Finding a suitable library size to call variants in RNA-Seq

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Abstract

Background: RNA sequencing allows the study of both gene expression changes and transcribed mutations, providing a highly effective way to gain insight into cancer biology. When planning the sequencing of a large cohort of samples, library size is a fundamental factor affecting both the overall cost and the quality of the results. Here we specifically address how overall library size influences the detection of somatic mutations in RNA-seq data in two acute myeloid leukaemia datasets.

Results: We simulated shallower sequencing depths by downsampling 45 acute myeloid leukaemia samples (100 bp PE) that are part of the Leucegene project, which were originally sequenced at high depth. We compared the sensitivity of six methods of recovering validated mutations on the same samples. The methods compared are a combination of three popular callers (MuTect, VarScan, and VarDict) and two filtering strategies. We observed an incremental loss in sensitivity when simulating libraries of 80M, 50M, 40M, 30M and 20M fragments, with the largest loss detected with less than 30M fragments (below 90%, average loss of 7%). The sensitivity in recovering insertions and deletions varied markedly between callers, with VarDict showing the highest sensitivity (60%). Single nucleotide variant sensitivity is relatively consistent across methods, apart from MuTect, whose default filters need adjustment when using RNA-Seq.

We also analysed 136 RNA-Seq samples from the TCGA-LAML cohort (50 bp PE) and assessed the change in sensitivity between the initial libraries (average 59M fragments) and after downsampling to 40M fragments. When considering single nucleotide variants in recurrently mutated myeloid genes we found a comparable performance, with a 6% average loss in sensitivity using 40M fragments.

Conclusions: Between 30M and 40M 100 bp PE reads are needed to recover 90–95% of the initial variants on recurrently mutated myeloid genes. To extend this result to another cancer type, an exploration of the characteristics of its mutations and gene expression patterns is suggested.

Keywords: Cancer RNA-Seq, Variant calling, Library size, Sequencing depth

Background

RNA sequencing (RNA-Seq) is routinely used to quantify transcripts, detect fusion genes and differential splicing. It can also be used to call mutations, a key component in the study of cancer genomes. This makes RNA-Seq a cost effective choice in cancer...
research. However, calling variants in RNA-Seq cancer samples is often overlooked due to the large number of possible sources of bias. We are only able to call variants if they are transcribed, and gene expression variation causes the transcriptome-wide depth to be highly heterogeneous. The variant allele frequency (VAF) in cancer samples can also be affected by other sources of variation, such as normal tissue contamination, differing clonality and copy number changes. When working with human material we are often limited by the number of samples available, and this makes decisions about the sequencing depth (or library size) especially critical. Previous research has highlighted the importance of sequencing depth in many fields of genome research, including transcriptome sequencing, but typically this has considered differential expression (DE) analysis, transcript discovery and differential splicing [1]. In that study a staged sequencing approach was presented as a useful tool for determining the parameters of the sequencing experiment (e.g. the number of replicates or the number of mapped reads). Numerous papers have been published around the power to detect DE genes in RNA-Seq, but the discussion has mainly concerned the number of replicates needed and the statistical tools applied [2–4]. The number of transcripts that can be identified and the number of potentially false positive DE genes increases steadily as sequencing depth increases [5]. Earlier work on the power to detect DE genes based on the number of replicates, sequencing depth and analytical tools used suggests that the number of replicates is more important than the read depth, and that going beyond 20M fragments does not increase the power [6]. Sensitivity analysis for variant calling from RNA-Seq data has received less attention.

Previously, staged sequencing approaches have been used to determine the depth of sequencing required for reliable detection of germline single nucleotide polymorphisms (SNPs) in whole exome sequencing (WES) and whole genome sequencing (WGS) [7]. For these studies, a mean on target coverage of 40X was enough to reach 95% sensitivity for the detection of germline variants. More recent work has explored SNPs detection in RNA-Seq from lymphoblastoid cell lines [8]. After applying a range of different aligners and callers, they found that sensitivity remained reliably > 90% with a total read depth of > 10X at the variant site. Providing general advice regarding the depth of sequencing required to obtain optimal coverage for variant calling in RNA-Seq is challenging, because it depends on the expression level of the target genes. The motivation for the present study was the need to inform the in-house sequencing of a cohort of Core Binding Factor Acute Myeloid Leukaemia (CBF-AML) RNA-Seq samples in order to allow accurate DE analysis and variant calling. To investigate this question, we used a staged sequencing approach using 45 deeply sequenced CBF-AML RNA-Seq samples from the Leucegene study [9], where mutations had been validated on matched DNA samples. We used the validated variants in order to get estimates of the sensitivity at shallower depths and validated our findings on a larger independent AML cohort.

Results

Sensitivity in the Leucegene cohort

We used 45 CBF-AML RNA-Seq samples that were deeply sequenced with 100 base pair (bp) paired end (PE) reads to compute the sensitivity in recovering 88 validated mutations at lower levels of sequencing depth [9] (Table 1, Additional file 1: Figure
S1). This was done by simulating smaller library sizes by random downsampling of the reads in the initial samples. We will refer to these validated mutations as the **truth set**.

