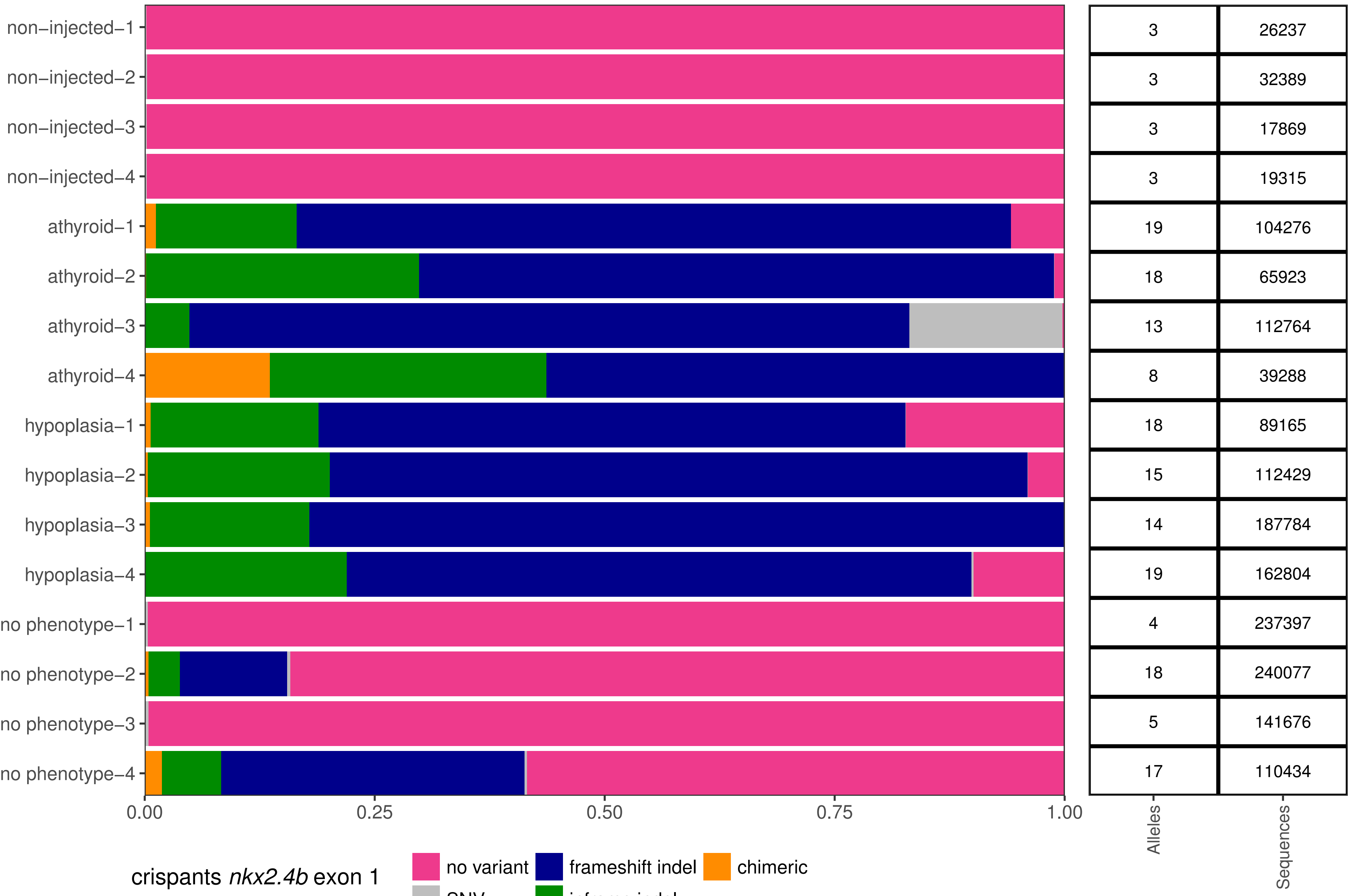
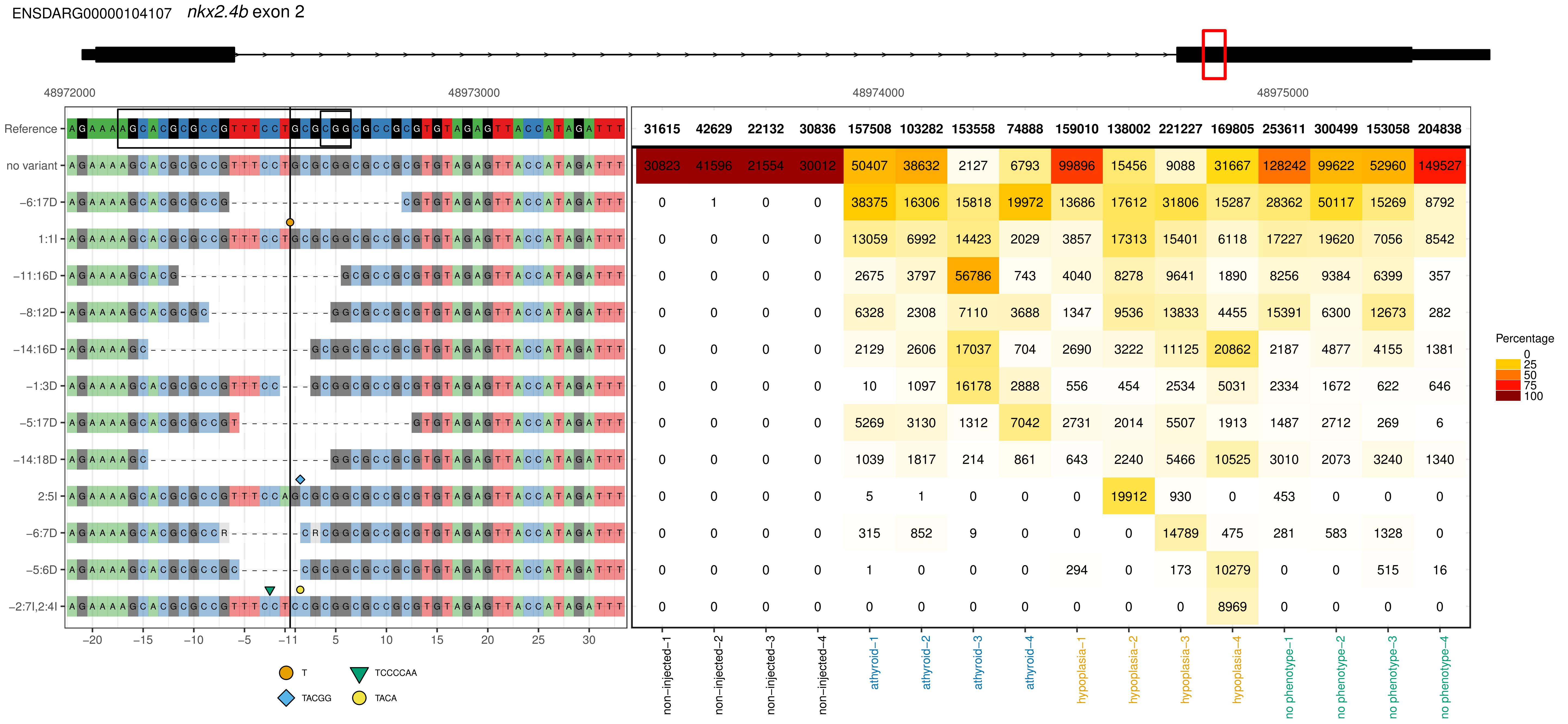
athyroid−2
athyroid−3
athyroid−4
hypoplasia−1
hypoplasia−2
hypoplasia−3
hypoplasia−4
no phenotype−1
no phenotype−2
no phenotype−3
no phenotype−4
0.00
0.25
0.50
0.75
1.00
1
26167
1
44513
1
30030
1
34308
12
89088
11
81618
11
85325
11
44990
12
104754
13
73228
11
215780
13
185316
1
259221
9
269631
11
173950
2
150280
Alleles
Sequences
pax2a crispants
no variant
frameshift indel
inframe indel

