nonacus

# GALEAS™ Bladder

# DNA Target Enrichment for Next Generation Sequencing

## Instructions for use (IFU) V1.2

**Date of issue: April 2026**

IVD UK CA For in vitro diagnostic use

Nonacus Limited
Quinton Business Park Unit 5, 11
Ridgeway, Quinton, Birmingham, UK
B32 1AF
support@nonacus.com

The instructions provided must be followed precisely and only by qualified, properly trained personnel to ensure the correct and safe use of the product(s). It is essential that the entire document is read and fully understood before using the product(s).

Failure to follow this Instructions for Use may result in incorrect product performance, invalid or unreliable results. Nonacus Ltd. accepts no liability for harm, loss, damage, or adverse outcomes resulting from misuse, off-label use, unauthorised modification, improper handling, or failure to follow this Instructions for Use.

# Revision history

| Revision | Date | Revision description |
|---|---|---|
| 1.0 | February 2026 | First release |
| 1.1 | April 2026 | Correction to product codes |
| 1.2 | April 2026 | Update to Clinical performance, Symbols, Storage, handling and disposal and Warning and precautions sections |

## Product identifier information

| GALEAS Bladder | Product code |
|---|---|
| GALEAS Bladder 96 Samples A | NGS_GAL_BCP_FR_96_A_UKCA |
| GALEAS Bladder 96 Samples B | NGS_GAL_BCP_FR_96_B_UKCA |
| GALEAS Bladder 96 Samples C | NGS_GAL_BCP_FR_96_C_UKCA |
| GALEAS Bladder 96 Samples D | NGS_GAL_BCP_FR_96_D_UKCA |

# Table of contents

# Intended purpose

GALEAS Bladder is an in vitro diagnostic system comprising reagents and proprietary GALEAS software for the detection and monitoring of bladder cancer through the identification and classification of somatic mutational signatures in 23 genes, using urine specimens collected from patients.

The system enables the preparation of sequencing-ready libraries from urine-derived DNA and the generation of sequencing data using Next Generation Sequencing (NGS) technology. The resulting sequencing data are processed by the GALEAS software to identify and classify somatic variants associated with bladder cancer.

Test results provide information on the presence or absence of somatic variants associated with bladder cancer and should be interpreted in conjunction with other clinical findings and diagnostic information. The device is not intended to be used as the sole basis for diagnosis or patient management.

The GALEAS Bladder system is intended for use in laboratories equipped with NGS capabilities by trained laboratory personnel. The GALEAS software is not supplied or intended for use as a standalone medical device.

# Performance characteristics – GALEAS Bladder

**Analytical performance**
Analytical performance studies were carried out under controlled laboratory conditions. All pre-specified acceptance criteria were met or exceeded.

| Analytical characteristic | Result |
|---|---|
| Limit of detection | 0.26 – 1.5 % VAF (≥ 95% CI) |
| Limit of blank | 0 % |
| Trueness / bias | $R^2 \geq 0.99$ |
| Precision (intra / inter-assay) | CV < 20 % |
| Linearity / Measuring range | $R^2 > 0.99$ across VAF range of 1-100% |
| Accuracy (derived) | $R^2 \geq 0.99$ |
| Cross-reactivity | Panel does not cross-react with non-human DNA<br>Panel off-target rate ≤50% |
| Interference | No effect on library preparation with ≤ 0.1 mM EDTA presence in reaction |
| Stability | 12 months confirmed (real time + in-use) |
| Minimum DNA input | 25 ng |

**Clinical performance**
Clinical performance aligns with Annex I §9.1 (b) using evidence from re-analysis of data presented in peer-reviewed publication (Ward et al., 2022), NHS real-world cohorts (> 900 patients), and guideline benchmarks (EAU 2025; NICE MIB250 2021; NCCN 2025). Cystoscopy (± histopathology) served as the diagnostic reference and urine cytology as the routine comparator.

<u>Haematuria triage</u>
In haematuria triage, GALEAS Bladder achieves diagnostic sensitivity and NPV equivalent to cystoscopy, with markedly greater sensitivity than cytology, supporting its use as a reliable diagnostic support tool.

| Observed performance (95 % CI) | | |
|---|---|---|
| Parameter | Re-analysis of Ward et al, 2022 (n=710) | NHS – real world evaluation (n=871) |
| Sensitivity | 91.8% (88.5%–94.2%) | 92.2% (84.0%–96.4%) |
| Specificity | 86.0% (82.0%–89.2%) | 91.1% (88.9%–92.9%) |
| PPV | 86.7% (82.9%–89.7%) | 50.0% (41.9%–58.1%) |
| NPV | 91.3% (87.8%–93.9%) | 99.2% (98.2%–99.6%) |
| LR + / LR – | 6.54 (5.05–8.47) / 0.1 (0.07–0.14) | 10.31 (8.18–12.99) /0.086 (0.040–0.185) |

Monitoring setting

In a monitoring setting the test maintains high sensitivity and very high NPV. Lower apparent specificity may reflect earlier molecular detection of disease before cystoscopic lesions become visible, as well as the limitations of cystoscopy and cytology as reference standards.

| Observed performance (95 % CI) | |
|---|---|
| Parameter | Re-analysis of Ward et al, 2022 (n=264) |
| Sensitivity | 92.3 % (75.9–97.9) |
| Specificity | 60.9 % (54.6–66.9) |
| NPV | 98.6 % (95.2–99.6) |
| PPV | 20.5 % (14.2–28.7) |
| LR + / LR – | 2.36 (1.95-2.87) / 0.13 (0.03-0.48) |

Clinical validity conclusion: Diagnostic sensitivity ≥ 85 % and NPV ≥ 95 % across intended uses, non-inferior (Δ ≤ 10 %) to the comparators.

# Performance characteristics – GALEAS Software

The performance of the GALEAS Software has been established through technical validation, clinical evaluation, and usability assessment.

**Technical Performance**

• Limit of Detection (LoD): The underlying GALEAS Bladder assay demonstrates detection of somatic variants at allele frequencies as low as 1% with ≥95% confidence at 1 million paired-end reads.
• Limit of Blank (LoB): No false positive variant calls were observed in wild-type control replicates, meeting acceptance criteria of specificity ≥95%.
• Analytical Specificity: No cross-reactivity was observed with common urinary tract flora or non-targeted genomic sequences.
• Precision / Reproducibility: Concordant variant calling was achieved across instruments, operators, and reagent lots, with ≥95% overall agreement.

**Clinical Performance**

• Diagnostic Accuracy: In a prospective, multicentre haematuria triage cohort, the GALEAS Bladder assay achieved sensitivity of 92.2 % (84.0-96.4%) and specificity of 92% (90.0-93.6%) relative to cystoscopy ± histopathology.
• Negative Predictive Value (NPV): In haematuria triage, the assay demonstrated an NPV of >95%, supporting its intended use as a non-invasive diagnostic support test in the triage and surveillance pathway.
• Intended Role: The software provides automated analysis, quality control, and reporting of GALEAS Bladder results, supporting clinical decisions in conjunction with standard of care.

**Usability and Reliability**

• Usability Testing: Simulated use studies with intended end-users (molecular laboratory staff) confirmed correct interpretation of software outputs and reports without critical errors.
• Software Reliability: Verification and validation testing confirmed consistent performance across supported operating systems, with >99% uptime and reproducibility of results from identical input data.
• Error Handling: The software includes automated QC flags for insufficient reads, coverage gaps, or discordant variant calls, directing users to repeat testing or seek orthogonal confirmation.

# Key features

**GALEAS Bladder**

- The GALEAS Bladder Panel contains the target-specific oligonucleotides necessary for amplification of clinically relevant genomic regions. The 23-bladder cancer–associated genes are: AKT1, BRAF, C3orf70, CDKN1A, CDKN2A, CREBBP, CTNNB1, ELF3, ERBB2, ERBB3, ERCC2, FBXW7, FGFR3, HRAS, KDM6A, KRAS, NRAS, PIK3CA, RHOB, RXRA, SF3B1, TERT promoter, TP53.
- The GALEAS Bladder panel is based on targeted NGS of DNA extracted from the cell pellet of urine specimens.
- Validated on 25 ng of urinary cell-pellet gDNA input.
- Single tube solution for library preparation reduces the number of bead clean-up steps, maximises yield and facilitates automation.
- Protocol supports library preparation with enzymatic fragmentation reagents for library preparation of gDNA, which avoids the need to physically shear gDNA by sonication.
- Illumina adapters containing Unique Dual Indexes (UDI) to identify and avoid sample index skipping.
- Unique Molecular Identifiers (UMI) 9 bp long for PCR/sequencing error removal and single molecule counting in bioinformatic analysis.
- Pooling of libraries prior to hybridization and capture limits the number of capture reactions and amount of panel required.
- Concentration of pre-capture pooled individual sample libraries by using Target Pure™ NGS clean-up beads avoid the requirement for a vacuum concentrator or freeze dryer.

**GALEAS Software**

- Clinically relevant tumour-associated variant detection:
- Automated Bioinformatics Pipeline:
    - Performs automated quality control, alignment to the human reference genome, somatic variant calling, annotation, and classification using validated algorithms and databases.
- Structured Reporting:
    - Generates a clinically interpretable report summarizing the presence or absence of tumour-associated variants, intended for review by qualified healthcare professionals.
- Data Input and Compatibility:
    - Accepts FASTQ files generated from the GALEAS Bladder assay. Operates via a secure online portal, uploader application, and user-specific Python scripts. Requires validated web browsers and stable internet connectivity.
- Qualitative Output:
    - Provides qualitative results (likely positive, likely negative, sample fail/retest) based on variant detection and classification, not quantitative measurements.
- No Reactive Components:
    - The software contains no biological reagents or reactive ingredients. It functions purely as a post-analytical bioinformatics tool.
- Use environment and users:
    - Intended for use in clinical laboratories with NGS capabilities. Operated by trained laboratory personnel experienced in NGS workflow.

# Warning and precautions

Warnings:

- For in vitro diagnostic use only
- Incorrect sample handling, contamination, or deviation from the procedure may result in inaccurate results
- Use of non-validated instruments or software may affect adversely affect device performance
- Cross-contamination may result in false positive results
    - GALEAS Bladder contains hazardous chemicals (formamide, tetramethylammonium chloride and sodium dodecyl sulphate)
    - Refer to Safety Data Sheets (SDS) for hazard information and safe handling

Precautions:

- Use only by trained laboratory personnel in clinical molecular diagnostic laboratories
- Wear appropriate personal protective equipment (PPE) at all times
- Follow all laboratory safety procedures when handling human samples and reagents
- Do not use reagents beyond their expiry date
- Ensure all quality control and sequencing performance criteria are met before reporting results

# Limitations and known interferences

The following section outlines the clinical limitations and known interferences associated with the GALEAS Bladder. This information is essential for correct interpretation of results and safe use of the device in clinical settings.

**GALEAS Bladder**

Limitations:

- GALEAS Bladder is validated only for genomic DNA (gDNA) extracted from urinary cell-pellets. Use with other sample types (e.g., blood, tissue) is not supported.
- The panel targets promoter and exonic regions of 23 genes. Mutations outside these regions will not be detected.
- Clinical performance data is limited on rare low-prevalence variants covered by the panel.
- Sequencing DNA libraries should only be performed on Illumina and Aviti NGS platforms.
- Sequencing ≥30,000x raw depth is required as lower coverage will result in missed low-frequency variants.
- GALEAS Software must be used for data analysis and interpretation.
- The test is not validated for prognosis or therapy selection.
- Clinical performance of the test in detecting minimal residual disease (MRD) to aid in the identification of recurrence in a surveillance setting has not been fully established in a real-world
- setting.
- Analytical sensitivity may be reduced in samples with:
- Low DNA shedding in low-grade tumours (e.g., sensitivity has been reported as 70.8% in grade 1 tumours)
- Low DNA input quality (e.g., where a DNA integrity score (DIN) of <6 is observed)
- Results may be affected by rare polymorphisms, structural variants, or technical artefacts not eliminated by internal quality control.
- Clinical performance estimates (sensitivity, specificity, predictive values) are influenced by disease prevalence and patient risk profile.
- Use of the assay should be restricted to trained laboratory professionals, and results should be interpreted in the context of all available clinical and diagnostic information.
- As the kit is shipped on dry ice and expected to reach users in a frozen condition to be considered functional, limited transport studies have been conducted.
- Kit expiry has been confirmed through real-time shelf-life studies on a single batch at defined storage conditions. Testing on additional batches is ongoing.

Known interferences:

- DNA in high EDTA concentration buffers (e.g., containing 1 mM EDTA) can inhibit enzymatic reactions. DNA must be purified and resuspended in low-EDTA or Tris-HCl buffer, to ensure that the concentration of EDTA in the

library preparation reaction is <0.1 mM.

- Residual salts, proteins, detergents, or RNA can interfere with library preparation. Only high-purity DNA should be used.

**GALEAS Software**

Limitations:

- GALEAS Software is an in vitro diagnostic medical device intended for professional use only.
- GALEAS Software must be operated by qualified healthcare personnel trained in its use and interpretation.
- GALEAS Software is designed to support diagnostic decision-making in bladder cancer risk stratification and must not be used as the sole basis for clinical decisions.
- Variants below the stated limit of detection may not be identifiable by the algorithm. Only SNVs are considered by the classifier.
- Clinical performance of the test in detecting minimal residual disease (MRD) to aid in the identification of recurrence in a surveillance setting has not been fully established in a real-world setting.
- It is essential that users verify the software is accessed within the validated IT infrastructure and meets the minimum hardware and cybersecurity requirements specified by the manufacturer.
- The software may be affected by environmental conditions such as network instability, which could compromise performance.
- Users must monitor signs of degradation, including unexpected outputs or interface anomalies, and report serious incidents to the manufacturer and relevant competent authority
- Disposal of associated hardware and data storage must follow local regulations to mitigate risks related to data protection and environmental safety. The software is not intended for self-testing or near-patient testing and must not be used outside of a controlled clinical environment.

# Materials provided

## GALEAS Bladder contents

| GALEAS Bladder component | Product code | Format |
|---|---|---|
| Library Preparation Kit V2 (b) | NGS_ACC_LV2_FR_96_UKCA | Box |
| UMIRC_AD01 – 96 (Adapter Plate) * | NGS_ACC_ADP_XX-XX_UKCA | Box |
| Hybridization and Capture Enrichment Kit V2 | NGS_ACC_HV2_12_UKCA | Box |
| GALEAS Bladder Panel | NGS_GAL_BCP_UKCA | Tube |

*NOTE: NGS_GAL_BCP_FR_96_A_UKCA includes adapter plate A (1-96 indexes), NGS_GAL_BCP_FR_96_B_UKCA includes adapter plate B (97-192 indexes), NGS_GAL_BCP_FR_96_C_UKCA includes adapter plate C (193-288 indexes) and NGS_GAL_BCP_FR_96_D_UKCA includes adapter plate D (289-384 indexes)

## Library Preparation Kit V2 (b): NGS_ACC_LV2_FR_96 _UKCA

| Reagent | Volume 96 samples | Product code | Storage (°C) | Reagent tube colour code |
|---|---|---|---|---|
| Fragmentation Enzyme | 2x 288 µl | C3TV2-FGE48 | -20 | Red |
| Fragmentation Buffer | 2x 192 µl | C3TV2-FGB48 | -20 | Red |
| Ligation Mix | 2x 960 µl | C3TV2-LIG48 | -20 | Blue |
| PreCap Amplification Mix | 2x 1.2 ml | C3TV2-PAM48 | -20 | Green |
| PreCap Primer Mix | 2x 240 µl | C3TV2-PPM48 | -20 | Black |
| UMIRC_AD01 – 96 (Adapter Plate)* | 01 – 96 | NGS_ACC_ADP_1-96_UKCA | -20 | - |

*NOTE: NGS_GAL_BCP_FR_96_A_UKCA includes adapter plate A (1-96 indexes), NGS_GAL_BCP_FR_96_B_UKCA includes adapter plate B (97-192 indexes), NGS_GAL_BCP_FR_96_C_UKCA includes adapter plate C (193-288 indexes) and NGS_GAL_BCP_FR_96_D_UKCA includes adapter plate D (289-384 indexes)

## Hybridization and Capture Enrichment Kit V2: NGS_ACC_HV2_12_UKCA

| Reagent | Volume 12rxns | Product code | Storage (°C) | Reagent tube colour code |
|---|---|---|---|---|
| Hybridization Buffer (2x) | 228 µl | C3TV2-THB12 | -20 | Blue |
| Hybridization Enhancer | 72 µl | C3TV2-THE12 | -20 | Brown |
| Stringent Wash Buffer (10x) | 480 µl | C3TV2-TSB12 | -20 | White (S) |
| Wash Buffer 1 (10x) | 360 µl | C3TV2-TW112 | -20 | White (1) |
| Wash Buffer 2 (10x) | 240 µl | C3TV2-TW212 | -20 | White (2) |
| Wash Buffer 3 (10x) | 240 µl | C3TV2-TW312 | -20 | White (3) |
| Bead Wash Buffer (2x) | 2x 1.5 ml | C3TV2-TWB12 | -20 | White (B) |
| Universal Blockers | 24 µl | C3TV2-TUB12 | -20 | Orange |
| COT-1 Human DNA | 90 µl | C3TV2-TCO12 | -20 | Red |
| PostCap Amplification Mix | 300 µl | C3TV2-PCM12 | -20 | Green |
| PostCap Primer Mix | 30 µl | C3TV2-TPO12 | -20 | Black |

