Illumina RNA Prep with Enrichment Kit: Automated on the Biomek NGeniuS System

Accelerating Answers

BECKMAN COULTER Life Sciences

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App Template Descriptions

App Template Illumina* RNA Prep with Enrichment

The Illumina* RNA Prep with Enrichment App Template on the Biomek NGeniuS Next Generation Library Prep System enables the generation of libraries compatible with Illumina sequencing platforms. The App Template is split into two parts, the Library Preparation App and the Target Enrichment App.

The **Illumina* RNA Prep with Enrichment: Library Preparation App** starts with the 'Normalize Samples' section then runs cDNA synthesis to clean up of tagmented libraries. A range between 4 to 24 libraries are produced in a single batch run. The App is optimized for use of 10–100 ng of purified total RNA sample input. Lower input amounts or use of RNA input from degraded or FFPE samples can reduce library yield. The user is required to specify the concentration of the starting material to enable normalization. The number of Tagmentation PCR cycles can also be adjusted between 14 and 17 cycles, and bead drying time following ethanol washes can be set between 2 and 5 minutes. 80% ethanol wash volumes have been reduced from 175 µl to 50 µl**.** In certain instances, the requested volume of reagent RVT may fall below recommended liquid level sensing threshold for the Biomek NGeniuS Next Generation Library Prep System, as the reagent dead volume has been reduced in order to ensure that the maximum number of reactions can be obtained from the kit.

Library quantification, normalization and enrichment pooling steps are to be performed off-deck.

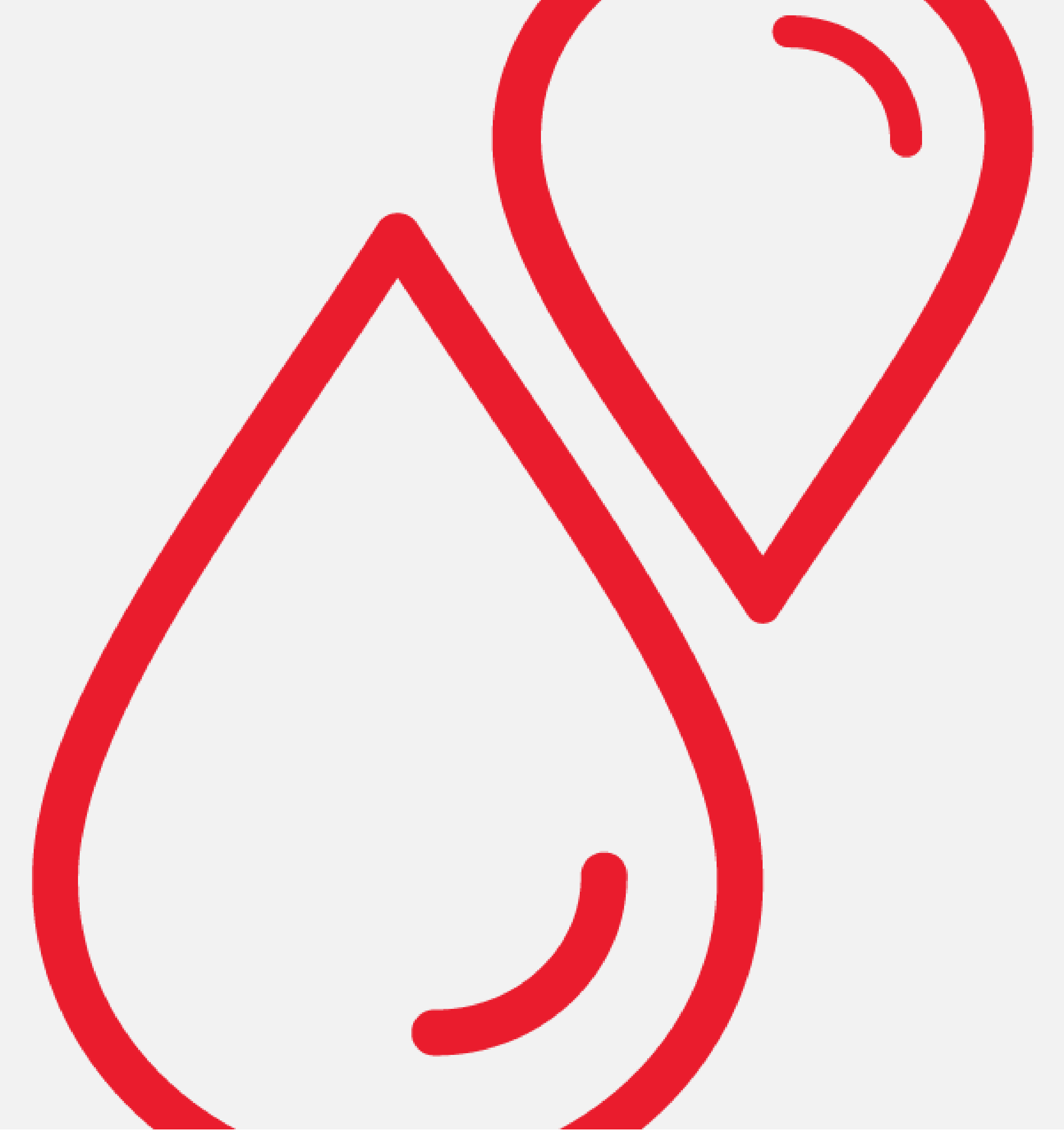
The **Illumina* RNA Prep with Enrichment: Target Enrichment App** runs from capture probe hybridization to clean up of enriched libraries and allows the user to produce between 4 to 16 pools in a single batch run. The user may utilize 200ng one-plex enrichment libraries or 600ng three-plex enrichment libraries as sample input. The hybridization time has been limited to the minimum time listed in the manual protocol (90 minutes) to reduce application run time and cannot be changed. 80% ethanol wash volumes have been reduced from 175 µl to 50 µl. The post enrichment amplification PCR program extension time has been increased from 30 seconds to 60 seconds to improve assay performance.

The App templates were designed using the Illumina RNA Prep with Enrichment (L) Tagmentation Reference guide (Document # 1000000124435 v04). The App Template utilizes the Illumina RNA Prep with Enrichment (L) Tagmentation (96 Samples) kit (Illumina Part Number 20040537) in conjunction with the IDT® for Illumina® DNA/RNA UD Indexes Sets A, B, C, or D (Illumina Part Numbers 20027213, 20027214, 20042666, or 20042667) or the Illumina DNA/RNA UD Indexes Sets A, B, C or D (Illumina Part Numbers 20091654, 20091656, 20091658, 20091660).

*Illumina is a trademark of Illumina, Inc. IDT is a registered trademark of Integrated DNA Technologies.

