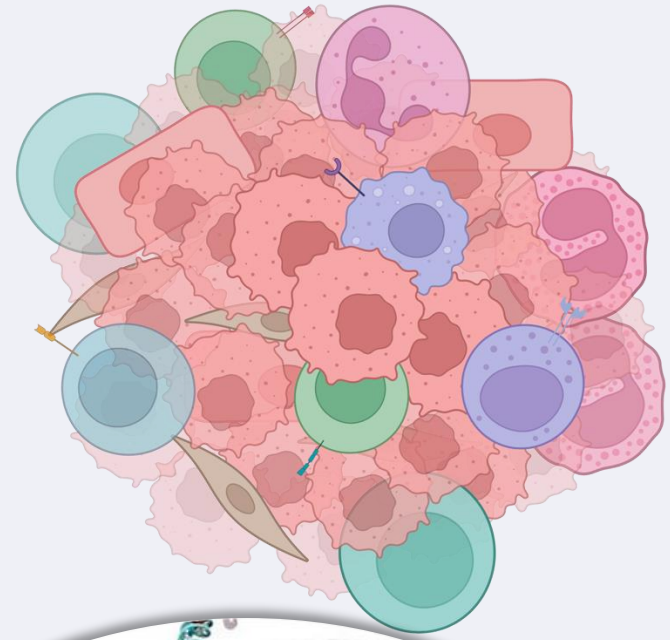
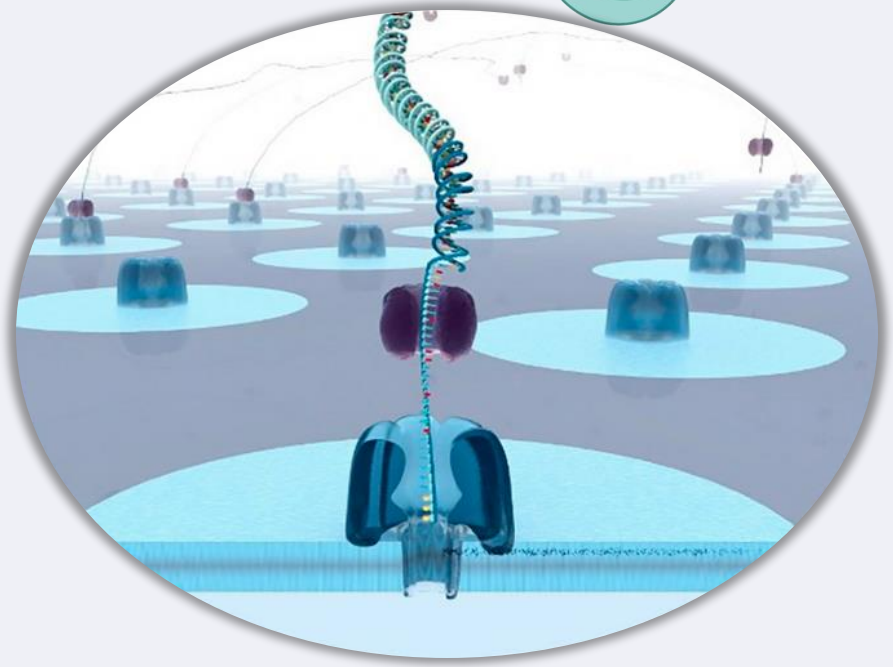
Multi-biomarker diagnostics using nanopore sequencing technology



In the context of BCG treatment of non-muscle invasive bladder cancer



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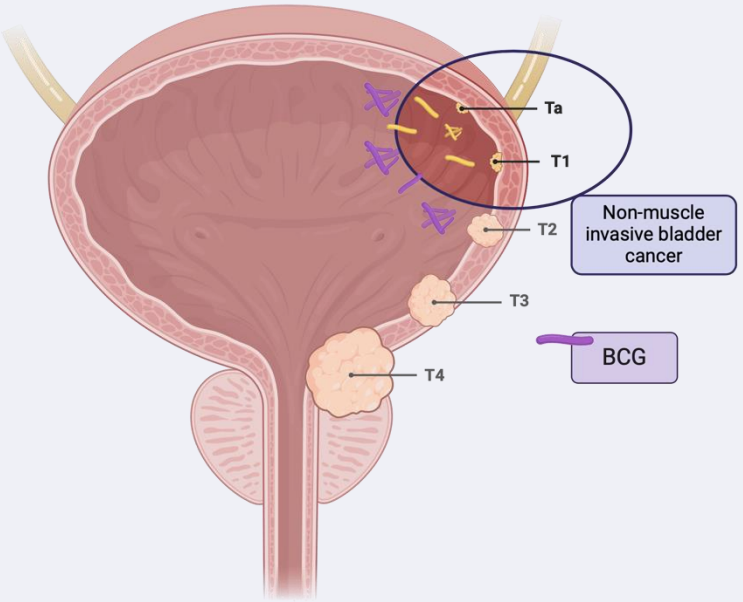
Overview

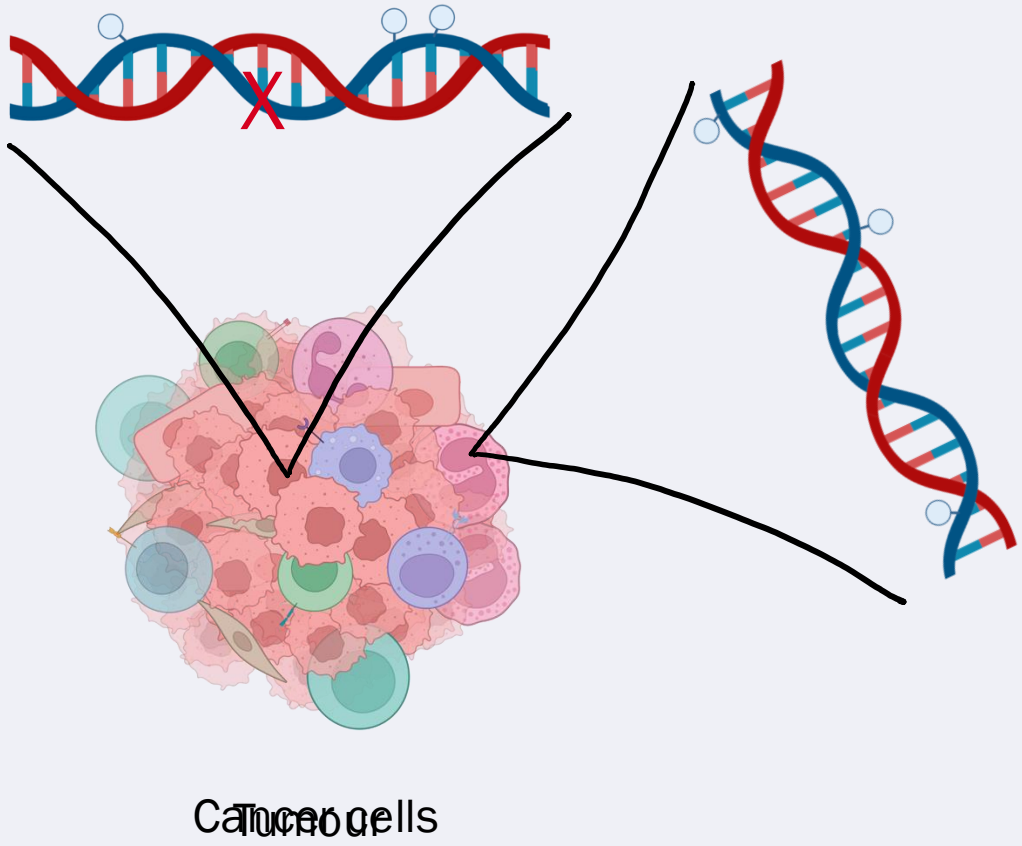
- Part One: Background
  - Bladder cancer and treatment background
  - Biomarkers
  - Nanopore sequencing
- Part Two: Our project
  - Diagnostic panel framework
  - Use in biomarker panel selection
  - Use in clinical testing for improved patient outcomes
- Part Three: Conclusion
  - Costs

Bladder cancer treatment with BCG

- Bladder cancer is the 9th most common cancer in NZ, with ~600 new cases a year
- BCG effective but intense treatment for ~40% of patients with non-muscle invasive bladder cancer
- BCG – derivative of *mycobacterium tuberculosis*
  - Tuberculosis vaccine - Bacillus Calmette-Guérin (BCG)
- If BCG treatment fails → cystectomy and/or chemo
- different types of biomarkers for predicting BCG treatment success have been researched, but are not widely used
- multi-biomarker panels can help



