IGHV Analysis Utilising the Ion GeneStudio™ S5 System and the Oncomine™ BCR IGH-LR Assay – Early Access Experience

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Background

In 1999 two studies found that patients with Chronic Lymphocytic Leukaemia (CLL) could be separated into two categories, those with mutated IGHV genes (<98% identity with the germline gene, M-CLL) having a more favourable prognosis than those with unmutated IGHV genes (U-CLL). IGHV gene mutational status has continued to be one of the most robust prognostic markers in CLL; it also has a strong predictive value for response to treatment with U-CLL displaying shorter progression-free survival after chemo-immunotherapy with the fludarabine, cyclophosphamide and rituximab (FCR) regimen compared to M-CLL.
The standard method for IGHV analysis has been to Sanger sequence the IGHV rearrangement of CLL B cells. Recently, Next Generation Sequencing (NGS) assays have been developed and here we describe our early access experience of the Oncomine™ BCR IGH-LR assay (Thermofisher).

Aim

To compare the standard Sanger sequencing method for IGHV analysis with the NGS Oncomine™ BCR IGH-LR assay, as part of an early access analysis.

Materials & Methods

- The Oncomine™ BCR IGH-LR assay is RNA-based and employs multiplex FR1 and isotype specific primers. Figure (1a) illustrates the NGS method workflow and (1b & 1c) the primer binding regions for NGS and Sanger respectively.
- We assessed 48 previously Sanger sequenced samples; 27 from the same time point and 21 with a median difference in time point of 36 months. 6 had been easy to sequence by Sanger, 8 had been difficult and 34 had borderline mutation identity (97-98.99%), this was to assess possible issues with mutational status assignment due to FR1 primers excluding the 5' sequence of the variable gene.
- RNA was extracted using the Ribopure kit and quantified with the Qubit RNA HS kit.
- 25ng of RNA was reverse transcribed using the Oncomine™ BCR IGH-LR provided reagents.
- Library preps were quantified using the Ion TaqMan® Quantification kit and either 25pM or 10pM each of 8 libraries were combined for each 530 chip.
- Table 1 compares the different steps of the workflow in Sanger sequencing and NGS for IGHV mutational analysis.

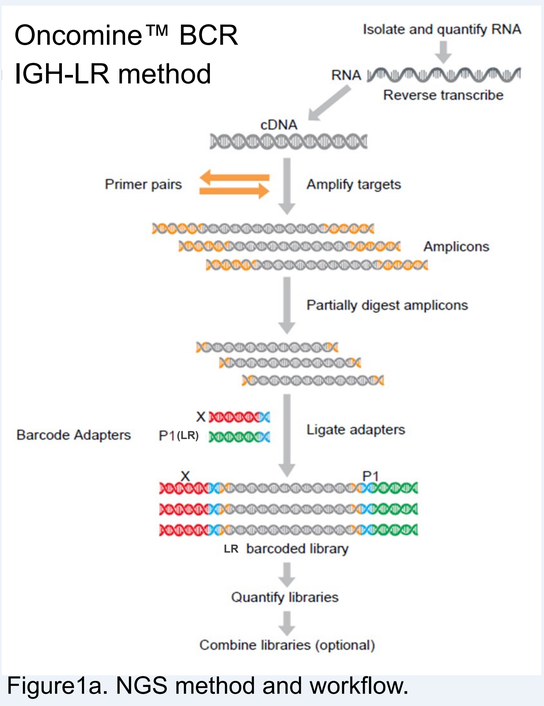

Figure1a. NGS method and workflow.

Sanger	NGS
Generally DNA	RNA & reverse transcription
3 or more PCRs per sample	1 PCR per sample
Gel & clean-up	Library prep & quantification
Can send away to a sequencing service at this point	Combine libraries
Sequencing reaction x2 per PCR	Load Chef for templating and chip loading
Clean-up sequencing reactions	Initialise sequencer
Load each sequence	Insert chip (8 samples/chip)

Table 1. Workflow comparison

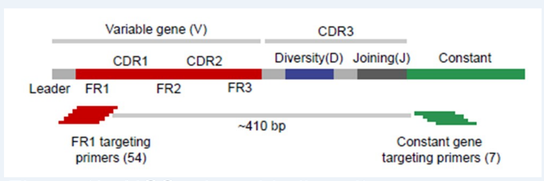

Figure 1b. NGS primer binding sites.

