

Identifying tumor biomarkers based on Nanopore sequencing DNA methylation data



Daria Meyer

TBI Winterseminar
14.02.2024

How to identify head neck cancer (oral cavity) tumor biomarkers for methylation specific PCR based on low coverage Nanopore sequencing DNA regarding CG-context 5mC methylation data



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Background: Screening for HNSCC (head neck squamous cell carcinoma)

- HNSCC specific DNA methylation (5mC) exist

Research Article

Cancer
Prevention
Research

New DNA Methylation Markers and Global DNA Hypomethylation Are Associated with Oral Cancer Development

Jean-Philippe Foy^{1,2,3}, Curtis R. Pickering⁴, Vassiliki A. Papadimitrakopoulou⁵, Jaroslav Jelinek⁶, Steven H. Lin⁷, William N. William Jr⁵, Mitchell J. Frederick⁴, Jing Wang⁸, Wenhua Lang⁵, Lei Feng⁹, Li Zhang⁸, Edward S. Kim¹⁰, You H. Fan⁵, Waun K. Hong¹¹, Adel K. El-Naggar¹², J. Jack Lee⁹, Jeffrey N. Myers⁴, Jean-Pierre Issa⁶, Scott M. Lippman¹³, Li Mao¹⁴, and Pierre Saintigny^{1,2,15,16}



International Journal of
Molecular Sciences



Article

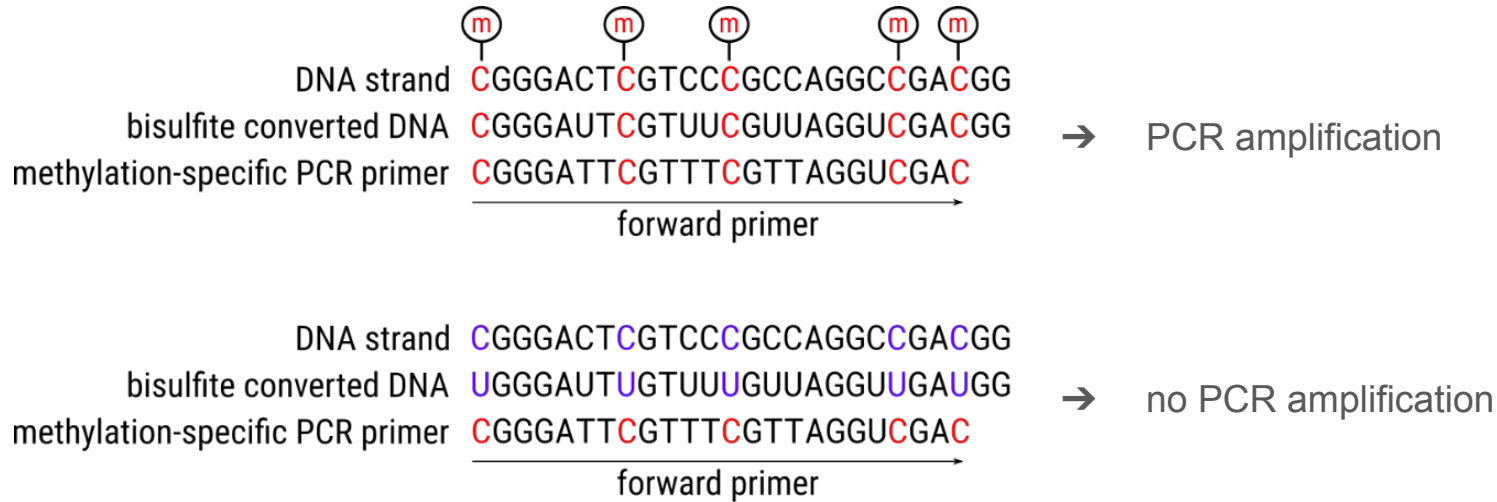
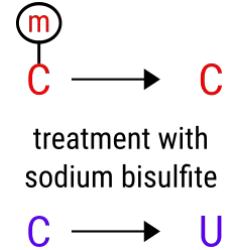
Pre-Operative Evaluation of DNA Methylation Profile in Oral Squamous Cell Carcinoma Can Predict Tumor Aggressive Potential

Davide B. Gissi^{1,†}, Viscardo P. Fabbri^{2,†}, Andrea Gabusi¹, Jacopo Lenzi³, Luca Morandi^{4,*}, Sofia Melotti², Sofia Asioli², Achille Tarsitano⁵, Tiziana Balbi⁶, Claudio Marchetti⁵ and Lucio Montebugnoli¹

How to identify biomarker for early detection of HNSCC?

