GALEAS™ Bladder Kit

DNA Target Enrichment for Next Generation Sequencing

Instructions for use (IFU) V1.0 (ENG)

Date of issue: October 2025

IVD UKCA For in vitro diagnostic use

REF NGS_GAL_BCP_FR_96

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GALEAS™/BLADDER

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 READ AND STRICTLY FOLLOW ALL INSTRUCTIONS MAY RESULT IN PRODUCT DAMAGE, PERSONAL INJURY (TO USERS OR OTHERS), PROPERTY DAMAGE, AND WILL VOID ANY APPLICABLE WARRANTY. NONACUS ACCEPTS NO LIABILITY FOR DAMAGES RESULTING FROM IMPROPER USE OF THE PRODUCT(S), INCLUDING ASSOCIATED COMPONENTS OR SOFTWARE.

Revision history

Revision	Date	Revision description
1.0	October 2025	First release

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Intended purpose

GALEAS Bladder (Panel) is an automated, qualitative in vitro diagnostic medical device intended for the detection and monitoring of bladder cancer by identifying and classifying the somatic mutational signature in 23 genes associated with the disease. The test is performed on a DNA sample extracted from cells contained in urine specimens collected from patients.

The device provides specific information related to:

- The presence or absence of bladder cancer, supporting diagnosis and clinical assessment.
- The monitoring of therapeutic measures, including surveillance for relapse and minimal residual disease (MRD) in patients previously diagnosed with bladder cancer.

Test results are intended to support healthcare professionals in clinical decision-making processes, including:

- Triage of patients presenting with haematuria, to determine the need for further invasive diagnostic procedures.
- Monitoring of patients with a previous diagnosis of bladder cancer, to assess treatment response and detect potential recurrence.

The GALEAS Bladder test utilises Next Generation Sequencing (NGS) technology and is therefore intended for use in laboratories equipped with NGS capabilities. GALEAS Bladder is intended for use by trained laboratory technicians who are proficient in performing molecular based tests, including NGS.

Performance characteristics

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 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.

Haematuria triage

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 to reduce unnecessary cystoscopies.

Observed performance (95 % CI)		
Parameter	**Re-analysis of Ward et al, 2022 (n=710)**	**NHS – real world evaluation (n=919)**
Sensitivity	91.8% (88.5%–94.2%)	90.6% (81.0%–95.6%)
Specificity	86.0% (82.0%–89.2%)	91.6% (89.5%–93.3%)
PPV	86.7% (82.9%–89.7%)	44.6% (36.3%–53.2%)
NPV	91.3% (87.8%–93.9%)	99.2% (98.4%–99.7%)
LR + / LR –	6.54 (5.05–8.47) / 0.1 (0.07–0.14)	10.76 (8.51–13.61) / 0.1 (0.05–0.22)

Surveillance / Recurrence Monitoring

In surveillance, the test maintains high sensitivity and very high NPV, supporting safe rule-out of recurrence. Apparent lower specificity reflects earlier molecular detection of disease prior to visible cystoscopic lesions and the inherent imperfection of cystoscopy and cytology comparators.

Observed performance (95 % CI)	
Parameter	**Re-analysis of Ward et al, 2022 (n=264)**
Sensitivity	95.0 % (76.4–99.1)
Specificity	60.9 % (54.6–66.9)
NPV	99.3 % (96.2–99.9)
PPV	17 % (11.1–25.0)
LR + / LR –	2.43 (2.01–2.93) / 0.08 (0.01–0.56)

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

Limitations and known interferences

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

Limitations:

- The GALEAS Bladder Kit is validated only for genomic DNA 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.
- 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.
- The GALEAS Bladder Software must be used for data analysis and interpretation.
- The test is not validated for prognosis or therapy selection.
- Analytical sensitivity may be reduced in samples with:
    - Low tumour DNA shedding (e.g., early low-grade tumours)
    - Low DNA input volume or quality
- The assay detects variants within the targeted regions only. Variants outside the Panel design will not be identified.
- 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.

Known interferences:

- DNA in high EDTA concentration buffers (e.g., containing 1mM 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.