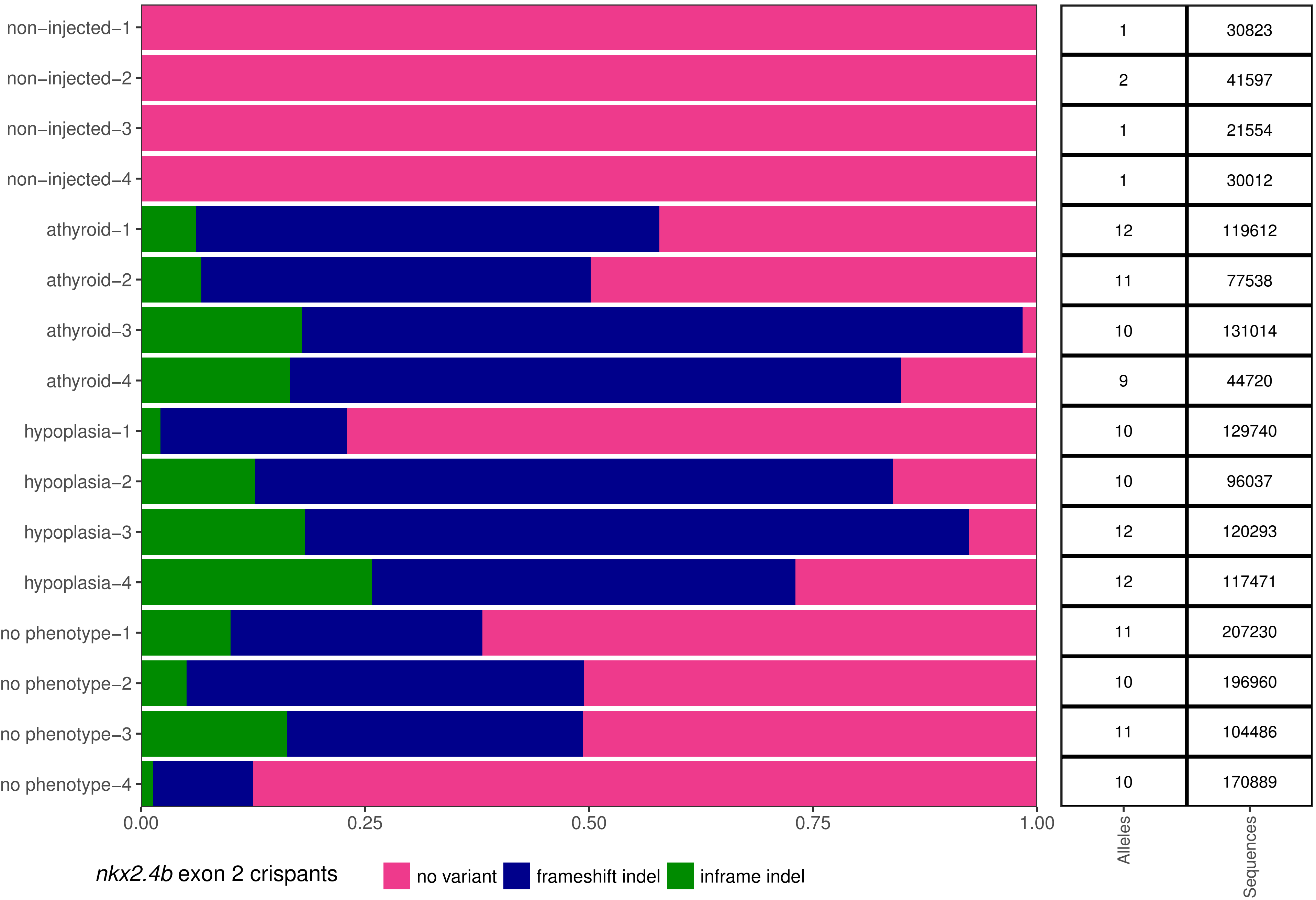


	non–injected–1	non–injected–2	non–injected–3	non–injected–4	athyroid–1	athyroid–2	athyroid–3	athyroid–4	hypoplasia–1	hypoplasia–2	hypoplasia–3	hypoplasia–4	no phenotype–1	no phenotype–2	no phenotype–3	no phenotype–4
Reference	27613	34221	18812	20300	159635	79904	134581	41104	123895	138024	226794	219202	250612	283064	150827	122062
no variant	26185	32304	17829	19272	6068	738	273	0	15376	4483	2	16098	236573	202035	141102	64544
SNV:−2	51	84	38	42	30	4	18742	0	57	24	1	418	822	911	567	260
−3:5D	0	0	0	0	37330	18559	44275	2469	26307	34774	64072	63423	1	14978	4	14427
−6:10D	0	0	0	0	26894	780	13801	8258	19672	19045	5594	16532	0	4212	0	6173
−3:11D	0	0	0	0	1018	32	1807	0	5211	5176	65121	4455	0	2071	1	2614
−12:15D	0	0	0	0	9463	2308	4257	3687	1275	249	29823	15837	0	3113	0	2100
−6:16D	0	0	0	0	5660	4573	2637	11403	3259	8164	14679	16324	0	1918	0	2820
1:1I	0	0	0	0	3118	1779	8442	0	1571	6418	4589	6065	0	3669	0	821
1:3D	0	0	0	0	2907	1045	0	468	3843	11555	0	8216	0	3991	0	377
−2:3D	0	0	0	0	573	16214	0	0	1259	44	0	1341	0	633	0	1944
−4:6D	0	0	0	0	2491	8	1241	0	1278	10424	1	3484	0	260	0	2283
1:3I	0	0	0	0	520	0	0	0	8620	9	2730	6815	0	224	0	392
−3:4D	0	0	0	0	1333	3	0	2	1	11621	68	1720	0	0	0	0
−2:2D	0	0	0	0	4038	10	9382	0	1	0	1	4	0	4	0	18
−12:17D	0	0	0	0	215	8297	0	0	452	0	0	1462	0	730	0	742
−2:16D	0	0	0	0	941	0	6	0	0	0	0	1	0	1	0	8827
2:1I	0	0	0	0	393	5	7900	0	4	77	17	25	0	15	0	3
1:27I	0	0	0	0	0	0	0	7659	0	0	0	0	0	0	0	0
−4:8D	0	0	0	0	6	5563	0	0	402	0	0	552	0	337	0	0
5:20I	0	0	0	0	0	5913	0	0	0	0	0	0	0	0	0	0
Other	1	1	2	1	1278	92	1	5342	577	366	1086	32	1	975	2	2089

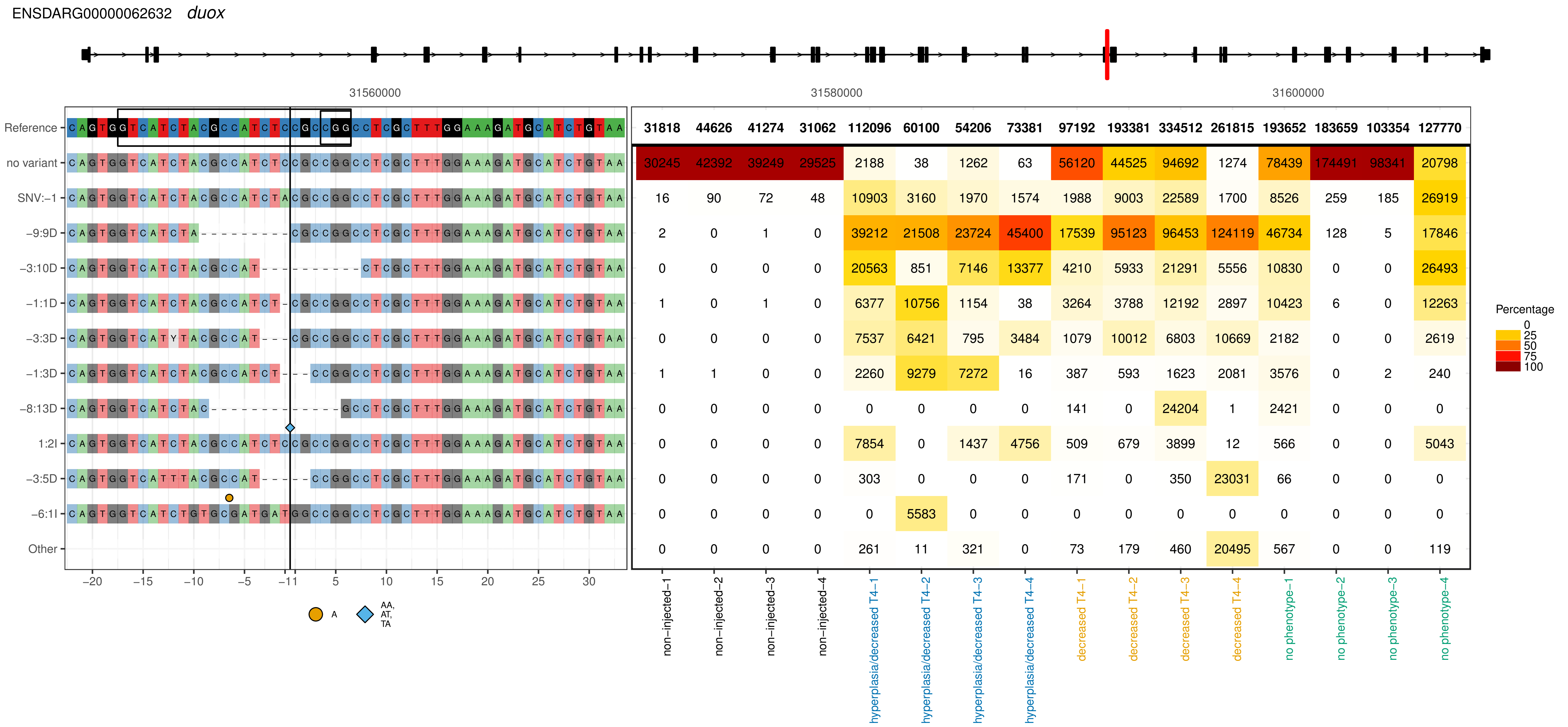


	Alleles	Sequences
non−injected−1	3	26237
non−injected−2	3	32389
non−injected−3	3	17869
non−injected−4	3	19315
athyroid−1	19	104276
athyroid−2	18	65923
athyroid−3	13	112764
athyroid−4	8	39288
hypoplasia−1	18	89165
hypoplasia−2	15	112429
hypoplasia−3	14	187784
hypoplasia−4	19	162804
no phenotype−1	4	237397
no phenotype−2	18	240077
no phenotype−3	5	141676
no phenotype−4	17	110434

