



BIOMEK WORKSTATIONS AND ECHO LIQUID HANDLERS IN GENOMIC APPLICATIONS



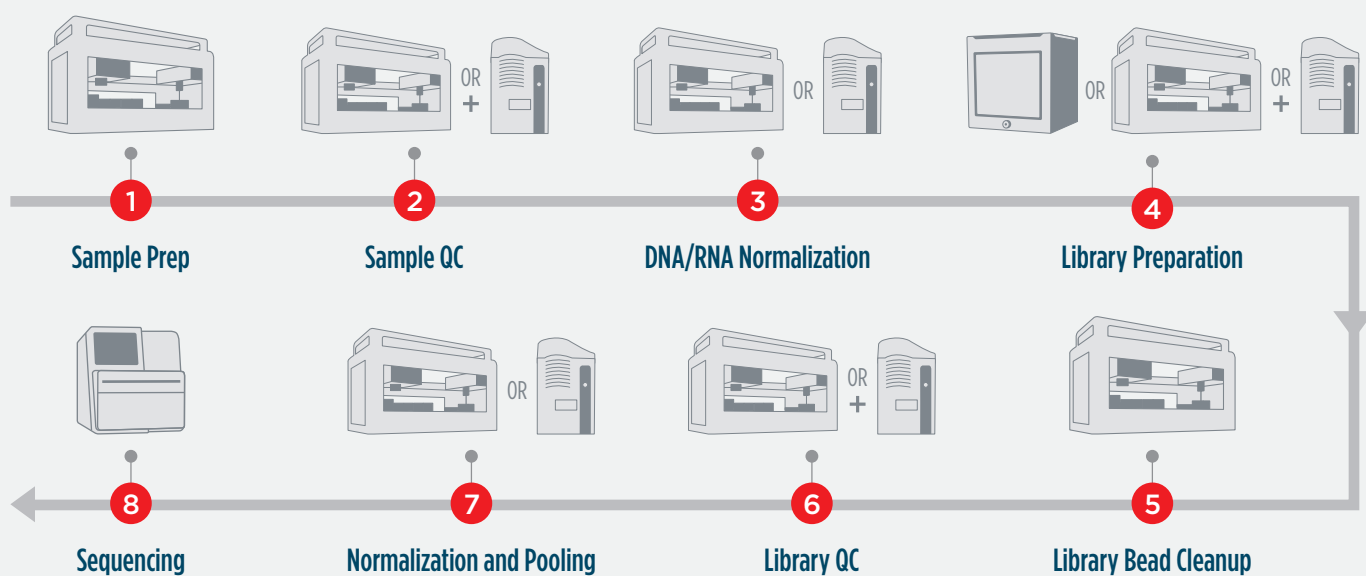
ACCELERATING
answers

 **BECKMAN
COULTER**
Life Sciences

Genomic applications vary in process complexity and throughput but share the need for high quality and consistent data. Quality is built into the Beckman Coulter Life Sciences instruments to enable your research, whether you need robust automation for a few samples, or need a truly high-throughput solution to increase your capacity and help lower the cost per sample, as well as decreasing the turnaround times.

Biomek Workstations are configurable to handle workflow needs from microliter to milliliter transfers for nucleic acid isolations from primary sample tubes, to preparing sequence-ready libraries. The Echo Acoustic Liquid Handlers (LHs) nanoliter to microliter transfers allow the reduction of reaction costs through reaction volume miniaturization and acoustic non-contact transfers.

A General Next Generation Sequencing Workflow



1 Sample Prep

Biomek Workstation and Beckman bead-based reagents to automate DNA and RNA extraction.

2 Sample QC

Use either a Biomek Workstation or a combination of Biomek Workstation and Echo LH, depending on your workflow needs.

3 DNA/RNA Normalization

Use either a Biomek Workstation or an Echo LH depending on your workflow needs.

4 Library Preparation

For standard volumes use a Biomek Workstation; or use combination of Echo LH and Biomek Workstation to reduce reaction volumes of precious reagents using the Echo LH, while doing larger volume transfers on the Biomek Workstation.

5 Library Bead Cleanup

Use a Biomek Workstation and Beckman bead-based reagents for 96- and 384- well bead clean ups.

6 Library QC

Use either a Biomek Workstation, or an Echo LH and Biomek Workstation depending on your workflow needs.

7 Normalization and Pooling

Use a Biomek Workstation for preparing the dilution plates and pooling of the libraries. Alternatively, use an Echo LH for “simultaneous normalization and pooling” for up to 97% faster processing time.

8 Sequencing

Load the pooled libraries onto a flow cell of a sequencer.