Key features

- The GALEAS Bladder panel is based on targeted next-generation sequencing (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 avoids the requirement for a vacuum concentrator or freeze dryer.

Additional accessories

Specimen collection

The GALEAS Bladder assay kit is recommended to be used with the Galeas Bladder Urine Collection Device (PRE_GAL_UCD_1). The device consists of a leak-proof collection container suitable for the collection of freshly voided urine specimens from human subjects. The collection device is designed to maintain specimen integrity during handling and transport prior to DNA extraction.

The following specifications apply:

- Specimen Type – Freshly voided urine specimens.
- Collection Device – Sterile urine collection container with secure closure, provided as part of the kit.
- Specimen Stability – Samples should be processed within 28 days from collection and should be stored at room temperature until then.
- Transport Conditions – Specimens may be transported in the supplied urine collection device under standard shipping conditions (no temperature control required).
- All specimen collection and transport must be conducted in accordance with local biosafety regulations and institutional procedures for handling human specimens.

Data analysis software

The GALEAS Bladder assay requires use of the GALEAS Software: Bladder (NGS_GAL_GBA_1) a Medical Device Software (MDSW) classified as an in vitro diagnostic medical device under Regulation (EU) 2017/746. The software is provided as a secure online portal and is an essential component of the diagnostic workflow.

The software accepts sequencing data (FASTQ format) generated from the GALEAS Bladder panel and validated next-generation sequencing platforms. It performs bioinformatics processing, including quality control, alignment, variant calling, and filtering, and generates a structured report of clinically relevant genomic alterations.

Symbols and abbreviations

Symbol	Meaning
CE	European Conformity
IVD	In vitro diagnostic medical device
REF	Product reference code
	Manufacturer
LOT	Unique batch code
#	Catalogue number
	Expiry date
-20°C	Storage temperature
eIFU Indicator	Electronic instructions for use available
	Harmful / irritant / skin sensitiser
	Carcinogen / germ cell mutagen / reproductive toxin
	Acute toxicity
	Dangerous for the aquatic environment
	Corrosive

Safety precautions

The GALEAS Bladder Kit reagents are intended for use by trained laboratory personnel in accordance with standard molecular biology practices and applicable safety regulations. Users must wear appropriate personal protective equipment (PPE), including lab coats, gloves, and eye protection, when handling reagents. All reagents should be used in a well-ventilated area and handled with care to avoid ingestion, inhalation, or skin contact. Do not pipette by mouth. Refer to the Safety Data Sheets (SDS) provided with each reagent for detailed hazard information and recommended handling procedures. In the event of accidental exposure or spillage, follow institutional emergency protocols and consult the SDS for appropriate first aid and cleanup measures. The GALEAS Bladder Kit reagents must not be used beyond their expiration date and should be stored and disposed of according to the manufacturer's instructions and local regulations.

