

Enabling complete transcriptome sequencing

QuantSeq 3' mRNA-Seq with integrated automatic data analysis - a complete workflow for most easy to use and cost efficient gene expression profiling

QuantSeq provides an easy protocol to generate highly strand-specific Next-Generation Sequencing (NGS) libraries close to the 3' end of polyadenylated RNAs within 4.5 hrs from high and low quality RNA (incl. FFPE). Only one fragment per transcript is generated, directly linking the number of reads mapping to a gene to its expression. QuantSeq reduces data analysis time and enables a higher level of multiplexing per run. Each QuantSeq kit also includes a complementary automated differential gene expression data analysis option. QuantSeq is the RNA sample preparation method of choice for accurate and affordable gene expression measurement and is the best alternative to using gene expression arrays.

With the rapid development of NGS technologies, RNA-Seq has become the new standard for transcriptome analysis. Although the price per base has been substantially reduced, sample preparation, sequencing, and data processing are major cost factors in high throughput screenings. QuantSeq's unique method reduces the expenditures in all these areas.

Sample Preparation. QuantSeq is a fast and simple protocol that generates NGS libraries of sequences close to the 3' end of poly(A) RNAs within 4.5 hrs with just 2 hrs of hands-on time. The kit requires only 0.1 – 2000 ng of total RNA input without the need for poly(A) enrichment or ribosomal RNA depletion. Because of its focus on the 3' end, QuantSeq is also highly suitable for formalin-fixed, paraffin-embedded (FFPE) samples.

Sequencing. QuantSeq generates only one fragment per transcript, and the number of reads mapped to a given gene is proportional to its expression. No complicated coverage-based quantification is required. Fewer reads are necessary for determining unambiguous gene expression values, allowing a higher level of multiplexing.

Data Processing. QuantSeq's high strand specificity (>99.9 %) enables the discovery and quantification of antisense transcripts and overlapping genes. QuantSeq data analysis pipeline has been automated on the Bluebee® genomics analysis platform, providing a fast and user friendly data processing tool.

The QuantSeq Workflow. Library generation begins with reverse transcription using an oligodT primer (Fig. 1a). Following first strand synthesis, the RNA template is removed and second strand synthesis initiated by random priming. Illumina- or Ion Torrent-specific linker sequences are introduced by the primers. The resulting double-stranded cDNA is purified with magnetic beads, rendering the protocol compatible with automation. Library PCR amplification then introduces the complete sequences required for cluster generation (Fig. 1b). Illumina libraries can be multiplexed with up to 384

dual indexed barcodes and are compatible with both single-read and paired-end sequencing reagents. The insert size is optimized for short reads (e.g., SR50 or SR100), however in-protocol options allow to adapt the library size also for longer read lengths. Ion Torrent libraries can be multiplexed using 48 in-line barcodes.

