

Automation and Miniaturisation of RNA-Seq Libraries

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Introduction

RNA sequencing enables genome-wide measurement of transcript abundance and is widely used to study gene expression, transcript structure and biological responses across sample groups. However, RNA library preparation remains relatively expensive and labour-intensive, particularly when ribosomal RNA depletion is required for degraded or lower-quality samples.

Miniaturising RNA library preparation has the potential to reduce reagent costs, while automation can improve throughput and reduce batch-to-batch variability. These advantages become especially important when processing large sample numbers, where manual handling is time-consuming and difficult to scale.

Study Aim

We evaluated whether automated, reduced-volume RNA-seq library preparation on the SPT Labtech firefly+ could produce usable libraries from low-input RNA while reducing reagent use and hands-on processing. Automated libraries were benchmarked against manually prepared libraries, with performance assessed by rRNA depletion, library complexity, mapping performance, reproducibility and sequencing cost.



SPT Labtech firefly+ liquid-handling platform

Library Prep Methods



Illumina NovaSeq X Plus Sequencer

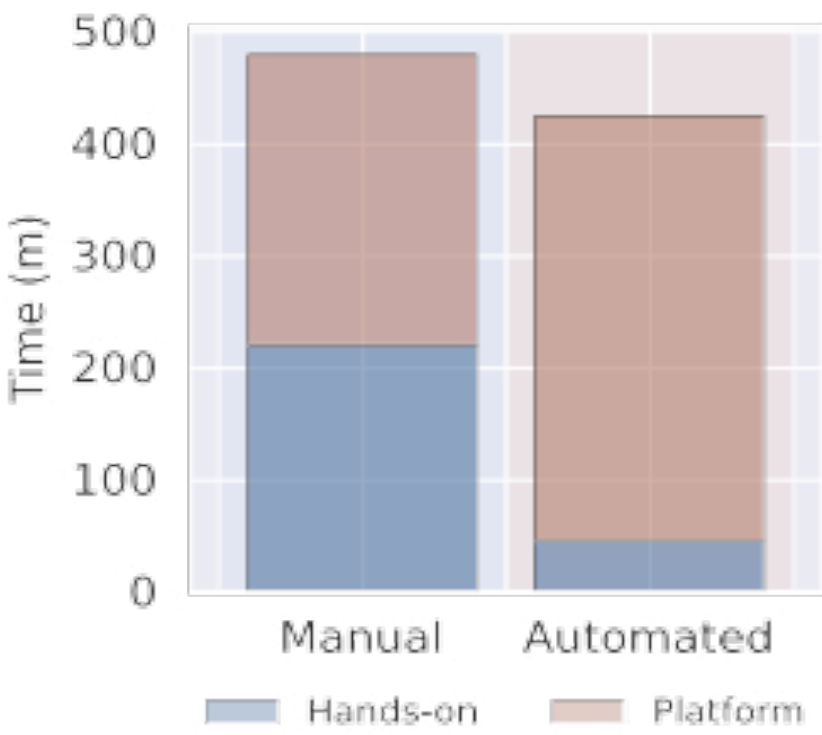
Human reference RNA was used as a standardised input to compare manual and automated workflows using QIAseq FastSelect ribosomal RNA depletion and the NEBNext Ultra II Directional RNA Library Prep kit. Final libraries were quantified using the Agilent Fragment Analyser before equimolar pooling. Sequencing was performed on one lane of a 25B flow cell using the Illumina NovaSeq X Plus.

Parameter	Automated	Manual
HMR ribosomal depletion bait dilution	1:5	None
Total RNA input	40ng	100ng
Library prep volume	1/5th miniaturised	Full volume
RNA fragmentation	7 mins	15 mins
PCR cycles	13	13