Workflow overview

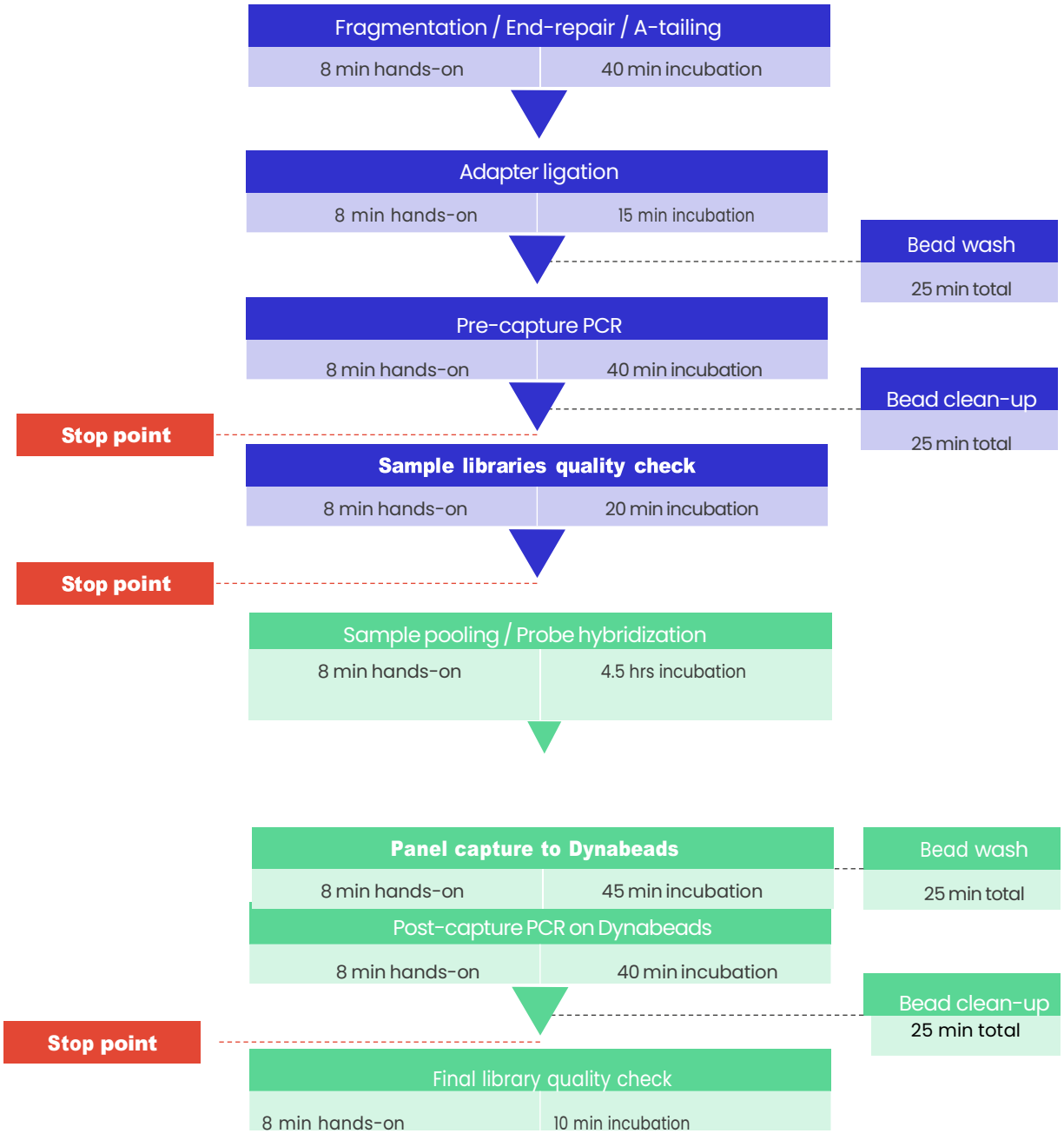

Figure 1. Flow chart outlining the main steps of the GALEAS Bladder kit workflow. Blue boxes refer to library preparation steps (3.5h); while green boxes refer to probe hybridization and capture steps (8h).

Product identifier information

Product codes and associated global trade item number (GTIN)

GALEAS Bladder Kit	Product code	GTIN number
GALEAS Bladder Kit 96 Samples A	NGS_GAL_BCP_FR_96_A	5061067370098
GALEAS Bladder Kit 96 Samples B	NGS_GAL_BCP_FR_96_B	5061067370104
GALEAS Bladder Kit 96 Samples C	NGS_GAL_BCP_FR_96_C	5061067370111
GALEAS Bladder Kit 96 Samples D	NGS_GAL_BCP_FR_96_D	5061067370128

Product identifier label barcode information

GALEAS Bladder Kit components	Product code	GTIN number
Library Preparation Kit V2 (b)	NGS_ACC_LV2_FR_96	5061067370036
UMIRC_AD01 – 96 (Adapter Plate)	NGS_ACC_ADP_1-96	5061067370050
UMIRC_AD01 97– 192 (Adapter Plate)	NGS_ACC_ADP_97-192	5061067370067
UMIRC_AD01 193– 288 (Adapter Plate)	NGS_ACC_ADP_193-288	5061067370074
UMIRC_AD01 289 – 384 (Adapter Plate)	NGS_ACC_ADP_289-384	5061067370081
GALEAS Bladder Panel	NGS_GAL_BCP_96	5061067370012
Hybridization and Capture Enrichment Kit V2	NGS_ACC_HV2_12	5061067370043