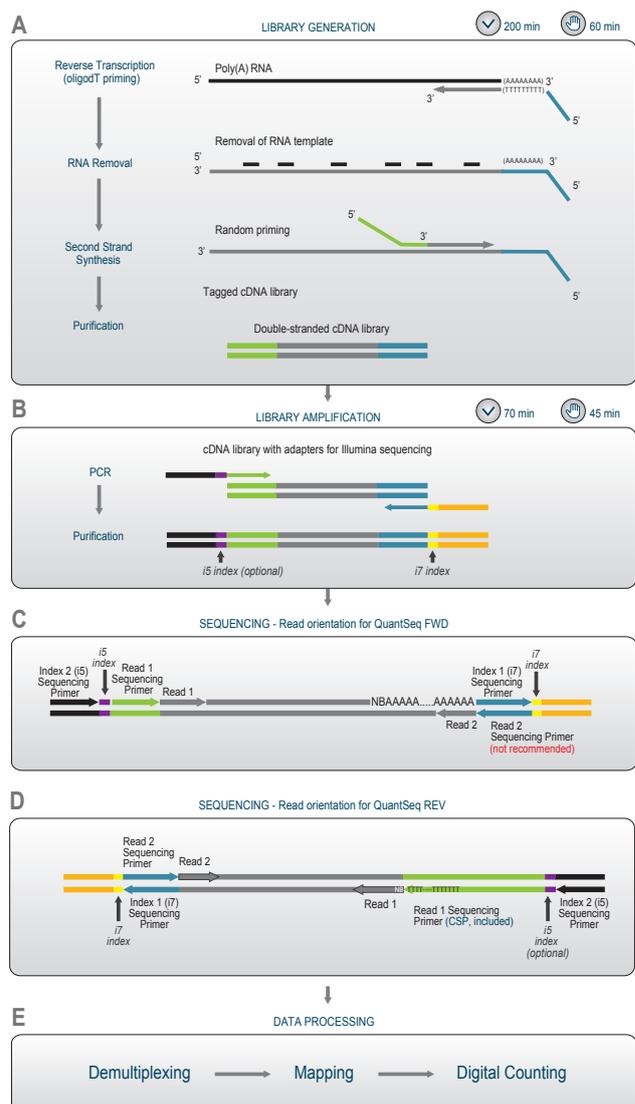
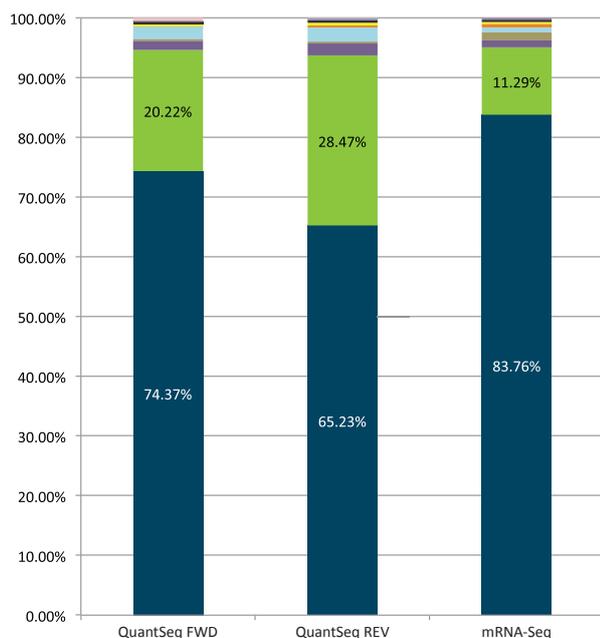


Figure 1 | Schematic overview of the QuantSeq FWD library preparation workflow. For QuantSeq REV the position of adapters for Read 1 (green) and Read 2 (blue) are switched.

Table 1 | Mapping statistics. Values depicted are averages from triplicates and given in percentage of all reads and percentage of uniquely mapping reads.

	QuantSeq FWD	QuantSeq REV		mRNA-Seq	
	A* ₁₋₂	A ₁₋₃	B ₁₋₃	A ₁₋₃	B ₁₋₃
Total Reads	6,181,833	21,938,757	12,829,269	10,069,397	11,902,438
% Mapping Reads	87.7 %	91.0 %	87.8 %	95.7 %	96.7 %
% Uniquely Mapping Reads	74.6 %	57.2 % ^a	59.8 % ^a	86.4 %	89.1 %
% ERCCs	1.5 %	4.2 % ^b	3.9 % ^b	0.7 %	1.0 %
% Strandedness ^c	99.9 %	99.9 %	99.9 %	93.4 %	97.8 %

A*₁₋₂: Universal Human Reference RNA + ERCC RNA Spike-In Mix 1 prepared in house, A₁₋₃: SEQC mixture of Universal Human Reference RNA (UHRR) and External RNA Controls Consortium (ERCC) ExFold Spike-In Mix 1, B₁₋₃: SEQC mixture of Human Brain Reference RNA (HBRR) and ExFold Spike-In Mix 2. ^a Common sequence motifs of the polyadenylation signal and the upstream sequence element limit the variability in the 3' region, thereby reducing the number of uniquely assignable reads in QuantSeq REV. ^b For further analysis, the number of ERCC reads was down-sampled to the common absolute denominator of 0.7 % and 1.0 % as seen in mRNA-Seq A₁₋₃ and B₁₋₃. ^c Strandedness was calculated on ERCC reads.