Cancer molecular biology

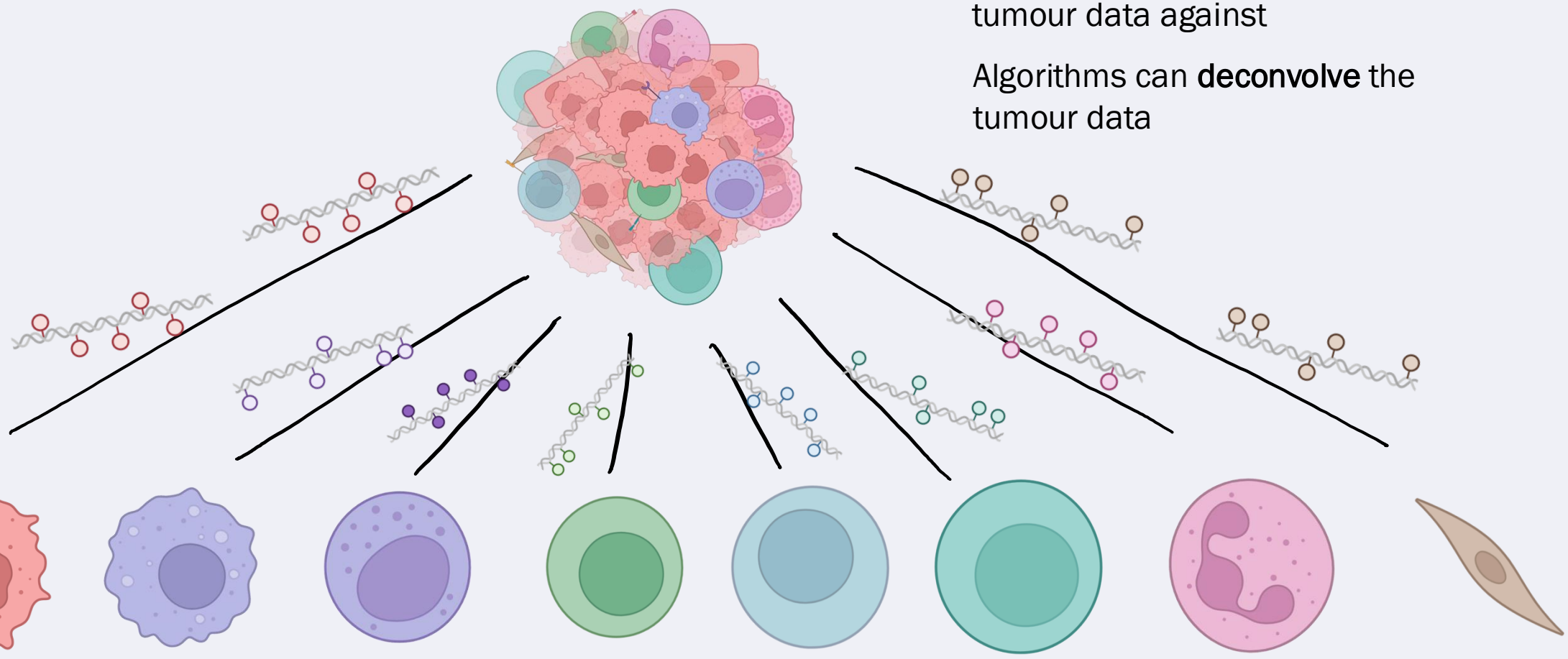


- Tumours are a mixture of cancer cells and other cells, namely immune cells
- Cancers have many DNA mutations
- All DNA has markers on it, the main one being **methyl** groups (methylation)
- Cancers have aberrant methylation
- Patterns of methylation are characteristic of cell type → **immune cell composition** of a tumour
- Different **immune cell compositions** can be a biomarker

Immune cell Infiltrate

Uses a reference matrix consisting of pure immune cell type data to match tumour data against

Algorithms can **deconvolve** the tumour data

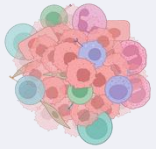


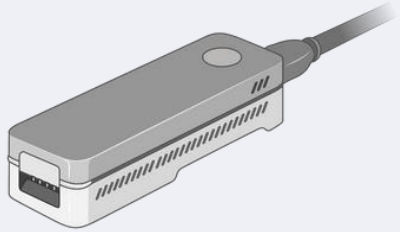
E.g.

56% Cancer	20% CD14	4% CD19	2% CD4	9% CD56	5% CD8	4% Neutrophils	12% Fibroblasts

Types of biomarkers

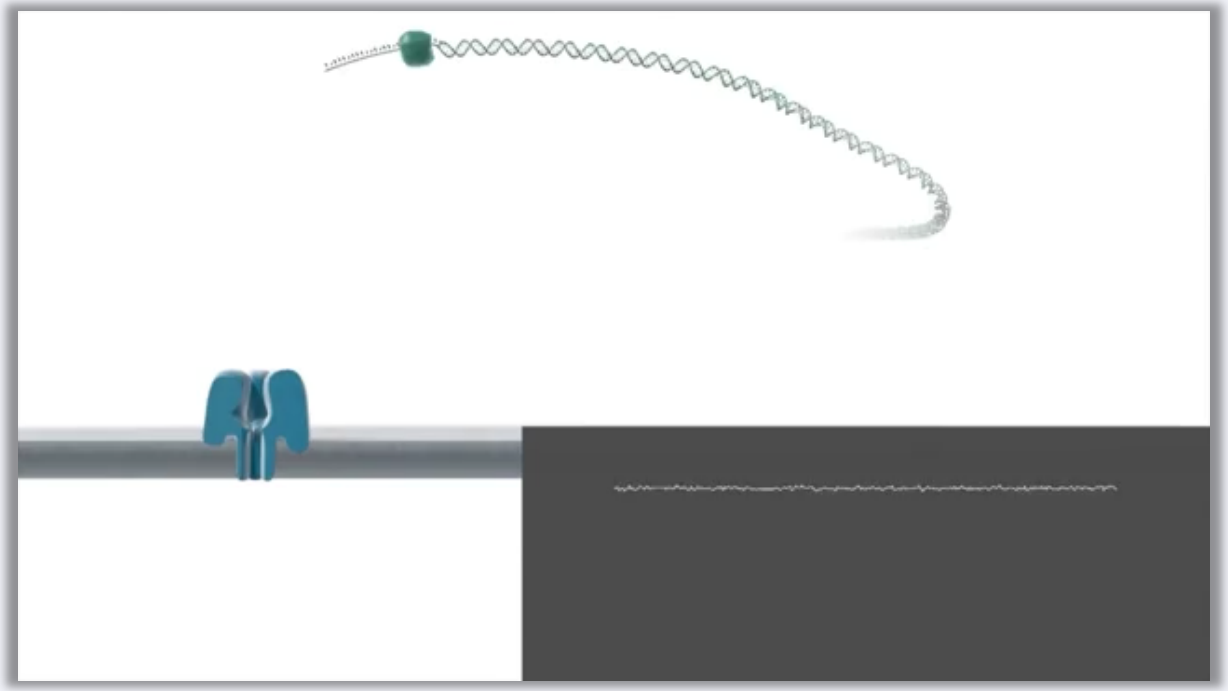
Types of biomarkers in an bladder tumour sample that predict treatment success:

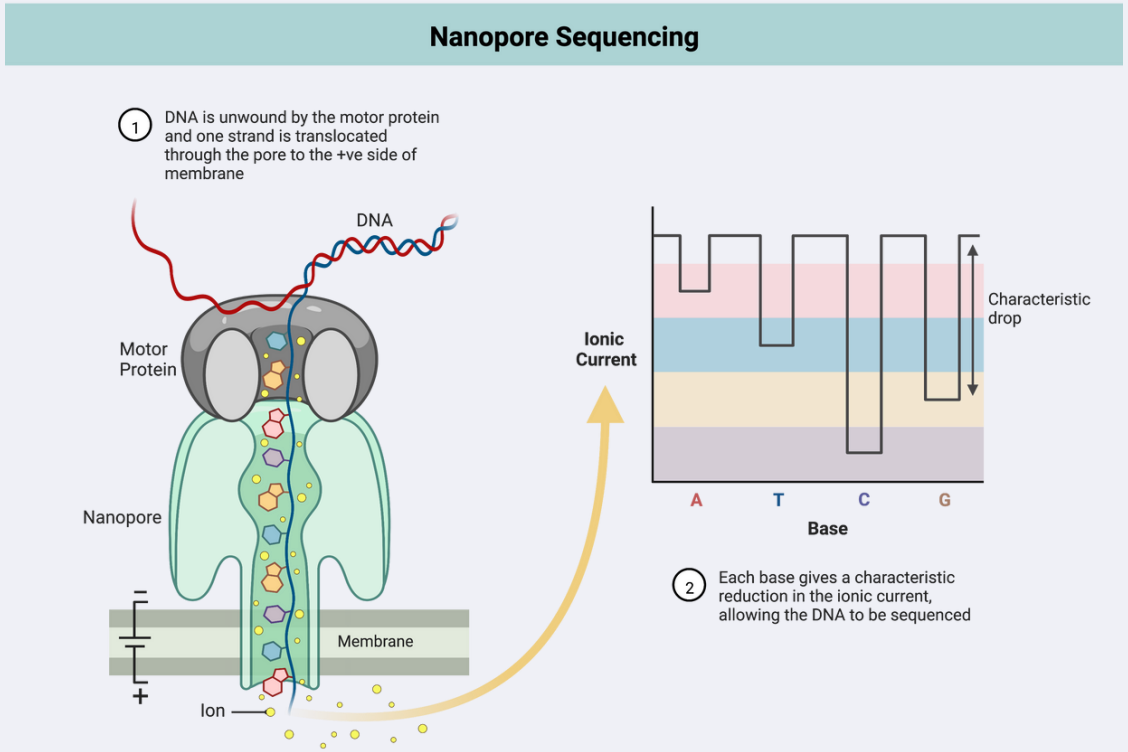
- DNA mutations 
- DNA methylation patterns 
- The **ratio of certain immune cells** present 



- Oxford Nanopore Technologies sequencing devices can detect **DNA mutations** and **methylation patterns** simultaneously
- Using methylation patterns we can estimate the **immune cell landscape**

How does it work?

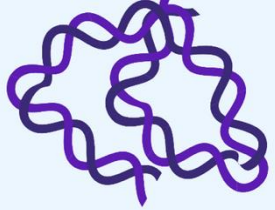




Sequencing can be **targeted**

Created with BioRender.com

Key takeaways



- Reads DNA sequence in real-time, allowing us to stop sequencing as soon as enough data is gathered (saves time and maybe **$$**)
- Can identify Single Nucleotide Variants (SNVs) in DNA sequence of tumour (a type of biomarker)
- Can read methylation marks on DNA, allowing a prediction of gene activity and providing a second biomarker
- Methylation information can be used to identify immune cell composition, providing a third type of biomarker

Nanopore sequencing in clinical context

- Dr Mathilde Filser
  - *Curie Institute, France*

- the team successfully identified complex SVs, such as inversions and duplications relevant to clinical management
- patient case → early-onset triple negative ductal carcinoma of the breast with no family history suggestive of hereditary breast-ovarian cancer syndrome. Routine tests revealed exon duplications but not pathogenicity of variant.
- Sequencing of long arm of chr17 determined duplication disrupted reading frame
- '*Within 10 days in total, we were able to go back to the clinicians and confirm … she was carrying a pathogenic variant in* BRCA1'

https://nanoporetech.com/blog/unravelling-the-complexity-of-cancer-genomics-and-predisposition:-nanopore-sequencing-and-the-potential-for-personalised-care

- Prof. Dr Pieter Wesseling
  - *Princess Máxima Center for Pediatric Oncology & Amsterdam University Medical Centers, The Netherlands*

- team developed an ultra-fast, deep-learning model called Sturgeon
- uses methylation data generated from rapid nanopore sequencing to classify CNS tumours in an intraoperative timescale.
- For most of the 47 CNS tumour research samples, the model delivered an accurate classification during surgery
- *'from the start of the sequencing until the answer, it's about 45 minutes'*

https://nanoporetech.com/blog/how-nanopore-sequencing-has-the-future-potential-for-rapid-brain-tumour-classification

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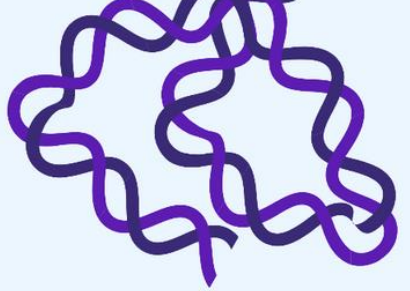
Goals



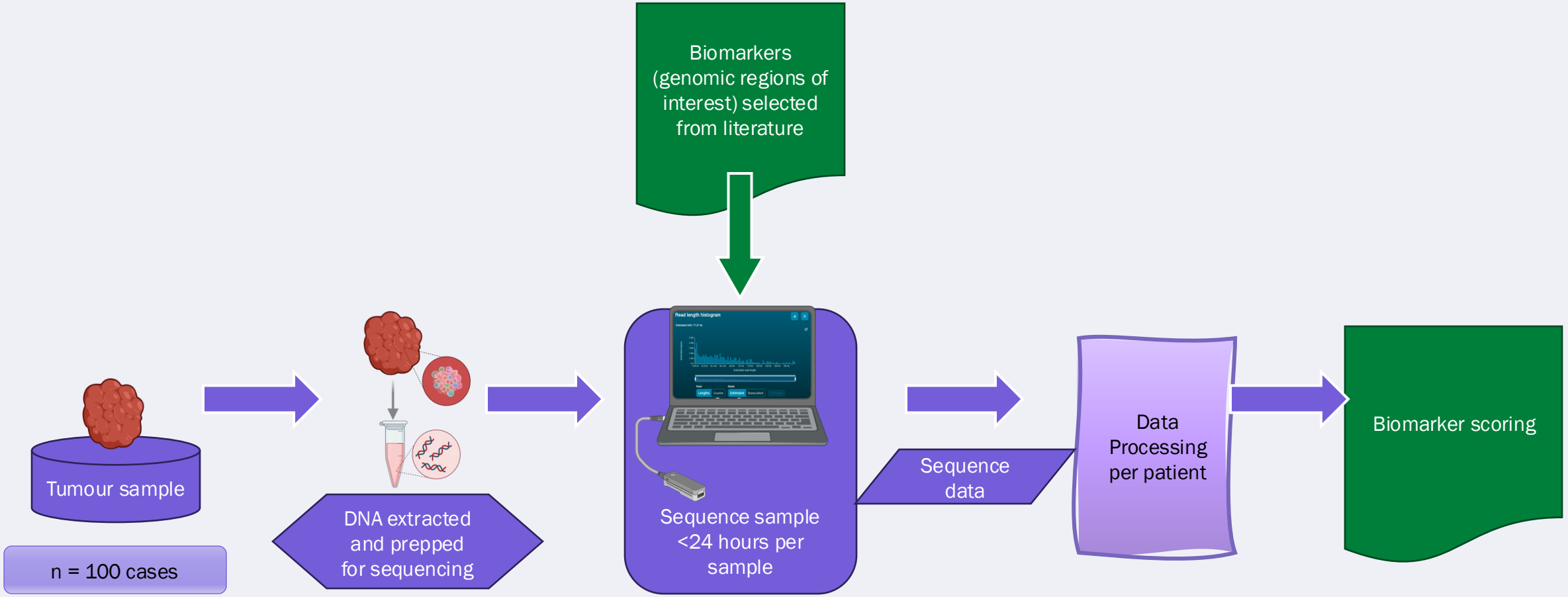
To develop a protocol and pipeline for fast detection of multiple types of biomarkers that can predict BCG efficacy using the MinION.

- Use adaptive sequencing functionality of MinION to target areas of the genome that could predict the efficacy of BCG treatment in bladder cancer.
- SNVs, methylation variants, immune status
- Should only take a day or so from DNA extraction to clinical result
- Proof-of-concept