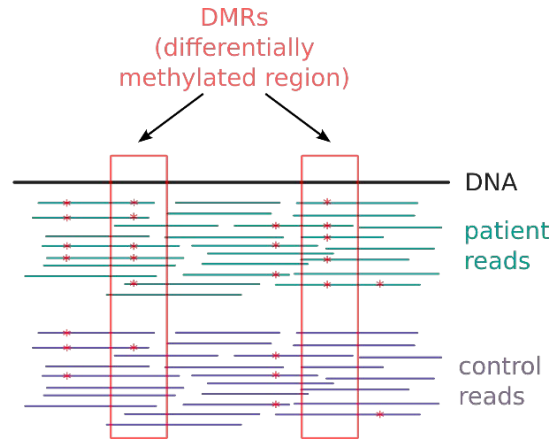
Background: Screening for HNSCC (head neck squamous cell carcinoma)

- HNSCC specific DNA methylation (5mC) exist
- Screening for methylation changes using Methylation Specific PCR (MSP)



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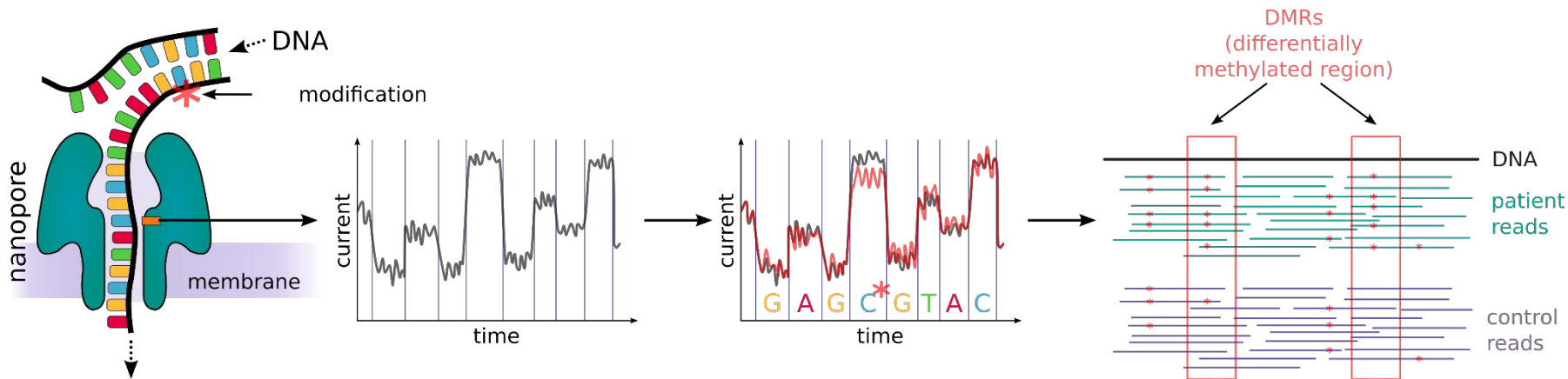


- Detect regions which show methylations in tumor samples but not in control samples
- Similar to detecting DMRs but:
 - **completely** unmethylated in controls (ideally)
 - further primer design constraints (primer length, distance, nucleotide composition,...)

Whole genome ONT sequencing (enriched for CpG islands)

Sample	CGI Coverage	sex	year of birth	tumor cells	origin
T0044C	12.81X	m	1967	80%	oral cavity
T0085C	14.62X	m	1946	80%	oral cavity
T0126C	11.14X	m	1961	70%	oral cavity
T0025N	25.00X	m	1961	0%	oral cavity
T0045N	18.05X	m	1998	0%	oral cavity
T0099N	16.74X	m	1963	0%	oral cavity

How to find PCR primer?



Whole genome ONT sequencing (enriched for CpG islands)

