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Illumina Urinary Pathogen ID/AMR Enrichment Kit

Reference Guide

ILLUMINA PROPRIETARY
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Overview

This guide explains how to prepare up to 384 uniquely dual-indexed libraries from DNA using the Illumina DNA Prep with Enrichment (IDPE) workflow and the Urinary Pathogen ID/AMR Panel (UPIP).

The IDPE workflow:

- Uses tagmentation, an enzymatic reaction, to fragment DNA and add adapter sequences in 15 minutes.

The Illumina Urinary Pathogen ID/AMR Panel:

- The Illumina Urinary Pathogen ID/AMR Panel detects up to 174 pathogens, including 121 bacteria, 35 viruses, 14 fungi, and 4 parasites, based on target-enriched next-generation sequencing (NGS) of microbial genome sequences.
- UPIP also detects over 3700 antimicrobial resistance (AMR) markers associated with resistance to 18 relevant drug classes and 69 uropathogens.

Recommendations for Illumina Sequencing Systems

The shortest recommended read length for each sample is 1 x 100 bp. Longer sequencing reads such as 1 x 150 bp, or paired-end reads such as 2 x 100 or 2 x 150, can improve specificity of the results. Whether using single-end or paired-end sequencing, we recommend producing sequencing data from a minimum of 1M clusters. This data equates to 1M single-end reads or 2M paired-end reads per sample. Choose your sequencing read length based on your budget, workflow, and other factors that can impact your laboratory. Additional recommendations are listed in *Dilute Libraries to the Starting Concentration* on page 41.

Illumina DNA Prep with Enrichment Workflow

The IDPE workflow uses a bead-based transposome complex to tagment genomic DNA. This complex fragments DNA and then tags the DNA with adapter sequences in one step. This fragmentation provides flexibility to use a wide DNA input range to generate normalized pre-enriched libraries of consistent tight fragment size distribution. Following tagmentation, a limited-cycle PCR adds additional adapter sequences and indexes to the ends of tagmented DNA fragments. This step enables compatibility across all Illumina sequencing systems. A subsequent target enrichment workflow is then applied. Enrichment is performed with pooled libraries (3-plex). Following pooling, the double-stranded DNA libraries are denatured and biotinylated oligonucleotide probes are hybridized to the denatured library fragments. After hybridization, Streptavidin Magnetic Beads 4 (SMB4) capture the targeted library fragments within the regions of interest. The captured and indexed libraries are eluted from the beads and further amplified before sequencing.

DNA Input Recommendations

The standard IDPE protocol is compatible with high-quality, double-stranded genomic DNA (gDNA). The protocol recommends inputs of 10–1000 ng for simple human gDNA samples or other low complexity DNA samples. Urine samples are highly complex and can contain both human genomic DNA and pathogen DNA. Therefore, normalized DNA input by equal mass is not recommended for this kit. Instead, this kit uses equal volume of extracted DNA per sample without normalization.

Assess gDNA Purity

Use one or more of the following strategies to assess gDNA purity. Make sure that the initial gDNA sample does not contain any organic contaminants, such as phenol and ethanol.

The input DNA must also contain less than 1 mM EDTA. These substances can interfere with the tagmentation reaction and result in assay failure.

- UV absorbance is a common method used for assessing the purity of a gDNA sample. The ratio of absorbance at 260 nm to 280 nm provides an indication of sample purity. This protocol is optimized for gDNA with A260/280 ratios of 1.8–2.0, which indicates a gDNA sample with high purity.
- For a secondary indication of sample purity, use an A260/230 ratio. Target an A260/230 ratio of 2.0–2.2. Values outside this range indicate the presence of contaminants. For a complete list of contaminants, including sources, avoidance, and effects on the library preparation, refer to the Illumina support site.
- **[Optional]** Dilute the starting material in 10 mM Tris-HCl, pH 7.5–8.5. Incomplete tagmentation caused by contaminants can cause library preparation failure, poor clustering, or low quality sequencing results. If the sample is diluted (and the EDTA concentration is < 1 mM), that addition of less input DNA into the tagmentation reaction can impact kit performance.

Consumables & Equipment

The IDPE protocol requires the following Illumina-supplied and user-supplied consumables and equipment.

The protocols have been optimized using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Make sure that you have the required consumables and equipment before starting the protocol.

Product Contents

Completing the Illumina DNA Prep with Enrichment protocol requires library prep and enrichment reagents, an enrichment probe panel, and index adapters. The number of index adapters required depends on the number of samples to be uniquely indexed for your experiment.

Component	Kit Options	Illumina Catalog #
Urinary Pathogen ID/AMR Enrichment Kit	Urinary Pathogen ID/AMR Enrichment Kit Set A, (RUO) (96 Indexes, 96 Samples)	20090308
	Urinary Pathogen ID/AMR Enrichment Kit Set B, (RUO) (96 Indexes, 96 Samples)	20090309
	Urinary Pathogen ID/AMR Enrichment Kit Set C, (RUO) (96 Indexes, 96 Samples)	20090310
	Urinary Pathogen ID/AMR Enrichment Kit Set D, (RUO) (96 Indexes, 96 Samples)	20090311

Urinary Pathogen ID/AMR Enrichment Kit Contents

Illumina Purification Beads are not included in these kits and must be purchased separately.

Illumina DNA/RNA Prep-Tagmentation (S) Beads, Store at 2°C to 8°C

Store the eBLT stock tube upright so that the beads are always submerged in the buffer.

Tube Quantity	Acronym	Reagent Name
4	eBLT	Enrichment Bead-Linked Transposomes
2	RSB	Resuspension Buffer

Illumina DNA/RNA Prep-Tagmentation Buffers, Store at 15°C to 30°C

The buffers are shipped at 2°C to 8°C. Promptly store reagents at the indicated temperature to ensure proper performance.

Tube Quantity	Acronym	Reagent Name
4	ST2	Stop Tagment Buffer 2
1	TWB	Tagmentation Wash Buffer

Illumina DNA/RNA Prep-Tagmentation PCR Reagents, Store at -25°C to -15°C

The following reagents are shipped at 2°C to 8°C. Promptly store reagents at the indicated temperature to ensure proper performance.

Tube Quantity	Acronym	Reagent Name
4	TB1	Tagmentation Buffer 1
4	EPM	Enhanced PCR Mix

Illumina DNA/RNA Fast Hyb-Enrichment Beads + Buffers, Store at 2°C to 8°C

Tube Quantity	Acronym	Reagent Name
2	SMB4	Streptavidin Magnetic Beads 4
1	RSB	Resuspension Buffer
1	EHB2	Enrichment Hyb Buffer 2
1	ET2	Elute Target Buffer 2

Illumina RNA Fast Hyb-Enrichment PCR + Buffers, Store at -25°C to -15°C

The following reagents are shipped at 2°C to 8°C. Promptly store reagents at the indicated temperature to ensure proper performance.

Tube Quantity	Acronym	Reagent Name
2	EE1	Enrichment Elution Buffer 1
4	EEW	Enhanced Enrichment Wash
1	PPC	PCR Primer Cocktail
1	HP3	2N NaOH
1	NHB2	Hyb Buffer 2 + IDT NXT Blockers
2	EPM	Enhanced PCR Mix

Urinary Pathogen ID/AMR Panel, Store at -25°C to -15°C

The following reagents are shipped at 2°C to 8°C. Promptly store reagents at the indicated temperature to ensure proper performance.

Tube Quantity	Acronym	Reagent Name
1	UPIP	Illumina Urinary Pathogen ID/AMR Panel

Illumina DNA/RNA UD Indexes, Store at -25°C to -15°C

Each Illumina Urinary Pathogen ID/AMR Enrichment Kit contains one of the following index plates.

For index adapter sequences, refer to Illumina Adapter Sequences.

Description	Part of Kit
Illumina DNA/RNA UD Indexes Set A (96 Indexes, 96 Samples)	20090308
Illumina DNA/RNA UD Indexes Set B (96 Indexes, 96 Samples)	20090309
Illumina DNA/RNA UD Indexes Set C (96 Indexes, 96 Samples)	20090310
Illumina DNA/RNA UD Indexes Set D (96 Indexes, 96 Samples)	20090311

Control Materials

The control materials described in this section are recommended for use with the Illumina Urinary Pathogen ID/AMR Panel.

External Control Recommendations

Positive Control Sample

Illumina recommends diluting ZymoBIOMICS Microbial Community Standard [1:1000] into 1X DNA/RNA Shield reagent (Zymo Research, catalog # R1100) to generate working positive control sample at the final defined sample volume. Lack of detection of one of the pathogens in this panel may serve as an indicator of workflow issues.