Classes Based on Uniquely Mapping Reads Sample A	QuantSeq FWD	QuantSeq REV	mRNA-Seq
protein_coding	74.37 %	65.23 %	83.76 %
no_feature	20.22 %	28.47 %	11.29 %
mt_rRNA	1.58 %	2.01 %	1.18 %
ambiguous	0.34 %	0.31 %	1.42 %
lincRNA	2.04 %	2.41 %	0.76 %
pseudogene	0.0007 %	0.32 %	0.55 %
processed_transcript	0.33 %	0.40 %	0.35 %
antisense	0.40 %	0.42 %	0.39 %
sense_overlapping	0.03 %	0.04 %	0.05 %
mt_tRNA	0.08 %	0.19 %	0.10 %
sense_intronic	0.08 %	0.09 %	0.07 %
3prime_overlapping_ncrna	0.0020 %	0.0038 %	0.0032 %
rRNA	0.0009 %	0.0097 %	0.0012 %
others*	0.519 %	0.099 %	0.069 %

Figure 2 | Gene and transcript biotypes. Uniquely mapped reads from QuantSeq FWD, QuantSeq REV, and mRNA-Seq libraries were assigned to biotypes based on the Ensembl annotation. * Includes miRNA, non-coding RNA, snRNA, snoRNA, IG and TR genes, and ncRNA-related pseudogenes.

QuantSeq is available in two editions with different read orientations. QuantSeq Forward (FWD, Cat. No. 015 for Illumina and Cat. No. 012 for IonTorrent), generates reads toward the poly(A) tail that correspond to the mRNA sequence during Read 1 sequencing (Fig. 1c). Longer reads may be required if the exact 3' end of the mRNA is of particular interest. QuantSeq Reverse (REV, Cat. No. 016 for Illumina only), generates reads corresponding to the cDNA sequence during Read 1 sequencing (Fig. 1d). Here, a Custom Sequencing Primer (CSP, included in the kit) is used that covers the oligodT stretch to achieve cluster calling on Illumina sequencers, which require a random base distribution within the first sequenced bases. Alternatively, a T-fill reaction can be carried out¹.

Comparison Between QuantSeq and Standard mRNA Sequencing

QuantSeq enables upscaling in multiplexing RNA-Seq experiments, rendering it highly suitable for differential gene expression analysis. Here we present a comparison between QuantSeq and a standard mRNA-Seq protocol, focusing on differential gene expression metrics. We performed QuantSeq REV library preparations on U.S. Food and Drug Administration (FDA) Sequencing Quality Control (SEQC) standard samples A and B in technical triplicates. Sample A is a mixture of Universal Human Reference RNA (UHRR) and External RNA

Controls Consortium (ERCC) ExFold Spike-In Mix 1. Sample B is a mixture of Human Brain Reference RNA (HBRR) and ExFold Spike-In Mix 2 (we received SEQC samples A and B from the FDA prepared according to the FDA/National Center for Toxicological Research SEQC RNA Sample Preparation and Testing SOP_20110804). After T-fill, these 6 libraries, referred to as QuantSeq REV A₁₋₃ and B₁₋₃, were sequenced in one Illumina HiSeq 2000 lane yielding 150 M single reads of 50 bp (SR50). Residual adapter sequences were removed, and the trimmed pass-filter reads were down-sampled to 10 M each to be comparable with an mRNA-Seq NGS experiment derived from the identical RNA input material. The mRNA-Seq data sets were made available by a laboratory that participated in a published Association of Biomolecular Resource Facilities (ABRF) NGS study². In that study, the researchers performed a stranded RNA-Seq library preparation with poly(A) enrichment in 2 technical triplicates, obtaining 50 bp paired-end (PE50) reads on an Illumina HiSeq 2000 (ref. 2; from the GSE48035 data set samples SRR903178-80 from GSM1166109 and SRR903210-12 from GSM1166113 were used in this comparison). We discarded Read 2 in those 6 data sets, referred to as mRNA-Seq A₁₋₃ and B₁₋₃, to obtain single-read data comparable to the QuantSeq REV data.

QuantSeq FWD data was generated from in house prepared UHRR reference RNA spiked with ERCC RNA Spike-In Mix 1.