Automated Workflow

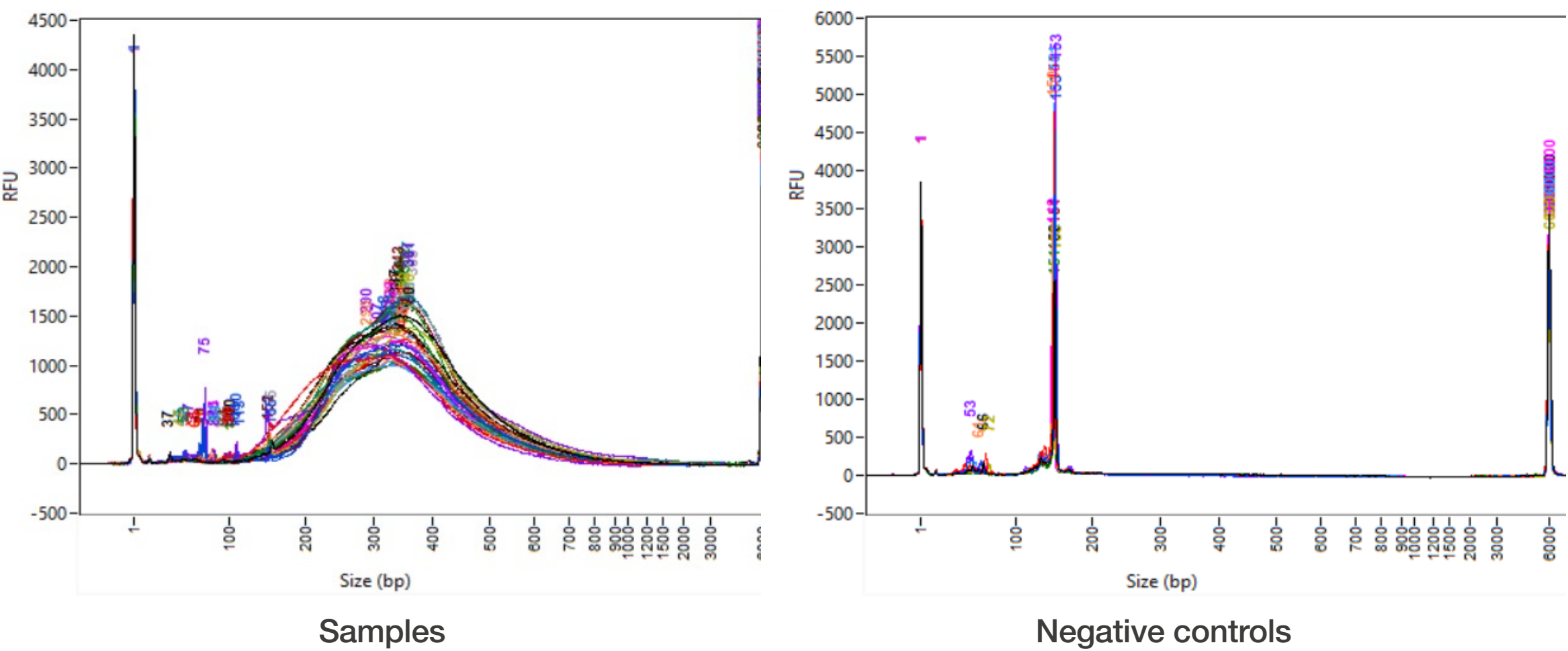
Automated library preparation was split into three walk-away protocols on the SPT Labtech firefly+, significantly reducing hands-on time relative to the manual protocols.

Protocol 1 3h 20m	Protocol 2 1h 30m	Protocol 3 2h 15m
- Ribosomal depletion - cDNA conversion - Bead clean	- End prep - Adaptor ligation - PCR plate setup	- Bead clean - PCR - Bead clean