UPIP does not target the *Lactobacillus fermentum* and *Saccharomyces cerevisiae* present in the ZymoBIOMICS Microbial Community Standard, and they are not detected. The other eight pathogens are targeted by UPIP and should be detected.

- Make sure that the External Control volume matches the final defined sample volume before performing DNA extraction in parallel with the test samples.

Negative Control Sample

- Urine Conditioning Buffer (UCB) is recommended for the UPIP negative control sample. No pathogens targeted by this kit should be detected in this negative control sample. Pathogen detection in this sample indicates potential sample contamination within your workflow.
- Make sure that the negative control volume is equal to the final defined sample volume before performing DNA extraction.

Blank Control Sample

- Molecular biology grade water is recommended for the UPIP blank control sample. No pathogens targeted by this kit should be detected in this negative control sample. Pathogen detection in this sample indicates potential sample contamination within your workflow.
- Make sure that the blank control volume is equal to the final defined sample volume before performing extraction.

Internal Spike-in Control Recommendations (Quantitation and process control for each unknown test sample)

Enterobacteria Phage T7 (T7 phage) Internal Control Sample

- Illumina recommends adding a T7 Bacteriophage Internal Spike-in Control to each test sample and External Control sample prior to starting DNA extraction to serve as a process control for nucleic acid extraction. In addition, the Explify UPIP Data Analysis pipeline uses T7 for absolute quantitation estimates of panel targets in each sample. The following procedure recommends to spike T7 phage Internal Spike-in Control at 1.21E+07 copies/ml of the sample before DNA extraction. The final spike-in can be modified based on the Internal Control chosen, stock concentration, budget, and other factors that may impact your workflow.
- The recommended T7 phage material must be purchased directly from the vendor. Contact Microbiologics to request a quote.

Running the recommended external controls consumes reagents and reduces the total number of unknown test samples that can be processed with this kit.

User-Supplied Consumables & Equipment

Make sure that you have the required consumables and equipment before starting the protocol.

Some items are required only for specific workflows. These items are specified in separate tables.

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

Consumables

Consumable	Supplier
Microcentrifuge tubes, 1.7 ml	General lab supplier
Pipette tips, 10 µl	General lab supplier
Pipette tips, 20 µl	General lab supplier
Pipette tips, 200 µl	General lab supplier
Pipette tips, 1000 µl	General lab supplier
96-well 0.8 ml polypropylene deep-well storage plate (MIDI plate)	Thermo Fisher Scientific, part # AB-0859
Nuclease-free water/Molecular Biology Grade	General lab supplier
Conical centrifuge tubes (15 ml or 50 ml)	General lab supplier
Eppendorf twin.tec 96-well LoBind PCR plate, skirted (or similar)	Eppendorf, catalog # 0030129512
Hard-Shell 96-well PCR plates	Bio-Rad, catalog # HSP-9601
Microseal 'B' adhesive seals	Bio-Rad, catalog # MSB-1001
Microseal 'F' foil seals	Bio-Rad, catalog # MSF-1001
RNase/DNase-free 8-tube strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR **[Optional]** catalog # 89094-658
Ethanol (EtOH) 100%, molecular biology grade (500 ml)	General lab supplier
Illumina Purification Beads	Illumina, 1 x 100 ml, catalog # 20060057 Illumina, 4 x 100 ml, catalog # 20060058
One of the following kits, depending on quantification method: • **[Bioanalyzer]** Agilent High Sensitivity DNA Kit • **[Fragment Analyzer]** High Sensitivity NGS Fragment Analysis Kit	One of the following suppliers, depending on instrument: • Agilent, catalog # 5067–4626* • Advanced Analytical, catalog # DNF-474-0500
Qubit Assay Tubes	Thermo Fisher Scientific, catalog # Q32856

Consumable	Supplier
One of the following kits for quantification: • Qubit dsDNA BR Assay Kit • KAPA Library Quantification Kit – Illumina qPCR Instrument / Reference Dye Universal	• Thermo Fisher Scientific, catalog # Q32850 or Q32853 • Roche, catalog # 07960140001, KAPA code KK4824
Tris-HCl 10 mM, pH 8.5	General lab supplier

* End of life announced. Refer to vendor site for more information.

DNA Extraction Consumables

Make sure that you have the required consumables before starting the DNA extraction protocol.

Consumable	Supplier
Collection tubes, 2.0 ml	Zymo Research, catalog # C1001
Microcentrifuge tubes, 1.5 ml	General lab supplier
Microcentrifuge tubes, 2.0 ml	General lab supplier
ZR BashingBead Lysis Tubes	Zymo Research, catalog # S6012-50
Zymo-Spin III-Filters	Zymo Research, catalog # C1057-50
Zymo-Spin IC-S Columns	Zymo Research, catalog # C1015
BashingBead Buffer	Zymo Research, catalog # D6001-3
Clearing Beads	Zymo Research, catalog # D3061-2-1
Genomic Lysis Buffer	Zymo Research, catalog # D3004-1

Consumable	Supplier
Urine Conditioning Buffer (UCB)	Zymo Research, catalog # D3061-1
Urine DNA Prep Buffer	Zymo Research, catalog # D3061-4-10
Urine DNA Wash Buffer (Concentrate)	Zymo Research, catalog # D3061-5-12
1X DNA/RNA Shield reagent	Zymo Research, catalog # R1100-50, or R1100-250
ZymoBIOMICS Microbial Community Standard	Zymo Research, catalog # D6300
Urine Conditioning Buffer (UCB)	Zymo Research, catalog # D3061-1
Molecular biology grade (MBG) water	General lab supplier
T7 Phage	Microbiologics, catalog # T7-UP-LEX

Equipment

Equipment	Supplier
Pipettes, multichannel, 10 µl	General lab supplier
Pipettes, multichannel, 20 µl	General lab supplier
Pipettes, multichannel, 200 µl	General lab supplier
Pipettes, single channel, 10 µl	General lab supplier
Pipettes, single channel, 20 µl	General lab supplier
Pipettes, single channel, 200 µl	General lab supplier

Equipment	Supplier
Pipettes, single channel, 1000 µl	General lab supplier
Magnetic Stand-96	Thermo Fisher Scientific, catalog # AM 10027
Microcentrifuge	General lab supplier
Microplate centrifuge	General lab supplier
Heat block (able to accommodate 2 ml microtubes and 60°C)	General lab supplier
Adhesive seal roller	General lab supplier
Biosafety Cabinet PCR instrument control computer	General lab supplier
Qubit Fluorometer 4.0	Thermo Fisher Scientific, catalog # Q33238 or Q33327
Vortexer	General lab supplier
One of the following analyzers: • Fragment Analyzer • 2100 Bioanalyzer Desktop System • 4150 TapeStation System • 4200 TapeStation System	Agilent Technologies catalog #: • Refer to web product pages for catalog # • G2939BA* or G2940CA • G2992AA • G2991BA

* No longer available for purchase.

DNA Extraction Equipment

Make sure that you have the required equipment before starting the DNA extraction protocol.

Equipment	Supplier
FastPrep-24 5G bead beating grinder and lysis system or FastPrep-24 Classic bead beating grinder and lysis system	MP Bio, catalog # 6005500 or MP Bio, catalog # 6004500
1000 µl pipettes	General lab supplier

Equipment	Supplier
QuickPrep adapter for 24 x 2 ml tube holder for FastPrep-24	MP Bio, catalog # 6005512

Equipment for Plate Workflow

Equipment	Supplier
Magnetic Stand-96	Thermo Fisher Scientific, catalog # AM10027
High-Speed Microplate Shaker	BioShake iQ High-Speed Thermal Mixer • Q Instruments, model # 1808-0506 BioShake XP High-Speed Thermal Mixer • Q Instruments, model # 1808-0505
Microplate centrifuge	General lab supplier

Thermal Cyclers

The following table lists recommended thermal cyclers or thermal cycler specifications. PCR thermal cyclers must be capable of supporting the sample volumes and temperature profiles used in this workflow, with appropriate thermal accuracy and block uniformity to ensure consistent incubation and amplification performance. Validate the thermal cycler before performing the protocol.

Performance may vary depending on the specific thermal cycler and consumables used. Minor workflow optimization may be required to account for instrument and consumable specific differences.

Thermal Cycler	Supplier

Thermal cycler with the following specifications:
• Heated lid
• Block ramp rate: ≥ 2.5°C/sec
• Temperature control range:
  • Min ≤ 4°C
  • Max ≥ 99°C
• Temperature accuracy: ±0.25°C
• Temperature uniformity: ±0.5°C
• Capable of supporting reaction volumes of 100 µl
• Compatible with 96-well PCR plates (full or semi-skirted), or suitable for the applicable workflow
	General lab supplier

DNA Extraction Protocol

Disclaimer

Due to the complex composition of urine samples, recommending a single DNA extraction kit or procedure that consistently extracts high quality DNA from a wide variety of organisms is difficult. The extraction protocol below is provided as a courtesy and has been tested and routinely used by Illumina in combination with this kit. Some reagents and materials must be purchased from non-authorized third-party suppliers, and Illumina does not guarantee or promise technical support for the performance of our products used with any of these reagents purchased from non-authorized third-party suppliers. Please contact the appropriate vendor for technical assistance with their respective products.