We pooled the mRNA-Seq data sets and aligned them to the GRCh 37.73 genome assembly including ERCC sequences using a splice-junction mapper, TopHat2, which required 2 h 50 min. In contrast, the pooled QuantSeq data sets were aligned in only 35 min using the short read aligner Bowtie2 on the same computer system. For gene expression quantification, standard mRNA-Seq relies on length normalization of the number of fragments per kilobase of exon per million fragments (FPKM) mapped, which depends on the correctness of read-to-transcript assignments. As QuantSeq generates only one fragment per transcript, length normalization is not required, and gene expression quantification is read-count based (Fig. 1e). QuantSeq showed comparable mapping statistics to mRNA-Seq data, but with superior strandedness (Tab. 1). Mapped reads were further categorized with HTSeq-count (Fig. 2).

QuantSeq reads map to intergenic “no_feature” regions to a higher degree than mRNA-Seq reads. This is largely due to the incompleteness of gene annotations at the 3’ end⁴. In a recent publication, Schwalb and colleagues demonstrated that transcription termination sites (TTS) were located within a termination window that extends from the last annotated polyadenylation site to an “ultimate TTS”. This termination window had a median width of ~3300 bp and could be up to 10 kbp wide. On average they detected four additional TTS per mRNA⁵. QuantSeq reads mapping correctly to such unannotated 3’ UTR regions and poly(A) sites do not count towards the “protein_coding” category. This effect is less pronounced for QuantSeq FWD reads since they map more proximal in the 3’ UTRs, and these regions might be part of an existing gene annotation. QuantSeq REV reads, however, extend from the nucleotide immediately upstream of the poly(A) tail towards the mRNA’s 5’ end, and this distal mapping site might be downstream of annotated TTS. Correction of an incomplete annotation (also using mRNA-Seq coverage data) or a sensible 3’ extension of the gene definition can address this discrepancy between the available annotation and the real, sequenced transcriptome⁶. Incorrect 3’ annotations also cause gene expression based on mRNA-Seq data to be calculated wrongly, since they are based on transcript length normalization.

Data sets were evaluated for ERCC spike-in abundances. To allow a direct comparison between mRNA-Seq and QuantSeq, all ERCC reads were down-sampled to identical ERCC read numbers. These subsets of ERCC reads were processed with routines embedded in the ERCC dashboard³. One major benefit of QuantSeq can be visualized by plotting the relative coverage across the normalized transcript length (Fig. 3). Standard mRNA-Seq distributes reads across the entire length of transcripts with underrepresentation of 3’ and 5’ ends, whereas QuantSeq covers the very 3’ end of transcripts. In fact, for gene expression and differential expression analysis, one read per transcript is sufficient. The additional sequencing space gained by focusing on the 3’ end can be used for a higher degree of multiplexing. In the present example, standard mRNA-Seq has a 12.4-fold higher relative sequence coverage (area under the curve (AUC) ratio for all genes (Fig. 3)), which in turn presents the maximal possible reduction in read depth when using QuantSeq while still determining gene expression accurately. We compared the results from the QuantSeq and mRNA-Seq ex-

periments focusing on differential gene expression³. The ability of a method to measure differentials can be evaluated using the predetermined fold changes between ERCC ExFold RNA Spike-In Mixes 1 and 2. When plotting the true-positive rate versus the false-positive rate, the AUC is a measure for the correct detection of differential gene expression (Fig. 4). The maximum mean AUC value, corresponding to optimal differential detection, is 1. When the number of reads is down-sampled from 10 M to 0.625 M, standard mRNA-Seq obtains mean AUC values of around 0.776 only, whereas QuantSeq maintains very high AUC values of around 0.860, although similar total numbers of ERCC spike-in RNAs were detected by both methods during the course of down-sampling.

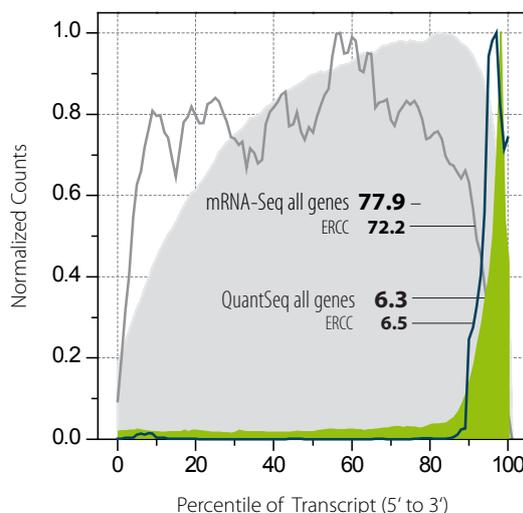


Figure 3 | Coverage versus normalized transcript length in QuantSeq REV and standard mRNA-seq. RSeQC-derived coverage is plotted for all transcripts (areas) and ERCC spike-in control mix only (lines) for QuantSeq (colored) and mRNA-seq (gray). Numbers give the AUC (area under the curve) values as a measure for sequence coverage.