After alignment, the initial samples have a mean of 113 million (M) mapped PE reads (min 77.8M–max 187.4M, 93.4% mean mapping rate). We downsampled the initial unaligned files at five fixed library sizes of 80M, 50M, 40M, 30M, and 20M PE reads (or fragments) to simulate shallower depths. Two samples have library size marginally below 80M and we used all the reads as the initial run. At each library size, the random downsampling was replicated more than once to account for downsampling variability (see “Downsampling strategy” section in Methods). At every stage we called variants using three different callers, previously used to call variants in RNA-Seq: MuTect2 [10], VarScan2 [11], and VarDict [12]. We will refer to MuTect2 as MuTect and to VarScan2 as VarScan. Figure 1 shows the sensitivity in recovering all the variants in the truth set. The sensitivity is computed using two different filtering strategies: (1) **default-filters** which are based on each caller’s default settings and a set of variants detected in a panel of normals (PON) comprising RNA-Seq samples from CD34+ cells; (2) **annotation-filters** which uses external databases, the PON variants and other quality filters (see details in “Variant filtering” section in Methods).

The sensitivity for detecting single nucleotide variants (SNVs, Fig. 1a left plot) is comparable across filtering strategies and callers apart from the unusual behaviour of MuTect with default-filters, where sensitivity increased as the library size decreased. MuTect behaviour is due to the **clustered events** filter which removes variants found on haplotypes where other variants are already detected (Additional file 1: Figure S2). This behaviour was also observed in a previous study comparing variants called from matched RNA-Seq and WES samples where the same flag was responsible for filtering the largest number of RNA variants [13].

Using knowledge from external databases allows retention of two NRAS SNVs present in COSMIC [14], which are discarded by default-filters as they are also present in the PON samples at very low frequency. The SNV sensitivity remains above 95% for all callers with the initial and 80M libraries and it incrementally decreases between the 50M and the 30M libraries, remaining around 90%. 100% of the initial variants are recovered with the initial and the 80M libraries using the annotation-filters. The largest drop in sensitivity is observed when moving from 30M to 20M fragments, where

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**Table 1 Types of variants in the Leucegene truth sets**

| Mutation type       | Min VAF | Mean VAF | Max VAF | N  |
|---------------------|---------|----------|---------|----|
| Composite indel     | 0.06    | 0.25     | 0.56    | 9  |
| Long insertion      | 0.41    | 0.5      | 0.64    | 3  |
| Short deletion      | 0.09    | 0.24     | 0.38    | 2  |
| Short insertion     | 0.07    | 0.33     | 0.84    | 15 |
| SNVs                | 0.05    | 0.37     | 0.97    | 58 |
| Indel-not reported  | 0.84    | 0.84     | 0.84    | 1  |

Variants used as the truth set were previously validated in a set of 45 CBF-AML RNA-Seq samples [9]. Variant types are inferred from the information in the published study and by the variant calls performed on the initial samples. A short indel (insertion/deletion) is an indel < 10 bp long; composite indels are mutations including both inserted and deleted nucleotides; SNVs are single nucleotide variants.
in some cases only 80% of the initial SNVs were recovered. With the default-filters, the median sensitivity for SNVs decreases by a maximum of 5% between the initial and the 30M libraries when using VarScan or VarDict, reaching a 10% loss with 20M fragments (Additional file 1: Figure S3). The drop in sensitivity using subsequently smaller library sizes is larger when using the annotation-filters, even though the sensitivity with larger libraries is higher using this strategy.

The sensitivity in recovering insertions and deletions (indels) (Fig. 1a right plot) varied markedly between callers, with VarDict calling consistently more indels than the other callers, but still only achieving a maximum of 60% recall. The large difference in indel sensitivity between callers is partly due to a bias in reporting indels. VarDict uses the same approach as the km [15] caller, used to create the truth set, and it is the only caller adopted here which was developed for both DNA and RNA. Indel sensitivity slightly increases with MuTect and VarScan if only a partial match with the allele in the truth set is required (Additional file 1: Figure S4). For these reasons, the choice of a suitable
library size will be based on SNV sensitivity, and indel sensitivity should be assessed using more appropriate and comparable callers. The unusual behavior of MuTect with default-filters is not observed for indels. This could be because a large number of indels are not detected by MuTect even with the initial samples. Therefore, the sensitivity curves do not appropriately reflect the change in the behaviour of the caller at different depths. The majority of the SNVs missed at shallower sequencing depths are either not reported by a caller or subsequently filtered by quality thresholds, especially when using annotation-filters (see flags of variant missed in Additional file 1: Figure S5). The quality filters are mainly affected by the hard threshold on the total and alternative depth at a variant site (see details in “Annotation filters” section in Methods).

The variants VAF remains stable across library sizes (Fig. 1b top plot), while the alternative and total depths at a variant site decrease steadily (Fig. 1b bottom plot, Additional file 1: Figure S6, distribution of the initial total depth and VAF in Additional file 1: Figure S7A), with low alternative depth characterizing a large part of the variants lost. The median alternative depth of the variants missed by a caller has a sharp drop when considering less than 50M fragments. The alternative depth varies between 30X and 50X when using libraries larger than 50M fragments and between 5 and 9X for smaller libraries (Additional file 1: Table S2). This is because the variants missed with larger libraries are not called due to presence in the PON, or mismatches with the alternative allele detected in the truth set. On the other hand, as the library size decreases, a larger number of variants present in smaller clones or lowly expressed loci, are lost. This is also observed in the bottom plot in Fig. 1b, where more red lines (variants lost) are noticeable with less than 50M fragments.