Document ID 2023-GBL-EN-104468-v3

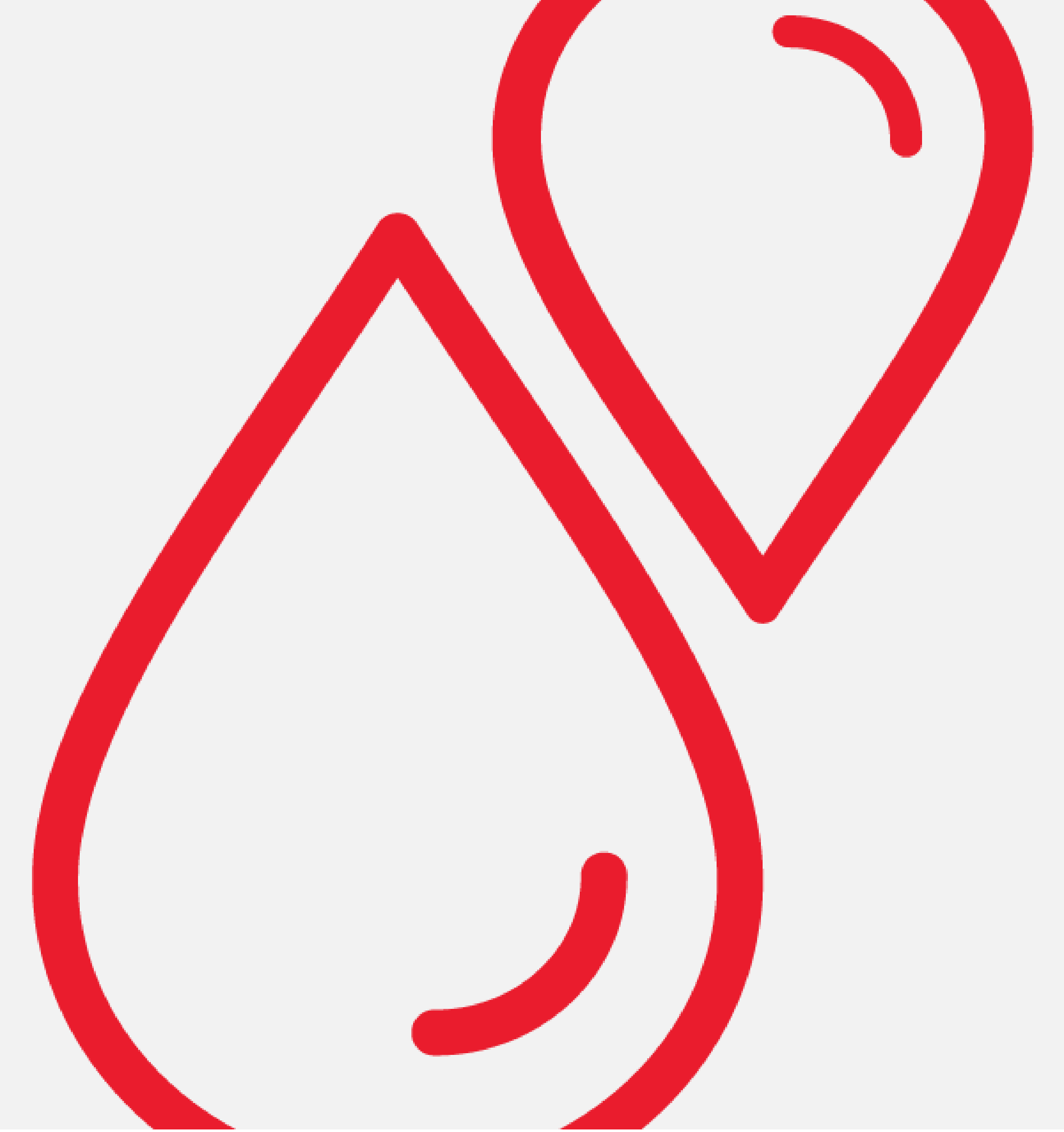
Scoping



Scoping

Scoping Parameter	
Author	Illumina scientists with support from Beckman Coulter Life Sciences
Kit	Illumina RNA Prep with Enrichment (L) Tagmentation Reference guide (Document # 1000000124435 v04)
Supported Features	Illumina RNA Prep with Enrichment: Library will support 4-24 libraries per batch. Illumina RNA Prep with Enrichment: Target Enrichment will support 4 to 16 wells representing either single-plex or three plex pools in each batch.
Excluded Features	Library pooling and concentration on instrument
Kit Part Numbers	• Illumina RNA Prep with Enrichment (L) Tagmentation (96 Samples) kit (Illumina Part Number 20040537) • IDT® for Illumina® DNA/RNA UD Indexes Sets A, B, C, or D (Illumina Part Numbers 20027213, 20027214, 20042666, or 20042667) or the Illumina DNA/RNA UD Indexes Sets A, B, C or D (Illumina Part Numbers 20091654, 20091656, 20091658, 20091660) • Multiple panels can be used with this kit. The Illumina Exome Panel – Enrichment Oligos Only (Illumina Part Number 20020183) and the TruSight RNA Pan-Cancer Oligo Panel (Illumina Part Number 20046104) were used to test the App Templates

App Details



Illumina RNA Prep with Enrichment kit (IRPE)

Illumina RNA Prep with Enrichment is designed to prepare and enrich libraries prepared from a variety of RNA types for sequencing on Illumina platforms. Notable features include:

- Compatibility with purified total RNA or degraded RNA from FFPE
- Inputs range from 10ng of total RNA (20ng for FFPE) to 100ng
- Compatibility with a variety of pre-made (CEX, RVOP, RPID panels from Illumina) or custom-made panels
- Useful for characterizing whole transcriptomes or respiratory pathogens
- Enrichments performed on either 1-plex or 3-plex pools

1 Denature RNA
Hands-on: 5 minutes
Total: 15 minutes
Reagents: EPH3

2 Synthesize First Strand cDNA
Hands-on: 5 minutes
Total: 45 minutes
Reagents: FSA, RVT

3 Synthesize Second Strand cDNA
Hands-on: 20 minutes
Total: 1.5 hours
Reagents: 80% EtOH, AMPure XP, RSB, SMM

Safe Stopping Point

4 Tagment cDNA
Hands-on: 20 minutes
Total: 1.5 hours
Reagents: EBLTL, EPM, ST2, TB1, TWB, UDP00XX

Safe Stopping Point

5 Clean Up Library
Hands-on: 10 minutes
Total: 20 minutes
Reagents: 80% EtOH, AMPure XP, RSB

Safe Stopping Point

6 Normalize Library
Hands-on: 15 minutes
Total: 15 minutes
Reagents: RSB

7 Hybridize Probes
Hands-on: 5 minutes
Total: 2 hours
Reagents: EHB2, enrichment oligos, NHB2

8 Capture Hybridized Probes
Hands-on: 20 minutes
Total: 70 minutes
Reagents: EE1, EEW, ET2, HP3, SMB3

Safe Stopping Point

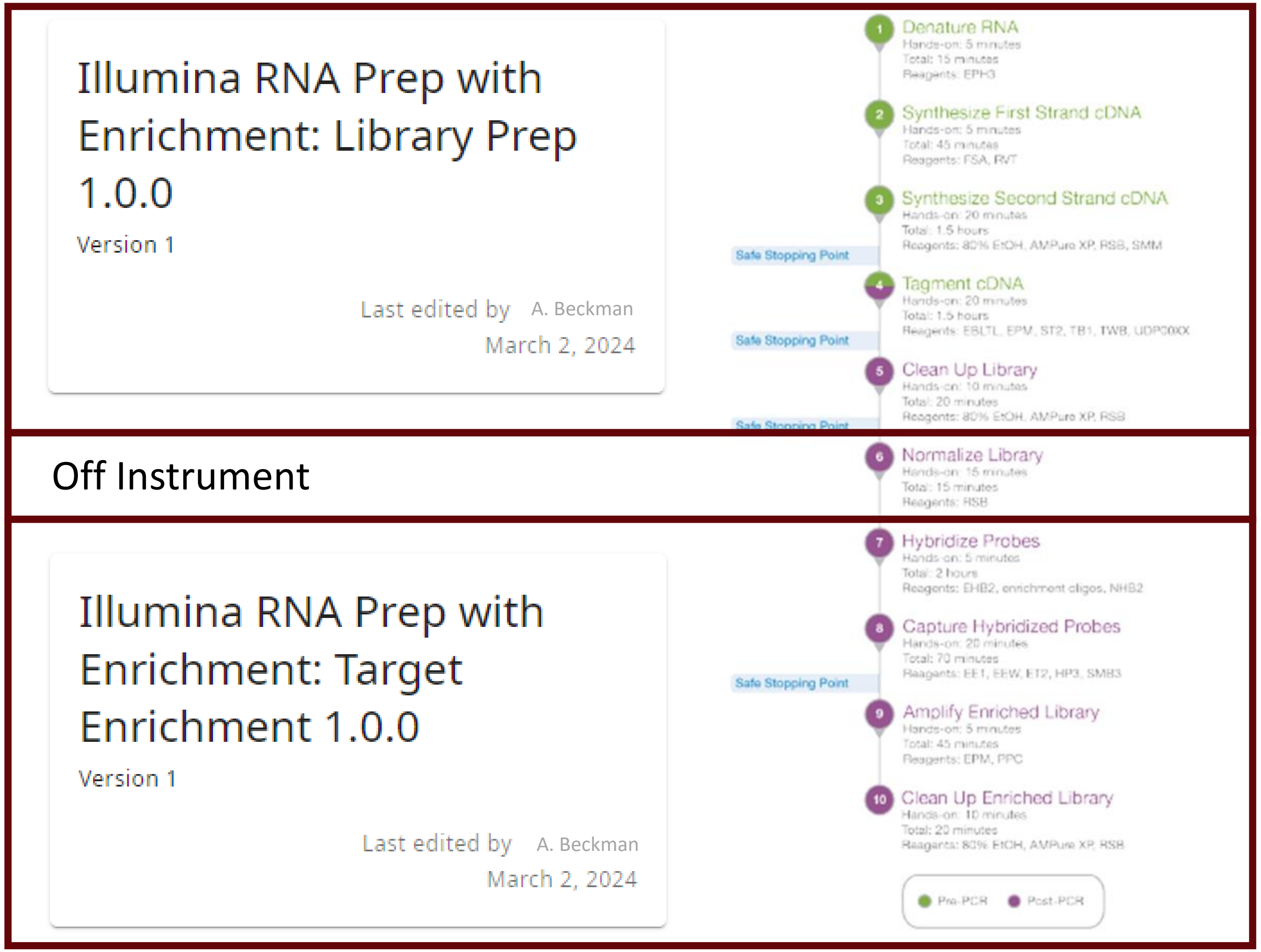
9 Amplify Enriched Library
Hands-on: 5 minutes
Total: 45 minutes
Reagents: EPM, PPC

10 Clean Up Enriched Library
Hands-on: 10 minutes
Total: 20 minutes
Reagents: 80% EtOH, AMPure XP, RSB

● Pre-PCR ● Post-PCR

Sections Automated

Both App Templates have a single loading operation, meaning they can both be set up to run unattended.