The DNA extraction protocol is optimized to obtain successful DNA extraction for all targets within each pathogen class (ie, bacteria, viruses, fungi, and parasites) from urine in a single procedure. Complete the steps outlined in this section to extract DNA in preparation for sequencing. Reagents used in this extraction method are part of the Quick-DNA Urine Kit (Zymo Research, Catalog # D3061). We recommend purchasing the individual consumables as listed below instead of the complete Quick-DNA Urine Kit to ensure all required components are available and in appropriate quantity. Technical support for Extraction should be directed to Zymo Research.

The recommended sample input is 1.8 ml of urine. Different kits may have different sample recommendations. Sample backgrounds other than urine (such as saline solution, synthetic urine, and molecular biology grade water), are not guaranteed to yield successful DNA extractions.

To avoid cross-contamination, complete the DNA extraction protocol in an appropriate biosafety cabinet, following recommended aseptic techniques.

Urine Conditioning Buffer (UCB) Treatment

Complete the following steps to perform UCB treatment, the first step to extract DNA before library preparation.

Always cap and seal tubes before centrifuge steps.

Consumables

- ZR BashingBead Lysis Tube
- Zymo-Spin III-Filter
- Zymo-Spin IC-S Column
- 2.0 ml collection tubes
- 1.5 ml microcentrifuge tube
- 2.0 ml microcentrifuge tube

- UCB (Urine Conditioning Buffer)
- Clearing Beads

Preparation

1. Remove samples and external control set from storage, and prepare as follows.
   a. Incubate at room temperature for 30 minutes.
   b. Vortex and centrifuge briefly to mix.
2. Remove T7 Phage stock from storage, and prepare as follows.
   a. Generate a Working stock concentration of 1.09E+10 copies/ml by diluting the purchased T7 Phage stock with water, if necessary.
   b. Vortex and centrifuge briefly to mix. Place on ice.
   c. **[Optional]** Dispense the T7 Phage Working stock into aliquots of 50 µl (or other volumes) and frozen to avoid multiple freeze thaws of the Working stock, and simplify usage in future experiments.
3. For each sample to be extracted, including the external control samples, prepare the following labeled tube sets.

Tube	Quantity
ZR BashingBead Lysis Tube	1
Zymo-Spin III-Filter	1
Zymo-Spin IC-S Column	1
2.0 ml microcentrifuge tube	2
1.5 ml microcentrifuge tube	1
2.0 ml collection tube	5

Procedure

1. Add 1.8 ml urine sample to a new 2.0 ml microcentrifuge tube.
2. Add 126 µl UCB to each microcentrifuge tube. Vortex to mix.
3. Add 10 µl Clearing Beads to each microcentrifuge tube. Vortex to mix.
4. Centrifuge the microcentrifuge tubes at 3000 × g for 15 minutes.
5. Without disturbing the bead pellet, use a single channel pipette to remove and discard 1500 µl of supernatant.
6. Vortex or pipette thoroughly to resuspend the pellet in the residual solution.

Sample Homogenization and Cell Lysis

Complete the following steps to perform sample homogenization and cell lysis, the second step to extract DNA before library preparation.

Always cap and seal tubes before centrifuge and homogenize steps.

Consumables

- Internal Control (T7 Phage Working stock at 1.09E+10 copies/ml)
- BashingBead Buffer
- ZR BashingBead Lysis Tube

Preparation

Save the following program (total running time is ~20 minutes) on the bead beating system (homogenizer):

1. Homogenize at 6m/sec for 1 minute.
2. Rest for 5 minutes.
3. Homogenize at 6m/sec for 1 minute.
4. Rest for 5 minutes.
5. Homogenize at 6m/sec for 1 minute.
6. Rest for 5 minutes.
7. Homogenize at 6m/sec for 1 minute.

Procedure

1. Combine the following volumes to prepare the Extraction Reagent Mix. Multiply each volume by the number of samples.
   Reagent overage is not included. We recommend making one extra Extraction Reagent Mix to compensate for pipetting variation.
   - Internal control T7 Phage (2 µl)
   - BashingBead Buffer (400 µl)
2. For each sample (including external controls), add 402 µl Extraction Reagent Mix into a new BashingBead Lysis Tube.
3. Add approximately 400 µl bead suspension from step 6 of *Urine Conditioning Buffer (UCB) Treatment* on page 12 to each BashingBead Lysis Tube.
4. Cap the BashingBead Lysis Tube.
5. Place BashingBead Lysis tubes on the homogenizer and run the saved program.
6. Centrifuge the BashingBead Lysis tubes at 10,000 × g for 2 minutes.

DNA Purification

Complete the following steps to perform DNA purification, the final step to extract DNA before library preparation.

Always cap and seal tubes before centrifuge steps.

Consumables

- Genomic Lysis Buffer
- Urine DNA Prep Buffer
- Urine DNA Wash Buffer
- MBG (Molecular Biology Grade) Water

Labeled Tube Sets from UCB Treatment Preparation:

- Zymo-Spin III-Filter
- Zymo-Spin IC-S Column
- 2.0 ml collection tubes
- 1.5 ml microcentrifuge tube
- 2.0 ml microcentrifuge tube

Procedure

1. Place Zymo-Spin III-Filter into a new labeled 2.0 ml microcentrifuge tube.
2. For each sample, transfer 400 µl homogenate lysate from step 6 of *Sample Homogenization and Cell Lysis* on page 13 onto the Zymo-Spin III-Filter.
   Avoid disturbing the bead volume.
3. Centrifuge at 10,000 × g for 1 minute.
4. Discard the Zymo-Spin III-Filter. Keep the flow-through.
5. Add 1.2 ml Genomic Lysis Buffer to each sample flow-through.
6. Vortex briefly to mix, and then centrifuge briefly.
7. Place Zymo-Spin IC-S Column into a new labeled 2.0 ml collection tube.
8. Transfer 800 µl of mixture to the Zymo-Spin IC-S Column.
9. Centrifuge at 10,000 × g for 1 minute.
10. Transfer Zymo-Spin IC-S Column to a new labeled 2.0 ml collection tube.
11. Discard the collection tube containing the flow-through.
12. Transfer the remaining sample filtrate volume from step 5 to the Zymo-Spin IC-S Column.
13. Centrifuge at 10,000 × g for 1 minute.
14. Transfer the Zymo-Spin IC-S Column to a new labeled 2.0 ml collection tube.
15. Discard the collection tube containing the flow-through.

16. Add 200 µl Urine DNA Prep Buffer to the Zymo-Spin IC-S Column.
17. Centrifuge at 10,000 × g for 1 minute.
18. Transfer Zymo-Spin IC-S Column to a new labeled 2.0 ml collection tube.
19. Discard the collection tube containing the flow-through.
20. Add 700 µl Urine DNA Wash Buffer to the Zymo-Spin IC-S Column.
21. Centrifuge at 10,000 × g for 1 minute.
22. Transfer Zymo-Spin IC-S Column to a new labeled 2.0 ml collection tube.
23. Discard the collection tube containing the flow-through.
24. Add 200 µl Urine DNA Wash Buffer to the Zymo-Spin IC-S Column.
25. Centrifuge at 10,000 × g for 1 minute.
26. Transfer Zymo-Spin IC-S Column to labeled 1.5 ml microcentrifuge tube.
27. Discard the collection tube containing the flow-through.
28. Add 70 µl MBG water to Zymo-Spin IC-S Column.
29. Incubate for 5 minutes at room temperature.
30. Centrifuge 1.5 ml microcentrifuge tube at 10,000 × g for 1 minute.
31. Reapply the elute (70 µl) to the Zymo-Spin IC-S Column.
32. Incubate for 5 minutes at room temperature.
33. Centrifuge 1.5 ml microcentrifuge tube at 10,000 × g for 1 minute.
    The final eluted volume contains purified sample DNA.

Protocol

This section describes the Illumina DNA Prep with Enrichment protocol.

- Review the planned complete sequencing workflow, from sample through analysis, to ensure compatibility of products and experiment parameters.
- Before proceeding, confirm kit contents and make sure that you have the required components, equipment, and consumables. This protocol uses the Urinary Pathogen ID/AMR Panel, library prep and enrichment reagents, and index adapters included in these kits.
- Follow the protocol in the order shown, using the specified volumes and incubation parameters.