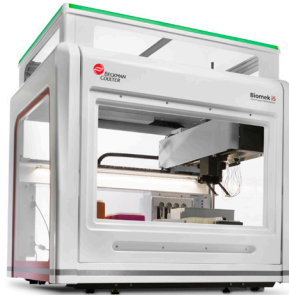
Biomek Workstation Solutions for Genomic Applications

Biomek NGenius Next Generation Library Prep System



- Purpose-built NGS sample preparation system with flexible application selection and the ability to control user access
- Work-from-anywhere batch setup, and reagent preparation made easy with software derived Work Aid*

Biomek i5 Automated Genomic Workstations



- 25 deck positions to support medium-throughput workflows
- Multichannel or Span-8 with gripper options

Biomek i7 Automated Genomic Workstations



- Spacious, open-platform with 45 deck positions for high-throughput applications
- Hybrid Workstation with Multichannel pod, Span-8 pod and two independent grippers (other pipetting configurations are possible as well)

Biomek NGenius Next Generation Library Prep System Hardware Features



Multichannel Liquid Level Sensing aids in the detection of insufficient reagents, alerting the operator before it becomes a processing issue

Reagent Carousel reduces the need to manually transfer reagents and automates most reagent kits designed for manual use

Temperature-controlled Reagent Storage protects reagents from thermal degradation and allows you to run end-to-end chemistries

Integrated Thermal Cycler extends walkaway time

Multi-functional Pipetting Pod

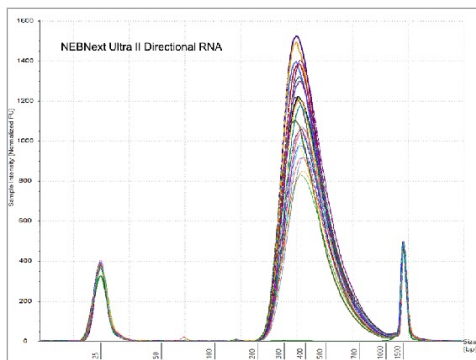
- **Pipetting Range** – 1 to 1000 μ L
- **Selective Tip Type** – ensures the right tip for the right volume without manual intervention
- **Dynamic DeckOptix** camera and labware recognition
- **Labware Transport** – enables longer walkaway time

*PC with Google Chrome or Microsoft Edge

Simplified Next Generation Sequencing Library Preparation

You asked for an easy-to-use, cost-effective, NGS sample prep system with built-in error reduction and the flexibility to run an array of library preparation kits from multiple vendors—without requiring in-depth programming knowledge. And now it's here.

Biomek NGenius Next Generation Library prep system



QC Data

Agilent TapeStation trace results from the libraries created on the Biomek NGenius Next Generation Library Prep System from 23 reference RNA samples and one negative control using the NEBNext Ultra II Directional RNA Library Prep Kit. Libraries have an average fragment size of 420 bp, averaged across all libraries created from these samples.

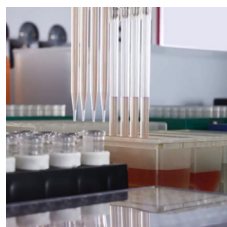
Input DNA Mass	Sample Type	Average Library Size (bp)	Qubit Conc. (ng/μL)	% >Q30	% Duplicates	Average % Reads Aligned	Average % Stranded
10 ng	RNA	420	8.37	93	7.4%	97.85%	99.4%
100 ng	RNA	432	8.75	93	3.68%	98.41%	99.42%
1000 ng	RNA	441	14.93	93	4.73%	98.42%	99.61%

Table 1. Sequencing results from the NEBNext Ultra II Directional RNA Library Prep Kit on the Biomek NGenius Library Prep System. Results fall within library parameters defined by NEB.¹

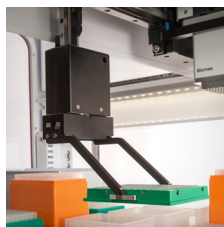
Biomek i-Series Hardware Features



60, 300 and 1200 μL Multichannel heads with 96 and 384 options for flexible transfer options including individual tip(s), column(s), row(s), patterns



Span-8 pod equipped with conductive Liquid Level Sensing (LLS), Allows sample volume tracking through Biomek software



“High-access” rotating gripper optimizes access to high-density decks, allows for direct device integrations.



Optional HEPA filter integration for environmental control. Optional enclosure to provide dust protection.

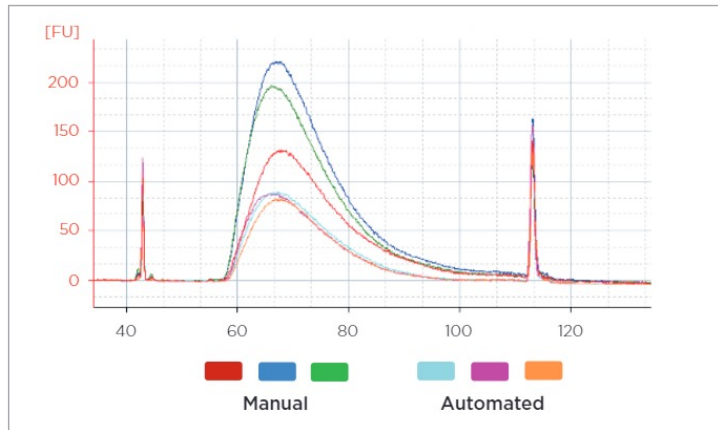


Integrated devices for process control – e.g. peltiers, shakers and thermocyclers

Successful Library Preparation on the Biomek i-Series Automated Liquid Handler

Obtaining reliable and repeatable results that meet the specifications of reagent vendor standards is crucial to successful automation. Therefore, we partner with customers and leading technology vendors to develop and deliver the best solutions to suit your needs. Our portfolio of Biomek-automated methods are demonstrated to generate quality data using real-world samples.

