Solid tumor cancer research

A convenient, solvent-free deparaffinization method for FFPE sample preparation

Introduction

Formalin-fixed paraffin-embedded (FFPE) tissue is commonly used in clinical oncology research. Before nucleic acid can be extracted from FFPE samples, they must be deparaffinized. Deparaffinization typically involves multiple rounds of washing with xylene and ethanol, which has several disadvantages. Tissue may be lost in the wash steps, and xylene is a flammable organic solvent that must be disposed of as chemical waste. The Occupational Safety and Health Administration (OSHA), the Environmental Protection Agency (EPA), and the Centers for Disease Control and Prevention (CDC) in the US have all identified xylene as a hazardous chemical that can be harmful when inhaled, ingested, or exposed to skin [1–3]. Some of the harmful effects of xylene detailed in a public health statement issued by the CDC and the Agency for Toxic Substances and Disease Registry (ATSDR) are listed in Table 1 [1–4].

Table 1. Potential toxicity due to xylene exposure.

Route of exposure	Affected organs	Symptoms
Inhalation	Nervous system	Dizziness, loss of balance, headache, confusion, and impaired memory
	Lungs	Irritation, chest pain, shortness of breath, and pulmonary edema (if exposed in a confined space)
External contact	Skin, eye, nose, and throat	Irritation and inflammation
Ingestion	Gastrointestinal tract	Discomfort, nausea, and vomiting
	Liver (rare)	Elevated serum transaminases and hepatocellular vacuolation
	Kidney (rare)	Distal renal tubular acidemia and abnormal chemistry

Deparaffinization can be performed without xylene using mineral oil, hot water, or dish washing soap [5–8], but the process is still tedious. We have developed a solvent-free method to deparaffinize FFPE samples using Applied Biosystems™ AutoLys M Tubes and Caps (Cat. No. A38738). AutoLys M Tubes and Caps eliminate the need for organic solvents and manual washing steps, and there are only two pipetting steps in the workflow. It requires less hands-on time than other deparaffinization methods, which makes the workflow more convenient for the operator and helps minimize tissue loss. AutoLys M Tubes and Caps have been validated and optimized using Thermo Scientific™ KingFisher™ purification systems and the Applied Biosystems™ MagMAX™ FFPE DNA/RNA Ultra Kit (Cat. No. A31881).

AutoLys M Tubes and Caps reduce processing time and simplify deparaffinization of FFPE samples for high-quality nucleic acid isolation. FFPE curls, slides, or cores can be added directly to the inner tubes for protease digestion (Figure 1). After digestion is complete, the inner tubes are lifted and locked in place for centrifugation to physically separate the wax and tissue by filtration. After centrifugation, the inner tubes are unlocked and removed. The lysates in the outer tubes are then ready for purification.

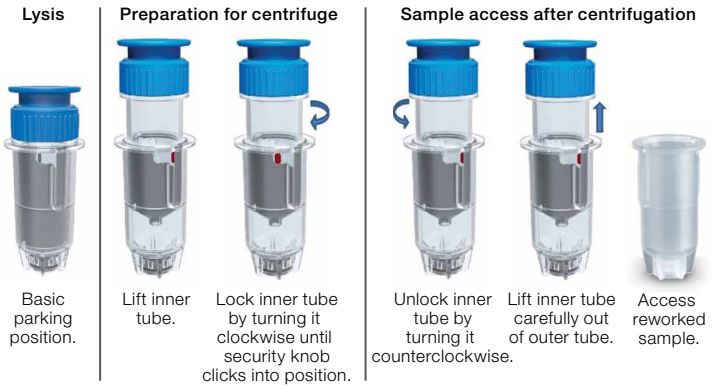

Figure 1. How to use AutoLys M Tubes and Caps.

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Deparaffinization workflow

An overview of the workflow for nucleic acid isolation from FFPE samples is shown in Figure 2. Deparaffinization is followed by nucleic acid isolation with the MagMAX FFPE DNA/RNA Ultra Kit on the Thermo Scientific™ KingFisher™ Flex or KingFisher™ Apex purification system.

