Quick steps towards a fast turnaround time for urgent diagnostic pharmacogenetic workflows

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Background

Genotypes in pharmacogenetic (PGx) passports guide a patient's therapeutic strategy, for example by advising on the tolerated type and dose of chemotherapy. In current diagnostic analyses, pharmacogenetic assays are generally performed by targeted short-read sequencing and/or qPCR, depending on the target of interest. While sequence-based approaches have analytical advantages, the turnaround times for these (days) are longer than PCR-based analysis (hours).

Aiming to reduce their respective turnaround times, we developed multiplex-PCR assays for the urgent fluoropyrimidine (*DPYD* and *UGT1A1*) and thiopurine (*TPMT* and *NUDT15*) workflows by sequencing the amplicons on the Minion (Oxford Nanopore Technologies).

Methods

Dividing the procedure into modules, we reduced the time for each step of the protocol.

1st step: Assay Design

Moving from a Molecular Inversion Probe capture method to PCR-based analysis, we designed custom multiplex PCRs amplicon targets of approximately 1kb. These targets include the recommended (tier 1) and optional (tier 2) variants across both gene panels.

To aid in interpretation of the zygosity of the variable TA-repeat *UGT1A1* (i.e. *28 *36 *37), the linked *80 SNP was included in the same amplicon.

Upon sequencing the first rounds, rebalancing of PCR primers in the multiplex was performed to obtain a uniform distribution across all targets.

2nd step: PCR:

Transitioning from a standard polymerase to DeCodiFi™ 2X All-in-One Mix High-Fidelity polymerase (Kura Biotech®) markedly reduced the time required for PCR.

Standard polymerase		DeCodiFi™ 2X All-in-One Mix
40 cycles	→	27 cycles
146 minutes		44 minutes

Following PCR, Amplified products of each multiplex reaction are quantified (Qubit) and pooled equimolarly prior to library preparation.

3rd step: Library preparation:

To speed up preparation of the sequencing library, we adapted the Nanopore Microbial Amplicon Barcoding Kit 24 protocol. In brief, our custom PCR reaction replaced the 16S amplification step in the protocol. Starting material of each multiplexed sample into the barcoding step was 50 fmol.

4th step: Sequencing:

Prepared libraries are sequenced on a Nanopore MinION (Mk1B or Mk1D) instrument to a minimal of 5000 sequencing reads per barcoded sample. Once the run is completed, the flowcell is washed and stored for re-use in future experiments.

5th step: Bioinformatic analysis

Bioinformatics is performed on a local high performance compute cluster with a GPU for basecalling.

The processing and analysis steps include:

- Nanopore basecalling (Dorado - super accuracy)
- Demultiplexing (Dorado demux)
- Mapping of reads to hg38 (Dorado aligner; minimap2)
- Sorting and indexing of alignment (samtools)
- Coverage statistics for QC (mosdepth)
- Star-allele genotyping (Aldy)