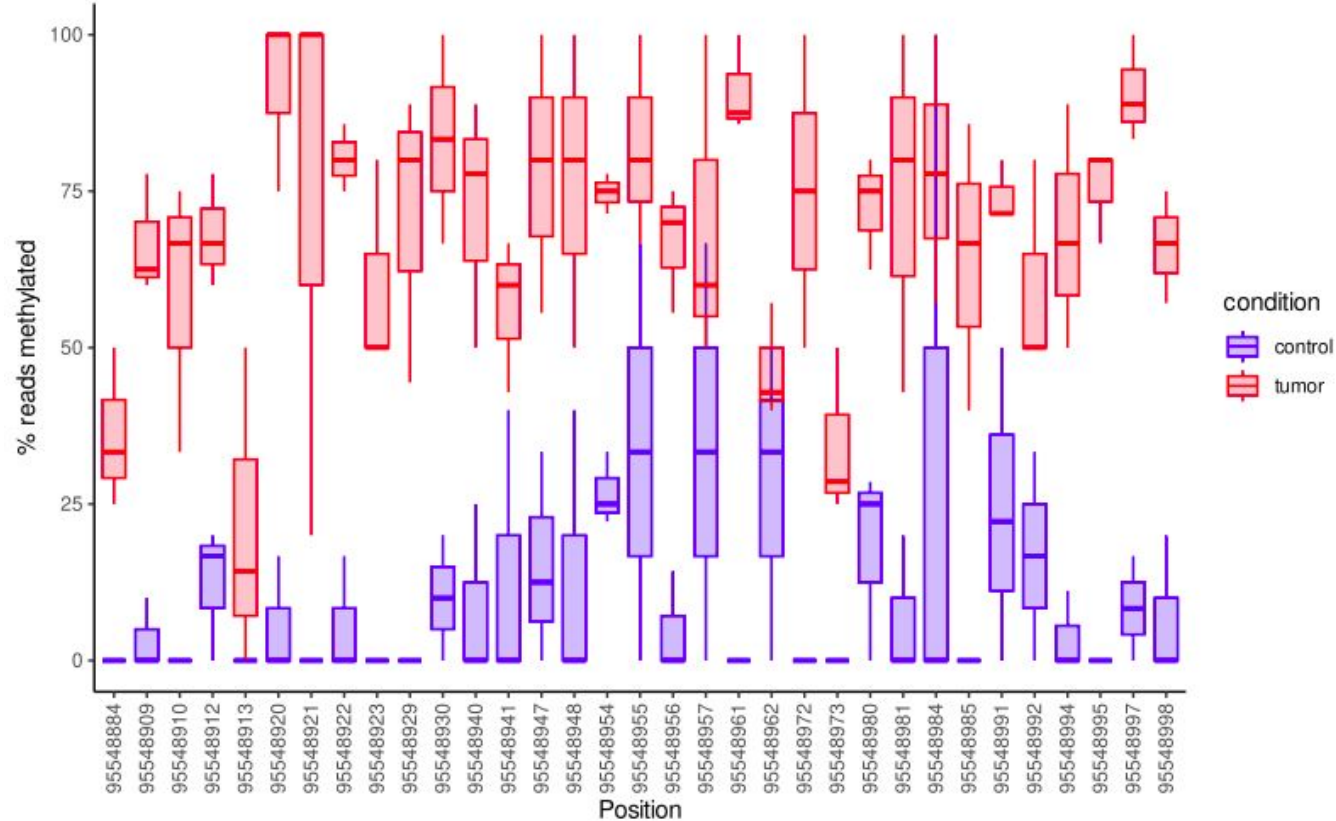
bedmethyl file format

chr3	194688040	194688041	5mC	666	+	194688040	194688041	0,0,0	12	87.50
chr3	194688048	194688049	5mC	833	+	194688048	194688049	0,0,0	12	50.00
chr3	194688053	194688054	5mC	750	+	194688053	194688054	0,0,0	12	44.44
chr3	194688061	194688062	5mC	583	+	194688061	194688062	0,0,0	12	85.71
chr3	194688064	194688065	5mC	666	+	194688064	194688065	0,0,0	12	87.50
chr3	194688067	194688068	5mC	666	+	194688067	194688068	0,0,0	12	75.00
chr3	194688079	194688080	5mC	500	+	194688079	194688080	0,0,0	12	50.00
chr3	194688090	194688091	5mC	750	+	194688090	194688091	0,0,0	12	77.78
chr3	194688105	194688106	5mC	583	+	194688105	194688106	0,0,0	12	85.71
