

Instructions for Use

IFU095

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 ASTX17.1(24)-IVD

ASTX17.1(24)-B-IVD

ASTX17.1(96)-A-IVD

ASTX17.1(96)-B-IVD

ASTX9.1(96)-A-IVD

ASTX9.1(96)-B-IVD







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1. Overview
   1. Principle

AlloSeq Tx is the product family name for targeted gene sequencing products designed to assist with determining the genetic compatibility between transplant patients and potential donors. AlloSeq Tx leverages hybrid capture technology to enrich the genes of interest from a whole genome library preparation. The use of hybrid capture technology, as opposed to traditional long-range PCR techniques, has workflow benefits, and enables the flexibility of variable gene/sequence content without the need for workflow changes.

The principle of the AlloSeq Tx assay workflow is summarized below:

Stage 1: Library Preparation: Simultaneous DNA fragmentation and tagmentation, indexing PCR, purification and size selection,

Stage 2: Hybrid Capture (Enrichment): Sample pooling 1 (original workflow or early pooling workflow), probe hybridization, capture probe-bound fragments with streptavidin magnetic beads, final enrichment PCR, purification and quantification,

Stage 3: Sequencing: Dilution and denaturing and sequencing on an Illumina sequencer,

Stage 4: Analysis: Genotyping in AlloSeq Assign software

1 The Early Pooling workflow pools samples immediately after indexing into a single tube for all subsequent steps, eliminating plate-based size selection and purification as well as the optional concentration step. This workflow allows the user to take samples through to sequencing in a single working shift. This workflow is well suited to laboratories who do not utilise automation, and prefer a protocol with less hands-on time, less consumable use, and 46% less pipetting steps compared to the original workflow.The plate-based format of the original workflow is well suited to laboratories who choose to automate this procedure.

The AlloSeq Tx kits include:

* Reagents to prepare whole genome libraries,
* Biotinylated probes complementary to capture sequence targets, and
* Reagents to enrich the captured targets for sequencing.

We recommend all users read the entire Instructions for Use, particularly the *Safety* section, prior to starting the procedure. Procedures for use of the AlloSeq Assign software can be found in the AlloSeq Assign Software Instructions for Use.

* 1. Intended Use

AlloSeq Tx is the product family name for targeted gene sequencing products designed to assist with determining the genetic compatibility between transplant patients and potential donors; including probes for targeted enrichment of up to 17 loci.

The AlloSeq Tx 17 typing kits are qualitative tests for the DNA typing of HLA-A, B, C, E, F, G, H, DRB1/3/4/5, DQA1, DQB1, DPA1, DPB1, MICA and MICB to assist in genetic matching for organ or stem cell transplantation.

The AlloSeq Tx 9 typing kits are qualitative tests for the DNA typing of HLA-A, B, C, DRB1/3/4/5, DQB1, and DPB1, to assist in genetic matching for organ or stem cell transplantation.

The product is intended for use with the Illumina MiSeq, MiniSeq and iSeq sequencers, along with the AlloSeq Assign interpretation software.

The device is intended for use by appropriately trained staff, with knowledge of the frequency of HLA types in their population, in appropriately regulated laboratories performing tissue (HLA) typing for matching donors and recipients for transplantation.

The AlloSeq Tx products are for professional use only and must not be used as the sole basis for making clinical decisions. AlloSeq Tx kits are not used for the diagnosis of disease.

* 1. AlloSeq Tx Targeted Gene Content

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Product Name** | **Product Code** | **Targeted Genes** | **Kit Size** | **Sequencing1** |
| AlloSeq Tx17 | ASTX17.1(24)-IVD | HLA-A, -B, -C, -E, -F, -G, -H, -DRB1/3/4/5, -DQA1, -DQB1, -DPA1, -DPB1, MICA and MICB | 24 library preparations  4 enrichments (between 6-24 samples per enrichment) | ≤24 samples on MiSeq Micro flow cell  ≤6 samples on MiSeq Nano flow cell  ≤24 samples on iSeq  ≤24 samples on MiniSeq Mid Output flow cell |
| ASTX17.1(24)-B-IVD |
| ASTX17.1(96)-A-IVD | HLA-A, -B, -C, -E, -F, -G, -H, -DRB1/3/4/5, -DQA1, -DQB1, -DPA1,  -DPB1, MICA and MICB | 96 library preparations  8 enrichments (between 12-96 samples per enrichment) | ≤96 samples on MiSeq v2 Standard flow cell  ≤24 samples on MiSeq Micro flow cell  ≤6 samples on MiSeq Nano flow cell  ≤24 samples on iSeq  ≤24 samples on MiniSeq Mid Output flow cell |
| ASTX17.1(96)-B-IVD |
| AlloSeq Tx9 | ASTX9.1(96)-A-IVD | HLA-A, -B, -C, -DRB1/3/4/5, -DQB1 and -DPB1 | 96 library preparations  8 enrichments (between 12-96 samples per enrichment) | ≤96 samples on MiSeq Standard flow cell  ≤24 samples on MiSeq Micro flow cell  ≤6 samples on MiSeq Nano flow cell  ≤24 samples on iSeq  ≤24 samples on MiniSeq Mid Output flow cell |
| ASTX9.1(96)-B-IVD |

1 The numbers of samples and flow cells shown in this table are based on AlloSeq Tx kit validation. These figures may be used as an indication and further verified by individual labs.

* 1. AlloSeq Tx Kit Contents and Storage Requirements

When stored at the temperature specifications below the kit components can be used until the expiry indicated on the outer kit containers and can tolerate up to 12 freeze-thaw cycles. After use, the kits/components should be returned immediately to storage conditions.

Kits are NOT to be used beyond their expiry date.

**Reagents Box 1 of 5, Store at -15 to -25°C**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Reagent** | **Quantity (24 test)** | **Tube size/ type (24 test)** | **Quantity (96 test)** | **Tube size/type (96 test)** |
| Tagmentation Beads | 1 | 0.5mL | 1 | 1.5mL |
| Tagmentation Buffer | 1 | 0.5mL | 1 | 1.5mL |
| PCR Mix-1 | N/A | N/A | 1 | 5mL |

**Reagents Box 2 of 5, Store at 15 to 30°C**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Reagent** | **Quantity (24 test)** | **Tube size/ type (24 test)** | **Quantity (96 test)** | **Tube size/type (96 test)** |
| Stop Buffer | 1 | 1.5mL | 2 | 2mL |
| Tagmentation Wash Buffer | 2 | 5mL | 1 | 50mL |

**Reagents Box 3 of 5, Store at -15 to -25°C**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Reagent** | **Quantity (24 test)** | **Tube size/ type (24 test)** | **Quantity (96 test)** | **Tube size/ type (96 test)** |
| AlloSeq Tx Index Primers | 1 set (10x) | FluidX tube | 1 plate | 96 well plate |

**Reagents Box 4 of 5, Store at -15 to -25°C**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Reagent** | **Quantity (24 test)** | **Tube size/ type (24 test)** | **Quantity (96 test)** | **Tube size/ type (96 test)** |
| Product Specific AlloSeq Tx Probes | 1 | 0.5mL | 1 | 0.5mL |
| PCR Mix | 1 | 1.5mL | N/A | N/A |
| PCR Mix-2 | N/A | N/A | 1 | 0.5mL |
| PCR Primers | 1 | 0.5mL | 1 | 0.5mL |
| Hybridisation Buffer 1 | 1 | 1.5mL, Conical | 1 | 1.5mL,  Conical |
| Capture Wash Buffer | 4 | 1.5mL, Amber Conical | 8 | 1.5mL, Amber Conical |
| Capture Elution Buffer 1 | 1 | 0.5mL | 1 | 0.5mL |
| 2N NaOH | 1 | 0.5mL | 2 | 0.5mL |

**Reagents Box 5 of 5, Store at 2-8°C**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Reagent** | **Quantity (24 test)** | **Tube size/ type (24 test)** | **Quantity (96 test)** | **Tube size/ type (96 test)** |
| Purification Beads | 1 | 5mL | N/A | N/A |
| Purification Beads-1 | N/A | N/A | 3 | 5mL |
| Purification Beads-2 | N/A | N/A | 1 | 0.5mL |
| Resuspension Buffer | 2 | 1.5mL | 2 | 5mL |
| Capture Beads | 1 | 1.5mL | 1 | 5mL |
| Hybridisation Buffer 2 | 1 | 0.5mL | 1 | 0.5mL |
| Capture Elution Buffer 2 | 1 | 0.5mL | 1 | 0.5mL |

* 1. Limitations and contraindications
* It is strongly recommended that these kits are validated by the user prior to implementation in the laboratory using samples whose genotype has been determined by other molecular based procedures.

• It is strongly recommended that the user follows all instructions provided via product labelling. Deviations from the described procedure are not recommended, may not be supported, and could lead to typing errors.

• It is recommended that a positive control (human DNA) and negative/no template control (using sterile water in place of DNA) be included on every library preparation run. The positive control must produce a quantifiable library (measured by Qubit or coverage sequencing metrics) and the resultant sequence must be concordant with the sample’s expected genotype. There must be no quantifiable library (measured by Qubit or reported as Low Coverage in Assign) in the negative template control for each experiment. If a quantifiable library is produced for the no template control, the run must be repeated.

* The AlloSeq Tx assay sequences DNA fragments with an average insert size of 500bp, meaning that polymorphisms greater than 500bp apart cannot be phased, which may result in heterozygous ambiguities.
  1. Sample Requirements

**Sample Type:**

High molecular weight human genomic DNA (suspended in Tris/EDTA buffer and OD260/280> 1.8) from whole blood specimens. Do NOT use whole blood specimens containing heparin.

The recommended amount of high molecular weight human genomic DNA is 100-1000 ng. Internal testing has shown that samples with DNA input as low as 50 ng can also be used. Correct genotypes were also obtained from poor quality or sheared DNA.

**Sample stability:**

Storage: Whole blood should be collected in ACD or EDTA anticoagulants. DNA can be isolated from samples as long as 2 weeks after the initial blood draw, although it is recommended that samples be processed within 2 to 3 days of draw. Frozen whole blood samples can be stored at -20°C to -70°C for at least 1 year without compromising the quality or quantity of the DNA isolated. (Ref ASHI Laboratory Manual Vol 2).

**DNA Extraction Methods:**

The AlloSeq Tx assay has been validated with QIAamp DNA Blood Mini Kit (Catalogue #51104), EZ1 DNA Blood 350 µl Kit (Catalogue #951054), and Promega Maxwell. Alternatively, DNA can be extracted using other methods and equipment that are user-validated to isolate high molecular weight DNA.

* 1. Analytical Specificity / Interfering Substances

CareDx Pty Ltd has identified all known potential interfering substances that could impact the test. See table below.

| **Inhibitor** | **Potential source** | **Risk** | **Comments** |
| --- | --- | --- | --- |
| EDTA | TE buffer, blood collection tubes | Very low | Resuspend DNA in Tris-HCl pH8 or TE with < 0.1mM EDTA. Use commercial Blood DNA preparation kits. Do not resuspend in EDTA > 0.1mM. |
| Alcohols | Ethanol, isopropanol, isoamyl alcohol | Low | Ensure DNA pellets or beads are air dried and visually inspected for ethanol droplets (1% ethanol = 1.25ul 80% ethanol in a 100ul PCR reaction). There are multiple 80% ethanol wash steps in the AlloSeq Tx protocol making inhibition due to ethanol carryover a low but slightly higher risk than other factors. |
| Excess salts | KCl, NaCl, CsCl, NaAc | Very low | Ensure thorough washing of DNA pellets or beads with 80% ethanol. Ensure OD 260/230 for starting genomic DNA is ~2 |
| Chaotropic salts | Guanidinium Cl; MgCl2; urea | Very low | Ensure thorough washing of DNA pellets or beads with 80% ethanol. Ensure OD 260/230 for starting genomic DNA is ~2 |
| Phenol: chloroform | Organic carryover | Very low | A component of the widely used commercial Trizol DNA extraction procedure. Ensure thorough washing of DNA pellets or beads with 80% ethanol. Ensure OD 260/230 for starting genomic DNA is ~2 |
| Proteins | BSA, PEG, blood albumin | Very low | Use commercial Blood DNA preparation kits. Ensure OD 260/280 of starting genomic DNA is >1.8 |
| Heme, hemoglobin, immunoglobulins | Blood | Very low | Avoid using blood samples exhibiting gross hemolysis. Use commercial Blood DNA preparation kits. Ensure OD 260/280 of starting genomic DNA is >1.8 |
| Detergents/DDT | Na deoxycholate, sarkosyl, SDS, NP40, Tween 20, Triton X-100, N-octyl glucoside | Very low | Ensure thorough washing of DNA pellets or beads with 80% ethanol. Ensure OD 260/230 of starting genomic DNA is ~2 |
| Proteases | Proteinase K, sample handling | Very low | Use commercial Blood or saliva DNA preparation kits. Wear gloves at all times |
| Nucleases | Sample handling, restriction enzymes, micrococcal nuclease | Very low | Use commercial Blood DNA preparation kits. Wear gloves at all times |
| Exogenous DNA/RNA | Carryover, contamination | Very low | Prepare genomic DNA in dedicated pre-PCR area |
| Carriers | RNA, heparin, glycogen | Very low | Use commercial Blood DNA preparation kits and/or avoid heparin blood collection tubes |
| Excess metal ions | Mg2+ from PCR buffer, Fe ions | Very low | Ensure thorough washing of DNA pellets or beads with 80% ethanol. Ensure OD 260/230 of starting genomic DNA is ~2 |
| Antiviral drugs (e.g., acyclovir) | Blood | Very low | Use commercial Blood DNA preparation kits. Ensure OD 260/280 of starting genomic DNA is >1.8 |
| Glove powder | Powdered gloves | Very low | Use powder free gloves |
| UV irradiated PCR tubes | UV treatment of PCR tubes | Very low | Avoid UV treatment of plasticware |
| Biotin | From pharmaceuticals interacting with streptavidin | Very  low | There are numerous purification washes between sample collection and the hybrid capture step within the protocol which will enable removal of the biotin molecules.  In the case of a hybrid capture assay, the presence of biotin (which as outlined above is unlikely) will not lead to an erroneous result. It could cause a reduced efficiency of enrichment which would be detected as a low enrichment yield or give no result. |

* 1. Performance Characteristics

Assay performance was assessed using a panel of DNA samples with known genotypes including internal CareDx control samples, International Histocompatibility Workshops (IHW) HLA Reference Standards and samples obtained from the International HLA DNA Exchange, University of California, Los Angeles (UCLA). Library preparation, enrichment and sequencing performance were evaluated against defined acceptance criteria.

* 1. Accuracy

The AlloSeq Tx product together with the AlloSeq Assign software are designed for compliance with the ASHI standard (American Society for Histocompatibility and Immunogenetics); as well as the EFI standard (European Federation of Immunogenetics) for HLA typing.

Summary of result obtained from Verification and Validation studies:

|  |  |  |  |
| --- | --- | --- | --- |
| **METRIC** | **PANEL** | **PANEL SIZE (n)** | **RESULT** |
| Genotyping Concordance | Internal | 190 | 100% |
| Genotyping Concordance | UCLA | 24 | 98.12% |
| Genotyping Concordance | IHW | 48 | 98.06% |
| Genotyping Concordance | External | 124 | 99.54% |
| **Total / Overall Concordance** | | | **99.49%** |

In all instances of discordance observed within the verification/validation studies, it is believed that the discordance arises from limitations in the previous typing method or technology.

* 1. Specificity

Data has shown that specificity as low as 12% has no impact on the assay, whilst typical specificity values fall in the range of 64 and 92%. Complete specificity is unlikely and would be an indicator of potential quality issues. Whilst the probe design strategy makes the probability of missing alleles unlikely, the sensitivity v specificity paradigm is likely to hold true. That is; some degree of reduced specificity will ensure complete sensitivity and alleles, including novel alleles, will be successfully sequenced.