## GALEAS Bladder Panel: NGS_GAL_BCP_UKCA

| Reagent | Volume 12rxns | Product code | Storage (°C) | No. of samples recommended for pre-capture pooling |
|---|---|---|---|---|
| GALEAS Bladder Panel | 54 µl | NGS_GAL_BCP_UKCA | -20 | 8 samples |

NOTE: See section 2.A Library pooling and probe hybridization, for more information

### GALEAS Bladder Panel: total covered region size (Mb)

| Design ID | Genome | Total Target Size (bp) | Total covered region size (bp) | Total covered region size (Mb) |
|---|---|---|---|---|
| GALEAS Bladder Panel | GRCh38 | 6994 | 18496 | 0.018496 |

### GALEAS Software

| Item | Reference |
|---|---|
| GALEAS Software | GAL_GBA_UKCA |

## Laboratory reagents and consumables required

| Item | Recommended source |
|---|---|
| Elution buffer (EB) | Qiagen, Cat # 19086 (or equivalent: 10 mM Tris-HCl, pH 8.0) |
| Digital electrophoresis system consumables | Agilent Technologies:<br>D1000 Reagents, Cat # 5067-5583; D1000<br>ScreenTape, Cat # 5067-5582<br>High Sensitivity D1000 Reagents, Cat # 5067-5585 High Sensitivity D1000 ScreenTape, Cat # 5067-5584<br>Genomic DNA ScreenTape, Cat # 5067-5365<br>Genomic DNA Reagents, Cat # 5067-5366<br>(Recommended: if not available, see appendix IV) |
| DNA low binding tubes, 1.5 ml PCR-clean | DNA LoBind 1.5 ml, Eppendorf, Cat # 022431021 |
| Dynabeads M-270 Streptavidin<br>IMPORTANT: we have validated our protocol with Dynabeads. Other beads are NOT recommended for use with the GALEAS Bladder protocol | Life Technologies, Cat # 65305 |
| Ethanol (absolute, 100%) | Various sources available |
| Fluorometer consumables | Invitrogen:<br>Qubit Assay Tubes, Cat # Q32856<br>Qubit dsDNA BR Assay kit, Cat # Q32853<br>Qubit dsDNA HS Assay kit, Cat # Q32854) |
| Quantitative / Real-Time PCR library quantification kit | KAPA Library Quantification Kit – Illumina/Universal kit, Roche (optional) |
| Nuclease-free, molecular biology grade water | Various sources available |
| PCR-clean 0.2 ml PCR tubes / 8-well tube strips with caps / 96-well plates with caps/seals | Various sources available |
| PCR-clean 1.5-2 ml microcentrifuge tubes | Various sources available |
| Target Pure NGS Clean-up Beads | Nonacus, Cat # NGS_ACC_CUB_10 |

## Laboratory equipment required

| Item | Source |
|---|---|
| Digital electrophoresis system | Agilent 4200 TapeStation, Agilent Technologies, Cat # G2965AA<br>(recommended: if not available, see appendix IV) |
| Fluorometer for DNA fluorometric quantitation | Qubit®3.0 Fluorometer, Invitrogen, Cat # Q33216<br>Qubit™ 4 Fluorometer, Invitrogen, Cat # Q33238 |
| Magnetic separation rack capable of accommodating 0.2 ml tubes / 8-well tube strips / 96-well plates | DynaMag™-96 Side Magnet, Invitrogen, Cat # 12331D<br>(Recommended: if not available, see appendix II) |
| Magnetic separation rack capable of accommodating 1.5-2 ml tubes | DynaMag™-2 Magnet, Invitrogen, Cat # 12321D<br>(Optional, if a 96-well magnetic separation rack is not available) |
| Micro-centrifuge capable of accommodating 1.5-2 ml tubes | Various sources available |
| Mini-centrifuge capable of accommodating 0.2 ml PCR tubes / 8-well tube strips | Various sources available |
| Multichannel pipettes (10, 100, 200 µl capacity) | Various sources available |
| Plate centrifuge capable of accommodating 0.2 ml 96-well plates | Various sources available |
| Single channel pipettes (10, 100, 200, 1000 µl capacity) | Various sources available |
| Thermocycler with heated lid capable of accommodating 96-well plates | Various sources available |
| Vacuum concentrator | Concentrator Plus, Eppendorf, Cat # 5305000304 or vacuum lyophiliser / freeze-dryer (such as the ScanVac CoolSafe, Labogene)<br>(Recommended: if not available, see appendix III) |
| Vortex mixer | Various sources available |

## Software equipment required

| Aspect | Description |
|---|---|
| Device access | GALEAS Software is accessed via a secure online portal, a secure uploader application, and user-specific Python scripts. Use requires compliance with specified technical conditions, including validated web browser versions, stable internet connectivity, secure user authentication, and submission of sequencing data in standard FASTQ format generated using the GALEAS Bladder assay workflow. |
| Software dependencies | GALEAS Software requires no third-party analytical tools, plugins, or local executables for operation. |
| Input data requirements | Input data must be provided in FASTQ format generated from the GALEAS Bladder assay workflow. |
| Operating system and hardware | Operating system and hardware requirements are limited to those necessary to run a supported web browser with stable internet access. |
| Intended use | GALEAS Software is intended to be used exclusively in combination with the GALEAS Bladder, which includes the library preparation kit and associated reagents for generating NGS sequencing data from urine specimens. |
| NGS platform compatibility | GALEAS Software requires use of a compatible NGS platform (e.g. Illumina sequencing instruments validated for the GALEAS Bladder assay) to generate raw sequencing data in FASTQ format. These sequencing instruments are commercially available devices not manufactured by Nonacus but are necessary for the complete workflow. |
| Uploader compatibility | GALEAS Software Uploader is compatible exclusively with Microsoft Windows operating systems. |
| User interface compatibility | GALEAS Software Interface (MyNonacus) is compatible with any modern internet browser (e.g. Edge, Chrome). |
| Data analysis details | Refer to Chapter 4 for data analysis information. |

## Materials required but not provided

| GALEAS Bladder components | Product Code | Manufacturer |
|---|---|---|
| Galeas Bladder Urine Collection Device | PRE_GAL_UCD_UKCA | Nonacus |

# Symbols

| Symbol | Meaning | Symbol | Meaning |
|---|---|---|---|
|  | In vitro diagnostic medical device |  | Manufacturer |
|  | UK Conformity Assessed |  | Product code |
|  | Batch code |  | Upper limit of temperature |
|  | Expiry date |  | Consult electronic instructions for use |

| Symbol | Meaning | Symbol | Meaning |
|---|---|---|---|
|  GHS08 | Severe health hazard |  GHS06 | Extremely Toxic |
|  GHS09 | Dangerous to the environment |  GHS05 | Corrosion |

# Storage, handling and disposal

**GALEAS Bladder Kit**

| GALEAS Bladder component | Product code | Kit storage (°C) |
|---|---|---|
| Library Preparation Kit V2 (b) | NGS_ACC_LV2_FR_96_UKCA | -20 |
| UMIRC_AD01 – 96 (Adapter Plate) * | NGS_ACC_ADP_XX-XX_UKCA | -20 |
| Hybridization and Capture Enrichment Kit V2 | NGS_ACC_HV2_12_UKCA | -20 |
| GALEAS Bladder Panel | NGS_GAL_BCP_UKCA | -20 |

*NOTE: NGS_GAL_BCP_FR_96_A includes adapter plate A (1-96 indexes), NGS_GAL_BCP_FR_96_B includes adapter plate B (97-192 indexes), NGS_GAL_BCP_FR_96_C includes adapter plate C (193-288 indexes) and NGS_GAL_BCP_FR_96_C includes adapter plate D (289-384 indexes)

- The GALEAS Bladder is shipped under temperature-controlled conditions using dry ice.
  If the shipment is received thawed or without dry ice, this may indicate a temperature excursion and potential compromise of product integrity. In such cases, do not use the product and contact Nonacus for further guidance **support@nonacus.com**.
- Upon receipt all kit components must be stored at -20°C, as stated in the table above.
- The following components must be thawed on ice and kept on ice during use:
  - Enzyme mixes and adapters in the Library Preparation Kit V2 (b)
  - PostCap Amplification Mix and PostCap Primer Mix in the Hybridisation and Capture Enrichment Kit V2
  - Probe set in the GALEAS Bladder Panel
- After thawing and prior to use, briefly vortex mix all reagents, except for the reagents below which should be mixed by light flicking of the tube or pipette mixing:
  - Fragmentation Enzyme
  - Ligation Mix
  - PreCap Amplification Mix
  - PostCap Amplification Mix
- All components should be briefly spun down in a microcentrifuge after mixing.
- GALEAS Bladder components have been validated to remain stable for up to five freeze–thaw cycles without adverse effects on assay performance or result integrity.
  All reagents, including those containing hazardous chemicals or biological materials, must be disposed of in accordance with applicable local, national, and international regulations.
  Users should consult their institution's environmental health and safety guidelines to ensure proper segregation, containment, and disposal of waste.
  Do not dispose of reagents via sinks or general waste bins.
- Used consumables (e.g. tubes, tips, plates) that have contained reagents must be treated as laboratory waste and disposed of through approved waste management procedures.
- Follow the SDS for each reagent for specific disposal instructions.

**GALEAS Software**

- To ensure secure and compliant disposal of patient data:
  - Automated Disposal:
  - Data uploaded to the GALEAS Software is automatically stored by the Nonacus system following report generation. Unless data retention is explicitly requested by the user's organisation, no further storage or archiving is performed.
- User Responsibility:
  - Clinical laboratories are responsible for ensuring that any locally stored data (e.g. FASTQ files or reports) are securely deleted or archived in accordance with their internal data governance policies.
- No Biological Waste:
  - As a software-only device, the GALEAS Software does not generate biological waste. Disposal of physical

specimens and reagents used in the GALEAS Bladder assay should follow the procedures outlined in the assay's technical documentation

# Laboratory workflow overview

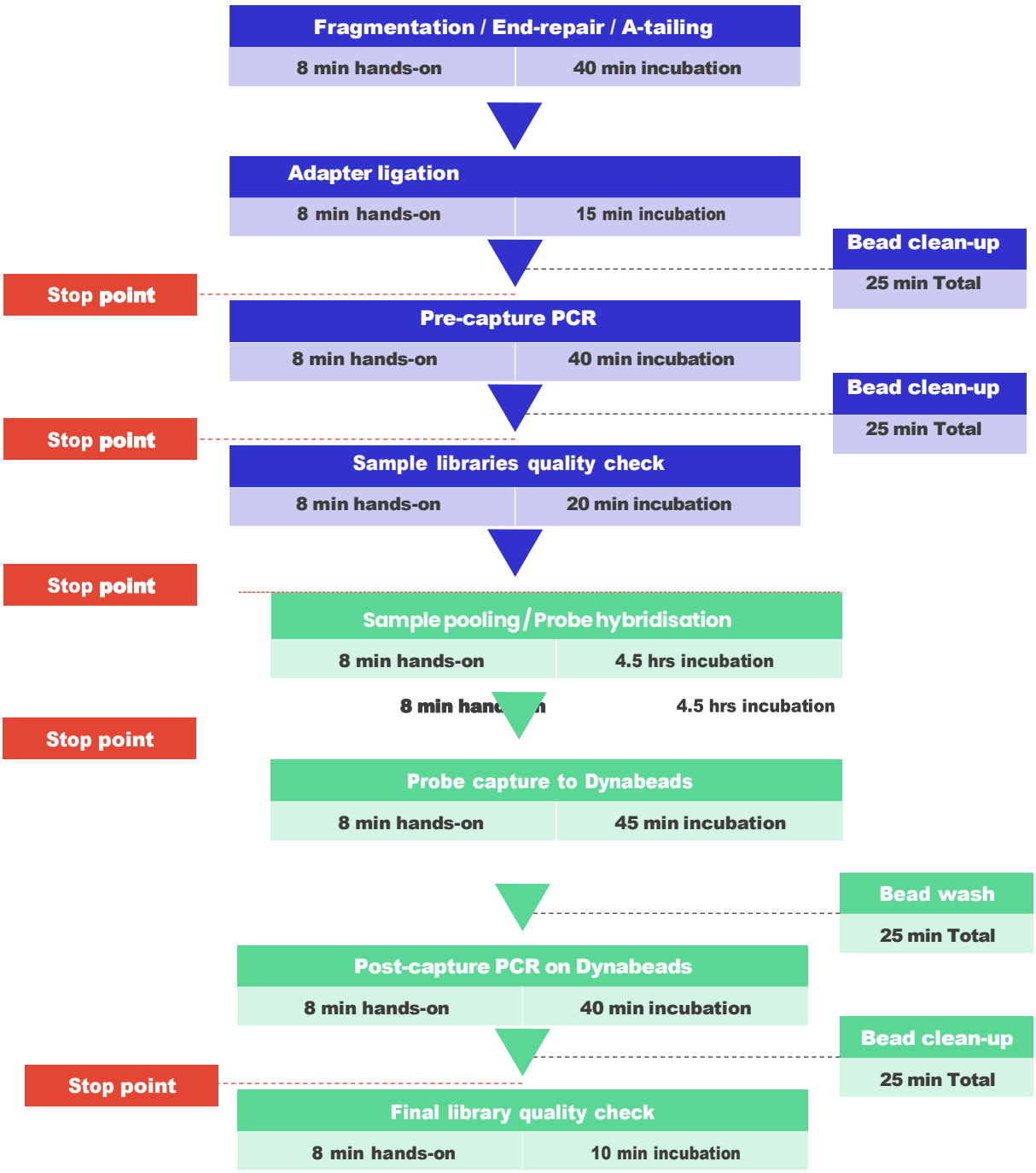



Figure 1. Flow chart outlining the main steps of the GALEAS Bladder workflow. Blue boxes refer to library preparation steps (3.5h); while green boxes refer to probe hybridization and capture steps (8h).

# Chapter 1: Library preparation

## Input DNA requirements

- Only high-purity DNA samples which are free of residual salts, proteins, detergents, or other contaminants should be used as input material. This protocol requires the use of 25 ng of gDNA extracted from urinary cell-pellets as input amount.
- Fluorometric methods (such as the Qubit assay, Invitrogen) are recommended to accurately determine DNA concentration, especially when using more than 100 ng of DNA as input.

IMPORTANT: We would advise against the use of a Nanodrop or similar spectrophotometry-based methods for DNA quantitation as these cannot accurately distinguish between DNA and RNA and have reduced sensitivity for <100 ng/µl concentrations.

- DNA samples that have been extracted using the GALEAS Bead Xtract: Urine gDNA kit (PRE_GAL_BXG_96) will have been resuspended in the correct buffer. If using an alternative extraction kit provider, ensure that the extracted DNA is resuspended in molecular biology grade water, a low EDTA concentration Tris-HCl buffer (such as 0.1 mM EDTA TE buffer) or a 10 mM TrisHCl pH 8.0 saline buffer (such as QIAGEN Buffer EB or equivalent).
- If DNA samples are kept in a high EDTA concentration buffer (such as 1x TE), DNA must be purified using a commercially available kit or DNA Purification Beads (such as Target Pure NGS clean-up beads) and resuspended in one of the above-mentioned buffers.

## How the UMI technology works

- UMIs are built into GALEAS Bladder adapters to enable PCR/sequencing error removal and high accuracy single molecule counting analysis. These 9 bp molecular tags are unique in sequence and positioned directly adjacent to the i7 index within the adapters, which are ligated to the end of DNA fragments during library preparation.
- Sequencing reads with the same UMI that map to the identical genomic location, are assumed to originate from the same DNA molecule and are considered to be PCR duplicates. They can be grouped together to form consensus reads (molecular families) allowing for PCR sequencing error correction and ultra-low frequency mutation calling.

## 1.A Enzymatic fragmentation and end-repair / A-tailing for intact gDNA samples

In this step, 25 ng of gDNA is sheared to a size of 180-200 bp by enzymatic fragmentation and the resulting fragments undergo end-repair and dA-tailing in a single reaction. This converts high molecular weight DNA into short 5'-phosphorylated and 3'-dA-tailed DNA fragments, enabling direct ligation of adapters.

**Before you start**

- Thaw the Fragmentation Buffer (red cap) from the Library Preparation Kit V2 (b) at room temperature and briefly vortex mix.
- Mix the Fragmentation Enzyme (red cap) and the Ligation Mix (blue cap) from the Library Preparation Kit (b) kit by light flicking of the tube or pipette mix.
- Briefly centrifuge all three reagents to collect the liquid to the bottom of the tubes and keep on ice.

IMPORTANT: All library preparation reaction setup procedures should be conducted on ice, unless stated otherwise.