The inner and outer tubes can be separated manually using Applied Biosystems™ AutoLys M Tube Pliers (Cat. No. A38261), which are useful when working with smaller sample sets (Figure 3). Applied Biosystems™ AutoLys M Tube Racks (Cat. No. A37955) hold up to 24 tubes, and the tubes can be spun down in any centrifuge with appropriate size plate adapters. A rack of 24 AutoLys M Tubes can be simultaneously lifted prior to centrifugation with the Applied Biosystems™ AutoLys M TubeLifter (Cat. No. A37956). The tube rack is returned to the AutoLys M TubeLifter after centrifugation to separate the inner and outer tubes.

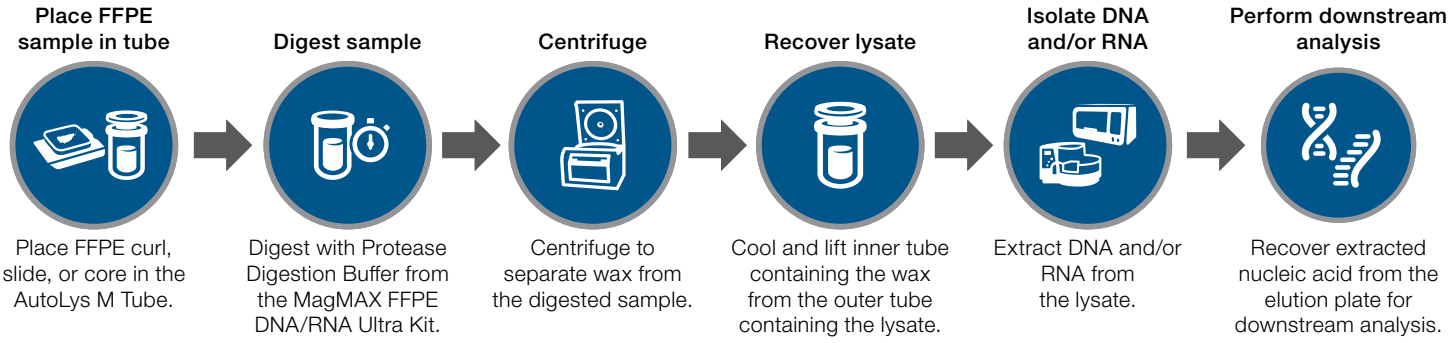

Figure 2. Workflow for deparaffinization and nucleic acid isolation using AutoLys M Tubes and Caps.

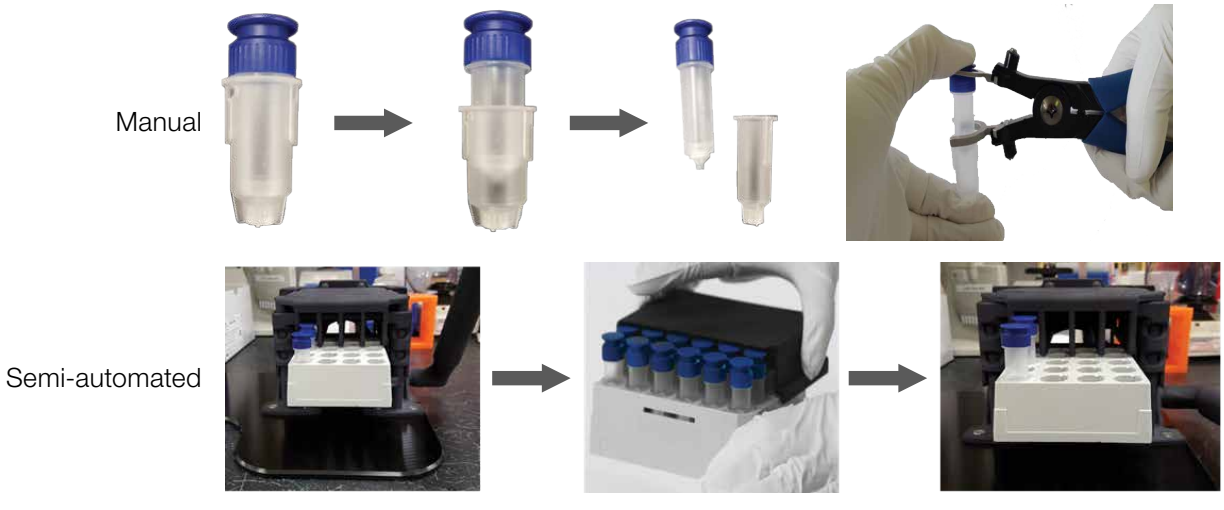

Figure 3. Manual or semi-automated use of AutoLys M Tubes and Caps.