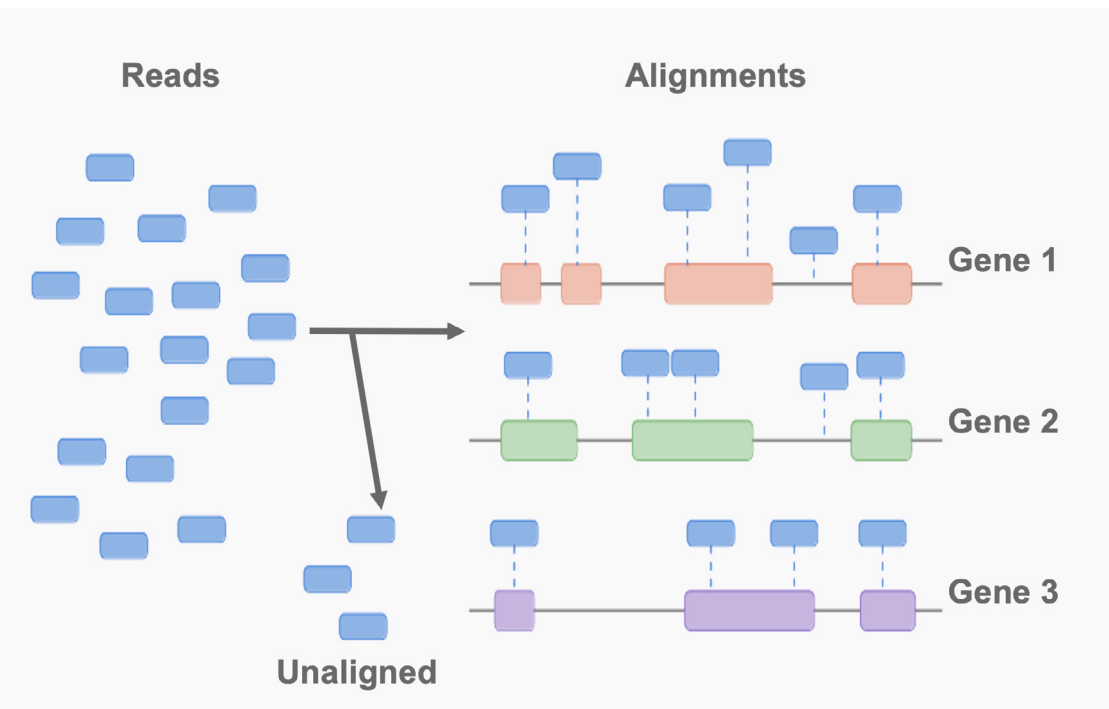
Library Quality

Fragment Analyser traces showed consistent final library size distributions across libraries, indicating successful preparation. Negative controls showed no detectable library peak, providing evidence of no or minimal contamination prior to sequencing.



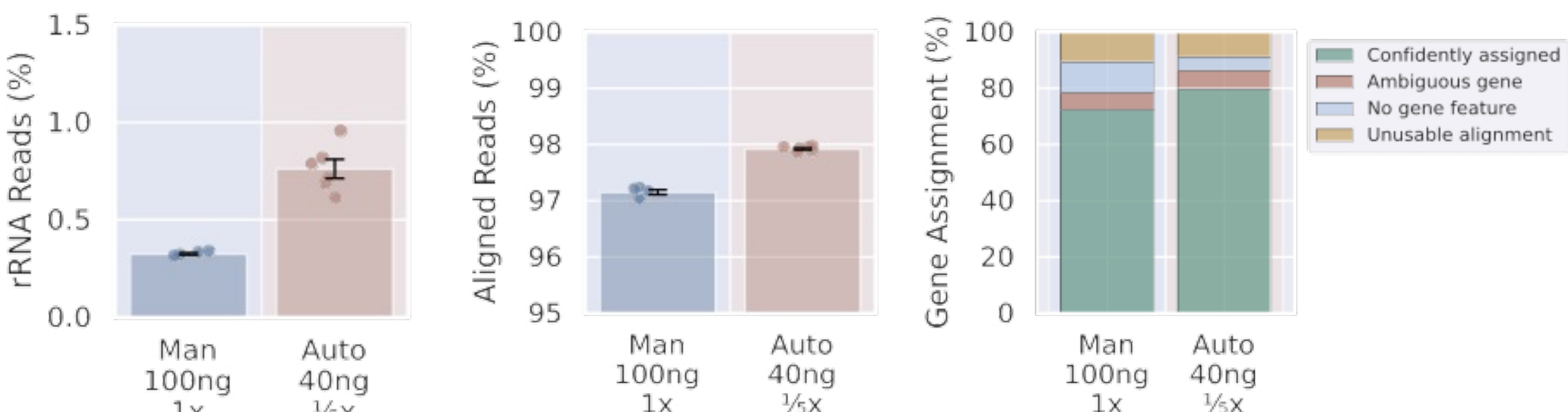
Informatics Methods

Reads were trimmed with **Cutadapt** and filtered for rRNA using **BBDuk** with the **SILVA** database. Non-rRNA reads were aligned to the human reference genome using **HISAT2**, and gene counts were generated with **HTSeq-count**. Library quality, transcript recovery, and complexity were assessed using alignment and assignment metrics, CPM-normalised gene counts, and **Preseq** rarefaction. Downstream analyses and visualisations were performed in **Python** and **R**.