Prepare for Pooling

If you plan to use different libraries plexities from what is advised in the subsequent protocol, record information about your samples before starting library prep.

- For low-plexity sequence pooling strategies (2-plex to 9-plex), refer to the Index Adapters Pooling Guide.
- For index adapter sequences and information about recording the sequences, refer to Illumina Adapter Sequences.

Supported Enrichment Plexities

The UPIP protocol for the UPIP is optimized for 3-plex enrichment. Although other enrichment plexities are possible, some plexities require additional pre-enrichment library prep and enrichment probe panel reagents.

Obtaining suitable enrichment yields for nonstandard enrichment plexities might require additional optimization. Optimal results are not guaranteed.

- **Enrichment plexity**—The number of pre-enriched libraries (3) pooled together in one enrichment reaction for hybridization with the enrichment probe panels. For example, combining 12 pre-enriched libraries together creates a 12-plex enrichment pool.
- **Enrichment reaction**—The number of unique enrichment reaction preparations, regardless of the number of pre-enriched libraries pooled per reaction.

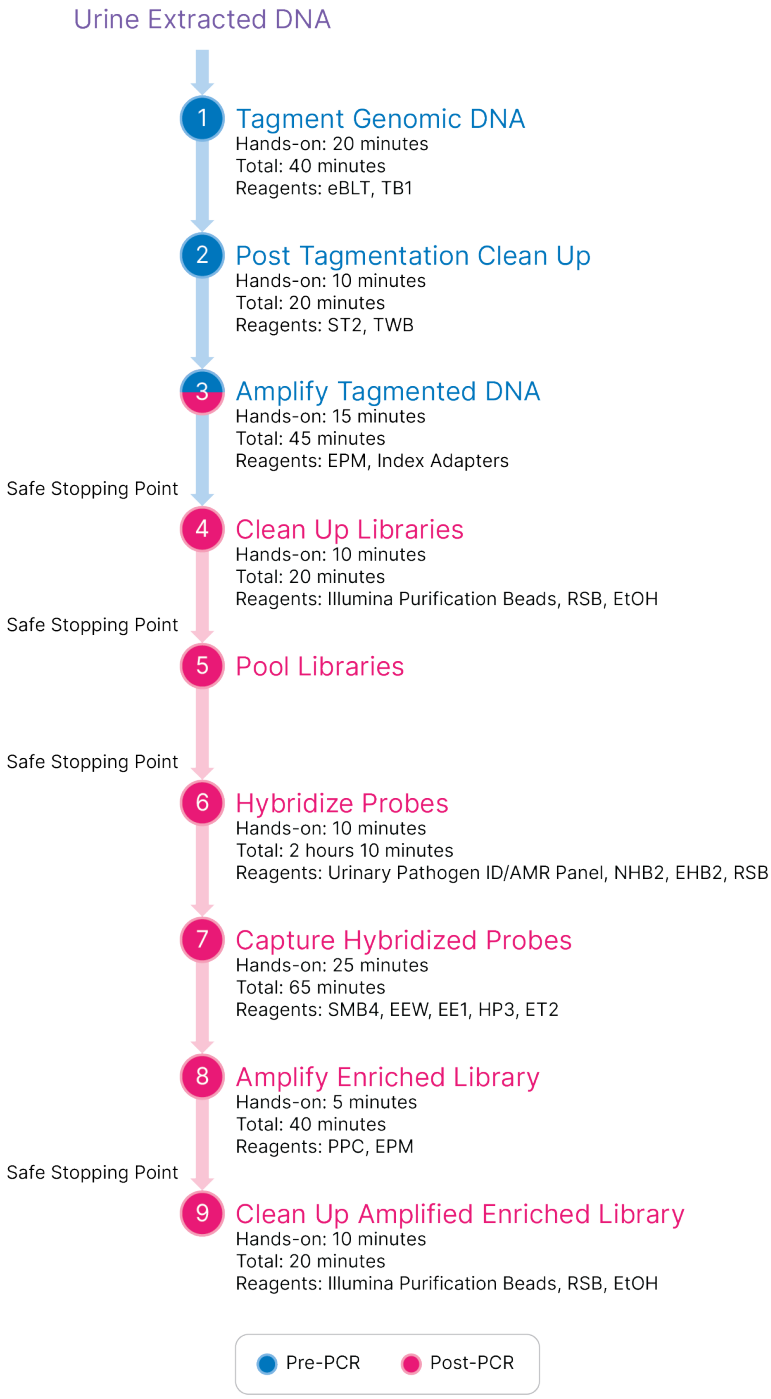
This kit enables the generation of 96 pre-enriched libraries, which are pooled together in 32 individual 3-plex enrichment reactions.

UPIP Library Prep and Enrichment Workflow

The following diagram illustrates this workflow.

- Safe stopping points are marked between steps.

- Time estimates are based on processing 12 samples at 3-plex enrichment.



Tips and Techniques

Safe Stopping Point

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

- When adding or transferring samples or reagent master mixes, change tips between ***each sample***.
- When adding index adapters with a multichannel pipette, change tips between ***each row*** or ***each column***. If using a single channel pipette, change tips between each sample.
- Record the samples that you plan to pool before starting Library prep.
- Illumina recommends using a PCR instrument control computer when adding or transferring samples or reagent master mixes. Perform all steps after amplification in a post-amplification area of the laboratory.
- Thoroughly clean and wipe the work area using a freshly prepared 10% bleach. Follow the bleach cleaning by wiping the area with water and a DNA degrading system. Finally, to avoid potential PCR inhibition, wipe the area with water (only) to clear all chemicals.
- Use personal protective equipment. Change gloves regularly during the decontamination process and before starting a new step in the process.
- When working with 96-well plates, avoid handling genetically related organisms sequentially. If spiking samples of known concentration, handle the diluted samples first, and the highly positive samples last.

Sealing the Plate

- Always seal the 96-well plate with the adhesive seal using a rubber roller to cover the plate before the following steps in the protocol:
  - Shaking steps
  - Thermal cycling steps
  - Centrifuge steps
- Microseal 'B' adhesive seals are effective at -40°C to 110°C and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' seals for thermal cycling or short-term storage.
- Microseal ‘F’ foil seals are effective at temperatures down to -70°C and are suitable for storing the 96-well plates containing the final libraries long term.

Handling Enrichment Bead-Linked Transposomes (eBLT)

- Store the eBLT stock tube upright in the refrigerator so that the beads are always submerged in the buffer.

- Vortex the eBLT stock tube thoroughly until the beads are resuspended before use. To avoid resettling the beads, centrifugation before pipetting is not recommended.
- If beads are adhered to the side or top of a 96-well plate, centrifuge at 280 × g for 3 seconds, and then pipette to resuspend.
- When washing beads:
  - Use the appropriate magnetic stand for the plate.
  - Keep the plate on the magnetic stand until the instructions specify to remove it.
  - Do not agitate the plate while it is on the magnetic stand.
  - Do not disturb the bead pellet.
  - If beads are aspirated into pipette tips, dispense back into the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
  - Dispense Tagmentation Wash Buffer (TWB) directly onto the beads.
  - If liquid becomes adhered to the side or top of the tube or well, centrifuge at 280 × g for 3 seconds to pull volume into liquid.

Preparing Illumina DNA/RNA Unique Dual (UD) Indexes Plate

- If using a NextSeq 500 system, the read lengths must be modified to accommodate 10 base pair indexes. Visit the compatible products page on the Illumina support site.

Prepare for Protocol

1. Remove reagents from storage.
2. Remove the reagents from the box and prepare as follows.

Table 1 15°C to 30°C Storage

Reagent	Box Name	Instructions
ST2	Illumina DNA/RNA Prep - Tagmentation Buffers	Use at room temperature.
TWB	Illumina DNA/RNA Prep - Tagmentation Buffers	Use at room temperature.

Table 2 2°C to 8°C Storage

Reagent	Box Name	Instructions
eBLT	Illumina DNA/RNA Prep - Tagmentation (S) Beads	Bring to room temperature.

Table 3 -25°C to -15°C Storage

Reagent	Box Name	Instructions
EPM	Illumina DNA/RNA Prep - Tagmentation PCR Reagents	Thaw on ice.
Index adapter plate	Illumina DNA/RNA UD Indexes	Thaw at room temperature.
TB1	Illumina DNA/RNA Prep - Tagmentation PCR Reagents	Bring to room temperature.

Tagment Genomic DNA

This step uses the Enrichment Bead-Linked Transposomes (eBLT) to tagment DNA, which is a process that fragments and tags the DNA with adapter sequences.