chr3	194688107	194688108	5mC	750	+	194688107	194688108	0,0,0	12	77.78
chr3	194688109	194688110	5mC	666	+	194688109	194688110	0,0,0	12	87.50
chr3	194688112	194688113	5mC	666	+	194688112	194688113	0,0,0	12	87.50
chr3	194688115	194688116	5mC	666	+	194688115	194688116	0,0,0	12	75.00
chr3	194688118	194688119	5mC	666	+	194688118	194688119	0,0,0	12	87.50
chr3	194688126	194688127	5mC	750	+	194688126	194688127	0,0,0	12	66.67
chr3	194688140	194688141	5mC	666	+	194688140	194688141	0,0,0	12	37.50
chr3	194688148	194688149	5mC	916	+	194688148	194688149	0,0,0	12	63.64

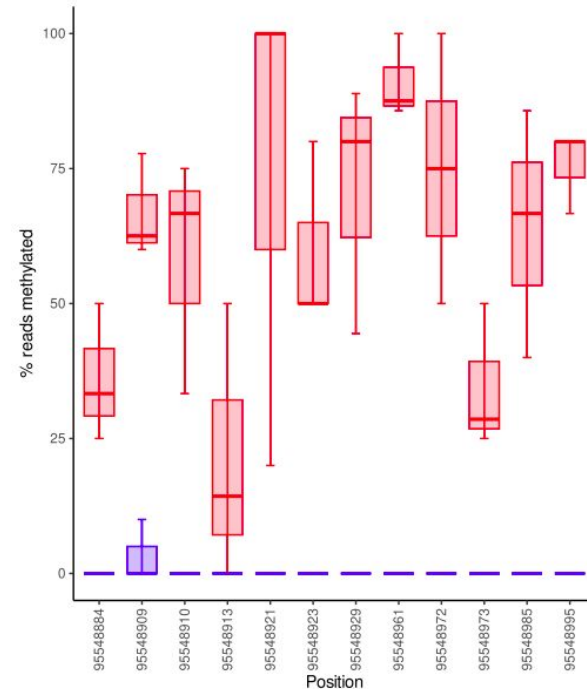
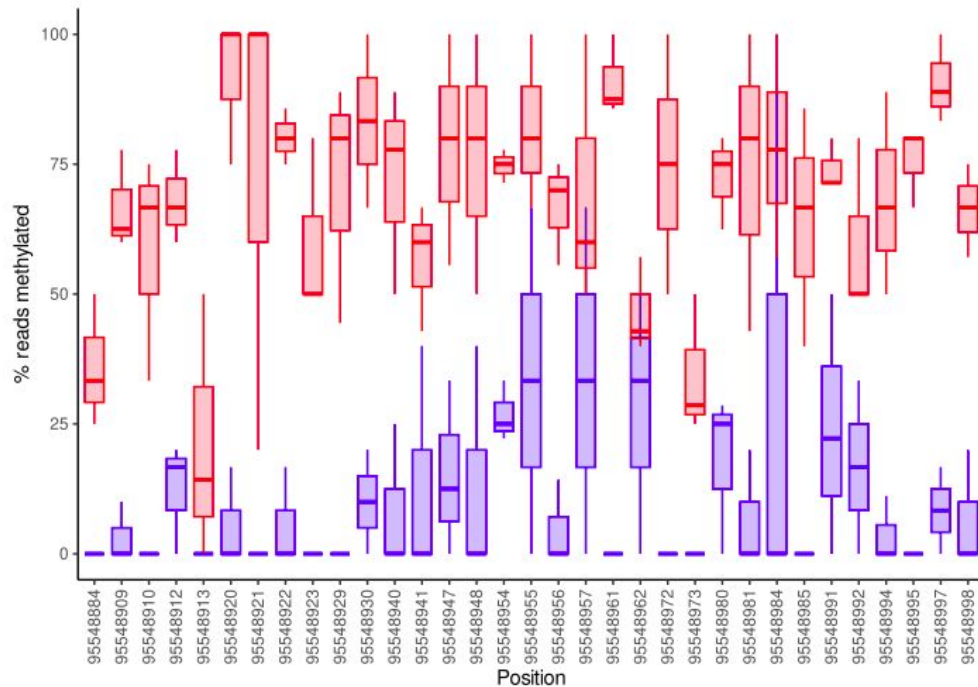
1. Remove uninteresting CpGs