Biomek i-Series Automated Data Demonstrated Methods



QC Data

Bioanalyzer traces showing consistent sizing between automated and manual libraries. The average size of the manual libraries was 267 bp, while the automated library average size was 266 bp.

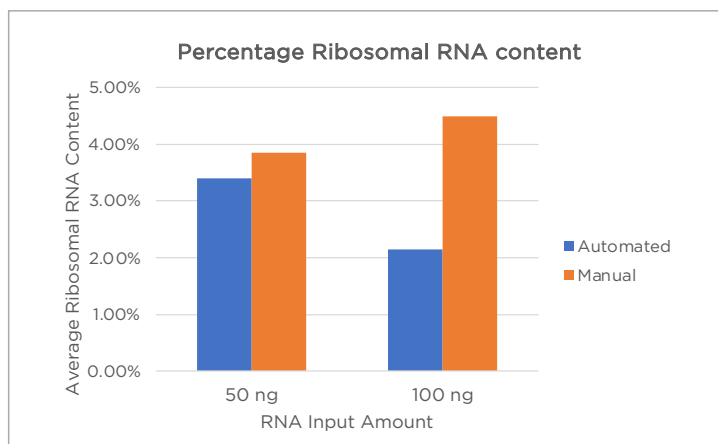
E.g. Biomek i7 automated Roche KAPA HyperPrep/HyperPlus method



Sequencing Data

Greater than 97 percent of sequenced reads that mapped to the reference genome.

E.g. Biomek i7 Automated Roche KAPA mRNA HyperPrep Kit



Sequencing Data

Low percentage of ribosomal RNA content in Biomek prepared libraries indicates an efficient capture of mRNA molecules.

E.g. Biomek i7 Automated Roche KAPA mRNA HyperPrep Kit



Complete Your Workflows With Our Genomic Reagents

Biomek automation can be complemented by a growing portfolio of genomic reagent kits from Beckman Coulter Life Sciences, which currently includes nucleic acid extraction and purification solutions for a range of input material. Our high-performance SPRI technology uses paramagnetic beads to selectively immobilize nucleic acids by type and size, and optimized binding conditions enable highly specific separation and cleanup protocols. Portfolio examples include:

Nucleic Acid Purification



AMPure XP Bead-Based Reagent

- Removes primers, unincorporated dNTPs, primer dimers, salts and other contaminants
- Eliminates fragments < 50 bp
- Recovers both double- and single-stranded DNA templates
- No PCR degradation after storage at 4° C for 7 days
- Captures DNA > 100 bp

Nucleic Acid Purification



RNAClean XP Reagent

- Certified RNase-free
- Complete removal of salts, unincorporated primers and dNTPs
- Used in RNA-Seq library preparations

Size Selection



SPRIselect Bead-Based Reagent

- Used for fragment size selection for library construction in NGS workflows
- Validated for target fragments between 150 bp to 800 bp in length
- Tight manufacturing specifications ensure run to run and lot to lot reproducibility

Plasmid Purification



CosMCPrep

- A SPRI paramagnetic bead-based system for all your plasmid purification needs
- Use a single protocol to purify a variety of high- and low-copy number template types
- Purify plasmids in a 96-well format for high-throughput sample processing

RNA Isolation



RNAdvance Extraction Kit

- Blood, Cell, Tissue and Viral kits
- Extract RNA from blood (PAXgene tubes), cultured eukaryotic cells, tissue, or saliva and swab transport media
- Produce high-quality RNA compatible with a variety of analysis techniques, such as NGS, microarray, or qRT-PCR

DNA Isolation



DNAdvance Extraction Kit

- A SPRI paramagnetic bead-based extraction system to isolate genomic DNA
- DNA from Tissue, Saliva, and Buccal swab
- Higher recovery and purity of gDNA comparing automated vs. manual protocols

DNA Isolation



GenFind V3 Extraction Kit

- Extraction and purification of gDNA from whole blood and serum
- Consistent and optimal purity from citrate, EDTA, or Heparin blood tubes or cultured mammalian cells
- High recovery of quality DNA for downstream applications, such as NGS library construction and PCR

DNA & RNA Isolation



FormaPure XL Total Extraction Kit

- An extraction kit to isolate DNA and RNA from formalin-fixed, paraffin-embedded (FFPE) tissue samples
- High performance for yield, integrity, and purity
- Single chemistry system compatible with manual and automated processing of DNA and RNA from a single sample



Echo Liquid Handlers for Genomic Applications

Echo acoustic liquid handling (LH) technology revolutionizes genomic applications by using sound energy to enable highly accurate, fully automated, non-contact dispensing of fluids in nL to μ L volumes. By reducing reaction volumes, the Echo LH offers significant cost-savings in reagents, and considerable amount of time-savings for barcoding/indexing and normalization and pooling while preserving data quality.