Product identifier label barcode information

GTIN	Expiry date	Lot number
506106737XXXX	YYYYMMDD	XXXXXXX

Kit contents

Library Preparation Kit V2 (b): NGS_ACC_LV2_FR_96

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	-20	-

NOTE: 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)

GALEAS Bladder Panel: NGS_GAL_BCP_96

Reagent	Volume 12rxns	Product code	Storage (°C)	No. of samples recommended for pre-capture pooling
GALEAS Bladder Panel	54 µl	NGS_GAL_BCP_96	-20	8 samples

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

Hybridization and Capture Enrichment Kit V2: NGS_ACC_HV2_12

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: 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

Required laboratory reagents and consumables not supplied

Item	Recommended source
Buffer Elution buffer (EB)	Qiagen, Cat # 19086 (or equivalent: 10 mM Tris-HCl, pH 8.0)
Digital electrophoresis system consumables	Agilent Technologies: D1000 Reagents, Cat # 5067-5583; D1000 ScreenTape, Cat # 5067-5582 High Sensitivity D1000 Reagents, Cat # 5067-5585 High Sensitivity D1000 ScreenTape, Cat # 5067-5584 Genomic DNA ScreenTape, Cat # 5067-5365 Genomic DNA Reagents, Cat # 5067-5366 (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 IMPORTANT: we have validated our protocol with Dynabeads. Other beads are NOT recommended for use with the GALEAS Bladder Kit protocol	Life Technologies, Cat # 65305
Ethanol (absolute, 100%)	Various sources available
Fluorometer consumables	Invitrogen: Qubit Assay Tubes, Cat # Q32856 Qubit dsDNA BR Assay kit, Cat # Q32853 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 or equivalent (such as Agencourt®)

Required equipment

Item	Source
Digital electrophoresis system	Agilent 4200 TapeStation, Agilent Technologies, Cat # G2965AA (recommended: if not available, see appendix IV)
Fluorometer for DNA fluorometric quantitation	Qubit®3.0 Fluorometer, Invitrogen, Cat # Q33216 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 (Recommended: if not available, see appendix II)
Magnetic separation rack capable of accommodating 1.5-2 ml tubes	DynaMag™-2 Magnet, Invitrogen, Cat # 12321D (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) (Recommended: if not available, see appendix III)
Vortex mixer	Various sources available

Storage, handling and disposal

All kit components must be stored at -20°C, as stated above. The enzyme mixes and Illumina UMI adapters in the Library Preparation Kit V2 (b); the PostCap Amplification Mix and PostCap Primer Mix in the Hybridisation and Capture Enrichment Kit V2; and the probe set in the GALEAS Bladder Panel must be thawed on ice and kept on ice during the relevant procedures. Briefly vortex mix all components after thawing and prior to use, except for the Fragmentation Enzyme, the Ligation Mix, PreCap Amplification Mix and PostCap Amplification Mix components, all of which should be mixed by light tapping. All components should be briefly spun down in a microcentrifuge after mixing.

All reagents used, including those containing hazardous chemicals or biological materials, must be disposed of in accordance with applicable local, national, and international regulations for chemical and biohazardous waste. Users should consult their institution's environmental health and safety guidelines to ensure proper segregation, containment, and disposal of waste materials. Do not dispose of reagents via sinks or general waste bins. Used consumables, such as tubes, tips, and plates, that have come into contact with reagents should be treated as laboratory waste and disposed of through approved waste management procedures. The manufacturer recommends following the SDS provided with each reagent for specific disposal instructions.

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 <100ng 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 <100ng/µl concentrations.

DNA samples that have been extracted using the GALEAS Bead Xtract: Urine gDNA (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 or equivalent; see 'Laboratory supplied reagents and consumables') and resuspended in one of the above-mentioned buffers.