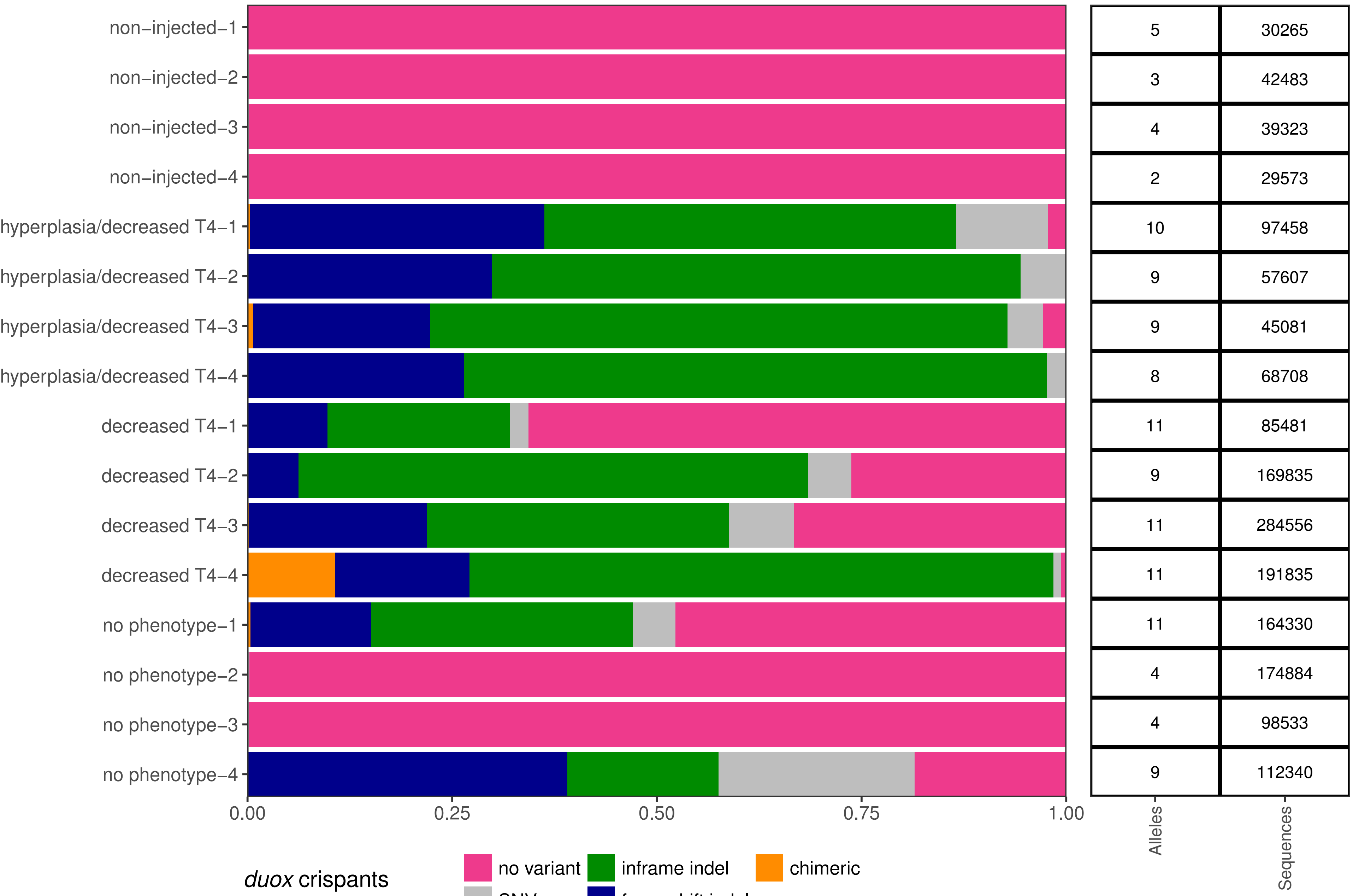

ENSDARG00000104107 *nkx2.4b* exon 2
48972000
48973000
48974000
48975000
Reference
no variant
−6:17D
1:1I
−11:16D
−8:12D
−14:16D
−1:3D
−5:17D
−14:18D
2:5I
−6:7D
−5:6D
−2:7I,2:4I
AGAAAAGCACGCGCCGTTTCCTGCGCGGCGCCGCGTGTAGAGTTACCATAGATTT
−20
−15
−10
−5
−1
1
5
10
15
20
25
30
T
TACGG
TCCCCAA
TACA
31615 42629 22132 30836 157508 103282 153558 74888 159010 138002 221227 169805 253611 300499 153058 204838
30823 41596 21554 30012 50407 38632 2127 6793 99896 15456 9088 31667 128242 99622 52960 149527
0 1 0 0 38375 16306 15818 19972 13686 17612 31806 15287 28362 50117 15269 8792
0 0 0 0 13059 6992 14423 2029 3857 17313 15401 6118 17227 19620 7056 8542
0 0 0 0 2675 3797 56786 743 4040 8278 9641 1890 8256 9384 6399 357
0 0 0 0 6328 2308 7110 3688 1347 9536 13833 4455 15391 6300 12673 282
0 0 0 0 2129 2606 17037 704 2690 3222 11125 20862 2187 4877 4155 1381
0 0 0 0 10 1097 16178 2888 556 454 2534 5031 2334 1672 622 646
0 0 0 0 5269 3130 1312 7042 2731 2014 5507 1913 1487 2712 269 6
0 0 0 0 1039 1817 214 861 643 2240 5466 10525 3010 2073 3240 1340
0 0 0 0 5 1 0 0 0 19912 930 0 453 0 0 0
0 0 0 0 315 852 9 0 0 0 14789 475 281 583 1328 0
0 0 0 0 1 0 0 0 294 0 173 10279 0 0 515 16
0 0 0 0 0 0 0 0 0 0 0 8969 0 0 0 0
non−injected−1
non−injected−2
non−injected−3
non−injected−4
athyroid−1
athyroid−2
athyroid−3
athyroid−4
hypoplasia−1
hypoplasia−2
hypoplasia−3
hypoplasia−4
no phenotype−1
no phenotype−2
no phenotype−3
no phenotype−4
Percentage
0
25
50
75
100



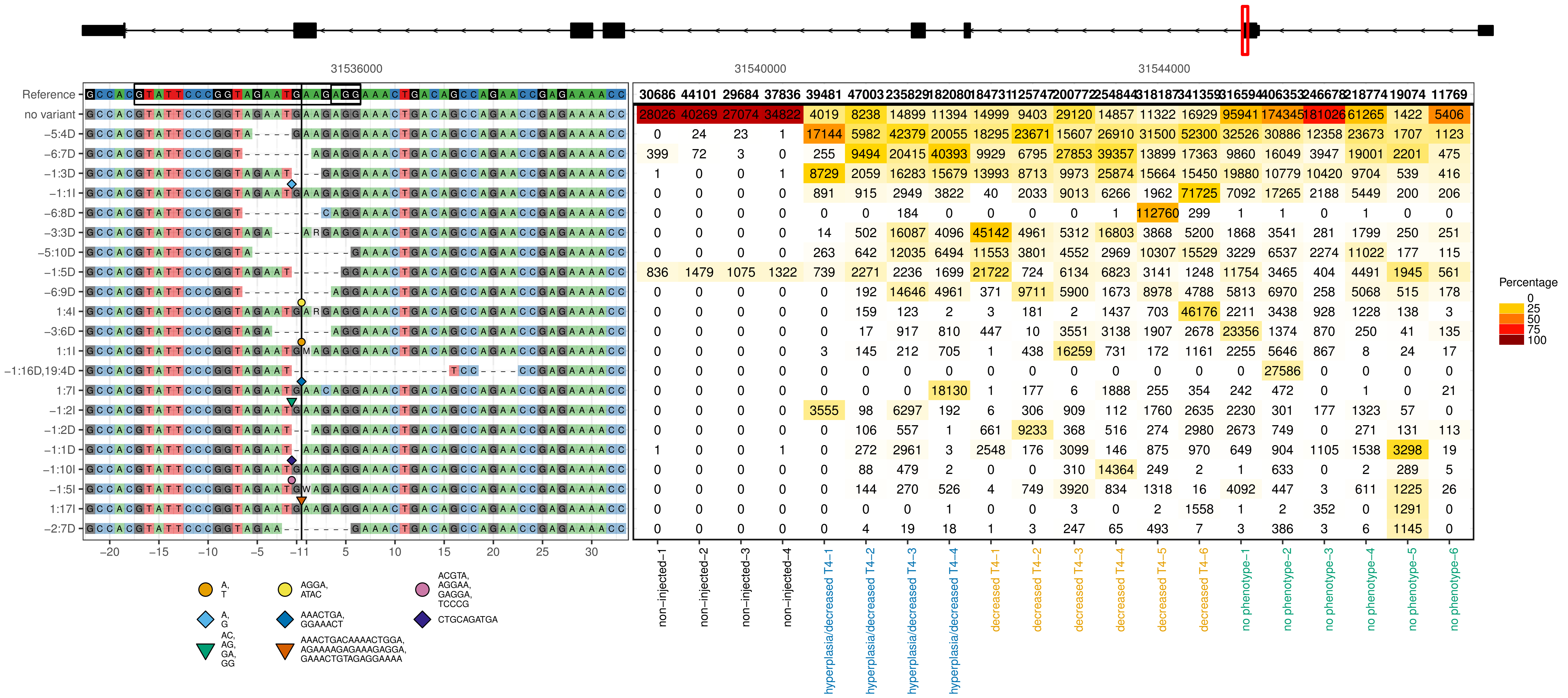
	Alleles	Sequences
non−injected−1	1	30823
non−injected−2	2	41597
non−injected−3	1	21554
non−injected−4	1	30012
athyroid−1	12	119612
athyroid−2	11	77538
athyroid−3	10	131014
athyroid−4	9	44720
hypoplasia−1	10	129740
hypoplasia−2	10	96037
hypoplasia−3	12	120293
hypoplasia−4	12	117471
no phenotype−1	11	207230
no phenotype−2	10	196960
no phenotype−3	11	104486
no phenotype−4	10	170889



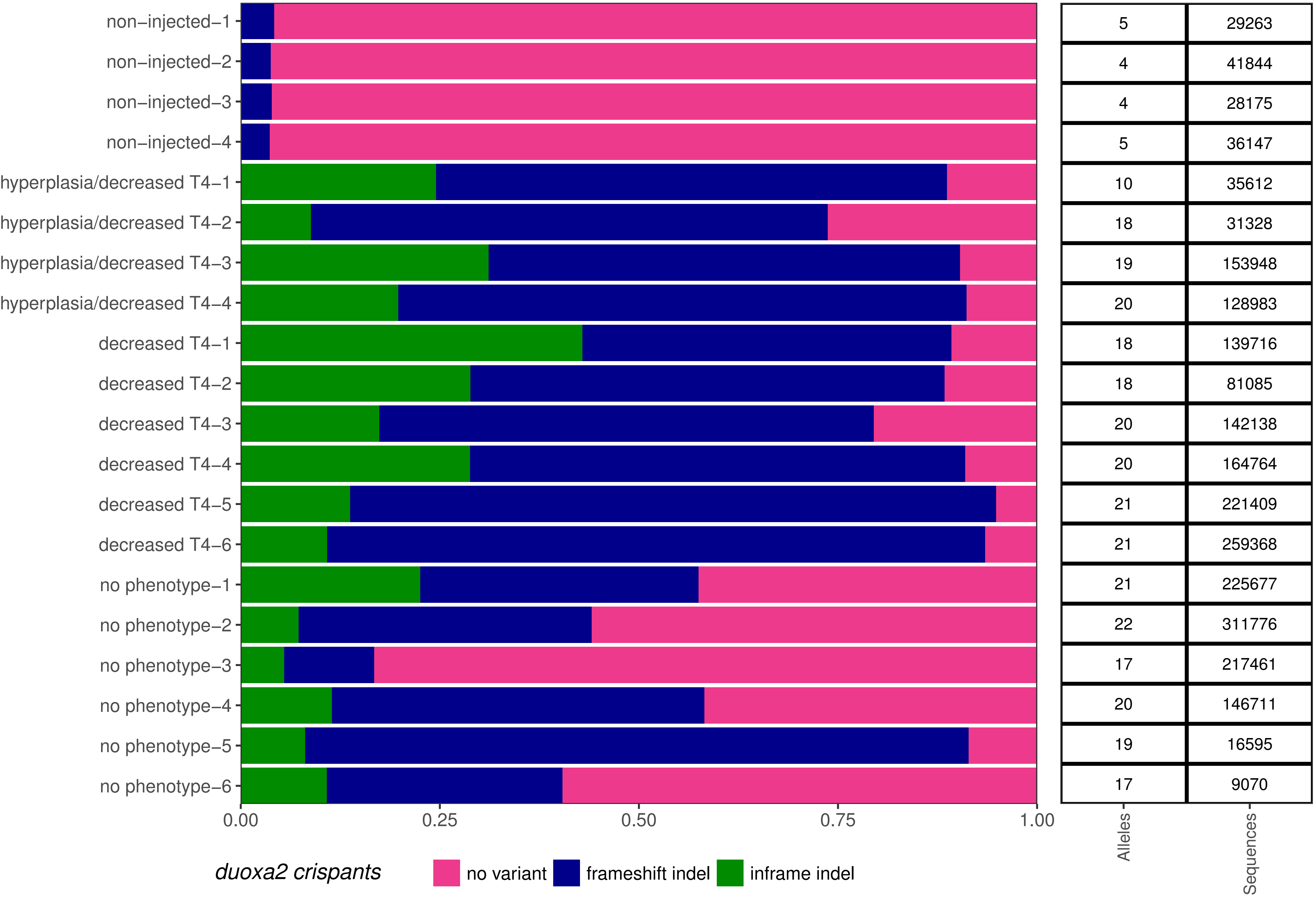
	non−injected−1	non−injected−2	non−injected−3	non−injected−4	hyperplasia/decreased T4−1	hyperplasia/decreased T4−2	hyperplasia/decreased T4−3	hyperplasia/decreased T4−4	decreased T4−1	decreased T4−2	decreased T4−3	decreased T4−4	no phenotype−1	no phenotype−2	no phenotype−3	no phenotype−4
Reference	31818	44626	41274	31062	112096	60100	54206	73381	97192	193381	334512	261815	193652	183659	103354	127770
no variant	30245	42392	39249	29525	2188	38	1262	63	56120	44525	94692	1274	78439	174491	98341	20798
SNV:−1	16	90	72	48	10903	3160	1970	1574	1988	9003	22589	1700	8526	259	185	26919
−9:9D	2	0	1	0	39212	21508	23724	45400	17539	95123	96453	124119	46734	128	5	17846
−3:10D	0	0	0	0	20563	851	7146	13377	4210	5933	21291	5556	10830	0	0	26493
−1:1D	1	0	1	0	6377	10756	1154	38	3264	3788	12192	2897	10423	6	0	12263
−3:3D	0	0	0	0	7537	6421	795	3484	1079	10012	6803	10669	2182	0	0	2619
−1:3D	1	1	0	0	2260	9279	7272	16	387	593	1623	2081	3576	0	2	240
−8:13D	0	0	0	0	0	0	0	0	141	0	24204	1	2421	0	0	0
1:2I	0	0	0	0	7854	0	1437	4756	509	679	3899	12	566	0	0	5043
−3:5D	0	0	0	0	303	0	0	0	171	0	350	23031	66	0	0	0
−6:1I	0	0	0	0	0	5583	0	0	0	0	0	0	0	0	0	0
Other	0	0	0	0	261	11	321	0	73	179	460	20495	567	0	0	119