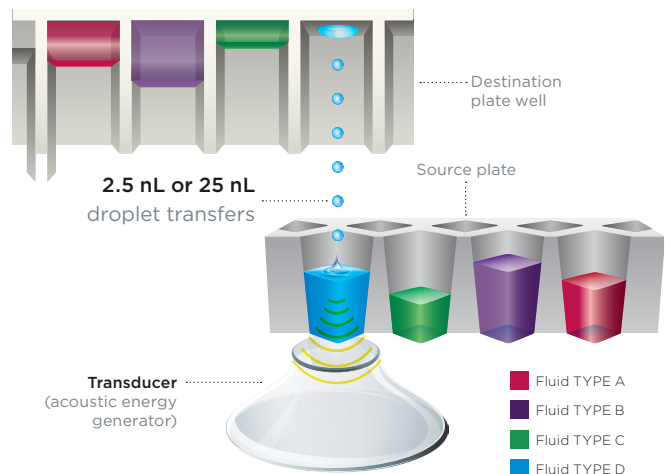
Echo 650 Liquid Handler



Echo 525 Liquid Handler

How Does the Echo LH Move Liquids with Sound?

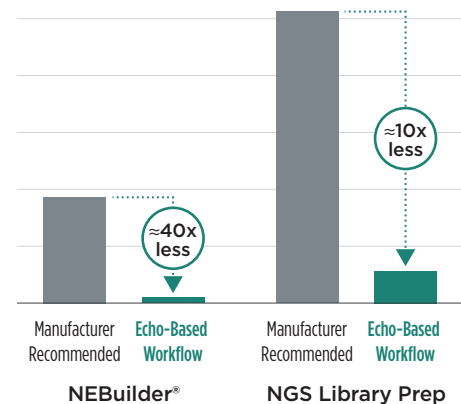
The heart of the Echo technology is a transducer which receives radio frequency energy and converts it to ultrasounds, which then are transferred to the liquid inside the source well. Using the proprietary dynamic fluid analysis algorithm, the Echo LH determines fluid composition and height, and the power needed to eject a precise volume of fluid into an inverted destination well. This analysis happens in milliseconds, enabling very fast, precise and accurate transfers of nanoliter droplets (less than 8% CV and less than 10% inaccuracy). The desired transfer volumes are achieved by rapidly transferring multiple droplets per second. Transfers can be made from any well in the source to any well in the destination in under a second.



Acoustic droplet ejection - The transducer is positioned below the source microplate well and emits focused sound energy repeatedly to the meniscus of the fluid to be transferred. A stream of 2.5 or 25 nL droplets (model dependent) is reliably ejected into a well of an inverted destination microplate

Low-Cost, Highly Efficient NGS Library Preparation

As the cost of sequencing has gone down, the cost of library preparation is becoming the bottleneck in many high-throughput NGS applications, such as high-throughput amplicon sequencing, or shotgun sequencing in microbial or single cell research. One way to reduce library prep cost is to reduce reaction volumes, therefore there is a need for a liquid handler than can go to sub microliter volumes accurately and precisely. Tip-less, Echo liquid handling systems offer up to 40-fold less reagent costs through miniaturization while ensuring high library quality and repeatability.¹





Biomek Echo One

High-Throughput Genomics Bundled Solution

The use of the Biomek Workstation with an Echo 525 instrument together provides the full workflow automation with the advantages of acoustic dispensing. This workflow solution features a sliding, rotating turntable that allows the Echo 525 instrument to be used in standalone mode for one-off quick runs or workflow development. For a comprehensive, walkaway solution, the Echo instrument is rotated to integrate with the Biomek i7 Workstation and control Echo application protocols. The Biomek Echo One is a fully integrated solution with included third-party devices such as an automated centrifuge, thermal cycler, and shaking-temperature control devices for walkway NGS library preparation. Additionally, the system is fully expandable to meet future workflow throughput needs.

Featured Highlights: Biomek Echo One For High-Throughput NGS

- **Dynamic Fluid Analysis:** Real-time adjustment to fluid properties enables users to rapidly normalize and pool libraries using non-contact acoustic transfer. No user calibration is required.
- **Nanoliter-scale Transfers:** Enables reduction of NGS library preparation costs by miniaturizing to 1/5th-1/20th the standard reaction volume.*
- **Echo Software:** Intuitive interface to quickly create transfer protocols with built-in simulators.
- **Biomek Software:** Simplicity so that you can focus on the science. Intuitive software for effortless operation.
- **Biomek Method Launcher:** Launch predefined methods through a secure interface and conduct system maintenance without affecting methods.
- **DeckOptix Final Check:** Analyzes the deck for common setup errors and alerts you prior to the start of a run.
- **Versatile Automation:** Multichannel pod, Span-8 pod and two independent grippers; integrated third-party devices for seamless method execution.
- **Run flexibility:** Echo transfer 'one-offs' can be quickly run in standalone mode. When ready to scale to multi-plate protocols, pivot to the Biomek Workstation for full automation scalability.
- **Expandability:** Add capabilities (e.g. readers, thermal cyclers) as needs evolve.

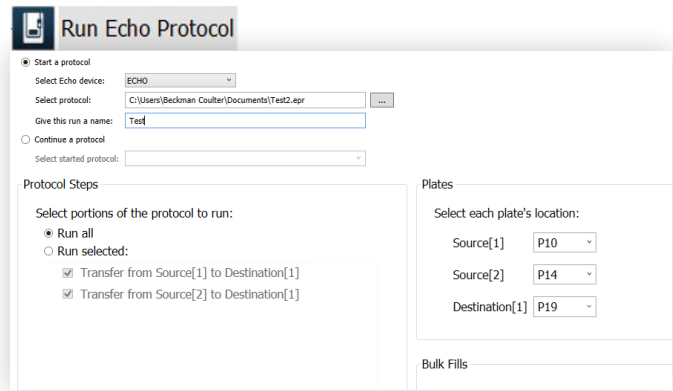


*Kit and chemistry-dependent.