**Procedure**

1. Set up the following thermocycler program:

| Step | Temperature (°C) | Time |
|---|---|---|
| 1 | 4 | Hold |
| 2 | 30 | 10 min |
| 3 | 65 | 30 min |
| 4 | 4 | Hold |

NOTE: Set the thermocycler heated lid to 105°C (if possible), the sample volume is 50 µl.

2. Prepare the DNA sample in a total volume of 40 µl (according to the input amount). Use nuclease-free water to dilute the DNA, if required. **Make sure to keep the reaction on ice during the whole procedure.**

3. Prepare the following reaction mix for each DNA sample as indicated in the table below. Vortex thoroughly to ensure appropriate mixing is achieved without formation of bubbles and briefly centrifuge.

| Components | Volume for 1 reaction (µl) |
|---|---|
| Fragmentation Buffer | 4 |
| Fragmentation Enzyme | 6 |
| DNA sample | X (25 ng) |
| Nuclease-free water | (40 – X) |
| Total | 50 |

NOTE: The Fragmentation Buffer and Fragmentation Enzyme can be combined as a master mix when processing

multiple samples. Include a 10% overage to allow accurate pipetting of 10 µL into 40 µL DNA sample. Vortex the master mix at moderate speed for 5 seconds to ensure thorough mixing without generating bubbles.

4. Add 10 µl of prepared End-repair/A-tailing reaction mix to each reaction for a total final volume of 50 µl. Mix thoroughly by vortex mixing and briefly centrifuge to collect the liquid at the bottom of the tube.

5. Immediately transfer the sample to the pre-chilled thermocycler (4°C) and "skip" to the next step in the program.

6. When the program finishes, and the thermocycler has returned to 4°C, remove the samples from the cycling block and place on ice. **Immediately proceed to the ligation step (1.B).**

## 1.B Ligation of Illumina UMI adapters

During the ligation step, adapters are ligated on both ends of the 5'-phosphorylated / 3'-dA-tailed DNA fragments. A clean-up step is performed immediately after adapter ligation using Target Pure NGS clean-up beads to purify the DNA library and remove residual non-ligated adapters, enzymes and buffers.

**Before you start**

- Equilibrate the Target Pure NGS clean-up beads to room temperature for 20-30 minutes ready for use in step 9.
- Remove the adapter-containing 96-well plate from the freezer and thaw on ice.
- Centrifuge the plate to collect the liquid at the bottom of the tubes.

NOTE: Refer to Appendix VI, Table-2 for the location of each adapter within the supplied adapter 96-well plate, (one adapter per sample library) containing wells with 16, 48 or 96 adapters.

**Procedure**

1. Set up the following thermocycler program.

| Step | Temperature (°C) | Time |
|---|---|---|
| 1 | 4 | Hold |
| 2 | 20 | 15 min |

Note: Set the lid to "not heated" (or leave the lid open), the sample volume is 75 µl.

2. While keeping the end-repaired / A-tailed DNA samples on ice, add 5 µl of the selected adapter to each sample and mix gently by pipette mixing or briefly vortex mixing.

IMPORTANT: Use only one adapter-containing well from the 96-well plate at a time by piercing the aluminium seal to access the adapter. Adapters are single use only.

3. Add 20 µl of Ligation Mix (blue cap) into each reaction for a total final volume of 75 µl. Keep on ice.

4. Mix well by pipetting up and down 10–15 times (do not vortex). Briefly centrifuge to collect all the liquid at the bottom of the tubes.

5. Immediately transfer the adapter ligated sample to the pre-chilled thermocycler (4°C) and "skip" to the next step in the program.

6. After the program finishes, proceed immediately to the clean-up step using Target Pure NGS clean-up beads.

**Clean-up of adapter ligated library**

7. Thoroughly vortex the room-temperature equilibrated Target Pure™ NGS clean-up beads.

8. Add 67.5 µl of thoroughly vortex mixed room-temperature equilibrated Target Pure NGS clean-up beads to a new 0.2 ml PCR tube / 8-well tube strip / 96-well plate for each sample.

NOTE: DNA clean-up with Target Pure NGS clean-up beads can also be performed in 1.5 ml tubes, as explained in Appendix II.

9. Transfer the whole 75 µl of adapter ligation reaction to the 67.5 µl of Target Pure NGS clean-up beads and mix well by pipetting up and down 15-20 times, taking care to avoid the formation of bubbles.

10. Incubate the mixture for 5 minutes at room temperature.

11. Prepare a solution of 80% ethanol / 20% molecular biology grade water (400 µl per sample is required for each clean-up step). For two washes and including an overage, 1000 µl per sample should be prepared for the entire library preparation procedure.

12. Place the sample on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tubes/wells.

13. Keeping the sample on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

14. Add 200 µl of 80% ethanol to the samples and incubate at room temperature for 30 seconds.

15. Repeat steps 13-14 for a total of two 80% ethanol washes.

16. Keeping the sample on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

17. Remove any residual liquid from the tubes/wells using a pipette.

18. Keeping the sample on the magnetic stand, incubate at room temperature with open lids for 3-5 minutes or until the beads are dry.

IMPORTANT: Avoid over-drying of beads, as this can result in a significant loss of DNA recovered. When dry, beads will appear matt in appearance, but should not be cracked

19. Remove the sample from the magnetic stand and resuspend the dried beads in 22 µl of molecular biology grade water by pipette mixing up and down 10-15 times, taking care to avoid the formation of bubbles.

20. Incubate the sample for 2 minutes at room temperature.

21. Place the sample on the magnetic stand for 2 minutes at room temperature to pellet the beads on the side of the tubes/wells.

22. Carefully recover 20 µl of supernatant and transfer it to a new 1.5 ml low-bind tube. **Proceed immediately to library amplification (1.C).**

## 1.C Library amplification

A high-fidelity amplification step is performed to ensure that sufficient library yield is available for the following targeted enrichment procedure. This is conducted using primers that bind to the adapter ligated DNA fragments at the start of the standard P5 and P7 sequences, which are present in all adapters.

**Before you start**

- Thaw the PreCap Amplification Mix (green cap) and the PreCap Primer Mix (**black** cap) from the Library Preparation Kit (b) on ice.
- Once thawed, lightly flick the tube or pipette mix the tube containing the PreCap Amplification Mix to ensure adequate mixing of the reagent (do not vortex).
- Briefly vortex mix the PreCap Primer Mix.
- Centrifuge all reagents to collect the liquid at the bottom of the tubes. Keep both tubes on ice for the whole procedure.
- Equilibrate the Target Pure NGS clean-up beads to room temperature for 20-30 minutes for use in step 6 and prepare 80% ethanol (500µl per sample to allow for overage), if not done so already in section 1.B, step 11.

**Procedure**

1. Set up the following thermocycler program.

| Step | Temperature (°C) | Time | Cycles |
|---|---|---|---|
| 1 | 98 | Hold | 1 |
| 2 | 98 | 45 sec | 1 |
| 3 | 98 | 15 sec | 7 |
| 4 | 60 | 30 sec | |
| 5 | 72 | 30 sec | |
| 6 | 72 | 1 min | 1 |
| 7 | 4 | Hold | 1 |

NOTE: Set the thermocycler heated lid to 105°C, the sample volume is 50 µl.

2. Prepare the following PCR master mix on ice in a separate 1.5 ml tube as indicated in the following table. Mix well by pipette mixing up and down 10 times or briefly vortex mix for 4 seconds. Centrifuge briefly to collect the liquid at the bottom of the tube.

NOTE: For multiple samples, prepare PCR master mix in a 1.5 ml tube by multiplying the volume of each reagent by the number of samples, add extra volumes (overage) to compensate for volume loss due to pipetting. Aliquot 30 µl of the prepared mix in a new 0.2 ml PCR tube / 8-well tube strip / 96-well plate for each sample.

IMPORTANT: Do not store the PCR master mix for periods of time exceeding 2 hours.

| Components | Volume for 1 reaction (µl) |
|---|---|
| PreCap Amplification Mix | 25 |
| PreCap Primer Mix | 5 |
| Total | 30 |

3. Transfer 20 µl of adapter-ligated and purified sample library to the 30 µl of PCR reaction master mix from step 2 and mix well by pipette mixing up and down 10 times or briefly vortex mixing for 4 seconds. Centrifuge briefly to collect liquid at the bottom of the tube.

4. Transfer the sample to the pre-heated thermocycler (98°C) and skip to the next step in the program.

5. When the program finishes, and the thermocycler has returned to 4°C, remove the samples from the cycling block and **proceed immediately to library clean-up using Target Pure NGS clean-up beads.**

**Clean-up of amplified library**

6. Thoroughly vortex the room-temperature equilibrated Target Pure™ NGS clean-up beads.

7. Add 50 µl of Target Pure NGS clean-up beads to a new 0.2 ml PCR tube / 8-well tube strip / 96-well plate for each sample.

NOTE: DNA clean-up with Target Pure NGS clean-up beads can also be performed in 1.5 ml tubes, as explained in Appendix II.

8. Transfer the entire 50 µl volume of PCR amplified library to the 50 µl of Target Pure NGS clean-up beads and mix well by pipette mixing up and down 15-20 times, taking care to avoid the formation of bubbles.

9. Incubate the mixture for 5 minutes at room temperature.

10. Place the sample on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tubes/wells.

11. Keeping the sample on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

12. Add 200 µl of 80% ethanol to the tube/well and incubate at room temperature for 30 seconds.

13. Repeat steps 11-12 for a total of two 80% ethanol washes.

14. Keeping the sample on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

15. Remove any residual liquid from the tubes/wells using a pipette.

16. Keeping the sample on the magnetic stand, incubate at room temperature with open lids for 3-5 minutes or until the beads are dry.

IMPORTANT: Avoid over-drying of beads, as this can result in a significant loss of DNA recovered. When dry, beads will appear matt in appearance but should not be cracked.

17. Remove the sample from the magnetic stand and resuspend the dried beads in 32.5 µl of molecular biology grade water by pipette mixing up and down 10-15 times, taking care to avoid the formation of bubbles.

18. Incubate the sample for 2 minutes at room temperature.

19. Place the sample on the magnetic stand for 2 minutes at room temperature to pellet the beads on the side of the tubes/wells.

20. Carefully recover 30 µl of supernatant and transfer it to a new 1.5 ml low-bind tube.

STOPPING POINT: At this point, amplified libraries can be stored at 4°C overnight or at -20°C for long term storage, if not proceeding immediately to the library quality check step.

## 1.D Library quality check

Libraries are assessed by determining:

- DNA quantity in terms of concentration (ng/µl) and total yield (ng)

- DNA quality in terms of expected fragment size distribution and absence of additional lower or higher molecular weight peaks (recommended)

**Library DNA quantity**

- Libraries prepared from high-purity DNA should yield >500 ng of total DNA (i.e., more than16 ng/µl in a volume of 30 µl). Use of fluorometric assays for dsDNA (such as the Qubit dsDNA BR assay kit, Invitrogen) is recommended for accurate determination of DNA concentration.
- If library yield is below the expected parameters, refer to the troubleshooting guide.

**Library DNA quality**

- Dual indexed adapters with UMIs add 144 bp to fragment length. This can be assessed by analysing libraries with digital electrophoresis systems (such as the Agilent 4200 TapeStation with D1000 reagents and ScreenTape, Agilent Technologies) and determining the peak size within the fragment distribution.
- Library yield can also be assessed using a digital electrophoresis system, but the measurement is not as accurate as that obtained with fluorometric assays (such as the Qubit), as it tends to underestimate DNA quantity. However, if the discrepancy between the measurement taken with a fluorometric assay and a digital electrophoresis assay is higher than 50%, then this might indicate PCR over-amplification of the library (refer to the troubleshooting guide to learn about this issue and how to fix it).
- Presence of carried-over adapters, adapter-dimers and primer-dimers can also be observed in the 60-160 bp range (refer to the troubleshooting guide). Note that adapter-dimers are generally removed during probe hybridization and therefore do not affect the targeted enrichment procedure. See example below for reference on how to check library quality.

- The Library Preparation Kit V2 (b) enables the preparation of libraries using high molecular weight gDNA. The enzymatic fragmentation procedure included in the kit shears the DNA to the required fragment length. Libraries successfully prepared using this kit show a single peak in the fragment size distribution graph (see Figure 2, below).

- Libraries which have not been completely sheared show a tail of variable size in the long fragment range (see Figure 3). In these cases, a repeat of the library preparation procedure is required.

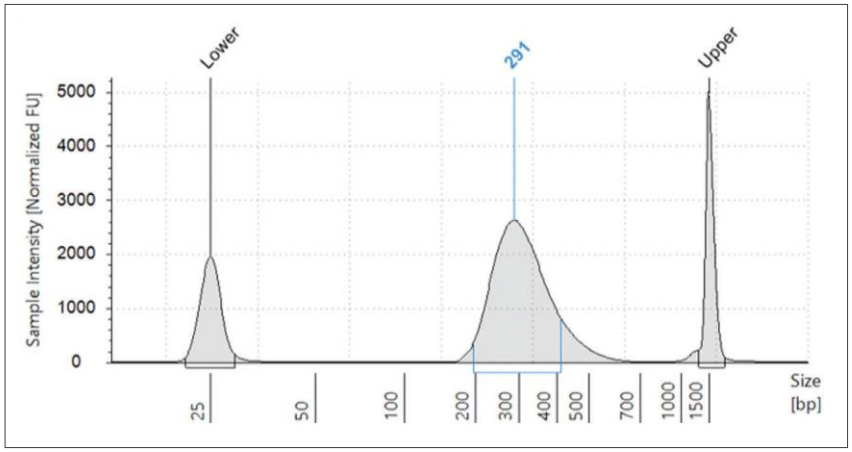



Figure 2. Fragment size distribution of library prepared with 25 ng of input high molecular weight gDNA extracted from urinary cell-pellet.

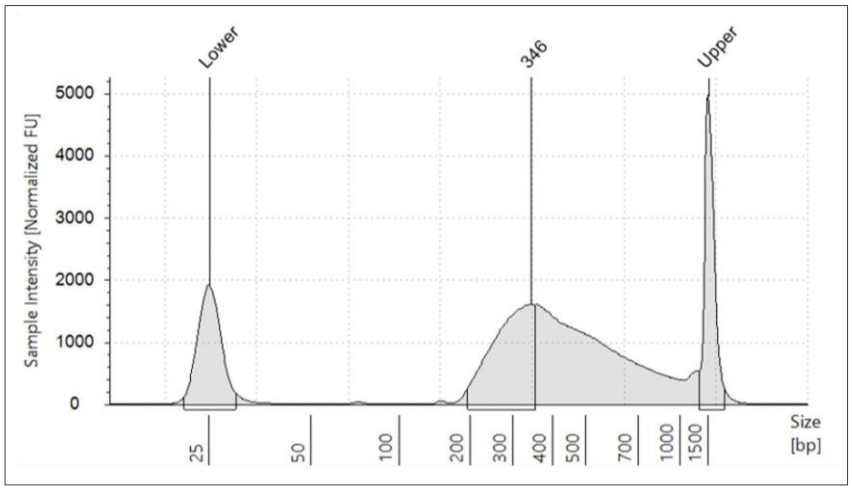



Figure 3. Fragment size distribution of unsuccessful library.

The presence of a tail in the long fragment size range suggests that the sample was not entirely sheared during enzymatic fragmentation.

STOPPING POINT: At this point, amplified libraries can be stored at 4°C overnight or at -20°C for long term storage, if not proceeding immediately to hybridisation and capture.

# Chapter 2: Probe hybridisation and capture enrichment

The Hybridization and Capture Enrichment Kit V2 enables probe hybridisation-based targeted enrichment of libraries prepared from gDNA as input material in combination with the GALEAS Bladder Panel.

## 2.A Library pooling and probe hybridisation

In this step, 8 individual libraries prepared with the Library Preparation Kit V2 (b) are pooled together in equal amounts and hybridized with DNA biotin-labelled probes, to enrich for the targeted region of interest.

IMPORTANT:

- No more than 8 samples per hybridization and capture reaction must be pooled.
- Enough reagents to perform a minimum of 8 libraries per capture are provided in the kit. If pooling less than 8 libraries per capture, not all the reactions in the kit will be utilised.

**Before you start**

- Switch on a vacuum concentrator and set the temperature to 70°C or lower. Alternatively, switch on a vacuum lyophiliser / freeze dryer. If this equipment is not available, pooled libraries can be concentrated using Target Pure™ NGS clean-up beads as described in Appendix III.
- Thaw the Hybridization Buffer (2x) (**blue** cap), the Hybridization Enhancer (**brown** cap), the Universal Blockers (**orange** cap) and the COT-1 Human DNA (**red** cap) from the Hybridization and Capture Enrichment Kit V2 – 4 or 12 reactions, at room temperature.
- Thaw the Galeas Bladder Panel on ice.
- Mix each component vigorously by vortex mixing, then centrifuge to collect the liquid at the bottom of the tube.

NOTE: Inspect the Hybridization Buffer (2x) (**blue** cap) for crystallization of salts. If crystals are present, heat the tube at 65°C in a heat block and vortex every few minutes until the buffer is completely homogenised (this may require heating for 30-60 minutes).