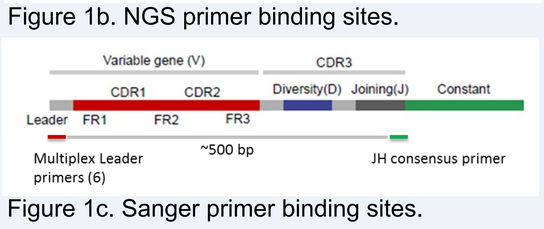

Figure 1c. Sanger primer binding sites.

Results

Results were initially assessed in the run report generated in the Torrent Suite (TS) software to check chip loading, read depth and quality of data. The data was then uploaded to Ion Reporter (IR), where IGHV rearrangement and mutational status can be analysed. Figures 2 and 3 illustrate how the results can be visualised in TS and IR respectively. Figure 3a is representative of a sample with one predominant clone, whereas 3b is an example of a case that was difficult to sequence by Sanger.

Barcode Name	Sample	Bases	>=Q20 Bases	Reads	Mean Read Length
No barcode	None	1,056,309,279	849,792,484	3,676,561	287 bp
IonDual_A1_0101	5833	430,294,446	363,894,746	1,255,507	342 bp
IonDual_B1_0102	5795	436,646,836	376,289,377	1,737,596	251 bp
IonDual_C1_0103	5637	137,447,726	115,535,717	602,434	228 bp
IonDual_D1_0104	5612	383,264,599	323,763,998	1,161,572	329 bp

Figure 2. Section from TS Run Report Summary showing the number of reads and mean read length for each sample.

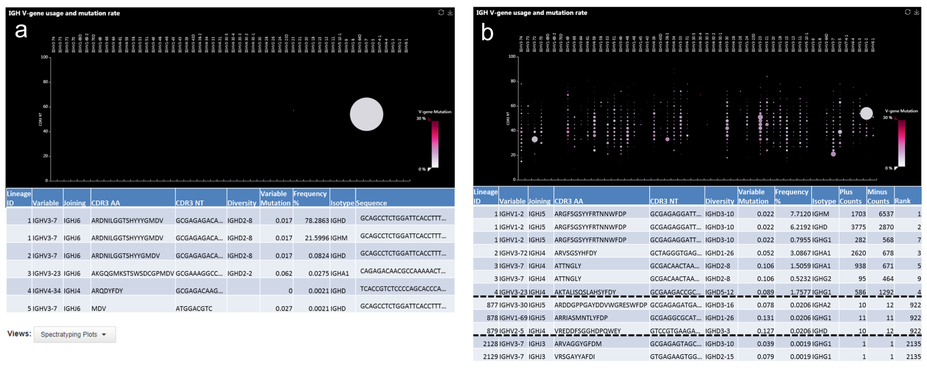

Figure 3. Example images of IR IGH BCR results; a) the large circle on the spectratyping plot indicates 1 predominant sequence, the clone summary can be downloaded in a table as shown under the spectratyping plot, b) a difficult sample to sequence by Sanger, likely due to the number of sequences detected, as seen in the NGS plot and highlighted in the clone summary below.

NGS & Sanger Compared

IGHV rearrangements detected by NGS

- NGS detected the same rearrangements as previously detected by Sanger in 47/48 cases and for 1 previously unproductive (UP) only result, NGS identified the productive (P) sequence.
- 10 NGS results, including 4 of the difficult cases, did show some variation between the two methods

1) 6/10 had the same major clone with further minor clones (1 with a minor P clone by NGS, but the same sequence was UP by Sanger due to a single bp insertion; Figure 4a)
2) 2 showed clonal variation over time
3) 1 was concordant but a 3bp deletion in the Sanger sequence was not evident in the NGS sequence; Figure 4b.
4) 1 with the predominant NGS sequence not detected by Sanger but two smaller clones were concordant; Figure 4c.

