Nucleic acid isolation

Detecting mutations in matched tissue and plasma liquid biopsies using optimized high-throughput sample preparation workflows

Introduction

Scientists are working to develop circulating cell-free DNA (cfDNA) profiling as a sensitive cancer research method and as a way to research cancer progression and relapse. The research focus has shifted to liquid biopsy as a companion to solid tumor biopsy, because liquid biopsy can be a viable alternative when traditional invasive biopsy is not feasible. In this study, we examined data quality and mutation-calling concordance by analyzing a small number of matched formalin-fixed, paraffin-embedded (FFPE) tissue samples and cell-free plasma liquid biopsy samples. Figure 1 shows an overview of the FFPE and plasma sample processing workflow for isolating cell-free nucleic acid with the Applied Biosystems™ MagMAX™ FFPE DNA/RNA Ultra Kit or the Applied Biosystems™ MagMAX™ Cell-Free Total Nucleic Acid Isolation and MagMAX™ Cell-Free DNA Isolation Kits.

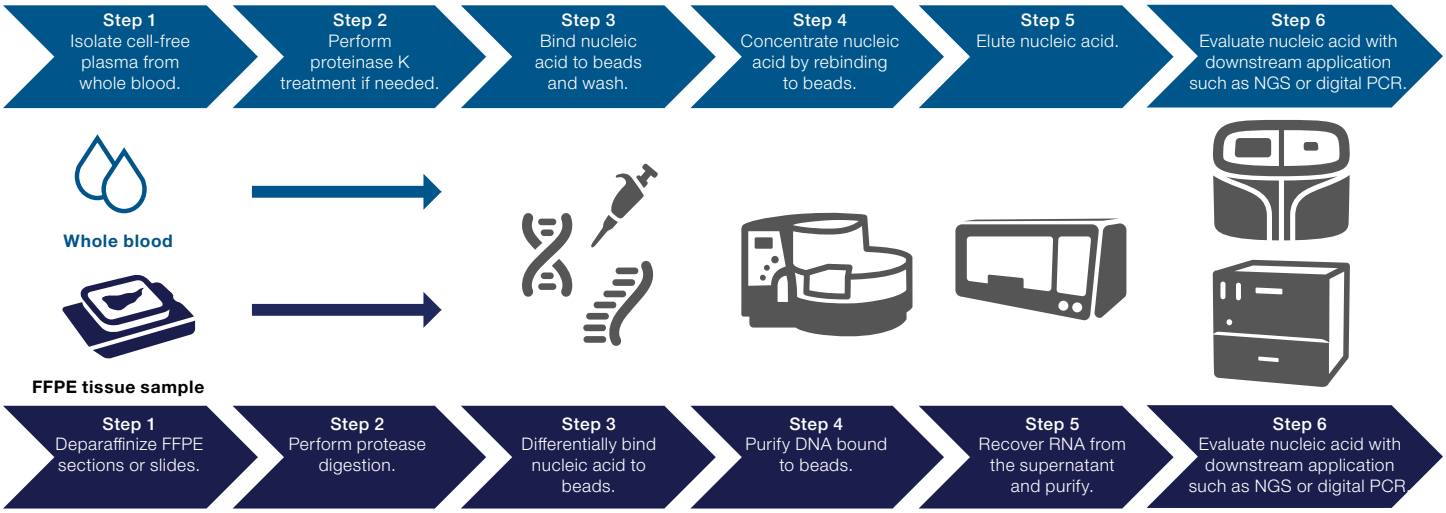

Figure 1. Nucleic acid isolation and purification workflow for MagMAX nucleic acid isolation kits.

Materials and methods

Samples

A total of 18 matched FFPE tissue blocks and plasma samples collected between 2015 and 2017 were obtained from Discovery Life Sciences. Information about the matched samples is summarized in Table 1.

Table 1. Information about matched FFPE tissue and cell-free plasma samples analyzed in the study.