**Procedure**

1. Set up the following thermocycler program.

| Step | Temperature (°C) | Time | Cycles |
|---|---|---|---|
| 1 | 95 | Hold | 1 |
| 2 | 95 | 30 sec | 1 |
| 3 | 65 | 4 hours | 1 |
| 4 | 65 | Hold | 1 |

NOTE: Set the thermocycler heated lid to 100°C, the sample volume is 17 µl.

2. If individual sample libraries were frozen, ensure that they are completely thawed and briefly vortex mixed.

3. Pool equal concentrations (in ng) of individual sample libraries into a fresh 1.5 ml low-bind tube to reach a total combined quantity of 1000 ng.

4. Add 5 µl (equivalent to 5 µg) of COT-1 Human DNA and 2 µl of Universal Blockers to the library pool. Briefly vortex mix and centrifuge to collect the liquid at the bottom of the tube.

5. Place the tube with the lid open in the vacuum concentrator or vacuum lyophiliser / freeze drier and press start.

NOTE: Depending on the amount of liquid present in the tube, the drying procedure may take from 10 to 60 minutes in a vacuum concentrator; and from 30 to 90 minutes in a vacuum lyophiliser / freeze drier. Ensure that all liquid has evaporated from the tube before proceeding to the next step.

NOTE: If a vacuum concentrator or vacuum lyophiliser / freeze drier are not available, pooled libraries can be concentrated using Target Pure™ NGS clean-up beads as described in Appendix III.

STOPPING POINT: At this point, the dried down library pool / COT-1 Human DNA / Universal Blockers can be stored overnight at 4°C, if not proceeding immediately to probe hybridization and capture enrichment.

6. Prepare the hybridization reaction mix in a new 1.5 ml tube as indicated in the table below.

| Components | Volume for 1 reaction (µl) |
|---|---|
| Hybridization Buffer (2x) | 8.5 |
| Hybridization Enhancer | 2.7 |
| GALEAS Bladder Panel | 4 |
| Nuclease-free water | 1.8 |
| Total | 17 |

7. Gently pipette the hybridisation reaction mix with the dried library pool, COT-1 Human DNA, and Universal Blockers. Mix thoroughly by pipetting up and down 10 times. Briefly centrifuge to collect the contents at the bottom of the tube, then incubate at room temperature for 10 minutes.

8. Transfer the whole volume of hybridisation reaction mix to a 0.2 ml PCR tube and briefly centrifuge to ensure that the liquid is collected at the bottom of the tube.

9. Place the 0.2 ml PCR tube containing the hybridisation reaction mix in the pre-heated thermocycler (95°C) and skip to the next step in the program.

10. Leave the hybridisation reaction mix at 65°C on the thermocycler to incubate for 4 hours.

NOTE: Alternatively, and if it aids the efficiency of the workflow, the hybridization reaction can be incubated for 16 hours or overnight.

## 2.B Probe capture on Streptavidin beads and washes

Biotin-labelled probes hybridized to their DNA targets are captured on streptavidin-coated beads. The beads are then washed multiple times to remove non-targeted DNA.

**Before you start**

- Equilibrate the Dynabeads M-270 Streptavidin to room temperature for 30 minutes for use in step 6.
- Thaw the Stringent Wash Buffer (10x) (white cap, S), the Wash Buffer 1 (10x) (white cap, 1), the Wash Buffer 2 (10x) (white cap, 2), the Wash Buffer 3 (10x) (white cap, 3) and the Bead Wash Buffer (2x) (white cap, B) from the Hybridization and Capture Enrichment Kit V2 at room temperature.
- Thoroughly vortex mix and centrifuge to collect the liquid at the bottom of the tube.
- Set a thermocycler to 65°C on hold with the heated lid set at 70°C in advance to prepare for steps 18 onwards.

NOTE: If necessary, heat the Wash Buffer 1 (10x) at 65°C in a heat block to completely resuspend precipitated particles.

NOTE: Dynabeads M-270 Streptavidin washes can also be performed in a 1.5 ml tube using a magnetic stand capable of accommodating 1.5-2 ml tubes, as outlined in Appendix II. In this case, turn on a heat block and set to 65°C.

**Preparation of wash buffers**

1. Dilute the following components for each capture reaction to prepare a 1x working solution in 1.5 ml tubes, as indicated in the table below. For multiple samples, prepare the buffers by multiplying the volume of each reagent by the number of samples, add extra volume (overage) to compensate for pipetting loss.

| Components | Stock solution (µl) | Nuclease-free water (µl) | Total (µl) |
|---|---|---|---|
| Stringent Wash Buffer (10x) | 40 | 360 | 400 |
| Wash Buffer 1 (10x) | 30 | 270 | 300 |
| Wash Buffer 2 (10x) | 20 | 180 | 200 |
| Wash Buffer 3 (10x) | 20 | 180 | 200 |
| Bead Wash Buffer (2x) | 250 | 250 | 500 |

2. Mix each diluted component thoroughly by vortex mixing and centrifuge to collect liquid at the bottom of the tube.

3. Transfer 100 µl of 1x Wash Buffer 1 into a fresh 0.2 ml PCR tube and pre-heat it in a thermocycler at 65°C for at least 15 minutes before use.

4. Split the 1x Stringent Wash Buffer into two 0.2 ml PCR tubes, transferring 200 µl in each tube, and pre-heat both aliquots in a thermocycler at 65°C for at least 15 minutes.

NOTE: Both the 100 µl aliquot of 1x Wash Buffer 1 and the two 200 µl aliquots of 1x Stringent Wash Buffer can be pre-heated on the same thermocycler where the hybridization reaction is taking place.

5. Store the 200 µl of 1x Wash Buffer 1 and the remaining 1x wash buffers at room temperature until needed.

**Preparation of Dynabeads M-270 Streptavidin**

6. After equilibration at room temperature, mix the Dynabeads M-270 Streptavidin thoroughly by vortex mixing for 15 seconds.

7. Aliquot 50 µl of Dynabeads M-270 Streptavidin per capture reaction into a fresh 1.5 ml tube.

NOTE: If preparing more than one capture reaction, up to 600µl of Dynabeads M-270 Streptavidin can be aliquoted into a single 1.5 ml tube for bead preparation.

8. Place the 1.5 ml tube in a magnetic stand and incubate 20-30 seconds or until all beads have separated from the supernatant and have pelleted on the side of the tube.

9. Carefully remove and discard the supernatant, taking care not to disturb the bead pellet.

10. Add 200 µl of 1x Bead Wash Buffer per capture reaction, remove the tube from the magnetic stand and vortex for 10 seconds.

11. Repeat steps 8-10 once more for a total of two washes.

12. Place the 1.5 ml tube in a magnetic stand and incubate 20-30 seconds or until all beads have separated from the supernatant and have pelleted on the side of the tube.

13. Carefully remove and discard the supernatant, taking care not to disturb the bead pellet.

14. Add 100 µl of 1x Bead Wash Buffer per capture reaction, remove the tube from the magnetic stand and vortex briefly.

15. Transfer 100 µl of resuspended beads into a new 0.2 ml PCR tube / 8-well tube strip for each capture reaction.

NOTE: At this stage, Dynabeads M-270 Streptavidin resuspended in 100 µl of Bead Wash Buffer can be transferred to a 1.5 ml tube to conduct bead capture and washes on a magnetic stand capable of accommodating 1.5-2 ml tubes, as explained in Appendix II.

NOTE: Washed Dynabeads M-270 Streptavidin can be kept in solution at room temperature. Proceed to the next step only when the hybridization (section 2.A, step 20) incubation ends.

**Procedure**

16. Set a thermocycler at 65°C on hold with the heated lid set at 70°C (If not already completed at the start of the procedure).

IMPORTANT: It is important that the heated lid is set to 70°C during the washes of Dynabeads® M-270 Streptavidin post-capture. Ensure that the hybridization reaction is kept at 65°C throughout the hybridization, capture and washes with 1x Stringent Wash Buffer steps to avoid unspecific binding of non-target DNA to the probes.

17. Place the tube on a magnetic stand and incubate for 1-2 minutes or until all beads have separated from the supernatant and have pelleted on the side of the tube/well.

18. Carefully remove and discard the supernatant, taking care not to disturb the bead pellet, and proceed immediately to the next step.

NOTE: Small amounts of residual 1x Bead Wash Buffer will not interfere with downstream binding of the biotin-labelled probes to the Dynabeads M-270 Streptavidin.

19. Transfer the whole amount of hybridization reaction mix (from section 2.A, step 20) to the 0.2 ml PCR tube / 8-well tube strip containing the pelleted Dynabeads M-270 Streptavidin.

20. Remove the 0.2 ml PCR tube / 8-well tube strip from the magnetic stand and mix the hybridization reaction mix with the Dynabeads M-270 Streptavidin by pipette mixing up and down 10 times.

21. Transfer the 0.2 ml PCR tube / 8-well tube strip back to the thermocycler set to 65°C (with the heated lid set to 70°C) and incubate for 45 minutes.

NOTE: At this stage, if bead capture and washes are conducted in 1.5-2 ml tubes, incubate the Dynabeads M-270 Streptavidin mixed with the hybridization reaction mix in a heat block set at 65°C, as explained in Appendix II.

22. Every 12 minutes during the 45-minute incubation at 65°C, carefully pipette mix whilst on the thermocycler. If it's not possible to pipette mix on the thermocycler, briefly vortex mix on a low speed to avoid splashing, for 3 seconds, to ensure the beads remain suspended in solution and then place back on the thermocycler.

23. Remove the reaction from the thermocycler and add 100 µl of pre-heated 1x Wash Buffer 1 (from step 3).

24. Pipette mix up and down 10 times and place the reaction on a magnetic stand for to allow the beads to separate from the supernatant and pellet on the side of the tube/well, this should happen within 2-5 seconds.

25. Once the liquid is clear, immediately remove the supernatant, taking care not to disturb the bead pellet. Remove the reaction from the magnetic stand and add 200 µl of pre-heated 1x Stringent Wash Buffer (from step 4).

26. Mix well by pipette mixing up and down 10 times, taking care to avoid the formation of bubbles.

27. Transfer the reaction to a thermocycler set to 65°C (with the heated lid set to 70°C) and incubate for 5 minutes.

28. After incubation, remove the reaction from the thermocycler and place on a magnetic stand to allow beads to separate from supernatant. and pellet on the side of the tube/well. As soon as the liquid is clear, remove the supernatant, this should happen within 2-5 seconds from placing samples on the magnet.

29. Repeat steps 25-28 for a total of two washes with pre-heated 1x Stringent Wash Buffer.

30. As soon as steps 25-29 are complete immediately remove the supernatant, taking care not to disturb the bead pellet.

31. Remove the reaction from the magnetic stand and add 200µl of room temperature 1x Wash Buffer 1.

32. Vortex mix thoroughly for 2 minutes and briefly centrifuge to collect the liquid at the bottom of the tube.

33. Place the reaction on a magnetic stand for 20-30 seconds to allow the beads to separate from the supernatant and pellet on the side of the tube/well.

34. Carefully remove the supernatant, taking care not to disturb the bead pellet.

35. Remove the reaction from the magnetic stand and add 200µl of room temperature 1x Wash Buffer 2.

36. Vortex mix thoroughly for 1 minute and briefly centrifuge to collect the liquid at the bottom of the tube.

37. Place the reaction on a magnetic stand for 20-30 seconds to allow the beads to separate from the supernatant and pellet on the side of the tube/well.

38. Carefully remove the supernatant, taking care not to disturb the bead pellet.

39. Remove the reaction strip from the magnetic stand and add 200 µl of room temperature 1x Wash Buffer 3.

40. Vortex mix thoroughly for 30 seconds and briefly centrifuge to collect the liquid at the bottom of the tube.

41. Place the reaction on a magnetic stand for 1-2 minutes to allow the beads to separate from the supernatant and pellet on the side of the tube/well.

42. Carefully remove the supernatant, taking care not to disturb the bead pellet.

43. Remove the reaction from the magnetic stand and resuspend the bead pellet in 24 µl of nuclease-free water by pipette mixing up and down 10-15 times.

## 2.C Captured library amplification and clean-up

Targeted library DNA sequences hybridized to the biotin-labelled probes and captured on Dynabeads M-270 Streptavidin are amplified by PCR using primers that specifically bind to the P5-P7 sequences on adapters. Target Pure NGS clean-up beads are then used to clean-up the amplified captured library.

**Before you start**

- Thaw the PostCap Amplification Mix (green cap) and the PostCap Primer Mix (**black** cap) from the Hybridization and Capture Enrichment Kit V2 on ice.
- Once thawed, lightly flick the tube containing the PostCap Amplification Mix to ensure adequate mixing of the reagent (do not vortex).
- Briefly vortex mix PostCap Primer Mix.
- Centrifuge all reagents to collect the liquid at the bottom of the tubes. Keep both tubes on ice for the whole procedure.
- Equilibrate the Target Pure™ NGS clean-up beads to room temperature for 20-30 minutes (for use in step 6) and prepare a solution of 80% Ethanol / 20% molecular biology grade water (500 µl required per capture reaction including overage, for use in step 11).

**Procedure**

1. Set up the following thermocycler program

| Step | Temperature (°C) | Time | Cycles |
|---|---|---|---|
| 1 | 98 | Hold | 1 |
| 2 | 98 | 45 sec | 1 |
| 3 | 98 | 15 sec | 17 |
| 4 | 60 | 30 sec | |
| 5 | 72 | 30 sec | |
| 6 | 72 | 1 min | 1 |
| 7 | 4 | Hold | 1 |

NOTE: Set the thermocycler heated lid to 105°C, the sample volume is 50 µl.

2. Prepare the PCR reaction mix in a new 1.5 ml tube on ice. For each capture reaction, prepare one PCR reaction mix in a 0.2 ml PCR tubes / 8-well tube strip as indicated in the table below.

3. Mix well by pipette mixing up and down 10 times or briefly vortex mixing. Centrifuge to collect the liquid at the bottom of the tube.

NOTE: For multiple samples, prepare the PCR master mix in a 1.5 ml tube by multiplying the volume of each reagent by the number of capture reactions, add extra volume (overage) to compensate for pipetting loss.

| Components | Volume for 1 reaction (µl) |
|---|---|
| PostCap Amplification Mix | 25 |
| PostCap Primer Mix | 2.5 |
| Total | 27.5 |

4. Transfer 22.5 µl of resuspended Dynabeads M-270 Streptavidin with captured library DNA (from section 2. B, step 43) to the 27.5 µl of PCR reaction master mix from step 2 and mix well by pipette mixing up and down 10-15 times.

5. Transfer the reaction to the pre-heated thermocycler (98°C) and skip to the next step in the program.

6. When the program finishes, and the thermocycler has returned to 4°C, remove the samples from the cycling block and proceed immediately to library clean-up using Target Pure NGS clean-up beads.

**Clean-up of amplified captured library**

7. Thoroughly vortex the room-temperature equilibrated Target Pure™ NGS clean-up beads.

8. Add 75 µl of Target Pure NGS clean-up beads to a new 0.2 ml PCR tube / 8-well tube strip for each captured library.

NOTE: DNA clean-up with Target Pure NGS clean-up beads can also be performed in 1.5 ml tubes, as explained in Appendix II.

9. Transfer the entire 50 µl of PCR product for each captured library to the 75 µl of Target Pure NGS clean-up beads and mix well by pipette mixing up and down 15-20 times, taking care to avoid the formation of bubbles.

10. Incubate the mixture for 5 minutes at room temperature.

11. Place the reaction on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tubes/wells.

12. Keeping the reaction on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

13. Add 200 µl of 80% ethanol to the tube/well and incubate at room temperature for 30 seconds.

14. Repeat steps 12-13 for a total of two 80% ethanol washes.

15. Keeping the reaction on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

16. Remove any residual liquid from the tubes/wells using a pipette.

17. Keeping the reaction on the magnetic stand, incubate at room temperature with open lids for 5 minutes or until the beads are dry.

IMPORTANT: Avoid over-drying of beads, as this can result in a significant loss of DNA recovered. When dry, beads will appear matt in appearance but should not be cracked.

18. Remove the reaction from the magnetic stand and resuspend the dried beads in 32.5 µl of EB or equivalent buffer saline solution (10 mM Tris-HCl, pH 8.0) by pipette mixing up and down 10-15 times, taking care to avoid the formation of bubbles.

19. Incubate the reaction for 2 minutes at room temperature.

20. Place the reaction on the magnetic stand for 2 minutes at room temperature to pellet the beads on the side of the tube/well.

21. Carefully recover 30µl of supernatant and transfer it to a fresh 1.5 ml low-bind tube.

STOPPING POINT: At this point, the captured DNA library can be stored at -20°C, if not proceeding immediately to the library quality check step.

## 2.D Captured library quality check

Libraries are assessed by determining:

- DNA quantity in terms of concentration (ng/µl) and total yield (ng)

- DNA quality in terms of expected fragment size distribution and absence of additional lower or higher molecular weight peaks

**Captured library DNA quantity**

- Captured libraries should yield 60-300 ng of total DNA. Use of high sensitivity fluorometric assays for dsDNA (such as the Qubit dsDNA HS assay kit, Invitrogen) is recommended for accurate determination of DNA concentration. If libraries yield <60 ng or >300 ng in total, refer to the troubleshooting guide.