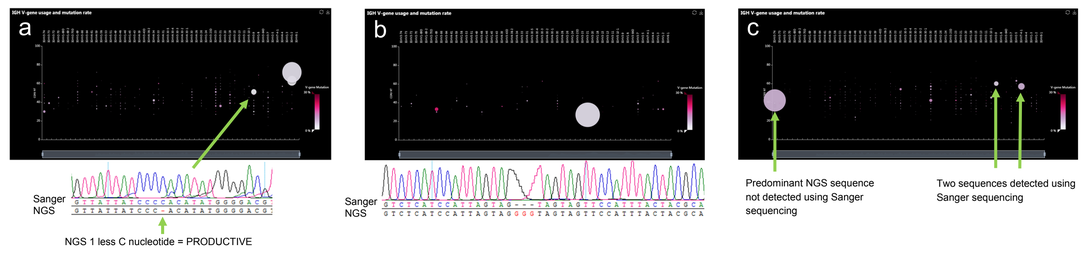

Figure 4. Highlights the variations between NGS and Sanger sequencing for 3 cases; a) Sanger identified a P and an UP sequence, the UP sequence was P by NGS due to the absence of a 1bp insertion detected by Sanger, b) concordant rearrangements detected but a 3bp deletion detected by Sanger was not evident by NGS and c) the predominant NGS sequence was not detected by Sanger, although the two minor NGS sequences were.

% identity of NGS sequences

- For 10/32 previously sequenced using leader primers where the entire FR1 region is used to calculate the % identity, the mutational status changed when calculated using the NGS assay (Table 2).
  - 3 unmutated > borderline
  - 6 borderline > mutated
  - 1 borderline > unmutated
- 13/14 previously sequenced using FR1 primers were concordant
  - 1 borderline > unmutated
- For 2 sequences the % identity had not been determined using Sanger due to poor quality sequence.

Sanger	NGS
98.26	97.75
98.26	97.77
98.25	97.72
97.64	96.97
97.57	96.85
97.55	96.85
97.19	96.83
97.22	96.43
97.22 / 97.56*	90.62 / 95.91
97.92**	98.21

Table 2. Changes in mutational status when using leader primers compared to FR1 NGS primers.
*2 minor sequences NGS, predominant not detected by Sanger.
** 2 mutations present in FR1.

Isotype Identification

- 9 CLL cases showed some evidence of class switching, 8/9 utilising an IGHV3 gene and 4 being predominantly switched
  - 2 IgG only (IGHV3-30, IGHV3-23)
  - 1 IgG/IgA (IGHV3-21)
  - 1 IgA/IgM/IgD (IGHV3-21)

Conclusions & Future work

Overall, our experience of the early access Oncomine™ BCR IGH-LR assay has been positive;

- For all productive rearrangements the same sequence was detected by NGS
- The observed differences in mutational status due to priming in FR1, should not be an issue going forward as a leader primed assay is currently in development.
- The 2 cases with variation in deletion-insertions will however, need further investigation.
- The main advantage of the assay is the detection of all rearrangements separately, which obviates the issues encountered with direct Sanger sequencing when there is more than one rearrangement or polyclonal B cells present.
- The assay will also enable the detection of a clone at very low frequency e.g. following treatment or at clonal evolution.
- In addition, isotype identification may be of interest in translational research and potentially provide information about the dynamics of the clone.

Acknowledgements & References Many thanks to Thermofisher for providing early access to the Oncomine™ BCR IGH-LR assay and support with the specific analysis methods. Hamblin *et al.* Blood 1999, Damle *et al.* Blood 1999, Thompson *et al.* Blood 2016.

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PosterSessionOnline.com