* 1. Reproducibility and Repeatability

The AlloSeq Tx product together with the AlloSeq Assign software is proven to give equivalent results across batches in a lot-to-lot verification study and across laboratories, users and instruments which was proven in verification and validation across four external sites. The AlloSeq Tx product has been verified to give equivalent results for the same sample in repeated runs.

* 1. Assumptions
* Instruments are properly calibrated, maintained and under a maintenance plan as needed.
* Standard Operating Procedures (SOPs) are in place and controlled.
* The kit is used by trained and authorised laboratory personnel.
* The reagents are used within their expiry dates stated.
* Reagents from different kit batches are NOT used together. This may impact the kit’s performance
* Only the reagents recorded as not included but required within this document are used.
* Care is taken to prevent cross-contamination of DNA specimens or sample mix-ups
* Care is taken at all stages of use to avoid spills
* The workbooks supplied by the manufacturer are used in conjunction with this document
  1. Safety

Follow general laboratory safety practices and clean room contamination prevention practices when performing this procedure. Through the CareDx Pty Ltd risk management process, all risks have been mitigated to an acceptable limit. Instructions for Use must be followed, , to prevent hazardous use scenarios. Hazardous materials are present in this kit. Please consult the Safety Data Sheets and take all required precautions in handling and disposal.

| **KIT COMPONENT** | **PICTOGRAMS** | **SAFETY WARNING** |
| --- | --- | --- |
| **TAGMENTATION BUFFER**  Contains N,N-Dimethylformamide |  | **Signal word:** Danger  **Hazard statements:**  H319 – Causes serious eye irritation  H332 – Harmful if inhaled  H350 – May cause cancer  H360 – May damage fertility or the unborn child  **Precautionary Statements – EU (§28, 1272/2008)**  P201 – Obtain special instructions before use  P202 – Do not handle until all safety precautions have been read and understood  P261 – Avoid breathing dust/fume/gas/mist/vapours/spray  P270 – Do not eat, drink or smoke when using this product  P280 – Wear protective gloves/protective clothing/eye protection/face protection  P264 – Wash face, hands and any exposed skin thoroughly after handling  P272 – Contaminated work clothing should not be allowed out of the workplace  P308 + P313 – IF exposed or concerned: Get medical advice/attention  P304 + P340 – IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing  P313 – Get medical advice/attention  P305 + P351 + P338 – IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing  P337 + P313 – If eye irritation persists: Get medical advice/attention  P405 – Store locked up  P501 – Dispose of contents/ container to an approved waste disposal plant |
| **PCR MIX**  Contains Tetramethylammonium chloride |  | **Signal word:** Danger  **Hazard statements**  H302 – Harmful if swallowed  H371 – May cause damage to organs  H412 – Harmful to aquatic life with long lasting effects  **Precautionary Statements – EU (§28, 1272/2008)**  P260 – Do not breathe dust/fume/gas/mist/vapours/spray  P270 – Do not eat, drink or smoke when using this product  P280 – Wear protective gloves/protective clothing/eye protection/face protection  P264 – Wash face, hands and any exposed skin thoroughly after handling  P272 – Contaminated work clothing should not be allowed out of the workplace  P273 – Avoid release to the environment  P308 + P313 – IF exposed or concerned: Get medical advice/attention  P301 + P312 – IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell  P330 – Rinse mouth  P405 – Store locked up  P501 – Dispose of contents/ container to an approved waste disposal plant |
| **2N NaOH**  Contains Sodium hydroxide |  | **Signal word:** Danger  **Hazard statements**  H314 – Causes severe skin burns and eye damage  H318 – Causes serious eye damage  **Precautionary Statements – EU (§28, 1272/2008)**  P260 – Do not breathe dust/fume/gas/mist/vapours/spray  P264 – Wash face, hands and any exposed skin thoroughly after handling  P280 – Wear protective gloves/protective clothing/eye protection/face protection  P301 + P330 + P331 – IF SWALLOWED: rinse mouth. Do NOT induce vomiting  P303 + P361 + P353 – IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/ shower  P363 – Wash contaminated clothing before reuse  P310 – Immediately call a POISON CENTER or doctor  P304 + P340 – IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing  P305 + P351 + P338 – IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing  P405 – Store locked up  P501 – Dispose of contents/container in accordance with local, regional, national, and international regulations as applicable |
| **CAPTURE BEADS**  Contains Formamide |  | **Signal word:** Danger  **Hazard statements**  H351 – Suspected of causing cancer  H360 – May damage fertility or the unborn child  H373 – May cause damage to organs through prolonged or repeated exposure  **Precautionary Statements – EU (§28, 1272/2008)**  P201 – Obtain special instructions before use  P202 – Do not handle until all safety precautions have been read and understood  P260 – Do not breathe dust/fume/gas/mist/vapours/spray  P270 – Do not eat, drink or smoke when using this product  P280 – Wear protective gloves/protective clothing/eye protection/face protection  P264 – Wash face, hands and any exposed skin thoroughly after handling  P272 – Contaminated work clothing should not be allowed out of the workplace  P308 + P313 – IF exposed or concerned: Get medical advice/attention  P405 – Store locked up  P501 – Dispose of contents/ container to an approved waste disposal plant |
| **HYBRIDISATION BUFFER 1**  Contains Formamide |  | **Signal word:** Danger  **Hazard statements**  H351 – Suspected of causing cancer  H360 – May damage fertility or the unborn child  **Precautionary Statements – EU (§28, 1272/2008)**  P201 – Obtain special instructions before use  P202 – Do not handle until all safety precautions have been read and understood  P261 – Avoid breathing dust/fume/gas/mist/vapours/spray  P270 – Do not eat, drink or smoke when using this product  P280 – Wear protective gloves/protective clothing/eye protection/face protection  P264 – Wash face, hands and any exposed skin thoroughly after handling  P272 – Contaminated work clothing should not be allowed out of the workplace  P308 + P313 – IF exposed or concerned: Get medical advice/attention  P405 – Store locked up  P501 – Dispose of contents/ container to an approved waste disposal plant |
| **STOP BUFFER**  Contains Sodium Dodecyl Sulfate |  | **Signal word:** Warning  **Hazard statements**  H319 – Causes serious eye irritation  **Precautionary Statements – EU (§28, 1272/2008)**  P264 – Wash face, hands and any exposed skin thoroughly after handling  P280 – Wear protective gloves/protective clothing/eye protection/face protection  P305 + P351 + P338 – IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to  do. Continue rinsing  P337 + P313 – If eye irritation persists: Get medical advice/attention |

**NOTE:** The component ‘Purification Beads’ contains Sodium Azide (<0.1%) which is not considered a hazardous concentration in accordance with EC 1272/2008 (CLP/GHS), EC Directives 1999/45EC and 67/548/EEC or US-OSHA (HCS 29 CFR 1910.1200) and UN GHS.

For additional detail on all hazardous materials contained in the AlloSeq Tx kit, please refer to TEC478\_AlloSeq Tx Safety Data Sheet at [www.caredx.com](file:///C:/s_drive/DocumentLibrary2/draft/Instructions%20for%20Use/AlloSeq%20Tx/www.caredx.com).

1. Library Preparation (Early Pooling Workflow)

2.0 Protocol Introduction

* Follow the AlloSeq Tx protocol below in the order shown using the specified parameters.
* Before proceeding, confirm kit contents and make sure that you have the required consumables and equipment.
* For ease of use, protocol steps for Library Preparation are also detailed in *IFU095-5\_AlloSeq Tx Early Pooling Workbook CE IVD*. References to the workbook in Chapter 2 pertain to this workbook.

2.1 Sample Preparation

1. Enter an experiment ID, description, operator, and the date into the workbook.
2. Select the AlloSeq probe set to be used for the experiment from the drop-down menu in the workbook.
3. Select the sequencer type to be used from the drop-down menu in the workbook. Once the sequencer type has been selected, the sequencing instructions will be displayed in the workbook.
4. Enter the ID of samples to be tested in the yellow section of the plate layout in the workbook, according to the desired configuration.

**NOTE:** Only alphanumeric characters can be entered. Duplicate sample IDs will be flagged in red to prompt user to correct. It is a requirement of the sequencer software to have only unique sample IDs on a run. Do not enter any information for wells which are not intended to contain any sample.

1. Select the desired index set from the orange dropdown under Plate Layout.
2. If required and using the tubed index format, the i7 index order can be modified, select the alternative i7 index to be used from the drop-down options for the required column. If duplicate i7 indexes are selected, then the cells will be flagged in red to prompt the user to correct.
3. If required and using the tubed index format, the i5 index order can be modified, select the alternative i5 index to be used from the drop-down options for the required row. If duplicate i5 indexes are selected, then the cells will be flagged in red to prompt the user to correct.
4. Once all the actions above have been completed, click on the 1.2 SampleSheet tab and review. Red text will be used to flag where information is still required. If no red text is evident then the SampleSheet tab can be saved as a CSV (Comma delimited) (\*.csv). Save the file as ‘SampleSheet.csv’. Saving in a csv format will only save the active tab. Open the saved SampleSheet.csv file in excel and delete any empty rows in the [Data] table, that is, any rows between 22 to 117 which do not contain sample information. Once this has been done, save the file. The sample sheet is then ready to be imported for sequencing on an Illumina sequencer.

2.2 Library Preparation

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Sample (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| Tagmentation Buffer | -15°C to -25°C  BOX 1 | 10 | Bring to room temperature. |
| Sterile Water | 15°C to 30°C  User supplied | 30 | No preparation required. |
| Tagmentation Beads | -15°C to -25°C  BOX 1 | 10 | **Bring to room temperature, at least 30 mins.** |
| Stop Buffer | 15°C to 30°C  BOX 2 | 10 | No preparation required. |
| Tagmentation Wash Buffer | 15°C to 30°C  BOX 2 | 300 | No preparation required. |
| PCR Mix (or PCR Mix-1 for 96 kit) | -15°C to -25°C  BOX 4 (or Box 1 for PCR Mix-1) | 20 | Thaw and keep on ice.  **Return to storage after use for subsequent steps.** |
| Indices:  **ASTX17.1(24)-IVD:** H503, H505, H506, H517, H705, H706, H707, H710, H711, H714  **ASTX17.1(24)-B-IVD:** H502, H507, H508, H521, H701, H702, H703, H704, H712, H715  **ASTX17.1(96)-A-IVD/ASTX9.1(96)-A-IVD:** H503, H505, H506, H517, H502, H507, H508, H521, H705, H706, H707, H710, H711, H714, H701, H702, H703, H704, H712, H715  **ASTX17.1(96)-B-IVD/ASTX9.1(96)-B-IVD:** H510, H511, H513, H522, H515, H516, H518, H520, H716, H718, H719, H720, H721, H722, H723, H724, H726, H727, H728, H729 | -15°C to -25°C  BOX 3 | 10 total | Thaw and keep on ice. |

1. Vortex and pulse-spin all reagents before use.
2. Gather the required 96-Well Half-Skirted Plate, Microseal B and 1.5 mL microcentrifuge tubes.

**NOTE:** There is no safe stopping point until the indexing PCR. Process takes approximately **1:50** hours (including 50 min PCR thermocycling).

1. For each DNA sample, aliquot 10 μL of 10-100 ng/μL sample into the appropriate well of a PCR plate according to plate layout on 1.0 Sample\_Prep sheet.
2. Prepare 40 µL Tagmentation Master Mix per sample using; 10 µL Tagmentation Buffer, 20 µL Sterile Water and 10 µL Tagmentation Beads.
3. Mix the above master mix by vortexing and then pulse-spin.
4. Pipette 40 μL of the Tagmentation Master Mix into each well that contains a DNA sample.
5. Seal the plate with Microseal B film.
6. Centrifuge the plate at 100 x g for 10 seconds to collect all reagents at the bottom of the well.
7. Use plate shaker to mix at 1800 rpm for 1 minute.
8. Visually inspect the plate:

a) If the beads are not evenly distributed in the well, repeat shaking as per step 10,

b) If material is not in the bottom of the well or has splashed onto the Microseal B film, pulse-spin and repeat shaking step 10.

1. Place plate in thermocycler and run the Tagmentation program using lid heated to 105°C, and reaction volume 50 µL:

| **Temperature** | **Time** |
| --- | --- |
| 55°C | 5 minutes |
| 10°C | 2 minutes |

1. Once the program has finished, immediately remove plate from thermocycler and leave at room temperature for 2 minutes.
2. Remove Microseal B film.
3. Add 10 μL of Stop Buffer to each reaction well.
4. Re-seal the plate with new Microseal B film.
5. Use plate shaker to mix at 1800 rpm for 1 minute.
6. Incubate the plate for an additional 5 minutes at room temperature.
7. During incubation remove PCR Mix (or PCR Mix-1 if using 96 kits) and Indexes from the freezer to thaw, and then place on ice/cold rack.
8. Visually inspect the plate, if material is not at the bottom of the well or has splashed on to the Microseal B film, pulse-spin the plate.
9. Wash three times with Tagmentation Wash Buffer as described below:

a) Remove Microseal B and place the plate on Magnetic Stand-96 for 30 seconds, allowing beads to collect in wells alongside magnet,

b) Using a pipette, aspirate and discard supernatant, leaving the beads in the wells alongside the magnet,

c) Slowly add 100 μL of Tagmentation Wash Buffer to each well,

d) Re-seal the plate with new Microseal B film, ensure that the film is properly attached,

e) Use plate shaker to mix at 1800 rpm for 2 minutes at room temperature,

f) **CAUTION:** If samples have splashed onto the seal, centrifuge at 100 x g for 10 secs before removing the seal,

g) Repeat steps a) through f) two more times for a total of 3 washes.

**NOTE:** Before discarding the supernatant of the third wash, prepare the PCR master mix as described below.

1. Prepare 40 µL PCR master mix using 20 µL Sterile Water and 20 µL PCR Mix. (or PCR Mix-1 if using 96 test kits).

**NOTE:** PCR Mix is used for subsequent steps in the protocol. Do not discard this vial.

1. Remove Microseal B and place the plate on Magnetic Stand-96 for 30 seconds, allowing beads to collect in wells alongside magnet.
2. Using a pipette set to 100 μL, aspirate and discard the supernatant from the final Tagmentation Wash.
3. Remove the plate from the magnetic stand.
4. Add 40 μL of the PCR master mix to each well.
5. Seal the plate with Microseal B film.
6. Use plate shaker to mix at 1800 rpm for 2 minutes at room temperature.
7. Centrifuge the plate at 100 x g for 10 seconds to ensure all beads are suspended within the PCR master mix.

**For index tubes (T),**

1. (T) Vortex and pulse-spin index tubes to ensure all volume is at the bottom of the tube.
2. (T) Remove the Microseal B film from the sample plate.
3. (T) Add 5 µL of the i7 index to each well, according to the plate layout on 1.0 Sample\_Prep sheet.
4. (T) Add 5 µL of the i5 index to each well, according to the plate layout on 1.0 Sample\_Prep sheet.

Resume to step 34.

**For index plate (P),**

30. (P) Use a plate shaker to mix the index plate at 1800 rpm for 1 minute.

31. (P) Centrifuge the index plate at 100 x g for 10 seconds to ensure all volume is at the bottom of the well.

32. (P) Remove the Microseal B film from the sample plate.

33. (P) **Confirm the correct orientation of the plate and correct index set. Do not peel the foil seal.** Pierce the foil seal of the index plate with a tip. With a new tip, transfer 10 µL of the combined indices from the index plate to each sample well, according to the plate layout on 1.0 Sample\_Prep sheet.

Resume to step 34.