Disclosure

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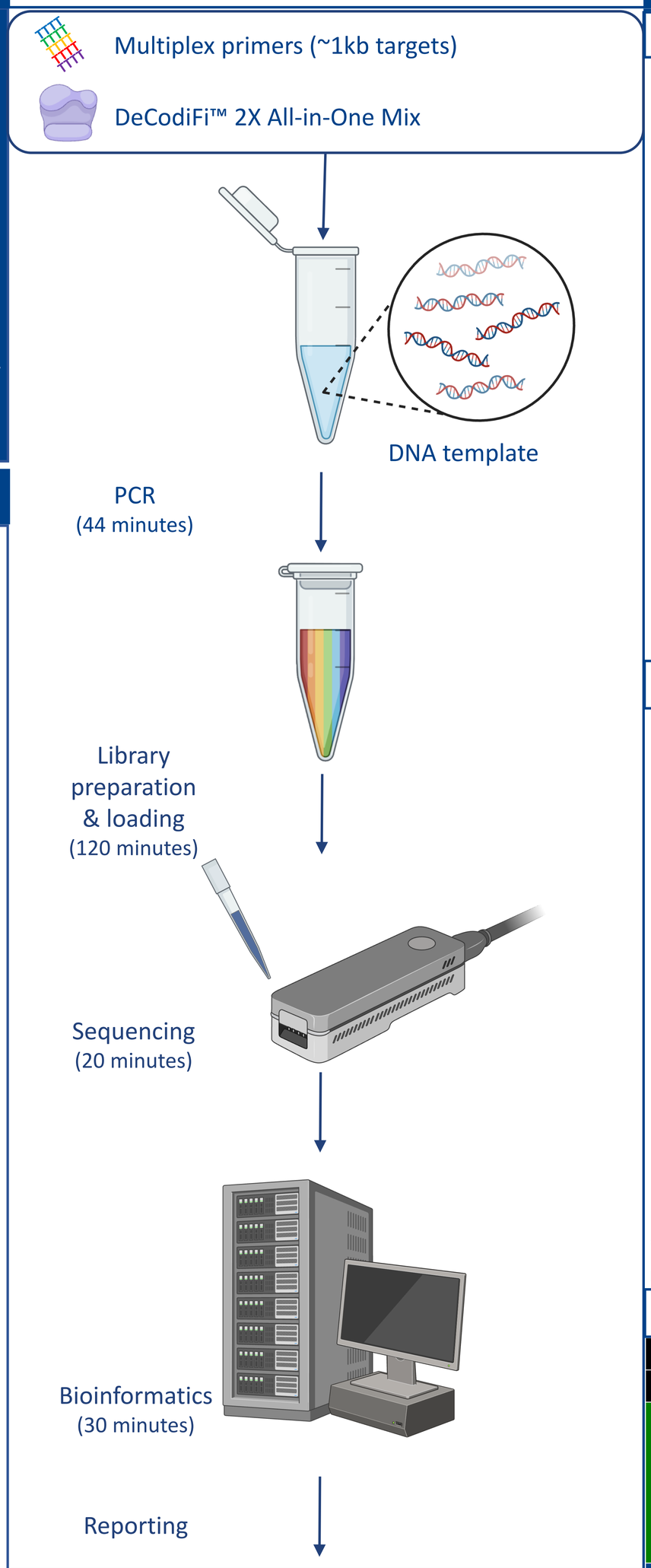
Workflow



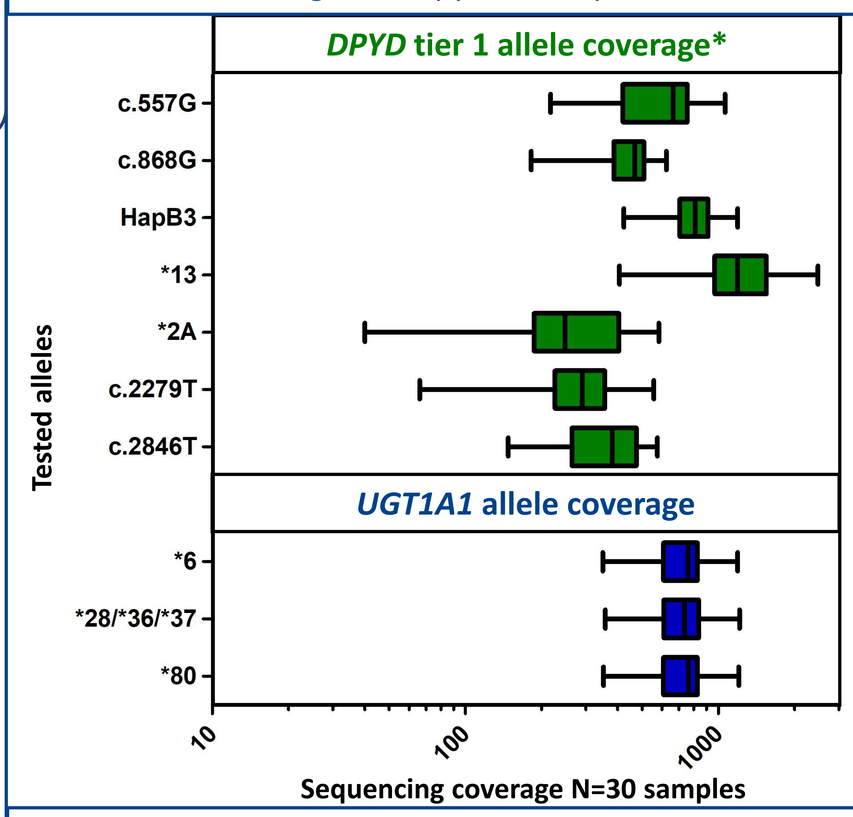
Pharmacogenetic pass (example)