Advantages of an Echo Liquid Handler Integrated to Biomek Workstation

Directly integrate the Echo LH to enable Biomek methods to process plates using Echo Applications version 1.8 protocols.

- Protocols developed using the Echo LH as a standalone instrument can be automated using the Biomek LH Methods
- Run one or more Echo LH protocols in a Biomek LH Method
- Run a full Echo LH protocol at one time, or run only selected plates within the Echo protocol
- Full use of all features in the Echo application software including identical well content and Echo applications reports
- Visually assign plates to the Echo protocols or use barcoded plates with an Echo picklist transfer file
- Echo LH well volume, dispense and exception data is assigned to plates handled in the Biomek LH Method for additional calculations and reporting



Included Major Components: Biomek Echo One for High-Throughput NGS

Acoustic Liquid Handler Components	Integrated Third-Party Devices	Biomek Workstation Components
Echo 525 Acoustic Liquid Handler	Automated Microplate centrifuge	Biomek i7 Hybrid Workstation – 384-well head*
Sliding-rotating turntable integration	ATC 384-well automated thermal cycler	Span-8 liquid displacement head
Echo Plate Reformat software	Shaking device (QInstruments BioShake 3000 elm)	384-well wash station
Echo Cherry Pick software	Cooling device (QInstruments ColdPlate Heater Cooler)	Biomek v 5.0 software
Insert swap station		DeckOptix Final Check
		Biomek Method Launcher

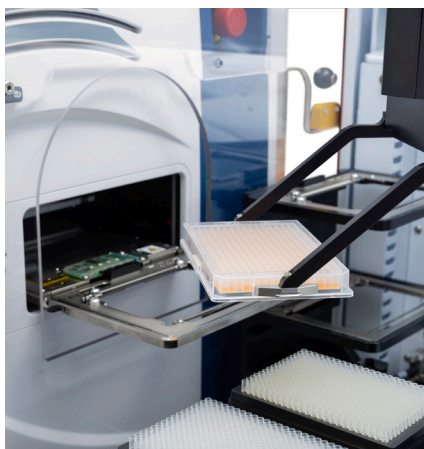
*96 well head available separately

One Workflow Evolving In Stages



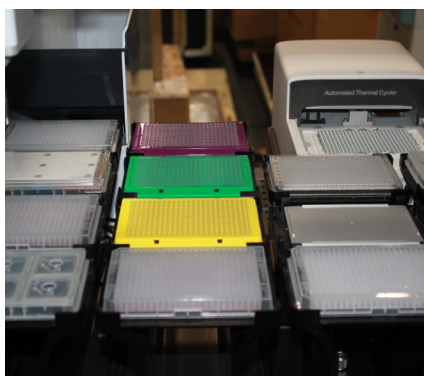
Designing

Imagine a not-so-far-fetched scenario. A new lab member is staring at an impressive (but intimidating to the uninitiated) Biomek Echo One integrated solution. No worries - we begin with a quick and simple workflow such as automating qPCR reaction setups with the Echo acoustic liquid handler. The new user can get used to operating the Echo instrument 'face forward' in standalone mode with our intuitive software applications.



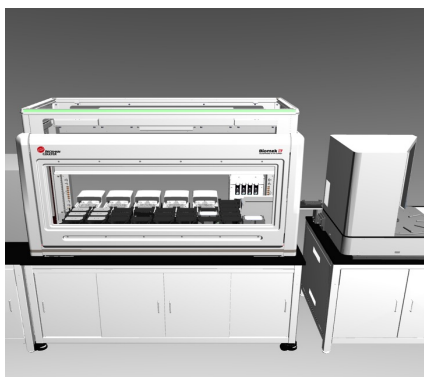
Building

The journey is now well underway. You've seen an amazing increase in workflow capacity and throughput by automating workflows such as PCR reaction setups, but the newly minted automation maverick has better things to do than load plates! It's time to unleash the walkaway capabilities of the Biomek Echo One solution. Just slide and rotate the Echo system to integrate with the Biomek workstation and execute a simple Biomek method that executes a multi-plate setup. Let the Biomek workstation load and unload plates as an Echo Plate Reformat protocol is being executed.