1. Seal with new Microseal B film.
2. Use plate shaker to mix at 1800 rpm for 1 minute at room temperature.
3. Centrifuge the plate at 100 x g for 10 seconds to collect all reagents at the bottom of the well. Visually inspect to ensure beads are still evenly distributed in solution. If beads aren't evenly distributed, repeat shaking as per step 35.
4. Place plate in thermocycler, run Indexing PCR program using lid heated to 105°C and reaction volume of 50 µL:

| **#** | **Step** | **Temperature** | **Time** | **Number of Cycles** |
| --- | --- | --- | --- | --- |
| 1 | Gap fill | 72°C | 3 minutes | 1 |
| 2 | Initial denature | 98°C | 3 minutes | 1 |
| 3 | Denaturation | 98°C | 20 seconds | 9 |
| 4 | Annealing | 60°C | 30 seconds |
| 5 | Extension | 72°C | 3 minutes |
| 6 | Final extension | 72°C | 3 minutes | 1 |
| 7 | Final hold | 10°C | Hold | 1 |

**NOTE:** Safe stopping point. Libraries may be stored at -15°C to -25°C for up to 1 week.

**NOTE:** If proceeding immediately to size selection and purification, ensure Purification Beads are brought to room temperature before use.

2.3 Size Selection and Purification

1. For optimal yield and size selection, the sample, bead and supernatant volume are varied according to run size. The table below outlines the volumes tested for the specified range of samples per run.

| **Reagent Volumes for Calculations** | **6 - 24  Samples/Pool** | **25 - 48 Samples/Pool** | **49 - 96 Samples/Pool** |
| --- | --- | --- | --- |
| No. of samples to be pooled (max. listed for ranges) | 24 | 48 | 96 |
| Volume of library to be pooled per sample (µL) | 10 | 5 | 2.5 |
| Volume of diluted beads per sample (µL) | 50 | 25 | 12.5 |
| Transfer volume of supernatant per sample (µL) | 55 | 27.5 | 13.75 |
| Volume of neat beads per sample, step 8 (µL) | 4.4 | 2.2 | 1.1 |

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Sample (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| Purification Beads (Purification Beads – 1 for 96 test kits) | 2°C to 8°C  BOX 5 | 24.7 | Bring to room temperature, at least 30 mins.  **Return to storage after use for subsequent steps.** |
| Sterile Water | 15°C to 30°C  User supplied | Varied | No preparation required. |
| 100% Ethanol | 15°C to 30°C  User supplied | 1920 | Prepare 80% ethanol, as described below. |
| Resuspension Buffer | 2°C to 8°C  BOX 5 | Varied | Bring to room temperature.  **Return to storage after use for subsequent steps.** |

1. Prepare diluted Purification Beads with Purification Beads and Sterile Water using volumes calculated by *IFU095-5\_AlloSeq Tx Early Pooling Workbook CE IVD***.** Ensure Purification Beads are thoroughly vortexed before use.
2. Prepare 2400 µL fresh 80% Ethanol per pool, enough for 2 washes, using 1920 µL 100% Ethanol and 480 µL Sterile Water.

**NOTE:** The number of pools per experiment is preset to 1 in the yellow cell in step 3 in the workbook. This can be manually overridden if required. Modification to this cell will update the number of pools for subsequent steps of this protocol/workbook. If more than 1 pool are to be performed on this experiment with the same number of samples per pool, the volume of Purification Beads should be increased accordingly (i.e., doubled for two pools), and the instructions below should be performed in full for each pool.

If multiple pools with different number of samples are required, this tab in the workbook should be duplicated and the cells in yellow updated to reflect the number of samples, so that the volumes of reagent required/transferred are correctly adjusted. In order to duplicate the tab, right click on any of the tabs and select 'Move or Copy', select '3.0 SS\_Purification' from the list, check the 'Create Copy' box and click 'OK'.

1. Vortex and pulse-spin all reagents before use.
2. Gather 1.5 mL tubes (**Optional:** 2mL tubes can be used if preferred, with increase of ethanol wash volume from 1200 µL to 1500 µL in step 20a below).

**NOTE:** Process takes approximately **1** hour.

1. Aliquot appropriate volume (see calculations table above or workbook) of diluted Purification Beads into the 1.5 mL tube.
2. Pipette mix the Tagmentation beads and supernatant from indexing PCR **or** shake the indexing PCR plate for 1 minute at 1800 rpm, and then add appropriate volume of each sample (see calculations table above or workbook) to tube containing diluted Purification Beads.
3. Vortex each tube at high speed for 10 seconds until the sample appears homogenous upon visual inspection.
4. Incubate at room temperature for 5 minutes. During this incubation, the largest fragments bind to the beads.
5. Quickly pulse-spin tube.
6. Place tube on magnet for 2.5 minutes, allowing beads to collect alongside the magnet. If the supernatant remains turbid remain on magnet until clear.
7. **Transfer** appropriate volume (see calculations table above or workbook) of the supernatant to a new tube.
8. Add appropriate volume (see calculations table above or workbook) of Purification Beads (undiluted) to the tube containing the supernatant.
9. Vortex each tube at high speed for 10 seconds.
10. Incubate at room temperature for 5 minutes. During this incubation, the target sized fragments bind to the beads.
11. Quickly pulse-spin tube.
12. Place tube on magnet for 2.5 minutes, allowing beads to collect alongside the magnet. If the supernatant remains turbid remain on magnet until clear.
13. Using a pipette, aspirate, and discard supernatant, leaving the beads in the tube alongside the magnet.
14. Keeping the tube on the magnet, wash twice with 80% ethanol:

a) Add 1200 µL of 80% ethanol to each tube,

b) Incubate at room temperature for 30 seconds,

c) Using a pipette, aspirate and discard all supernatant,

d) Repeat steps a) through c) for a total of 2 washes.

1. Remove all remaining supernatant with P20 pipette.
2. Air dry the tube for 5 minutes at room temperature, to allow residual ethanol to evaporate.
3. Take the tube off the magnet and add 37 µL of Resuspension Buffer to each tube to elute target fragments.
4. Vortex each tube at high speed for 10 seconds.
5. Incubate at room temperature for 5 minutes.
6. Quickly pulse-spin tube.
7. Place tube on magnet for 30 seconds, allowing beads to collect in tube alongside magnet.
8. **Transfer** 35 µL of supernatant to a new 1.5 mL tube for storage. This pool can continue through to Hybridization.

**NOTE:** Safe stopping point. Libraries may be stored at -15°C to -25°C for up to 1 month.

2.4 Qubit Quantification (Optional)

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Pool (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| Qubit BR buffer | -25°C to 30°C User supplied | 199 | No preparation required. |
| Qubit BR dye | -25°C to 30°C User supplied | 1 | No preparation required. |
| BR Standard #1 | 2°C to 8°C User supplied | 10 | Bring to room temperature. |
| BR Standard #2 | 2°C to 8°C User supplied | 10 | Bring to room temperature. |

1. Gather the required Qubit tubes and 1.5 mL or 5 mL tube for working solution preparation, depending on volume required.
2. Set up two assay tubes for the standards and one for each pool.
3. Prepare the 200 µL Qubit working solution using 199 µL Qubit buffer and 1 µL Qubit dye per pool/standard to be quantified.
4. Vortex working solution for 2-3 seconds, and then pulse-spin.
5. Aliquot **190 μL** of working solution into each of the Standard tubes.
6. Aliquot **198 μL** of working solution into each of the pool tubes.
7. Aliquot **10 μL** of Standard solution into each of the respective Standard tubes.
8. Aliquot **2 μL** of each pool into the respective tube.
9. Vortex all tubes for 2-3 seconds, and then pulse-spin.
10. Incubate the tubes for 2 minutes at room temperature.
11. Insert the tubes in the Qubit Fluorometer and take readings (see Qubit manufacturer’s protocol for further information).
12. Record Qubit readings in the table in the workbook to calculate the per pool average.

**NOTE:** Expected library yield is approximately 30-100 ng/L but may vary dependent on DNA quality and input. A yield of 10 ng/L or greater is expected to provide satisfactory enrichment results.

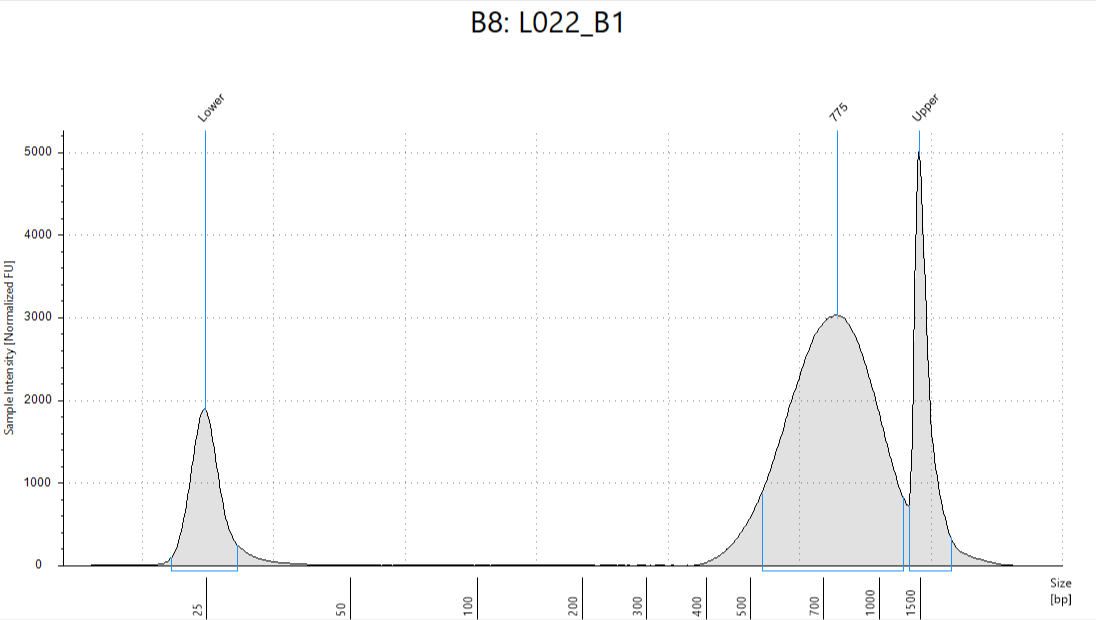
2.5 TapeStation Visualisation (Optional)

**NOTE:** Alternative systems for fragment visualisation such as Fragment Analyzer, Bioanalyzer or similar may be used following user validation.

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Sample (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| D1000 Ladder | 2°C to 8°C User supplied | 1 | Bring to room temperature. |
| D1000 Sample Buffer | 2°C to 8°C User supplied | 3 | Bring to room temperature. |

1. Gather the required D1000 ScreenTape, TapeStation optical tube strips and caps.
2. Transfer 1 μL of each pre-enriched library to a new tube.
3. Add 1 μL of D1000 Ladder into a reference tube.
4. Add 3 μL of D1000 sample buffer to each pre-enriched library tube and reference tube.
5. Seal all tubes with caps.
6. Vortex all tubes thoroughly using IKA vortex at 2000 rpm for 1 minute.
7. Centrifuge briefly to ensure that all samples are at the bottom of the tubes.
8. Uncap and load sample tubes into the 2200 TapeStation instrument.
9. Select the required tubes on the 2200 TapeStation Controller Software and run the samples (see TapeStation user manual for further information).
10. Once the run is complete, start TapeStation Analysis Software to view results (see TapeStation user manual for further information).
11. Record results in workbook.



**Figure 2.5.1**: Representative image of TapeStation trace for libraries.

1. Hybrid Capture (Early Pooling Workflow)

3.0 Protocol Introduction

* Follow the AlloSeq Tx protocol below in the order shown using the specified parameters.
* Before proceeding, confirm kit contents and make sure that you have the required consumables and equipment.
* For ease of use, protocol steps for Hybrid Capture are also detailed in *IFU095-5\_AlloSeq Tx Early Pooling Workbook CE IVD*. References to the workbook in Chapter 3 pertain to this workbook.

3.1 Probe Hybridisation

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Pool (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| AlloSeq Tx Probe Panel | -15°C to -25°C BOX 4 | 10 | Bring to room temperature. |
| Hybridisation Buffer 1 | -15°C to -25°C BOX 4 | 50 | Place in Hybex at 58°C for 15 minutes. Vortex and visually inspect, if precipitate remains then incubate at 58°C for another 15 minutes. |
| Hybridisation Buffer 2 | 2°C to 8°C BOX 5 | 10 | Bring to room temperature. |

1. Vortex and pulse-spin all reagents before use.
2. Gather the required PCR tubes/strips and caps.

**NOTE:** There is no safe stopping point until after the capture protocol. Sample pools must proceed straight from the 62°C hold step of the hybridisation thermocycling reaction to the bead capture and heated wash steps.

**NOTE:** Process takes approximately 20 minutes to set up, and minimum of 1.5 hours and maximum of 18 hours in thermocycler (reactions left overnight, or up to 18 hours, must be held at a temperature of 62°C at the final hold step of reaction).

1. For each hybridisation reaction, combine the following reagents in the order listed below into a PCR tube/strip:

| **Reagent** | **Volume per Pool (µL)** |
| --- | --- |
| Pool of Sample Libraries | 30 |
| AlloSeq Tx Probe Panel | 10 |
| Hybridisation Buffer 1 | 50 |
| Hybridisation Buffer 2 | 10 |
| Total | 100 |

1. With a pipette set to 70 μL, pipette mix each hybridisation reaction well 10 times, cap and then pulse-spin.
2. If the solution remains cloudy, pipette mix a further 6-8 times, cap and then pulse spin
3. Place tube/strip in thermocycler and run the Hybridisation program using lid heated to **100°C**, and reaction volume of 100 µL:

| **#** | **Step** | **Temperature** | **Time** | **No. of Cycles** |
| --- | --- | --- | --- | --- |
| 1 | Denaturation | 98°C | 5 minutes | 1 |
| 2 | Ramp Down | 98°C - 62°C, decreasing 2°C/cycle | 1 minute | 1 |
| 3 | Go to step 2 for 18 more cycles (total of 19 cycles) decreasing 2°C/cycle. | | | |
| 4 | Hybridisation | 62°C | **60 minutes** | 1 |
| 5 | Final Hold | 62°C | Hold (do not exceed 18 hours at 62°C, inclusive of step #4) | 1 |

1. Leave the tube/strip in the thermocycler until ready to proceed with the capture. Ensure capture beads have reached room temperature, and that Capture Wash Buffer and Hybex are heated to 58°C.

3.2 Capture

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Pool (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| Capture Beads | 2°C to 8°C BOX 5 | 250 | **Bring to room temperature, at least 30 mins.** |
| Capture Wash Buffer | -15°C to -25°C BOX 4 | 800 | **Pre-warm to 58 ͦC before use.** |
| Capture Elution Buffer 1 | -15°C to -25°C BOX 4 | 28.5 | Bring to room temperature. |
| 2N NaOH | -15°C to -25°C BOX 4 | 1.5 | Bring to room temperature.  **Return to storage after use for subsequent steps.** |
| Capture Elution Buffer 2 | 2°C to 8°C BOX 5 | 4 | Bring to room temperature. |

1. Prepare an elution master mix of the following reagents per pool to be captured:

| **Reagent** | **Volume per Pool (µL)** |
| --- | --- |
| Capture Elution Buffer 1 | 28.5 |
| 2N NaOH (fresh) | 1.5 |

**NOTE:** NaOH solution will readily absorb CO2 from the atmosphere, altering the pH and performance of the reagent. Ensure the 2N NaOH tube is sealed when not in use.

1. Vortex and pulse-spin all reagents before use.
2. Gather the required 1.5 mL microcentrifuge tubes, PCR tubes/strips and caps.