The genes mutated in the Leucegene cohort tended to be highly expressed, with the majority of the genes having total read counts above 4 log2RPKM (Additional file 1: Figure S7B). The mutations were also detected across a wide range of VAFs, and it is clear that reliably detecting mutations with VAFs below 0.2 remains challenging, even when using more than 30 million reads (Fig. 1c).

**Sensitivity in the TCGA-LAML cohort using validated WGS and WES variants**

The analysis with the Leucegene samples showed that there is limited sensitivity below 30M fragments, with an average decrease in recall of 7% when sequencing 20M compared to 30M fragments (Fig. 1). Increasing the library size to > 30M fragments induces incremental small gains in sensitivity (between 0.5 and 2%) and at 40M all callers recover > 90% of the initial variants. Therefore, we decided to analyse the loss in sensitivity within the critical range 30M–20M fragments in an independent AML cohort, namely 136 50 bp PE RNA-Seq samples from the TCGA-LAML cohort [16]. Given the difference in read length between the two cohorts, a comparable coverage between them is obtained when considering double the number of fragments in the TCGA-LAML cohort compared to the Leucegene cohort (100 bp PE). The initial TCGA-LAML samples have an average of 58.7M mapped reads (min 36.7M–max 69.6M, 75.6% mean mapping rate) which corresponds to roughly 30M fragments in the Leucegene samples. To replicate 20M PE reads in the Leucegene cohort we choose 40M fragments as the target library size and analysed the loss in sensitivity between the initial TCGA-LAML and the downsampled libraries.
The available BAM files were downsampled at different proportions depending on their mapping rate, in order to obtain a number of mapped reads similar to that obtained with the 40M downsampled Leucegene libraries (see “Downsampling strategy” section in Methods). We called variants with VarDict, MuTect and VarScan on the initial and downsampled BAM files, and evaluated the sensitivity in recovering SNVs from two truth sets created from the list of validated WES and WGS variants [16]. The truth sets are: Set1, including 1,643 SNVs from the published variants after removing intergenic and intronic SNVs (Fig. 2a left plot); Set2, which is a subset of Set1 including 169 SNVs from recurrently mutated myeloid genes (Fig. 2a right plot, Additional file 1: Table S3, details in “TCGA truth sets” section in Methods). Both the default-filters and the annotation-filters were used for variant filtering with some minor exceptions from the Leucegene analysis (see “TCGA truth sets” section in Methods). Due to the challenges induced by different indel representation across callers described in the previous section, we only assessed SNVs sensitivity.

![Image](image_url)

**Fig. 2** Sensitivity using the TCGA-LAML truth sets. **a** 2D Density plots of the RNA VAF (x-axis) against the total depth on a logarithmic scale (y-axis) of the SNVs in TCGA-LAML validated sets of variants: Set1 (all SNVs after removing intronic and intergenic variants) on the left and Set2 (SNVs on recurrently mutated AML genes) on the right. **b** Violin plots of the log2RPKM of the genes with variants detected in Set1 and Set2. The gene expression distributions are provided for the TCGA-LAML and the Leucegene libraries using the initial samples, before downsampling. **c** Sensitivity in recovering SNVs from the TCGA-LAML cohort in the initial and downsampled libraries using a combination of the two truth sets, callers and filtering strategies.
Genes whose variants belong to Set2 tend to be more expressed than genes in Set1 in both the Leucegene and the TCGA-LAML cohorts (Fig. 2b). This is also reflected at the variant level, where a large number of variants from Set1 has very low total depth at the variant site in the RNA samples (Fig. 2a left plot), while when only restricting to myeloid genes, there is only a low number of variants with very low total depth (Fig. 2a right plot). This implies that the genes commonly mutated in AML also tend to be more expressed. Figure 2c summarizes the sensitivity with the initial and the downsampled TCGA-LAML data. The default-filters always outperform the annotation-filters. As expected, the sensitivity in recalling the variants in Set1 (blue shaded dots) is quite low, ranging between 22 and 38% across callers, with marginal differences between library sizes, (average 4% decrease in sensitivity). The recall rate improves if only variants on recurrently mutated myeloid genes are considered (green shaded dots). VarDict with default-filters has the highest sensitivities, recovering 89% and 88% of the SNVs with the initial and the downsampled libraries respectively. Within this truth set, the filtering strategy used has a larger impact on the sensitivity. The decrease in sensitivity between the initial and the 40M libraries is on average 6%, with the annotation-filters showing a consistent poorer performance across callers (average decrease of 9%) and default-filters returning more stable recall values (average decrease of 3%). Many of the variants are filtered by the hard thresholds on the total and alternative depth at a variant site as well as because of their proximity to exon boundaries (see flags and alternative depth of missed variants in Additional file 1: Figures S8).