**Captured library DNA quantification by quantitative PCR (Optional)**

- Quantitative PCR (qPCR) is widely regarded as the most accurate way of measuring library concentration.
- This assumption is based on the principle that only DNA fragments correctly ligated with the Illumina P5 and P7 adapters will amplify in the qPCR reaction and will therefore be quantified. Therefore, the calculated DNA concentration is relevant only to the fraction of properly adapted DNA fragments which can be sequenced.
- Library quantification kits by qPCR are commercially available, such as the KAPA Library Quantification – Illumina/ Universal kit (Roche). To ensure an accurate measurement of library DNA concentration when using these kits, follow the manufacturer's guidelines and use a 1:10,000 – 1:40,000 dilution of the captured library as input material.

**Captured library DNA quality**

- A high sensitivity digital electrophoresis system (such as the Agilent 4200 TapeStation with High Sensitivity D1000 reagents and ScreenTape, Agilent Technologies) should be used to determine the peak size within the fragment distribution and the average fragment size. The latter is required to calculate the molar concentration of the captured library, which is essential for final library dilution and preparation for sequencing. See examples of captured libraries below for reference.

Library obtained after targeted enrichment with the GALEAS Bladder Panel and Hybridization and Capture Enrichment Kit V2.

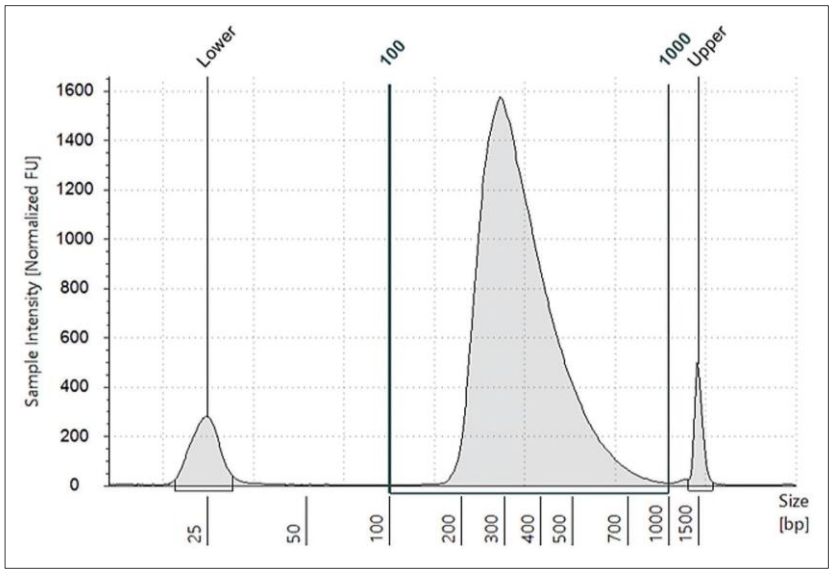



Figure 4. Fragment size distribution showing the range of 100-1000 bp within which the average fragment size is calculated. Average fragment size: 354 bp.

# Chapter 3: Sequencing of captured libraries

Libraries enriched by targeted capture using GALEAS Bladder Target technology are ready for sequencing.

## 3.A Calculate captured library molar concentration

An accurate molar concentration can be calculated in the following ways:

- In combination with fluorometric assay reading: use the following formula to calculate molarity.

$$\text{concentration in nM} = \frac{\text{concentration in ng/µl}}{\left(660 \frac{g}{mol} \times \text{average library size in bp}\right)} \times 10^6$$

- In combination with the KAPA Library Quantification – Illumina/Universal kit or equivalent: insert the average fragment size in bp into the required field of the KAPA Library Quantification Data Analysis worksheet (or equivalent from other supplier) to determine library molar concentration.

## 3.B Sequencing requirements for Illumina and Element Biosciences platforms

Libraries generated with the GALEAS Bladder panel are suitable for sequencing on Illumina and Element Biosciences platforms. Paired-end sequencing is required with dual indexing, where the i7 index requires 17 cycles (8 cycles for the UDI index and 9 for the UMI sequence) and the i5 index requires 8 cycles (for the UDI index). Each library requires 1 Gb of data output to achieve >30,000x targeted depth of coverage.

# Troubleshooting guide

The following guide is meant to address the most common issues which might arise during library preparation and targeted capture enrichment. For further guidance, please contact us at **support@nonacus.com**.

## A) Individual sample library yield <500 ng

- Library preparation reaction setup must be conducted on ice, to ensure that enzymatic activity does not start before all components have been added to the reaction mix.
- After thawing, all components must be thoroughly vortex mixed or tubes tapped for enzyme mixes, (as indicated in the protocol) to ensure that salts and/or enzymes are homogenously mixed and in solution.
- Prior to incubation, all reaction mixes must be thoroughly vortex mixed, or pipette mixed (as indicated in the protocol) to ensure maximum enzymatic activity.
- Ensure that Target Pure NGS clean-up beads have been equilibrated at room temperature for 20-30 minutes prior to use, as the beads DNA binding capacity is reduced at low temperatures.
- During bead clean-up steps, 80% ethanol solution must be prepared fresh on the same day, as evaporation of ethanol over time can increase the water fraction and cause elution of DNA from the Target Pure NGS clean-up beads during washes.
- Over-drying of bead pellet during bead clean-up can significantly reduce DNA recovery in eluate. After drying beads at room temperature for 5 minutes, inspect the bead pellet frequently to ensure it does not over-dry. Bead pellets that show signs of cracking have been dried for too long; the pellet should have a matt appearance.

## B) Larger than expected fragment size in individual sample library from gDNA input

- If the DNA sample is kept in buffer containing >0.1 mM EDTA, use a bead or column clean-up procedure and elute the DNA sample in nuclease-free water or a 10 mM Tris-HCl, pH 8.0 solution (such as EB, Qiagen), then repeat the library preparation procedure.
- Ensure that the fragmentation reaction is thoroughly mixed, prior to incubation on the thermocycler.

## C) Discrepancy between Qubit and TapeStation measured sample library yield

- The sample library may have been over-amplified.
- When a considerable discrepancy between Qubit and digital electrophoresis measurement of sample library yield is observed, this may be due to inconsistencies in DNA concentration measurements, leading to more input material being added to the library preparation reaction. In these cases, the PCR reaction becomes depleted of primers quicker, leading to multiple denaturation / annealing cycles to happen without amplification. This causes un-paired single stranded library fragments to hybridize on the adapter sequences, but not on the insert sequence, forming 'bubble-like' structures that do not migrate properly during electrophoresis. When run on a TapeStation D1000 ScreenTape, this effect can be noticed as a dark band appearing above the upper marker resulting in a 'shoulder' signal to the right of the upper marker peak in the electropherogram, as shown in figure 5 below.
- Sample library over-amplification does not have a negative impact on the yield of viable DNA fragments used in the hybridization and capture stage but will cause under-estimation of the sample library yield by Qubit and digital electrophoresis methods, which will impact on the amount of sample library pooled in the hybridization reaction. Ensuring that the DNA concentration of samples is accurately measured will resolve this issue.

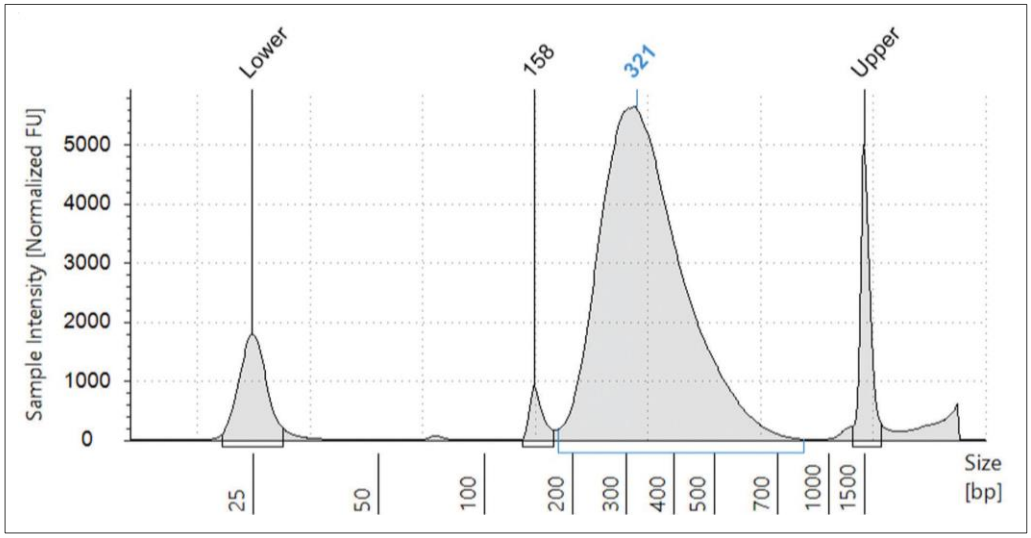



Figure 5. D1000 screen tape electropherogram of an over-amplified sample library.

## D) Low molecular weight peaks present in individual sample library

- A low molecular weight peak of 150-160 bp in size indicates the presence of adapter-dimers carried over from the adapter ligation reaction. Adapter-dimers are generally lost during the targeted capture enrichment procedure and therefore will not affect downstream processes.
- Make sure that the DNA concentration of samples is accurately measured prior to library preparation, to ensure that the right ratio of DNA sample to adapter is maintained to avoid this issue.
- Make sure that the correct amount of Target Pure NGS clean-up beads is used in the clean-up of amplified library step (see section 1.C). Use of a higher bead to sample volume ratio leads to the additional purification of smaller DNA fragments, such as adapters and adapter-dimers from the ligation reaction step (see section 1.B); and primer-dimers from the library amplification step (see section 1.C)

## E) Captured library yield is lower than expected

- Ensure that individual sample libraries are eluted in nuclease-free water and not in saline solutions, such as EB or TE, during the clean-up of amplified library step (section 1.C). Use of saline buffers to elute library DNA at this stage may interfere with probe hybridization (see section 2.A).

- Follow protocol recommendations when capturing hybridized probes to Dynabeads M-270 Streptavidin and target DNA to Target Pure NGS clean-up beads. Make sure that Dynabeads M-270 Streptavidin are equilibrated to room temperature for 20-30 minutes prior to use, as the biotin binding capacity is reduced at low temperatures. Ensure that target DNA clean-up using Target Pure NGS clean-up beads is conducted as recommended in the protocol (see relevant tips for DNA clean-up outlined in section A of the troubleshooting guide).

- Ensure that Dynabeads M-270 do not over-dry during the capture wash procedure (see section 2.B), as this will result in a loss of functionality of the beads, which will translate into a loss of captured library yield.

## F) Low molecular weight peaks present in the captured library

- A low molecular weight peak of 150-160 bp in size indicates the presence of adapter-dimers, which are formed during the ligation reaction step in the library preparation procedure (see section 1.B). Adapter dimers should not hybridize to the probes and therefore are usually removed during the probe capture step (see section 2.B). Make sure 1x Wash Buffer 1 and 1x Stringent Wash Buffer are pre-heated at 65°C prior to use in steps 21-28 in section 2.B; and that these steps are performed as quickly as possible to ensure that the capture reaction does not considerably cool down below 65°C. This is to ensure the removal of non-hybridized DNA fragments, including adapter-dimers.

- Make sure that the correct amount of Target Pure NGS clean-up beads is used in the clean-up of amplified captured library step (see section 2.C), as explained in section D of the troubleshooting guide.

# Chapter 4: GALEAS Software Report Generation

## 1. Introduction

Chapter 4 explains how to setup on the MyNonacus software and submit samples for processing.

It covers:

### Initial One-Time Setup:

- Account Registration: Guidance on creating an account on MyNonacus including login procedures.
- Software Installation: Steps to download and install the necessary desktop applications, such as the MyNonacus Uploader and file upload/download scripts.
- Configuration: Instructions for completing/creating required files, installing python scripts, prerequisites like Python and necessary libraries and preparing templates like the Analysis Request File (ARF).

| One Time Steps |
| --- |
| • Create a MyNonacus account<br>• Download the Desktop sample upload application<br>• Download example ARF template<br>• Download File upload script<br>• Download Batch result files download script |

### Batch Sample Submission:

- Detailed procedures for preparing and submitting batches of samples using the GALEAS software.
- Instructions on how to complete and use the ARF for batch uploads.
- Steps for monitoring batch processing, receiving email notifications, and downloading results once processing is complete.
- Guidelines on using the Batch Management tab, downloading individual results or automating downloads with the download script.

| Batch Sample Processing |
| --- |
| • Login via Desktop sample upload application<br>• Create an ARF for the batch of samples<br>• Use the Desktop application to upload samples<br>• Read the Batch notification email<br>• Login into MyNonacus<br>• Download the results of the sample processing |

## 2. Account Registration

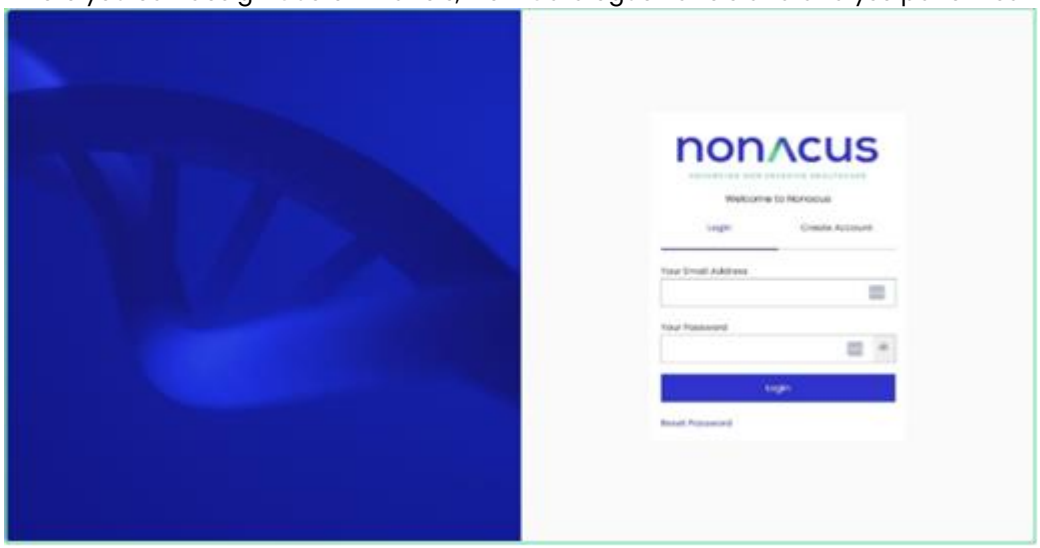
To register for a 'MyNonacus' account on www.nonacus.com, open your preferred web browser and navigate to the site. At the top right of the homepage, click the 'My NONACUS' logo.


Select the Create Account Tab. Fill out the registration form with your personal details, such as your name, email address, and a secure password, then submit the form. Follow any subsequent prompts, such as email verification, to complete your registration.

After you have registered, you can request access to GALEAS in your Account by sending a mail to **support@nonacus.com**. Your account will be associated with the GALEAS software, which enables you to upload & process patient samples (in FASTQ format) and download the bioinformatic results.

## 3. Login to My Nonacus

Once the registration complete, go to **mynonacus.nonacus.com** via your preferred web browser to login, where you can design Custom Panels, view Catalogue Panels and analyse patient samples

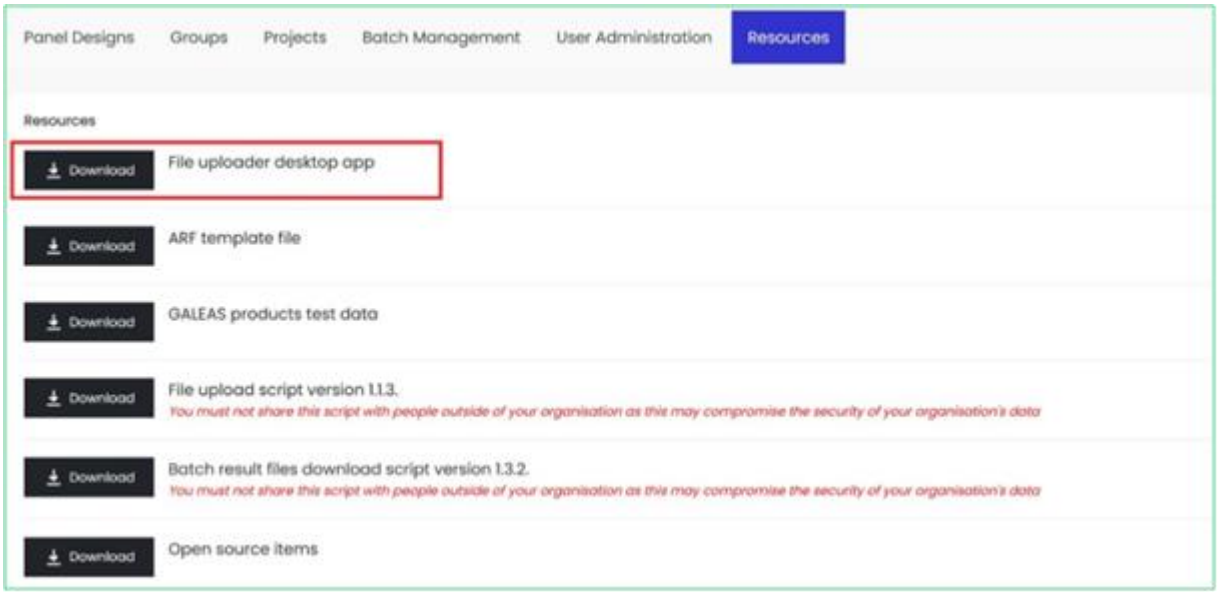



## 4. Software and Analysis Request File (ARF) Download

### 4.1 Download and Install the File uploader desktop app

To upload FASTQ files, start by downloading the Desktop File Uploader from the Resources tab on the MyNoncus GUI, open the Resources tab and locate the Download button for File uploader desktop app. Click Download to begin downloading the installation file to your computer.