**NOTE:** This process takes approximately **1** hour.

1. For each hybridisation reaction, add 250 μL of Capture Beads to a fresh 1.5 mL tube.
2. **Transfer** 100 μL of each hybridisation reaction to the respective tube containing Capture Beads.
3. Vortex tube at high speed for 10 seconds. Do not centrifuge or pulse-spin.
4. Incubate tube at 58°C in Hybex for 15 minutes.
5. Quickly pulse-spin tube.
6. Immediately place tube on magnet for 30 seconds, allowing beads to collect alongside magnet.
7. Using a pipette, aspirate and discard supernatant, leaving the beads in the tube alongside the magnet.
8. Wash three times with heated Capture Wash Buffer as described below:

**NOTE:** When not in use, keep the Capture Wash Buffer in the Hybex to maintain 58°C temperature. Only remove from the Hybex immediately before adding to reaction in step 12b and 14. Work quickly when performing the heated wash steps to minimise the time the sample pool/buffer are at room temperature.

1. Remove tube from the magnet,
2. Add 200 μL of heated Capture Wash Buffer (58°C),
3. Vortex tube at high speed for 10 seconds. Do not centrifuge or pulse-spin.
4. Incubate tube at 58°C in Hybex for 5 minutes.
5. Pulse-spin and then immediately place tube on magnet for 30 seconds, allowing beads to collect alongside magnet.
6. Using a pipette, aspirate and discard supernatant, leaving the beads in the tube alongside the magnet.
7. Repeat steps a) through f) two more times for a total of 3 washes.
8. Remove tube from the magnet.
9. Add 200 μL of heated Capture Wash Buffer (58°C).
10. Vortex tube at high speed for 10 seconds. Do not centrifuge or pulse-spin.
11. **Transfer** the entire contents (wash solution and beads) to a new 1.5 mL tube.

**NOTE:** This transfer step is critical to remove PCR inhibitors which may impact downstream performance.

1. Incubate tube at 58°C in Hybex for 5 minutes.
2. Immediately pulse-spin and then place tube on magnet for 30 seconds, allowing beads to collect alongside magnet.
3. Using a pipette, aspirate and discard supernatant, leaving the beads in the tube alongside the magnet.
4. Quickly pulse-spin tube, and then immediately place tube on magnet for 30 seconds, allowing beads to collect alongside magnet.
5. Using a P20 pipette, aspirate and discard any remaining supernatant, leaving the beads in the tube alongside the magnet.
6. Vortex the elution master mix prepared above, and then take the reaction tube off the magnet and add 23 μL of elution master mix to each tube.
7. Vortex tube at high speed for 10 seconds.
8. Incubate at room temperature for 2 minutes.
9. Quickly pulse-spin tube.
10. Place tube on magnet for 30 seconds, allowing beads to collect alongside magnet.
11. **Transfer** 21 μL of supernatant to a new PCR tube/strip.
12. Add 4 μL of Capture Elution Buffer 2.
13. Pipette mix 6-8 times. Final volume is 25 μL.

**NOTE:** Safe stopping point. Libraries may be stored at -15°C to -25°C for up to 24 hours.

3.3 Post Enrichment PCR

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Pool (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| PCR Primers | -15°C to -25°C BOX 4 | 5 | Thaw on ice. Invert to mix, then centrifuge briefly. |
| PCR Mix (or PCR Mix–2 for 96 test kits) | -15°C to -25°C BOX 4 | 20 | Thaw at room temperature and then place on ice. |

1. Vortex and pulse-spin all reagents before use.
2. Gather the required PCR tubes containing capture libraries from step 3.2 Capture.

**NOTE:** This process takes approximately 5 minutes to set up, **1:40** hours in thermocycler.

1. Add 5 μL of PCR Primers to the captured libraries in the PCR tube.
2. Add 20 μL of PCR Mix (or PCR Mix-2 if using 96 kits) to the tube.
3. Pipette mix 10 times.
4. Quickly pulse-spin tube.
5. Place tube/strip in thermocycler and run the post enrichment PCR program using lid heated to 105°C, and reaction volume of 50 µL:

| **#** | **Step** | **Temperature** | **Time** | **Number of Cycles** |
| --- | --- | --- | --- | --- |
| 1 | Denaturation | 98°C | 30 seconds | 1 |
| 2 | Denaturation | 98°C | 1 minute | 17 |
| 3 | Annealing | 60°C | 30 seconds |
| 4 | Extension | 72°C | 3 minutes |
| 5 | Final extension | 72°C | 5 minutes | 1 |
| 6 | Final hold | 10°C | Hold | 1 |

**NOTE:** Safe stopping point. Libraries may be stored at -15°C to -25°C for up to 1 week.

**NOTE:** If proceeding immediately to purification, ensure Purification Beads are brought to room temperature before use.

3.4 Post Enrichment PCR Purification

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Pool (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| Purification Beads  (Purification Beads-2 for 96 kit) | 2°C to 8°C BOX 5 | 27 | Bring to room temperature, at least 30 mins. |
| Sterile Water | 15°C to 30°C User supplied | 80 | No preparation required. |
| 100% Ethanol | 15°C to 30°C User supplied | 320 | Prepare 80% ethanol, as described below. |
| Resuspension Buffer | 2°C to 8°C BOX 5 | 32 | Bring to room temperature.  **Return to storage after use for subsequent steps.** |

1. Prepare fresh 80% ethanol (2 washes per pool) using 480µL 100% Ethanol and 120µL Sterile Water (an excess volume is included).
2. Vortex and pulse-spin all reagents before use.
3. Gather the required 1.5 mL microcentrifuge tubes.

**NOTE:** Process takes approximately **30** minutes.

1. For each purification reaction, add 27 μL of thoroughly vortexed Purification Beads to a fresh 1.5 mL tube.
2. **Transfer** 45 μL of each post-enrichment PCR reaction to the respective tube containing Purification Beads.
3. Vortex each tube at high speed for 10 seconds.
4. Quickly pulse-spin tube.
5. Incubate at room temperature for 5 minutes.
6. Place tube on magnet for 30 seconds, allowing beads to collect alongside magnet.
7. Using a pipette, aspirate and discard supernatant, leaving the beads in the tube alongside the magnet.
8. Keeping the tube on the magnet, wash twice with 80% ethanol:
9. Add 200 μL of 80% ethanol to each tube,
10. Incubate at room temperature for 30 seconds,
11. Using a pipette, aspirate and discard all supernatant,
12. Repeat steps a) through c) for a total of 2 washes.
13. Remove all remaining supernatant with P20 pipette.
14. Air dry the tube for 5 minutes at room temperature, to allow residual ethanol to evaporate.
15. Take the tube off the magnet and add 32 μL of Resuspension Buffer to each tube to elute target fragments.
16. Vortex each tube at high speed for 10 seconds.
17. Incubate at room temperature for 5 minutes.
18. Quickly pulse-spin tube.
19. Place tube on magnet for 30 seconds, allowing beads to collect in tube alongside magnet.
20. **Transfer** 30 μL of supernatant to a new 1.5 mL tube for storage.

**NOTE:** Safe stopping point. Libraries may be stored at -15°C to -25°C for up to 1 month.

3.5 Qubit Quantification

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Pool (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| Qubit BR buffer | -25°C to 30°C User supplied | 199 | No preparation required. |
| Qubit BR dye | -25°C to 30°C User supplied | 1 | No preparation required. |
| BR Standard #1 | 2°C to 8°C User supplied | 10 | Bring to room temperature. |
| BR Standard #2 | 2°C to 8°C User supplied | 10 | Bring to room temperature. |

1. Gather the required Qubit tubes and 1.5 mL or 5 mL tube for working solution preparation, depending on volume required.
2. Set up two assay tubes for the standards and one for each pool.
3. Prepare the 200 µL Qubit working solution using 199 µL Qubit buffer and 1 µL Qubit dye per pool/standard to be quantified.
4. Vortex working solution for 2-3 seconds, and then pulse-spin.
5. Aliquot **190 μL** of working solution into each of the Standard tubes.
6. Aliquot **198 μL** of working solution into each of the Pool tubes.
7. Aliquot **10 μL** of Standard solution into each of the respective Standard tubes.
8. Aliquot **2 μL** of each pool into the respective tube.
9. Vortex all tubes for 2-3 seconds, and then pulse-spin.
10. Incubate the tubes for 2 minutes at room temperature.
11. Insert the tubes in the Qubit Fluorometer and take readings (see Qubit manufacturer’s protocol for further information).
12. Record Qubit readings in the table in the workbook to calculate average concentration.

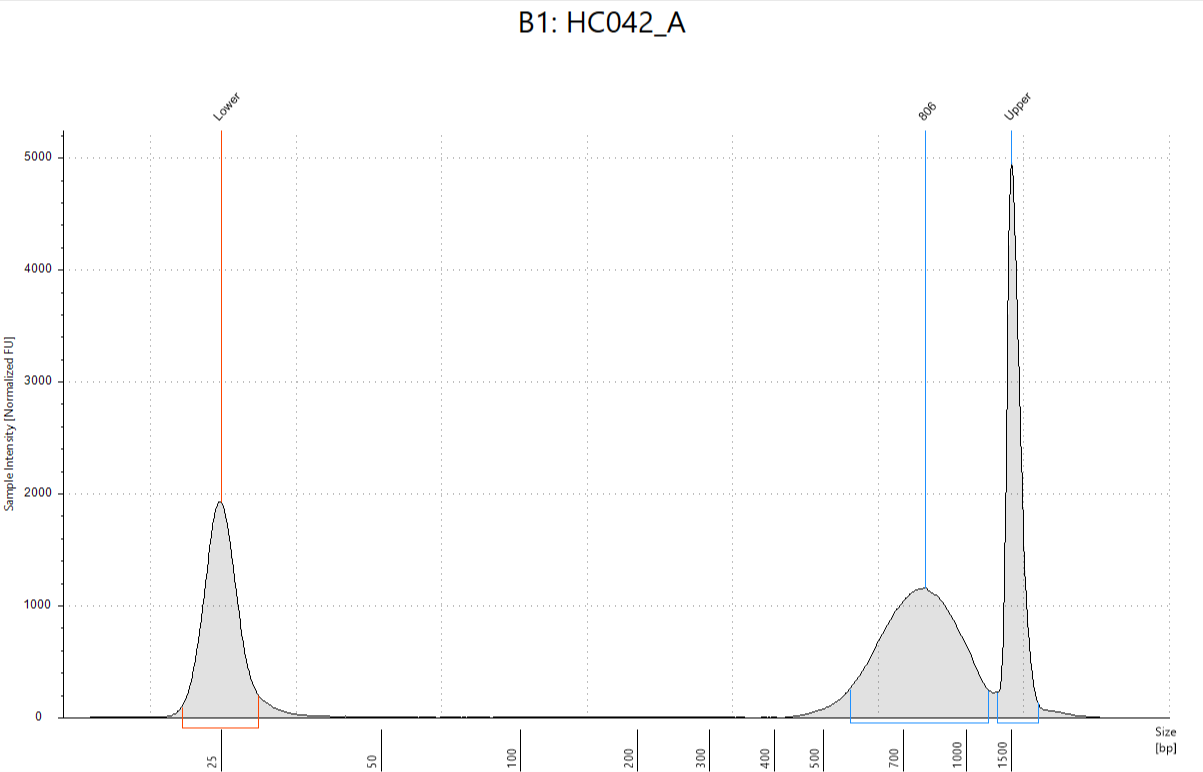
3.6 TapeStation Visualisation (Optional)

**NOTE:** Alternative systems for fragment visualisation such as Fragment Analyzer, Bioanalyzer or similar may be used following user validation.

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Pool (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| D1000 Ladder | 2°C to 8°C User supplied | 1 | Bring to room temperature. |
| D1000 Sample Buffer | 2°C to 8°C User supplied | 3 | Bring to room temperature. |

1. Gather the required D1000 ScreenTape, TapeStation optical tube strips and caps.
2. Transfer 1 μL of each pool to a new tube.
3. Add 1 μL of D1000 Ladder into a reference tube.
4. Add 3 μL of D1000 sample buffer to each pool tube and reference tube.
5. Seal all tubes with caps.
6. Vortex all tubes thoroughly using IKA vortex at 2000 rpm for 1 minute.
7. Centrifuge briefly to ensure that all samples are at the bottom of the tubes.
8. Uncap and load sample tubes into the 2200 TapeStation instrument.
9. Select the required tubes on the 2200 TapeStation Controller Software and run the samples (see TapeStation user manual for further information).
10. Once the run is complete, start TapeStation Analysis Software to view results (see TapeStation user manual for further information).
11. Record results in the table in the workbook.



**Figure 3.6.1**: Representative image of TapeStation trace for final enriched library pool.

1. Library Preparation (Original workflow)

4.0 Protocol Introduction

* Follow the AlloSeq Tx protocol below in the order shown using the specified parameters.
* Before proceeding, confirm kit contents and make sure that you have the required consumables and equipment.
* For ease of use, protocol steps for Library Preparation are also detailed in *IFU095-1\_AlloSeq Tx Library Preparation Workbook CE IVD*. References to the workbook in Chapter 4 pertain to this workbook.

4.1 Sample Preparation

1. Enter an experiment ID, description, operator and the date into the workbook.
2. Select the AlloSeq probe set to be used for the experiment from the drop-down menu in the workbook.
3. Select the sequencer type to be used from the drop-down menu in the workbook.
4. Enter the ID of samples to be tested in the yellow section of the plate layout in the workbook, according to the desired configuration.

**NOTE:** Only alphanumeric characters can be entered. Duplicate sample IDs will be flagged in red to prompt user to correct. It is a requirement of the sequencer software to have only unique sample IDs on a run. Do not enter any information for wells which are not intended to contain any sample.

1. Select the desired index set from the orange dropdown under Plate Layout in the workbook.
2. If required and using the tubed index format, the i7 index order can be modified, select the alternative i7 index to be used from the drop-down options for the required column. If duplicate i7 indexes are selected, then the cells will be flagged in red to prompt the user to correct.
3. If required and using the tubed index format, the i5 index order can be modified, select the alternative i5 index to be used from the drop-down options for the required row. If duplicate i5 indexes are selected, then the cells will be flagged in red to prompt the user to correct.
4. Once all the actions above have been completed, click on the 1.2 SampleSheet tab and review. Red text will be used to flag where information is still required. If no red text is evident then the SampleSheet tab can be saved as a CSV (Comma delimited) (\*.csv). Save the file as 'SampleSheet.csv'. Saving in a csv format will only save the active tab. Open the saved SampleSheet.csv file in excel and delete any empty rows in the [Data] table, that is, any rows between 22 to 117 which do not contain sample information. Once this has been done, save the file. The sample sheet is then ready to be imported for sequencing on an Illumina sequencer.

4.2 Library Preparation

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Sample (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| Tagmentation Buffer | -15°C to -25°C  BOX 1 | 10 | Bring to room temperature. |
| Sterile Water | 15°C to 30°C  User supplied | 30 | No preparation required. |
| Tagmentation Beads | -15°C to -25°C  BOX 1 | 10 | **Bring to room temperature, at least 30 mins.** |
| Stop Buffer | 15°C to 30°C  BOX 2 | 10 | No preparation required. |
| Tagmentation Wash Buffer | 15°C to 30°C  BOX 2 | 300 | No preparation required. |
| PCR Mix (or PCR Mix-1 for 96 kits) | -15°C to -25°C  BOX 4 (or BOX 1 for 96 kit) | 20 | Thaw and keep on ice.  **Return to storage after use for subsequent steps.** |
| Indexes:  **ASTX17.1(24)-IVD:** H503, H505, H506, H517, H705, H706, H707, H710, H711, H714  **ASTX17.1(24)-B-IVD:** H502, H507, H508, H521, H701, H702, H703, H704, H712, H715  **ASTX17.1(96)-A-IVD/ASTX9.1(96)-A-IVD:** H503, H505, H506, H517, H502, H507, H508, H521, H705, H706, H707, H710, H711, H714, H701, H702, H703, H704, H712, H715  **ASTX17.1(96)-B-IVD/ASTX9.1(96)-B-IVD:** H510, H511, H513, H522, H515, H516, H518, H520, H716, H718, H719, H720, H721, H722, H723, H724, H726, H727, H728, H729 | -15°C to -25°C  BOX 3 | 10 total | Thaw and keep on ice. |

1. Vortex and pulse-spin all reagents before use.
2. Gather the required 96-Well Half-Skirted Plate, Microseal B and 1.5 mL microcentrifuge tubes.