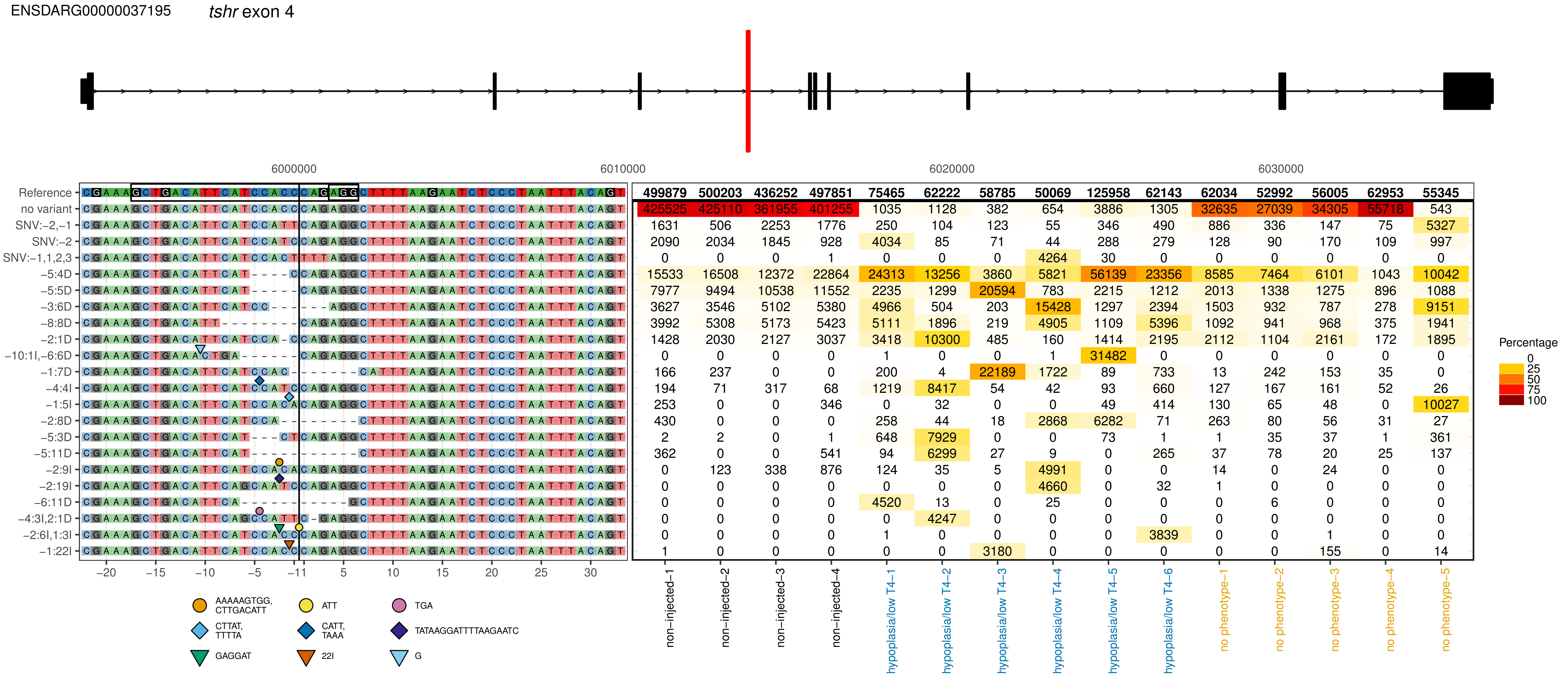

non−injected−1
non−injected−2
non−injected−3
non−injected−4
hyperplasia/decreased T4−1
hyperplasia/decreased T4−2
hyperplasia/decreased T4−3
hyperplasia/decreased T4−4
decreased T4−1
decreased T4−2
decreased T4−3
decreased T4−4
no phenotype−1
no phenotype−2
no phenotype−3
no phenotype−4
0.00
0.25
0.50
0.75
1.00
5
30265
3
42483
4
39323
2
29573
10
97458
9
57607
9
45081
8
68708
11
85481
9
169835
11
284556
11
191835
11
164330
4
174884
4
98533
9
112340
Alleles
Sequences
duox crispants
no variant
inframe indel
chimeric
SNV
frameshift indel