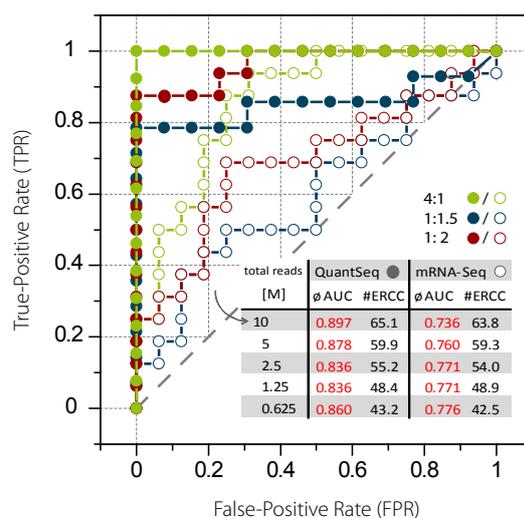


Figure 4 | Differential gene expression performance of QuantSeq REV and mRNA-Seq. The predetermined fold changes (4:1 ●/○, 1:1.5 ●/○, 1:2 ●/○) between ERCC ExFold Spike-In Mix 1 and 2 were used to assess TPRs and FPRs. The receiver operator characteristic- derived AUC value is one measure for the correct detection of differential gene expression. AUC values were assessed together with the number of ERCC RNAs detected (#ERCC) for reads down-sampled from 10 M to 0.625 M. The averaged values of the 6 samples \bar{A}_{1-3} and \bar{B}_{1-3} each are presented in the insert table.

QuantSeq yields good correlation between high and low quality (FFPE) samples

The suitability of QuantSeq when using highly degraded samples (e.g., formalin-fixed, paraffin-embedded (FFPE) material) was evaluated by comparing two samples derived from one source but of different RNA qualities.

A xenograft of the MOLP-8 human tumor cell line was split into two pieces, which were subsequently processed either as fresh frozen cryo-block or embedded FFPE material, leading to different RNA qualities from the same original sample. To determine RNA quality often the RIN (RNA Integrity Number) is used, with an RIN of >8 indicating high RNA quality. For heavily degraded samples this is not a sensitive measure, and hence the DV₂₀₀ value (distribution value of RNA fragments >200 nucleotides) should rather be used. Low RNA integrity correlates with low DV₂₀₀ values.

After RNA extraction, the FFPE sample had a DV₂₀₀ of 87 % (RIN of 2.8), while the cryo sample yielded a RIN of 8.3. The libraries were generated with the QuantSeq 3' mRNA-Seq FWD kit using 50 ng total RNA input. QuantSeq libraries were also successfully generated of FFPE-derived RNA with a DV₂₀₀ of down to 52 % (data not shown). For the FFPE sample the protocol recommendations for low quality RNA input were followed, for the cryo sample the standard protocol was applied. The libraries were sequenced on a HiSeq 2500 instrument at 1x50 bp read length.

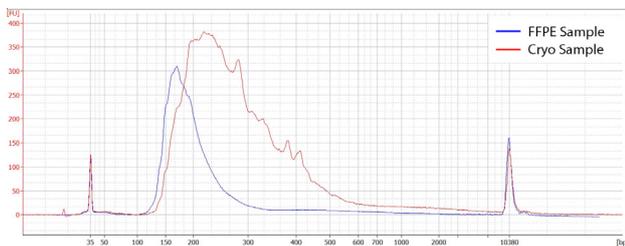


Figure 5 | Bioanalyzer 2100 HS DNA chip traces of QuantSeq 3' mRNA-Seq FWD prepared libraries using FFPE (blue) or cryo RNA input (red). For the degraded input, the resulting library shows a smooth size distribution with no visible linker-linker by-products but only a shift towards shorter fragments. Average library size is 204 bp (FFPE) and 286 bp, respectively.