Illumina RNA Prep with Enrichment: Library Prep 1.0.0

Version 1

Last edited by A. Beckman
March 2, 2024

1 Denature RNA
Hands-on: 5 minutes
Total: 15 minutes
Reagents: EPH3

2 Synthesize First Strand cDNA
Hands-on: 5 minutes
Total: 45 minutes
Reagents: FSA, RVT

3 Synthesize Second Strand cDNA
Hands-on: 20 minutes
Total: 1.5 hours
Reagents: 80% EtOH, AMPure XP, RSB, SMM

Safe Stopping Point

4 Tagment cDNA
Hands-on: 20 minutes
Total: 1.5 hours
Reagents: EBLTL, EPM, ST2, TB1, TWB, UDP00XX

Safe Stopping Point

5 Clean Up Library
Hands-on: 10 minutes
Total: 20 minutes
Reagents: 80% EtOH, AMPure XP, RSB

Safe Stopping Point

Off Instrument

6 Normalize Library
Hands-on: 15 minutes
Total: 15 minutes
Reagents: RSB

Illumina RNA Prep with Enrichment: Target Enrichment 1.0.0

Version 1

Last edited by A. Beckman
March 2, 2024

7 Hybridize Probes
Hands-on: 5 minutes
Total: 2 hours
Reagents: EHB2, enrichment oligos, NHB2

8 Capture Hybridized Probes
Hands-on: 20 minutes
Total: 70 minutes
Reagents: EE1, EEW, ET2, HP3, SMB3

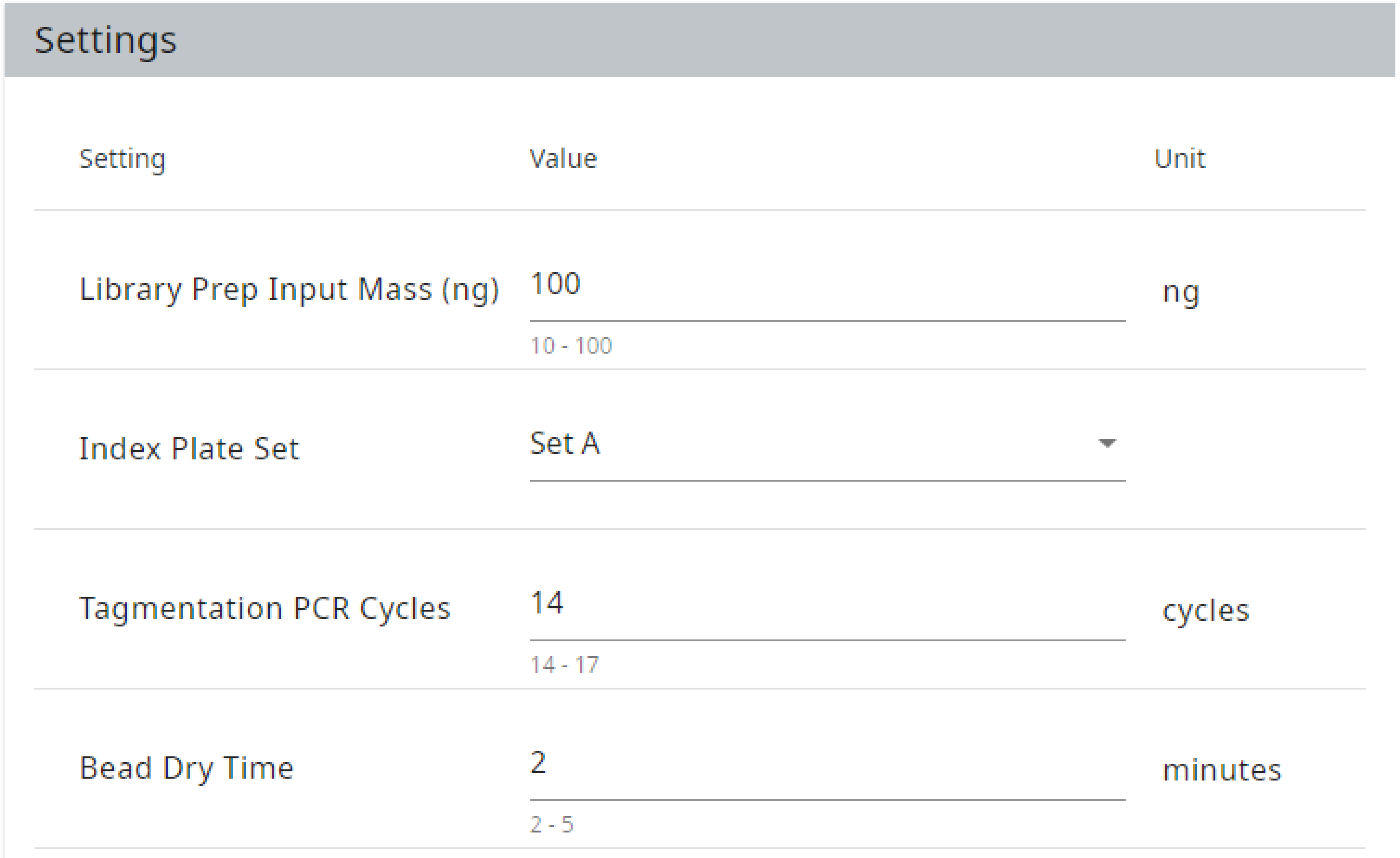
Safe Stopping Point

9 Amplify Enriched Library
Hands-on: 5 minutes
Total: 45 minutes
Reagents: EPM, PPC

10 Clean Up Enriched Library
Hands-on: 10 minutes
Total: 20 minutes
Reagents: 80% EtOH, AMPure XP, RSB

● Pre-PCR ● Post-PCR

Illumina RNA Prep with Enrichment: Library Prep App Settings



Settings

Setting	Value	Unit
Library Prep Input Mass (ng)	100 10 - 100	ng
Index Plate Set	Set A	
Tagmentation PCR Cycles	14 14 - 17	cycles
Bead Dry Time	2 2 - 5	minutes

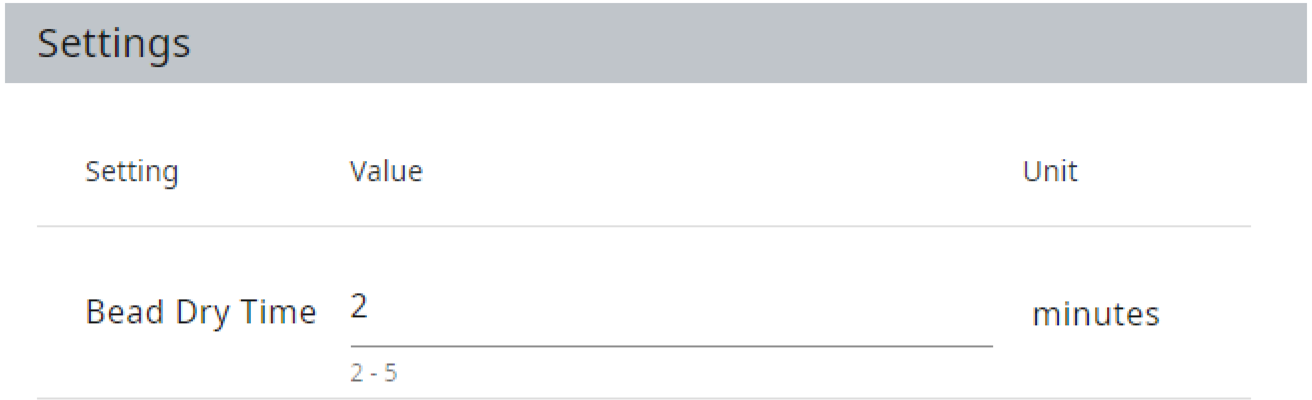
High-quality RNA with DV200 > 80%: 10-100 ng
FFPE and RNA with DV200 < 80%: 20-100 ng

Index Plate used. Options include Set A, Set B, Set C, or Set D

High-quality RNA with DV200 > 80%: 14 cycles
FFPE and RNA with DV200 < 80% or Extracted Viral RNA: 17

Adjust drying time according to laboratory conditions

Illumina RNA Prep with Enrichment: Target Enrichment App Settings



Settings

Setting	Value	Unit
Bead Dry Time	2 2 - 5	minutes

Adjust drying time according to laboratory conditions

Illumina RNA Prep with Enrichment: Library Prep Volumes Requested by Batch Size

Reagent	Stated Vial Volume (µL)*	Vials Per Kit	4 Samples Requested Volume (µL)	8 Samples Requested Volume (µL)	16 Samples Requested Volume (µL)	24 Samples Requested Volume (µL)
EBLTL	290	4	72	114	198	282
EPH3	241	4	55.7	91.4	162.8	234.2
EPM	610	4	132	224	408	592.01
FSA	238	4	58.28	87.95	147.27	206.6
RSB (-20°C)	1647	2	121.9	203.8	367.6	531.4
RSB (4°C)	1647	2	171.4	242.8	385.6	528.4
RVT†	96	1	19.12	22.42	29.02	35.592
SMM	630	4	120	220	420	620
ST2	350	4	82	124	208	292
TB1	290	4	72	114	198	282
TWB**	45100	1	4260	5520	8040	10560

* All values in µL, consumed volumes are less than requested due to source labware dead volume requirements

**TWB comes in a 50 mL conical and is stored in a Bulk Reservoir

†RVT reagent is supplied in a limiting fashion. A full 96 reactions will not be possible due to the dead volume demands of the automation platform.

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Illumina RNA Prep with Enrichment: Target Enrichment Volumes Requested by Batch Size

Reagent	Stated Vial Volume (µL)*	Vials Per Kit	4 Pools Requested Volume (µL)	8 Pools Requested Volume (µL)	16 Pools Requested Volume (µL)
EE1	580	2	209.6	329.31	568.71
EEW**	4100	4	3466	4432	6364
EHB2	200	1	40.5	51	72
EPM	610	2	100	180	340
ET2	200	1	56.8	73.6	107.2
HP3	200	1	68.4	74.71	87.3
NHB2	440	1	67.5	120	225
Oligos†	80	1	27.5	38	59
PPC	320	1	61	82	124
RSB (4°C)	1510	1	234.4	368.8	637.6
SMB3	1200	2	322.5	585	1110

* All values in µL, consumed volumes are less than requested due to source labware dead volume requirements

**EEW is stored in a Bulk Reservoir

†Oligos (CEX, RPO, or other panel) are supplied in limiting fashion. A full 32 reactions (listed on the panel) will not be possible due to the dead volume demands of the automation platform.

Illumina RNA Prep with Enrichment: Reagents Required But Not Supplied

Reagent	Supplier	Part Number
AMPureXP, 60 mL	Beckman Coulter	A63881
Ethyl Alcohol, pure (500 mL)	Sigma Aldrich	E7023
Ultrapure water, nuclease-free	General Lab Supplier	N/A

See the Illumina RNA Prep with Enrichment (L) Tagmentation Reference guide (Document # 1000000124435 v04) Consumables and Equipment list for additional reagents and equipment needed for operations performed off the Biomek NGeniuS System

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Illumina RNA Prep with Enrichment: Library Prep Batches Per Kit

Batch Size	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
Batches per kit	9	8	7	7	6	6	6	5	5	5	4	4	4	4	4	4	3	3	3	3	3
Samples	36	40	42	49	48	54	60	55	60	65	56	60	64	68	72	76	60	63	66	69	72
Largest batch with leftover volume	-	-	6	-	7	-	-	7	-	-	13	9	5	-	-	-	16	13	10	7	4
Total samples from kit	36	40	48	49	55	54	60	62	60	65	69	69	69	68	72	76	76	76	76	76	76

- The ***Batch size*** can be run ***Batches per kit*** times, leaving enough reagent volume to do one additional batch with ***Largest batch with leftover volume*** samples.
- Run combinations calculated based on **published** reagent vial volumes.