ENSDARG00000078962 *duoxa2*

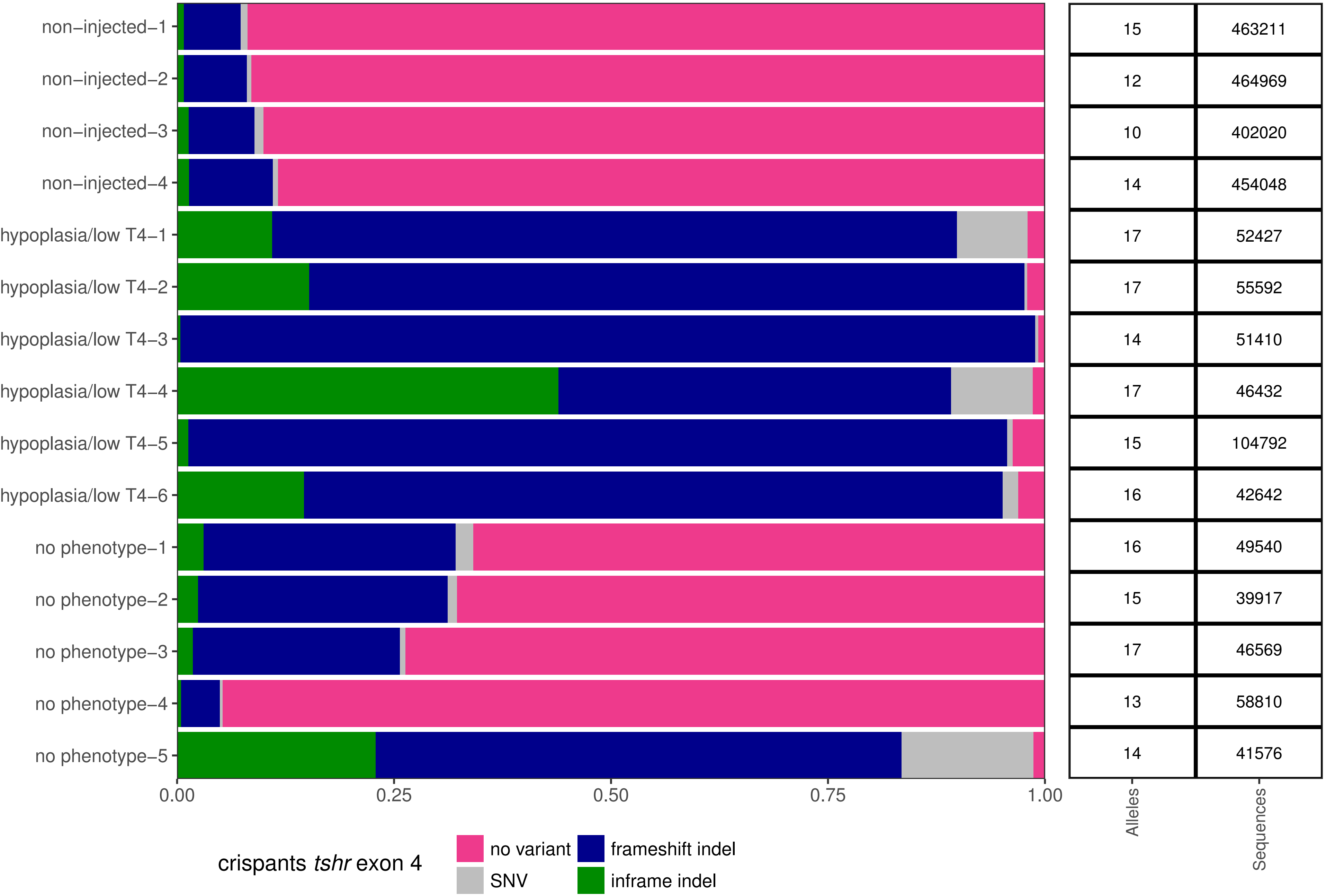


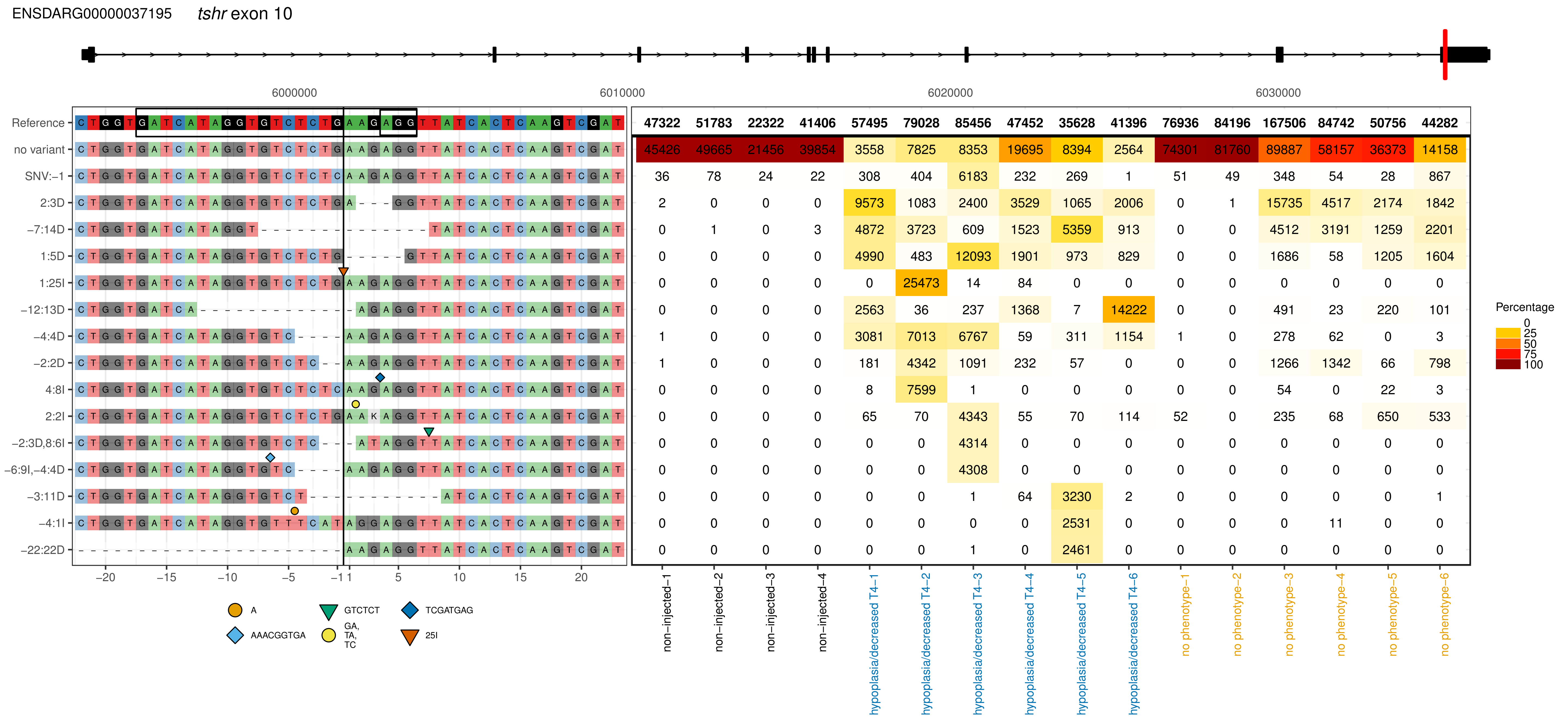
	non−injected−1	non−injected−2	non−injected−3	non−injected−4	hyperplasia/decreased T4−1	hyperplasia/decreased T4−2	hyperplasia/decreased T4−3	hyperplasia/decreased T4−4	decreased T4−1	decreased T4−2	decreased T4−3	decreased T4−4	decreased T4−5	decreased T4−6	no phenotype−1	no phenotype−2	no phenotype−3	no phenotype−4	no phenotype−5	no phenotype−6
Reference	30686	44101	29684	37836	39481	47003	235829	182080	184731	125747	200772	254844	318187	341359	316594	406353	246678	218774	19074	11769
no variant	28026	40269	27074	34822	4019	8238	14899	11394	14999	9403	29120	14857	11322	16929	95941	174345	181026	61265	1422	5406
−5:4D	0	24	23	1	17144	5982	42379	20055	18295	23671	15607	26910	31500	52300	32526	30886	12358	23673	1707	1123
−6:7D	399	72	3	0	255	9494	20415	40393	9929	6795	27853	39357	13899	17363	9860	16049	3947	19001	2201	475
−1:3D	1	0	0	1	8729	2059	16283	15679	13993	8713	9973	25874	15664	15450	19880	10779	10420	9704	539	416
−1:1I	0	0	0	0	891	915	2949	3822	40	2033	9013	6266	1962	71725	7092	17265	2188	5449	200	206
−6:8D	0	0	0	0	0	0	184	0	0	0	0	1	112760	299	1	1	0	1	0	0
−3:3D	0	0	0	0	14	502	16087	4096	45142	4961	5312	16803	3868	5200	1868	3541	281	1799	250	251
−5:10D	0	0	0	0	263	642	12035	6494	11553	3801	4552	2969	10307	15529	3229	6537	2274	11022	177	115
−1:5D	836	1479	1075	1322	739	2271	2236	1699	21722	724	6134	6823	3141	1248	11754	3465	404	4491	1945	561
−6:9D	0	0	0	0	0	192	14646	4961	371	9711	5900	1673	8978	4788	5813	6970	258	5068	515	178
1:4I	0	0	0	0	0	159	123	2	3	181	2	1437	703	46176	2211	3438	928	1228	138	3
−3:6D	0	0	0	0	0	17	917	810	447	10	3551	3138	1907	2678	23356	1374	870	250	41	135
1:1I	0	0	0	0	3	145	212	705	1	438	16259	731	172	1161	2255	5646	867	8	24	17
−1:16D,19:4D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	27586	0	0	0	0
1:7I	0	0	0	0	0	0	0	18130	1	177	6	1888	255	354	242	472	0	1	0	21
−1:2I	0	0	0	0	3555	98	6297	192	6	306	909	112	1760	2635	2230	301	177	1323	57	0
−1:2D	0	0	0	0	0	106	557	1	661	9233	368	516	274	2980	2673	749	0	271	131	113
−1:1D	1	0	0	1	0	272	2961	3	2548	176	3099	146	875	970	649	904	1105	1538	3298	19
−1:10I	0	0	0	0	0	88	479	2	0	0	310	14364	249	2	1	633	0	2	289	5
−1:5I	0	0	0	0	0	144	270	526	4	749	3920	834	1318	16	4092	447	3	611	1225	26
1:17I	0	0	0	0	0	0	0	1	0	0	3	0	2	1558	1	2	352	0	1291	0
−2:7D	0	0	0	0	0	4	19	18	1	3	247	65	493	7	3	386	3	6	1145	0


non−injected−1
non−injected−2
non−injected−3
non−injected−4
hyperplasia/decreased T4−1
hyperplasia/decreased T4−2
hyperplasia/decreased T4−3
hyperplasia/decreased T4−4
decreased T4−1
decreased T4−2
decreased T4−3
decreased T4−4
decreased T4−5
decreased T4−6
no phenotype−1
no phenotype−2
no phenotype−3
no phenotype−4
no phenotype−5
no phenotype−6
0.00
0.25
0.50
0.75
1.00
5 29263
4 41844
4 28175
5 36147
10 35612
18 31328
19 153948
20 128983
18 139716
18 81085
20 142138
20 164764
21 221409
21 259368
21 225677
22 311776
17 217461
20 146711
19 16595
17 9070
Alleles
Sequences
duoxa2 crispants
no variant
frameshift indel
inframe indel

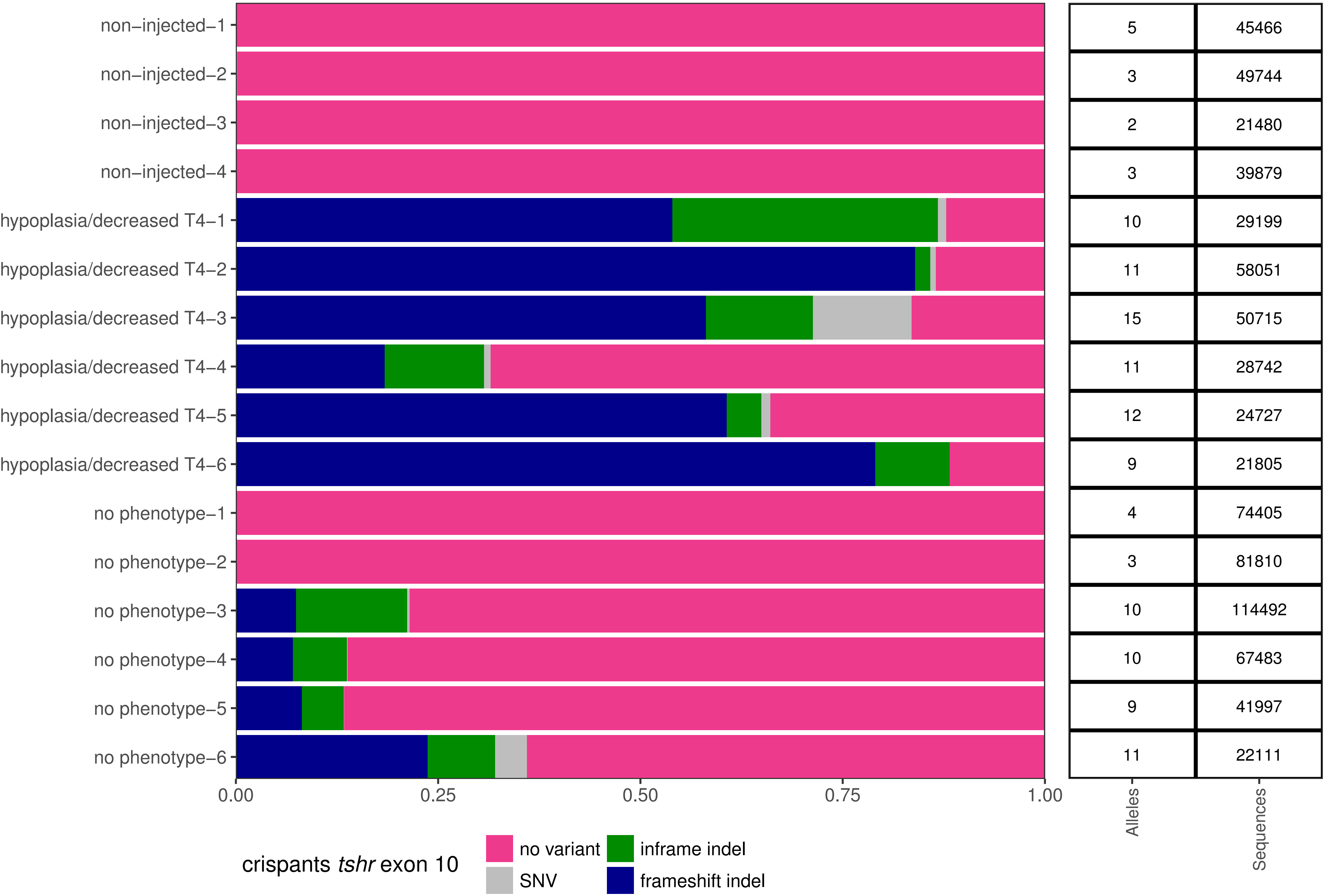


	non-injected-1	non-injected-2	non-injected-3	non-injected-4	hypoplasia/low T4-1	hypoplasia/low T4-2	hypoplasia/low T4-3	hypoplasia/low T4-4	hypoplasia/low T4-5	hypoplasia/low T4-6	no phenotype-1	no phenotype-2	no phenotype-3	no phenotype-4	no phenotype-5
Reference	499879	500203	436252	497851	75465	62222	58785	50069	125958	62143	62034	52992	56005	62953	55345
no variant	425525	425110	361955	401255	1035	1128	382	654	3886	1305	32635	27039	34305	55718	543
SNV:−2,−1	1631	506	2253	1776	250	104	123	55	346	490	886	336	147	75	5327
SNV:−2	2090	2034	1845	928	4034	85	71	44	288	279	128	90	170	109	997
SNV:−1,1,2,3	0	0	0	1	0	0	0	4264	30	0	0	0	0	0	0
−5:4D	15533	16508	12372	22864	24313	13256	3860	5821	56139	23356	8585	7464	6101	1043	10042
−5:5D	7977	9494	10538	11552	2235	1299	20594	783	2215	1212	2013	1338	1275	896	1088
−3:6D	3627	3546	5102	5380	4966	504	203	15428	1297	2394	1503	932	787	278	9151
−8:8D	3992	5308	5173	5423	5111	1896	219	4905	1109	5396	1092	941	968	375	1941
−2:1D	1428	2030	2127	3037	3418	10300	485	160	1414	2195	2112	1104	2161	172	1895
−10:1I,−6:6D	0	0	0	0	1	0	0	1	31482	0	0	0	0	0	0
−1:7D	166	237	0	0	200	4	22189	1722	89	733	13	242	153	35	0
−4:4I	194	71	317	68	1219	8417	54	42	93	660	127	167	161	52	26
−1:5I	253	0	0	346	0	32	0	0	49	414	130	65	48	0	10027
−2:8D	430	0	0	0	258	44	18	2868	6282	71	263	80	56	31	27
−5:3D	2	2	0	1	648	7929	0	0	73	1	1	35	37	1	361
−5:11D	362	0	0	541	94	6299	27	9	0	265	37	78	20	25	137
−2:9I	0	123	338	876	124	35	5	4991	0	0	14	0	24	0	0
−2:19I	0	0	0	0	0	0	0	4660	0	32	1	0	0	0	0
−6:11D	0	0	0	0	4520	13	0	25	0	0	0	6	0	0	0
−4:3I,2:1D	0	0	0	0	0	4247	0	0	0	0	0	0	0	0	0
−2:6I,1:3I	0	0	0	0	1	0	0	0	0	3839	0	0	1	0	0
−1:22I	1	0	0	0	0	0	3180	0	0	0	0	0	155	0	14