Gene	Outcome	Effect	Tested variants
DPYD	*1/c.2946T	Intermediate (IM)	*2A, *7, *8, *13, HapB3, c.557G, c.868G, c.1314G, c.1475T, c.1774T, c.2279T, c.2639T, c.2864T
UGT1A1	*1/*28	Normal (NM)	*6, *28, *36, *37, *80
TPMT	*1/*3A	Intermediate (IM)	*2, *3A-C, *4-18, *29, *42
NUDT15	*1/*1	Normal (NM)	*2-6, *9,*14

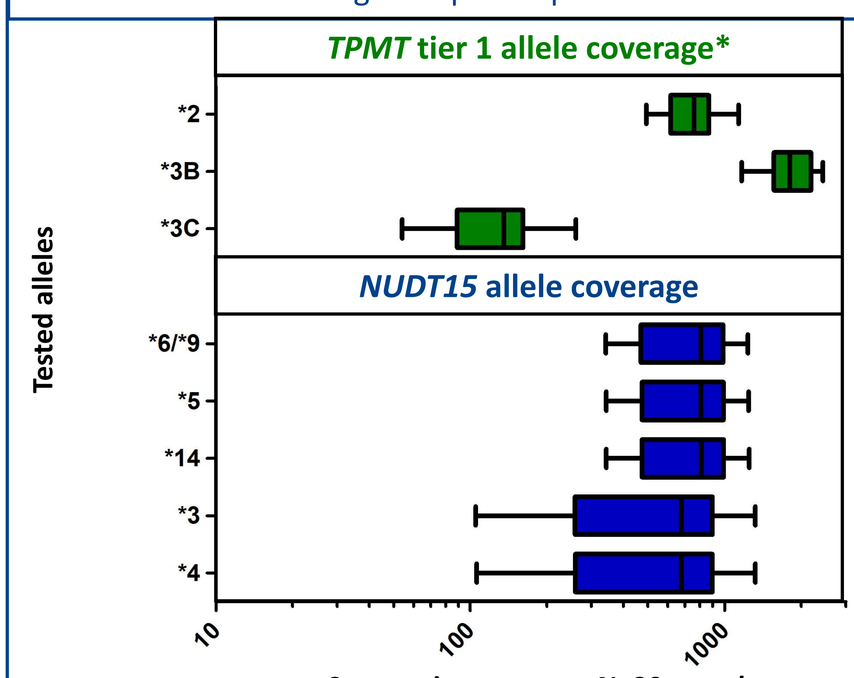
Pharmacy
Informed therapeutic strategy

Results

Coverage fluoropyrimidine panel



Coverage thiopurine panel



* Coverage distribution for the tier 2 variants in *DPYD* and *TPMT* are not shown due to space constraints

Variant detection panel

Fluoropyrimidines		Detected variants		
Gene	**Variant**	**wt**	**het**	**hom**
DPYD	*7	14	1	0
	HapB3	12	3	0
	*13	14	1	0
	*2A	13	1	1
	C.2846T	12	3	0
UGT1A1	*36	14	1	0
	*28	5	5	5
	*37	14	1	0
	*80	5	5	5

Thiopurines		Detected variants		
Gene	**Variant**	**wt**	**het**	**hom**
TPMT	*2	9	1	0
	*9	9	1	0
	*3B	7	3	0
	*8	9	1	0
	*3C	6	4	0
NUDT15	*9	9	1	0
	*6	8	2	0
	*3	9	1	0
	*4	9	1	0

Conclusions

- Concordance; all previously identified variants were detected
- Our new amplicon-based workflow is (nearly) ready for implementation
- Optimising each step of the workflow resulted in short turnaround times for urgent diagnostic tests

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