**NOTE:** There is no safe stopping point until the indexing PCR. Process takes approximately **1:50** hours (including 50 min PCR thermocycling).

1. For each DNA sample, aliquot 10 μL of 10-100 ng/μL sample into the appropriate well of a PCR plate according to plate layout on 1.0 Sample\_Prep sheet.
2. Prepare 40 µL Tagmentation Master Mix per sample using; 10 µL Tagmentation Buffer, 20 µL Sterile Water and 10 µL Tagmentation Beads.
3. Mix the above master mix by vortexing and then pulse-spin.
4. Pipette 40 μL of the Tagmentation Master Mix into each well that contains a DNA sample.
5. Seal the plate with Microseal B film.
6. Centrifuge the plate at 100 x g for 10 seconds to collect all reagents at the bottom of the well.
7. Use plate shaker to mix at 1600 rpm for 1 minute.
8. Visually inspect the plate:

a) If the beads are not evenly distributed in the well, repeat shaking as per step 10,

b) If material is not in the bottom of the well or has splashed onto the Microseal B film, pulse-spin and repeat shaking step 10.

1. Place plate in thermocycler and run the Tagmentation program using lid heated to 105°C, and reaction volume 50 µL:

| **Temperature** | **Time** |
| --- | --- |
| 55°C | 5 minutes |
| 10°C | 2 minutes |

1. Once the program has finished, immediately remove plate from thermocycler and leave at room temperature for 2 minutes.
2. Remove Microseal B film.
3. Add 10 μL of Stop Buffer to each reaction well.
4. Re-seal the plate with new Microseal B film.
5. Use plate shaker to mix at 1600 rpm for 1 minute.
6. Incubate the plate for an additional 5 minutes at room temperature.
7. During incubation remove PCR Mix (or PCR Mix-1 if using 96 kits) and Indexes from the freezer to thaw, and then place on ice/cold rack.
8. Visually inspect the plate, if material is not at the bottom of the well or has splashed on to the Microseal B film, pulse-spin the plate.
9. Wash three times with Tagmentation Wash Buffer as described below:

a) Remove Microseal B and place the plate on Magnetic Stand-96 for 1 minute, allowing beads to collect in wells alongside the magnet,

b) Using a pipette, aspirate and discard supernatant, leaving the beads in the wells alongside the magnet,

c) Slowly add 100 μL of Tagmentation Wash Buffer to each well,

d) Re-seal the plate with new Microseal B film, ensure that the film is properly attached,

e) Use plate shaker to mix at 1800 rpm for 2 minutes at room temperature,

f) **CAUTION:** If samples have splashed onto the seal, centrifuge at 100 x g for 10 seconds before removing the seal,

g) Repeat steps a) through f) two more times for a total of 3 washes.

**NOTE:** Before discarding the supernatant of the third wash, prepare the PCR master mix as described below.

1. Prepare 40 µL PCR master mix using 20 µL Sterile Water and 20 µL PCR Mix (or PCR Mix-1 if using 96 test kits).

**NOTE:** PCR Mix is used for subsequent steps in the protocol. Do not discard this vial.

1. Remove Microseal B and place the plate on Magnetic Stand-96 for 1 minute, allowing beads to collect in wells alongside the magnet.
2. Using a pipette set to 100 μL, aspirate and discard the supernatant from the final Tagmentation Wash.
3. Remove the plate from the magnetic stand.
4. Add 40 μL of the PCR master mix to each well.
5. Seal the plate with Microseal B film.
6. Use plate shaker to mix at 1800 rpm for 2 minutes at room temperature.
7. Centrifuge the plate at 100 x g for 10 seconds to ensure all beads are suspended within the PCR master mix.

**For index tubes (T),**

1. (T) Vortex and pulse-spin index tubes to ensure all volume is at the bottom of the tube.
2. (T) Remove the Microseal B film from the sample plate.
3. (T) Add 5 µL of the i7 index to each well, according to the plate layout on 1.0 Sample\_Prep sheet.
4. (T) Add 5 µL of the i5 index to each well, according to the plate layout on 1.0 Sample\_Prep sheet.

Resume to step 34.

**For index plate (P),**

30. (P) Use a plate shaker to mix the index plate at 1800 rpm for 1 minute.

31. (P) Centrifuge the index plate at 100 x g for 10 seconds to ensure all volume is at the bottom of the well.

32. (P) Remove the Microseal B film from the sample plate.

33. (P) **Confirm the correct orientation of the plate and correct index set**. **Do not peel the foil seal.** Pierce the foil seal of the index plate with a tip. With a new tip, transfer 10 µL of the combined indices from the index plate to each sample well, according to the plate layout on 1.0 Sample\_Prep sheet.

Resume to step 34.

1. Seal with new Microseal B film.
2. Use plate shaker to mix at 1800 rpm for 1 minute at room temperature.
3. Centrifuge the plate at 100 x g for 10 seconds to collect all reagents at the bottom of the well. Visually inspect to ensure beads are still evenly distributed in solution. If beads aren't evenly distributed, repeat shaking as per step 35.
4. Place plate in thermocycler, run Indexing PCR program using lid heated to 105°C and reaction volume of 50 µL:

| **#** | **Step** | **Temperature** | **Time** | **Number of Cycles** |
| --- | --- | --- | --- | --- |
| 1 | Gap fill | 72°C | 3 minutes | 1 |
| 2 | Initial denature | 98°C | 3 minutes | 1 |
| 3 | Denaturation | 98°C | 20 seconds | 9 |
| 4 | Annealing | 60°C | 30 seconds |
| 5 | Extension | 72°C | 3 minutes |
| 6 | Final extension | 72°C | 3 minutes | 1 |
| 7 | Final hold | 10°C | Hold | 1 |

**NOTE:** Safe stopping point. Libraries may be stored at -15°C to -25°C for up to 1 week.

**NOTE:** If proceeding immediately to size selection and purification, ensure Purification Beads are brought to room temperature before use.

4.3 Size Selection and Purification

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Sample (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| Purification Beads (Purification Beads – 1 for 96 test kits) | 2°C to 8°C  BOX 5 | 110.8 | Bring to room temperature, at least 30 mins.  **Return to storage after use for subsequent steps.** |
| Sterile Water | 15°C to 30°C  User supplied | 215 | No preparation required. |
| 100% Ethanol | 15°C to 30°C  User supplied | 320 | Prepare 80% ethanol, as described below. |
| Resuspension Buffer | 2°C to 8°C  BOX 5 | 17 | Bring to room temperature.  **Return to storage after use for subsequent steps.** |

1. Prepare diluted Purification Beads using 90 µL Purification Beads and 135 µLSterile Water, per sample. Ensure Purification Beads are thoroughly vortexed before use.
2. Prepare 600 µL fresh 80% Ethanol per sample, enough for 2 washes, using 480µL 100% Ethanol and 120µL Sterile Water (an excess volume is included).
3. Vortex and pulse-spin all reagents before use.
4. Gather the required MIDI plate, Microseal B and PCR plates.

**NOTE:** Process takes approximately **1** hour.

1. Aliquot 225 μL of diluted Purification Beads into the appropriate wells of a MIDI plate (according to plate layout on 1.0 Sample\_Prep sheet).
2. Pipette mix the tagmentation beads and supernatant from indexing PCR **or** shake the indexing PCR plate for 1 minute at 1800 rpm, and then add 45 µL of each mixture to the relevant well of the MIDI plate containing diluted Purification Beads.
3. Seal MIDI plate with Microseal B film.
4. Use plate shaker to mix at 1800 rpm for 2 minutes.
5. Incubate plate for 5 minutes at room temperature. During this incubation the largest fragments bind to the beads.
6. Centrifuge plate at 280 x g for 1 minute.
7. Remove Microseal B and place the plate on Magnetic Stand-96 for 5 minutes, allowing beads to collect in wells alongside magnet.
8. **Transfer 260 μL of the supernatant to a new MIDI plate or clean wells in the same plate.**
9. Add 20.8 μL of thoroughly vortexed Purification Beads (undiluted) to each sample. Seal with Microseal B film.
10. Use plate shaker to mix at 1800 rpm for 1 minute.
11. Incubate plate for 5 minutes at room temperature. During this incubation the target sized fragments bind to the beads.
12. Remove Microseal B and place the plate on Magnetic Stand-96 for 5 minutes, allowing beads to collect in wells alongside magnet.
13. Using a pipette, aspirate and discard supernatant, leaving the beads in the wells alongside the magnet.
14. Keeping the plate on the magnet, wash twice with 80% ethanol:

a) Add 200 μL of 80% ethanol to each sample,

b) Incubate at room temperature for 30 seconds,

c) Using a pipette, aspirate and discard all supernatant,

d) Repeat steps a) through c) for a total of 2 washes.

1. Remove all remaining supernatant with P20 pipette.
2. Air dry the plate for 5 minutes at room temperature, to allow residual ethanol to evaporate.
3. Add 17 μL of Resuspension Buffer to each well to elute target fragments.
4. Seal plate with Microseal B film.
5. Use plate shaker to mix at 1800 rpm for 2 minutes.
6. Incubate plate for 5 minutes at room temperature.
7. Centrifuge plate at 280 x g for 30 seconds.
8. Remove Microseal B and place the plate on Magnetic Stand-96 for 5 minutes, allowing beads to collect in wells alongside magnet.
9. **Transfer** 15 μL of supernatant to a new PCR plate for storage.

**NOTE:** Safe stopping point. Libraries may be stored at -15°C to -25°C for up to 1 month.

4.4 Qubit Quantification (Optional)

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Sample (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| Qubit BR buffer | -25°C to 30°C  User supplied | 199 | No preparation required. |
| Qubit BR dye | -25°C to 30°C  User supplied | 1 | No preparation required. |
| BR Standard #1 | 2°C to 8°C  User supplied | 10 | Bring to room temperature. |
| BR Standard #2 | 2°C to 8°C  User supplied | 10 | Bring to room temperature. |

1. Gather the required Qubit tubes and 1.5 mL or 5 mL tube for working solution preparation, depending on volume required.
2. Set up two assay tubes for the standards and one for each sample.
3. Prepare the 200 µL Qubit working solution using 199 µL Qubit buffer and 1 µL Qubit dye per sample/standard to be quantified.
4. Vortex working solution for 2-3 seconds, and then pulse-spin.
5. Aliquot **190 μL** of working solution into each of the Standard tubes.
6. Aliquot **198 μL** of working solution into each of the Sample tubes.
7. Aliquot **10 μL** of Standard solution into each of the respective Standard tubes.
8. Aliquot **2 μL** of each sample into the respective tube.
9. Vortex all tubes for 2-3 seconds, and then pulse-spin.
10. Incubate the tubes for 2 minutes at room temperature.
11. Insert the tubes in the Qubit Fluorometer and take readings (see Qubit protocol for further information).
12. Record Qubit readings in the table in the workbook to calculate the per sample average.

**NOTE:** Expected library yield is approximately 30 ng/L but may vary dependent on DNA quality and input. A yield of 10 ng/L or greater is expected to provide satisfactory enrichment results.

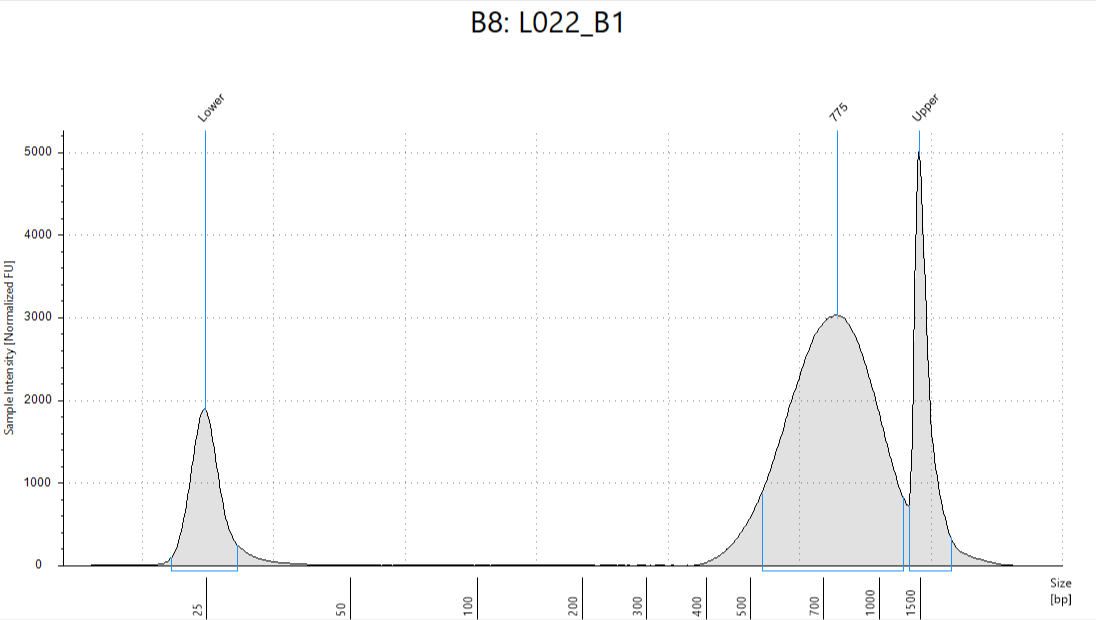
4.5 TapeStation Visualisation (Optional)

**NOTE:** Alternative systems for fragment visualisation such as Fragment Analyzer, Bioanalyzer or similar may be used following user validation.

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Sample (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| D1000 Ladder | 2°C to 8°C User supplied | 1 | Bring to room temperature. |
| D1000 Sample Buffer | 2°C to 8°C User supplied | 3 | Bring to room temperature. |

1. Gather the required D1000 ScreenTape, 96-well Sample Plate (thin-walled) and Foil seal.
2. Transfer 1 μL of each pre-enriched library to a new 96-well PCR plate.
3. Add 1 μL of D1000 Ladder into a reference well.
4. Add 3 μL of D1000 sample buffer to each pre-enriched library well and reference well.
5. Seal the plate with foil seal.
6. Vortex using IKA vortex at 2000 rpm for 1 minute.
7. Centrifuge briefly to ensure that all samples are at the bottom of the wells.
8. Load sample plate into the 2200 TapeStation instrument.
9. Select the required wells on the 2200 TapeStation Controller Software and run the samples (see TapeStation user manual for further information).
10. Once the run is complete, start TapeStation Analysis Software to view results (see TapeStation user manual for further information).
11. Record results in workbook.



**Figure 4.5.1**: Representative image of TapeStation trace for libraries.

1. Hybrid Capture (Original workflow)

5.0 Protocol Introduction

* Follow the AlloSeq Tx protocol below in the order shown using the specified parameters.
* Before proceeding, confirm kit contents and make sure that you have the required consumables and equipment.
* For ease of use, protocol steps for Hybrid Capture are also detailed in *IFU095-2\_AlloSeq Tx Hybrid Capture Workbook CE IVD*. References to the workbook in Chapter 5 pertain to this workbook.