Illumina RNA Prep with Enrichment: Target Enrichment Batches Per Kit

Batch Size	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Batches per kit	4	4	3	3	3	2	2	2	2	2	1	1	1	1	1	1
Samples	4	8	9	12	15	12	14	16	18	20	11	12	13	14	15	16
Largest batch with leftover volume	-	-	-	-	-	6	4	2	-	-	10	9	8	7	6	5
Total samples from kit	4	8	9	12	15	18	18	18	18	20	21	21	21	21	21	21

- The ***Batch size*** can be run ***Batches per kit*** times, leaving enough reagent volume to do one additional batch with ***Largest batch with leftover volume*** samples.
- For this app template, a batch consists of the number of enrichment pools being run.
- Run combinations calculated based on **published** reagent vial volumes.

Illumina RNA Prep with Enrichment: Library Prep Estimated Time of Completion

Samples	4 Libraries (hr:min:sec)	8 Libraries (hr:min:sec)	16 Libraries (hr:min:sec)	24 Libraries (hr:min:sec)
Index Aliquot	00:04:06	00:04:06	00:04:04	00:04:02
Reagent Aliquot	00:23:29	00:37:13	00:45:51	00:55:13
Processing	06:46:56	07:27:22	10:14:12	12:57:44
Total ETC	07:14:32	08:08:42	11:04:08	13:56:59

Times (hours:minutes:seconds) calculated based on default 2-minute bead dry time, with all plate-based indices contiguous starting with well A1. Does not include times needed for manual interactions (*e.g*., reagent thawing, placing labware into Biomek NGeniuS System).

Illumina RNA Prep with Enrichment: Target Enrichment Estimated Time of Completion

Samples	4 Pools (hr:min:sec)	8 Pools (hr:min:sec)	16 Pools (hr:min:sec)
Reagent Aliquot	00:30:13	00:33:57	00:39:42
Processing	05:59:32	06:28:19	08:20:22
Total ETC	06:29:45	07:02:17	09:00:04

Times (hours:minutes:seconds) calculated based on default 2-minute bead dry time. Hybridization time for Target Enrichment App locked at the minimum 90-minute hold time listed in the IFU. Does not include times needed for manual interactions (*e.g*., reagent thawing, placing labware into Biomek NGeniuS System).

Illumina RNA Prep with Enrichment: Library Prep Consumables

		Batch Size (Libraries)			
Consumable	**Part number**	**4**	**8**	**16**	**24**
RVs	C62705	8	8	8	8
Bulk Reservoirs	C62707	2	2	2	2
Lids	C62706	5	5	5	5
Millitips (boxes)	C59585	44 (1)	78 (1)	142 (2)	198 (3)
Microtips (boxes)	C62712	207 (1)	389 (2)	757 (2)	1133 (3)
Seal plate	C70665	1	1	1	1
Price Per Sample ($)*	-	$30.10	$18.40	$12.03	$11.02

* Costs assume using fresh tip boxes. Some clean tips will remain each run, reducing cost of subsequent runs. Costs do not include empty tip boxes for tip disposal. Costs calculated as of May 2024, subject to change.

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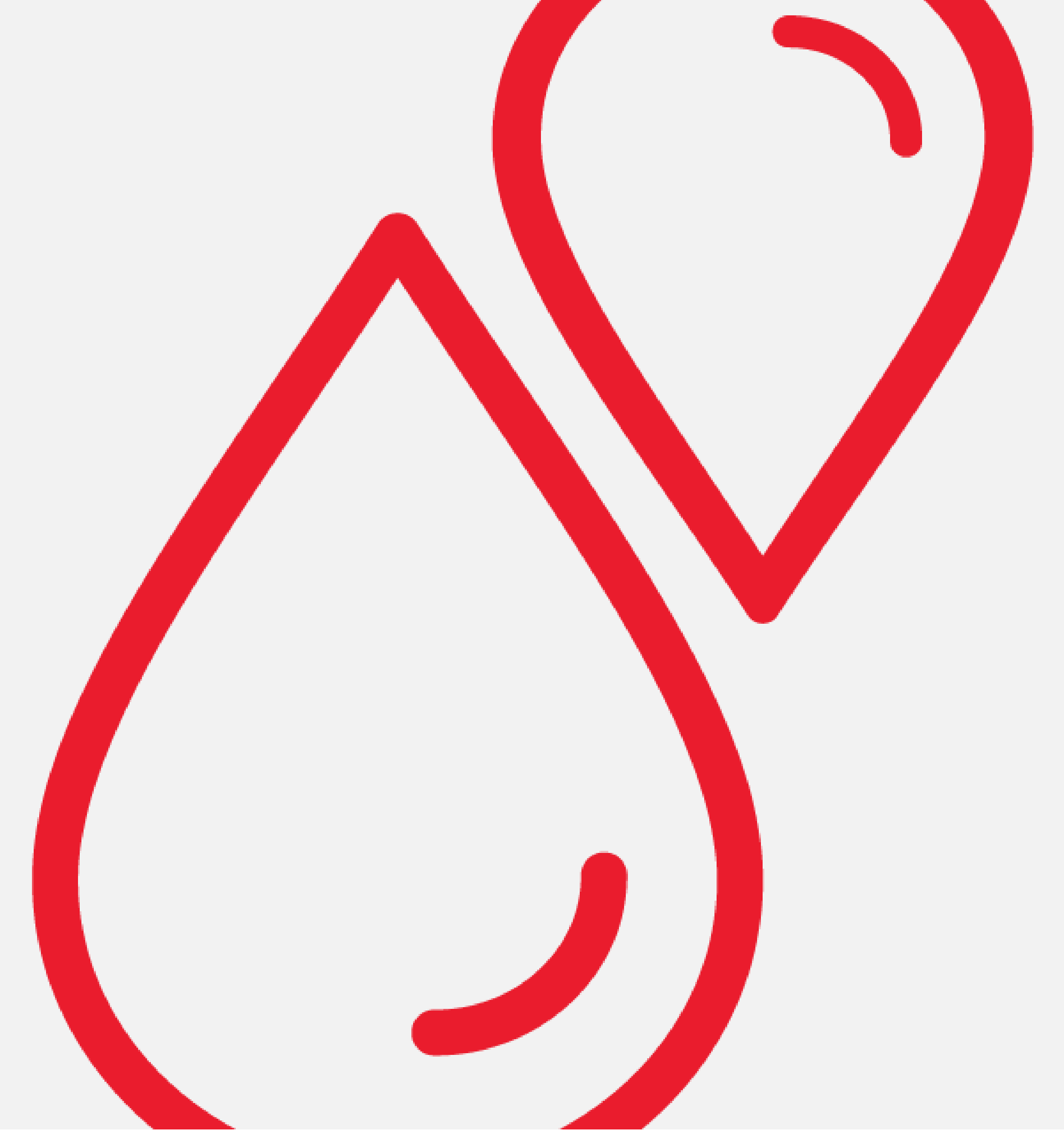
Illumina RNA Prep with Enrichment: Target Enrichment Consumables

		Batch Size (Pools)		
Consumable	**Part number**	**4**	**8**	**16**
RVs	C62705	8	8	8
Bulk Reservoirs	C62707	2	2	2
Lids	C62706	5	5	5
Millitips (boxes)	C59585	44 (1)	84 (1)	148 (2)
Microtips (boxes)	C62712	148 (1)	276 (1)	548 (2)
Seal plate	C70665	1	1	1
Price Per Sample ($)*	-	$30.10	$15.05	$12.03

* Costs assume using fresh tip boxes. Some clean tips will remain each run, reducing cost of subsequent runs. Costs do not include empty tip boxes for tip disposal. Costs calculated as of May 2024, subject to change.

Demonstration Data (App Template Version 1.0.0)



Illumina Enrichment Panels

Metric	CEX (Illumina Exome Panel)	RPO (RNA Pan-Cancer)
Panel Size (Mb)	45	0.403
Number of Probes	425,437	57,010
Number of Target Regions	214,126	21,043
Number of Target Genes	21,415	1,385

Demonstration Runs

Experiment	Library Number	Pool Number	Input Mass (ng)	Panel	App Settings	Instrument	Sequencing	Analysis Workflow
1	16	6 (3-plex and 1-plex)	100	CEX	Tagmentation PCR Cycles: 14 Ethanol Dry Time: 2 minutes	Illumina UK	(CEX): NovaSeq 6000 S4 flowcell 2x100 bp PE run (RPO): NovaSeq 6000 SP flowcell 2x74 bp PE run	FASTQ Toolkit v2.2.0 (Down sampled to 25 M reads) Enrichment v3.1.0
2	16	6 (3-plex and 1-plex)	10	CEX	Tagmentation PCR Cycles: 14 Ethanol Dry Time: 2 minutes	Illumina UK	NovaSeq 6000 SP flowcell 2x100 bp PE run	FASTQ Toolkit v2.2.0 (Down sampled to 25 M reads) Enrichment v3.1.0
3	4	4 (1-plex)	100	CEX	Tagmentation PCR Cycles: 14 Ethanol Dry Time: 2 minutes	Illumina San Diego	NovaSeq 6000 S2 flowcell 2x100 bp PE run	FASTQ Toolkit v2.2.0 (Down sampled to 25 M reads) Enrichment v3.1.0
4	24	8 (3-plex)*	100	RPO	Tagmentation PCR Cycles: 14 Ethanol Dry Time: 2 minutes	Illumina UK	NovaSeq 6000 S4 flowcell 2x74 bp PE run	FASTQ Toolkit v2.2.0 (Down sampled to 4 M reads) RNA Seq Alignment v1.1.1
5	15	15 (1-plex)	10	RPO	Tagmentation PCR Cycles: 14 Ethanol Dry Time: 2 minutes	Illumina San Diego	NovaSeq 6000 S2 flowcell 2x74 bp PE run	FASTQ Toolkit v2.2.0 (Down sampled to 4 M reads) RNA Seq Alignment v1.1.1
6	24	8 (3-plex)*	10	RPO	Tagmentation PCR Cycles: 14 Ethanol Dry Time: 2 minutes	Illumina UK	NovaSeq 6000 SP flowcell 2x74 bp PE run	FASTQ Toolkit v2.2.0 (Down sampled to 4 M reads) RNA Seq Alignment v1.1.1
7	16	6 (3-plex and 1-plex)	100	CEX	Tagmentation PCR Cycles: 14 Ethanol Dry Time: 2 minutes	Beckman Coulter	NovaSeq 6000 S4 flowcell 2x100 bp PE run	FASTQ Toolkit v2.2.0 (Down sampled to 25 M reads) Enrichment v3.1.0