Consumables

- eBLT (Enrichment Bead-Linked Transposomes)
- TB1 (Tagmentation Buffer 1)
- Nuclease-free water
- 8-tube strip
- 96-well PCR plate
- Microseal 'B' adhesive seal

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, refer to the SDS at support.illumina.com/sds.html.

About Reagents

- eBLT
  - Once thawed, eBLT must be stored upright so that the beads are always submerged in the buffer.
  - Do not use eBLT that has been stored below 2°C.

Preparation

1. Prepare the following consumables:
   - eBLT—Vortex to mix. Do not centrifuge before pipetting.
   - TB1—Vortex to mix.
2. Save the following TAG program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 55°C for 5 minutes
   - Hold at 10°C

Procedure

1. Add 30 µl DNA to each well of a 96-well PCR plate.
2. Vortex eBLT for 10 seconds to resuspend. Repeat as necessary.
3. For each sample, combine the following volumes to prepare the Tagmentation Master Mix. Multiply each volume by the number of samples being processed.
   - eBLT (11.5 µl)
   - TB1 (11.5 µl)

   These volumes produce 23 µl Tagmentation Master Mix per sample, which includes extra volume to ensure accurate pipetting.
4. Vortex the Tagmentation Master Mix for 10 seconds to resuspend.
5. Divide the Tagmentation Master Mix volume equally into an 8-tube strip.
6. Using a multichannel pipette, transfer 20 µl Tagmentation Master Mix from the 8-tube strip to each well of the plate containing a sample.
   Use fresh tips for each sample column.
7. Discard the 8-tube strip after the Tagmentation Master Mix has been dispensed.
8. Using a multichannel pipette set to 40 µl, pipette each sample 10 times to resuspend, and then seal the plate.
9. Place on the preprogrammed thermal cycler and run the TAG program.
10. Wait until the TAG program has reached the 10°C hold temperature before removing the plate and proceeding.

Post Tagmentation Clean Up

This step washes the adapter-tagged DNA on the eBLT before PCR amplification.

Consumables

- ST2 (Stop Tagment Buffer 2)
- TWB (Tagmentation Wash Buffer)
- 8-tube strip
- Microseal 'B' adhesive seal

About Reagents

- TWB
  - Pipette slowly to minimize foaming.
  - A deliberately slow pipetting technique minimizes the potential of foaming to avoid incorrect volume aspiration and incomplete mixing.

Preparation

1. Prepare the following consumables:
   - ST2—If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved.
   - TWB—Use at room temperature. Vortex to mix.

Procedure

1. Let the 96-well PCR plate stand at room temperature for 2 minutes.
2. Add 10 µl ST2 to each well of the plate. If you are using a multichannel pipette, pipette ST2 into an 8-tube strip, and then transfer the 10 µl volumes.
3. Using a 200 µl pipette set to 50 µl, slowly pipette each well 10 times to resuspend the beads, and then seal. Alternatively, seal the plate and use a plate shaker at 1800 rpm for 1 minute. Repeat as needed to resuspend the beads.
4. Make sure that the plate is sealed and centrifuge the samples for approximately 2 seconds.
5. Incubate the samples at room temperature for 5 minutes.
6. Place the samples on a magnetic stand, and then wait until the liquid is clear (~3 minutes).
7. **[≤ 48 samples]** Wash as follows.
   a. Using a 200 µl multichannel pipette set to 60 µl, remove and discard supernatant.
   b. Remove from the magnetic stand.
   c. Use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads.
   d. Pipette slowly until beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.

e. Place the plate on the magnetic stand and wait until the liquid is clear (~3 minutes).
f. Using a 200 µl multichannel pipette set to 100 µl, remove and discard supernatant.
g. Repeat steps b–f for a **second** wash.

8. **[> 48 samples]** Wash as follows.
   a. To avoid excessive drying of the beads, perform steps b–d in one or two column increments until all columns have been processed.
   b. Using a 200 µl multichannel pipette set to 60 µl, remove and discard supernatant.
   c. Remove from the magnetic stand.
   d. Use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads.
   e. Pipette slowly until beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
   f. Place the plate on the magnetic stand and wait until the liquid is clear (~3 minutes).
   g. To avoid excessive drying of the beads, perform steps h–k in one or two column increments until all columns have been processed.
   h. Using a 200 µl multichannel pipette set to 100 µl, remove and discard supernatant.
   i. Remove from the magnetic stand.
   j. Use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads.
   k. Repeat steps e–j for a **third** wash.
9. Pipette slowly until beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
10. Place the plate on the magnetic stand and wait until the liquid is clear.
11. Keep on the magnetic stand and proceed to *Amplify Tagmented DNA* on page 24.
    The TWB remains in the wells to prevent drying of the beads.

Amplify Tagmented DNA

This step amplifies the tagmented DNA using a limited-cycle PCR program. The PCR step adds Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for sequencing cluster generation. To confirm the indexes of libraries have the appropriate color balance, refer to the *Index Adapters Pooling Guide*.

For a list of compatible index adapters for use with this protocol, refer to *Product Contents* on page 3.

Consumables

- EPM (Enhanced PCR Mix)
- Microcentrifuge tubes, 1.7 ml
- Eppendorf Lo Bind PCR Plate

- Microseal 'B' adhesive seal
- Nuclease-free water
- Index adapter plate (A, B, C, or D plates)

About Reagents

- Index adapter plates
  - A well can contain > 10 µl index adapters.
  - Do not add samples to the index adapter plate.
  - Each well of the index plate is single use only.

Preparation

1. Prepare the following consumables:
   - EPM—Invert to mix, then centrifuge briefly.
   - Index adapter plates
2. Save the following eBLT PCR program on a thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 72°C for 3 minutes
   - 98°C for 3 minutes
   - 18 cycles of:
     - 98°C for 20 seconds
     - 60°C for 30 seconds
     - 72°C for 1 minute
   - 72°C for 3 minutes
   - Hold at 10°C

Procedure

1. For each sample, combine the following volumes to prepare the PCR Master Mix. Multiply each volume by the number of samples being processed. Reagent overage is included in the volume to ensure accurate pipetting.
   - EPM (23 µl)
   - Nuclease-free water (23 µl)
2. Vortex, and then centrifuge the PCR Master Mix at 280 × g for 10 seconds.

3. With the plate on the magnetic stand, use a 200 µl multichannel pipette to remove and discard supernatant.
   Foam that remains on the well walls does not adversely affect the library.
4. Remove from the magnetic stand.
5. Immediately add 40 µl PCR Master Mix directly onto the beads in each sample well.
6. Immediately pipette to mix until the beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1800 rpm for 1 minute.
7. Seal the sample plate and centrifuge at 280 × g for 3 seconds.
8. Centrifuge the index adapter plate at 1000 × g for 1 minute.
9. Prepare the index adapter plate.
   - **[< 96 samples]** Pierce the foil seal on the index adapter plate with a new pipette tip for each well for only the number of samples being processed.
   - **[96 samples]** Align a new Eppendorf PCR plate above the index adapter plate and press down to puncture the foil seal. Discard the Eppendorf PCR plate used to puncture the foil seal.
10. Using a new pipette tip, add 10 µl pre-paired Index 1 (i7) and Index 2 (i5) index adapters to each well.
11. Using a pipette set to 40 µl, pipette 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1800 rpm for 1 minute.
12. Seal the plate with Microseal 'B', and then centrifuge at 280 × g for 30 seconds.
13. Place on the preprogrammed thermal cycler and run the eBLT PCR program.

SAFE STOPPING POINT

If you are stopping, store at -25°C to -15°C for up to 30 days.

Prepare for Protocol

1. Remove reagents from storage.
2. Remove the reagents from the box and prepare as follows.

Table 4 15°C to 30°C Storage

Reagent	Box Name	Instructions
IPB	IPB	Bring to room temperature.

Table 5 2°C to 8°C Storage

Reagent	Box Name	Instructions
RSB	Illumina DNA/RNA Prep - Tagmentation (S) Beads	Bring to room temperature.

Clean Up Libraries

This step uses double-sided bead purification procedure to purify the amplified and indexed libraries.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- EtOH (Freshly prepared 80% ethanol)
- Nuclease-free water
- 96-well 0.8 ml Polypropylene Deepwell Storage Plate (MIDI plate) (2)
- 96-well PCR plate
- 1.7 ml microcentrifuge tubes
- Microseal 'B' adhesive seal
- Microseal 'F' foil seal

About Reagents

- IPB
  - Must be at room temperature before use.
  - Vortex to resuspend before each use.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. RSB—Vortex to mix.
2. For each sample, prepare 400 µl fresh 80% EtOH from absolute ethanol. Including an overage of 20% is recommended.