Usable data yields maintained

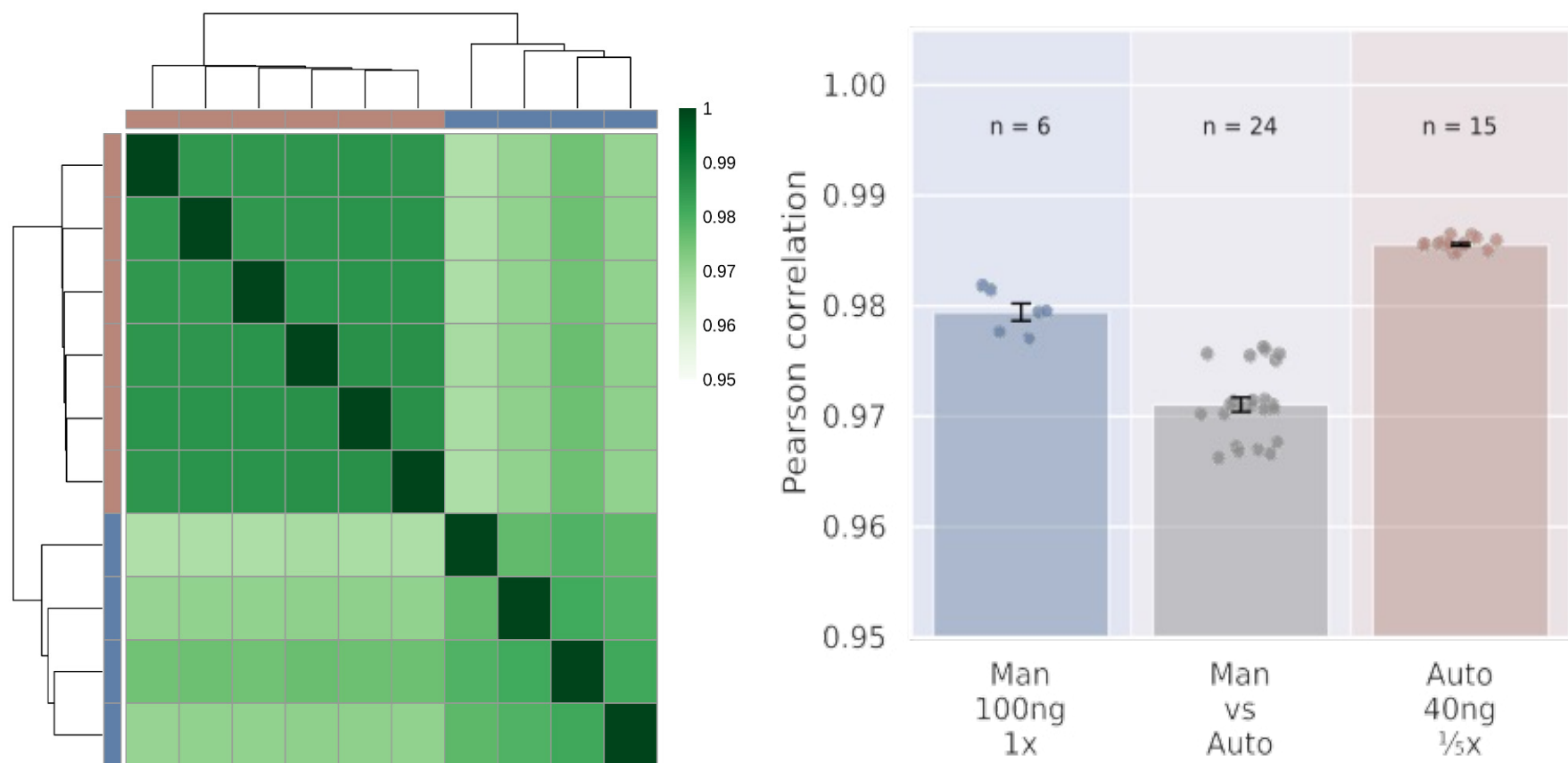
Aim: Libraries were assessed for rRNA content, genome alignment rate, and gene-assigned reads, key indicators of how much sequencing data is biologically useful.