- methylation in control > 10% -> remove CpG
- median methylation of tumor below max methylation in control -> remove CpG

Workflow: Remove uninteresting CpGs



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- methylation in control > 10% -> remove CpG
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2. Find potential primers

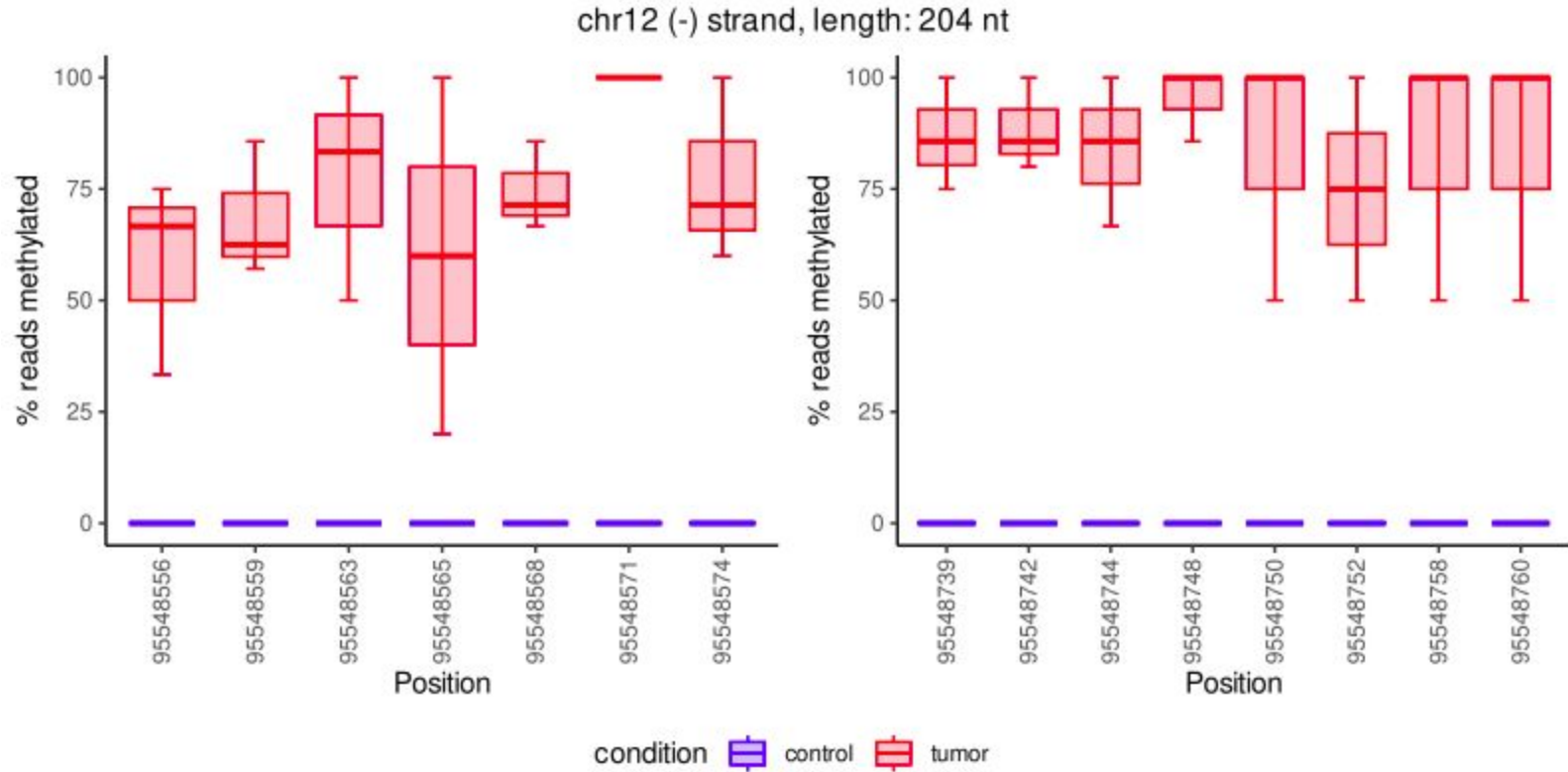
- max 24nt, min 3 CpGs

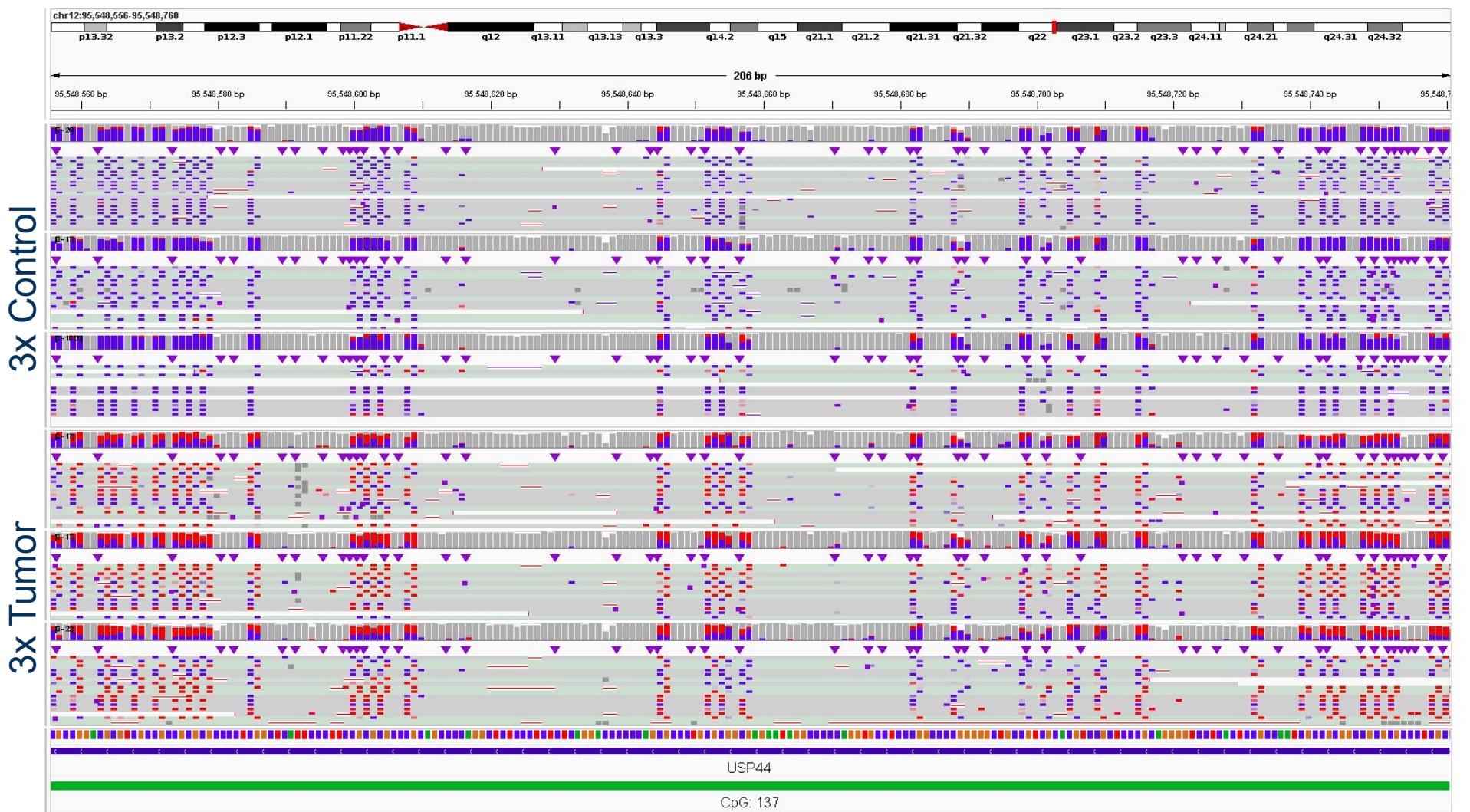
$$\text{score}_{\text{Primer}} = \sum_{\substack{i \text{ in} \\ \text{tumor} \\ \text{samples}}} \sum_{\substack{j \text{ in} \\ \text{primer} \\ \text{CGs}}} [(\text{Cov}_{ij}/\text{Norm}_i) * \text{Meth}_{ij}], \text{ with } \text{Norm}_i = \sum_{\substack{k \text{ in} \\ \text{CpGs in} \\ \text{tumor sample } i}} \text{Cov}_k$$