Please note that this application only works on Microsoft Windows platforms.

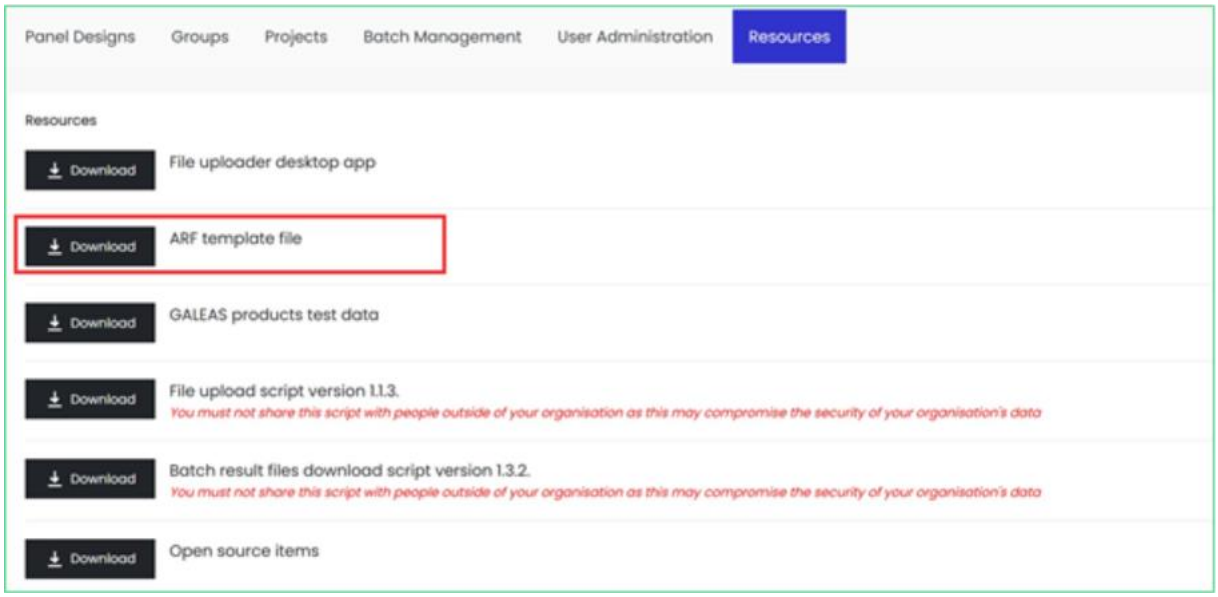


Once the download is complete, open the file to start the installation process. Follow the on-screen prompts to install the Nonacus Uploader application onto your PC. After installation, you can launch the application by clicking the shortcut icon on your PC:

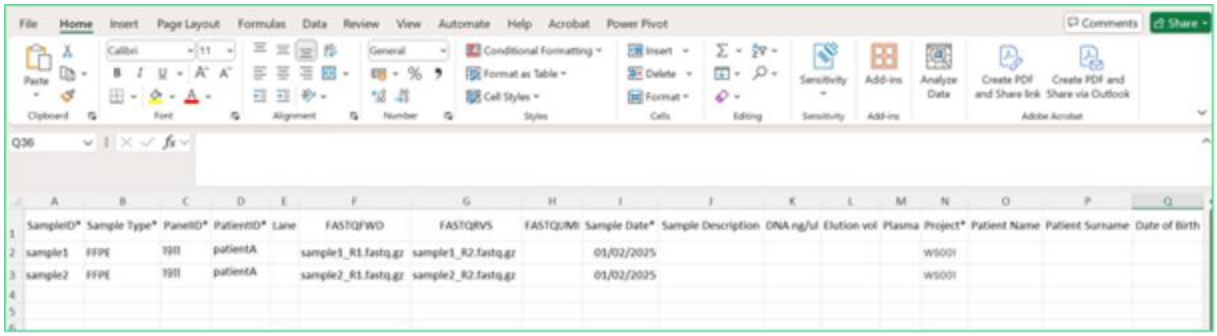

Login page for Nonacus Uploader as below, same login credentials used for MyNonacus.


## 4.2 Download Example Analysis Request File (ARF) file

A SampleSheet instructs an Illumina sequencer on how to process physical samples, whereas the Analysis Request File (ARF) informs the GALEAS software which FASTQ files to upload, how to associate them with specific patients in the system and run the appropriate bioinformatics pipeline. The ARF file is essential for performing a batch upload. An example of an ARF file can be downloaded from the MyNonacus GUI, by clicking on the resources tab and then clicking on "ARF Template file" 'download' link. An ARF file is required for a batch upload.



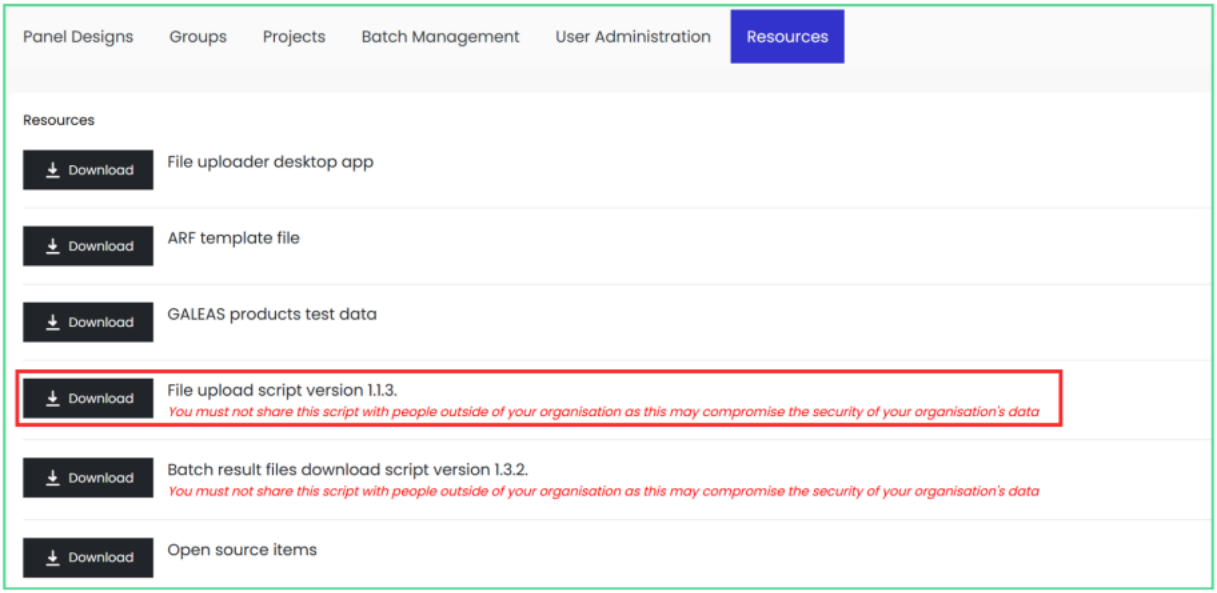
A screenshot of the ARF is as follows;



| SampleID* | Sample Type* | PanelID* | PatientID* | Lane | FASTQFWD | FASTQRVS | FASTQUMI | Sample Date* | Sample Description | DNA ng/ul | Elution vol | Plasma | Project* | Patient Name | Patient Surname | Date of Birth |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| sample1 | FFPE | 1111 | patientA | | sample1_R1.fastq.gz | sample1_R2.fastq.gz | | 01/02/2025 | | | | | W5001 | | | |
| sample2 | FFPE | 1111 | patientA | | sample2_R1.fastq.gz | sample2_R2.fastq.gz | | 01/02/2025 | | | | | W5001 | | | |

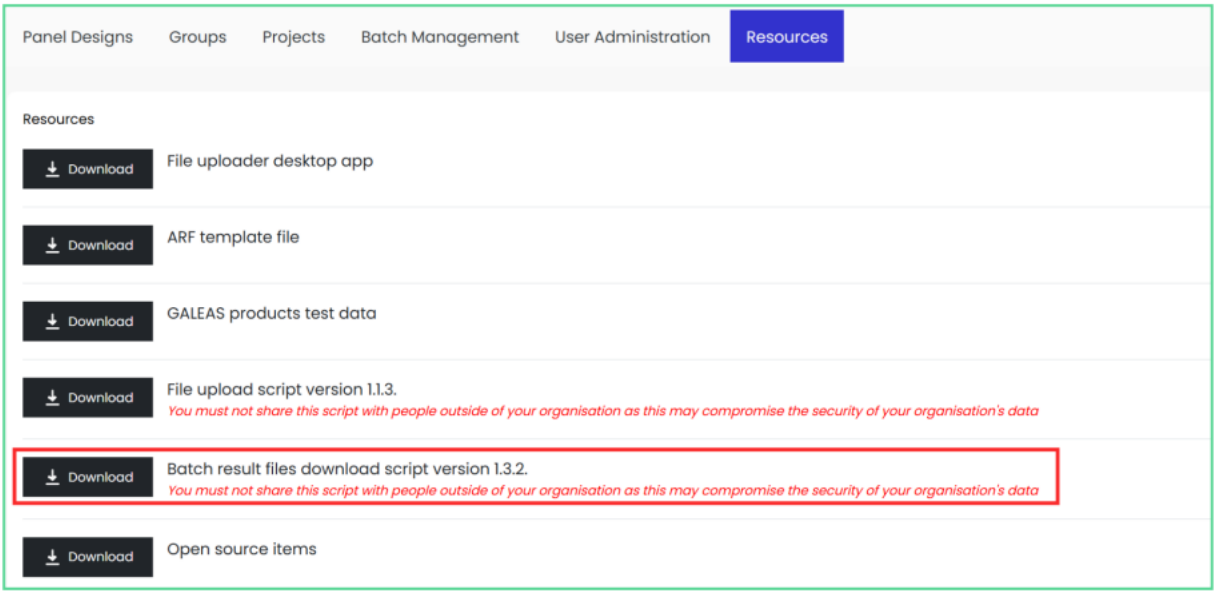
## 4.3 Download File download script: ARFUpload.py

- Click Download button as shown below for Batch result files download script ARFUpload.py
- Depending on your browser settings, you may be prompted to choose a location to save the file, or it may automatically download to your default Downloads folder. If prompted, select a preferred directory on your computer where you want to save the script.
- Click Save to confirm the download location.




## 4.4 Download File download script: GALEASDownload.py

- Click Download button as shown below for Batch result files download script GaleasDownload.py

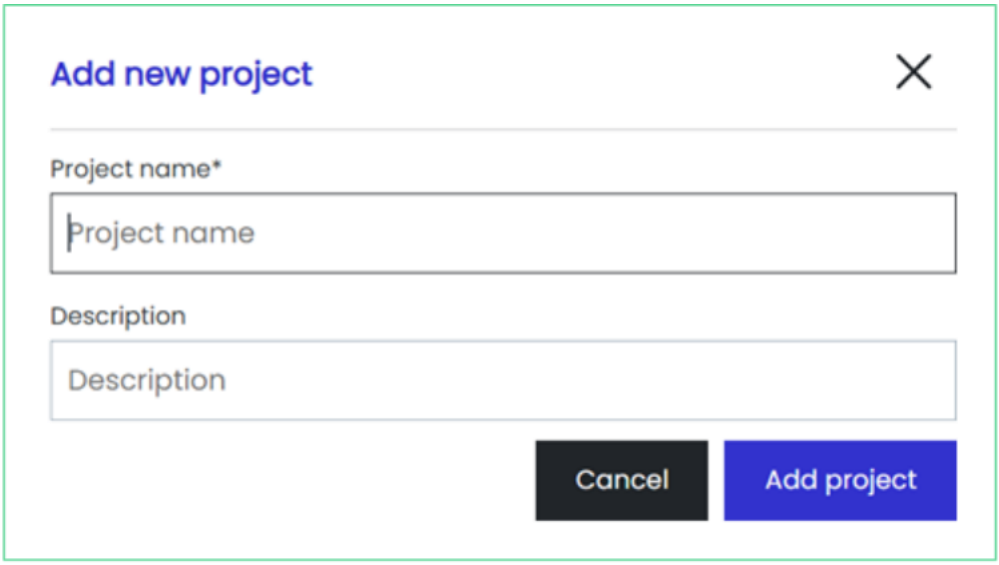



- Depending on your browser settings, you may be prompted to choose a location to save the file or it may automatically download to your default Downloads folder. If prompted, select a preferred directory on your computer where you want to save the script.

- Click Save to confirm the download location.

## 5.Create a project

Projects group patients together and are associated with a customer in the GALEAS system, allowing any user belonging to that customer to view all related projects. Each patient can belong to ONLY one project, and while a 'default' project exists for each customer, it is recommended to create additional projects to keep patients well organized.
To create a new project, click on the "Projects" tab. On this page, you will see a list of any existing projects as well as a list of patients for the most recently selected project, if applicable. Locate and click the "+ Add Project" button to start creating a new project. Follow the on-screen prompts to name and configure the project as needed. This process will help you effectively group and manage patients under the appropriate projects within the system.



## 5.1 Create an ARF for Batch Sample Processing

Overview ARF File

The Analysis Request File (ARF) instructs the MyNonacus software which FASTQ files to upload and how to associate them with a specific patient in the system. Below is a detailed explanation of the ARF format, including its structure and required fields, followed by an example of a completed ARF.

Patient Privacy

It is essential to exclude any Personally Identifiable Information (PII) from the ARF. Use only nonidentifiable IDs to protect patient privacy and comply with data protection regulations.

ARF File Generation

The ARF follows a specific format with defined fields.

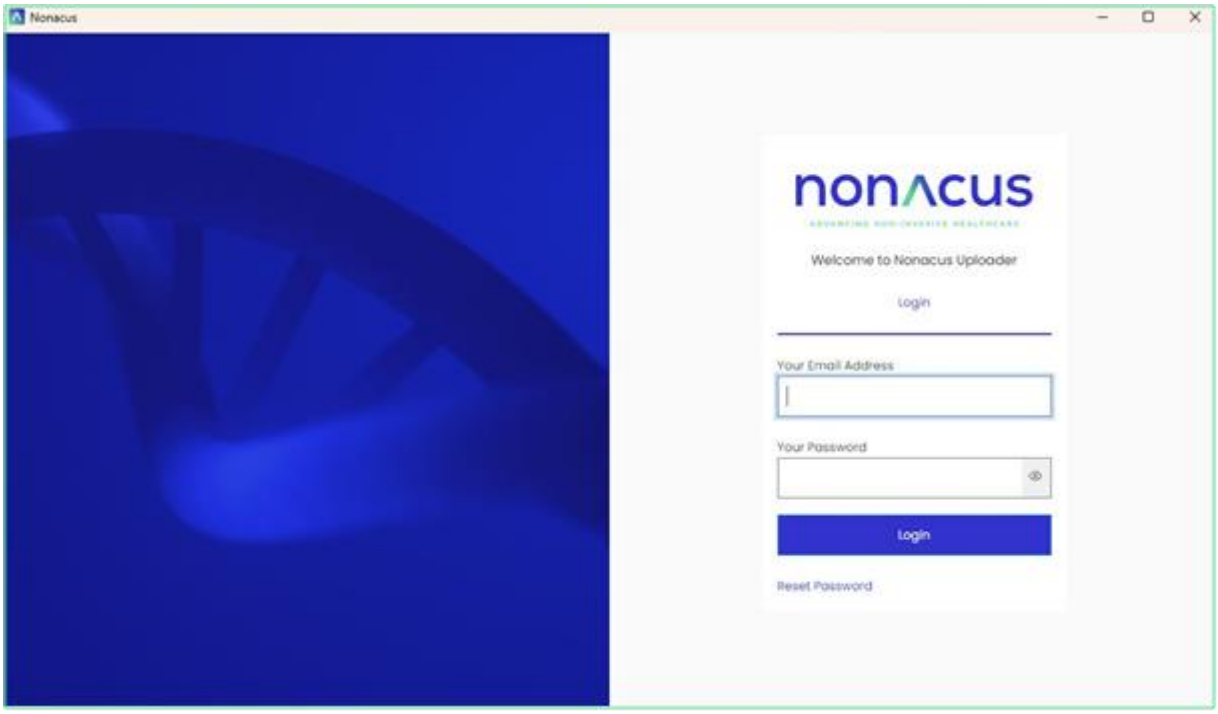
- Use the ARF template downloaded in section 4B. By ensuring that all mandatory fields are included and correctly formatted.
- Save fie as with excel format (.xlsx) A format ARF is shown below.