Type of cancer		
Colon	n = 10	55.6%
Breast	n = 6	33.3%
Lung	n = 2	11.1%
Cancer stage		
<III	n = 9	50.0%
III	n = 8	44.4%
IV	n = 1	5.6%
Smoking history		
Current or past	n = 2	11.1%
Never	n = 16	88.9%
Alcohol history		
Current or past	n = 1	5.6%
Never	n = 17	94.4%

FFPE sample preparation and nucleic acid quality assessment

A 1 x 5 µm section from each FFPE tissue block was deparaffinized using either a standard xylene deparaffinization protocol or Applied Biosystems™ AutoLys M Tubes and Caps. The deparaffinized tissues were then subjected to protease digestion. DNA and RNA were sequentially isolated from each sample using the MagMAX FFPE DNA/RNA Ultra Kit (Cat. No. A31881) on the Thermo Scientific™ KingFisher™ Presto Purification System integrated with a Microlab™ NIMBUS™ automated pipetting station (Hamilton). Invitrogen™ Qubit™ dsDNA and RNA assays were used for quantification. RNA integrity was assessed by performing on-chip electrophoresis on a 2100 Bioanalyzer™ instrument (Agilent). RNA integrity numbers (RINs) below 2.5 indicated degradation, although the RNA fragments were relatively large.

Isolation and assessment of nucleic acid from cell-free plasma liquid biopsies

Cell-free DNA (cfDNA) and cell-free total nucleic acid (cfTNA) were isolated from 2–4 mL plasma samples with the MagMAX Cell-Free DNA Isolation Kit (Cat. No. A29319) and the MagMAX Cell-Free Total Nucleic Acid Isolation Kit (Cat. No. A36716). The samples were eluted in small volumes of 15 µL to obtain concentrated nucleic acid for preparing next-generation sequencing (NGS) libraries. All samples were analyzed on the Bioanalyzer electrophoresis instrument with a high-sensitivity kit to assess cfDNA peaks and genomic DNA (gDNA) contamination.

Library preparation and NGS

The Ion Torrent™ Oncomine™ Breast cfDNA Research Assay v2 and the Ion Torrent™ Oncomine™ Colon cfDNA Assay were used to prepare libraries with 10 ng of gDNA isolated from each of the FFPE breast and colon tissue samples. cfTNA isolated from the two lung cancer plasma samples was used to prepare libraries with the Ion Torrent™ Oncomine™ Lung Cell-Free Total Nucleic Acid Research Assay. The Oncomine Lung cfTNA panel has 12 targets, and cfDNA and cfRNA are both used as input materials. The final libraries were quantified by quantitative PCR (qPCR) using the Ion Library TaqMan™ Quantitation Kit, and the Ion Chef™ Instrument was used for template preparation and chip loading. Sequencing was performed on the Ion GeneStudio™ S5 System using an Ion™ 530 Chip or Ion™ 540 Chip. After sequencing was complete, the data were analyzed with Applied Biosystems™ Variant Reporter™ Software using the appropriate Oncomine liquid biopsy or tumor analysis plug-in. A concordance heatmap was generated to identify shared mutations and mutations that were unique to each sample type.

Results and discussion

RNA and DNA extracted from the FFPE blocks with the MagMAX FFPE DNA/RNA Ultra Kit were quantitated using Qubit RNA and dsDNA assays to determine total nucleic acid yields. DNA yields were greater than 500 ng on average, with a maximum yield near 2,200 ng depending on the donor (Figure 2). The RNA yields from colon, lung, and breast tissue were all greater than 1,000 ng and reached a maximum of 4,500 ng.

RNA extracted from the FFPE tissue samples using the MagMAX FFPE DNA/RNA Ultra Kit was loaded onto an Agilent™ RNA Nano chip in 1 µL volumes. The RINs were all below 2.5, but 85% of the samples contained fragments that were ≥200 nucleotides in length (Figure 3). Ribosomal RNA was detected in some samples. cfDNA extracted from plasma with the MagMAX Cell-Free DNA Isolation Kit was loaded onto a high-sensitivity DNA chip in 1 µL volumes. The most prominent cfDNA peaks appeared between 100 bp and 225 bp (Figure 4). Fragments longer than ~1,000 bp were assumed to be gDNA.