Procedure

1. Use a plate shaker to shake the 96-well PCR plate at 1800 rpm for 1 minute.
2. Place the plate on the magnetic stand and wait until the liquid is clear (~5 minutes).
3. Transfer 45 µl supernatant from each well of the PCR plate to the corresponding well of a new MIDI plate.
4. Resuspend IPB as follows.
   a. To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 seconds.

b. If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.

5. For urine, perform the following steps.
   a. Add 77 µl nuclease-free water to each well-containing supernatant.
   b. Add 88 µl IPB to each well-containing supernatant.
   c. Pipette each well 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1800 rpm for 1 minute.
   d. Seal the plate and incubate at room temperature for 5 minutes.
   e. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
   f. During incubation, thoroughly vortex and resuspend the IPB, and then add 20 µl to each well of a *new* MIDI plate.
   g. Remove seal and transfer 200 µl supernatant from each well of the first plate to the corresponding well of the new MIDI plate containing 20 µl IPB.
   h. Pipette each well in the MIDI plate 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1800 rpm for 1 minute.
   i. Discard the first plate.
6. Incubate the sealed MIDI plate at room temperature for 5 minutes.
7. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
8. Without disturbing the beads, remove and discard supernatant.
9. Wash beads as follows.
   a. With the plate on the magnetic stand, add 200 µl fresh 80% EtOH without mixing.
   b. Incubate for 30 seconds.
   c. Without disturbing the beads, remove and discard supernatant.
10. Wash beads a **second** time.
11. Use a 20 µl pipette to remove and discard residual EtOH.
12. Air-dry on the magnetic stand for 5 minutes.
13. Remove from the magnetic stand.
14. Add 17 µl RSB to the beads.
15. Seal the plate, and then use a plate shaker at 1800 rpm for 2 minutes.
16. Incubate at room temperature for 2 minutes.
17. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
18. Transfer 15 µl supernatant to a new 96-well PCR plate.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal, and store at -25°C to -15°C for up to 30 days.

Pool Pre-Enriched Libraries

The UPIP protocol recommends volume-based pooling of pre-enriched libraries in 3-plex library pool format. Unlike similar kits, UPIP pre-enriched libraries are not quantified before the pooling event.

To achieve optimal performance, only pool pre-enriched library samples prepared by the same user, reagent lot, and index adapter plate.

1. Using the sample tracking method you chose in *Prepare for Pooling* on page 17, record the indexes for the libraries you plan to pool in this step.
2. Pool pre-enriched libraries based on the sample volumes in the following table.

Library Pool Plexity	Each Pre-Enriched Library Volume (µl)	Total Volume per enriched reaction (µl)
3-plex	2.5	7.5 (2.5 per sample)

SAFE STOPPING POINT

If you are stopping, cap the 1.5 ml microcentrifuge tube and store at -25°C to -15°C for up to 30 days.

Prepare for Protocol

1. Remove reagents from storage.
2. Remove the reagents from the box and prepare as follows.

Table 6 2°C to 8°C Storage

Reagent	Box Name	Instructions
EHB2	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature.
ET2	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature for 30 minutes before the NF-HYB thermal cycler program ends.
SMB4	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature for 30 minutes before the NF-HYB thermal cycler program ends, and vortex to resuspend.

Table 7 -25°C to -15°C Storage

Reagent	Box Name	Instructions
Enrichment Probe Panel (one of the following):		

Reagent	Box Name	Instructions
• TruSight Cancer	Not applicable	Bring to room temperature.
• TruSight Hereditary Cancer	Not applicable	Bring to room temperature.
• Illumina Exome Panel (CEX)	Not applicable	Bring to room temperature.
• Illumina Custom Enrichment Panel	Not applicable	Bring to room temperature.
• Illumina Custom Enrichment Panel v2	Not applicable	Bring to room temperature.
EE1	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	If proceeding to *Capture Hybridized Probes* on page 33 immediately after the NF-HYB thermal cycler program 90 minute hold time, thaw at room temperature. Otherwise, if extending the hold time overnight, wait until 30 minutes before the program ends to thaw at room temperature, and vortex to resuspend. Refer to *[Optional] Extend the hold to a maximum of 24 hours* on page 33 for more information.
EEW	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	If proceeding to *Capture Hybridized Probes* on page 33 immediately after the NF-HYB thermal cycler program 90 minute hold time, thaw at room temperature. Otherwise, if extending the hold time overnight, wait until 30 minutes before the program ends to thaw at room temperature, and vortex to resuspend. Refer to *[Optional] Extend the hold to a maximum of 24 hours* on page 33 for more information.

Reagent	Box Name	Instructions
EPM	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	If proceeding to *Capture Hybridized Probes* on page 33 immediately after the NF-HYB thermal cycler program 90 minute hold time, thaw on ice. Otherwise, if extending the hold time overnight, wait until 30 minutes before the program ends to thaw on ice, and vortex to resuspend. Refer to *[Optional] Extend the hold to a maximum of 24 hours* on page 33 for more information.
HP3	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	If proceeding to *Capture Hybridized Probes* on page 33 immediately after the NF-HYB thermal cycler program 90 minute hold time, thaw at room temperature. Otherwise, if extending the hold time overnight, wait until 30 minutes before the program ends to thaw at room temperature, and vortex to resuspend. Refer to *[Optional] Extend the hold to a maximum of 24 hours* on page 33 for more information.
NHB2	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	If proceeding to *Capture Hybridized Probes* on page 33 immediately after the NF-HYB thermal cycler program 90 minute hold time, thaw at room temperature. Otherwise, if extending the hold time overnight, wait until 30 minutes before the program ends to thaw at room temperature, and vortex to resuspend. Refer to *[Optional] Extend the hold to a maximum of 24 hours* on page 33 for more information.
PPC	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	If proceeding to *Capture Hybridized Probes* on page 33 immediately after the NF-HYB thermal cycler program 90 minute hold time, thaw at room temperature. Otherwise, if extending the hold time overnight, wait until 30 minutes before the program ends to thaw at room temperature, and vortex to resuspend. Refer to *[Optional] Extend the hold to a maximum of 24 hours* on page 33 for more information.

Hybridize Probes

This step binds targeted regions of the DNA with the UPIP capture probes.

Consumables

- UPIP (Urinary Pathogen ID/AMR Panel)
- EHB2 (Enrichment Hyb Buffer 2)
- NHB2 (Hyb Buffer 2 + IDT NXT Blockers)
- RSB (Resuspension Buffer)
- One of the following containers:
  - **[Plate]** 96-well PCR plate
  - **[Tube]** 8-tube strip
- One of the following seals:
  - **[Plate]** Microseal 'B' adhesive seal
  - **[Tube]** 8-tube strip caps

About Reagents

- NHB2 precipitates and separates during storage. Follow the NHB2 preparation instructions before first use.

Preparation

1. Prepare the following consumables:
   - UPIP—Vortex to mix. Use a total probe volume of 2.5 µl per hybridization reaction.
   - EHB2:
     a. Vortex to mix.
     b. If crystals and cloudiness are observed, repeat vortex, or pipette up and down to mix well until the solution is clear.
   - NHB2:
     a. When at room temperature, preheat to 50°C on a microheating system for 5 minutes.
     b. Vortex at maximum speed 3 times for 10 seconds each to resuspend.
     c. Centrifuge briefly.
     d. Pipette up and down from the bottom of the tube. If crystals and cloudiness are observed, repeat vortex, or pipette up and down to mix well until the solution is clear.
     Use while warm to prevent precipitate from reforming.

- SMB4—If proceeding to *Capture Hybridized Probes* on page 33 immediately after the NF-HYB thermal cycler program 90 minute hold time, thaw at room temperature. Otherwise, if extending the hold time overnight, wait until 30 minutes before the program ends to thaw at room temperature, and vortex to resuspend. Refer to *[Optional] Extend the hold to a maximum of 24 hours* on page 33 for more information.

2. Save the following NF-HYB program on the thermal cycler.
   - Choose the preheat lid option and set to 100°C
   - Reaction volume is 25 µl
   - 95°C for 5 minutes
   - 18 cycles of 1 minute each, starting at 94°C for the first cycle, then decreasing 2°C per cycle
   - Hold for 90 minutes at 58°C.
   - **[Optional]** Extend the hold to a maximum of 24 hours

   Total running time is ~115 minutes.

Procedure

1. Add the following reagents to each well of a new PCR plate or 8-tube strip in the order listed. Creating a master mix of NHB2 and EHB2, or failing to add the reagents in the ***exact*** order and volumes listed, negatively impacts hybridization.
   - Pre-enriched library pool (3-plex) (7.5 µl)
   - NHB2 (blue cap) (12.5 µl)
   - UPIP panel (2.5 µl)
   - EHB2 (2.5 µl)
2. Using a pipette set to 20 µl, pipette each enrichment reaction 10 times to mix.
3. Centrifuge as follows.
   - **[Plate]** Seal the plate with Microseal 'B' and centrifuge at 280 × g for 30 seconds.
   - **[Tube]** Cap the tubes and centrifuge at 280 × g for 30 seconds.
4. Place the sample plate or tube on the preprogrammed thermal cycler and run the NF-HYB program.
5. Proceed immediately to the next procedure before the NF-HYB program hold temperature time ends.