non−injected−1
non−injected−2
non−injected−3
non−injected−4
hypoplasia/low T4−1
hypoplasia/low T4−2
hypoplasia/low T4−3
hypoplasia/low T4−4
hypoplasia/low T4−5
hypoplasia/low T4−6
no phenotype−1
no phenotype−2
no phenotype−3
no phenotype−4
no phenotype−5
0.00
0.25
0.50
0.75
1.00
Alleles
Sequences
15 463211
12 464969
10 402020
14 454048
17 52427
17 55592
14 51410
17 46432
15 104792
16 42642
16 49540
15 39917
17 46569
13 58810
14 41576
crispants *tshr* exon 4
no variant
SNV
frameshift indel
inframe indel



	non–injected–1	non–injected–2	non–injected–3	non–injected–4	hypoplasia/decreased T4–1	hypoplasia/decreased T4–2	hypoplasia/decreased T4–3	hypoplasia/decreased T4–4	hypoplasia/decreased T4–5	hypoplasia/decreased T4–6	no phenotype–1	no phenotype–2	no phenotype–3	no phenotype–4	no phenotype–5	no phenotype–6
Reference	47322	51783	22322	41406	57495	79028	85456	47452	35628	41396	76936	84196	167506	84742	50756	44282
no variant	45426	49665	21456	39854	3558	7825	8353	19695	8394	2564	74301	81760	89887	58157	36373	14158
SNV:−1	36	78	24	22	308	404	6183	232	269	1	51	49	348	54	28	867
2:3D	2	0	0	0	9573	1083	2400	3529	1065	2006	0	1	15735	4517	2174	1842
−7:14D	0	1	0	3	4872	3723	609	1523	5359	913	0	0	4512	3191	1259	2201
1:5D	0	0	0	0	4990	483	12093	1901	973	829	0	0	1686	58	1205	1604
1:25I	0	0	0	0	0	25473	14	84	0	0	0	0	0	0	0	0
−12:13D	0	0	0	0	2563	36	237	1368	7	14222	0	0	491	23	220	101
−4:4D	1	0	0	0	3081	7013	6767	59	311	1154	1	0	278	62	0	3
−2:2D	1	0	0	0	181	4342	1091	232	57	0	0	0	1266	1342	66	798
4:8I	0	0	0	0	8	7599	1	0	0	0	0	0	54	0	22	3
2:2I	0	0	0	0	65	70	4343	55	70	114	52	0	235	68	650	533
−2:3D,8:6I	0	0	0	0	0	0	4314	0	0	0	0	0	0	0	0	0
−6:9I,−4:4D	0	0	0	0	0	0	4308	0	0	0	0	0	0	0	0	0
−3:11D	0	0	0	0	0	0	1	64	3230	2	0	0	0	0	0	1
−4:1I	0	0	0	0	0	0	0	0	2531	0	0	0	0	11	0	0
−22:22D	0	0	0	0	0	0	1	0	2461	0	0	0	0	0	0	0



	Alleles	Sequences
non−injected−1	5	45466
non−injected−2	3	49744
non−injected−3	2	21480
non−injected−4	3	39879
hypoplasia/decreased T4−1	10	29199
hypoplasia/decreased T4−2	11	58051
hypoplasia/decreased T4−3	15	50715
hypoplasia/decreased T4−4	11	28742
hypoplasia/decreased T4−5	12	24727
hypoplasia/decreased T4−6	9	21805
no phenotype−1	4	74405
no phenotype−2	3	81810
no phenotype−3	10	114492
no phenotype−4	10	67483
no phenotype−5	9	41997
no phenotype−6	11	22111

Hiseq sequencing data tables and reads statistic.

A)

		e)	
i)	h)	f)	a)
			b)
		g)	c)
			d)

B)

		nkx2.4b ex1	
pool 1	barcode 1	27634	311923
			290449
		10%	284435
			6014

A) Scheme representing the organisation of the data tables: a) Raw number of total reads pairs generated during Hiseq sequencing experiment. b) Number of sequences extracted from the raw data and used for analysis. c) Number of sequences successfully mapped on the genomic regions of interest. d) Number sequences unsuccessfully mapped on the genomic regions of interest. e) Genomic region of interest. f) Number of mapped reads corresponding to the genomic region of interest in (e). g) Proportion of a specific genomic region mapped reads on the total of Hiseq sequencing run reads. h) Barcode library reference for a specific set of samples. i) Hiseq sequencing run reference.

B) This example coming from the 1st barcode library generated and used in the first run of NGS experiment illustrates how one can read the data tables presented here. This NGS run generated 311923 reads, among those, 290449 could be used for analysis. Analysis showed that 284435 reads were mapped on the different regions of interest while 6014 could not be mapped. Out of those 284435 mapped reads 27634 could be mapped on the nkx2.4b exon1 targeted region. This number represents around 10% of the mapped reads from this experiment.

Important note on the analysis:

1) Note that the number of mapped reads reported in the following tables might be slightly higher than the total number of reads reported in CrispRVariant plots (first row). This is due to CrispRVariant analyses that excludes reads that do not fully span the target window used to summarize mutations for a given amplicon.
2) Note that the sum of the mapped reads might be slightly lower that the total number of mapped reads. This is due to off-target reads that could be mapped on the genome of the zebrafish but not on any of our genomic regions of interest.

		nkx2.4b ex1	*nkx2.4b* ex2	*pax2a*	*Duox* ex23	*duoxa* ex2	*tshr* ex10	*tshr* ex4	Unrellated genes	
Hiseq sequencing pool number 1	barcode1	27634	31620	26897	31979	30837	47331	N/A	87680	311923 290449
		10%	11%	9%	11%	11%	17%	N/A	31%	284435 6014
	barcode2	34248	42630	45534	44827	44120	51791	N/A	103777	383709 369209
		9%	12%	12%	12%	12%	14%	N/A	28%	367165 2044
	barcode3	18827	22136	30775	41469	29698	22327	N/A	79966	251393 246266
		8%	9%	13%	17%	12%	9%	N/A	33%	245383 883
	barcode4	20309	30839	35138	31209	37855	41416	N/A	94699	305698 294573
		7%	11%	12%	11%	13%	14%	N/A	32%	291627 2946
	barcode5	N/A	N/A	N/A	N/A	39548	N/A	N/A	237629	300855 279833
		N/A	N/A	N/A	N/A	14%	N/A	N/A	86%	277513 2320
	barcode6	N/A	N/A	N/A	N/A	47499	N/A	N/A	372064	517319 464391
		N/A	N/A	N/A	N/A	11%	N/A	N/A	87%	428781 35610
	barcode7	N/A	N/A	N/A	N/A	19555	N/A	N/A	146163	178411 168333
		N/A	N/A	N/A	N/A	12%	N/A	N/A	88%	166187 2146
	barcode8	N/A	N/A	N/A	N/A	11929	N/A	N/A	82630	104141 97050
		N/A	N/A	N/A	N/A	13%	N/A	N/A	87%	94703 2347

		nkx2.4b ex1	*nkx2.4b* ex2	*pax2a*	*Duox* ex23	*duoxa* ex2	*tshr* ex10	*tshr* ex4	Unrellated genes	
Hiseq sequencing pool number 2	barcode1	167333	157614	158825	113132	241650	66333	N/A	574859	1515679 1483691
		11%	11%	11%	8%	16%	4%	N/A	39%	1480170 3521
	barcode2	118677	103410	119374	60543	185064	80453	N/A	560086	1251645 1230863
		10%	8%	10%	5%	15%	7%	N/A	46%	1227690 3173
	barcode3	141879	153593	134503	54679	185980	94678	N/A	570641	1359848 1339316
		11%	11%	10%	4%	14%	7%	N/A	43%	1336161 3155
	barcode4	61284	75101	94298	73743	143437	48962	N/A	362124	887505 861800
		7%	9%	11%	9%	17%	6%	N/A	42%	859002 2798
	barcode5	132492	160579	180481	97663	203510	37577	N/A	525620	1358958 1340590
		10%	12%	13%	7%	15%	3%	N/A	39%	1338015 2575
	barcode6	162274	138317	160662	194754	256055	43682	N/A	547955	1530808 1508150
		11%	9%	11%	13%	17%	3%	N/A	36%	1503778 4372
	barcode7	233132	222020	94298	336642	329571	76950	N/A	861336	2382353 2337036
		10%	10%	4%	14%	14%	3%	N/A	37%	2330502 6534
	barcode8	230917	170524	132492	264755	359011	84203	N/A	919718	2331461 2288821
		10%	7%	6%	12%	16%	4%	N/A	40%	2282616 6205
	barcode9	250798	254568	97663	194652	318695	170679	N/A	944916	2436898 2405437
		10%	11%	4%	8%	13%	7%	N/A	39%	2399410 6027
	barcode10	285564	300872	202930	184546	412454	84971	N/A	1058048	2664509 2620212
		11%	12%	8%	7%	16%	3%	N/A	40%	2614461 5751
	barcode11	150956	153261	44130	103825	247152	50893	N/A	571775	1496017 1472009
		10%	10%	3%	7%	17%	3%	N/A	39%	1468389 3620
	barcode12	128164	205185	160662	128456	220169	46332	N/A	501095	1409077 1386484
		9%	15%	12%	9%	16%	3%	N/A	36%	1382764 3720

		nkx2.4b ex1	*nkx2.4b* ex2	*pax2a*	*Duox* ex23	*duoxa* ex2	*tshr* ex10	*tshr* ex4	Unrellated genes	
Hiseq sequencing pool number 3	barcode1	N/A	N/A	N/A	N/A	N/A	N/A	501161	N/A	540387 503599
		N/A	N/A	N/A	N/A	N/A	N/A	100%	N/A	501264 2335
	barcode2	N/A	N/A	N/A	N/A	N/A	N/A	500742	N/A	556699 504044
		N/A	N/A	N/A	N/A	N/A	N/A	100%	N/A	500843 3201
	barcode3	N/A	N/A	N/A	N/A	N/A	N/A	436873	N/A	474455 438540
		N/A	N/A	N/A	N/A	N/A	N/A	100%	N/A	436924 1616
	barcode4	N/A	N/A	N/A	N/A	N/A	N/A	498319	N/A	552900 501444
		N/A	N/A	N/A	N/A	N/A	N/A	100%	N/A	498645 2799
	barcode5	N/A	N/A	N/A	N/A	N/A	N/A	75723	258786	352047 339806
		N/A	N/A	N/A	N/A	N/A	N/A	23%	77%	334673 5133
	barcode6	N/A	N/A	N/A	N/A	N/A	N/A	62429	283350	400162 374983
		N/A	N/A	N/A	N/A	N/A	N/A	18%	82%	346415 28568
	barcode7	N/A	N/A	N/A	N/A	N/A	N/A	58862	311639	378762 370965
		N/A	N/A	N/A	N/A	N/A	N/A	16%	84%	370550 415
	barcode8	N/A	N/A	N/A	N/A	N/A	N/A	50357	250810	318162 302204
		N/A	N/A	N/A	N/A	N/A	N/A	17%	83%	301209 995
	barcode9	N/A	N/A	N/A	N/A	N/A	N/A	128219	262062	412041 393245
		N/A	N/A	N/A	N/A	N/A	N/A	33%	67%	390403 2842
	barcode10	N/A	N/A	N/A	N/A	N/A	N/A	63494	263287	337536 327778
		N/A	N/A	N/A	N/A	N/A	N/A	19%	81%	326823 955
	barcode11	N/A	N/A	N/A	N/A	N/A	N/A	62188	298405	371673 361404
		N/A	N/A	N/A	N/A	N/A	N/A	17%	83%	360630 774
	barcode12	N/A	N/A	N/A	N/A	N/A	N/A	53214	338873	412222 392883
		N/A	N/A	N/A	N/A	N/A	N/A	14%	86%	392125 758
	barcode13	N/A	N/A	N/A	N/A	N/A	N/A	56137	268006	333838 324973
		N/A	N/A	N/A	N/A	N/A	N/A	17%	83%	324234 739
	barcode14	N/A	N/A	N/A	N/A	N/A	N/A	63064	256022	330028 320154
		N/A	N/A	N/A	N/A	N/A	N/A	20%	80%	319160 994
	barcode15	N/A	N/A	N/A	N/A	N/A	N/A	55541	262144	324996 318001
		N/A	N/A	N/A	N/A	N/A	N/A	17%	83%	317731 270