5.1 Sample Pooling

1. Enter an experiment ID into the appropriate yellow field of the workbook.
2. Enter the number of pools to be processed in the appropriate yellow field of the workbook.
3. Copy the appropriate sample information from the 1.1 LinearView tab of *IFU095-1\_AlloSeq Tx Library Preparation Workbook CE IVD* and paste into the Library Pool List in the *IFU095-2\_AlloSeq Tx Hybrid Capture Workbook CE IVD* using the paste special "Values & Number Formatting" option.
4. If pooling ≤ 12 samples, add 2.5 μL of each library to be enriched into a PCR tube/strip, and add the appropriate volume of Resuspension Buffer to total 30 μL, according to table below.
5. If pooling > 12 samples, add 2.5 μL of each library to be enriched into a 1.5 mL microcentrifuge tube, and proceed to concentration step (1.1 of workbook *IFU095-2\_AlloSeq Tx Hybrid Capture Workbook CE IVD*).
6. If processing multiple pools, duplicate this tab and follow steps 1-5 above, uniquely identifying each pool.

**NOTE:** Lower yield libraries, may benefit from using a larger library input volume (not exceeding the 30 L combined total library input volume). Processing of low yield libraries in a separate enrichment pool from higher yield (genomic) libraries is recommended, where possible. For specific advice contact your local technical representative.

5.2 Library Pool Concentration (Optional)

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Pool (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| Purification Beads (Purification Beads-1 for 96 test kits) | 2°C to 8°C BOX 5 | 58.5 - 108 | Bring to room temperature, at least 30 mins.  **Return to storage after use for subsequent steps.** |
| Sterile Water | 15°C to 30°C  User supplied | 480 | No preparation required. |
| 100% Ethanol | 15°C to 30°C  User supplied | 1,920 | Prepare 80% ethanol, as described below. |
| Resuspension Buffer | 2°C to 8°C BOX 5 | 32 | Bring to room temperature. **Return to storage after use for subsequent steps.** |

1. Prepare fresh 80% ethanol (2 washes per sample) using 1920 µL 100% Ethanol and 480 µL Sterile Water (an excess volume is included).
2. Vortex and pulse-spin all reagents before use.
3. Gather the required 1.5 mL microcentrifuge tubes.

**NOTE:** Process takes approximately 30 minutes.

1. Pool sample libraries, in a 1.5 mL tube, according to 1.0 Sample Pooling workbook instructions.
2. Add appropriate volume (1.8x pooled sample volume or see calculations in workbook) of thoroughly vortexed Purification Beads to the 1.5 mL tube containing pooled libraries.
3. Vortex each tube at high speed for 10 seconds, 3 times.
4. Quickly pulse-spin tube.
5. Incubate at room temperature for 5 minutes.
6. Place tube on magnet for 1 minute (up to 2.5 minutes for 96 sample libraries), allowing beads to collect alongside magnet. If the supernatant remains turbid remain on magnet until clear.
7. Using a pipette, aspirate and discard supernatant, leaving the beads in the tube alongside the magnet.
8. Keeping the tube on the magnet, wash twice with 80% ethanol:
9. Add 800 μL of 80% ethanol to each tube,
10. Incubate at room temperature for 30 seconds,
11. Using a pipette, aspirate and discard all supernatant,
12. Repeat steps a) through c) for a total of 2 washes.
13. Remove all remaining supernatant with P20 pipette.
14. Air dry the tube for 5 minutes at room temperature, to allow residual ethanol to evaporate.
15. Add 32 μL of Resuspension Buffer to tube to elute libraries.
16. Vortex each tube at high speed for 10 seconds, 3 times.
17. Incubate at room temperature for 5 minutes.
18. Quickly pulse-spin tube.
19. Place tubes on magnet for 1 minute, allowing beads to collect in tubes alongside magnet.
20. **Transfer** 30 μL of supernatant to a new PCR tube/strip for hybridisation.

**NOTE:** Safe stopping point. Libraries may be stored at -15°C to -25°C for up to 1 week.

5.3 Probe Hybridisation

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Pool (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| AlloSeq Tx Probe Panel | -15°C to -25°C BOX 4 | 10 | Bring to room temperature. |
| Hybridisation Buffer 1 | -15°C to -25°C BOX 4 | 50 | Place in Hybex at 50°C for 15 minutes. Vortex and visually inspect; if precipitate remains then incubate at 50°C for another 15 minutes. |
| Hybridisation Buffer 2 | 2°C to 8°C BOX 5 | 10 | Bring to room temperature. |

1. Vortex and pulse-spin all reagents before use.
2. Gather the required PCR tubes/strips and caps.

**NOTE:** There is no safe stopping point until after the capture protocol. Sample pools must proceed straight from the 62°C hold step of the hybridisation thermocycling reaction to the bead capture and heated wash steps.

**NOTE:** Process takes approximately 20 minutes to set up, and minimum of 2 hours and maximum of 18 hours in thermocycler (reactions left overnight, or up to 18 hours, must be held at a temperature of 62°C at the final hold step of reaction).

1. For each hybridisation reaction, combine the following reagents in the order listed below into a PCR tube/strip:

| **Reagent** | **Volume per Pool (µL)** |
| --- | --- |
| Pool of Sample Libraries | 30 |
| AlloSeq Tx Probe Panel | 10 |
| Hybridisation Buffer 1 | 50 |
| Hybridisation Buffer 2 | 10 |
| Total | 100 |

1. With a pipette set to 70 μL, pipette mix each hybridisation reaction well 10 times, seal and then pulse-spin.
2. If the solution remains cloudy, pipette mix a further 6-8 times.
3. Place tube/strip in thermocycler and run the Hybridisation program using lid heated to **100°C**, and reaction volume of 100 µL:

| **#** | **Step** | **Temperature** | **Time** | **No. of Cycles** |
| --- | --- | --- | --- | --- |
| 1 | Denaturation | 98°C | 5 minutes | 1 |
| 2 | Ramp Down | 98°C - 62°C, decreasing 2°C/cycle | 1 minute | 1 |
| 3 | Go to step 2 for 18 more cycles (total of 19 cycles) decreasing 2°C/cycle. | | | |
| 4 | Hybridisation | 62°C | 90 minutes | 1 |
| 5 | Final Hold | 62°C | Hold (do not exceed 18 hours at 62°C, inclusive of step #4) | 1 |

1. Leave the tube/strip in the thermocycler until ready to proceed with the capture. Ensure capture beads have reached room temperature, and that Capture Wash Buffer and Hybex are heated to 58°C.
2. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Pool (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| Capture Beads | 2°C to 8°C BOX 5 | 250 | **Bring to room temperature, at least 30 mins.** |
| Capture Wash Buffer | -15°C to -25°C BOX 4 | 800 | **Pre-warm to 58**°**C before use.** |
| Capture Elution Buffer 1 | -15°C to -25°C BOX 4 | 28.5 | Bring to room temperature. |
| 2N NaOH | -15°C to -25°C BOX 4 | 1.5 | Bring to room temperature.  **Return to storage after use for subsequent steps.** |
| Capture Elution Buffer 2 | 2°C to 8°C BOX 5 | 4 | Bring to room temperature. |

5.4 Capture

1. Prepare an elution master mix of the following reagents per pool to be captured:

| **Reagent** | **Volume per Pool (µL)** |
| --- | --- |
| Capture Elution Buffer 1 | 28.5 |
| 2N NaOH (fresh) | 1.5 |

**NOTE:** NaOH solution will readily absorb CO2 from the atmosphere, altering the pH and performance of the reagent. Ensure the 2N NaOH tube is sealed when not in use.

1. Vortex and pulse-spin all reagents before use.
2. Gather the required 1.5 mL microcentrifuge tubes, PCR tubes/strips and caps.

**NOTE:** This process takes approximately **1** hour.

1. For each hybridisation reaction, add 250 μL of Capture Beads to a fresh 1.5 mL tube.
2. **Transfer** 100 μL of each hybridisation reaction to the respective tube containing Capture Beads.
3. Vortex tube at high speed for 10 seconds, 3 times. Do not centrifuge or pulse-spin.
4. Incubate tube at 58°C in Hybex for 15 minutes.
5. Quickly pulse-spin tube.
6. Immediately place tube on magnet for 1 minute, allowing beads to collect alongside magnet.
7. Using a pipette, aspirate and discard supernatant, leaving the beads in the tube alongside the magnet.
8. Wash three times with heated Capture Wash Buffer as described below:

**NOTE:** When not in use, keep the Capture Wash Buffer in the Hybex to maintain 58°C temperature. Only remove immediately before adding to reaction in step 12b and 14. Work quickly when performing the heated wash steps to minimize the time the sample pool/buffer are at room temperature.

1. Remove tube from the magnet,
2. Add 200 μL of heated Capture Wash Buffer (58°C),
3. Vortex tube at high speed for 10 seconds, 3 times. Do not centrifuge or pulse-spin.
4. Incubate tube at 58°C in Hybex for 5 minutes.
5. Pulse-spin and then immediately place tube on 1.5mL magnet for 1 minute, allowing beads to collect alongside magnet.
6. Using a pipette, aspirate and discard supernatant, leaving the beads in the tube alongside the magnet.
7. Repeat steps a) through f) two more times for a total of 3 washes.
8. Remove tube from the magnet.
9. Add 200 μL of heated Capture Wash Buffer (58°C).
10. Vortex tube at high speed for 10 seconds, 3 times. Do not centrifuge or pulse-spin.
11. **Transfer** the entire contents (wash solution and beads) to a new 1.5 mL tube.

**NOTE:** This transfer step is critical to remove PCR inhibitors which may impact downstream performance.

1. Incubate tube at 58°C in Hybex for 5 minutes.
2. Immediately pulse-spin and then place tube on magnet for 1 minute, allowing beads to collect alongside magnet.
3. Using a pipette, aspirate and discard supernatant, leaving the beads in the tube alongside the magnet.
4. Quickly pulse-spin tube, and then immediately place tube on magnet for 1 minute, allowing beads to collect alongside magnet.
5. Using a P20 pipette, aspirate and discard any remaining supernatant, leaving the beads in the tube alongside the magnet.
6. Vortex the elution master mix prepared above, and then take the reaction tube off the magnet and add 23 μL of elution master mix to each tube.
7. Vortex tube at high speed for 10 seconds, 3 times.
8. Incubate at room temperature for 2 minutes.
9. Quickly pulse-spin tube.
10. Place tube on magnet for 1 minute, allowing beads to collect alongside magnet.
11. **Transfer** 21 μL of supernatant to a new PCR tube/strip.
12. Add 4 μL of Capture Elution Buffer 2.
13. Pipette mix 6-8 times. Final volume is 25 μL.

**NOTE:** Safe stopping point. Libraries may be stored at -15°C to -25°C for up to 24 hours.

5.5 Post Enrichment PCR

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Pool (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| PCR Primers | -15°C to -25°C BOX 4 | 5 | Thaw on ice. Invert to mix, then centrifuge briefly. |
| PCR Mix (or PCR Mix-2 for 96 kits) | -15°C to -25°C BOX 4 | 20 | Thaw at room temperature and then place on ice. |

1. Vortex and pulse-spin all reagents before use.
2. Gather the required PCR tubes containing capture libraries from step 5.4 Capture.

**NOTE:** This process takes approximately 5 minutes to set up, **1:40** hours in thermocycler.

1. Add 5 μL of PCR Primers to the captured libraries in the PCR tube.
2. Add 20 μL of PCR Mix (or PCR Mix-2 if using 96 kits) to the tube.
3. Pipette mix 10 times.
4. Quickly pulse-spin tube.
5. Place tube/strip in thermocycler and run the post-enrichment PCR program using lid heated to 105°C, and reaction volume of 50 µL:

| **#** | **Step** | **Temperature** | **Time** | **Number of Cycles** |
| --- | --- | --- | --- | --- |
| 1 | Denaturation | 98°C | 30 seconds | 1 |
| 2 | Denaturation | 98°C | 1 minute | 17 |
| 3 | Annealing | 60°C | 30 seconds |
| 4 | Extension | 72°C | 3 minutes |
| 5 | Final extension | 72°C | 5 minutes | 1 |
| 6 | Final hold | 10°C | Hold | 1 |

**NOTE:** Safe stopping point. Libraries may be stored at -15°C to -25°C for up to 1 week.

**NOTE:** If proceeding immediately to purification, ensure Purification Beads are brought to room temperature before use.

5.6 Post Enrichment PCR Purification

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Pool (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| Purification Beads  (Purification Beads-2 for 96 kit) | 2°C to 8°C BOX 5 | 27 | Bring to room temperature, at least 30 mins. |
| Sterile Water | 15°C to 30°C User supplied | 80 | No preparation required. |
| 100% Ethanol | 15°C to 30°C User supplied | 320 | Prepare 80% ethanol, as described below. |
| Resuspension Buffer | 2°C to 8°C BOX 5 | 32 | Bring to room temperature. **Return to storage after use for subsequent steps.** |

1. Prepare fresh 80% ethanol (2 washes per sample) using 480µL 100% Ethanol and 120µL Sterile Water (an excess volume is included).
2. Vortex and pulse-spin all reagents before use.
3. Gather the required 1.5 mL microcentrifuge tubes.

**NOTE:** Process takes approximately **30** minutes.

1. For each purification reaction, add 27 μL of thoroughly vortexed Purification Beads to a fresh 1.5 mL tube.
2. **Transfer** 45 μL of each post enrichment PCR reaction to the respective tube containing Purification Beads.
3. Vortex each tube at high speed for 10 seconds, 3 times.
4. Quickly pulse-spin tube.
5. Incubate at room temperature for 5 minutes.
6. Place tube on magnet for 1 minute, or until all beads have collected on magnet.
7. Using a pipette, aspirate and discard supernatant, leaving the beads in the tube alongside the magnet.
8. Keeping the tube on the magnet, wash twice with 80% ethanol:
9. Add 200 μL of 80% ethanol to each tube,
10. Incubate at room temperature for 30 seconds,
11. Using a pipette, aspirate and discard all supernatant,
12. Repeat steps a) through c) for a total of 2 washes.
13. Remove all remaining supernatant with P20 pipette.
14. Air dry the tube for 5 minutes at room temperature, to allow residual ethanol to evaporate.
15. Take the tube off the magnet and add 32 μL of Resuspension Buffer to each tube to elute target fragments.
16. Vortex each tube at high speed for 10 seconds, 3 times.
17. Incubate at room temperature for 5 minutes.
18. Quickly pulse-spin tube.
19. Place tubes on magnet for 1 minute, allowing beads to collect in tube alongside magnet.
20. **Transfer** 30 μL of supernatant to a new 1.5 mL tube for storage.

**NOTE:** Safe stopping point. Libraries may be stored at -15°C to -25°C for up to 1 month.

5.7 Qubit Quantification

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Pool (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| Qubit BR buffer | -25°C to 30°C User supplied | 199 | No preparation required. |
| Qubit BR dye | -25°C to 30°C User supplied | 1 | No preparation required. |
| BR Standard #1 | 2°C to 8°C User supplied | 10 | Bring to room temperature. |
| BR Standard #2 | 2°C to 8°C User supplied | 10 | Bring to room temperature. |

1. Gather the required Qubit tubes and 1.5 mL or 5 mL tube for working solution preparation, depending on volume required.
2. Set up two assay tubes for the standards and one for each pool.
3. Prepare the 200 µL Qubit working solution using 199 µL Qubit buffer and 1 µL Qubit dye per sample/standard to be quantified.
4. Vortex working solution for 2-3 seconds, and then pulse-spin.
5. Aliquot **190 μL** of working solution into each of the Standard tubes.
6. Aliquot **198 μL** of working solution into each of the Pool tubes.
7. Aliquot **10 μL** of Standard solution into each of the respective Standard tubes.
8. Aliquot **2 μL** of each pool into the respective tube.
9. Vortex all tubes for 2-3 seconds, and then pulse-spin.
10. Incubate the tubes for 2 minutes at room temperature.
11. Insert the tubes in the Qubit Fluorometer and take readings (see Qubit manufacturer’s protocol for further information).
12. Record Qubit readings in the table in the workbook to calculate average concentration.