Figure 3a offers a breakdown of the per gene sensitivities using default-filters and considering only genes whose variants are in Set2. MuTect’s poor performance on the top recurrently mutated genes, FLT3, IDH1, and IDH2 is again caused by the clustered events filter, discussed in the “Sensitivity in the Leucegene cohort” section and sensitivity improves when applying annotation-filters (Additional file 1: Figure S9). Both VarDict and VarScan recover almost all events on these genes which are highly expressed across the TCGA-LAML samples (log$_2$RPKM > 3.5 across the three genes, log$_2$RPKM > 4.5 for FLT3 and IDH2). VarDict is the caller with the highest sensitivity, reaching > 90% recovery rate with the top six mutated genes (DNMT3A, IDH2, RUNX1, IDH1, FLT3 and TP53) using both the initial and the 40M PE reads libraries. The genes with the lowest sensitivities are U2AF1, KDM6A, RAD21 and STAG2. Apart from U2AF1, not many variants are present on the other genes and several SNVs are filtered out due to low quality of the alternative alleles and low total depth. U2AF1 has the lowest sensitivities across all genes in Set2, apart when using VarScan. Not surprisingly, this gene also has the lowest expression levels among all myeloid genes considered (log$_2$RPKM < 2.5, Fig. 3b). As the gene expression decreases there is an increased discordance between callers (red and darker shaded dots in Fig. 3b) and only around 50% of the variants are detected on average with log$_2$RPKM < 3.

**Sensitivity by total depth at a variant site**

We estimated the sensitivity as a function of the total depth at a variant site for both the TCGA-LAML samples using variants from Set1 (Fig. 4a) and the Leucegene samples using the published truth set (Fig. 4b). For both cohorts, the same SNVs detected across
downsampled runs are used to compute the sensitivity curves (see “Computation of sensitivity” section in Methods).

Across all strategies and in both datasets, the changes in sensitivities stabilise and remain above 75% when the total depth is larger than 20X, and they stay on average above 80% when larger than 30X. The low sensitivities obtained with the TCGA-LAML samples using the variants in Set1 are due to 67% of the variants having total depth in RNA below 20X (60% between 0 and 10X, 7.4% between 10 and 20X, Fig. 4a, Additional file 1: Figure S10) as the truth set was obtained from DNA variants. The difference in sensitivity between the initial and the downsampled TCGA-LAML libraries is small at any total depth interval, with the exceptions of MuTect, delivering lower and more discordant sensitivities at higher depths. When stratifying the Leucegene sensitivities by library size, the majority of the variants lost derives from the 20M and 30M libraries, using either filtering strategies (Additional file 1: Figure S11). This is because the Leucegene variants were detected from RNA and are therefore well expressed. Indeed, all variants in the truth set have total depth > 90X in the initial libraries (Additional file 1: Figure S12).

Figure 4c shows the cumulative sensitivity of each caller using the annotation-filters, and applying increasingly higher thresholds, $d$, on the total read depth at a
variant site. Only variants with total depth $\geq d$ are classified as called. The annotation-filters were used to allow a fair comparison with MuTect due to the bias with its default filters. However, the two strategies have a comparable performance (Additional file 1: Figure S13). The results confirm the poor performance when using only 20M fragments. Indeed, using this library size the sensitivity remains below 90% at any total depth threshold. Not until the 40M library sizes, and borderline with 30M, can MuTect and VarScan recover at least 95% of the variants when requiring $d \geq 20$. VarDict recall is slightly lower than the other callers, recovering 90% of the variants when setting $d \geq 20$. When increasing the threshold $d$ above 20, the calls are progressively more discordant between different library sizes.
Discussion

Our study complements previous research which has assessed the mean on-target depth necessary to recover SNPs in RNA-Seq, WES and WGS. In those studies, different depths at a variant site were specified, ranging between 10X and 40X, depending on the technology being applied [7, 8]. Previous work has suggested 20M PE reads is enough for accurate detection of DE genes [6], but our results suggest higher levels of coverage are required for robust variant detection. Here we have focused on the influence of the total library size, to provide some guidance when planning the design of a sequencing study. Our study used acute myeloid leukaemia as an example, and it provides a direct connection between the total library size and the on-site variant features (VAF and total depth). We applied a range of different variant calling methods and focused on the ability to recall a set of validated variants in key AML genes. We did not assess the specificity of variant calls. The advice provided here is general, and it is important to consider that the optimal library size may need to be adapted depending on the type of cancer under study, the expression level of key target genes, or individual sample characteristics, like tumour purity or the degree of intratumoural heterogeneity.

We suggest that between 30M and 40M fragments are required to guarantee 90–95% sensitivity in recovering variants in myeloid genes, which is approximately 50–100% greater than the suggested library size for DE analysis. While the largest loss in sensitivity is often observed when sequencing less than 30M fragments, it is not clear how to define a fixed library size suitable for all types of samples. This is because of the incremental decrease in sensitivity as the library size gets smaller. Nonetheless, using the Leucegene samples, we saw a negligible reduction in the sensitivity to recover validated variants when sequencing 80M compared to more than 100M PE reads (Fig. 1). Following this, there are similar modest losses of sensitivity when going from 80M to 50M, from 50M to 40M, and from 40M to 30M reads. The loss only becomes more noticeable with library sizes below 30M fragments. A similar conclusion was reached with the TCGA-LAML cohort, where the average drop in sensitivity (6%) in recovering SNVs between 60M and 40M 50bp PE reads on recurrently mutated myeloid genes is comparable to that one of the Leucegene cohort between the critical range of 30M to 20M 100 bp PE reads (7%) (Fig. 2c). We also found a total depth at a variant site larger than 20X to be a critical threshold to stabilize the sensitivity above 75%, for both AML cohorts (Fig. 4a). However, the highest recall rates are obtained for variants with total depth larger than 50X.