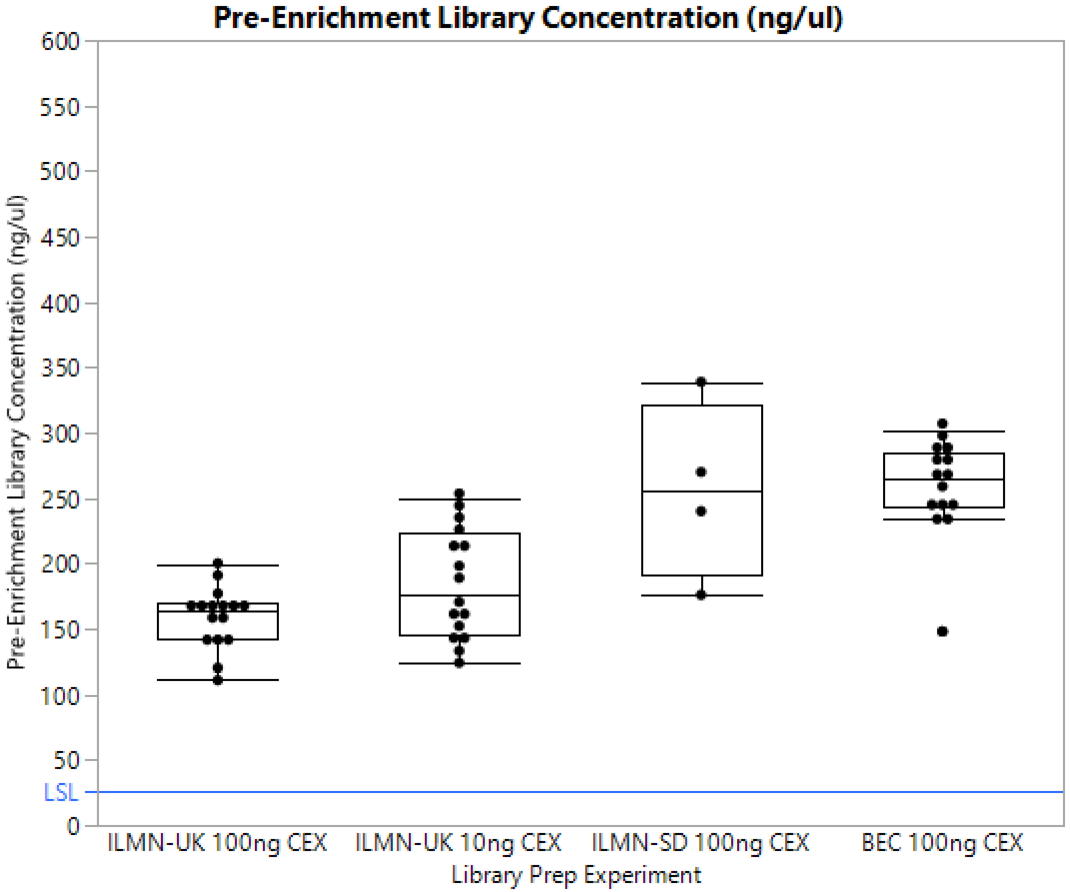
*one non-template control (water) used on the run

Demonstration Acceptance Criteria

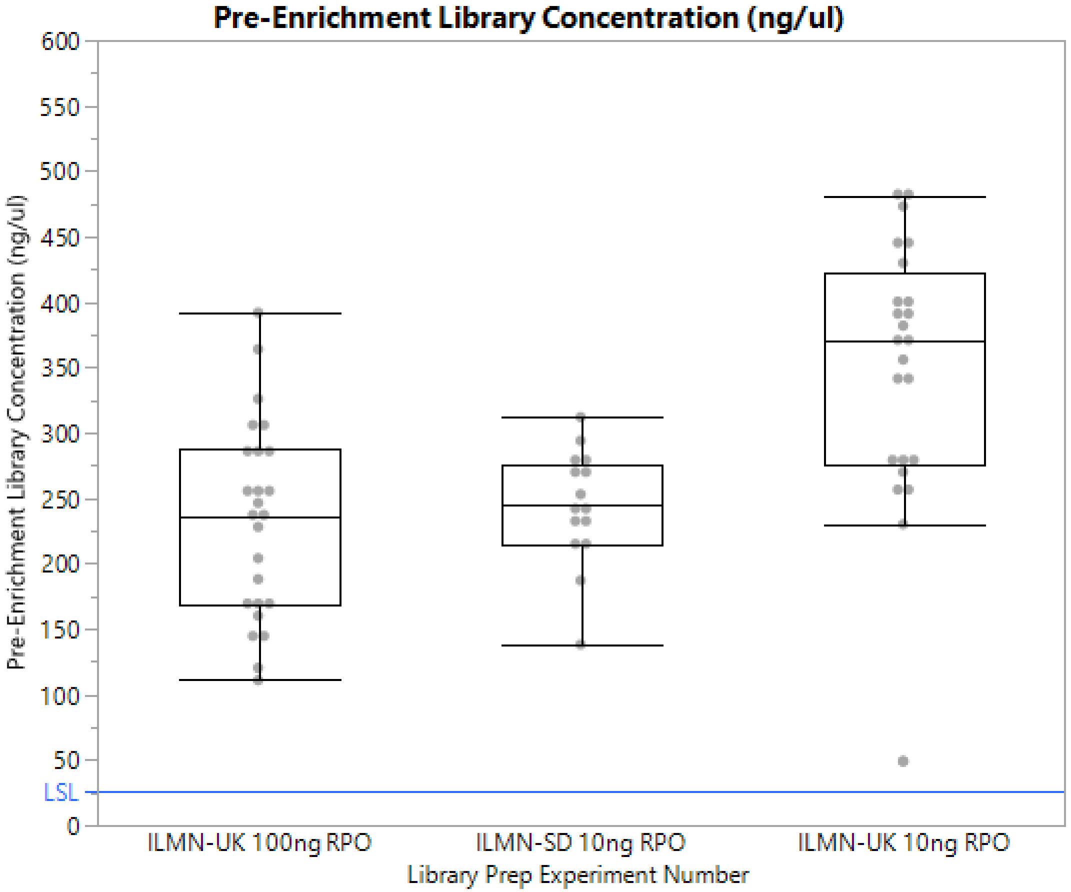
Metric	Criteria
Pre-Enrichment Library Concentration	Pre-Enrichment library concentration should be higher than 26.66 ng/µl as determined by Qubit.
Average Percent Aligned Reads	Average percent aligned reads should be equal to or higher than 90% regardless of enrichment panel tested for each run.
Average Percent Padded Read Enrichment	Average padded read enrichment should be equal to or higher than 90% regardless of enrichment panel tested for each run.
Percent Duplicates	Percent duplicates should be less than or equal to 32%.