Do not allow the hybridization reactions to cool. Precipitation occurs if the temperature of the hybridization reaction falls below room temperature.

Capture Hybridized Probes

This step uses magnetic beads to capture probes hybridized to the target regions of interest within the libraries. Heated washes remove nonspecific binding from the beads. The enriched library is then eluted from the beads.

Consumables

- EE1 (Enrichment Elution Buffer 1)
- EEW (Enhanced Enrichment Wash)
- ET2 (Elute Target Buffer 2)
- HP3 (2N NaOH)
- SMB4 (Streptavidin Magnetic Beads 4)
- One of the following containers:
  - **[Plate]** 96-well MIDI plate and 96-well PCR plate
  - **[Tube]** 8-tube strip
- One of the following seals:
  - **[Plate]** Microseal 'B' adhesive seal
  - **[Tube]** 8-tube strip caps
- One of the following magnetic stands:
  - **[Plate]** Magnetic Stand-96
  - **[Tube]** Magnetic Stand (8-tube strip)

About Reagents

- EEW
  - Can be cloudy after reaching room temperature.
  - Can appear yellow.
  - Heat before use as instructed.
- SMB4
  - Make sure to use SMB4 and not Illumina Purification Beads for this procedure.
  - Must be at room temperature before use.

Preparation

1. Prepare the following consumables:
   - EE1—Pipette to mix. Centrifuge briefly before use.
   - EEW—Vortex three times for 30 seconds each. The reagent is heated during the procedure.
   - ET2—Vortex to mix. Centrifuge briefly before use.

- HP3—Vortex to mix. Centrifuge briefly before use.
- SMB4—Vortex to resuspend. If precipitate or the bead pellet is present, make sure to reach room temperature, pipette up and down to release the pellet, and then vortex to resuspend.

2. Preheat a minimum of one microheating system with a heat block insert to incubate the sample plate to 58°C. An optional second microheating system can be used to preheat EEW.
3. Place the EEW (amber tube) on its side on the heat block.
   The tube remains on the heat block until the *Wash on page 35*.
4. If the thermal cycler has an incubator option, set it to 58°C. If it does not have an incubator option, save the following program:
   - Choose the preheat lid option and set to 70°C.
   - Reaction volume is 100 µl.
   - Hold at 58°C.

Procedure

Capture

1. Centrifuge the sample plate or tube at 280 × g for 10 seconds.
2. Vortex SMB4 to resuspend, and then add 62.5 µl to each well or tube, and then mix thoroughly as follows:
   - **[Plate]** Seal the plate and shake at 1200 rpm for 4 minutes.
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
3. Place the sample plate or tube in the thermal cycler, close the lid, and incubate for 15 minutes at 58°C.
   The thermal cycler runs continuously through the capture and four washes.
   Proceed to step 4 while the sample incubates.
4. Centrifuge the sample plate or tube at 280 × g for 10 seconds.
5. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
6. Remove and discard all supernatant from each well or tube.

Wash

1. Remove from the magnetic stand.
2. Add 50 µl preheated EEW to each well or microcentrifuge tube, and then mix thoroughly as follows.
   - If using a tube, cap the tube and then vortex at high speed three times for 10 seconds each. Do not centrifuge.
   - If using a plate, seal and shake at 2400 rpm for 4 minutes.
3. Return unused EEW to the microheating system and keep heated.

4. Place the sample plate or tube in the thermal cycler, close the lid, and incubate for 5 minutes at 58°C.
5. Centrifuge at 280 × g for 3 seconds.
6. Immediately place the plate or microcentrifuge tube on a magnetic stand and wait until the liquid is clear (~2 minutes).
7. Remove and discard all supernatant from each well or tube.
8. Repeat steps 1–7 two additional times for a total of three washes.

Transfer Wash

1. Remove the plate or tube from the magnetic stand.
2. Add 50µl preheated EEW to each well or microcentrifuge tube, and then mix thoroughly as follows.
   - If using a tube, cap the tube and then vortex at high speed three times for 10 seconds each. Do not centrifuge.
   - If using a plate, seal and shake at 2400 rpm for 4 minutes.
3. Transfer 50 µl to a new MIDI plate or a new tube, and seal accordingly.

   Transferring the reagent minimizes carryover of residual reagents that can inhibit downstream PCR.

4. Place the sample plate or tube in the thermal cycler, close the lid, and incubate for 5 minutes at 58°C.
5. Centrifuge at 280 × g for 3 seconds.
6. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
7. Remove and discard all supernatant from each well or tube.
8. Centrifuge the plate or the tube at 280 × g for 30 seconds.
9. Place on a magnetic stand for 10 seconds.
10. Use a 20 µl pipette to remove and discard residual liquid from each well or from the tube.
11. Immediately proceed to *Elute* on page 36 to prevent excessive drying of the beads and library yield loss.

Elute

1. Combine the following volumes to prepare an Elution Master Mix. Multiply each volume by the number of samples being processed.
   Additional reagent is included in the volume to ensure accurate pipetting due to the potential of reagent foaming.
   - EE1 (28.5 µl)
   - HP3 (1.5 µl)
2. Pipette the Elution Master Mix thoroughly to mix, and then set aside at room temperature.
3. Remove the sample plate or tube from the magnetic stand.

4. Add 23 µl Elution Master Mix to each well or tube, and then mix thoroughly as follows.
    - **[Plate]** Seal plate and shake at 1800 rpm for 2 minutes.
    - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
5. Incubate the plate or tube at room temperature for 2 minutes.
6. Centrifuge at 280 × g for 30 seconds.
7. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
8. Transfer 21 µl supernatant from the MIDI plate or tube strip to the corresponding well of a new 96-well PCR plate or a new 8-tube strip.
9. Add 4 µl ET2 to each well or to the tube containing 21 µl supernatant.
10. Set pipette to 20 µl and slowly pipette each well or the tube 10 times to mix.
11. Centrifuge the sample plate or the tube at 280 × g for 30 seconds.

Amplify Enriched Library

This step uses PCR to amplify the targeted UPIP library.

Consumables

- EPM (Enhanced PCR Mix)
- PPC (PCR Primer Cocktail)
- **[Plate]** Microseal 'B' adhesive seal
- **[Tube]** 8-tube strip caps

Preparation

1. Prepare the following consumables:
    - EPM—Invert to mix, then centrifuge briefly.
    - PPC—Invert to mix, then centrifuge briefly.
2. Save the following AMP program on the thermal cycler.
    - Choose the preheat lid option and set to 100°C
    - Set the reaction volume to 50 µl
    - 98°C for 30 seconds
    - 17 cycles of:
        - 98°C for 10 seconds
        - 60°C for 30 seconds
        - 72°C for 30 seconds

- 72°C for 5 minutes
- Hold at 10°C

Total running time is ~45 minutes.

Procedure

1. Add 5 µl PPC to each well or tube.
2. Add 20 µl EPM to each well or tube and mix thoroughly as follows.
   - **[Plate]** Seal plate and shake at 1200 rpm for 1 minute.
   - **[Tube]** Pipette 10 times to mix, and then cap the 8-tube strip.
3. Centrifuge the plate or 8-tube strip at 280 × g for 30 seconds.
4. Place on the preprogrammed thermal cycler and run the AMP program.

SAFE STOPPING POINT

If you are stopping, store at 2°C to 8°C for up to two days. Alternatively, leave on the thermal cycler for up to 24 hours.

Prepare for Protocol

1. Remove reagents from storage.
2. Remove the reagents from the box and prepare as follows.

Table 8 15°C to 30°C Storage

Reagent	Box Name	Instructions
IPB	IPB	Bring to room temperature.

Table 9 2°C to 8°C Storage

Reagent	Box Name	Instructions
RSB	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature.

Clean Up Amplified Enriched Library

This step uses IPB to purify the enriched library pools and remove unwanted products.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)

- Freshly prepared 80% ethanol (EtOH)
- **[Plate]** Microseal 'B' adhesive seals
- One of the following containers:
  - **[Plate]** 96-well MIDI plate and 96-well PCR plate
  - **[Tube]** 8-tube strip
- One of the following magnetic stands:
  - **[Plate]** Magnetic Stand able to accommodate 96-well PCR plate
  - **[Tube]** Magnetic stands able to accommodate PCR tube strips

About Reagents

- IPB
  - Must be at room temperature before use.
  - Resuspend before each use.
  - Resuspend frequently to make sure the beads are evenly distributed.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. RSB—Vortex to mix.
2. For each sample, prepare 400 µl fresh 80% EtOH from absolute ethanol. Including an overage of 20% is recommended.