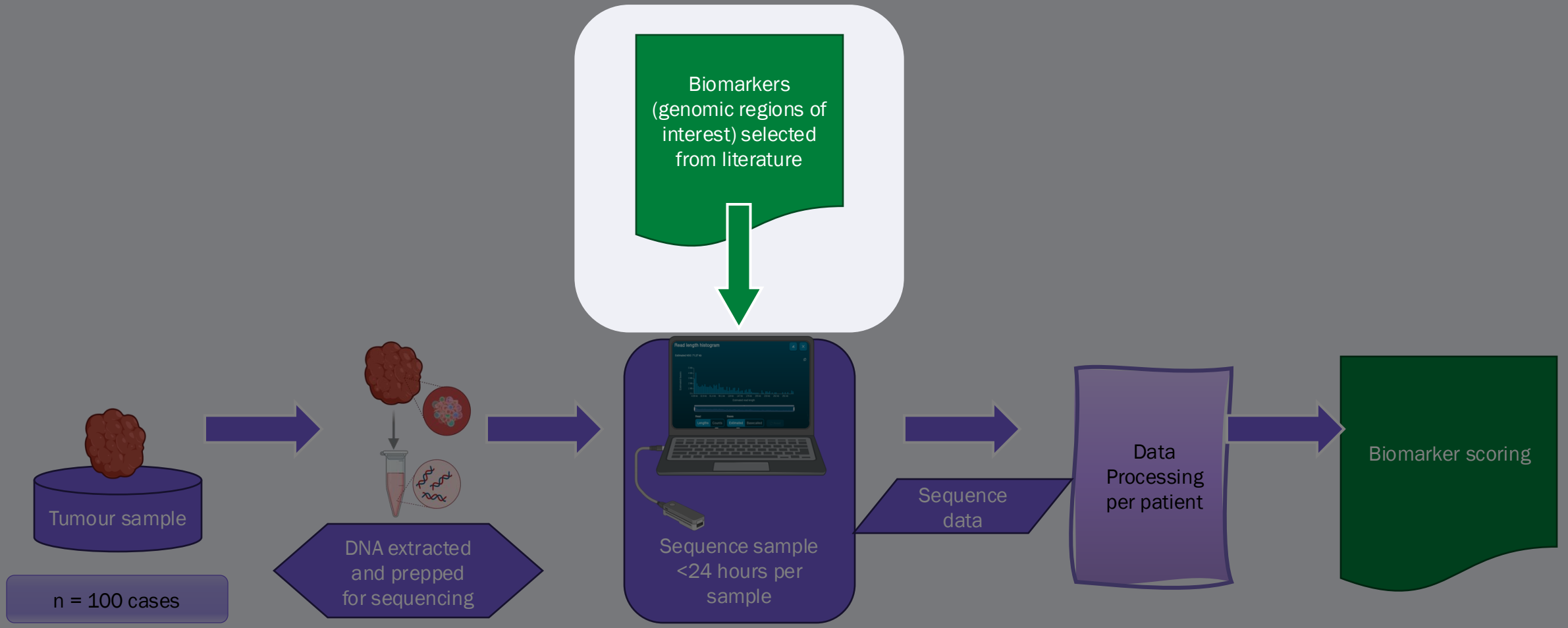
Two stages to using this pipeline after proof-of-concept development:

1. Biomarker panel scoring development (pre-clinical trial)
2. Clinical testing of patient samples to predict likely response to BCG

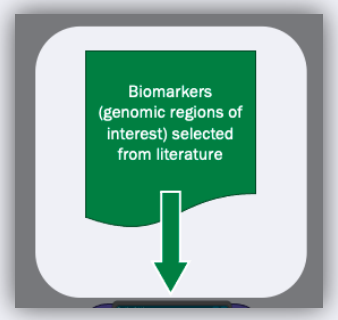
Scoring Development



Scoring Development

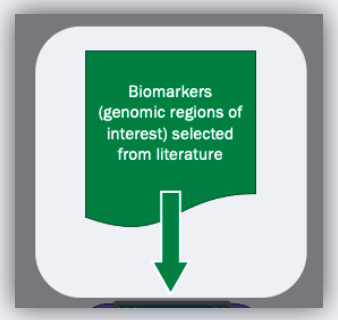


Input: Panel Design



- Input: need this information per biomarker:
  - Genomic location
    - e.g. “chr14 : 96,311,131 - 96,311,131”
  - Type
    - “SNV” or “Methylation Variant” or “Promoter region methylation”)
  - Response/non-response characteristic
    - response = “increased methylation by >10%”
    - non-response = “decreased methylation >10%”
- Required positions for immune infiltrate data will be included by default

Panel example – my current top 10



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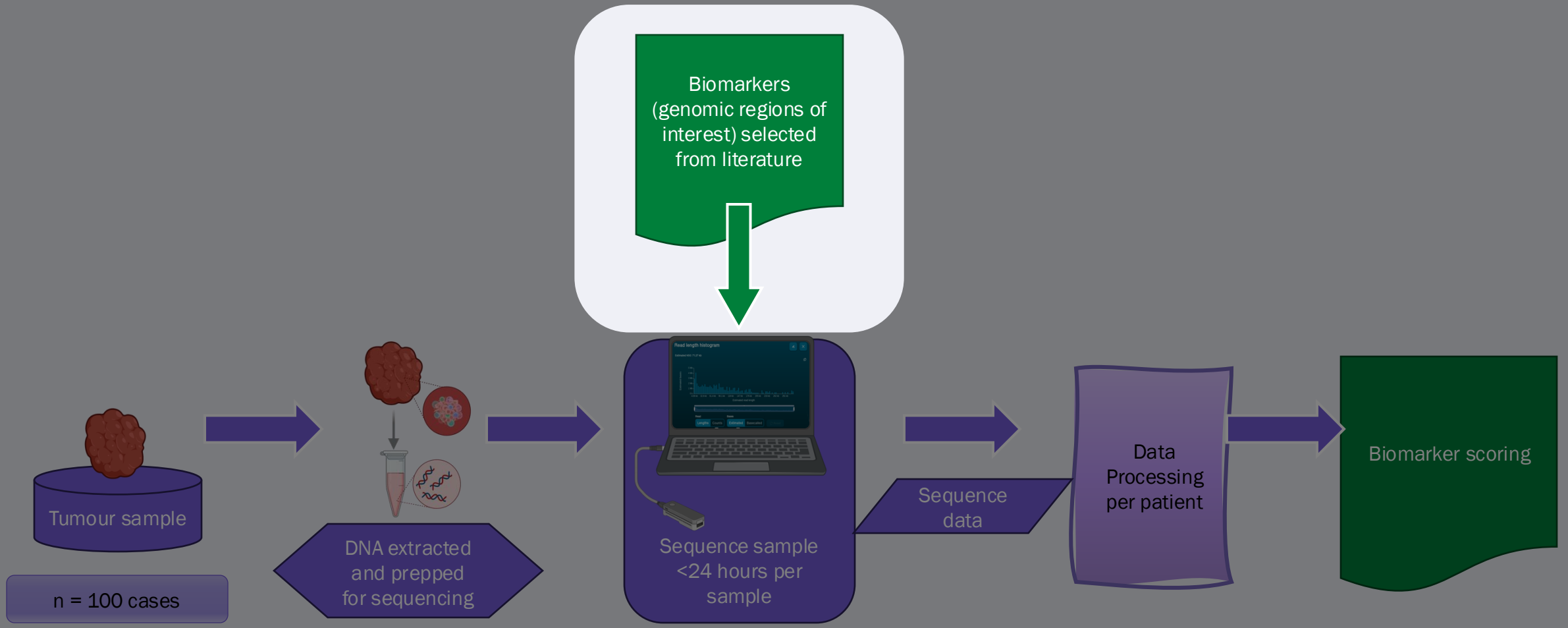
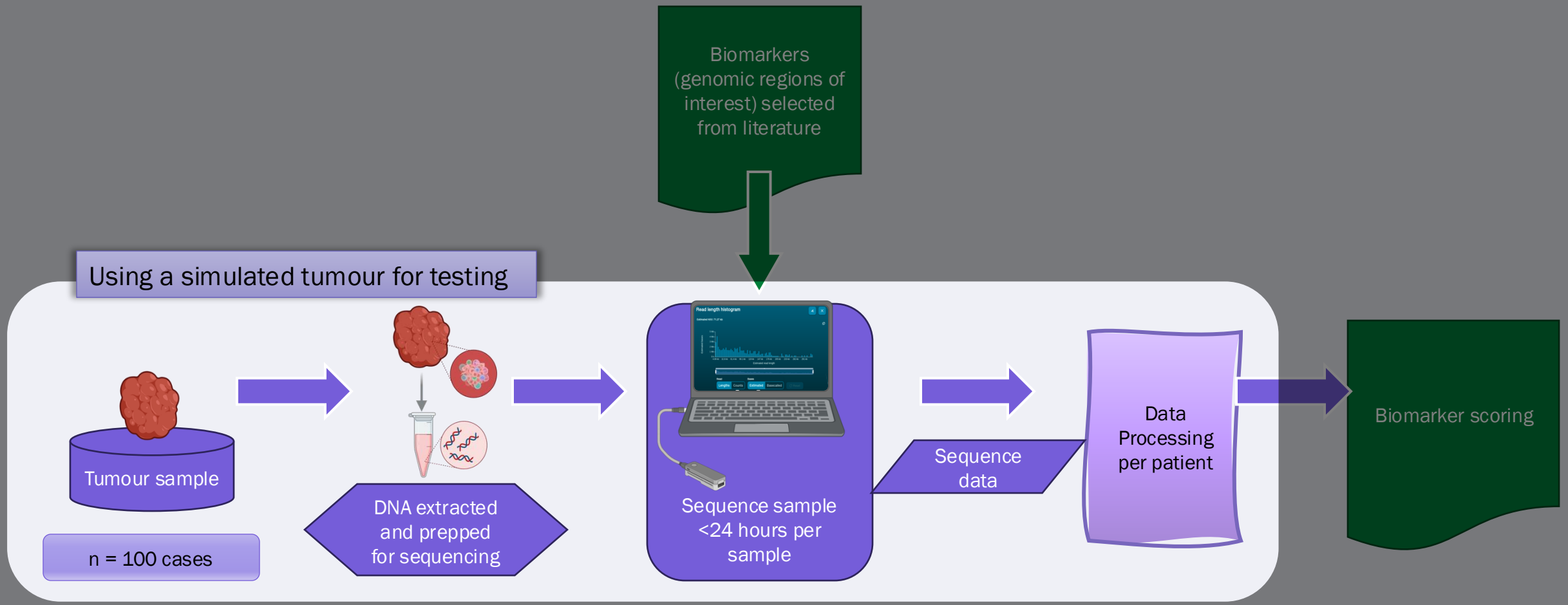
002	chr17:96,311,131	NOS2	No		(CCTTT)n		<13 repeats	?		
004	chr7:96,311,131	NOS3	Yes	rs1799983	T/A/G		GG	?		
005	chr7:96,311,131	NOS3	No	rs2070744	C/T		TT	?		
007	chr13:96,311,131	FLT1	No			promoter	reduced methylation by >10%	?	increased methylation by >10%	?
008	chr1:96,311,131	ARID1A	whole gene				wildtype	?	any mutation	?
009	chr12:96,311,131	CHST11	N/A			promoter			increased methylation by >15%	?
010	chrX:96,311,131	KLF8	N/A			single site			increased methylation by >12%	?