Pass metrics provided by Illumina

Pre-Enrichment Library Concentration

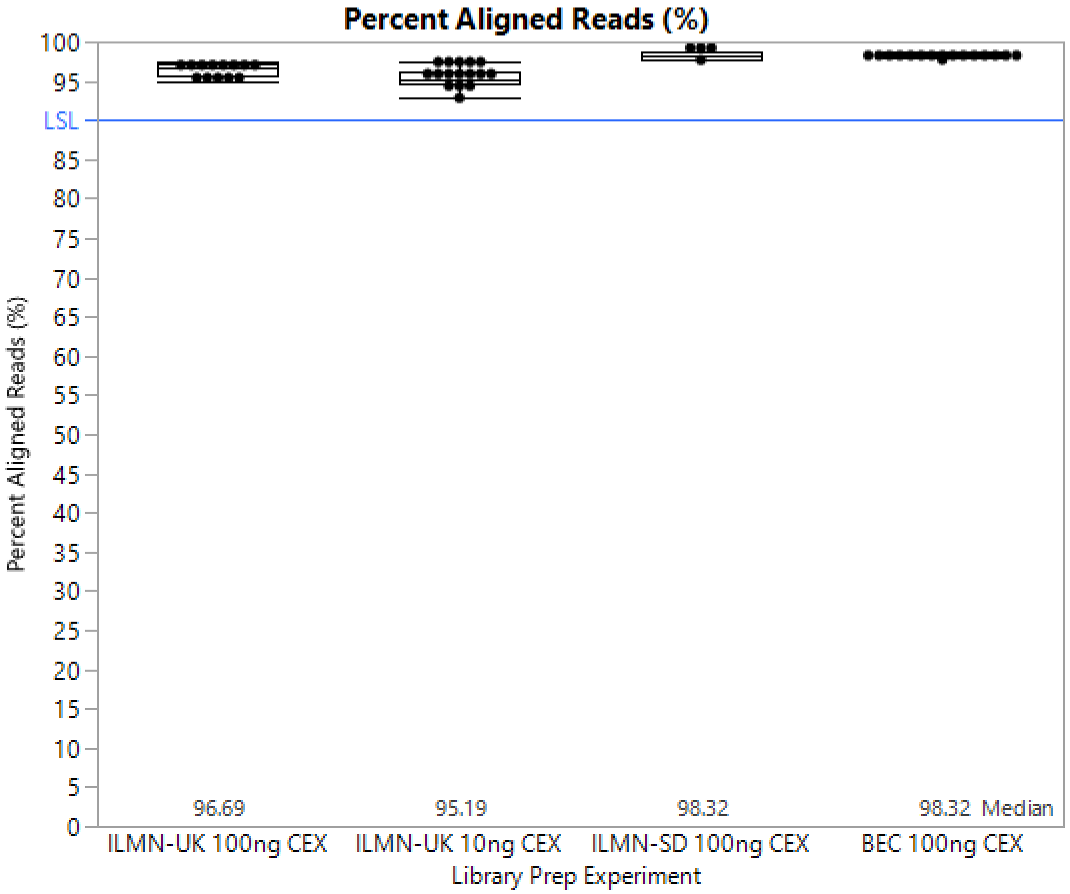


CEX Panel (45 Mb) Runs

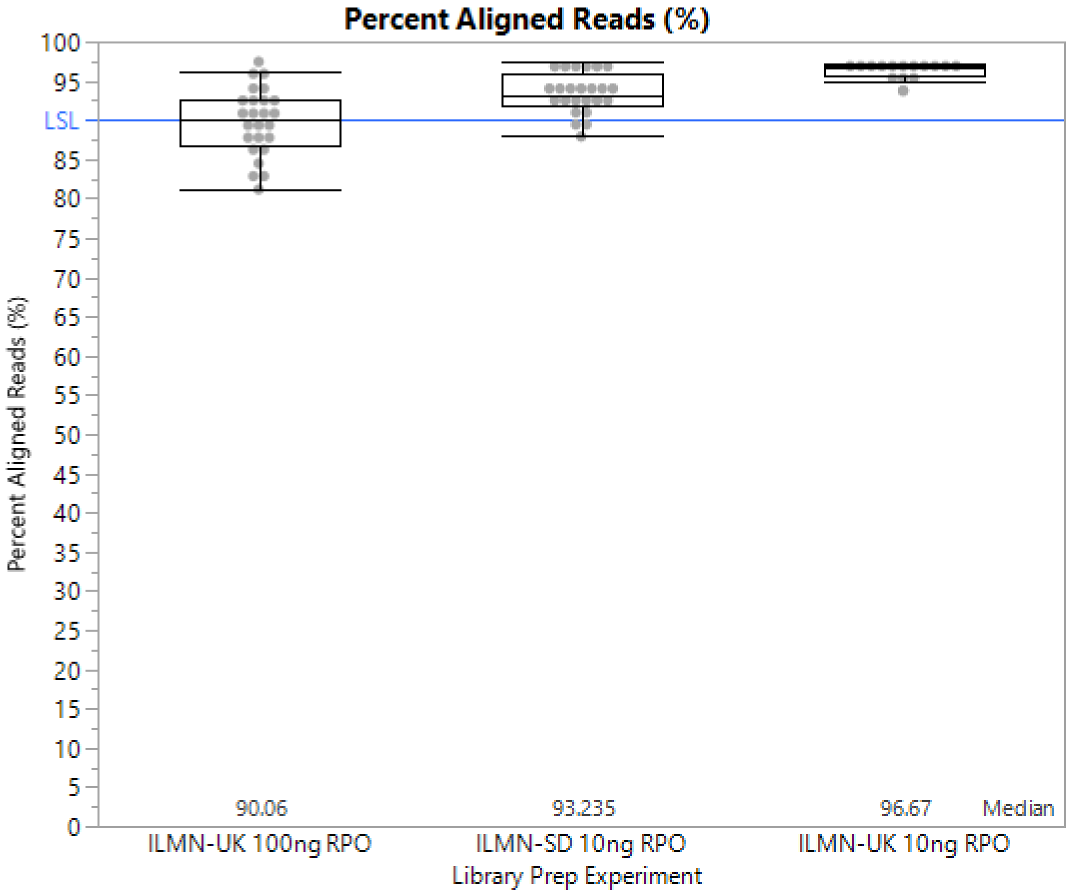


RPO Panel (0.4 Mb) Runs

Average Percent Aligned Reads

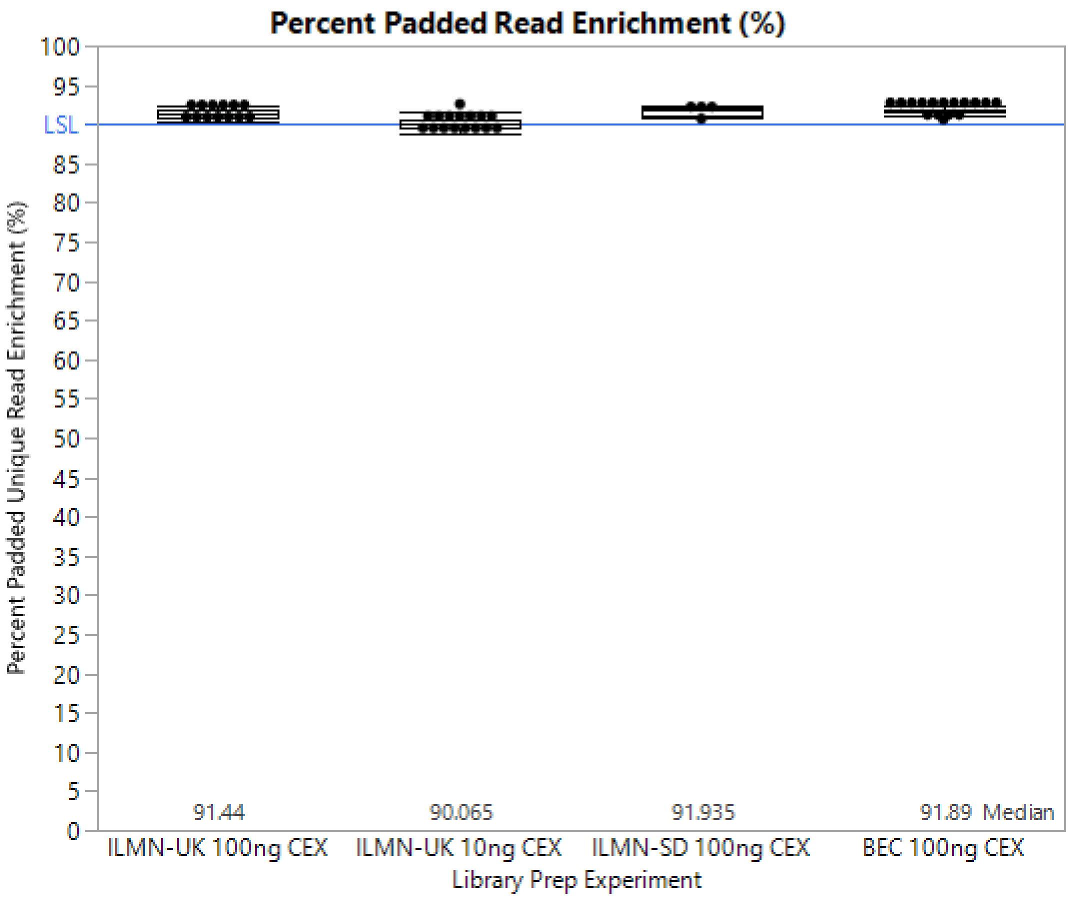


CEX Panel (45 Mb) Runs

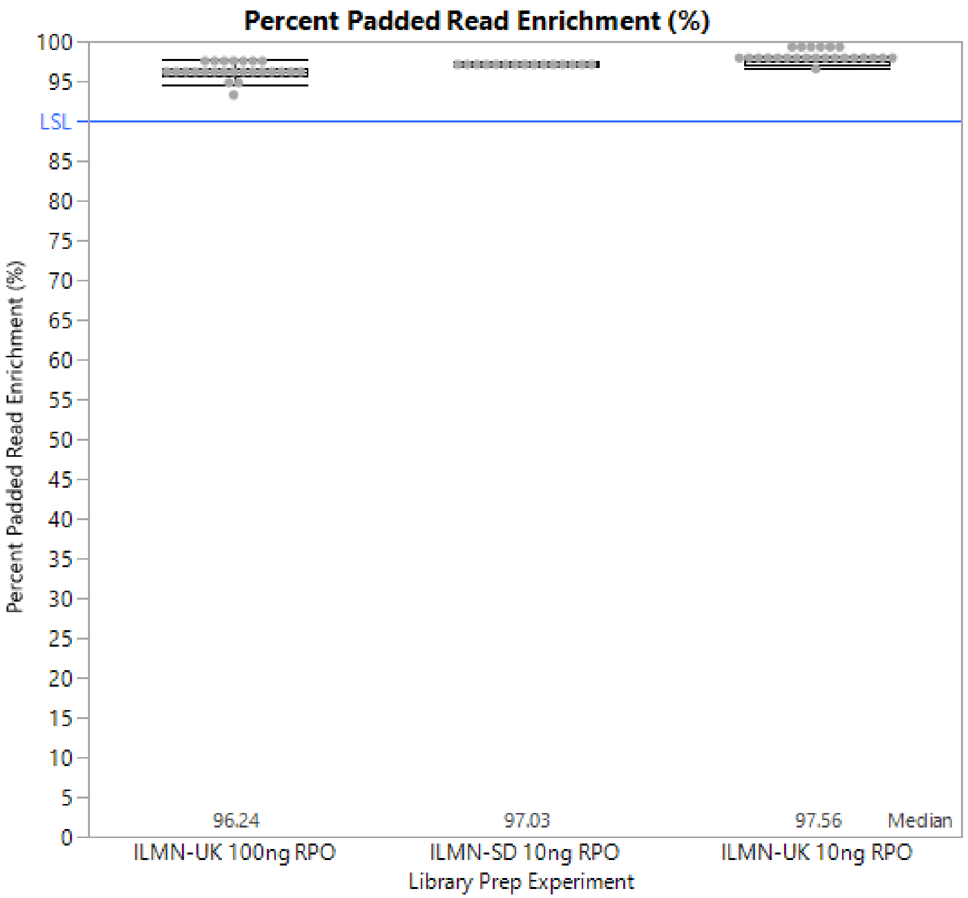


RPO Panel (0.4 Mb) Runs

Averaged Padded Read Enrichment

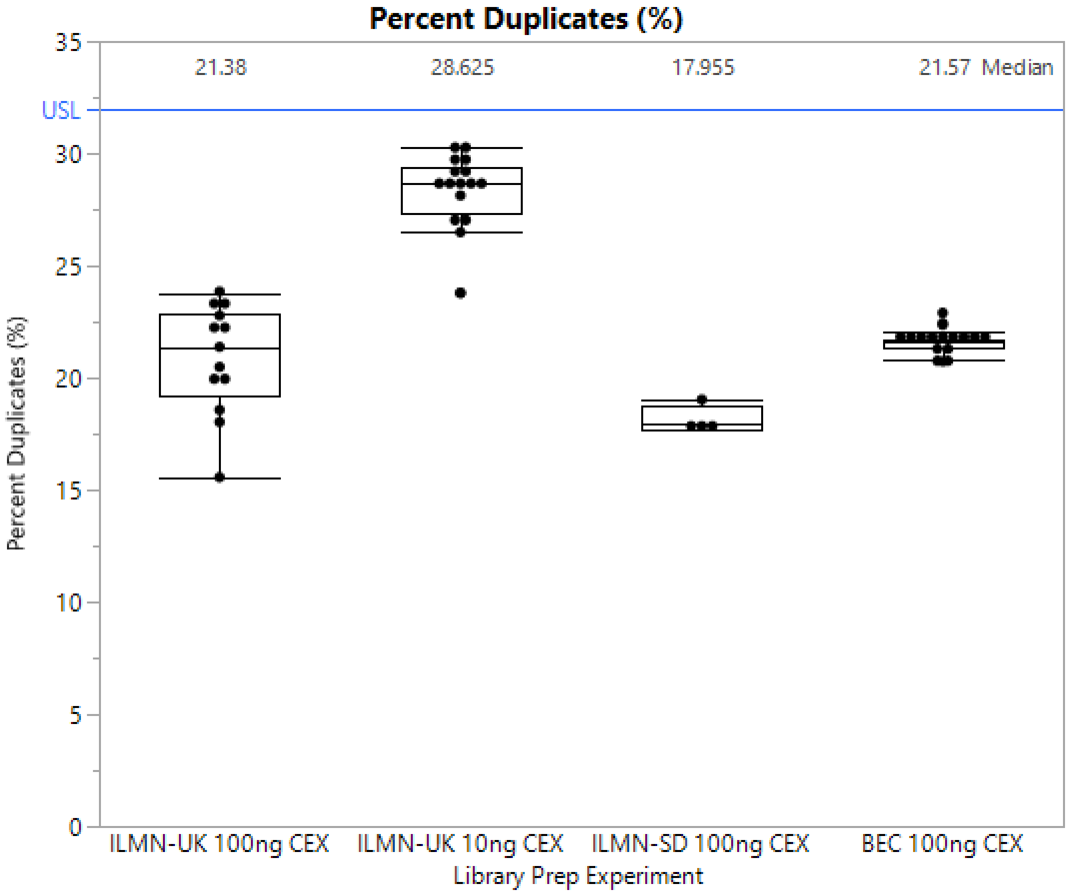


CEX Panel (45 Mb) Runs

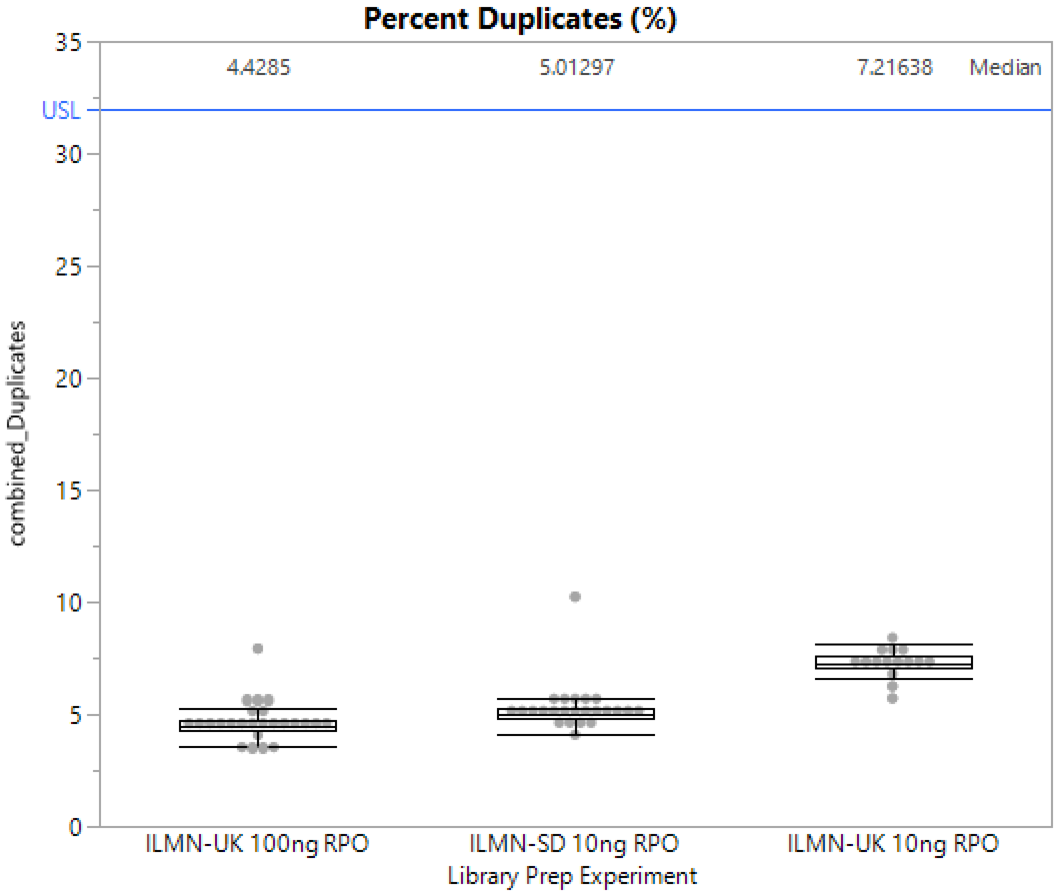


RPO Panel (0.4 Mb) Runs

Percent Duplicates

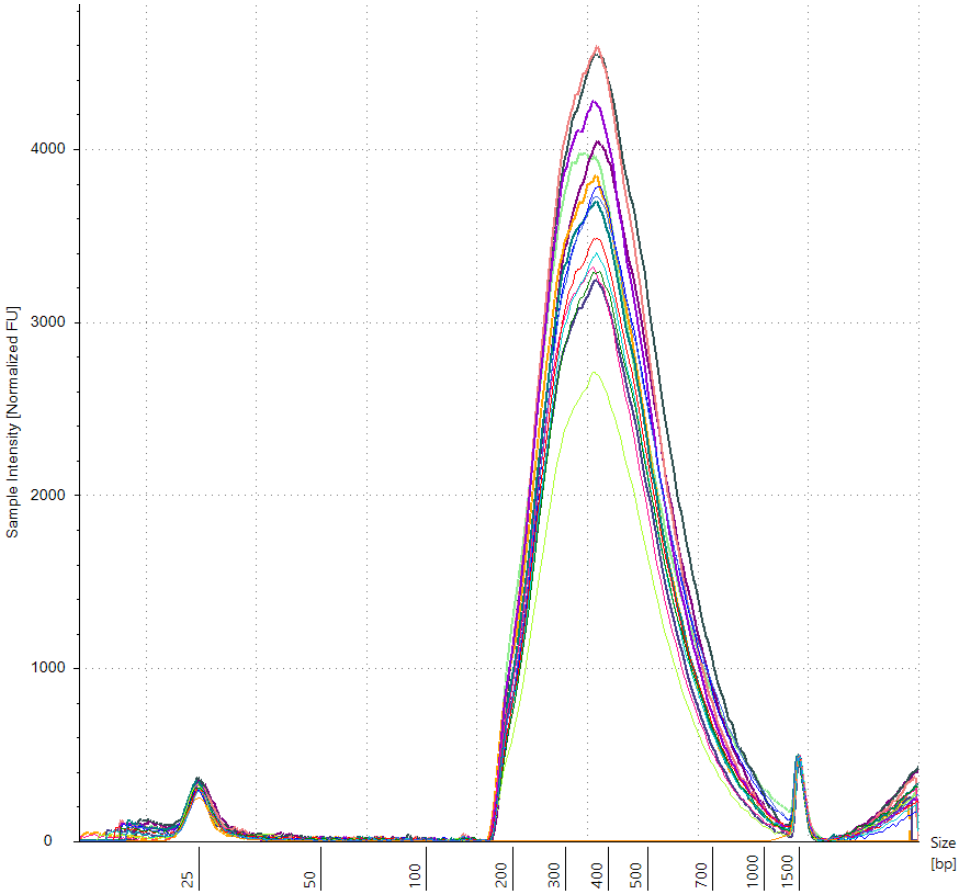


CEX Panel (45 Mb) Runs

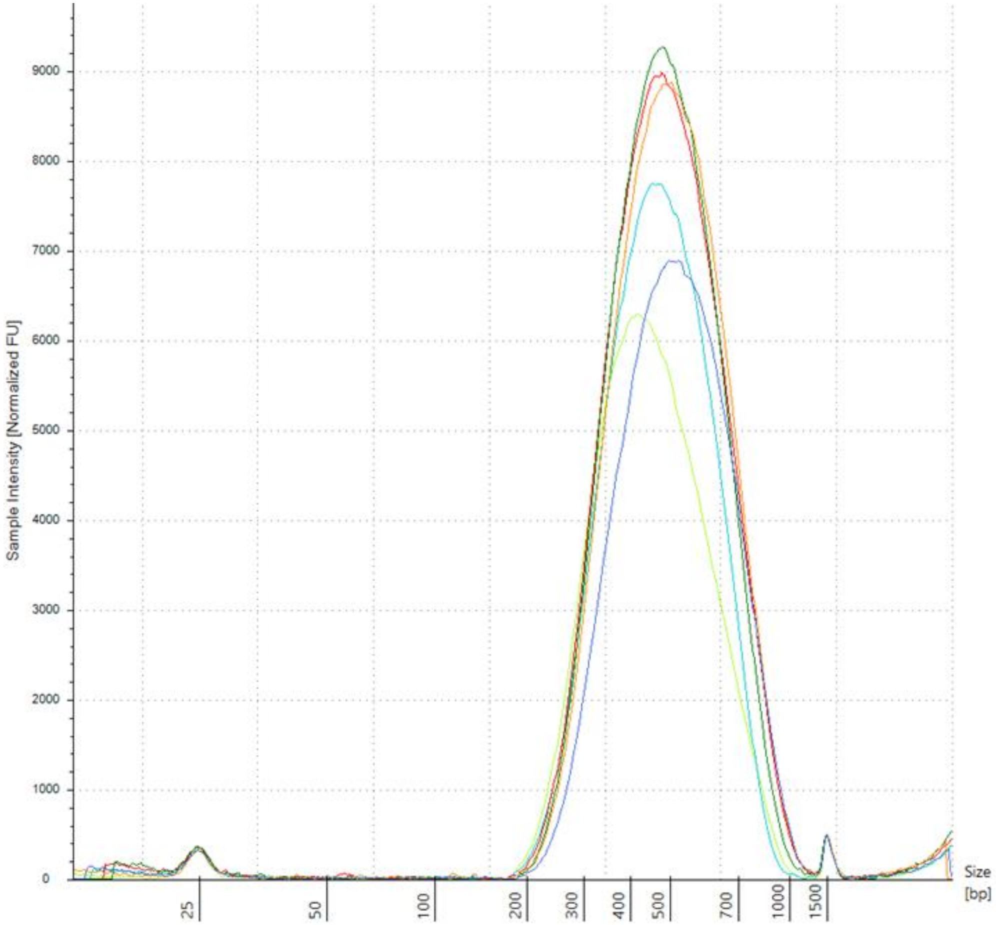


RPO Panel (0.4 Mb) Runs

Example Agilent TapeStation Traces: BEC 100ng CEX Panel Run

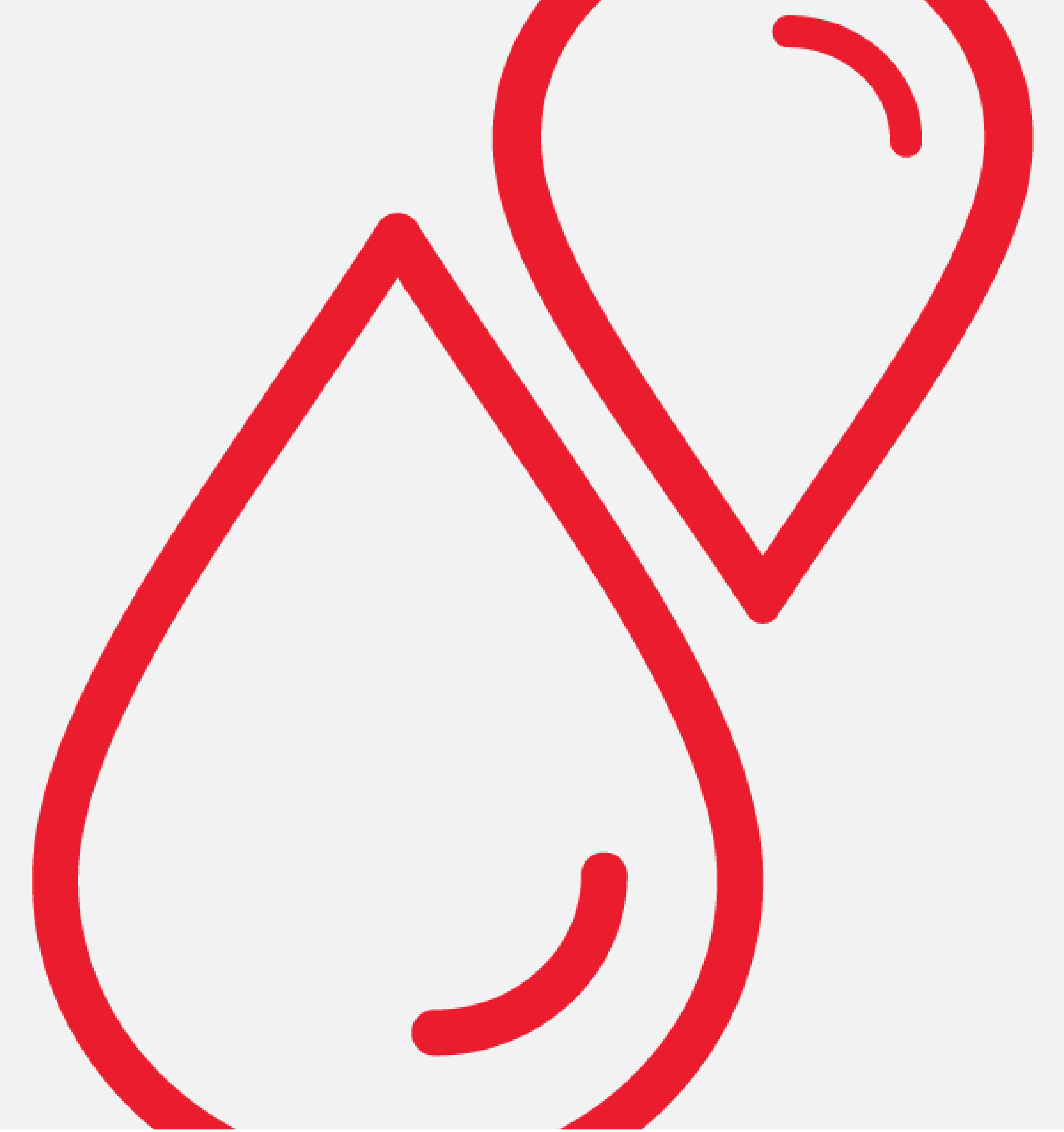


Above: Pre-Enrichment Library Traces for 15 libraries and one negative control.



Above: Post Enrichment traces for five 3-plex pools and one 1-plex pool.

Demonstration Summary



Demonstration Summary

Metric	Criteria	Results