Plotting the relative coverage across the normalized transcript length shows that coverage is focused on the transcripts' 3' end, independent of the input RNA quality (Fig. 6). However, as QuantSeq FWD libraries are sequenced towards the poly(A) site coverage is dependent on library size and sequencing length.

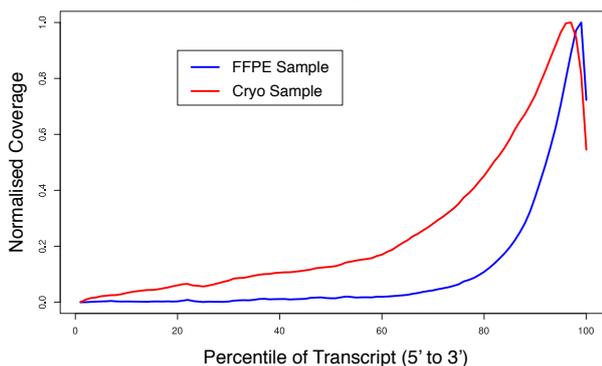


Figure 6 | QuantSeq read coverage versus normalized transcript length of NGS libraries derived from FFPE RNA (blue) and cryo-preserved RNA (red).

The FFPE libraries were significantly shorter than the cryo sample, therefore the ends of the transcripts were reached more frequently, which is reflected in the coverage plots (Fig. 6).

Gene expression correlation between libraries derived from FFPE and cryo-preserved RNA is high (R^2 0.86) and indicates that QuantSeq performs consistently well on samples of different RNA quality (Fig. 7).

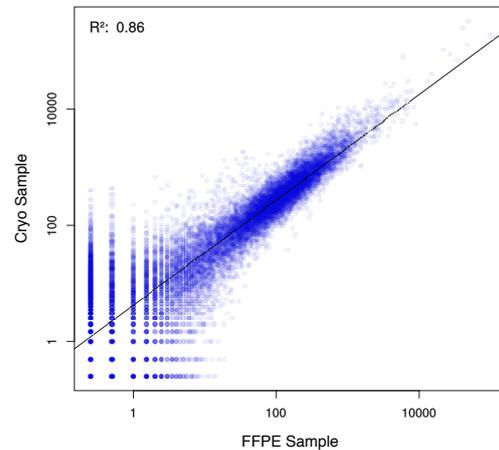


Figure 7 | Correlation of gene counts of FFPE and cryo samples.

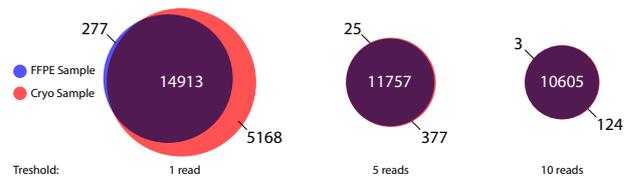


Figure 8 | Venn diagrams of genes detected by QuantSeq at a uniform read depth of 2.5 M reads in FFPE and cryo samples with 1, 5, and 10 reads/gene thresholds.

QuantSeq has a very high sensitivity, at 26.5 M reads, 25,842 genes were detected in the intact Cryo RNA sample (data not shown). At uniformly 2.5 M reads, 20,081 genes were detected in the Cryo sample with at least 1 read, compared to 15,190 genes in the FFPE samples, which represents a 24 % difference (Fig. 8). However, increasing the detection level to 5 or 10 reads/gene reduces the difference to 3 % and 1 %, respectively. These alignments show that QuantSeq reliably detects gene expression in both cryo and FFPE samples; the difference in detection of lowly expressed genes is due to the higher susceptibility of their low copy transcripts to degradation during FFPE treatment, storage, and recovery.

QuantSeq is based on oligodT priming of the reverse transcription and only generates one fragment per transcript. This enables accurate gene expression quantification independent of the RNA quality (including FFPE samples). Standard mRNA-Seq protocols aim to cover the whole transcript, but will result in a heavy 3' bias when used on degraded RNA input. Therefore, QuantSeq 3' mRNA-Seq is an efficient tool to generate NGS libraries from low quality samples compared to other mRNA-Seq protocols using poly(A) selection.