NGS ORF alignments

pax2a exon 2 HiSeq sequencing most frequent alleles alignment:

```
a) ref rna: ATG|GAT|ATT|CAC|TGC|AAA|GCA|GAC|CCC|TTC|TCG|GCG|ATG|CAC|CGG|CAC|GGC|GGT|GTG|AAC|CAG|CTA|GGA|GGG|GTG|TTT|GTG|AAT|GGC|AGA|CCC|CTA|CCT|GAC|GTG|GTC|AGG|CAA|
   ref aa:   M   D   I   H   C   K   A   D   P   F   S   A   M   H   R   H   G   G   V   N   Q   L   G   G   V   F   V   N   G   R   P   L   P   D   V   V   R   Q
1) mut rna: ATG|GAT|ATT|CAC|TGC|AAA|GCA|GAC|CCC|TTC|TCG|GCG|ATG|CAC|CGG|CAC|GGC|GGT|GTG|AAC|CAG|GAG|GGG|TGT|TTG|TGA
   mut aa:   M   D   I   H   C   K   A   D   P   F   S   A   M   H   R   H   G   G   V   N   Q   E   G   C   L   *
2) mut rna: ATG|GAT|ATT|CAC|TGC|AAA|GCA|GAC|CCC|TTC|TCG|GCG|ATG|CAC|CGG|CAC|GGC|GGT|GTG|AAC|CAG|GGG|TGT|TTG|TGA
   mut aa :  M   D   I   H   C   K   A   D   P   F   S   A   M   H   R   H   G   G   V   N   Q   G   C   L   *
3) mut rna: ATG|GAT|ATT|CAC|TGC|AAA|GCA|GAC|CCC|TTC|TCG|GCG|ATG|CAC|CGG|CAC|GGC|GGT|GTG|AAC|CAG|CTT|AGG|AGG|GGT|GTT|TGT|GAA|TGG|CAG|ACC|CCT|ACC|TGA
   mut aa :  M   D   I   H   C   K   A   D   P   F   S   A   M   H   R   H   G   G   V   N   Q   L   R   R   G   V   C   E   W   Q   T   P   T   *
```

a) Reference transcript (ENSDARG00000028148).
1) Deletion of 4nt (AGCT). Occurrence: 14-50%.
2) Deletion of 7nt (CAGCTAGG). Occurrence: 1-13%.
3) Insertion of 1nt (T). Occurrence: 1-6%.

tshr exon 4 HiSeq sequencing most frequent alleles alignment:

```
a) ref rna:...AAG|CTG|ACA|TTC|ATC|CAC|CCA|GAG|GCT|TTT|AAG|AAT|CTC|CCT|AAT|TTA|CAG|TAT|CTA|GGA|TTA|TTC|AAC| ...
   ref aa:  ...K   L   T   F   I   H   P   E   A   F   K   N   L   P   N   L   Q   Y   L   G   L   F   N   ...
1) mut rna:...AAG|CTG|ACA|TTC|ATC|CAG|AGG|CTT|TTA|AGA|ATC|TCC|CTA|ATT|TAC|AGT|ATC|TAG
   mut aa:  ...K   L   T   F   I   Q   R   L   L   R   I   S   L   I   Y   S   I   *
2) mut rna:...AAG|CTG|ACA|TTC|ATC|AGA|GGC|TTT|TAA
   mut aa: ... K   L   T   F   I   R   G   F   *
3) mut rna:...AAG|CTG|ACA|TTC|ATC|CAC|CAT|TTA|AGA|ATC|TCC|CTA|ATT|TAC|AGT|ATC|TAG
   mut aa: ... K   L   T   F   I   H   H   L   R   I   S   L   I   Y   S   I   *
```

a) Reference transcript (ENSDARG00000037195).
1) Deletion of 4nt (CCAC). Occurrence: 6.6-44.6%.
2) Deletion of 5nt (CCACC). Occurrence: 1.6-35%.
3) Deletion of 7nt (CCAGAGG). Occurrence: 0-37.8%.

tshr exon 10 HiSeq sequencing most frequent alleles alignment:

```
a) ref rna:... TTG|AGT|GAT|AAC|CTC|TTC|AGA|GAC|ACC|TAT|GAT|CAC|CAG|GAG|TTT|TTC|GGC|AAC|GGA|CAT|TAT|CCC|ACT|TAT|TTT|GAC|AGA|CAT|GCA|GAT|GAG|AAC|ATG|GGT|TTC|GGT...
   ref aa: ...  L   S   D   N   L   F   R   D   T   Y   D   H   Q   E   F   F   G   N   G   H   Y   P   T   Y   F   D   R   H   A   D   E   N   M   G   F   G ...
1) mut rna:... TTG|AGT|GAT|AAC|CTC|    |AGA|GAC|ACC|TAT|GAT|CAC|CAG|GAG|TTT|TTC|GGC|AAC|GGA|CAT|TAT|CCC|ACT|TAT|TTT|GAC|AGA|CAT|GCA|GAT|GAG|AAC|ATG|GGT|TTC|GGT...
   mut aa: ...  L   S   D   N   L       R   D   T   Y   D   H   Q   E   F   F   G   N   G   H   Y   P   T   Y   F   D   R   H   A   D   E   N   M   G   F   G ...
2) mut rna:... TTG|AGT|GAT|AAC|CTA|TGA
   mut aa: ...  L   S   D   N   L   *
3) mut rna:... TTG|AGT|GAT|AAC|CAG|AGA|CAC|CTA|TGA
   mut aa: ...  L   S   D   N   Q   R   H   L   *
4) mut rna:... TTG|AGT|GAT|AAC|CTC|TTA|TCA|CTC|AGA|GAC|ACC|TAT|GAT|AAC|CAG|AGA|CAC|CTA|TGA
   mut aa: ...  L   S   D   N   L   L   S   L   R   D   T   Y   D   N   Q   R   H   L   *
5) mut rna:... TTG|AGT|GAT|AAC|CTC|TTG|ATC|ACC|AGG|AGT|TTT|TCG|GCA|ACG|GAC|ATT|ATC|CCA|CTT|ATT|TTG|ACA|GAC|ATG|CAG|ATG|AGA|ACA|TGG|GTT|TCG|GTG|AAA|CAC|TGA
   mut aa: ...  L   S   D   N   L   L   I   T   R   S   F   S   A   T   D   I   I   P   L   I   L   T   D   M   Q   M   R   T   W   V   S   V   K   H   *
```

a) Reference transcript (ENSDARG00000037195).
1) Deletion of 3nt (TCT), no frameshift induced. Occurrence: 1.4-16.7%.
2) Deletion of 14nt (ACCTCTTCAGAGAC). Occurrence: 0.8-15%.
3) Deletion of 5nt (CTCTT). Occurrence: 0.6-14.2%.
4) Insertion of 25nt (ATCACTCAGAGACACCTATGATAAC). Occurrence: 0-32.2%.
5) Deletion of 12nt (TCAGAGACACCTA). Occurrence: 0.1-34.4%

duox exon 23 HiSeq sequencing most frequent alleles alignment:

```
a) ref rna:...GTC|ATC|TAC|GCC|ATC|TCC|GCC|GGC|CTC|GCT|TTG|GAA|AGA|TGC|ATC|TAT|TAT|GGT|TTA|CAG|GCT|CAC|TCC|AGT|GGC|ATC|CCA|GAG|ACC|TCT|ATG|GTG|GGT|GTG|TTG|GTG|TCT|CGT
   ref aa: ... V   I   Y   A   I   S   A   G   L   A   L   E   R   C   I   Y   Y   G   L   Q   A   H   S   S   G   I   P   E   T   S   M   V   G   V   L   V   S   R
   ref rna:   GGC|TCA|GCG|GCC|GCC|ATT|TCG|TTC|CTG|TTC|CCC|TAC|ATG|CTG|CTG|ACT|GTG|TGC|CGA|AAC| ...
   ref aa:     G   S   A   A   A   I   S   F   L   F   P   Y   M   L   L   T   V   C   R   N   ...
1) mut rna:   GTC|ATC|TAC|GCC|GGC|           |CTC|GCT|TTG|GAA|AGA|TGC|ATC|TAT|TAT|GGT|TTA|CAG|GCT|CAC|TCC|AGT|GGC|ATC|CCA|GAG|ACC|TCT|ATG|GTG|GGT|GTG|TTG|GTG|TCT|CGT
   mut aa:     V   I   Y   A   G               L   A   L   E   R   C   I   Y   Y   G   L   Q   A   H   S   S   G   I   P   E   T   S   M   V   G   V   L   V   S   R
   mut rna:   GGC|TCA|GCG|GCC|GCC|ATT|TCG|TTC|CTG|TTC|CCC|TAC|ATG|CTG|CTG|ACT|GTG|TGC|CGA|AAC| ...
   mut aa:     G   S   A   A   A   I   S   F   L   F   P   Y   M   L   L   T   V   C   R   N   ...
2) mut rna:...GTC|ATC|TAC|GCC|ATC|TCG|CTT|TGG|AAA|GAT|GCA|TCT|ATT|ATG|GTT|TAC|AGG|CTC|ACT|CCA|GTG|GCA|TCC|CAG|AGA|CCT|CTA|TGG|TGG|GTG|TGT|TGG|TGT|CTC|GTG|GCT|CAG|CGG
   mut aa: ... V   I   Y   A   I   S   L   W   K   D   A   S   I   M   V   Y   R   L   T   P   V   A   S   Q   R   P   L   W   W   V   C   W   C   L   V   A   Q   R
   mut rna:   CCG|CCA|TTT|CGT|TCC|TGT|TCC|CCT|ACA|TGC|TGC|TGA
   mut aa:     P   P   F   R   S   C   S   P   T   C   C   *
3) mut rna:...GTC|ATC|TAC|GCC|ATC|TCG|CCG|GCC|TCG|CTT|TGG|AAA|GAT|GCA|TCT|ATT|ATG|GTT|TAC|AGG|CTC|ACT|CCA|GTG|GCA|TCC|CAG|AGA|CCT|CTA|TGG|TGG|GTG|TGT|TGG|TGT|CTC|GTG
   mut aa: ... V   I   Y   A   I   S   P   A   S   L   W   K   D   A   S   I   M   V   Y   R   L   T   P   V   A   S   Q   R   P   L   W   W   V   C   W   C   L   V
   mut rna:   GCT|CAG|CGG|CCG|CCA|TTT|CGT|TCC|TGT|TCC|CCT|ACA|TGC|TGC|TGA
   mut aa:     A   Q   R   P   P   F   R   S   C   S   P   T   C   C   *
```

a) Reference transcript (ENSDARG00000062632)
1) Deletion of 9nt (CGCCATCTC), no frameshift induced. Occurrence: 18-61.9%.
2) Deletion of 10nt (CTCCGCCGGC). Occurrence: 1.5-18.4%
3) Deletion of 1nt (C). Occurrence: 0-18%