Pre-Enrichment Library Concentration	Pre-Enrichment library concentration should be higher than 26.66 ng/µL as determined by Qubit.	Pre-Enrichment library concentration was greater than 26.66 ng/µL for all libraries. **CEX Panel library average was 200 ng/µl. RPO Panel library average was 259 ng/µL.**
Average Percent Aligned Reads	Average percent aligned reads should be equal to or higher than 90% regardless of enrichment panel tested for each run.	Average percent aligned reads was 90% or higher for all runs (7 of 7).
Average Percent Padded Read Enrichment	Average padded read enrichment should be equal to or higher than 90% regardless of enrichment panel tested for each run.	Average percent padded read enrichment was 90% of higher for all runs (7 of 7).
Percent Duplicates	Percent duplicates should be less than or equal to 32%.	Percent duplicates was 32% or lower for all libraries across all runs (7 of 7).

Data reviewed and approved by Illumina

General Automation Considerations

- Please read and understand Biomek NGeniuS System IFU, C43212
- Spin down index plate before use to make sure indices are at the bottom of wells
- Do not use unsupported index plates (see slide 6 for part numbers)
    - If the plate geometry is not the same, it could result in an instrument crash
- Make sure foil of each index well is widely opened to prevent tip-friction binding and lifting of Index Plate
    - Use a *new* P200 or P1000 to pierce and widen *each* well being used
- Do not use other sized kits, as their reagents might come in an unexpected tube size
    - Chance for misread OCR token on vial
    - Chance for failed chemistry
    - Chance for damaged instrument
- Avoid bubbles in reagent tubes to ensure accurate liquid level sensing and aliquoting
    - ELBTL, TB1, and TWB are bubbly
- The Work Aid requests more volume than what is consumed
    - Dead volume is needed in source tubes to ensure enough is available due to tolerance stack-ups
- Dead volume will be left behind in some storage wells
    - The nature of automation, tolerance stack-ups, and environment necessitates some overage
- Make sure bulk reagents wet the entire length of reservoir
    - Ensures accurate liquid volume sensing
- Start with sample volumes ≥ 2 μL.
    - Biomek NGeniuS System will calculate needed sample volume based on provided sample concentration
    - System *will* accept smaller input volumes, but more variation is observed

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