Taking all the above results together, we can consider a general formula to inform the target library size, based on the expression level (RPKM) of key genes and the total depth at a variant site:

\[
\text{Library size} = \frac{(\text{Mean read depth at a variant site} \times 1K \times 1M)}{(\text{Average Gene RPKM} \times \text{Read length} \times 2)}
\]  

(1)

For example, if we want the average total depth at a variant site to be 30X, in order to ensure good sensitivity in a gene with RPKM of 4 (slightly above the lowest expression levels of genes in Set2, Fig. 2b), and we consider 100 bp PE reads, the required library size is 37.5M fragments. If we set the total depth to 20X, then the library size drops to 25M fragments. By using the above equation and knowledge about the transcriptome
features of the tissue under investigation one can explore whether detecting mutations from RNA-Seq is worthwhile.

Our validation set included somatic mutations across a wide range of VAFs and expression levels. The genes identified in the Leucegene study tended to have higher expression than those in the TCGA-LAML cohort [5/15 genes with reported mutations had total read counts above 4 log2RPKM for Leucegene, compared with 13/28 genes in TCGA-LAML (Fig. 3b, Additional file 1: Figure S7B)]. This likely reflects the fact that mutation detection for TCGA-LAML included assessment of DNA, where discovery would be independent of expression. While the Leucegene genes were highly expressed, the truth set includes somatic mutations with low VAF, and our downsampling approach showed that robust detection of these variants remains challenging.

We showed that the sensitivity in recovering validated SNVs in the Leucegene RNA-Seq samples is independent of the caller used, with the exception of MuTect with default settings, whose behavior should be adjusted when applied to RNA-Seq. However, the choice of a caller has a greater impact when calling indels, and targeted approaches are required to guarantee a good sensitivity. The number of bioinformatics tools developed recently to improve indel detection in RNA-seq is a demonstration of the increasing interest in exploiting RNA for variant calling, and the need for better algorithms and benchmarking [17, 18]. In particular, AML genomes often harbour hot spot indels whose detection is of clinical importance. These are internal tandem duplications (ITDs) found in FLT3 and KIT [19, 20] as well as a 4bp insertion in NPM1. The km algorithm was recently published [15] which performs targeted variant detection. The sensitivity and precision of the caller were studied using FLT3-ITDs and NPM1 insertions detected from the Leucegene and the TCGA-LAML cohorts, reaching more than 90% sensitivity for both lesions and making it an appealing caller for complex indels.

In this study, no single method to call and filter SNVs always outperformed the others. Prioritizing cancer variants based on external databases, like COSMIC, rescued NRAS somatic mutations which were found at very low VAF in one sample in the reference PON. NRAS is commonly mutated in CBF-AML and it is not surprising if the same mutation is present with low VAF in normal haematopoietic stem cells. The hard thresholds used in combination with the annotation-filters appeared too stringent and induced sharper decreases in sensitivities compared to using default-settings in the Leucegene samples. Also, while MuTect and VarDict are better choices to detect complex indels (Fig. 1a right plot), VarScan is quicker than MuTect; it allows genome-wide calls; and has comparable sensitivity to the other callers in recovering SNVs. A suggested workflow when calling variants in cancer RNA-seq would be to choose a SNV caller and adapt its filters to the specific characteristics of the cohort and samples available. For example, it is advisable to carefully check for highly recurrent filters to avoid losing interesting variants as was found with the clustered events filter in MuTect. In general, MuTect should not be used in tumor-only mode but this does raise the problem of the availability of suitable matched normal samples for tumour RNA-Seq. Differences in gene expression between tissues may complicated variant detection, and with blood cancers it can be challenging to obtain a normal sample, free from contaminating cancer cells. If normal DNA is available, it can be used to filter artefacts and germline variants [21, 22]. It is also
useful to adopt different callers to detect different types of variants, e.g. using a targeted caller for indels to increase sensitivity and a fast genome-wide caller for SNVs.

Conclusions
In conclusion, this study offers a starting point to help design an informative and cost-effective analysis of cancer transcriptomes. It is important to consider that cancers are extremely heterogeneous, and that a rigorous assessment of the cohort characteristics is necessary to determine the optimal library size for variant detection.