How the UMI technology works

Unique molecular identifiers (UMIs) are built into GALEAS Bladder Kit 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 genomic DNA 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 Illumina sequencing 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 V2 (b) kit by lightly tapping the tube. Briefly centrifuge all three reagents in a microcentrifuge to collect the liquid to the bottom of the tubes and keep on ice.

IMPORTANT: All library preparation reaction setup procedures should be conducted while keeping tubes / 8-well tube strips / 96-well plates 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 105°C is not possible, set the highest temperature available for the instrument), the sample volume is 50 µl.

2. Prepare the following reaction mix for each DNA sample in a 0.2 ml PCR tube / 8-well tube strip / 96-well plate as indicated in the table below, keeping the reaction on ice during the whole procedure. Mix well by briefly vortex mixing or pipette mixing 10-15 times and briefly centrifuge the 0.2 ml PCR tube / 8-well tube strip / 96-well plate to collect the liquid at the bottom of the tube.

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 in a master mix prior to adding the DNA samples when processing multiple samples at the same time. Ensure that the master mix includes 10% overage to enable consistent pipetting of 10 µl to the 40 µl of DNA sample. The master mix should be vortexed at moderate speed for 5 seconds to ensure appropriate mixing is achieved without formation of bubbles.

3. Immediately transfer the tube / 8-well tube strip / 96-well plate to the pre-chilled thermocycler (4°C) and "skip" to the next step in the program.

4. 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, Illumina UMI 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 Illumina UMI adapter-containing 96-well plate from the freezer and thaw on ice. Centrifuge the plate in a plate centrifuge to collect the liquid at the bottom of the tubes.

Refer to Appendix VI, Table-2 for the location of each adapter within the supplied Illumina UMI Adapter 96-well plate, (one adapter per sample library) containing wells with 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 tubes / 8-well tube strip / 96-well plate containing the end-repaired / A-tailed DNA samples on ice, add 5 µl of the selected Illumina UMI adapter to each sample and mix gently by pipette mixing or briefly vortex mixing.

IMPORTANT: Use only one Illumina UMI 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 the tubes in a microcentrifuge or the 96-well plate in a plate centrifuge to collect all the liquid at the bottom of the tubes.

5. Immediately transfer the tube / 8-well tube strip / 96-well plate 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. 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.

8. 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.

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

10. 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.

11. Place the tubes / 8-well tube strip / 96-well plate on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tubes/wells.

12. Keeping the tubes / 8-well tube strip / 96-well plate 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 13-14 for a total of two 80% ethanol washes.

15. Keeping the tubes / 8-well tube strip / 96-well plate on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

16. Use a 10 µl multichannel or single channel pipette to remove any residual liquid from the tubes/wells.

17. Keeping the tubes / 8-well tube strip / 96-well plate 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

18. Remove the tubes / 8-well tube strip / 96-well plate 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.

19. Incubate the tubes / 8-well tube strip / 96-well plate for 2 minutes at room temperature.

20. Place the tubes / 8-well tube strip / 96-well plate on the magnetic stand for 2 minutes at room temperature to pellet the beads on the side of the tubes/wells.

21. 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 the provided Illumina adapters.

Before you start

Thaw the PreCap Amplification Mix (green cap) and the PreCap Primer Mix (**black** cap) from the Library Preparation Kit V2 (b) on ice. Once thawed, lightly tap 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 in a microcentrifuge to collect the liquid at the bottom of the tube. 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 using a microcentrifuge to collect liquid at the bottom of the tube.

4. Transfer the 0.2 ml PCR tubes / 8-well tube strip / 96-well plate 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. Add 50 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.

7. 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.

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

9. Place the tubes / 8-well tube strip / 96-well plate on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tubes/wells.

10. Keeping the tubes / 8-well tube strip / 96-well plate on the magnetic stand, slowly remove and discard

the supernatant, taking care not to disturb the pelleted beads.