Procedure

1. Centrifuge the PCR samples at 280 × g for 30 seconds.
2. Transfer 50 µl supernatant from each well of the PCR plate or from the 8-tube strip, to the corresponding well of a new MIDI plate or a new 8-tube strip.
3. Resuspend IPB as follows.
   a. To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 seconds.
   b. If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.
4. Add 90 µl IPB to each well or tube, and then mix thoroughly as follows.
   - **[Plate]** Seal the plate and shake at 1800 rpm for 1 minute.
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
5. Incubate the plate or tube at room temperature for 5 minutes.

6. Centrifuge at 280 × g for 1 minute.
7. Place on a magnetic stand and wait until liquid is clear (~5 minutes).
8. Using a pipette set to 140 µl, remove and discard all supernatant from each well or tube.
9. Wash beads as follows.
    a. Keep on the magnetic stand, add 200 µl fresh 80% EtOH without mixing.
    b. Wait for 30 seconds.
    c. Without disturbing the beads, remove and discard supernatant.
10. Wash beads a **second** time.
11. Use a 20 µl pipette to remove and discard residual EtOH from each well or tube.
12. Air-dry on the magnetic stand for 5 minutes.
13. Remove from the magnetic stand and add 32 µl RSB to each well or tube.
14. Mix thoroughly as follows.
    - **[Plate]** Seal the plate and shake at 1800 rpm for 1 minute.
    - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
15. Incubate the plate or tube at room temperature for 5 minutes.
16. Centrifuge at 280 × g for 30 seconds.
17. Place on a magnetic stand and wait until liquid is clear (~2 minutes).
18. Transfer 30 µl supernatant from the MIDI plate or from the 8-tube strip, to the corresponding well of a new MIDI plate or a new 8-tube strip.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal, or cap the tube, and store at -25°C to -15°C for up to 7 days.

Check Enriched Libraries

Perform the following to check the concentration and quality of the enriched libraries.

1. Quantify 1 µl enriched libraries to determine library concentration. Follow the manufacturer's recommendations.
2. Run 1 µl pooled library or the individual libraries on a Bioanalyzer using a high sensitivity DNA kit. Expect a mean fragment size ~350 bp and distribution of DNA fragments with a size range from ~200 bp to ~1000 bp.

Figure 1 Bioanalyzer Trace: Example 1

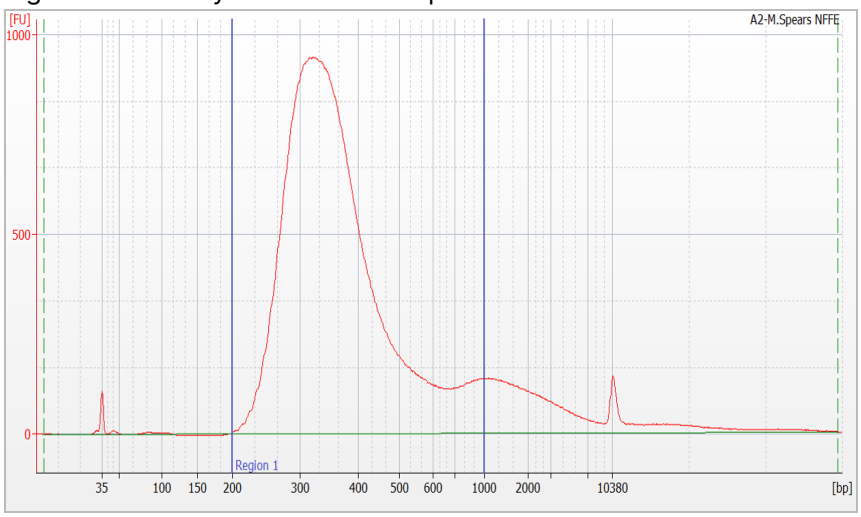
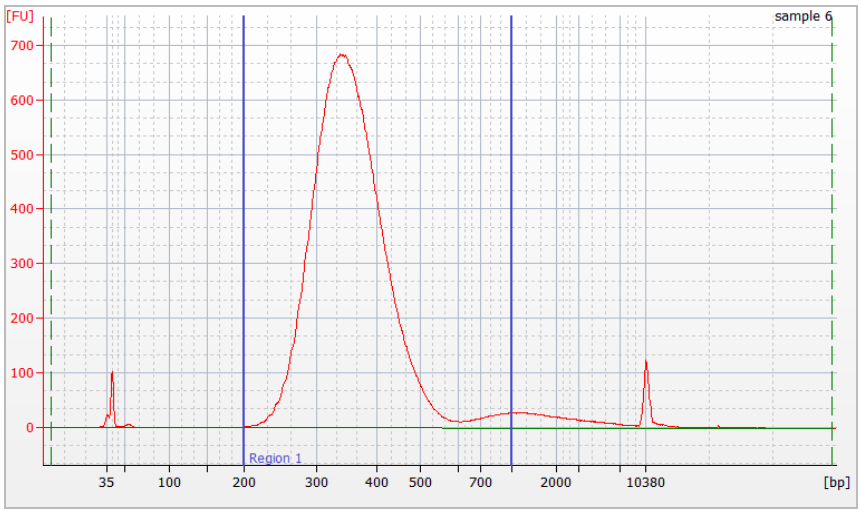


Figure 2 Bioanalyzer Trace: Example 2



Dilute Libraries to the Starting Concentration

This step dilutes libraries to the starting concentration for your sequencing system and is the first step in a serial dilution. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration.

For sequencing, Illumina recommends a minimum 1 x 100 bp Sequencing and 10 cycles per Index Read. If you want optional and additional overlapped reads/raw coverage, you can sequence up to 1 x 150, 2 x 100, or 2 x 150. For single-end reads, analyze 1 million total reads per sample; for paired-end reads analyze 2 million total reads per sample.

1. Calculate the molarity value of the library or pooled libraries using the following formula.
    - For libraries qualified on a Bioanalyzer, use the average size obtained for the library.

- For all other qualification methods, use 350 bp as the average library size.

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

2. Using the molarity value, calculate the volumes of RSB and library needed to dilute libraries to the starting concentration for your system.

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)
iSeq 100 (v1 reagents)	2	100
iSeq 100 (v2 reagents)	2	75
MiniSeq	2	2
MiSeq (v3 reagents)	4	10–12
MiSeq i100*	0.8	80
NextSeq 550 and NextSeq 500	2	1.4–1.5
NextSeq 1000 and NextSeq 2000	2	1000

* Denaturation performed onboard. Refer to the system guide.

3. Dilute libraries using RSB:
   - **Libraries quantified as a multiplexed library pool**—Dilute the pool to the starting concentration for your system.
     Add 10 µl each diluted library to a tube to create a multiplexed library pool.
4. Follow the denature and dilute instructions for your system to dilute to the final loading concentration.
   - Refer to the Illumina Denature and Dilute protocol generator and the Illumina support site for pool and denature instructions.
   - The final loading concentrations are a starting point and general guideline. Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.

Resources & References

The Illumina support site provides software, training resources, product compatibility information, and the following documentation. Always check support pages for the latest versions.

Additional Resources

Resource	Description
Index Adapters Pooling Guide	Provides pooling guidelines and dual-index strategies for using the 10-base pair Illumina DNA/RNA UD Indexes or 8-base pair Nextera XT and Nextera XT v2 Indexes with the Illumina DNA Prep with Enrichment kit.
Illumina Adapter Sequences	Provides the nucleotide sequences that comprise Illumina oligonucleotides used in Illumina sequencing technologies.

Revision History

Document	Date	Description of Change
Document # 200028760 v04	September 2026	Updated name of tagmentation bead box from Illumina DNA Prep – Tagmentation (S) Beads to Illumina DNA/RNA Prep – Tagmentation (S) Beads. Standardized thermal cycler specifications. Clarified SMB4 preparation timing based off of thermal cycler hold timing.
Document # 200028760 v03	October 2025	Updated SMB3 to SMB4. Updated SMB4 thaw and preparation instructions. Updated thermal cyclers. Updated DNA/RNA UD Indexes naming. Added MiSeq i100.
Document # 200028760 v02	November 2023	Restructured from a deviation document, to a complete stand-alone document. Removed EOL hardware. Removed EOL IDT for Illumina Nextera DNA Unique Dual (UD) from additional resources.
Document # 200028760 v01	October 2022	Updated language for AMR detection. Updated unit of measure for pipette volume in the equipment table.
Document # 200028760 v00	October 2022	Initial release.

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