Methods
Leucegene CBF-AML RNA-Seq: alignment and pre-processing
The R package GEOquery [23] and the SRA Toolkit [24] were used to download the SRA files from GEO and to convert them to FASTQ files. FastQC [25] 0.11.5 was used to check the quality of the initial FASTQ files and no samples was removed due to low quality. Sample SRX381851 was excluded from the analysis due to a small initial library size of only 44.5M PE reads. The GNU Parallel command-line utility [26] was used to parallelize the FastQC runs. The FASTQ files were aligned against the UCSC hg19 reference genome to resemble the analysis performed in the original publication [9]. Alignment was performed with STAR [27] 2.5 in two-pass mode. The splice junctions from the 45 CBF-AML samples collected from the first pass were used to inform the alignment in the second pass. STAR was chosen for several reasons: its speed; its good performance in the correct alignment of indels [28]; and since it is the suggested choice in the GATK [29] Best Practices for RNA-Seq variant calling. Read groups were added to the aligned BAM files using AddOrReplaceReadGroups from Picard tools [30] 2.9.4 and PCR duplicates were marked with sambamba [31] 0.6.6 markdup. Duplicate reads were not removed from the BAM files but reads marked as duplicates are ignored at the variant calling step. The quality of the BAM files were validated with ValidateSamFile from Picard Tools and no errors were found in any processed library. Gene counts were obtained with featureCounts using the hg19 inbuilt RefSeq annotation available in Rsubread [32]. The same pipeline was used for both the initial and every downsampled run as well the PON samples.

TCGA-LAML data: bamfile pre-processing
The TCGA-LAML cohort comprises 151 50bp PE RNA-Seq bamfiles of which 17 are CBF-AML. The bamfiles were already aligned to the hg38 genome reference genome using STAR in two-pass mode. Read groups had already been added using STAR. We flagged PCR duplicates with sambamba markdup and obtained gene counts with featureCounts and the hg38 inbuilt RefSeq annotation. Both sambamba markdup and MarkDuplicates from Picard Tools failed in processing sample TCGA-AB-2931 which was removed from the rest of the analysis. Eight bamfiles failed the downsampling step due to some internal features of the bamfiles. These samples are: TCGA-AB-2821, TCGA-AB-2870, TCGA-AB-2884, TCGA-AB-2925, TCGA-AB-2950, TCGA-AB-2991, TCGA-AB-2994, and TCGA-AB-2995 and they were excluded from the rest of the analysis. Out of the 142 bamfiles left, 139 had mutations validated through targeted capture
and manual review in the original publication [16]. After excluding indels from the truth set, 136 RNA-Seq samples with available variants were used for sensitivity analysis.

**Downsampling strategy**

The FASTQ files from the Leucegene CBF-AML data were downsampled using the seqtk toolkit for FASTA/Q files [26]. Every fixed downsampled library size was obtained five times (only 3 times for the 80M library size). Five seeds were used to allow reproducibility of the results: 100, 26880, 56745, 7234, 9999. Only BAM files were available for the TCGA-LAML cohort and they were downsampled only once using the DownsampleSam function from Picard Tools. This function extracts a proportion of the reads out of the initial library size. We adjusted the sampling proportions for the TCGA-LAML samples in order to simulate a setting with approximately 40M sequenced fragments and a > 90% mapping rate. Adjustment was needed since the mean proportion of mapped reads in the TCGA-LAML samples was lower then for the Leucegene samples, defining a ratio of 1.24 (93.4% in the Leucegene and 75.6% in TCGA samples respectively). Therefore, we first obtained the total number of reads in each TCGA-LAML BAM file using samtools flagstat [33], where this number includes both mapped and unmapped reads, and increased the sampling proportion of each sample by 1.24. This adjustment should make the results comparable and it is based on the assumption that, in the future, the mapping quality for bulk RNA-Seq samples will more likely resemble the Leucegene quality. This led to a mean of 37.3M mapped fragments in the downsampled TCGA-LAML cohort (37.4M mapped with the Leucegene data). Additional file 2: Table S4 contains the downsampling proportions used for the TCGA-LAML RNA-Seq samples.

**TCGA truth sets**

The sensitivity analysis with the TCGA-LAML samples was performed using previously published validated variants as the truth set [16]. From the initial table of variants we removed 6,460 mutations falling outside of gene bodies; 8,319 variants in untranslated and intronic regions; and only kept variants belonging to samples whose RNA-Seq BAM files were available. Eventually, 1,643 SNVs were kept for sensitivity analysis (Additional file 3: Table S5). From these SNVs, two truth sets were defined: 1) Set1: including all 1,643 SNVs; 2) Set2: a subset of Set1 including the published significantly mutated myeloid genes [16], out of which FAM5C and HNRNPK were removed by previous filters, and on top of which 7 genes were added as they were mutated in the Leucegene CBF-AML samples (KMT2C, JAK2, GATA2, CSF3R, ASXL1, NF1, KDM6A) [9] (list of genes in Additional file 1: Table S3). We created 1k symmetric windows around the starting positions of the variants in the final truth set and we used those regions to perform variant calling. The genomic position obtained from the original table were lifted up from the hg18 to the hg38 reference genome using the UCSC Genome Browser [34]. When matching the TCGA-LAML variants called by a caller with the variants in the truth sets, we did not use transcript information. This was because the transcripts reported in the original table derive from old annotations as well as they come from a mix of ensembl and RefSeq annotations. We decided to not consider transcript information to avoid wrong assignments or removal of variants due to missing transcript information.
Choice of callers and BAM file preparation for variant calling