3. Combine Primer Pairs into PCR Products

- build all potential combinations

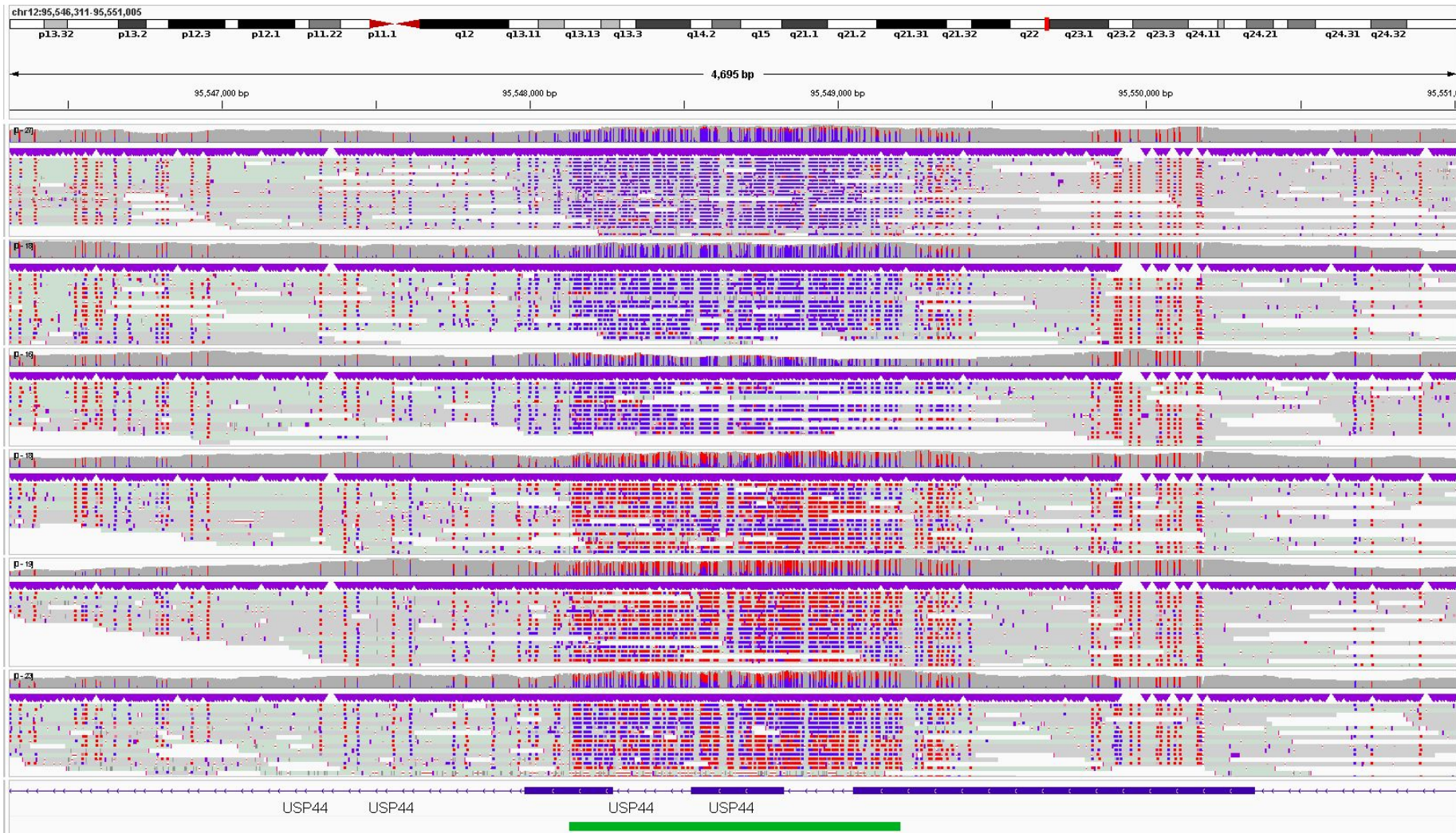
Results: Top hit on chr 12 (USP44)





3x Control

3x Tumor



Results: Many regions known from literature

www.nature.com/nc

Oncogene



ARTICLE



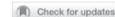
<https://doi.org/10.1038/s41467-022-28158-2>

OPEN

USP44 regulates irradiation-induced DNA double-strand break repair and suppresses tumorigenesis in nasopharyngeal carcinoma

Yang Chen^{1,3}, Yin Zhao^{1,3}, Xiaojing Yang^{1,3}, Xianyue Ren^{2,3}, Shengyan Huang¹, Sha Gong¹, Xirong Tan¹, Junyan Li¹, Shiwei He¹, Yingqin Li¹, Xiaohong Hong¹, Qian Li¹, Cong Ding¹, Xueliang Fang¹, Jun Ma¹ & Na Liu¹✉

ARTICLE



N⁶-methyladenosine demethylase ALKBH5 suppresses malignancy of esophageal cancer by regulating microRNA biogenesis and RAI1 expression

Pengxiang Chen^{1,2,7}, Song Li^{3,7}, Ke Zhang⁴, Renchang Zhao⁵, Jianfeng Cui⁶, Wei Zhou¹, Yuchen Liu¹, Lin Zhang¹✉^{1,5,8} and Yufeng Cheng¹✉^{1,5,8}



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Research Article

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Outlook

Biological:

- Design PCR primers based on predicted regions
- Compare PCR results with ONT data
- Check reproducibility in saliva and swap samples

Computational:

- Check for overlap with CpG islands
- Check for overlap with promoter regions
- Improve the runtime

Thank you for your attention.