- rRNA carryover was very low in both workflows, but marginally higher after automation. Alignment and gene-assignment rates were slightly higher in automated libraries.
- Conclusion:** Automated miniaturised preparation maintained high usable data yields.

Technical reproducibility improved

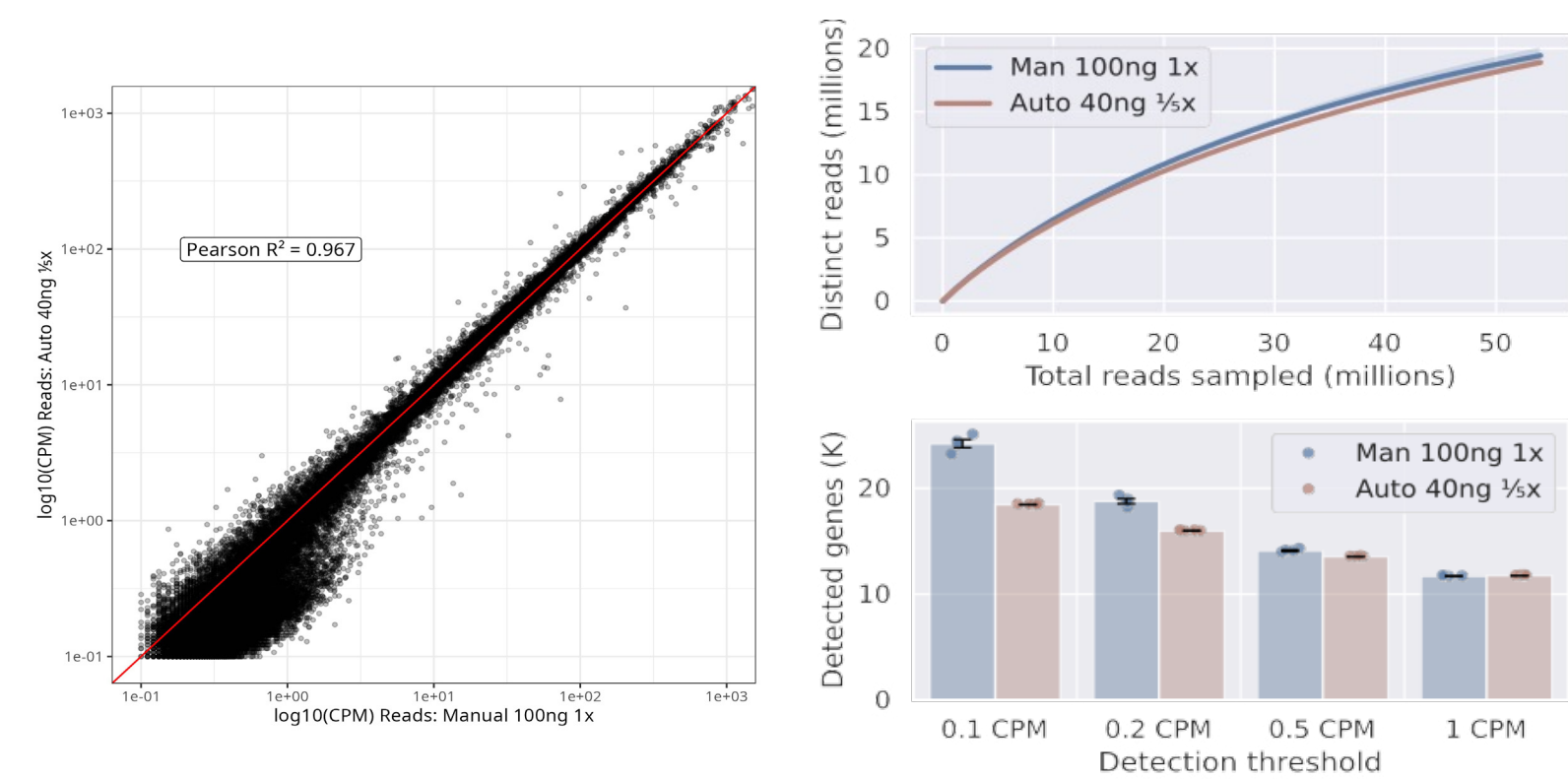
Aim: Technical reproducibility was assessed by comparing sample expression profiles within and between preparation workflows.