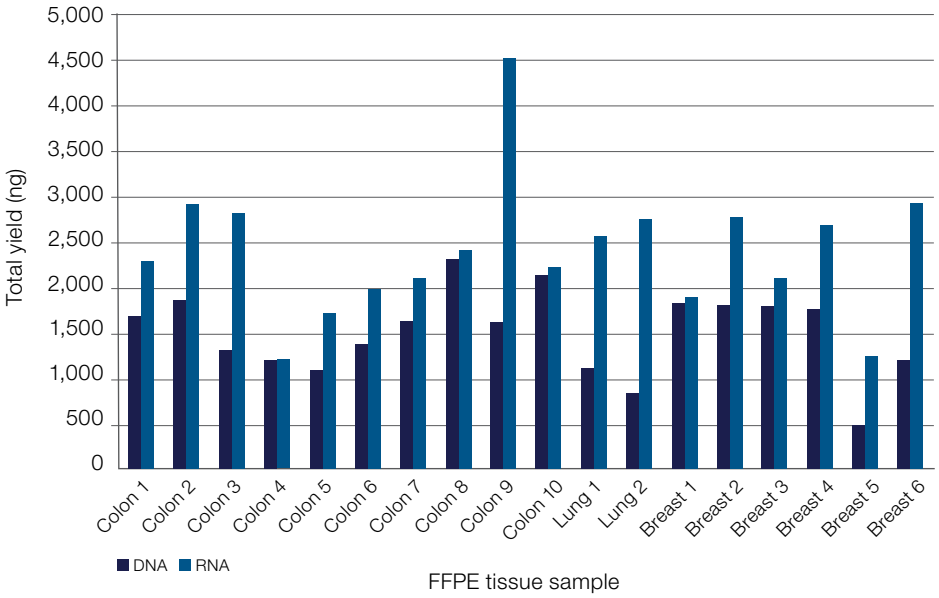

Figure 2. DNA and RNA yields obtained from 1 x 5 µm FFPE tissue samples. DNA and RNA were sequentially isolated from colon, lung, and breast FFPE samples using the MagMAX FFPE DNA/RNA Ultra Kit. The total DNA and RNA yields were determined by Qubit RNA and dsDNA assays.

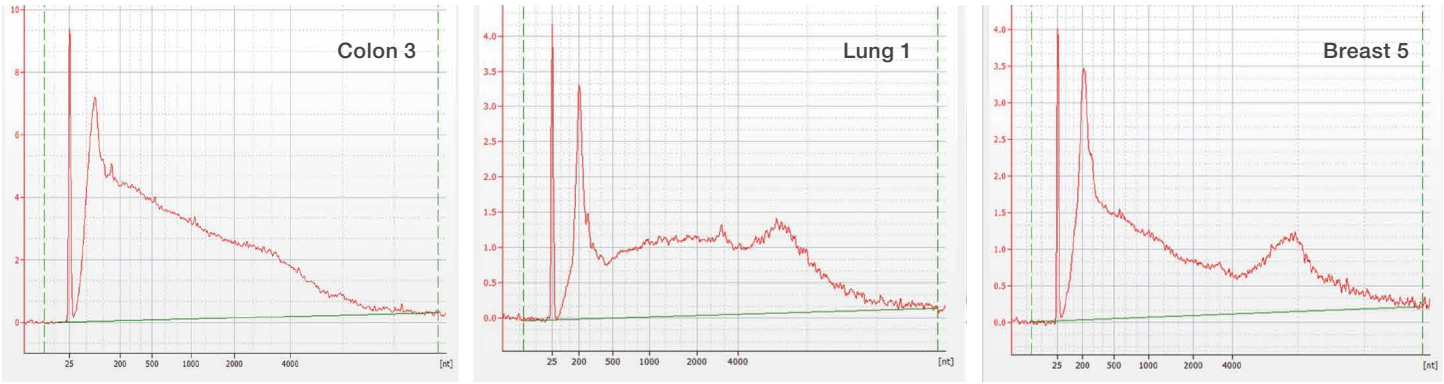

Figure 3. RNA traces for FFPE tissue samples.