Special thanks to:

AG Marz
Manja

Martina Schmitz
Alfred Hansel



Funded by Thüringen-Stipendium

Backup-Slides

Workflow: Remove uninteresting CpGs

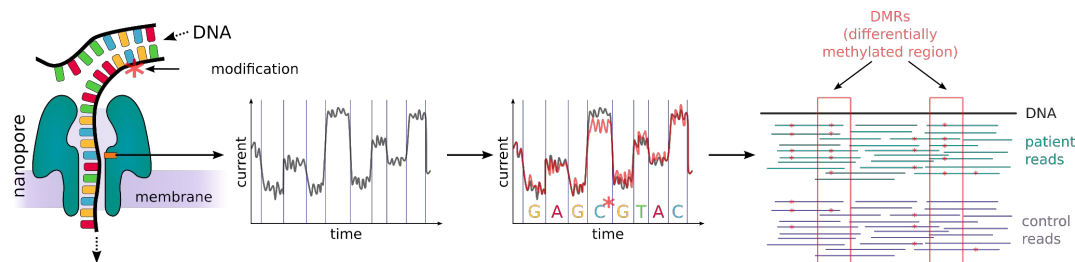
workflow image bam

- bedmethyl introduce file format
 - remove uninteresting CGs
 - build primers
 - score primers
 - build primer combinations
- bedmethyl introduce file format
 - boxplot with overlapping CGs
 - build primers → image??
 - Formula
 - build primer combinations

$$\text{score}_{\text{Primer}} = \sum_{\substack{i \text{ in} \\ \text{tumor} \\ \text{samples}}} \sum_{\substack{j \text{ in} \\ \text{primer} \\ \text{CGs}}} [(\text{Cov}_{ij}/\text{Norm}_i) * \text{Meth}_{ij}] \quad , \quad \text{with } \text{Norm}_i = \sum_{\substack{k \text{ in} \\ \text{CpGs in} \\ \text{tumor sample } i}} \text{Cov}_k$$

Methylation Calling Algorithms

- Different methylation caller exist
- On the context of CpG islands and high GC-content especially Nanopolish and Megalodon are described as well performing methylation callers¹



¹Liu, Y., et al. DNA methylation-calling tools for Oxford Nanopore sequencing: a survey and human epigenome-wide evaluation. *Genome Biol* **22**, 295 (2021).

²Simpson, Jared T., et al. "Detecting DNA cytosine methylation using nanopore sequencing." *nature methods* 14.4 (2017): 407-410.

³Oxford Nanopore Technologies, <https://github.com/nanoporetech/megalodon>.

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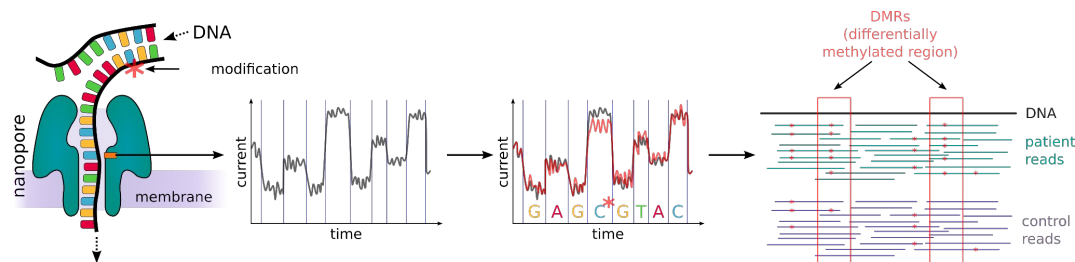
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Nanopolish²:

- HMM approach
- Returns log-likelihood per read and position

Megalodon³:

- Recurrent neural network
- Returns a score per position and read



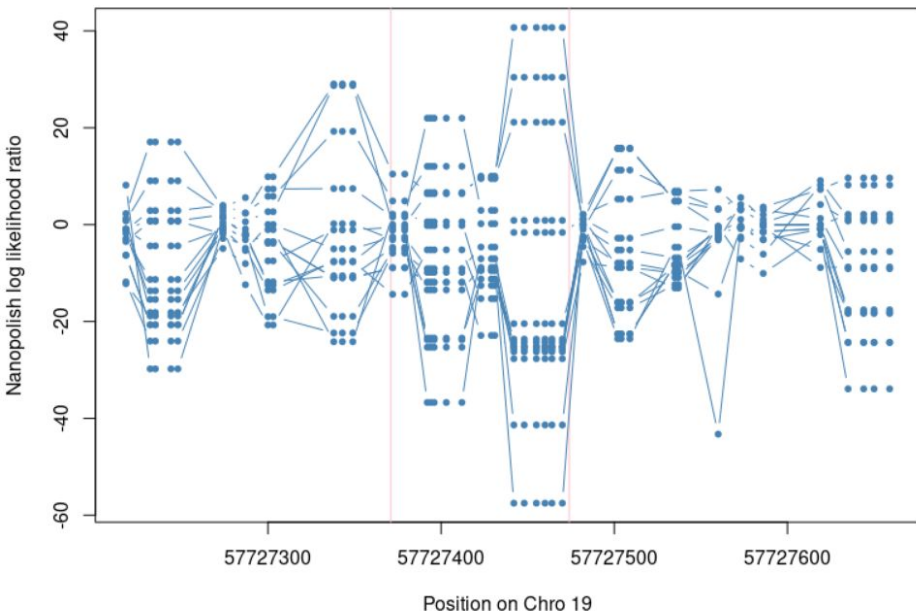
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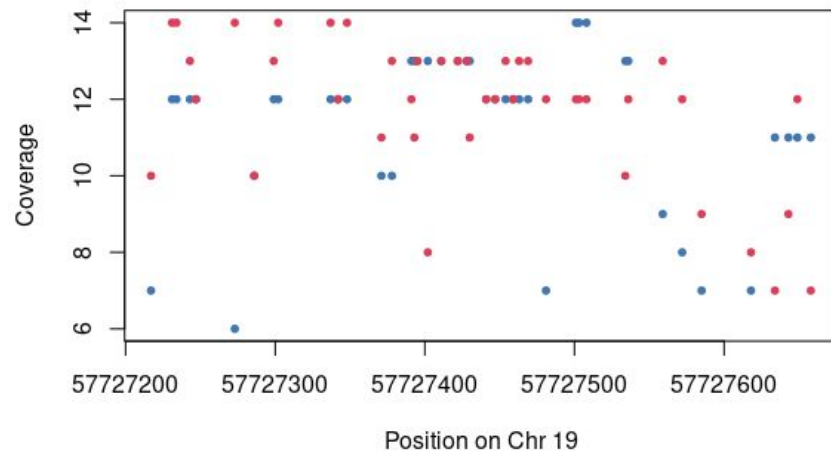
³Oxford Nanopore Technologies, <https://github.com/nanoporetech/megalodon>.