Automated Data Analysis

For your convenience, QuantSeq data analysis pipeline has been integrated on the Bluebee® genomics analysis platform (www.bluebee.com/quantseq) and is accessible free of charge in conjunction with the QuantSeq kit (Illumina compatible kits only). This data analysis pipeline enables fully automated data analysis without bioinformatics involvement (Fig. 9).

Bluebee genome analysis platform is a highly secure cloud-based solution, which connects the sequencer infrastructure with the bioinformatics workflow in a transparent and a very efficient way. Bluebee's private cloud operates in multiple geographic regions and provides users with elaborate functionality for audit trails, encryption, data storage, and retrieval.

Uploading Your Data

The input to start QuantSeq data analysis pipeline is zipped fastq file. Uploading and downloading data is handled by the Bluebee Service Connector. The Bluebee Service Connector is a lightweight piece of software enabling several key functionalities and security features for use of the Bluebee service. The Connector uniquely identifies the client and facilitates end-to-end process auditing. It collects data coming straight from the sequencer and initiates the transfer of raw data in an automated way (no manual intervention required). Additionally, the software encrypts, compresses the raw sequence data and drastically accelerates the data transfer to the appropriate Bluebee compute centre.

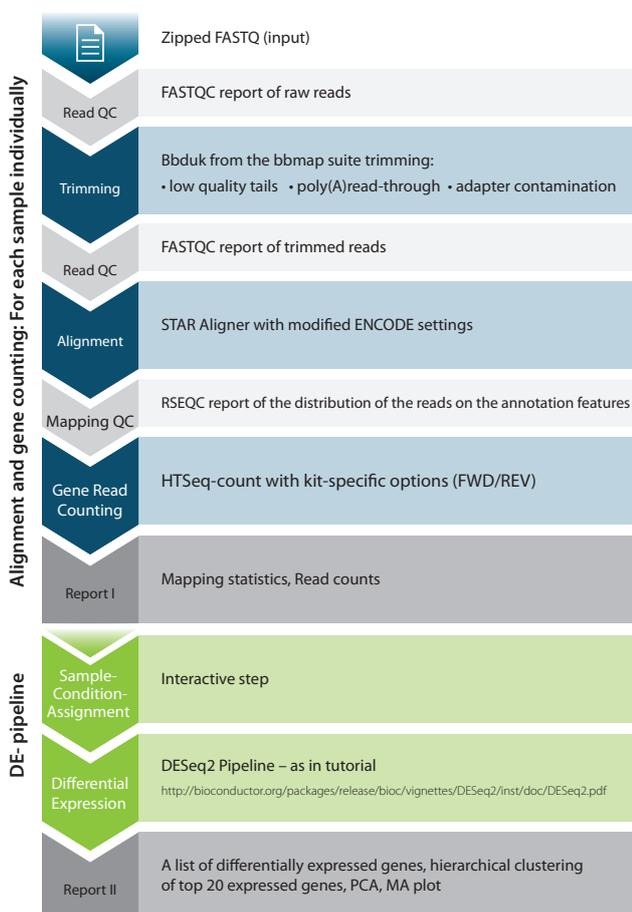


Figure 9 | Automated data analysis pipeline on Bluebee genomic analysis platform.

Processing Your Data

Based on the kit code that has been assigned to your QuantSeq kit the correct data analysis pipeline (FWD or REV) will be initiated automatically.

Viewing and Retrieving Your Data

The Bluebee Service Connector also caters for the download of the results. This can be fully automated by defining download rules. Alternatively, the users can login to the platform via the web GUI to view the results online.

Defining Access to Data (for e.g., Core Facilities)

Access to data is controlled through the concept of projects whereby fine grained access to data can be defined. Data is always owned by a specific account within the system, the administrators of this account can setup specific sub-projects and link the appropriate data to become available to designated users.

Once a sub-project has been setup, individual users of different accounts can be invited to join the project as a viewer (view only) or contributor (allowing the person to upload and process additional data).

Security and Compliance

Bluebee takes security very seriously. The platform has been designed from the bottom up with confidential patient information and multi-layered data security in mind. The result is to ensure an environment that covers the strictest of security controls. The Bluebee platform data centres are compliant with all applicable security and regulation standards. This guarantees state-of-the-art data security as well regulatory compliance. The compliance standards Bluebee adheres to include ISO 27001, HIPAA, and EU Model Clauses (for regional data storage and processing).