- High sample-sample correlations were observed across both workflows, with automated libraries showing particularly tight clustering (left).
 - Within-workflow correlations were highest after automated preparation, indicating improved technical reproducibility (right).
- Conclusion:** Automated miniaturised preparation improved technical reproducibility, while agreement with manual libraries was maintained.

Expression profiles preserved above 1 CPM

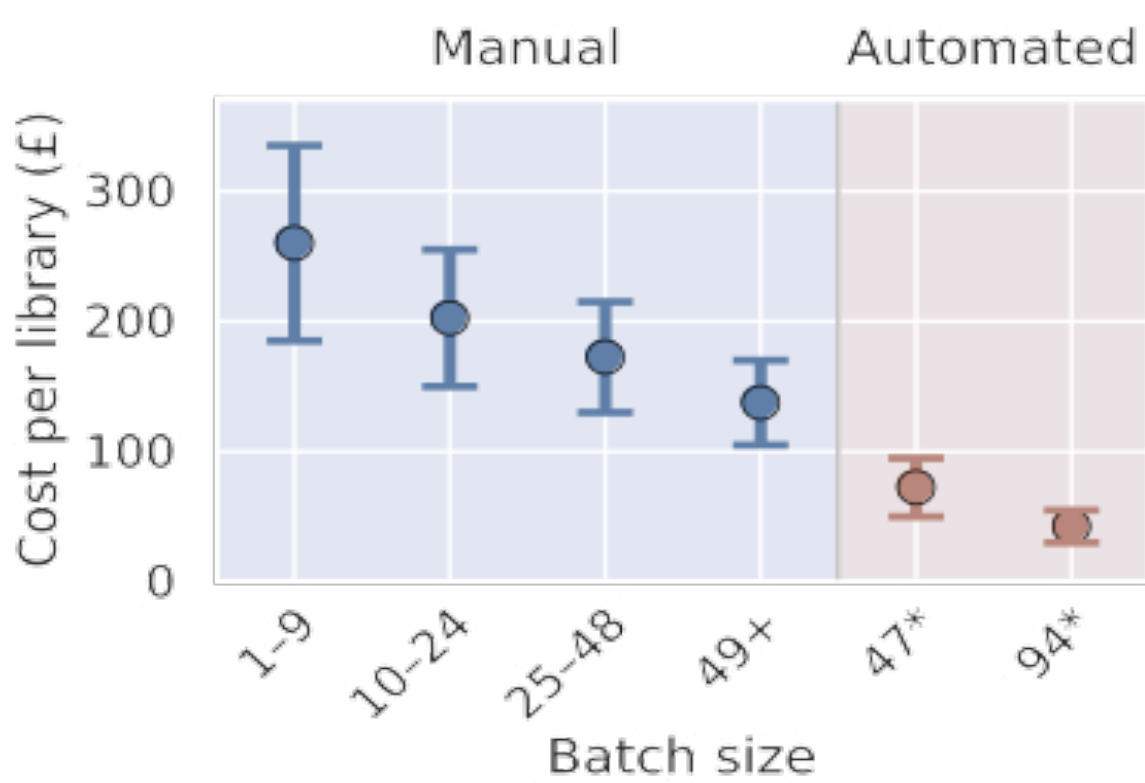
Aim: Libraries were assessed to determine whether automated preparation preserved gene-expression levels, low-abundance gene detection, and distinct-read recovery.



- Gene-expression levels were well preserved overall, with reduced detection mainly limited to very low-abundance genes below 1 CPM (left; bottom right).
 - Distinct-read recovery was similar, with only a small reduction after automation (top-right).
- Conclusion:** Automated preps preserved standard gene-expression profiles, with reduced sensitivity mainly for very low-abundance genes

Per-library cost cut by ~60-70%

Miniaturisation substantially reduced per-library costs while preserving the main gene-expression signals and improving technical reproducibility. Despite some sensitivity loss for very low-abundance genes, the reduced cost enables larger studies within the same library-preparation budget.



Acknowledgements

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