Several variant callers are compatible with RNA-Seq [35]: RADIA [21], Seurat [22], SNPir [36], eSNV-detect [37], VarScan [11] and VarDict [12]. The first two callers, RADIA and Seurat, integrate tumor-normal RNA and DNA and were not considered since only RNA is available. SNPir is a caller specifically developed for RNA-Seq but for normal tissue. It is based on GATK [29] pre-processing and the Haplotypecaller [38] and implements a series of RNA-Seq specific filters. eSNV-detect implements an ensemble approach by combining the calls performed with SAMtools using two different aligners. Here we compared the performance of three popular callers: VarScan 2.4.0 (which requires samtools mpileup [33] output), VarDict 1.5.1 and MuTect2 from GATK 3.7.0, which is the GATK choice for tumor samples. Only VarDict was developed to call variants from both RNA and DNA. VarDict can call variants in both tumor-only and matched tumor-normal settings, whereas VarScan and MuTect were designed for somatic variant calling. All three callers were run in tumor-only mode and a reference of PON samples was created for filtering (see details “Variant filtering” section). Prior to calling variants with MuTect and VarScan the BAM files were pre-processed following the GATK best practices, which include splitting reads that contain N’s in the CIGAR string and base quality recalibration. VarDict can handle spliced reads without pre-processing. VarDict can only call variants on subsets of the genome whereas both VarScan and MuTect call variants genome-wide. This last difference will not introduce any bias into our comparison since variants are called only in target regions of interest throughout the whole analysis (see regions in Additional file 1: Table S1). The three callers call both SNVs and indels but only VarDict and MuTect adopt local realignment, which should give better accuracy around indels. Samtools mpileup performs base alignment quality (BAQ) which aims at reducing SNVs miscalled due to nearby indels.

Variant calling, annotation and standardised output

To allow a fair comparison between variant callers, variants were called with MuTect, Samtools mpileup + VarScan and VarDict using their default settings. Variant calling is always performed in tumour-only mode. Variants were then annotated with the Variant Effect Predictor (VEP) 89.0 [39]. The genome assemblies GRCh37 and GRCh38 were used for the Leucegene and the TCGA-LAML samples respectively. The annotated VCF files were parsed using the parsing functions included in the varikondo [40] R package to produce a standardized output across callers containing the relevant information for the analysis.

Variant filtering

We adopted two variant filtering strategies, namely default-filters and annotation-filters. The default-filters strategy is based on each caller’s default filters while the annotation-filters strategy exploits external databases and applies the same quality measures across callers. The current analysis aims at reproducing a general framework to be used in tumor-only RNA-seq variant calling, and we do not wish to “over-fit” a caller’s settings to benefit this specific type of data. What matters in this study
is to compare the performance of variant calls under the same circumstances while varying the overall library size.

**Default filters**

We used only variants flagged with PASS in the VCF output to estimate sensitivity and specificity based on each caller’s default settings. We also used a PON samples to remove likely artefact variants that are called across many normal independent samples. In particular, we used a set of 17 RNA-Seq libraries of CD34+CD45RA- cord blood cells from 17 non-pooled individuals. We created a PON variants separately for every caller (see details in Additional file 1: “Variant filtering” section in Additional Methods). A variant is classified as present in normals if it is found in more than two normal samples or in less than two normal samples but with VAF > 0.03. We created PON variants using both the hg19 and hg38 reference human genomes.

**Annotation filters**

We set up a series of filters based on variant quality measures, public databases, features of the genome known to be challenging (repeat regions, homopolymers, splice junctions), and PON samples. Some of these filters, including variants in homopolymers, RNA editing sites [41], variants in repeat regions [42] and variants near splice junctions, have been previously shown to be successful in reducing the number of false positives [36, 41]. Variant annotation using VEP adds information from external databases of mutations such as COSMIC [14], ExAC [43] and dbSNP [44]. Details about how to download the databases mentioned here and how we used them to flag variants can be found in Additional file 1: “Variant filtering” section in Additional Methods. Flags and quality filters are then used for filtering. In particular:

- Variants are removed if they are found in the PON (using the same strategy as explained in the “Default-filters” section) and they are not found in COSMIC.
- Variants are removed if they are not found in COSMIC but they are present in dbSNP and ExAC.
- Even if present in COSMIC, variants are removed if they overlap with exon boundaries, homopolymer stretches or repeated regions. Overlap with exon boundaries is defined if the variant falls within 4 bp upstream of an exon start site or downstream of an exon end site. Exon boundaries were obtained from the hg19 and hg38 inbuilt RefSeq Rsubread annotation. We considered as homopolymers, stretches of the same nucleotide longer than 5 bp as obtained from the hg19 and hg38 reference human genomes (more details provided in Additional file 1: “Annotation databases and genomic features” section in Additional Methods).
- Finally, a variant is kept only if it has an average base quality > 18, a minimum alternative allele depth of 5, a minimum total depth of 15 and a minimum VAF of 0.03. This means, for example, that in order to keep a rare variants with VAF < 0.03 the mutation needs to be covered by at least 167 reads.
Matching alternative alleles