Testing

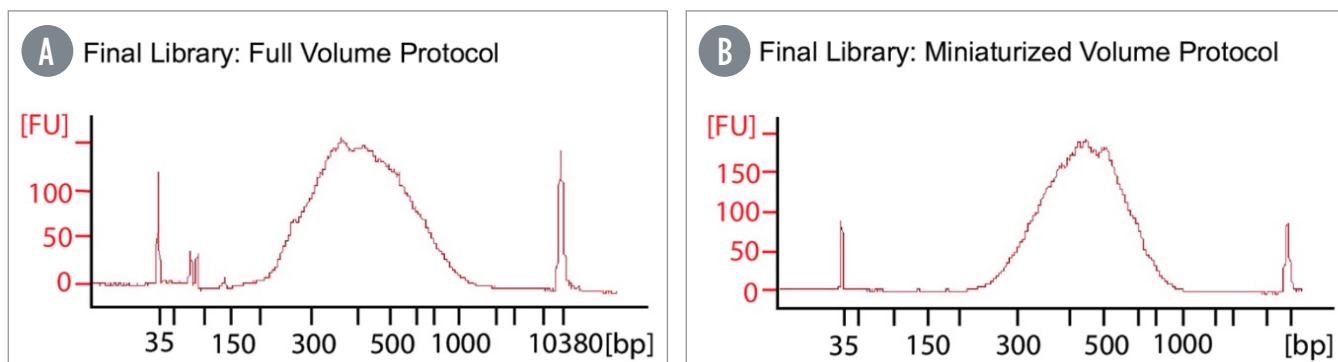
While the Biomek Echo One solution for genomics applications can be used for partial work steps, the goal is a fully automated and miniaturized library creation from sample preparation to sequencing-ready. You now have a system capable of over 960 libraries per day. Consult with our field team as well as our NGS solutions partners to automate library prep at 384 well-scale and at miniaturization levels that pay off fast!



Growing

It's a good problem to have but please do note that success brings along the drive to push the bounds further. However, you, your well-trained staff and your partners at Beckman Coulter Life Sciences will be ready to take on increasing throughput. You can expand the Biomek Echo One solution with devices such as added thermal cyclers, integrated robotic arms, as well as plate peelers and sealers for continuously expanded capabilities.

Reduced Reaction Volume for Cost and Time Savings



Metagenomic NGS libraries were prepared at 10-times smaller reaction volumes, using the Echo 525 liquid handler. Final libraries produced by the full volume and miniaturized protocols have similar fragment distributions. (A) Bioanalyzer trace of a 5 ng HeLA RNA final library prepared with (A) full volume protocol using manual methods, (B) the miniaturized protocol on Echo 525 LH.²

Mayday MY, Khan LM, Chow ED, Zinter MS, DeRisi JL (2019) Miniaturization and optimization of 384-well compatible RNA sequencing library preparation. PLOS ONE 14(1): e0206194. <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0206194> is licensed under <https://creativecommons.org/licenses/by/4.0/> Images used with no changes.

Cost savings of reduced reaction volume

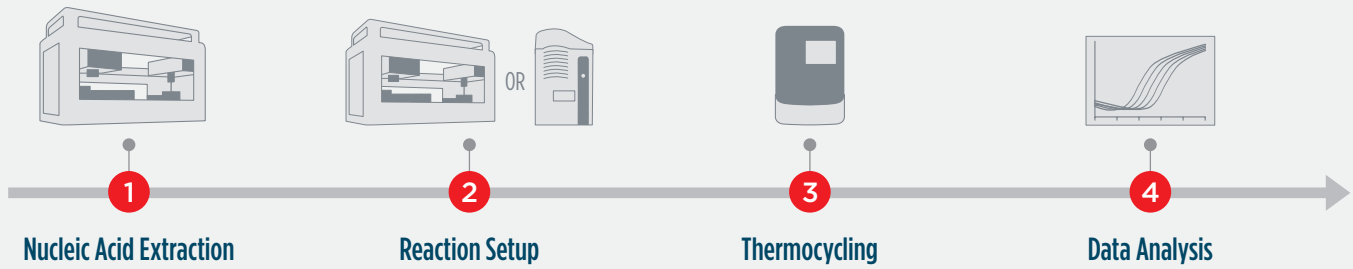
	FULL VOLUME PROTOCOL	MINIATURIZED PROTOCOL
Reagents	\$13,427	\$1824
Other reagents	\$2024	\$282
Tips	\$1085	\$1020
Plates/tubes	\$112	\$14
Source plate	N/A	\$21
TOTAL	\$16,648	\$3,161

Time savings of reduced reaction volume

	FULL VOLUME PROTOCOL	MINIATURIZED, AUTOMATED PROTOCOL
Calculations/planning	120 minutes	10 minutes
Aliquoting RNA	120 minutes	120 minutes
Drying RNA	N/A	25 minutes
Master mix preparation	120 minutes	10 minutes
Dispensing master mixes	215 minutes	45 minutes
Incubations	5808 minutes	242 minutes
Bead cleanups	2880 minutes	120 minutes
Quality check	Using the fragment analyzer and iSeq, QC time is equivalent for 384 samples	
Pooling	720 minutes	20 minutes
TOTAL	9,883 minutes (~166 hours)	592 minutes (~10 hours)

~80% cost savings and ~90% time savings for reduced reaction volumes (10 times smaller) on the Echo LH compared to the manual and full volume methods.