| **Column Name** | **Optional/Mandatory** | **Description** |
|---|---|---|
| Patient ID * | Mandatory | A mandatory field this is the unique ID for the patient – it can be an existing ID, in which case the sample will be associated with that patient in the system, or it can be a new ID, in which case a new patient will be created with the details supplied in the ARF. |
| Sample ID * | Mandatory | A mandatory field, that must be unique, to identify the sample in the system.<br>The FASTQ filename must contain the sampleID in it, to enable the application to 'find' the FASTQ file(s) and associated it with this sample when the filenames are not explicitly provided in the 3 filename columns below (FASTQ Forward filename etc.).<br>The system will automatically work out whether the r1, r2 (and r3) are forward, reverse or UMI files. |
| Sample Date * | Mandatory | A mandatory field to specify when the sample was taken by the patient |
| Panel ID * | Mandatory | A mandatory field to indicate which panel should be used for the indicated sample. For GALEAS Bladder, the required panel ID is 1821. |
| Project * | Mandatory | This field is optional if a patientID is already associated with a Project.<br>If the patientID is new (i.e. not an existing patient) then this is a Mandatory field specifying the project to associate the patient with. If the project provided does not exist, then it will create a new one. A project must include only alphanumeric characters, hyphens, underscores and spaces. |
| FASTQ Forward * | Mandatory | Field which specifies the filename of the FASTQ file for forward reads e.g. S0086.1.fastq.gz |
| FASTQ Reverse * | Mandatory | Field which specifies the filename of the FASTQ file for reverse reads e.g. S0086.3.fastq.gz |
| FASTQ UMI | Optional | Optional field which specifies the filename of the FASTQ file for UMI e.g. S0086.2.fastq.gz |
| Lane | Optional | Optional field to be used when the sequencer produces FASTQ files for each lane, in which case the filenames below must be provided for each lane specified here (lanes 1-4).<br>If the sequencer produces multi-lane files (most common) then this field can be left blank. |
| Sample Type | Optional | An optional field which can be selected depending on the type of panel being used |
| Sample Description | Optional | Optional description field for the sample |
| Received Date | Optional | Date the sample was received by the lab |

| | | |
|---|---|---|
| Pipeline Version | Optional | The version of the pipeline to use. |
| Repeat Run | Optional | Optional field to indicate if the run is a technical repeat |
| Tumor Purity Level | Optional | An optional field between 1 and 100, representing the tumor purity level of the sample (e.g. as assessed by the pathologist) |
| DNA (ng/ul) | Optional | Optional field specifying the amount of DNA in ng per ul |
| Elution Vol (ul) | Optional | Optional field specifying the elution volume |
| Plasma | Optional | Optional field specifying the plasma volume |
| Patient Forename | Optional | Optional field specifying the patient first name |
| Patient Surname | Optional | Optional field specifying the patient surname |
| Date of Birth | Optional | Optional field specifying the DoB of the patient |
| Cancer Diagnosis or Conditions | Optional | Optional field, specifying the disease/condition of the patient |
| Patient Comment | Optional | Optional field for a patient comment |
| Clinician Forename | Optional | Optional field for the requesting clinician first name |
| Clinician Surname | Optional | Optional field for the requesting clinician surname |
| Clinician Address Line 1 | Optional | Optional field for the requesting clinician address (1st line) |
| Clinician Address Line 2 | Optional | Optional field for the requesting clinician address (2nd line) |
| Clinician City | Optional | Optional field for the requesting clinician city |
| Clinician Country Name | Optional | Optional field for the requesting clinician country |
| Clinician Postcode | Optional | Optional field for the requesting clinician postcode |

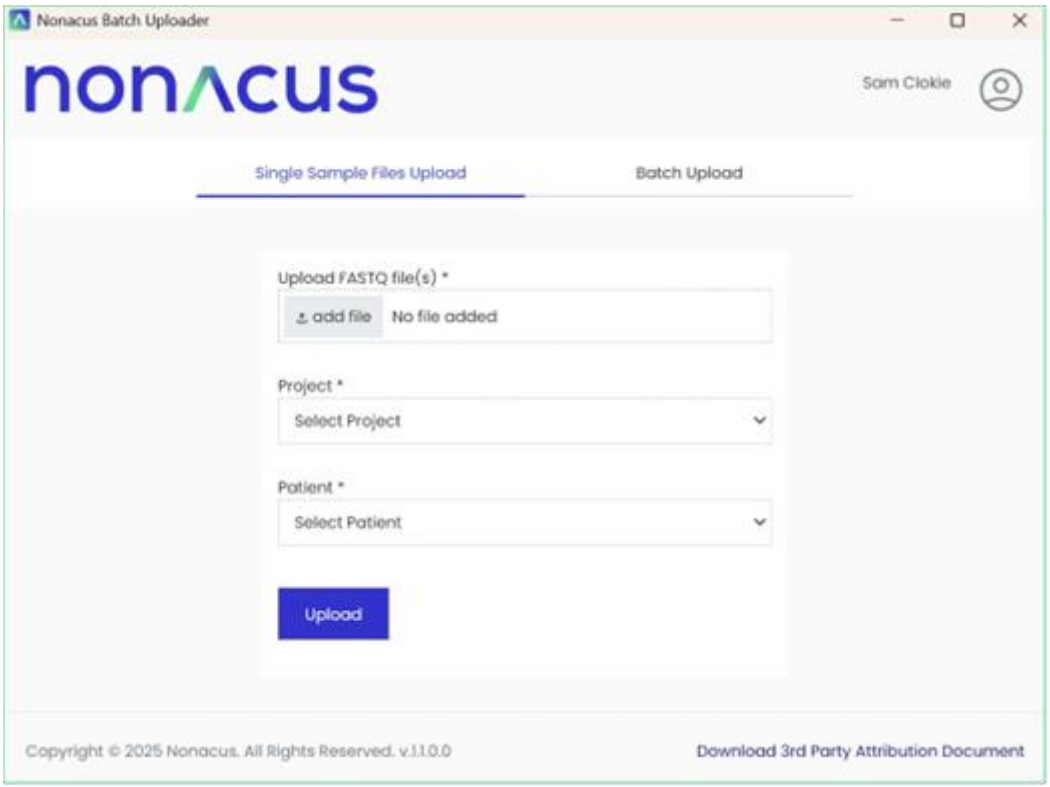
## 5.2 Upload Sample to MyNonacus

Using Nonacus Uploader requires you to login refer to steps in section 3 in this document.
The desktop application will then allow you to upload a single sample ("Single Sample Upload tab") or a batch of samples using the Batch Upload tab – see next section "Choose the 'batch-upload' tab for multiple files using the ARF".



## 5.3 Uploading a Batch of Samples using Nonacus Uploader

Choose the **Batch Upload** tab in the Nonacus Batch Uploader
Under **Analysis Request File—add file**, select your ARF to upload. Then under **Sample Files Folder –add file** to select the folder of the FASTQ files for uploading.
Finally click on **Upload** button. MyNonacus will then perform some checks, such as ensuring all the samples you've listed in the ARF have corresponding FASTQ file(s) in the location you've specified. If there are any errors, a message will be displayed informing you what the issue is and how to fix it.
Uploading even a small number of FASTQ files can take hours, depending on the nature of the samples (e.g. small panel versus Exome) and your network/internet speed. Please leave the application running until it says it has completed the upload.
Once the samples have been uploaded, the MyNonacus system will process the files, and again this process could take several hours.
You will receive an email notification when the processing is complete – see section "Batch Notification Email."




## 5.4 Batch upload using the File upload script

To streamline and automate sample processing, you can use the File Upload Script obtained in section 4C
This script can be executed from the command line, allowing you to process batches of samples without manual interaction with the desktop application.
NOTE: The script is designed to work on both Windows and Linux machines

5.4.1 Pre-requisites for Running the File Upload Script
5.4.11 Install Python (v3 or later)

Download and install the latest version of Python from https://www.python.org/downloads/ Follow the installation instructions for your specific operating system (Windows, macOS, or Linux).

5.4.12 Install Required Python Dependencies

Install the necessary Python packages using pip if not already installed in your system, run the following ``` commands:

```
python -m pip install requests
pip install openpyxl
pip install numpy
pip install boto3
```

After completing these steps, your environment should be ready to run the File Upload Script with the ARF file and sample folder paths as arguments.

5.4.2 Get the Script

The script is customer specific for security reasons, so to get your copy refer to section 4.3 in this document, you need to

- Login to the customer site
- Go to the resources tab
- Click the download button

5.4.3 Run the Script

5.4.3.1 Prepare Your Files:

- Ensure you have a completed Analysis Request File (ARF) that specifies which FASTQ files to upload and how to associate them with patients.
- Place all relevant sample files in a designated folder on your computer.

5.4.3.2 Open the Command Line Interface:

- On Windows, you might use Command Prompt or PowerShell.
- On macOS or Linux, open the Terminal.

5.4.3.3 Navigate to the Script Location:

- Use cd to navigate to the directory where the File Upload Script is located or ensure the script's directory is included in your system's PATH for easier access.

```
cd /path/to/ARFUpload.py
```

5.4.3.4 Execute the Script with Required Arguments:

- Run the script by providing two arguments:
- The path to the ARF file.
- The path to the folder containing your sample files.

```
python ARFUpload.py -a /path/to/your/ARF_file.xlsx /path/to/your/sample_folder -s path_to_sample_files_folder
```

5.4.4 Monitor the Process

After executing the command, the script will begin processing the samples as defined in the ARF file and uploading the corresponding FASTQ files.

```
GALEAS UPLOAD SCRIPT, VERSION 1.1.2
Uploading ARF file...
Uploading samples...
Getting remote file locations
Checking if files have been uploaded for sample sample_name
Updating sample file paths for sample sample_name
Sample sample_name uploaded to GALEAS successfully, 1 sample now uploaded.
Processing started.
```

Watch the command line output for any messages or errors that might require your attention.

5.4.5 Verify Upload Completion
Once the script finishes executing, verify that all intended files have been uploaded correctly via logging in to the MyNonacus GUI and that the sample associations in the system are correct.

## 6. Batch notification email

Once the GALEAS solution has finished processing your batch of samples, you will receive an email notification. This email contains a link that directs you to the “Batch Download Pages” within GALEAS. Clicking the link may prompt you to log in; once authenticated, the link will take you directly to the appropriate page. On this page, you can download the processing results, such as the generated report, for your batch of samples.


nonacus
ADVANCING NON-INVASIVE HEALTHCARE

**The sample files you requested are now available to download.**

Hello,

Sample files ready for download

Download

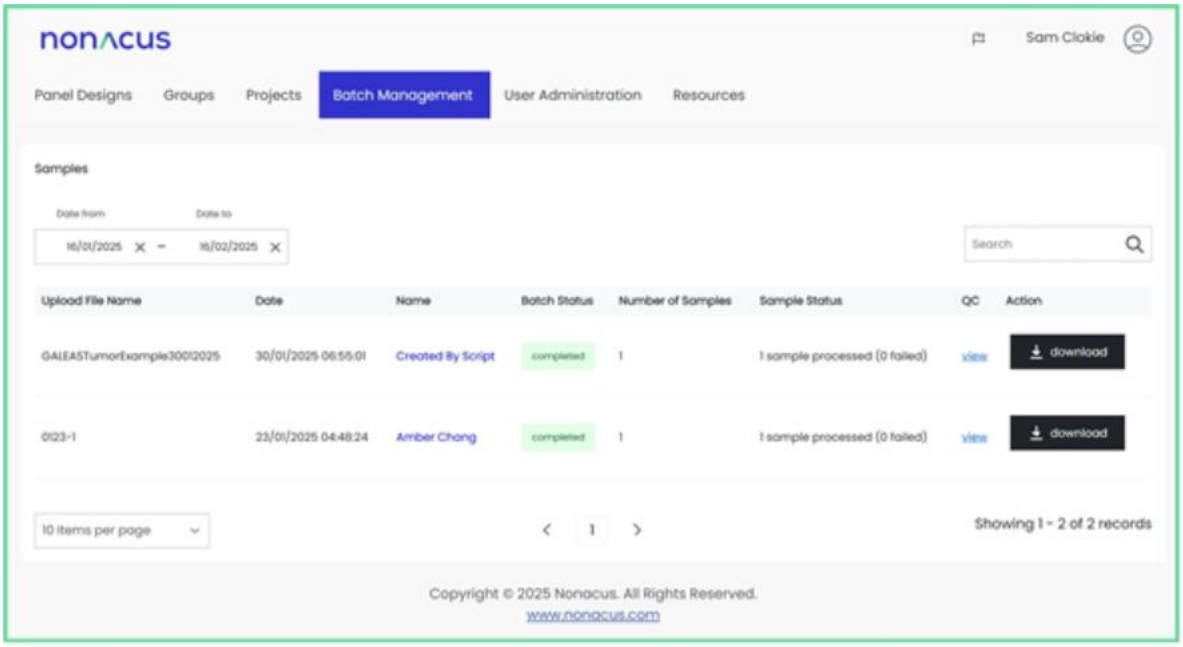
Kind regards,
Nonacus.

Quinton Business Park, Unit 5 11 Ridgeway, Quinton Birmingham West Midlands B32 1AF United Kingdom

## 7.Download the Result for the batch(es)

### 7.1 Download via MyNonacus GUI

Once the processing is complete, the link provided in the email will take you directly to the “Batch Management” tab in the GALEAS software. This page displays all the batches that have been processed for your customer, with each row representing a single sample batch processing run.
To download the results for a specific batch, locate the desired row and click the Download button. This action will download all the results for each sample within that batch to your computer’s default ‘Downloads’ folder.




## 7.2 Download Using Download Script

The download script enables users to automatically download the results of a specific batch run, streamlining and automating their NGS workflows. This tool simplifies retrieving data for further analysis and integration into existing pipelines. For detailed instructions on how to download and set up the script, please refer to section 2 of this chapter.

## 7.3 Running the script

7.3.1 Instructions for Preparing and Using the Download Script

- Move the Python script into your desired folder. The script saves downloaded files in the same directory where it resides. For example, if you place the GaleasDownload.py file in your Documents folder, all downloads executed by the script will also be saved in the Documents folder.
- Ensure that the batch you intend to download is in a completed state. The script can only download results for batches that have finished processing. Batches still in the processing stage cannot be downloaded.

7.3.2 Prerequisites for running Python

Install Python (v3 or later)

Download and install the latest version of Python from https://www.python.org/downloads/ Follow the installation instructions for your specific operating system (Windows, macOS, or Linux).

7.3.3 Execute the Script with Required Arguments

Run the script by providing two arguments:

- Batch name: the name of the ARF that was uploaded for this batch.
- <file types>: a list of file types to be included in the download (case insensitive). The following file types are supported:

```
python GaleasDownload.py -b “<batch name>” <file types>

python GaleasDownload.py -b “Batch upload1” PDF JSON MAF

#For download BAM files
python“GaleasDownload.py” -b “<batchName>” -s “<sampleID>” BAM
```

The following file types are supported:

PDF VCF BAM JSON QC MAF TSV TXT ALL (include all files)

# 8.Description of Download GALEAS Bladder contents

The download bundle for a GALEAS Bladder sample contains the following.

- PDF of the report
- JSON encoding of the report
- multiQC.html – web page containing a large variety of sample QC data obtained from MultiQC

# Appendix

The following information is intended to help users with the technical procedures described in this guide. For further support, please email us at **support@nonacus.com**.

## I. Alternative procedure for magnetic bead clean-up steps

All handling of magnetic beads described in this protocol (i.e. Dynabeads M-270 Streptavidin and Target Pure NGS clean-up beads) requires the use of a magnetic rack capable of accommodating 0.2 ml PCR tubes, 8-well tube strips or 96-well plates. Alternatively, all clean-up steps performed with Target Pure NGS clean-up beads (see sections 1.B, 1.C and 2.C) and Dynabeads M-270 Streptavidin capture and bead washes (see section 2.B) can be performed in 1.5 ml tubes on a magnetic rack capable of accommodating 1.5-2 ml tubes.

For Dynabeads M-270 Streptavidin capture and washes at 65°C, set a heat block at 65°C and incubate for 45 minutes for capture (see section 2.B, step 21); and for 5 minutes for washes (see section 2.B, step 28). Centrifuge the 1.5-2 ml tube containing the Dynabeads M-270 Streptavidin before vortex mixing during capture (see section 2.B, 22) to ensure that any condensation present on the cap is recovered at the bottom of the tube. After each 5-minute incubation of the Dynabeads M-270 Streptavidin during washes with Stringent Wash Buffer (see section 2.B, 26), centrifuge the 1.5-2 ml tube to ensure that any condensation present on the cap is recovered at the bottom of the tube.

## II. Index sequences of Illumina UMI adapters

Table 2: List of adapters contained in the Library Preparation Kit V2 (b). i7 index and i5 index sequences are listed for each adapter. The reverse and complement sequence of the i5 index is also shown for the relevant Illumina platforms. Sequences are unique in the i5 and i7 position to detect sample index skipping. The 9 bp "NNNNNNNNN" sequence stands for the UMI, which is sequenced on the same read as the i7 index and allows PCR/sequencing error removal and single molecule counting.