Duoxa (duox2) exon 2 HiSeq sequencing most frequent alleles alignment:

```
a) ref rna:...GTC|AGT|TTC|CTC|TTC|ATT|CTA|CCG|GGA|ATA|CGT|GGC|AGA|TCG|AGA|TGG|TTT|TGG|ATG|TTC|CGG|ATA|TTC|ATT|AGC|TTA|TTT|ATT|GGA|GTT|GTT|TTA|GTT|GTG|CTT|AAT| ...
   ref aa: ... V   S   F   L   F   I   L   P   G   I   R   G   R   S   R   W   F   W   M   F   R   I   F   I   S   L   F   I   G   V   V   L   V   V   L   N   ...
1) mut rna:...GTC|AGT|TTC|CTC|TTC|TAC|CGG|GAA|TAC|GTG|GCA|GAT|CGA|GAT|GGT|TTT|GGA|TGT|TCC|GGA|TAT|TCA|TTA|GCT|TAT|TTA|TTG|GAG|TTG|TTT|TAG
   mut aa: ... V   S   F   L   F   Y   R   E   Y   V   A   D   R   D   G   F   G   C   S   G   Y   S   L   A   Y   L   L   E   L   F   *
2) mut rna:...GTC|AGT|TTC|CTC|TAC|CGG|GAA|TAC|GTG|GCA|GAT|CGA|GAT|GGT|TTT|GGA|TGT|TCC|GGA|TAT|TCA|TTA|GCT|TAT|TTA|TTG|GAG|TTG|TTT|TAG
   mut aa: ... V   S   F   L   Y   R   E   Y   V   A   D   R   D   G   F   G   C   S   G   Y   S   L   A   Y   L   L   E   L   F   *
3) mut rna:...GTC|AGT|TTC|CTC|   |ATT|CTA|CCG|GGA|ATA|CGT|GGC|AGA|TCG|AGA|TGG|TTT|TGG|ATG|TTC|CGG|ATA|TTC|ATT|AGC|TTA|TTT|ATT|GGA|GTT|GTT|TTA|GTT|GTG|CTT|AAT| ...
   mut aa: ... V   S   F   L       I   L   P   G   I   R   G   R   S   R   W   F   W   M   F   R   I   F   I   S   L   F   I   G   V   V   L   V   V   L   N   ...
4) mut rna:...GTC|AGT|TTC|CTC|TTC|TAT|TCT|ACC|GGG|AAT|ACG|TGG|CAG|ATC|GAG|ATG|GTT|TTG|GAT|GTT|CCG|GAT|ATT|CAT|TAG
   mut aa: ... V   S   F   L   F   Y   S   T   G   N   T   W   Q   I   E   M   V   L   D   V   P   D   I   H   *
5) mut rna:...GTC|AGT|TTC|CTC|CTT|   |CTA|CCG|GGA|ATA|CGT|GGC|AGA|TCG|AGA|TGG|TTT|TGG|ATG|TTC|CGG|ATA|TTC|ATT|AGC|TTA|TTT|ATT|GGA|GTT|GTT|TTA|GTT|GTG|CTT|AAT| ...
   mut aa: ... V   S   F   L   L       L   P   G   I   R   G   R   S   R   W   F   W   M   F   R   I   F   I   S   L   F   I   G   V   V   L   V   V   L   N   ...
```

a) Reference transcript (ENSDARG00000078962)
1) Deletion of 4nt (GAAT). Occurrence: 9.9-43.4%.
2) Deletion of 7nt (AGAATGA). Occurrence: 0.6-22.2%.
3) Deletion of 3nt (GAA), no frameshift induced. Occurrence: 4.4-22.1%
4) Insertion of 1nt (T). Occurrence: 0-21.1%.
5) Deletion of 3nt (ATG), no frameshift induced. Occurrence: 0-24.4%.

nkx2.4b exon 1 HiSeq sequencing most frequent alleles alignment:

```
a) Ref rna:... GGC|GCC|ACG|GCC|ACG|GCT|TGG|TAC|GGC|TCG|AAC|CCG|GAG|CCG|AGA|TAC|CCA|ACA|ATC|TCC|AGG|TTT|ATG|GGT|CCC|TCG|GCG|GGC|ATG|AAC|ATG|GGC|ACG|TTA|CCG|GGA|ATG|GAC|GCC|AGT...
   ref aa: ...  G   A   T   A   T   A   W   Y   G   S   N   P   E   P   R   Y   P   T   I   S   R   F   M   G   P   S   A   G   M   N   M   G   T   L   P   G   M   D   A   S ...
1) mut rna:... GGC|GCC|ACG|GCC|ACG|GTA|CGG|CTC|GAA|CCC|GGA|GCC|GAG|ATA|CCC|AAC|AAT|CTC|CAG|GTT|TAT|GGG|TCC|CTC|GGC|GGG|CAT|GAA|CAT|GGG|CAC|GTT|ACC|GGG|AAT|GGA|CGC|CAG|TAA
   mut aa: ...  G   A   T   A   T   V   R   L   E   P   G   A   E   I   P   N   N   L   Q   V   Y   G   S   L   G   G   H   E   H   G   H   V   T   G   N   G   R   Q   *
2) mut rna:... GCC|ACG|GCC|ACG|GCT|CGA|ACC|CGG|AGC|CGA|GAT|ACC|CAA|CAA|TCT|CCA|GGT|TTA|TGG|GTC|CCT|CGG|CGG|GCA|TGA
   mut aa: ...  A   T   A   T   A   R   T   R   S   R   D   T   Q   Q   S   P   G   L   W   V   P   R   R   A   *
3) mut rna:... AGA|AAC|GGC|GCC|ACG|GTA|CGG|CTC|GAA|CCC|GGA|GCC|GAG|ATA|CCC|AAC|AAT|CTC|CAG|GTT|TAT|GGG|TCC|CTC|GGC|GGG|CAT|GAA|CAT|GGG|CAC|GTT|ACC|GGG|AAT|GGA|CGC|CAG|TAA
   mut aa: ...  R   N   G   A   T   V   R   L   E   P   G   A   E   I   P   N   N   L   Q   V   Y   G   S   L   G   G   H   E   H   G   H   V   T   G   N   G   R   Q   *
4) mut rna:... AGA|AAC|GGC|GCC|ACG|GCT|CGA|ACC|CGG|AGC|CGA|GAT|ACC|CAA|CAA|TCT|CCA|GGT|TTA|TGG|GTC|CCT|CGG|CGG|GCA|TGA
   mut aa: ...  R   N   G   A   T   A   R   T   R   S   R   D   T   Q   Q   S   P   G   L   W   V   P   R   R   A   *
```

a) Reference transcript (ENSDARG00000104107).
1) Deletion of 5nt (GGCTT). Occurrence: 6-32.9%.
2) Deletion of 10nt (ACGGCTTGGT). Occurrence: 1-20%.
3) Deletion of 11nt (GGCCACGGCTT). Occurrence: 0-28.7%.
4) Deletion of 16nt (ACGGCCACGGCTTGGT). Occurrence: 2-27.7%.

nkx2.4b exon 2 HiSeq sequencing most frequent alleles alignment:

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a) ref rna:... CAC|GCG|GCG|CCG|CGC|AGG|AAA|CGG|CGC|GTG|CTT|TTC|TCC|CAA|GCG|CAG|GTA|TAC|GAG|CTG|GAG|CGC|CGC|TTC|AAG|CAG|CAA|AAA|TAC|CTG|TCG|GCC|CCG|GAG|AGG|GAA|CAT|TTG|GCC|AGC|
   ref aa: ...  H   A   A   P   R   R   K   R   R   V   L   F   S   Q   A   Q   V   Y   E   L   E   R   R   F   K   Q   Q   K   Y   L   S   A   P   E   R   E   H   L   A   S
   ref rna:    ATG|ATC|CAT|CTA|ACC|CCG|ACG|CAG|GTC|AAG|ATC|TGG|TTC|CAG|AAC|CAC|AGG|TAC|AAA|ATG|AAG|CGG|GAG|GCG|AAG|GAT|AAA|GCG|TCC|CAG|CAG|CAG| ...
   ref aa:      M   I   H   L   T   P   T   Q   V   K   I   W   F   Q   N   H   R   Y   K   M   K   R   E   A   K   D   K   A   S   Q   Q   Q   ...
1) mut rna:... CAC|GCG|GCG|CGT|GCT|TTT|CTC|CCA|AGC|GCA|GGT|ATA|CGA|GCT|GGA|GCG|CCG|CTT|CAA|GCA|GCA|AAA|ATA|CCT|GTC|GGC|CCC|GGA|GAG|GGA|ACA|TTT|GGC|CAG|CAT|GAT|CCA|TCT|AAC|CCC|
   mut aa: ...  H   A   A   R   A   F   L   P   S   A   G   I   R   A   G   A   P   L   Q   A   A   K   I   P   V   G   P   G   E   G   T   F   G   Q   H   D   P   S   N   P
   mut rna:    GAC|GCA|GGT|CAA|GAT|CTG|GTT|CCA|GAA|CCA|CAG|GTA|CAA|AAT|GAA|GCG|GGA|GGC|GAA|GGA|TAA
   mut aa:      D   A   G   Q   D   L   V   P   E   P   Q   V   Q   N   E   A   G   G   E   G   *
2) mut rna:... CAC|GCG|GCG|CCG|CGC|AAG|GAA|ACG|GCG|CGT|GCT|TTT|CTC|CCA|AGC|GCA|GGT|ATA|CGA|GCT|GGA|GCG|CCG|CTT|CAA|GCA|GCA|AAA|ATA|CCT|GTC|GGC|CCC|GGA|GAG|GGA|ACA|TTT|GGC|CAG|
   mut aa: ...  H   A   A   P   R   K   E   T   A   R   A   F   L   P   S   A   G   I   R   A   G   A   P   L   Q   A   A   K   I   P   V   G   P   G   E   G   T   F   G   Q
   mut rna:    CAT|GAT|CCA|TCT|AAC|CCC|GAC|GCA|GGT|CAA|GAT|CTG|GTT|CCA|GAA|CCA|CAG|GTA|CAA|AAT|GAA|GCG|GGA|GGC|GAA|GGA|TAA
   mut aa:      H   D   P   S   N   P   D   A   G   Q   D   L   V   P   E   P   Q   V   Q   N   E   A   G   G   E   G   *
3) mut rna:... CAC|GCG|GCG|CCG|TGC|TTT|TCT|CCC|AAG|CGC|AGG|TAT|ACG|AGC|TGG|AGC|GCC|GCT|TCA|AGC|AGC|AAA|AAT|ACC|TGT|CGG|CCC|CGG|AGA|GGG|AAC|ATT|TGG|CCA|GCA|TGA
   mut aa: ...  H   A   A   P   C   F   S   P   K   R   R   Y   T   S   W   S   A   A   S   S   K   N   T   C   R   P   R   R   G   N   I   W   P   A   *
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a) Reference transcript (ENSDARG00000104107).
1) Deletion of 17nt (CGGCGCCGCGCAGGAAA). Occurrence: 8.6-26.7%
2) Insertion of 1nt (T). Occurrence: 2.4-12.5%
3) Deletion of 16nt (CGCGCAGGAAACGGCG). Occurrence: 1-37%