A General qPCR Workflow



1 Nucleic Acid Extraction

Automated nucleic acid extraction on Biomek Workstation with Beckman Coulter Life Sciences genomics reagents

2 Reaction Setup

Biomek Workstation: PCR setup at standard volume (master mix preparation, reagent and sample addition), Echo LH: Tip-less, fast and low volume reaction setup (reagent and sample addition) for cost-savings through reducing reaction volumes while preserving data quality

3 Thermocycling

96- or 384-well plate thermocycler, standalone or integrated to Biomek

4 Data Analysis

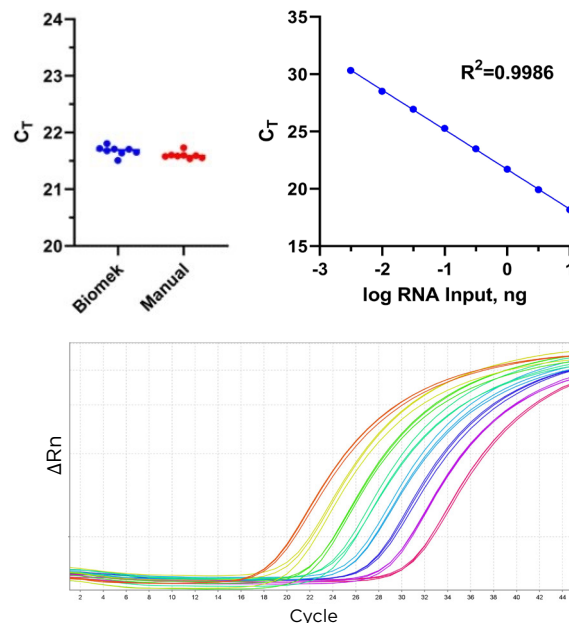
Measuring the Ct values

Automated qPCR on Biomek: Flexibility to Automate to Meet Your Throughput and Walkaway Needs

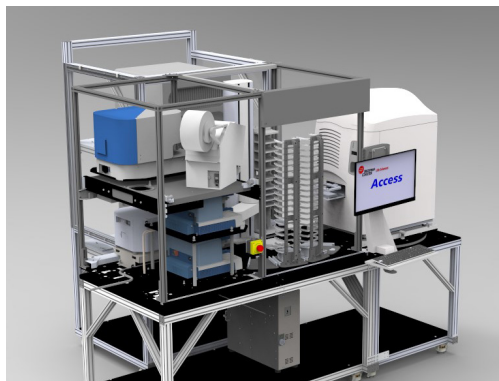
Automation on Biomek Workstations replaces repetitive pipetting steps in PCR/qPCR setup. Biomek allows the flexibility to work with multiple plate formats including 96 and 384, with any combination of reaction components. On-deck temperature control by Peltier maintains sensitive reagents at the appropriate temperature, minimizing the possibility of assay failure. Complete workflow automation is achieved through integration of automated thermocyclers, reducing hands-on time and associated human errors.

Biomek i7 Automated GAPDH qPCR Assay

The calculated CT values are in excellent agreement between manual reaction preparation (21.60 ± 0.06) and Biomek automated reaction prep (21.68 ± 0.08) for GAPDH qPCR assay. As expected, a concentration dependent shift in the fluorescence trace was observed. The highest amount of RNA input (10 ng) produced a CT value of 18.2 ± 0.1 , while the lowest concentration tested (3 pg) had a CT value of 30.33 ± 0.09 . When the calculated CT value was plotted as a function of RNA input an excellent linearity ($R^2 = 0.9986$) was observed.



Integrating the Echo LH into an Access System for Robotic Plate Handling

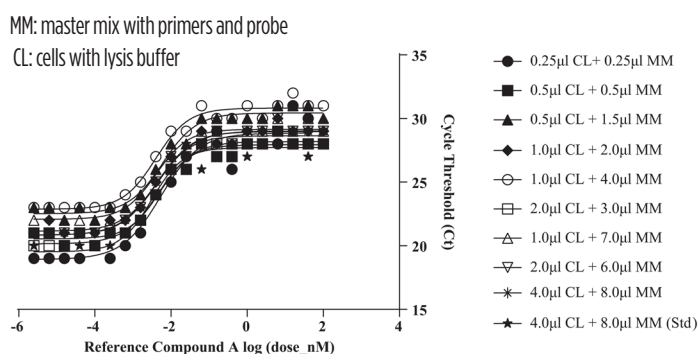


An example Access Workstation with third-party devices for peeling, centrifugation, bulkfilling, sealing and qPCR cycling, to automate reaction setup for qPCR screening around an Echo Liquid Handler.

The benefits of using the Echo Liquid Handler can be a solution for a number of assays. The Access Systems are designed to be modular and configurable workflow automation. Using robotic plate handling and third-party devices, the systems optimize the workflow scheduling around the Echo protocols. Integrate two Echo LHs to allow simultaneous use of different instruments to assemble various assays. The Access Workstation is a benchtop robotic platform for most any application. And the Access Single Robot System and Dual Robot System (Access SRS and DRS) are designed with docking carts where many third-party devices are integrated vertically in a small footprint for greater capability, with optional HEPA air handling or low humidity HVAC attachment for the appropriate environment in the enclosure for your assays.