Scoring development - This panel will be used in Regression Analysis to identify scoring – similar to polygenic risk scores

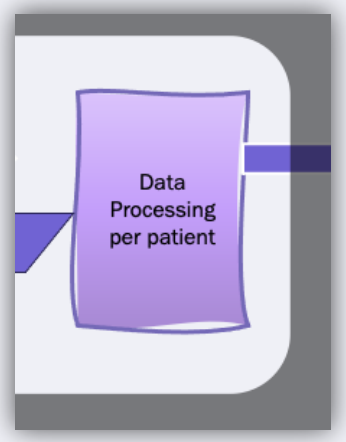
Scoring Development



Scoring Development



Data Processing



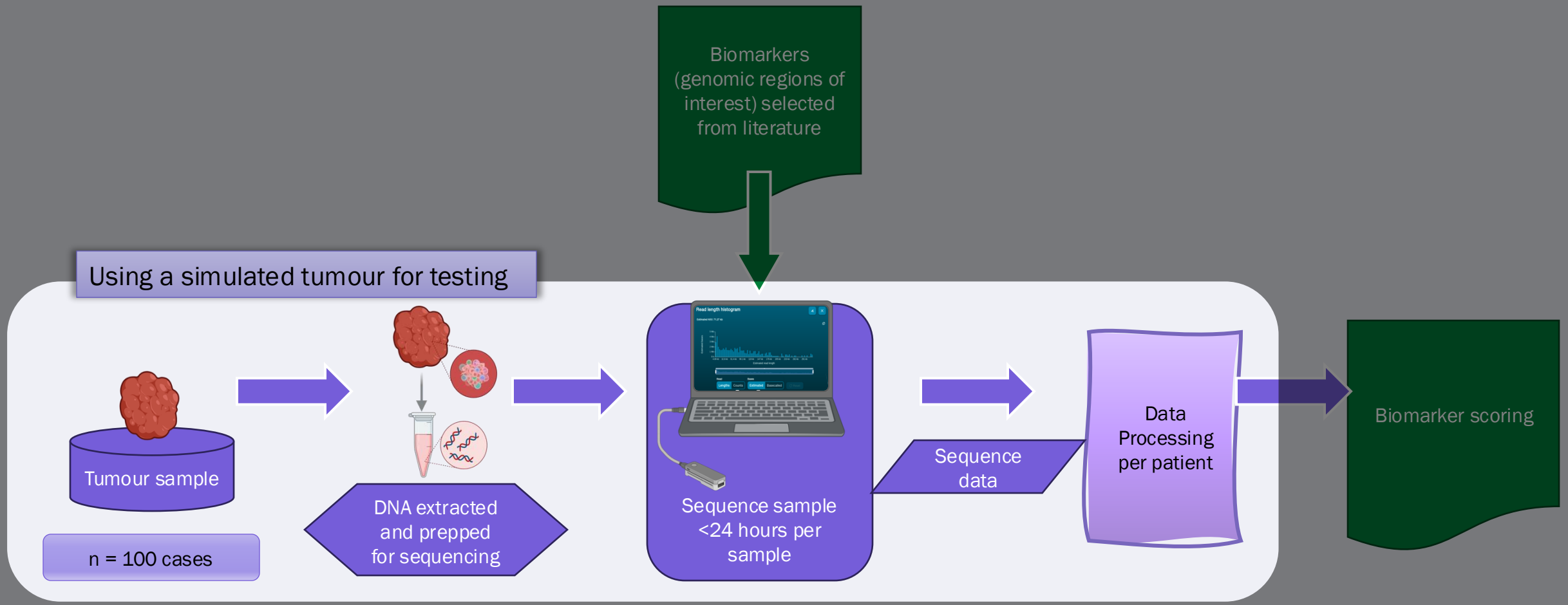
Workflow from ONT ready for use

- SNP annotation by ClinVar
- Structural variant (SV) calling by SNPEff – identifies repeat expansions
- Methylation identified
- Phasing is performed – some BCG response information is allelic

Framework I'm working on

- Match SNP, SV and methylation results to panel selection
- Process methylation to get immune infiltrate and immune sections of panel