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

12. Repeat steps 10-11 for a total of two 80% ethanol washes.

13. Keeping the tubes / 8-well tube strip / 96-well plate on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

14. Use a 10 µl multichannel or single channel pipette to remove any residual liquid from the tubes/wells.

15. Keeping the tubes / 8-well tube strip / 96-well plate 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.

16. Remove the tubes / 8-well tube strip / 96-well plate 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.

17. Incubate the tubes / 8-well tube strip / 96-well plate for 2 minutes at room temperature.

18. Place the tubes / 8-well tube strip / 96-well plate on the magnetic stand for 2 minutes at room temperature to pellet the beads on the side of the tubes/wells.

19. 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., >16 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

By ligating dual indexed adapters containing UMIs to DNA fragments, the library preparation procedure adds 144 bp to the 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 genomic DNA. 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.

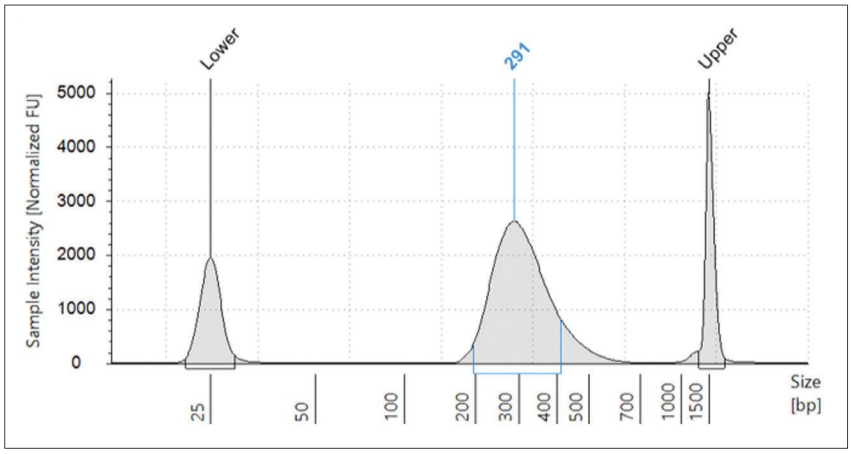

Figure 2. Fragment size distribution of library prepared with 25 ng of input high molecular weight genomic DNA extracted from urinary cell-pellet.

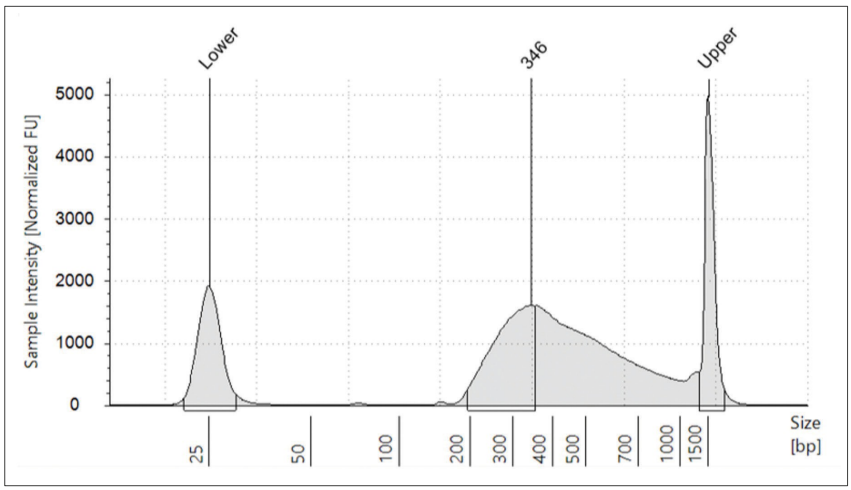

Figure 3. Fragment size distribution of unsuccessful library.

- 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 hybridization and capture.

Chapter 2: Probe hybridization and capture enrichment

The Hybridization and Capture Enrichment Kit V2 enables probe hybridization-based targeted enrichment of Illumina sequencing libraries (i.e., containing Illumina adapters) prepared from gDNA as input material in combination with the GALEAS Bladder Panel.

2.A Library pooling and probe hybridization

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

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 (400 µl required per capture reaction, for use in step 10).

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 at room temperature. Thaw the GALEAS Bladder Panel on ice. Mix each component vigorously by vortex mixing, then microcentrifuge 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	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. 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

4. 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.

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

6. 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.