Materials and methods

We performed up-front deparaffinization of FFPE samples using AutoLys M Tubes and Caps to evaluate their performance for standard sequential nucleic acid isolation. Six 1 x 10 µm curls were cut from each FFPE lung, colon, and breast cancer sample block. Each curl was deparaffinized using the AutoLys M Tubes and Caps workflow [9], a xylene-based method [10], or organic phase separation (MN). Each deparaffinization method was evaluated with a unique FFPE sample to analyze differences in performance. The AutoLys M TubeLifter was used to handle the AutoLys M Tubes and Caps after protease digestion.

After deparaffinization, DNA and RNA were sequentially extracted from each sample using the MagMAX FFPE DNA/RNA Ultra Kit on the KingFisher Flex Purification System. The eluates were prepared for DNA or RNA quantitation using Invitrogen™ Qubit™ dsDNA HS (high-sensitivity) or Qubit™ RNA High Sensitivity Assay Kits, respectively. The nucleic acid concentrations were then measured on an Invitrogen™ Qubit™ fluorometer.

Real-time PCR (qPCR) was performed using an Applied Biosystems™ TaqMan™ GAPDH assay on the Applied Biosystems™ QuantStudio™ 12K Flex Real-Time PCR System to evaluate the quality of the extracted nucleic acids. Invitrogen™ SuperScript™ IV VILO™ reagents were used for reverse transcription of RNA samples. The quality of the isolated nucleic acids was also assessed by next-generation sequencing (NGS). DNA and RNA sequencing libraries were prepared using the Ion AmpliSeq™ Cancer Hotspot Panel v2 and the Ion AmpliSeq™ RNA Cancer Panel, respectively, on the Ion Chef™ Instrument. This was followed by targeted NGS on the Ion GeneStudio™ S5 System.

Results and discussion

The DNA and RNA concentrations measured after deparaffinization using AutoLys M Tubes and Caps were comparable to or higher than the concentrations measured after deparaffinization with the organic separation (MN) and xylene-based methods (Figure 4). Variability in the yields obtained from curls cut from the same FFPE block was attributed to minor variations across replicates. The qPCR results indicate the deparaffinization workflows were largely comparable for processing FFPE curls from lung, colon, and breast tissue samples (Figure 5).

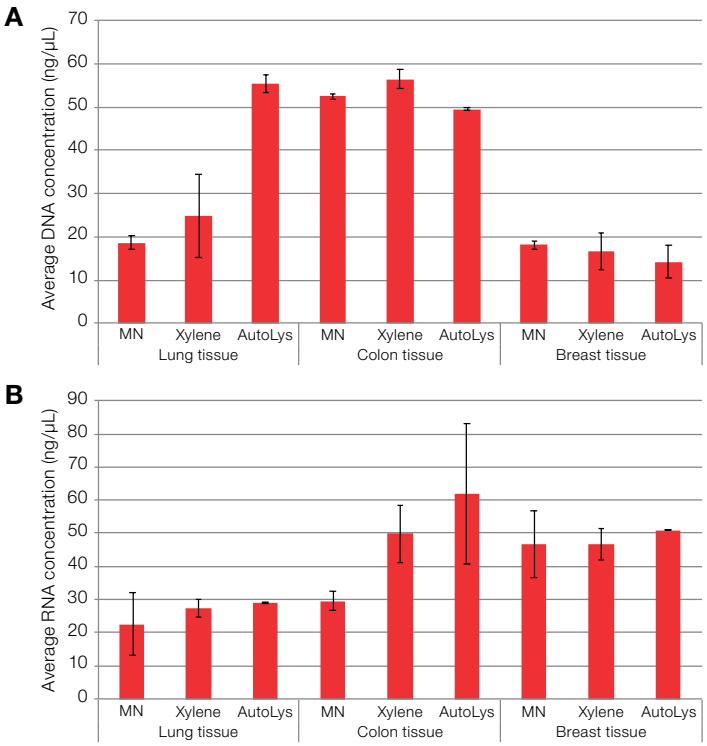

Figure 4. Concentrations of (A) DNA and (B) RNA after deparaffinization of FFPE lung, colon, and breast tissue samples using the organic separation (MN), xylene-based, and AutoLys M Tubes and Caps workflows.