In both strategies a variant is classified as called in a downsampled library only if the alternative allele matches the alternative allele in the truth set, if available, or the one obtained when calling variants using the initial deeply sequenced libraries. The alternative alleles were available for the TCGA-LAML dataset but not for the Leucegene cohort. The three callers were consistent in reporting the same alternative alleles for all the SNVs called with the Leucegene cohort but not for indels. To reduce callers differences in reporting indels, we first ran the variant normalization tool vt normalize [45]. To take into account persisting differences we considered a match if an indel lied within 100 bp (+/-50 bp) of the position reported in the Leucegene truth set. Manual curation was then needed to remove false positives. An extra level of complexity derives from the fact that callers report composite events differently, making it hard to assess a match with the alternative allele. MuTect and VarScan output indels as separate SNVs and short indels rather than as block substitutions, while VarDict, like the km algorithm, outputs composite variants in the same line. This is why we also explored whether a partial match with the reference and alternative alleles would increase sensitivity for MuTect and VarScan. A partial match required at least 3 bp overlap with the alleles reported in the truth set.

Computation of sensitivity

At every downsampled run, the sensitivity was computed as in Equation 2 below:

$$Sensitivity_{ijt} = \frac{TP_{ijt}}{TP_{ijt} + FN_{ijt}}$$

(2)

where $TP_{ijt}$ is the number of variants called by caller $i$ with library size $j$ that belong to truth set $t$, and $FN_{ijt}$ is the number of variants in truth set $t$ missed by caller $i$ at the library size $j$. A variant is reported as a match with respect to a truth set, if it is called by a caller and it matches the genomic information (chromosome, position etc..) reported in the truth set.

The sensitivity by intervals on total depth is computed as in Eq. 2 but stratifying by intervals on the total depth. The intervals are set from 0X to the maximum total depth observed in the data by gaps of 10 until 100X. When total depth $>100X$ only two intervals are considered, 100X–130X and 130X to the maximum total depth.

The sensitivity as a function of the depth $d$ is computed as in Eq. 3 below:

$$Sensitivity_{ij \mid tot\ depth \geq d} = \frac{TP_{ij \mid tot\ depth \geq d}}{TP_{ij \mid tot\ depth \geq d} + FN_{ij \mid tot\ depth \geq d}}$$

(3)

where $TP_{ij \mid tot\ depth \geq d}$ is the number of variants called by caller $i$ with library size $j$ that has total depth $\geq d$ and that belong to the truth set. $FN_{ij \mid tot\ depth \geq d}$ is the number of variants with total depth $\geq d$ present in the truth set but missed by caller $i$ with library size $j$.

R packages used in the study

The variant calling workflow was developed using the package optparse [46]. The packages foreach [47] and doParallel [48] were used to parallelize the parsing of the variant
annotation fields added by VEP. Variants output were standardised across callers using the package varikondo [40], available on GitHub. The Bioconductor package GenomicRanges [49] was used to create the files needed to annotate variants with respect to genomic features (see Additional file 1: Additional Methods for more details). The library seqinr [50] was used to read FASTA files into R in order to detect stretches of homopolymers used for variant filtering. The package samplepower [51], only available on GitHub, contains the functions used to compute sensitivities throughout the analysis (more details in Additional file 1: Additional Methods). Data manipulation to parse variants output and to produce summary of the sensitivity results was obtained using the R packages readr [52], dplyr [53], tidyr [54], stringr [55]. All figures in this paper were produced with the libraries ggplot2 [56] and cowplot [57]. All analysis in R were run using R3.5.2.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s12859-020-03860-4.

**Additional files**

Additional file 1. Supplementary figures, tables and methods.

Additional file 2: Table S4. Downsampling proportions for the TCGA-LAML samples.

Additional file 3: Table S5. TCGA-LAML truth set.

**Abbreviations**

AML:: Acute myeloid leukaemia; CBF-AML:: Core binding factor acute myeloid leukemia; DE:: Differential expression; Indels:: Insertions and deletions; M:: Million; PE:: Paired end; PON:: Panel of normals; RPKM:: Read per kilobase per million; RNA-Seq:: RNA sequencing; SNP:: Single nucleotide polymorphism; SNV:: Single nucleotide variant; VAF:: Variant allele frequency; WES:: Whole exome sequencing; WGS:: Whole genome sequencing.

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**Authors’ contributions**

AQ performed all the analyses and wrote the manuscript. CF provided guidance through several steps of the development of the RNA-Seq variant calling workflow and reviewed the manuscript. IM conceived the initial idea and provided suggestions and revision for the manuscript. TS contributed with suggestions during the conception and development of the study and reviewed the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

The FASTQ files of the 45 Leucegene CBF-AML RNA-Seq samples were downloaded from GEO at the accession numbers GSE49642, GSE52656, GSE62198, GSE66917, and GSE67039. The FASTQ files of the 17 RNA-Seq samples of CD34+CD45 RA-cord blood cells used to create the PON variants were downloaded from GEO at accession number GSE48846. The TCGA-LAML BAM files were downloaded after permission was granted from the GDC Data Portal. The software settings and supporting scripts used for downsampling, preprocessing, alignment, variant calling and annotations are available on GitHub https://github.com/annaquaglieri16/Supporting-scripts-library-size-RNA-Seq.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.
Competing interests
The authors declare that they have no competing interests.

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