RT-qPCR on Echo 525 LH: 0.5 μ L RT-qPCR, Similar Performance to 12 μ L

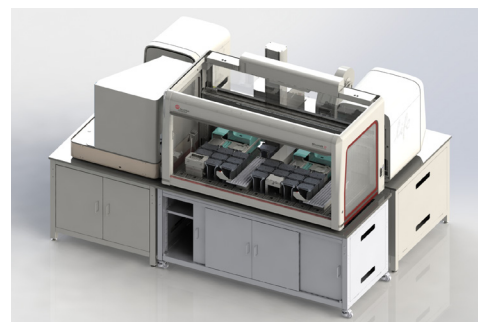
Effect of reference compound A on hepatitis C virus (HCV) replicon RNA synthesis in human hepatoma (Huh7) cells was studied using RT-qPCR (TaqMan) analysis of the HCV replicon RNA level (C_T). Huh7 cells were treated with increasing concentrations of reference compound A for 72 h. Increases in C_T values indicate decreasing replicon RNA levels; each C_T reflects a two-fold change in RNA level from baseline. Same performance was observed between reduced volume reactions, as compared to standard reaction volumes, set up using traditional methods.



Agrawal S, Cifelli S, Johnstone R, Pechter D, Barbey DA, Lin K, Allison T, Agrawal S, Rivera-Gines A, Milligan JA, Schneeweis J, Houle K, Struck AJ, Visconti R, Sills M, Wildey MJ, Utilizing Low-Volume Aqueous Acoustic Transfer with the Echo 525 to Enable Miniaturization of qRT-PCR (Vol. 21(1)) pp 57-63, copyright © 2015 by Society for Laboratory Automation and Screening, Reprinted by Permission of SAGE Publications

Integrating the Biomek Workstation into a Complete, Multi-Device Robotic System

Genomic applications using the Biomek Workstation can include robotic plate handling to obtain full walkaway solutions, for sample to results processing and minimizing errors that can come from manual processing. The engineers in the Access Solutions Group at Beckman Coulter Life Sciences can help create a solution to meet these needs, who have historically integrated more than 400 third-party devices from over 75 manufacturers in different laboratory settings. Our SAMI EX scheduling software works to plan workflows and batching to maintain incubation times for reproducibility of results.

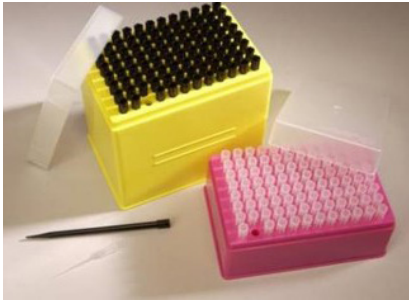


An example laboratory system with Biomek i7 Workstation and integrated devices to fully automate nucleic acid isolation from sample tubes, amplification reaction setup and qPCR/RTqPCR automation, in one system. Users load the sample tubes and needed consumables, and the system processes the samples to generate results.



Consumables

Biomek and Echo Qualified for Genomic Applications



Biomek Pipetting Tips

- **RNase- and DNase-free***: Ensures high-quality nucleic acid purification and reliable testing results.
- **DNA-free (human & mouse) and PCR inhibitor-free**: Confirms the absence of any contaminants (microbial or mammalian) that contribute to erroneous results and interfere with PCR.
- **Pyrogen-free**: Addresses pyrogen-sensitive applications, such as cell transformations and preparative protocols that use extracted biological material for in vivo experimentation.
- **Trace metal-free**: Reduces chemical interference due to chelation and denaturation.



*Bio-certification "free of" claims are defined as the lower limit of detection based on the sensitivity of the test method or instrumentation used.

Echo Qualified Source Microplates ensure precise, accurate liquid transfers, delivering the best possible results in miniaturized assays. Echo qualified source microplates are deionized and are packaged in anti-static bags to ensure precise drop placement. Microplates are available with low dead volume, custom barcode, DNase and RNase free or sterile options.



The MicroClimate Environmental Lid minimizes edge effects and preserves the concentrations of solutions in microplate wells. The novel fluid-absorbing matrix inside the lid can be loaded with water or a compatible solvent to create a vapor barrier, protecting the sample from evaporation and greatly reducing edge effects, and therefore increasing the microplate efficiency by 37%.



Acoustic Sample Tubes (sold by Azenta Life Sciences) transform workflows by enabling acoustic dispensing directly from tubes, on Echo 650 Series LHs. The tubes can be accessed individually – ideal for applications that require subsets of large reagent or sample libraries to be accessed frequently.

Beckman Coulter Life Sciences Service and Support



Maximize Your Instrument Performance

Timely service and preventive maintenance are essential for optimal instrument performance and data quality. Beckman Coulter Life Sciences offers a wide range of service contract options for the Biomek Workstations, Echo Liquid Handlers and Access Laboratory Workstations and Systems to fit every lab's needs and budget.

Our application scientists have extensive experience with genomic workflows and provide support and training on all our product offerings to help you maximize the use of your systems.

[beckman.com](https://www.beckman.com)

REFERENCES

1. Shapland EB, Holmes V, Reeves CD, et al. Low-Cost, High-throughput Sequencing of DNA Assemblies Using a Highly Multiplexed Nextera Process. Shapland et al., ACS Synth. Biol., 2015. doi.org/10.1021/sb500362n
2. Mayday MY, Khan LM, Chow ED, Zinter MS, DeRisi JL (2019) Miniaturization and optimization of 384-well compatible RNA sequencing library preparation. PLoS ONE 14(1): e0206194. <https://doi.org/10.1371/journal.pone.0206194>

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