Scoring Development



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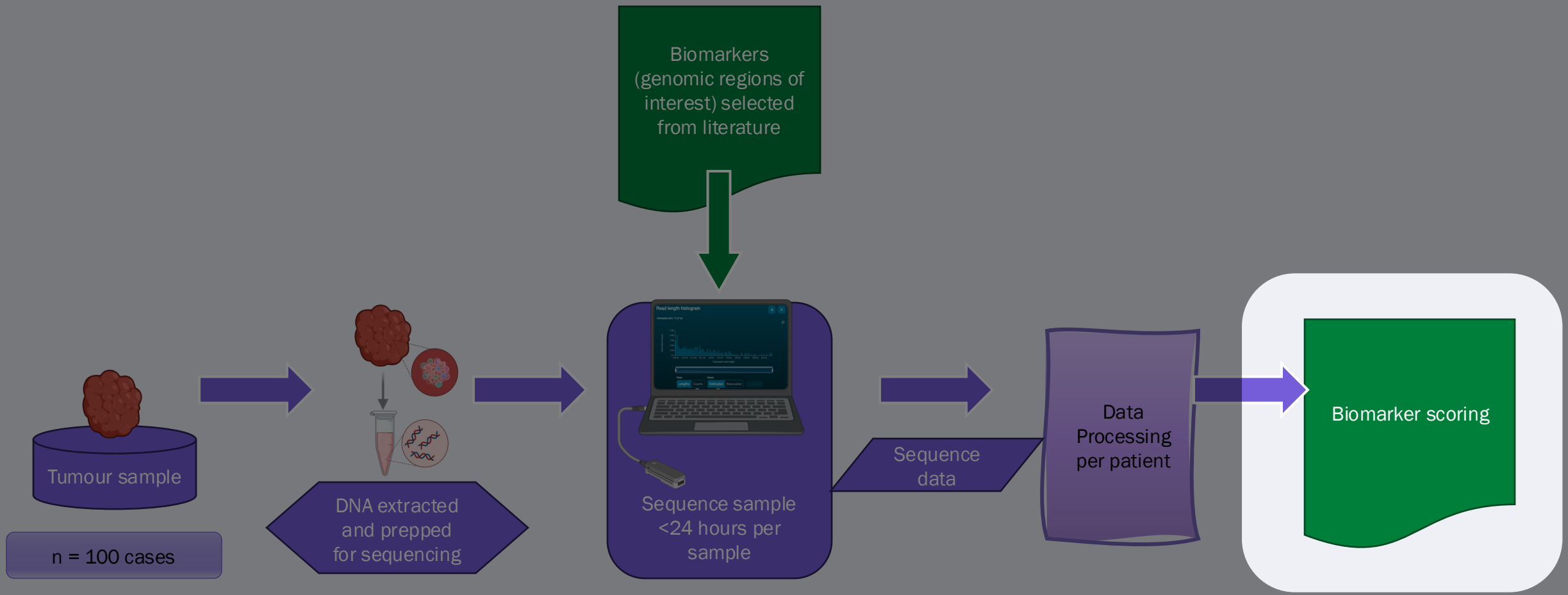
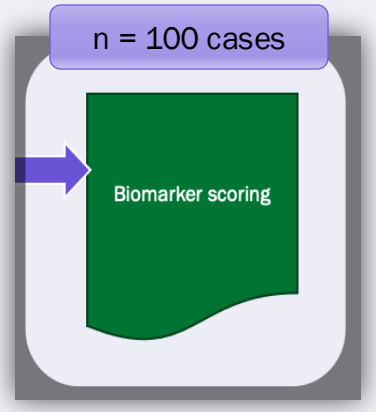
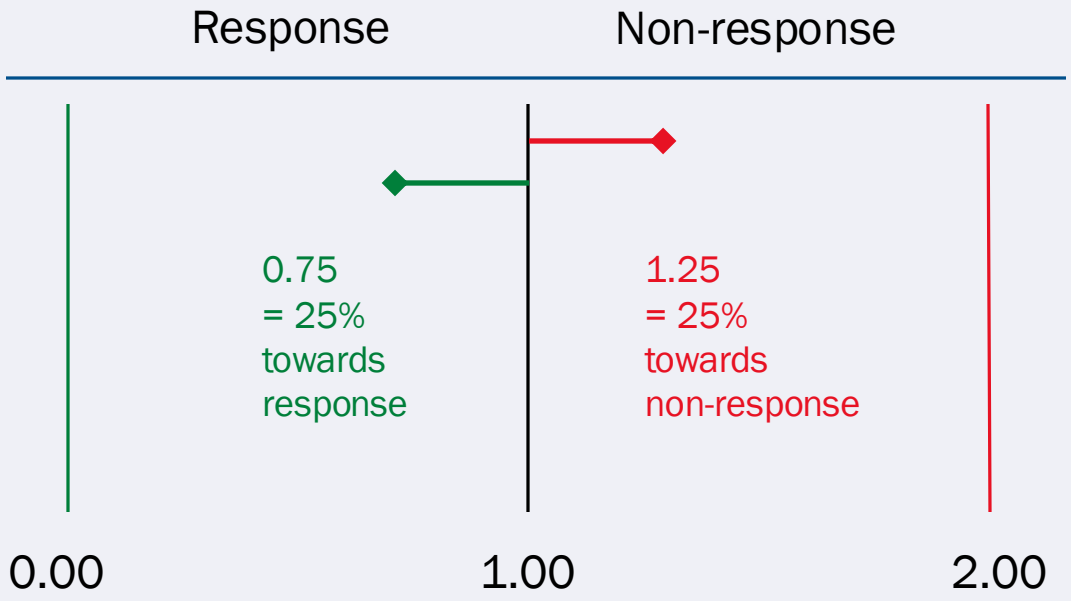


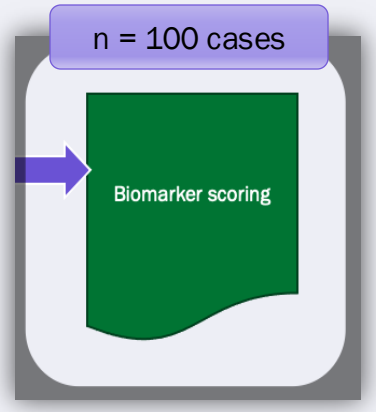
Biomarker Scoring



- A few different methods
  - Machine learning methods
  - LASSO – similar to linear regression for risk analysis
- Hazard ratio and standard deviation or similar
  - Can code this into my framework



Panel example – After Regression Analysis



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002	chr17:96,311,131	NOS2	No		(CCTTT)n		<13 repeats	?		
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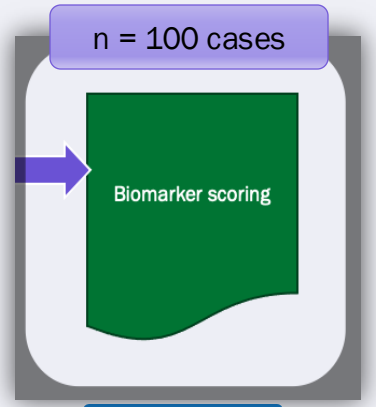

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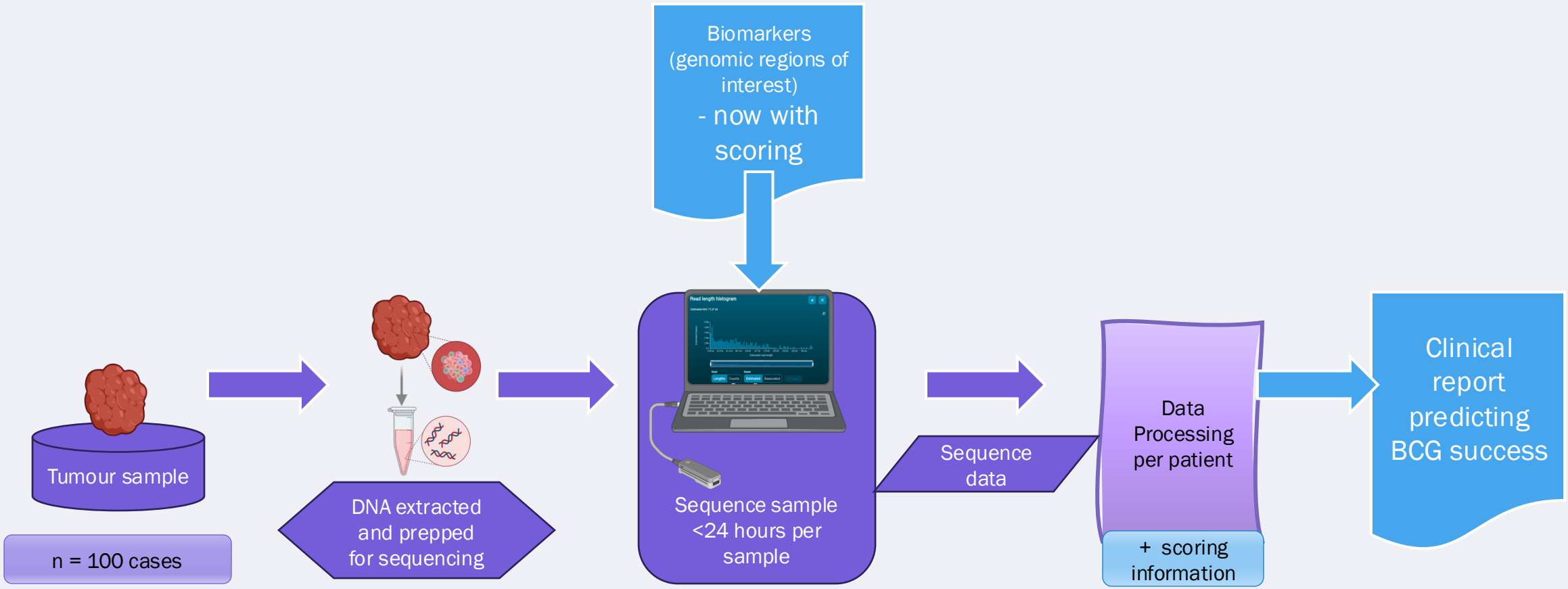
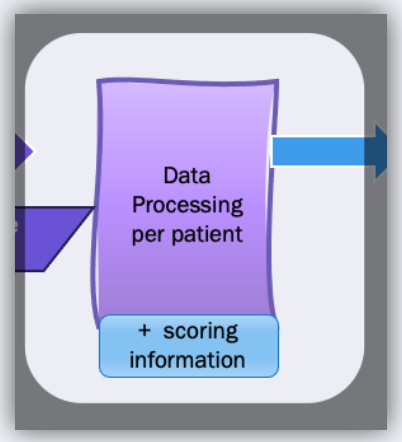
002	chr17:96,311,131	NOS2	No		(CCTTT)n		<13 repeats	0.91		
004	chr7:96,311,131	NOS3	Yes	rs1799983	T/A/G		GG	0.96		
005	chr7:96,311,131	NOS3	No	rs2070744	C/T		TT	0.72		
007	chr13:96,311,131	FLT1	No			promoter	reduced methylation by >10%	0.83	increased methylation by >10%	1.11
008	chr1:96,311,131	ARID1A	whole gene				wildtype	0.88	any mutation	1.25
009	chr12:96,311,131	CHST11	N/A			promoter			increased methylation by >15%	1.34
010						single site			increased methylation by >12%	1.19

This panel now has scoring to use for patient prediction to BCG treatment

Clinical Diagnostic Test



Data Processing



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- SNP annotation by ClinVar
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- Methylation identified
- Phasing is performed – some BCG response information is allelic

Framework I'm working on

- Match SNP, SV and methylation results to panel selection
- Process methylation to get immune infiltrate and immune sections of panel
- **Process biomarker scoring results to obtain a single overall score**

BCG susceptibility panel

This test combines the results for relevant single nucleotide variants, methylation variants, and immune infiltrate prediction to reach a final score for prediction of BCG treatment success for NMIBC.