Per CpG methylation within a CpG island

CpG island cgi25860 containing ZNF671

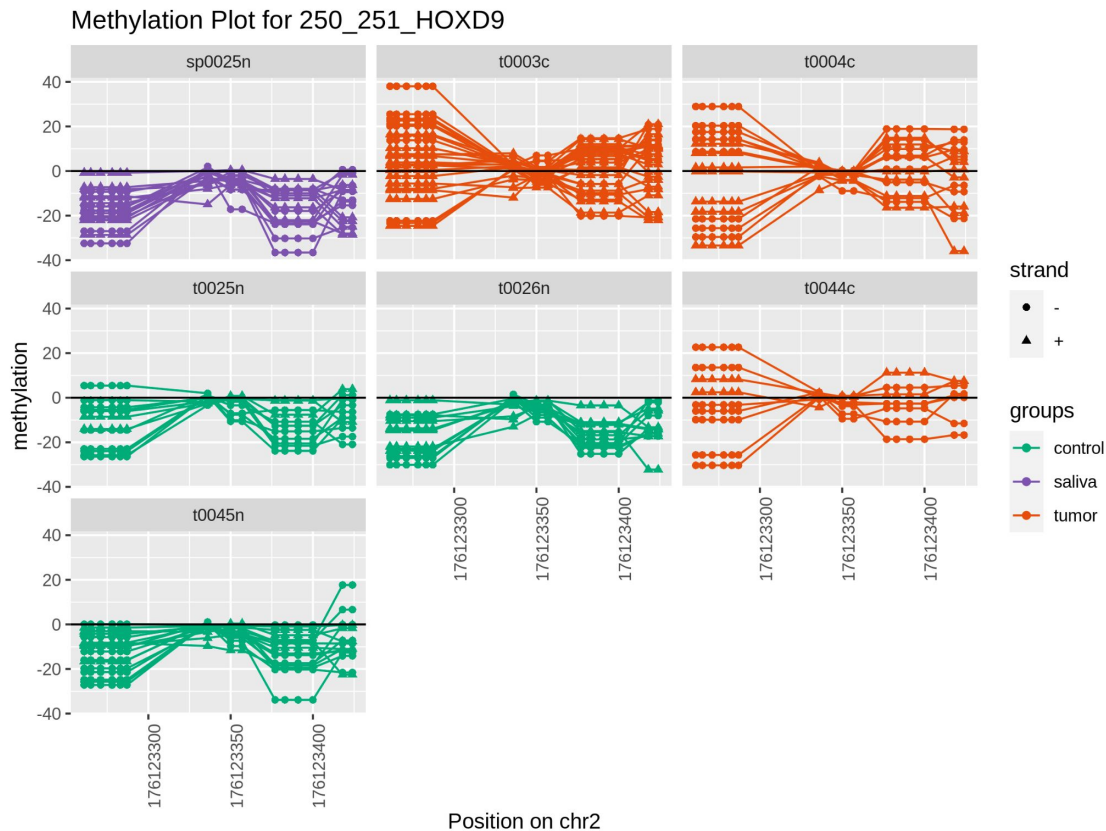


Coverage for CpG island cgi25860 containing ZNF671



Comparison with saliva

- HOXD9 has already been used before as marker
- results are consistent with PCR data



Comparison with PCR assay

Kontrollen	Diagnose	Lokalisation	Probennr.	ZIC1	ZNF833	ACTB	PAX6-1	HOXA9	ACTB	ZNF671	ACTB
T-0025-N	Kontrolle	Uvula	T-0025-N	32,90	35,19	25,35	31,48	29,00	25,41	34,43	25,31
SP0025	Kontrolle	Uvula	SP0025	0,00	0,00	26,25	0,00	34,89	26,05	38,66	26,01

Paar	ΔCq (Marker-ACTB)					$\Delta Cut-off$ ZIC1	$\Delta Cut-off$ ZNF833	$\Delta Cut-off$ PAX6-1	$\Delta Cut-off$ HOXA9	$\Delta Cut-off$ ZNF671	$\Sigma 3$ aus 5 positiv	HNOPOSITIVITÄT
	ΔCq ZIC1	ΔCq ZNF833	ΔCq PAX6-1	ΔCq HOXA9	ΔCq ZNF671							
T-0025-N	7,55	9,84	6,07	3,59	9,12	0	0	0	0	0	0	0
SP0025	keine Angabe	keine Angabe	keine Angabe	8,84	12,65	0	0	0	0	0	0	0
						G=SP	G=SP	G=SP	G=SP	G=SP	G=SP	G=SP