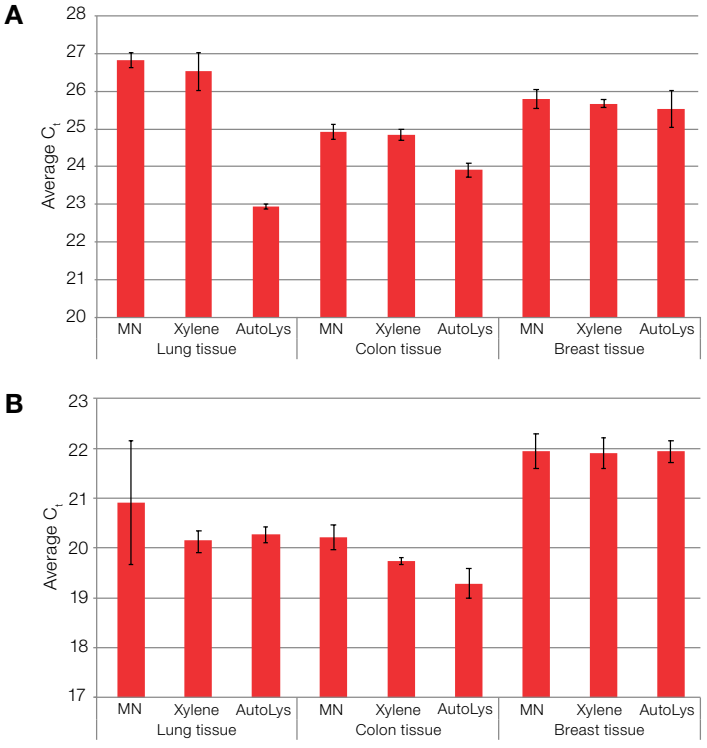

Figure 5. qPCR for the GAPDH gene from (A) DNA and (B) RNA samples isolated from FFPE curls after deparaffinization using the organic separation (MN), xylene-based, and AutoLys M Tubes and Caps workflows.

Sequencing results obtained using the Ion AmpliSeq Cancer Hotspot Panel v2 (DNA) and Ion AmpliSeq RNA Cancer Panel were analyzed using the coverage analysis and variant calling analysis software plug-ins. The metrics for DNA and RNA library coverage are shown by tissue type and deparaffinization workflow in Table 2. The mean read lengths, on-target rates, and coverage uniformity were comparable for both sets of libraries across deparaffinization workflows.

Table 3 lists allelic frequencies for 8 gene targets detected from the lung, colon, and breast tissue samples for the three deparaffinization methods. The allelic frequencies were largely concordant across workflows. Gene expression correlation plots for RNA obtained from samples deparaffinized with the xylene-based and AutoLys M Tubes and Caps workflows demonstrate that results appear to be comparable across all tissues (Figure 6). Specifically, R^2 values for lung, colon, and breast tissue samples are 0.977, 0.982, and 0.983, respectively.

Table 2. DNA and RNA library coverage analysis for FFPE lung, colon, and breast tissue samples deparaffinized using the organic separation (MN), xylene-based, and AutoLys M Tubes and Caps workflows.

	DNA library				
Tumor tissue	**Deparaffinization workflow**	**Mapped reads**	**Mean read length (bp)**	**On-target rate (%)**	**Uniformity (%)**
Lung	MN	1,265,341	113	95	98
	Xylene	1,518,161	112	96	99
	AutoLys M Tubes and Caps	2,049,273	114	99	99
Colon	MN	1,294,640	111	95	99
	Xylene	1,453,346	112	99	100
	AutoLys M Tubes and Caps	1,622,482	113	98	100
Breast	MN	1,007,587	112	97	98
	Xylene	1,165,427	111	93	99
	AutoLys M Tubes and Caps	1,173,460	112	97	98
	RNA library				
Tumor tissue	**Deparaffinization workflow**	**Mapped reads**	**Mean read length (bp)**	**On-target rate (%)**	**Uniformity (%)**
Lung	MN	651,627	141	100	100
	Xylene	649,535	150	100	100
	AutoLys M Tubes and Caps	673,427	149	99	100
Colon	MN	625,569	135	100	98
	Xylene	694,485	135	100	98
	AutoLys M Tubes and Caps	936,485	136	100	98
Breast	MN	875,669	132	100	98
	Xylene	648,966	139	100	100
	AutoLys M Tubes and Caps	660,510	132	100	100

Table 3. Allelic variants detected following deparaffinization using the organic separation (MN), xylene-based, and AutoLys M Tubes and Caps workflows.