Clinical Details

Bladder lesion, transurethral resection:
Papillary urothelial carcinoma, high grade (grade 3/3 - WHO 1973)
Noninvasive, pTa

Specimen: TURBT biopsy

Overall Result and Interpretation

Tumour is 78% likely to respond to treatment.

Comments:

The result is an aggregate score obtained by combining the scores of biomarkers that reach read depth threshold.

Guidance: Result is a scale of 0% – 100%. 0% will not respond and 100% will respond to BCG treatment. Most results will fall between 25% and 75%. We recommend treatment with BCG when the result is >50% for patients who are otherwise healthy, and >60% for all other patients.

Methodology

Nanopore sequencing was used to detect single nucleotide variants, methylation status and immune status (by methylation) of the tumour sample.

Standard Panel results

N/A = Not Applicable
N/Av = Not Available

SNVs

39 out of 40 predictive SNVs reached read depth threshold and used in final score composition. ClinVar annotations were found for 6 of these.

Panel ID	Gene name	dbSNP ID	BCG response characteristic	BCG non-response characteristic	RESULT	SCORE
001	ATG2B	rs3759601	G	CC	G	0.82
002	NOS2		<13 repeats		<13 repeats	0.91
004	NOS3	rs1799983	GG	N/Av	GG	0.96

Methylation

39 out of 40 predictive methylation variants reached read depth threshold and used in final score composition. Hypomethylation of promotor regions predict expression of the associated gene.

Panel ID	Gene name	Methylation region type	BCG response characteristic	BCG non-response characteristic	RESULT	SCORE
007	FLT1	Promoter	Reduced methylation by >10%	Increased methylation by >10%	Reduced methylation by >10%	0.83
010	KLF8	Single site		Increased methylation by >12%	Normal methylation	1

Immune Infiltrate Ratios

This table shows immune infiltrate ratios predictive of BCG efficacy and used in final score composition.

Immune Ratio	Description	BCG response characteristic	BCG non-response characteristic	RESULT	SCORE
LMR	Lymphocyte to Monocyte	N/A	>3.25[1]	1.61	0.68
NLR	Neutrophil to Lymphocyte	N/A	>2.5[2]	0.30	0.79

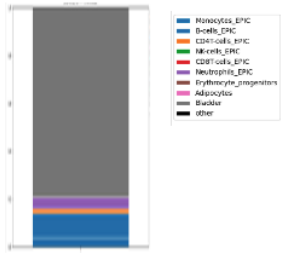
Immune Infiltrate percentage per cell type

Percentage of each of the 11 cell types detected in the tumour sample.

Cell type	BCG response characteristic	BCG non-response characteristic	RESULT (% of cells)	SCORE
Monocytes	>7	<7	7%	1
B cells	>10	<10	5%	1.15
CD4+	>20	<20	8%	1.20
NK cells	>7	<7	4%	1.05
CD8+	>20	<20	21%	0.92
Neutrophils	>12	<12	5%	1.17
Th1	<10	>10	5%	0.75
M1 type macrophage	>15	<15	10%	1.21
Dendritic cells	>3	<3	1%	1.03
Adipocytes	<2	>2	1%	0.89
Bladder -type cells	<70	>70	73%	1.10

Supplementary

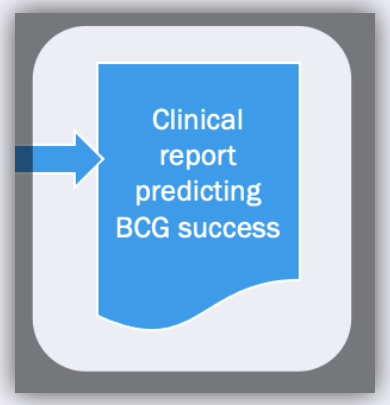
Immune Infiltrate Plot:



Method

Sequencing library was prepared from extracted DNA using the Ligation sequencing DNA V14 (SQK-LSK114) kit and sequenced on the MinION device with R10.4.1 flow cells (Oxford Nanopore Technologies). Analysis used software packages: *Clair 3 v2.0, modkit v3.1.*

Report Format



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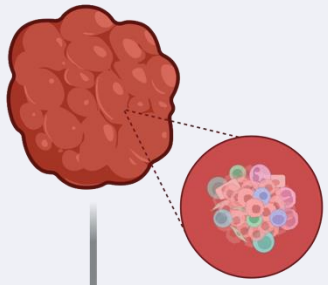
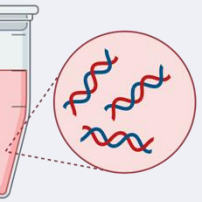
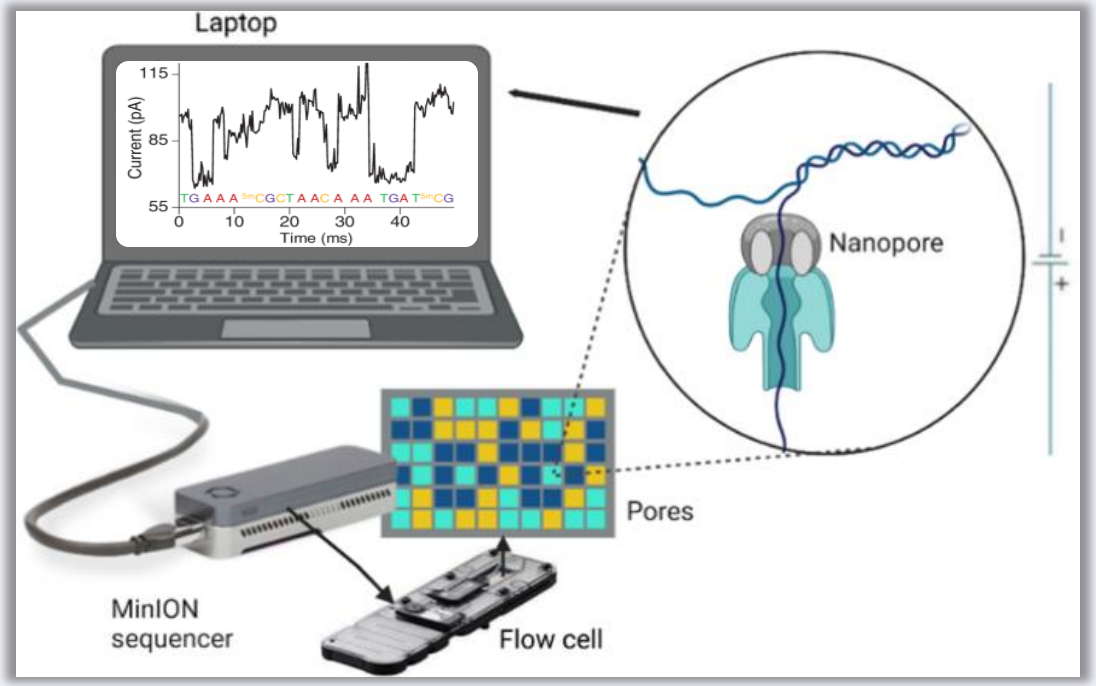
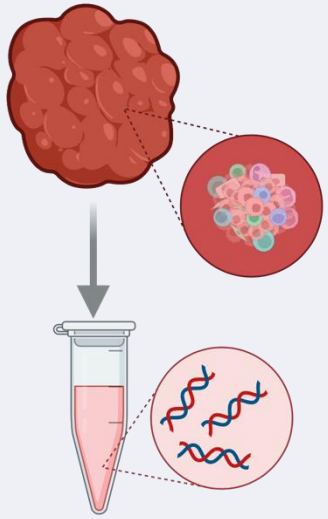
What do you need?