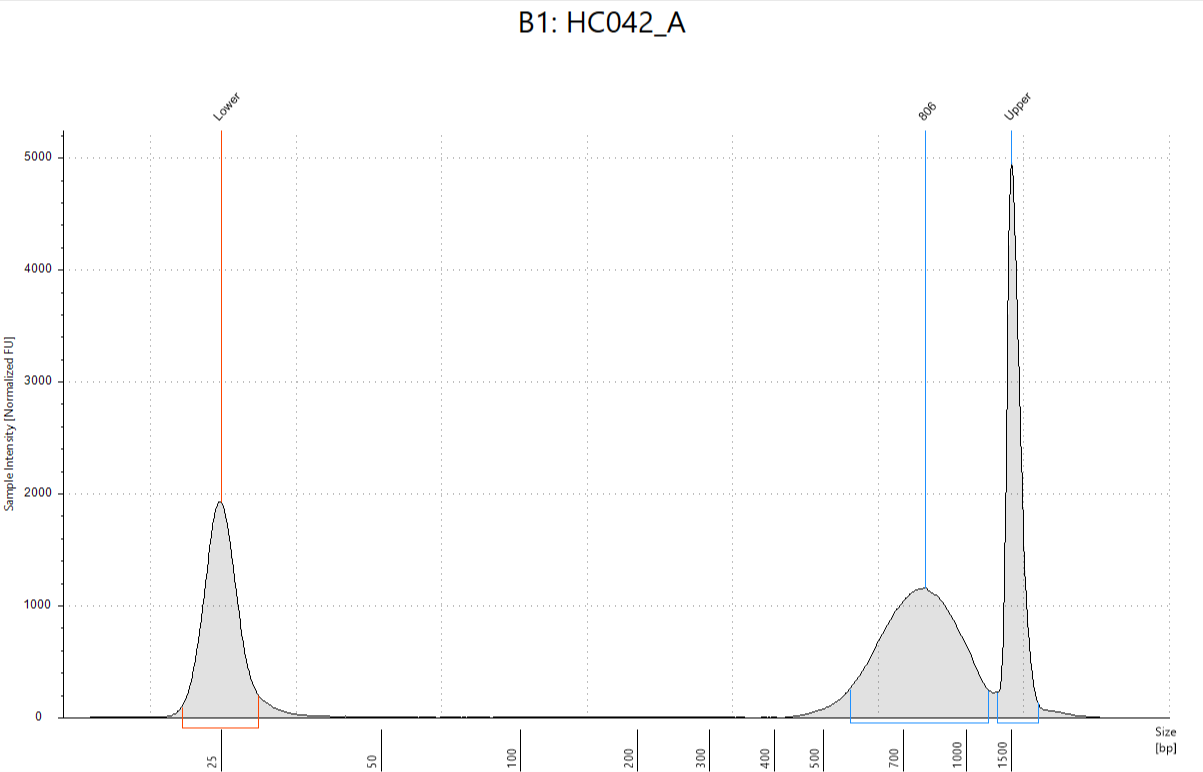
5.8 TapeStation Visualisation (Optional)

**NOTE:** Alternative systems for fragment visualisation such as Fragment Analyzer, Bioanalyzer or similar may be used following user validation.

1. Gather the required reagents (volume specified in table below) and prepare according to table:

| **Reagent** | **Storage Conditions** | **Volume per Pool (µL)** | **Preparation Required** |
| --- | --- | --- | --- |
| D1000 Ladder | 2°C to 8°C User supplied | 1 | Bring to room temperature. |
| D1000 Sample Buffer | 2°C to 8°C User supplied | 3 | Bring to room temperature. |

1. Gather the required D1000 ScreenTape, TapeStation optical tube strips and caps.
2. Transfer 1 μL of each pool to a new tube.
3. Add 1 μL of D1000 Ladder into a reference tube.
4. Add 3 μL of D1000 sample buffer to each pool tube and reference tube.
5. Seal all tubes with caps.
6. Vortex all tubes thoroughly using IKA vortex at 2000 rpm for 1 minute.
7. Centrifuge briefly to ensure that all samples are at the bottom of the tubes.
8. Uncap and load sample tubes into the 2200 TapeStation instrument.
9. Select the required tubes on the 2200 TapeStation Controller Software and run the samples (see TapeStation user manual for further information).
10. Once the run is complete, start TapeStation Analysis Software to view results (see TapeStation user manual for further information).
11. Record results in the table in the workbook.



**Figure 5.8.1**: Representative image of TapeStation trace for final enriched library pool.

1. Sequencing

6.0 Protocol Introduction

AlloSeq Tx libraries are validated for sequencing that is performed on Illumina sequencers including MiSeq, MiniSeq or iSeq, where the resulting sequence data is output in fastq file format. The number of samples added to each enriched pool will determine the necessary sequencer flow cell as listed in the *1.3 AlloSeq Tx Targeted Gene Content* section of this IFU.

* Follow the AlloSeq Tx protocol below in the order shown using the specified parameters.
* Before proceeding, confirm kit contents and make sure that you have the required consumables and equipment.
* For ease of use, protocol steps for Sequencing are also detailed in *IFU095-5\_AlloSeq Tx Early Pooling Workbook CE IVD (*Early Pooling Workflow) and *IFU095-3\_AlloSeq Tx Sequencing Workbook CE IVD* (Original Workflow). References to the workbook in Chapter 6 pertain to these workbooks*.*
* Sequencers should be loaded according to the instrument protocol, as indicated in the instructions below.
* If using a MiSeq instrument, it is recommended to perform the template line bleach (sodium hypochlorite) wash according to the MiSeq user guide instructions for optimal performance.

**NOTE:** The Illumina formatted sequencing samplesheet import .csv file may be generated and saved as described in *IFU095-5\_AlloSeq Tx Early Pooling Workbook CE IVD* (Worksheet “1.0 Sample\_Prep”), from “1.2 SampleSheet” *(*Early Pooling Workflow) or *IFU095-1\_AlloSeq Tx Library Preparation Workbook CE IVD* (Worksheet “1.0 Sample\_Prep”), from “1.2 SampleSheet” (Original Workflow).

6.1 PhiX Preparation

1. Gather the required reagents and prepare according to table:

| **Reagent** | **Storage Conditions** | **Preparation Required** |
| --- | --- | --- |
| 10 nM PhiX | -15°C to -25°C User supplied | Thaw and then keep on ice. |
| 2N NaOH | -15°C to -25°C BOX 4 | Bring to room temperature. |
| Sterile Water | 15°C to 30°C User supplied | No preparation required. |
| Resuspension Buffer | 2°C to 8°C BOX 5 | Bring to room temperature. |
| HT1 (with Sequencing cartridge) | -15°C to -25°C User supplied | Thaw and then keep on ice. |

1. Prepare 0.2 NaOH working solution using 45 µL Sterile Water and 5 µL 2N NaOH.

**NOTE:** NaOH solution will readily absorb CO2 from the atmosphere, altering the pH and performance of the reagent. Ensure the 2N NaOH tube is sealed when not in use.

1. Vortex and pulse-spin all reagents before use.
2. Gather the required 1.5 mL microcentrifuge tubes.

6.1.1 Dilute and Denature PhiX for MiSeq and MiniSeq Runs

1. Add 3 μL of Resuspension Buffer to a fresh tube.
2. Add 2 μL of 10 nM PhiX to the tube.
3. Add 5 μL of 0.2N NaOH working solution (as diluted above) to the tube.
4. Vortex and pulse-spin the tube.
5. Incubate at room temperature for 5 minutes.
6. Add 990 μL prechilled HT1 to the tube containing denatured PhiX.
7. **Invert to mix** (do NOT vortex) and then pulse-spin the tube.

**NOTE:** This results in 1 mL of 20 pM PhiX which is ready for use on MiSeq runs. For MiniSeq runs perform the additional dilution set to 5 pM described below. Denatured PhiX may be stored at -15°C to -25°C for up to 1 month.

6.1.2 For MiniSeq Runs, Further Dilute PhiX to 5 pM

1. Enter the desired dilution volume into the workbook in order to calculate the appropriate dilution for the PhiX.
2. Dilute PhiX to 5 pM by combining the volume of reagent (see workbook) in a microcentrifuge tube.
3. **Invert to mix** (do NOT vortex) and then pulse-spin the tube.

**NOTE:** Denatured PhiX may be stored at -15°C to -25°C for up to 1 month.

6.1.3 For iSeq Runs, Dilute PhiX to 20pM (Without Denaturing)

1. Enter the desired dilution volume into the workbook in order to calculate the appropriate dilution for the PhiX.
2. Dilute PhiX to 20 pM by combining the volume of reagent (see workbook) in a microcentrifuge tube.
3. **Invert to mix** (do NOT vortex) and then pulse-spin the tube.

**NOTE:** Denatured PhiX may be stored at -15°C to -25°C for up to 1 month.

6.2 Dilute and Denature for MiSeq

1. Gather the required reagents and prepare according to table:

| **Reagent** | **Storage Conditions** | **Preparation Required** |
| --- | --- | --- |
| AlloSeq Tx Enriched Sample Pool | -15°C to -25°C User prepared | Thaw and then keep on ice. |
| 2N NaOH | -15°C to -25°C BOX 4 | Bring to room temperature. |
| Sterile Water | 15°C to 30°C User supplied | No preparation required. |
| HT1 (with MiSeq cartridge) | -15°C to -25°C User supplied | Thaw and then keep on ice. |
| Resuspension Buffer | 2°C to 8°C BOX 5 | Bring to room temperature. |
| 20 pM PhiX (previously diluted) | -15°C to -25°C User supplied | Thaw and then keep on ice. |

1. Prepare 0.2N NaOH working solution using 45 µL Sterile Water and 5 µL 2N NaOH.

**NOTE:** NaOH solution will readily absorb CO2 from the atmosphere, altering the pH and performance of the reagent. Ensure the 2N NaOH tube is sealed when not in use.

1. Vortex and pulse-spin all reagents before use.
2. Gather the required 1.5 mL microcentrifuge tubes.
3. Enter the enriched sample pool concentration, fragment size (expect approximately 800 bp) and desired total volume (recommended 30 µL) at 4 nM into the workbook in order to calculate the appropriate dilution for the library.
4. Dilute the sample pool to 4 nM by combining the reagents (see workbook) in a microcentrifuge tube.
5. Vortex and pulse-spin diluted sample pool before further use.
6. Add 5 μL of 4 nM enriched sample pool to a fresh tube.
7. Add 5 μL of 0.2N NaOH working solution (as diluted above) to the tube.
8. Vortex and pulse-spin the tube.
9. Incubate at room temperature for 5 minutes.
10. Add 990 μL prechilled HT1 to the tube containing denatured enriched sample pool.
11. **Invert to mix** (do NOT vortex) and then pulse-spin the tube.

**NOTE:** This results in 1 ml of 20 pM sample pool. Pools may be stored at -15°C to -25°C for up to 1 month.

1. Enter the number of pools to be combined for sequencing, loading concentration (recommended 12pM), desired % PhiX spike in (recommended 1% minimum) and loading volume (minimum of 600 μL) into the workbook, in order to calculate the appropriate dilution for the library.
2. Dilute the sample pool to loading concentration by combining the reagents (see workbook) in a microcentrifuge tube.
3. **Invert to mix** (do NOT vortex) and then pulse-spin the tube.
4. Set aside on ice until ready to be loaded onto the MiSeq reagent cartridge for sequencing.

**NOTE:** Pools may be stored at -15°C to -25°C for up to 48 hours prior to sequencing.

1. Proceed to load MiSeq according to instrument protocol.

6.3 Dilute and Denature for MiniSeq

1. Gather the required reagents and prepare according to table:

| **Reagent** | **Storage Conditions** | **Preparation Required** |
| --- | --- | --- |
| AlloSeq Tx Enriched Sample Pool | -15°C to -25°C User prepared | Thaw and then keep on ice. |
| 2N NaOH | -15°C to -25°C BOX 4 | Bring to room temperature. |
| Sterile Water | 15°C to 30°C User supplied | No preparation required. |
| HT1 (with sequencing cartridge) | -15°C to -25°C User supplied | Thaw and then keep on ice. |
| Resuspension Buffer | 2°C to 8°C BOX 5 | Bring to room temperature. |
| 200 mM Tris-HCl, pH 7.0 | 15°C to 30°C User supplied | No preparation required. |
| 5 pM PhiX (previously diluted) | -15°C to -25°C User supplied | Thaw and then keep on ice. |

1. Prepare 0.1N NaOH working solution using 95 µL Sterile Water and 5 µL 2N NaOH.

**NOTE:** NaOH solution will readily absorb CO2 from the atmosphere, altering the pH and performance of the reagent. Ensure the 2N NaOH tube is sealed when not in use.

1. Vortex and pulse-spin all reagents before use.
2. Gather the required 1.5 mL microcentrifuge tubes.
3. Enter the enriched sample pool concentration, fragment size (expect approximately 800 bp) and desired total volume (recommended 100 µL) at 1 nM into the workbook in order to calculate the appropriate dilution for the library.
4. Dilute the sample pool to 1 nM by combining the reagents (see workbook) in a microcentrifuge tube.
5. Vortex and pulse-spin diluted sample pool before further use.
6. Add 5 μL of 1 nM enriched sample pool to a fresh tube.
7. Add 5 μL of 0.1N NaOH working solution (as diluted above) to the tube.
8. Vortex and pulse-spin the tube.
9. Incubate at room temperature for 5 minutes.
10. Add 5 μL of 200 mM Tris-HCl, at pH 7.0.
11. Vortex and pulse-spin the tube.
12. Add 985 μL prechilled HT1 to the tube containing denatured enriched sample pool.
13. **Invert to mix** (do NOT vortex) and then pulse-spin the tube.

**NOTE:** This results in 1 mL of 5 pM sample pool. Pools may be stored at -15°C to -25°C for up to 1 month.

1. Enter the number of pools to be combined for sequencing, loading concentration (recommended 1.6 pM), desired % PhiX spike in (recommended 1% minimum) and loading volume (minimum of 500 μL) into the workbook, in order to calculate the appropriate dilution for the library.
2. Dilute the sample pool to loading concentration by combining the reagents (see workbook) in a microcentrifuge tube.
3. **Invert to mix** (do NOT vortex) and then pulse-spin the tube.
4. Set aside on ice until ready to be loaded onto the MiniSeq reagent cartridge for sequencing.

**NOTE:** Pools may be stored at -15°C to -25°C for up to 48 hours prior to sequencing.

1. Proceed to load MiniSeq according to instrument protocol.

6.4 Dilute for iSeq

1. Gather the required reagents and prepare according to table:

| **Reagent** | **Storage Conditions** | **Preparation Required** |
| --- | --- | --- |
| AlloSeq Tx Enriched Sample Pool | -15°C to -25°C User prepared | Thaw and then keep on ice. |
| Resuspension Buffer | 2°C to 8°C BOX 5 | Bring to room temperature. |
| 20 pM PhiX (previously diluted) | -15°C to -25°C User supplied | Thaw and then keep on ice. |

1. Vortex and pulse-spin all reagents before use.
2. Gather the required 1.5 mL microcentrifuge tubes.
3. Enter the enriched sample pool concentration, fragment size (expect approximately 800 bp) and desired total volume (recommended 100 µL) at 1 nM into the workbook, in order to calculate the appropriate dilution for the library.
4. Dilute the sample pool to 1 nM by combining the reagents (see workbook) in a microcentrifuge tube.
5. Vortex and pulse-spin diluted sample pool before further use.
6. Enter the number of pools to be combined for sequencing, loading concentration (recommended 200 pM), desired % PhiX spike in (recommended 1% minimum) and loading volume (minimum of 100 μL) into the workbook in order to calculate the appropriate dilution for the library.
7. Dilute the sample pool to loading concentration by combining the reagents (see workbook) in a microcentrifuge tube.
8. **Invert to mix** (do NOT vortex) and then pulse-spin the tube.
9. Set aside on ice until ready to be loaded onto the iSeq reagent cartridge for sequencing.