		Allelic frequency (%)			
Tumor tissue	**Variant**	**MN**	**Xylene**	**AutoLys M Tubes and Caps**	**Mutation**
Lung	*IDH1*	43	46	45	G to A
	KRAS	12	12	17	C to G
	SMARCB1	46	52	48	G to A
Colon	*PIK3CA*	29	29	26	G to A
	PDGFRA	49	54	51	C to T
	FBXW7	31	27	29	C to T
	HRAS	47	51	48	A to G
	KRAS	50	49	43	C to T
	SMAD4	30	27	29	C to T
Breast	*HRAS*	15	16	17	A to G
	SMARCB1	40	40	41	G to A

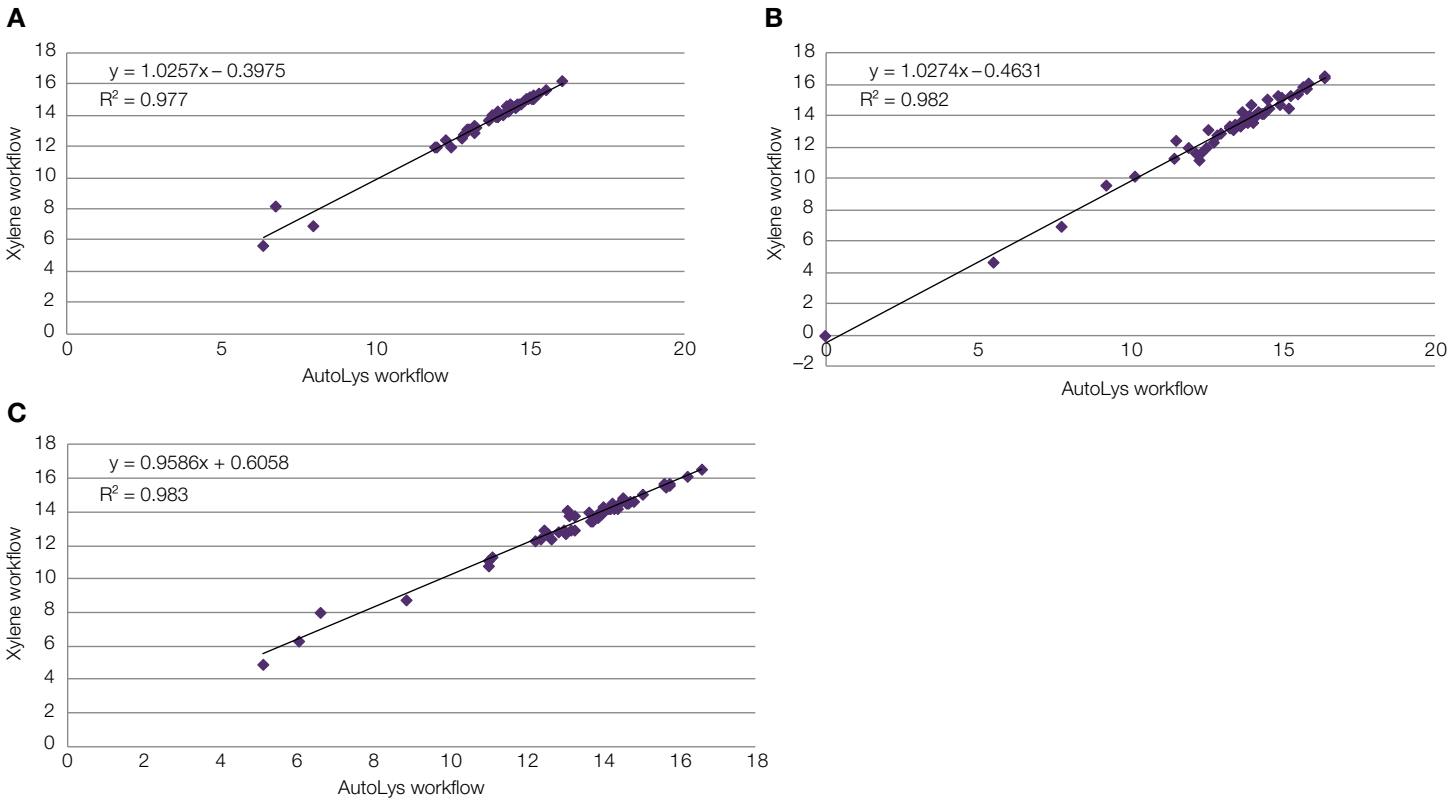

Figure 6. Gene expression correlation plots for FFPE (A) lung, (B) colon, and (C) breast tissue samples. RNA was extracted from FFPE samples after deparaffinization using the xylene-based and AutoLys M Tubes and Caps workflows.