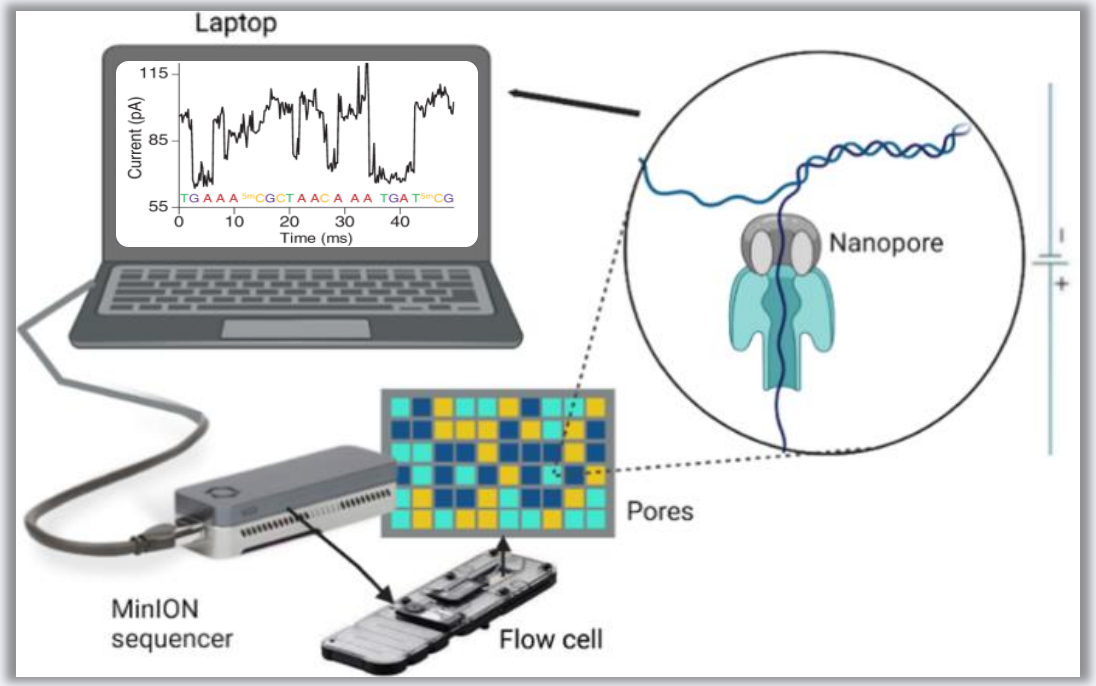




Consumables $$ per sample



$7



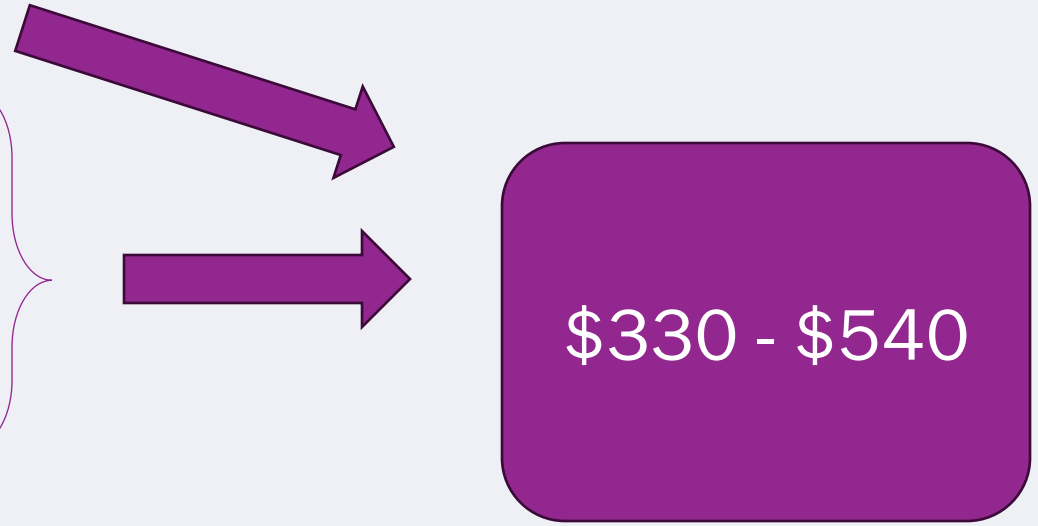
$150

$70

$160

Cost Considerations

- Consumables: $237 - $387 per sample*
- Flongle ~1-2 Gb per run,
  - $150 (NZD) per flow cell
- MinION ~ 10-20 Gb per run
  - $720 - $1120 (NZD) per flow cell
  - lowest price $92 per sample if multiplexing for 1Gb per sample**
  - dependent on size of packs
  - A MinION starter pack is around $1700
- 3 – 6 hours hands-on time



* with/out G-tube fragmentation
**incl. multiplex kit price here

I want to hear from you!

- Relevant scoring techniques for multiple types of biomarkers?
- Hurdles to clinical implementation?
  - especially in Aotearoa
- Collaboration/consultation anyone?

More details

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- Department of Pathology and Molecular Medicine Research Team
  - Dr. Katharina Robichon
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  - Alex Bloomberg (PhD student)
  - Catherine Schwabe (Honours student)
  - Rosa Latton (Honours student)
- Clinical Advisor: Mr. Andrew Kennedy-Smith
  Wellington Urology

University of Otago
ŌTĀKOU WHAKAIHU WAKA
NEW ZEALAND

Oxford NANOPORE Technologies

I want to hear from you!

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  - especially in Aotearoa
- Collaboration/consultation anyone?

More details

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Nanopore accuracy

- In general ~99% accurate basecalling with latest chemistry + software*
- ~99% accurate methylation calling on Cytosine
- Accounted for as part of the panel development, will adjust for individual accuracy scores per target region

*as at November 2024

Report Format (continued)

Extended Panel Results

This table displays the results of the additional regions requested. Analysis is only carried out on regions that meet the read depth threshold.

Panel ID	Gene name	Requested test	Reached 25 read depth analysis threshold?	Result		Interpretation
				Genome region	Variant result	
301	Ki67	Methylation expression indication	Yes	chr14: 96,311,131-96,312,131	Hypomethylated	Gene region has low methylation, Ki67 is likely expressed.
				chr14: 96,311,142-96,311,143	Unmethylated	
302	PTEN	Methylation expression indication	Yes			
303	TP53	Mutations	Yes	chr17: 96,311,142-96,311,143	C46T	TP53 has multiple mutations indicative of gene inactivation
304	RB	Mutations	Yes			

Comments

N/A

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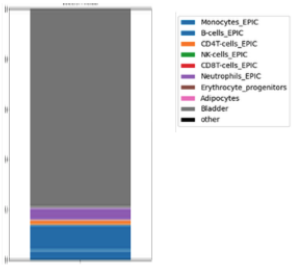
Supplementary

Immune Infiltrate Plot:



Method

Sequencing library was prepared from extracted DNA using the Ligation sequencing DNA V14 (SQK-LSK114) kit and sequenced on the MinION device with R10.4.1 flow cells (Oxford Nanopore Technologies). Analysis used software packages: *Clair 3 v2.0, modkit v3.1*.

References

1. Adamkiewicz, M. *et al.* Lymphocyte-to-Monocyte Ratio Is the Independent Prognostic Marker of Progression in Patients Undergoing BCG-Immunotherapy for Bladder Cancer. *Frontiers in Oncology* **11**, 655000 (2021).
2. Ziani, I. *et al.* Role of preoperative neutrophil to lymphocyte ratio in prediction of recurrence, progression, and BCG failure in non-muscle invasive bladder cancer: a retrospective study. *Pan Afr Med J* **44**, 145 (2023).

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Abstract for NZIMLS

Multi-biomarker diagnostics using nanopore sequencing technology.

Lucy Picard[1], *Logan Walker*[2], *Aaron Stevens*[1]

[1] University of Otago, Wellington, Department of Pathology and Molecular Medicine
[2] University of Otago, Christchurch, Department of Pathology and Biomedical Science

The MinION device, developed by Oxford Nanopore Technologies (ONT), is a mobile-phone-sized DNA sequencing tool ideally suited for integration into diagnostic settings. This technology has the potential to simplify the entire process from patient samples to clinical results. However, bioinformatic tools need to be developed to enhance ease of use.

We are developing a bioinformatic pipeline for informing BCG treatment efficacy for non-muscle-invasive bladder cancer, based on simultaneous detection of relevant DNA mutations and DNA methylation. This information also gives us critical information on the immune status of the tumour, a key component that can be used to predict treatment success.
This comprehensive multi-biomarker approach, combines mutations, methylation, and immune status and offers greater potential than current single biomarker approaches. This has been developed in the context of BCG treatment in patients with BCG-resistant tumours with the aim of decreasing the time to effective treatment, however, it is designed for easy adaptation to other diseases.