**NOTE:** Pools may be stored at -15°C to -25°C for up to 48 hours prior to sequencing.

1. Proceed to load 20 µL of diluted sample pool into the iSeq cartridge according to instrument protocol.
2. Sequence Analysis

Resulting Fastq files are to be analysed using the AlloSeq Assign software. Procedures for use of the AlloSeq Assign software can be found in the *IFU094\_AlloSeq Assign IFU CE IVD*.

1. Troubleshooting Guide

| **PROBLEM** | **POSSIBLE CAUSE(S)** | **Solution** |
| --- | --- | --- |
| Low or no yield from library preparation (detected by Qubit quantification) | Poor quality or low concentration input DNA | Assess DNA quality by gel electrophoresis. Intact DNA should be approx. 3kb with little or no evidence of smearing on gel. Re-extract DNA and repeat procedure where possible. |
| Incorrect primary sample type used. | Avoid the use of whole blood specimens containing heparin. Re-extract DNA from ACD or EDTA preserved whole blood and repeat procedure, where possible. |
| Libraries lost during capture | Consult protocol. Ensure that Purification steps that retain supernatant; and steps that discard supernatant are correctly followed.  Ensure that ethanol concentration is correct. Using water only or excessive-water content may elute DNA prematurely. |
| Libraries not eluted from capture beads | Consult protocol. Ensure correct order of elution buffers employed.  Ensure adequate removal of reagents/buffers prior to resuspension and elution.  Avoid over-drying of DNA or bead-bound DNA pellets during drying steps. Prolonged drying of DNA pellets may impede resuspension and subsequent yield. |
| Failure to add critical reagent(s) (i.e. bead, index primer, PCR master mix etc.); or failure to use in correct order. | Consult protocol. Ensure reagents were added in the correct order and at the correct volume. Check residual reagent volume(s) for excessive remainder. Repeat procedure, if indicated. |
| Incorrect incubation or cycling conditions | Review thermal cycler conditions:  Tagmentation program  Indexing PCR program |
| Low or no yield from enrichment (detected by Qubit quantification) | Libraries lost during capture | Review protocol and ensure that steps requiring retained supernatant and those discarding supernatant are correctly followed.  Ensure that diluted Purification Beads are at the correct concentration; and that undiluted Purification Beads (not residual of diluted beads) are employed following size selection. |
| Libraries were not eluted from the capture beads | Ensure correct order of elution buffers employed.  Ensure adequate removal of reagents/buffers prior to resuspension and elution.  Avoid over-drying of DNA or bead-bound DNA pellets during drying steps. Prolonged drying of DNA pellets may reduce ability to resuspend solution and subsequent yield. |
| Incorrect incubation or cycling conditions | Review thermal cycler conditions:  Hybridisation program  Post enrichment PCR program |
| Incorrect sized fragments following library preparation or enrichment (detected via fragment analysis) | Incorrect ratio of sample to Purification Beads. | Repeat protocol. Ensure the correct bead concentration is used during the size selection and purification steps of the protocol. |
| Failed to sequence |  | Check that the protocol was followed.  Consult user respective manual for sequencer model used. |
| Low coverage for targeted loci in Assign despite high enrichment yield. | Non-specific fragments captured due to lower temperature during capture and heated wash steps. | Ensure that the Hybex used for heated wash steps is calibrated and maintained.  Work quickly during heated wash steps to ensure the temperature of the pool does not drop substantially. |

1. Supporting Information

The protocol described in this guide assumes that you have reviewed the contents of this section, confirmed and obtained all required consumables and equipment.

License

The AlloSeq Tx kits contain Nextera Flex for Enrichment reagents which are manufactured by Illumina Inc. for distribution by CareDx Pty Ltd.

Consumables and Equipment Required but Not Supplied

The consumables and equipment listed below are required for performing the assay but are not included in the AlloSeq Tx Kit.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

| **Consumable** | **Supplier/Catalog #** | |
| --- | --- | --- |
| PCR-grade water | Sigma-Aldrich, W3500 |
| Absolute ethanol for molecular biology | General lab supplier |
| Qubit™ dsDNA BR Assay Kit | Thermo Fisher Scientific, Q32850 or Q32853 |
| Qubit™ Assay Tubes | Thermo Fisher Scientific, Q32856 |
| D1000 ScreenTape (optional) | Agilent Technologies, 5067-5584 |
| D1000 Reagents (optional) | Agilent Technologies, 5067-5585 |
| 96-Well Sample Plates (optional) | Agilent Technologies, 5067-5150 |
| 96-well Plate Foil Seal (optional) | Agilent Technologies, 5067-5154 |
| Optical tube strips (8x Strip) (optional) | Agilent Technologies, 401428 |
| Optical tube strip caps (8x Strip) (optional) | Agilent Technologies, 401425 |
| **One of the following sequencing reagent kits:**   * MiSeq Reagent Kit v2 (300-cycles) * MiSeq Reagent Micro Kit v2 (300-cycles) * MiSeq Reagent Nano Kit v2 (300-cycles) * MiniSeq Mid Output Kit (300-cycles) * iSeq 100 i1 Reagent v2 (300 cycles) | * Illumina, MS-102-2002 * Illumina, MS-103-1002 * Illumina, MS-103-1001 * Illumina, FC-420-1004 * Illumina, 20031371 |
| PhiX Control v3 | Illumina, FC-110-3001 |
| 20μl barrier pipette tips | General lab supplier |
| 200μl barrier pipette tips | General lab supplier |
| 1000μl barrier pipette tips | General lab supplier |
| 1.5mL microcentrifuge tubes | General lab supplier |
| 5mL tubes | General lab supplier |
| 15mL conical centrifuge tubes | General lab supplier |
| 25mL reagent reservoirs | General lab supplier |
| 0.2mL PCR 8-tube strips with caps | General lab supplier |
| 96-well PCR Plates | General lab supplier |
| Microseal 'B' adhesive seals | Bio-Rad, MSB1001 |
| Abgene™ 96 Well 0.8mL Polypropylene Deepwell Storage Plate (MIDI plate, Original workflow only) | Thermo Fisher, AB0859 or AB0765 |
| Sodium hypochlorite (NaOCl) for post run sequencing wash (optional) | Sigma, 239305 |
|  |  |
| **Equipment** | **Supplier/Catalog #** | |
| 20μl pipette | General lab supplier | |
| 200μl pipette | General lab supplier | |
| 1000μl pipette | General lab supplier | |
| 20μl multichannel pipettes | General lab supplier | |
| 200μl multichannel pipettes | General lab supplier | |
| Microcentrifuge | General lab supplier | |
| Microplate centrifuge | General lab supplier | |
| Vortexer | General lab supplier | |
| Sealing film roller | General lab supplier | |
| Index Fixture Plate | Illumina, FC-130-1005 | |
| One of the following magnetic racks:   * DynaMag™-2 Magnet * MagJET Separation Rack, 2 x 1.5mL tube | Thermo Fisher, 12321D  Thermo Fisher, MR01 | |
| Magnetic Stand-96 | Thermo Fisher, AM10027 | |
| BioShake iQ, or  BioShake XP | BioShake iQ, 1808-0506  BioShake XP, 1808-0505 | |
| Agilent 2200 TapeStation System (optional) | Agilent Technologies | |
| Qubit Fluorometer | Thermo Fisher | |
| One of the following 96-well thermal cyclers:   * Veriti™ 96-Well Thermal Cycler * Mastercycler Pro S Thermal Cycler   Or other PCR thermal cycler with comparable performance. The minimum requirements of the alternate PCR thermal cycler include a heated lid function at 105°C for Indexing PCR program and a 0.2 mL tubes/96-well plate block format. | Applied Biosystems, 4375786  Eppendorf, 6325 | |
| Hybex System | SciGene, 1057-30-2 | |
| Hybex 1.5mL tube block. (32 x 1.5 ml tubes). | SciGene, 1057-34-0 | |
| One of the following sequencers:   * Illumina MiSeq * Illumina MiniSeq * Illumina iSeq | Illumina, SY-410-1003  Illumina, SY-420-1001  Illumina, 20021532 | |
|  |  | |

1. Contact Information

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**CH-REP:**

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CHRN: CHRN-AR-20002058

**Technical Support and Reporting serious incidents:**

Email: [techsupport-global@caredx.com](mailto:techsupport-global@caredx.com)

Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State or local Ministry of Health in which the user and/or the patient is established

For more information please refer to the CareDx website (<https://www.caredx.com/contact-us/>).

**Related Products:**

CE marked IVDs:

AlloSeq Assign

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3. Illumina Technical Note. **DNA/RNA isolation considerations for Illumina library preparation kits.** (12/21/18)
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**Method history**

| **Version** | **Date** | **Modification** | **Reference/ Justification** |
| --- | --- | --- | --- |
| 1.0 | 19Feb20 | First version of the AlloSeq Tx IFU CE. Issued by E. Naughton on 19Feb20 |  |
| 1.1 | 28Apr20 | Added the AlloSeq Tx assay sequences DNA fragments with an average size of 700bp, meaning that polymorphisms greater than 700bp apart cannot be phased, which may result in heterozygous ambiguities to the limitations section. Issued by E. Naughton 29Apr20 |  |
| 1.2 | 08May20 | Updated Interfering substances. Reissued by E. Naughton on 08May20 |  |
| 1.3 | 13May20 | Added Biotin to Interfering substances table. Added control sample details to section 1.9. Reissued by E. Naughton on 14May20 |  |
| 1.4 | 20May20 | Updated 1.4 AlloSeq Tx Kit Contents and Storage Requirements section with the following corrections: 2x Tubes for Tagmentation Wash Buffer, 2x Tubes for Capture Wash Buffer. Reissued by E. Naughton on 20May20 |  |
| 1.5 | 05Jun20 | Made the following changes:  - Corrected H714 in reagent table in section 2.2,  - Corrected reference to Hybrid Capture in the 3.0 section,  - Added reference to PhiX in Consumables  - Added reference to workbooks for point 3.1.5 and 3.2.5.  Reissued by E. Naughton on 05Jun20 |  |
| 1.6 | 20Oct20 | Added a note on NaOH usage. Reissued by E. Naughton on 20Oct20 |  |
| 2.0 | 26Mar21 | Updated distributor from Vienna to Stockholm in section 8.0 as per CR 2020-097. Updated iSeq reagent v1 as discontinued & replaced with v2 in section 7.0 as per CR 2020-077. Corrected 25 to 12 freeze thaw cycles in section 1.4. Reissued by S. Antony on 09Apr2021 |  |
| 3.0 | 6May21 | Updated the following:   * Section 1: Supporting workbooks section moved to section 9; IFU095-5 added. Included explanation regarding the Original vs Early Pooling workflow. Added ASTX17.1(24)-B-IVD targeted gene content. Updated formatting of Tx kit contents table. Added buccal swab reference (RUO). Corrected limit of detection to reflect DNA not DNA concentration. Added H318 warning for 2N NaOH, H351 and H373 hazard warnings for Capture Beads, H351 warning for Hybridisation Buffer 1; added Stop Buffer safety information to table. * Section 2 – added Library Preparation (Early Pooling workflow) section * Section 3 – added Hybrid Capture (Early Pooling workflow) section * Section 4.1 – updated description of sample preparation to align with workbook * Section 4.2 – added Set B indices in table, updated time estimate from 2:45 hours to 1:50 hours, changed centrifuge step from 280 x g for 30 seconds to 100 x g for 10 seconds step 9, 21f, changed centrifuge step from 280 x g for 1 minute to 100 x g for 10 seconds step 29, changed centrifuge step from 280 x g for 10 seconds to 100 x g for 10 seconds, step 36, added comment ‘repeat shaking as per step 35’ at step 36 * Sections 4.4/5.7 – included Broad Range (BR) clarification in all Qubit reagent tables * Section 5.3 - Included clarification comment, "Hold (do not exceed 18 hours at 62°C, inclusive of step #4)" in hybridisation program table * Section 5.8 – corrected TapeStation plate/seal references to tube/caps * Section 6.0 – removed table. This table is in Section 1.1 * Section 6.2 – desired MiSeq total volume changed from 100uL to 30uL to match the workbook * Section 6.4 – comment to clarify only 20uL of 100uL diluted pool is loaded onto the iSeq * Section 9 – added “Original workflow only” disclaimer on MIDI plate, added Sodium hypochlorite (NaOCl) for post run sequencing wash * Section 9 – Changed MiSeq reagent names to align with Illumina ordering details. Added MiSeq Reagent Kit v3 (600-cycle) for AlloSeq Tx 8, added Qubit tube and TapeStation tube/cap/plate/foil ordering information   General: Added ASTX17.1(24)-B-IVD on cover page. Added “**Return to storage after use for subsequent steps.”** Recommendations for reagents that need to be retained for subsequent steps, minor formatting, grammar, punctuation and spelling mistakes correct throughout the document. Added additional guidance to ‘thoroughly vortex’ all instances of neat Purification Bead use following field team feedback. Corrected all instances of ‘hybex’ to ‘Hybex’ and ‘QuBit’ to ‘Qubit’ and ‘pulse spin’ to ‘pulse-spin’. Updated Section 2.2 & 4.2 in table for Stop Buffer and corrected Preparation Required to ‘No preparation required’. Added “imported by” and symbol as per ISO 15223-1-2021 and IVDR. Reissued by L.Langley 13 Oct 21 |  |
| 4.0 | N/A | Version 4.0 not issued. |  |
| 5.0 | 28Jan22 | Corrected grammar. Corrected limitations statement about quantification of controls. Corrected performance characteristics to match package insert. Corrections made in response to ZD-2445. Based on the Summative evaluation, Section 2.3 steps 8, 12, 18 and Section 4.3 step 7 have been updated with added detail for mixing Tagmentation beads and Purification beads. Updated box contents to reflect updated kit configuration CR2020-096 (SCN 2021-08-13). |  |
| 31Mar22 | Added Tx 17 (96) Set A and B to product codes, Targeted Gene Content, Kit Contents. Detail added throughout IFU to instruct for the use of the pre-plated index, in particular, section 2.2 and 4.2. Issued by E. Naughton on 27Apr22 |  |
| 6.0 | 13Jun22 | Added Tx 9 (96) Set A and B to product codes, Targeted Gene Content and intended use. Removed ™ from the AlloSeq Tx logo. Corrected the OD values in Interfering Substances. Aligned phrasing between workbook and IFU. Section 5.2, point 12 ; change volume of ethanol from 200 μL to 800 μL. Section 10: Addition of vigilance reporting requirements.  Reissued by Hira Meraj 26-Jul-22 |  |
| 7.0 | 25 Jan 23 | Update copyright date. Additional detail to 1.1 Principle, remove duplicated information. Section 1.3 update table to reflect verification studies performed by CareDx. Section 1.4 remove “accelerated studies are ongoing”. Update section 1.6 Sample Requirements; remove buccal swab, add sample type, sample stability and DNA extraction methods. Update section 1.7 -Add “analytical specificity” to section header, add clarity on EDTA as an interfering substance. Section 1.9 and 1.10, remove information not required. Remove “limit of detection” (information added to section 1.6 Sample Requirements” . Update section 6 with other thermal cycler parameters for use with Tx assay. | Process Improvement |
| 8.0 | 03Nov23 | Addition of Swiss authorised representative. | N/A |

**Authorship and approvals**

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| --- | --- | --- | --- |
| Author signs to confirm technical content | | | |
| Prepared by: | Job title: | Signature: | Date: |
| Subject matter expert reviewer signs to confirm technical content | | | |
| Reviewed by: | Job title: | Signature: | Date: |
| Quality representative signs to confirm document complies with quality management system | | | |
| Authorised by: | Job title: | Signature: | Date: |
| Document review date |  | | |