Conclusions

We demonstrated that with AutoLys M Tubes and Caps, FFPE sample deparaffinization is convenient. AutoLys M Tubes and Caps work seamlessly for nucleic acid isolation with the MagMAX FFPE DNA/RNA Ultra Kit on KingFisher instruments for throughput versatility. The new workflow requires less hands-on time than manual deparaffinization with organic solvents and does not involve hazardous chemicals. It also helps minimize tissue loss by eliminating tedious washing steps.

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References

1. US Occupational Safety and Health Administration. Xylene, all isomers (dimethylbenzene). Occupational Chemical Database. Updated April 6, 2021. **https://www.osha.gov/chemicaldata/228**.
2. US Environmental Protection Agency. Xylenes; CASRN 1330-20-7. Integrated Risk Information System (IRIS) chemical assessment summary. Updated February 21, 2003. **https://iris.epa.gov/static/pdfs/0270_summary.pdf**.
3. US Centers for Disease Control and Prevention. Xylene. National Institute for Occupational Safety and Health (NIOSH). **https://www.cdc.gov/niosh/topics/xylene/default.html**, accessed January 19, 2023.
4. US Department of Health and Human Services (2007) Public Health Statement: Xylene. Agency for Toxic Substances and Disease Registry. **https://www.atsdr.cdc.gov/ToxProfiles/tp71-c1-b.pdf**.
5. Heikal N, Nussenzveig RH, Agarwal AM (2014) Deparaffinization with mineral oil: a simple procedure for extraction of high-quality DNA from archival formalin-fixed paraffin-embedded samples. *Appl Immunohistochem Mol Morphol* 22(8):623–626.
6. Kalantari N, Bayani M, Ghaffari T (2016) Deparaffinization of formalin-fixed paraffin-embedded tissue blocks using hot water instead of xylene. *Anal Biochem* 507:71–73.
7. Mansour A, Chatila R, Bejjani N et al. (2014) A novel xylene-free deparaffinization method for the extraction of proteins from human derived formalin-fixed paraffin embedded (FFPE) archival tissue blocks. *MethodsX* 1:90–95.
8. Negi A, Puri A, Gupta R et al. (2013) Biosafe alternative to xylene: a comparative study. *J Oral Maxillofac Pathol* 17(3):363–266.
9. Thermo Fisher Scientific. 2018. User Guide: MagMAX FFPE DNA/RNA Ultra Kit—Automated or manual sequential isolation of DNA and RNA from FFPE samples using AutoLys tubes (**MAN0017541**).
10. Thermo Fisher Scientific. 2021. User Guide: MagMAX FFPE DNA/RNA Ultra Kit—Automated or manual sequential isolation of DNA and RNA from FFPE samples (**MAN0015877**).

Ordering information

Product	Quantity	Cat. No.
AutoLys M tubes, caps, and accessories		
AutoLys M Tubes and Caps	25 each	A38738
AutoLys M TubeLifter	1 each	A37956
AutoLys M Tube Racks	5 racks	A37955
AutoLys M Tube Locking Lid	1 each	A37954
AutoLys M Tube Pliers	1 each	A38261
Assays and reagents		
MagMAX FFPE DNA/RNA Ultra Kit	1 kit	A31881
Ion AmpliSeq Cancer Hotspot Panel v2	8 reactions	4475346
Ion AmpliSeq Comprehensive Cancer Panel	8 reactions	4477685
SuperScript IV First-Strand Synthesis System	50 reactions	18091050
Instruments		
KingFisher Flex Purification System, with 96 Deep-Well Head	1 system	5400630
KingFisher Apex Purification System, with 96 Deep-Well Head	1 system	5400930
Qubit 4 Fluorometer, with WiFi	1 fluorometer	Q33238
QuantStudio 12K Flex Real-Time PCR System, OpenArray block, Accufill System	1 system	4471090
Ion Chef Instrument	1 system	4484177
Ion GeneStudio S5 System	1 system	A38194

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