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

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

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

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

11. Repeat steps 9-10 for a total of two 80% ethanol washes.

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

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

14. 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

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

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

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

18. 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.

19. 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.

20. Incubate overnight and proceed to section 2.B the next day.

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 in a microcentrifuge to collect the liquid at the bottom of the tube.

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 in a microcentrifuge 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.

16. Place the tube on a magnetic stand capable of accommodating 0.2 ml PCR tubes / 8-well tube strips 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.

17. 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.

Procedure

18. Set a thermocycler at 65°C on hold with the heated lid set at 70°C.

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.

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, remove the 0.2 ml PCR tube / 8-well tube strip from the thermocycler, quickly vortex for 3 seconds to ensure the beads remain in solution and place back on the thermocycler.

23. Remove the 0.2 ml PCR tube / 8-well tube strip 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 0.2 ml PCR tube / 8-well tube strip 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 0.2 ml PCR tube / 8-well tube strip 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 0.2 ml PCR tube / 8-well tube strip 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 0.2 ml PCR tube / 8-well tube strip 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 25-29 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 0.2 ml PCR tube / 8-well tube strip 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 0.2 ml PCR tube / 8-well tube strip 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 0.2 ml PCR tube / 8-well tube strip 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 0.2 ml PCR tube / 8-well tube strip 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 0.2 ml PCR tube / 8-well tube 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 0.2 ml PCR tube / 8-well tube strip 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 0.2 ml PCR tube / 8-well tube strip 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 Illumina 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. Briefly vortex mix and centrifuge 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. 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. Mix well by pipette mixing up and down 10 times or briefly vortex mixing. Centrifuge in a microcentrifuge to collect the liquid at the bottom of the tube.

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

3. 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.

4. Transfer the 0.2 ml PCR tube / 8-well tube strip 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 captured library

6. Add 75 µl of thoroughly vortexed room-temperature equilibrated 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.

7. 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.

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

9. Place the 0.2 ml PCR tube / 8-well tube strip on the magnetic stand for 5 minutes at room temperature to pellet the beads on the side of the tubes/wells.

10. Keeping the 0.2 ml PCR tube / 8-well tube strip on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

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

12. Repeat steps 10-11 for a total of two 80% ethanol washes.

13. Keeping the 0.2 ml PCR tube / 8-well tube strip on the magnetic stand, slowly remove and discard the supernatant, taking care not to disturb the pelleted beads.

14. Use a 10 µl pipette to remove any residual liquid from the tube/well.

15. Keeping the 0.2 ml PCR tube / 8-well tube strip 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.

16. Remove the 0.2 ml PCR tube / 8-well tube strip 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.

17. Incubate the 0.2 ml PCR tube / 8-well tube strip for 2 minutes at room temperature.

18. Place the 0.2 ml PCR tube / 8-well tube strip on the magnetic stand for 2 minutes at room temperature to pellet the beads on the side of the tube/well.

19. 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.

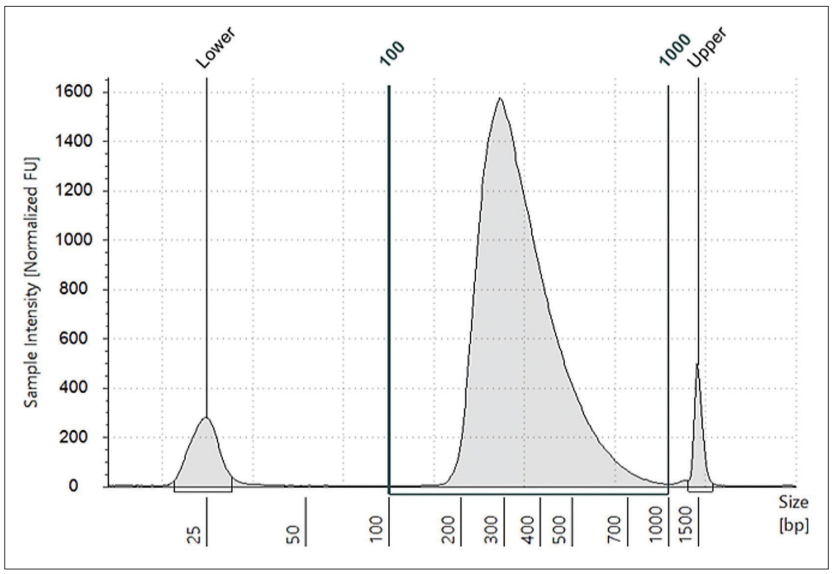

Figure 4. Fragment size distribution showing the range of 100-1000 bp within which the average fragment size is calculated. Average fragment size: 354 bp.