**IMPORTANT for demultiplexing samples on Illumina platforms:**

- If demultiplexing with bcl2fastq2 or bcl-convert, do not include the 'NNNNNNNNN' sequence in the i7 index.
- If using bcl2fastq to demultiplex, use a v1 Sample Sheet. If using Dragen or bcl-convert to demultiplex, then use a v2 Sample Sheet.

**IMPORTANT for demultiplexing samples on Element Biosciences platforms:**

- The AVITI platform uses a "Run Manifest" CSV file with different structure than Illumina sample sheets. The key sections are [Settings] and [Samples] with columns like SampleName, Index1, Index2, Project, Lane, and ExternalID.
- Use the following Adapter and UMI settings in the Run Manifest:

```
[SETTINGS],,,
SettingName,Value,Lane,
R1Adapter,AAAAAAAAAAAAAAAAAAA,1+2,
R1AdapterTrim,FALSE,1+2,
R2Adapter,TTTTTTTTTTTTTTTTTTT,1+2,
R2AdapterTrim,FALSE,1+2,
# Index mask is set to index length with no FASTQ generated for Lanes 1 and 2.,,,
I1Mask,I1:Y8N*,1+2,
I1Fastq,FALSE,1+2,
I1MismatchThreshold,1,1+2,
I2Mask,I2:Y*,1+2,
I2Fastq,FALSE,1+2,
I2MismatchThreshold,1,1+2,
# UMI mask is set to nothing with no FASTQ generated for Lanes 1 and 2.,,,
UmiMask,I1:N8Y9N*, 1+2,
```

| Well position | Adapter ID | i7 index | i5 index forward | i5 index reverse |
|---|---|---|---|---|
| A1 | UMIRC_AN01 | CTGATCGTNNNNNNNNN | ATATGCGC | GCGCATAT |
| B1 | UMIRC_AN02 | ACTCTCGANNNNNNNNN | TGGTACAG | CTGTACCA |
| C1 | UMIRC_AN03 | TGAGCTAGNNNNNNNNN | AACCGTTC | GAACGGTT |
| D1 | UMIRC_AN04 | GAGACGATNNNNNNNNN | TAACCGGT | ACCGGTTA |
| E1 | UMIRC_AN05 | CTTGTCGANNNNNNNNN | GAACATCG | CGATGTTC |
| F1 | UMIRC_AN06 | TTCCAAGGNNNNNNNNN | CCTTGTAG | CTACAAGG |
| G1 | UMIRC_AN07 | CGCATGATNNNNNNNNN | TCAGGCTT | AAGCCTGA |
| H1 | UMIRC_AN08 | ACGGAACANNNNNNNNN | GTTCTCGT | ACGAGAAC |
| A2 | UMIRC_AN09 | CGGCTAATNNNNNNNNN | AGAACGAG | CTCGTTCT |
| B2 | UMIRC_AN10 | ATCGATCGNNNNNNNNN | TGCTTCCA | TGGAAGCA |
| C2 | UMIRC_AN11 | GCAAGATCNNNNNNNNN | CTTCGACT | AGTCGAAG |
| D2 | UMIRC_AN12 | GCTATCCTNNNNNNNNN | CACCTGTT | AACAGGTG |
| E2 | UMIRC_AN13 | TACGCTACNNNNNNNNN | ATCACACG | CGTGTGAT |
| F2 | UMIRC_AN14 | TGGACTCTNNNNNNNNN | CCGTAAGA | TCTTACGG |
| G2 | UMIRC_AN15 | AGAGTAGCNNNNNNNNN | TACGCCTT | AAGGCGTA |
| H2 | UMIRC_AN16 | ATCCAGAGNNNNNNNNN | CGACGTTA | TAACGTCG |
| A3 | UMIRC_AN17 | GACGATCTNNNNNNNNN | ATGCACGA | TCGTGCAT |
| B3 | UMIRC_AN18 | AACTGAGCNNNNNNNNN | CCTGATTG | CAATCAGG |
| C3 | UMIRC_AN19 | CTTAGGACNNNNNNNNN | GTAGGAGT | ACTCCTAC |
| D3 | UMIRC_AN20 | GTGCCATANNNNNNNNN | ACTAGGAG | CTCCTAGT |
| E3 | UMIRC_AN21 | GAATCCGANNNNNNNNN | CACTAGCT | AGCTAGTG |
| F3 | UMIRC_AN22 | TCGCTGTTNNNNNNNNN | ACGACTTG | CAAGTCGT |
| G3 | UMIRC_AN23 | TTCGTTGGNNNNNNNNN | CGTGTGTA | TACACACG |
| H3 | UMIRC_AN24 | AAGCACTGNNNNNNNNN | GTTGACCT | AGGTCAAC |
| A4 | UMIRC_AN25 | CCTTGATCNNNNNNNNN | ACTCCATC | GATGGAGT |
| B4 | UMIRC_AN26 | GTCGAAGANNNNNNNNN | CAATGTGG | CCACATTG |
| C4 | UMIRC_AN27 | ACCACGATNNNNNNNNN | TTGCAGAC | GTCTGCAA |
| D4 | UMIRC_AN28 | GATTACCGNNNNNNNNN | CAGTCCAA | TTGGACTG |

| Well position | Adapter ID | i7 index | i5 index forward | i5 index reverse |
|---|---|---|---|---|
| E4 | UMIRC_AN29 | GCACAACTNNNNNNNNN | ACGTTCAG | CTGAACGT |
| F4 | UMIRC_AN30 | GCGTCATTNNNNNNNNN | AACGTCTG | CAGACGTT |
| G4 | UMIRC_AN31 | ATCCGGTANNNNNNNNN | TATCGGTC | GACCGATA |
| H4 | UMIRC_AN32 | CGTTGCAANNNNNNNNN | CGCTCTAT | ATAGAGCG |
| A5 | UMIRC_AN33 | GTGAAGTGNNNNNNNNN | GATTGCTC | GAGCAATC |
| B5 | UMIRC_AN34 | CATGGCTANNNNNNNNN | GATGTGTG | CACACATC |
| C5 | UMIRC_AN35 | ATGCCTGTNNNNNNNNN | CGCAATCT | AGATTGCG |
| D5 | UMIRC_AN36 | CAACACCTNNNNNNNNN | TGGTAGCT | AGCTACCA |
| E5 | UMIRC_AN37 | TGTGACTGNNNNNNNNN | GATAGGCT | AGCCTATC |
| F5 | UMIRC_AN38 | GTCATCGANNNNNNNNN | AGTGGATC | GATCCACT |
| G5 | UMIRC_AN39 | AGCACTTCNNNNNNNNN | TTGGACGT | ACGTCCAA |
| H5 | UMIRC_AN40 | GAAGGAAGNNNNNNNNN | ATGACGTC | GACGTCAT |
| A6 | UMIRC_AN41 | GTTGTTCGNNNNNNNNN | GAAGTTGG | CCAACTTC |
| B6 | UMIRC_AN42 | CGGTTGTTNNNNNNNNN | CATACCAC | GTGGTATG |
| C6 | UMIRC_AN43 | ACTGAGGTNNNNNNNNN | CTGTTGAC | GTCAACAG |
| D6 | UMIRC_AN44 | TGAAGACGNNNNNNNNN | TGGCATGT | ACATGCCA |
| E6 | UMIRC_AN45 | GTTACGCANNNNNNNNN | ATCGCCAT | ATGGCGAT |
| F6 | UMIRC_AN46 | AGCGTGTTNNNNNNNNN | TTGCGAAG | CTTCGCAA |
| G6 | UMIRC_AN47 | GATCGAGTNNNNNNNNN | AGTTCGTC | GACGAACT |
| H6 | UMIRC_AN48 | ACAGCTCANNNNNNNNN | GAGCAGTA | TACTGCTC |
| A7 | UMIRC_AN49 | GAGCAGTANNNNNNNNN | ACAGCTCA | TGAGCTGT |
| B7 | UMIRC_AN50 | AGTTCGTCNNNNNNNNN | GATCGAGT | ACTCGATC |
| C7 | UMIRC_AN51 | TTGCGAAGNNNNNNNNN | AGCGTGTT | AACACGCT |
| D7 | UMIRC_AN52 | ATCGCCATNNNNNNNNN | GTTACGCA | TGCGTAAC |
| E7 | UMIRC_AN53 | TGGCATGTNNNNNNNNN | TGAAGACG | CGTCTTCA |
| F7 | UMIRC_AN54 | CTGTTGACNNNNNNNNN | ACTGAGGT | ACCTCAGT |
| G7 | UMIRC_AN55 | CATACCACNNNNNNNNN | CGGTTGTT | AACAACCG |
| H7 | UMIRC_AN56 | GAAGTTGGNNNNNNNNN | GTTGTTCG | CGAACAAC |
| A8 | UMIRC_AN57 | ATGACGTCNNNNNNNNN | GAAGGAAG | CTTCCTTC |
| B8 | UMIRC_AN58 | TTGGACGTNNNNNNNNN | AGCACTTC | GAAGTGCT |
| C8 | UMIRC_AN59 | AGTGGATCNNNNNNNNN | GTCATCGA | TCGATGAC |
| D8 | UMIRC_AN60 | GATAGGCTNNNNNNNNN | TGTGACTG | CAGTCACA |
| E8 | UMIRC_AN61 | TGGTAGCTNNNNNNNNN | CAACACCT | AGGTGTTG |
| F8 | UMIRC_AN62 | CGCAATCTNNNNNNNNN | ATGCCTGT | ACAGGCAT |
| G8 | UMIRC_AN63 | GATGTGTGNNNNNNNNN | CATGGCTA | TAGCCATG |
| H8 | UMIRC_AN64 | GATTGCTCNNNNNNNNN | GTGAAGTG | CACTTCAC |
| A9 | UMIRC_AN65 | CGCTCTATNNNNNNNNN | CGTTGCAA | TTGCAACG |
| B9 | UMIRC_AN66 | TATCGGTCNNNNNNNNN | ATCCGGTA | TACCGGAT |
| C9 | UMIRC_AN67 | AACGTCTGNNNNNNNNN | GCGTCATT | AATGACGC |
| D9 | UMIRC_AN68 | ACGTTCAGNNNNNNNNN | GCACAACT | AGTTGTGC |
| E9 | UMIRC_AN69 | CAGTCCAANNNNNNNNN | GATTACCG | CGGTAATC |
| F9 | UMIRC_AN70 | TTGCAGACNNNNNNNNN | ACCACGAT | ATCGTGGT |
| G9 | UMIRC_AN71 | CAATGTGGNNNNNNNNN | GTCGAAGA | TCTTCGAC |

| Well position | Adapter ID | i7 index | i5 index forward | i5 index reverse |
|---|---|---|---|---|
| H9 | UMIRC_AN72 | ACTCCATCNNNNNNNNN | CCTTGATC | GATCAAGG |
| A10 | UMIRC_AN73 | GTTGACCTNNNNNNNNN | AAGCACTG | CAGTGCTT |
| B10 | UMIRC_AN74 | CGTGTGTANNNNNNNNN | TTCGTTGG | CCAACGAA |
| C10 | UMIRC_AN75 | ACGACTTGNNNNNNNNN | TCGCTGTT | AACAGCGA |
| D10 | UMIRC_AN76 | CACTAGCTNNNNNNNNN | GAATCCGA | TCGGATTC |
| E10 | UMIRC_AN77 | ACTAGGAGNNNNNNNNN | GTGCCATA | TATGGCAC |
| F10 | UMIRC_AN78 | GTAGGAGTNNNNNNNNN | CTTAGGAC | GTCCTAAG |
| G10 | UMIRC_AN79 | CCTGATTGNNNNNNNNN | AACTGAGC | GCTCAGTT |
| H10 | UMIRC_AN80 | ATGCACGANNNNNNNNN | GACGATCT | AGATCGTC |
| A11 | UMIRC_AN81 | CGACGTTANNNNNNNNN | ATCCAGAG | CTCTGGAT |
| B11 | UMIRC_AN82 | TACGCCTTNNNNNNNNN | AGAGTAGC | GCTACTCT |
| C11 | UMIRC_AN83 | CCGTAAGANNNNNNNNN | TGGACTCT | AGAGTCCA |
| D11 | UMIRC_AN84 | ATCACACGNNNNNNNNN | TACGCTAC | GTAGCGTA |
| E11 | UMIRC_AN85 | CACCTGTTNNNNNNNNN | GCTATCCT | AGGATAGC |
| F11 | UMIRC_AN86 | CTTCGACTNNNNNNNNN | GCAAGATC | GATCTTGC |
| G11 | UMIRC_AN87 | TGCTTCCANNNNNNNNN | ATCGATCG | CGATCGAT |
| H11 | UMIRC_AN88 | AGAACGAGNNNNNNNNN | CGGCTAAT | ATTAGCCG |
| A12 | UMIRC_AN89 | GTTCTCGTNNNNNNNNN | ACGGAACA | TGTTCCGT |
| B12 | UMIRC_AN90 | TCAGGCTTNNNNNNNNN | CGCATGAT | ATCATGCG |
| C12 | UMIRC_AN91 | CCTTGTAGNNNNNNNNN | TTCCAAGG | CCTTGGAA |
| D12 | UMIRC_AN92 | GAACATCGNNNNNNNNN | CTTGTCGA | TCGACAAG |
| E12 | UMIRC_AN93 | TAACCGGTNNNNNNNNN | GAGACGAT | ATCGTCTC |
| F12 | UMIRC_AN94 | AACCGTTCNNNNNNNNN | TGAGCTAG | CTAGCTCA |
| G12 | UMIRC_AN95 | TGGTACAGNNNNNNNNN | ACTCTCGA | TCGAGAGT |
| H12 | UMIRC_AN96 | ATATGCGCNNNNNNNNN | CTGATCGT | ACGATCAG |

NOTE: To view the list of all 384 adapters available with Library Preparation Kit V2 (b), please download it at www.nonacus.com.

## III. Alternative procedure for concentrating pooled sample libraries prior to hybridization using Target Pure™ NGS clean-up beads

If a vacuum concentrator or vacuum lyophiliser / freeze drier are not available for use, pooled sample libraries can be concentrated using Target Pure™ NGS clean-up beads. However, please note that this procedure does introduce a minor GC bias.

Before you start, equilibrate the Target Pure™ NGS clean-up beads to room temperature for 20-30 minutes (for use in step 4) and prepare a solution of 80% Ethanol / 20% molecular biology grade water (400 µl required per capture reaction, for use in step 8).

## Proceeding from section 2.A, step 2:

1. Prepare the hybridization reaction mix (for use in step 13 below) in a new 1.5 ml tube as indicated in the table below.

| Components | Volume for 1 reaction (µl) |
|---|---|
| Hybridization Buffer (2x) | 9.5 |
| Hybridization Enhancer | 3 |
| Universal Blockers | 2 |
| GALEAS Bladder Panel | 4.5 |
| Total | 19 |

2. Pool equal concentrations (in ng) of individual sample libraries into a fresh 1.5 ml low-bind tube to reach a total combined quantity of 1000 ng.

3. Add 7.5 µl (equivalent to 7.5 µg) of COT-1 Human DNA to the library pool. Briefly vortex mix and centrifuge to collect the liquid at the bottom of the tube.

4. Add 1.8x volume of Target Pure™ NGS clean-up beads and mix thoroughly by pipette mixing 15-20 times, taking care to avoid the formation of bubbles.

5. Incubate the mixture for 10 minutes at room temperature.

6. Place the tube on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tube.

7. Keeping the tube on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

8. Add enough volume of ethanol to ensure the bead pellet is fully submerged, then incubate at room temperature for 30 seconds

9. Keeping the tube on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

10. Use a 10 µl multichannel or single channel pipette to remove any residual liquid from the tube.

11. Keeping the tube on the magnetic stand, incubate at room temperature with open lids for 5 minutes or until the beads are dry.

IMPORTANT: Avoid over-drying of beads, as this can result in a significant loss of DNA recovered. When dry, beads will appear matt in appearance but should not be cracked.

12. Remove the tube from the magnetic stand and resuspend the dried beads in 19 µl of hybridization reaction mix.

13. Incubate the tube for 5 minutes at room temperature.

14. Place the tube on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tubes/wells.

15. Carefully recover 17 µl of supernatant and transfer it to a fresh 0.2 ml PCR tube / 8-well tube strip / 96 well plate. NOTE: make sure to avoid bead carryover during the transfer process.

16. Place the 0.2 ml PCR tube / 8-well tube strip / 96 well plate containing the hybridization mix into the pre-heated (95°C) thermocycler and skip to the next step on the program.

17. Incubate for 4 hours until the thermocycler program reaches the hold step.

18. Proceed to section 2.B in the protocol.

## Incident reporting

Users of GALEAS Bladder are encouraged to report any suspected serious incidents, including unexpected results or adverse health outcomes potentially linked to the use of this product, to Nonacus Ltd. and/or the relevant national competent authority. Timely reporting supports post-market surveillance and ensures the continued safety and performance of GALEAS Bladder.