Overall Data Security

As genomics related data is very sensitive, a wide range security features are a natural part of the platform.

- Two factor authentication including step up authentication for sensitive actions.
- Login policies enforce strong passwords, renewal period, inactivity timeouts, etc.
- Anonymization, patient data can be stored alongside the actual files and can be used throughout the processing chain or only a reference is provided which links back to patient data held by the clients themselves.
- Data classification is applied to all data (e.g., components, pipelines, datasets, etc.). By default, any data item belongs to the user who introduced it (owner) in the system, it is the owner's exclusive privilege to make the data accessible to other user, companies and/or communities.
- Audit trailing is a concept applied to all objects within the platform, any action including view are recorded assuring regulatory requirements are met.
- Four eyes control on sensitive actions (e.g., modifying a pipeline, uploading/configuring a component, etc.) are combined with step up authentication. Sensitive actions must be approved by another user having the appropriate access rights.
- Role based access defines who can do what on which data. A complex matrix allows the clients administrators to setup very granular security definitions to fit their requirements.
- A PKI infrastructure is used to ensure the integrity of data flowing across the entire platform, going from the upload of the data to the retrieval of the processing results. The combination of digital signatures and encryption techniques are used to ensure the origin and integrity of the data.
- All data are encrypted when in transit (SSL/TLS), both over the internet and internally in the cloud, and at rest (AES 256).

On top of having the data encrypted in flight and at rest, the integrity of the data is checked before any action like: downloading the data, using the data as input for a pipeline (data sealing), any breach will be alerted to the Bluebee security officers and data will be quarantined, once the root cause has been defined appropriate actions will be taken.

"We have successfully used the Lexogen QuantSeq FWD kit for the preparation of RNAseq libraries over the last year. The kit provides us with a highly cost effective and reliable, fast solution to generate RNAseq libraries that we subsequently use to assess gene expression levels and most importantly to rapidly compile and assess alternative cleavage and polyadenylation profiles. QuantSeq is a cornerstone for our RNAseq based research, in particular as multiplexing allows us to combine it with cost effective sequencing on the Ion Torrent platform."



André Furger, Department of Biochemistry, University of Oxford, UK

"Using the QuantSeq 3' mRNA-Seq library prep kits, we were able to multiplex >40 samples per sequencing lane and obtain between 2 to 5 million reads per sample. This enabled us to analyze numerous different strains with various exosome and roadblocking factors inactivated, showing that inactivating roadblocks shifted the window of NNS termination downstream."



Kevin Roy, Lars Steinmetz Lab, Department of Genetics Stanford University School of Medicine, USA

"We have used the QuantSeq 3' mRNA-Seq library prep kit for generating over 2000 libraries. Preparing 96 samples at a time is easy and can be done in one day. We multiplexed 48 samples on a HiSeq lane and thereby obtained 3 million reads per sample on average. Using this shallow sequencing approach, we were highly impressed by the quality of the data and robustness of the method."



Bianca Gopp, Ludwig Institute for Cancer Research, Oxford, UK

"To compare gene expression measures between 3'-RNA-seq and RNA-seq technologies, we used data from a subset of 20 samples that were previously used in a RNA-seq study of feed efficiency. The correlation of the log10 (fold-change) for gene expression (high- vs. low-feed efficiency birds) between these two methods was 0.90. In conclusion, 3'-RNA-seq is a cost effective method amenable to global gene expression studies at population-level, e.g., expression QTL (eQTL) mapping. Also, it allows for accurate detection of the 3'-end of transcripts, enabling verification of the current gene model annotations and global characterization of alternative polyadenylation."



Behnam Abasht, University of Delaware, USA

"I found the QuantSeq pipeline on Bluebee platform to be user-friendly with step-by-step video guide. I contacted Lexogen to inquire further about the pipeline after I got my results and was replied with sufficient explanation and advise."



Kai Ling Liang, Ghent University, Belgium

"Bluebee makes validated and well-documented open-access programs accessible while maintaining transparency. It is simple to use and clearly guides you through the steps in the QuantSeq data analysis pipeline."



Sheina B. Sim and Scott Geibb
University of Hawaii, USDA-ARS Daniel K. Inouye US PBARC, USA

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QUANTTM
SEQ
Sequencing that counts