Chapter 3: Sequencing of captured libraries

Libraries enriched by targeted capture using GALEAS Bladder Kit 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.

$$concentration\ in\ nM = \frac{concentration\ in\ ng/\mu l}{(660\ \frac{g}{mol}\ x\ average\ library\ size\ in\ bp)}\ x\ 10^6$$

- 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 Kit 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 genomic DNA 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.

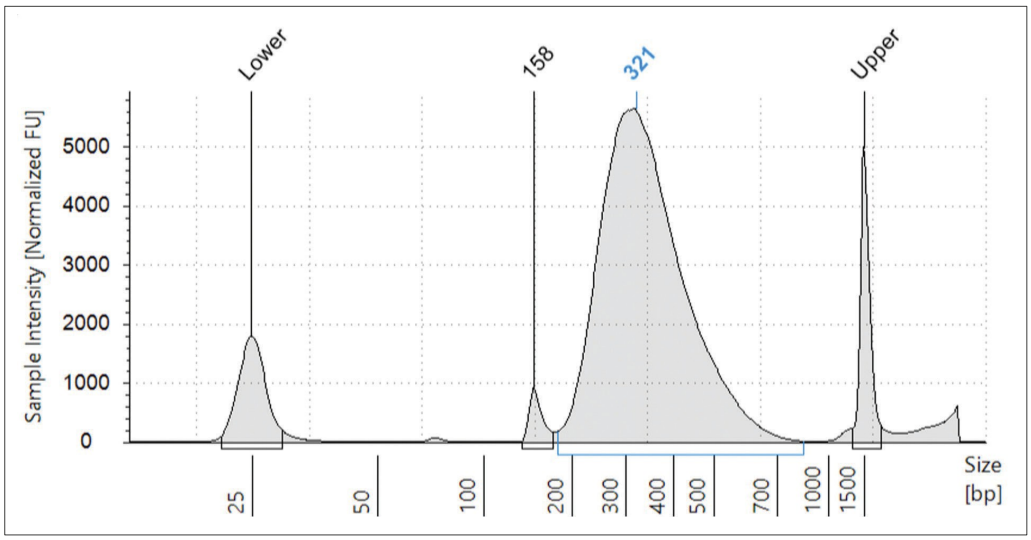

Figure 5. D1000 screen tape electropherogram of an over-amplified sample library.

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.

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,AAAAAAAAAAAAAAAAAA,1+2,
R1AdapterTrim,FALSE,1+2,
R2Adapter,TTTTTTTTTTTTTTTTTT,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.

Electronic Instructions for Use (eIFU)

The eIFU for the GALEAS Bladder kit is available online, free of charge, to ensure timely access to the most current and compliant documentation, including updates and revisions. Users may download or print the eIFU as needed. Access to the eIFU is intended to support safe and effective use of the device and does not replace the obligation to consult the IFU prior to use. If required, printed copies of the IFU can be requested from the manufacturer free of charge, ensuring that users are informed, supported, and compliant with applicable safety and performance requirements.

This IFU is provided in English. Translations into other EU languages are available upon request.

Incident reporting

This device is CE-marked and complies with Regulation (EU) 2017/746 for in vitro diagnostic medical devices (IVDR). Users of the GALEAS Bladder test 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 the GALEAS Bladder test.

EU declaration of conformity

GALEAS Bladder Kit is a Class C device is classified as CE marked (CE-IVD) under the In Vitro Diagnostic Regulation (EU) 2017/746 and complies with the relevant General Safety and Performance Requirements.

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GALEAS™/BLADDER