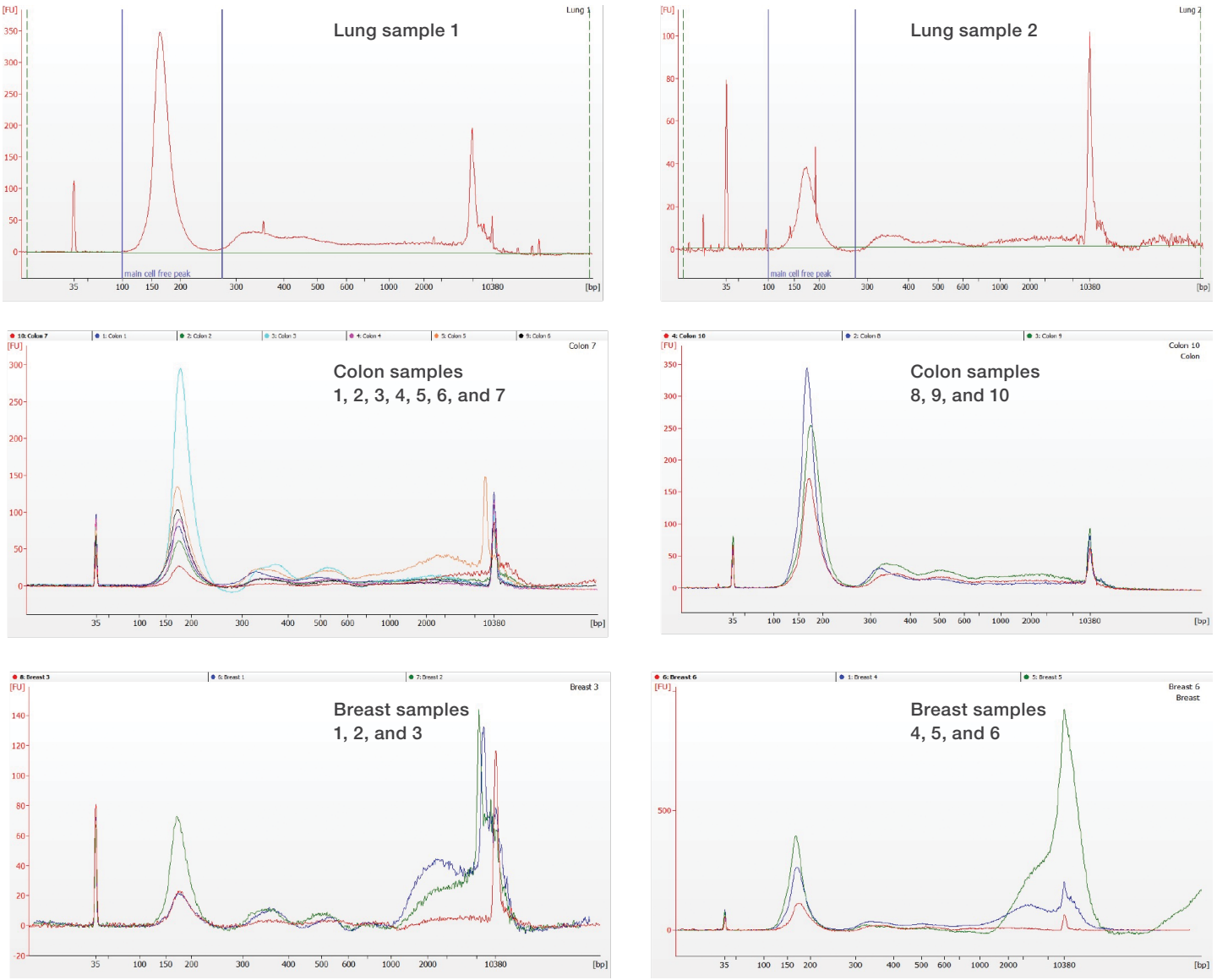

Figure 4. DNA traces for plasma samples. The samples were extracted using the MagMAX Cell-Free DNA Isolation Kit. The DNA was loaded onto a high-sensitivity DNA chip in 1 µL volumes.

Oncomine libraries were prepared for all 18 samples and sequenced. A detailed concordance summary for the full sample set is shown in Table 2. All detected hotspot mutations were recorded by the change in gene and amino acid. The allelic frequencies observed in the FFPE tissue and plasma samples were also recorded. Truly concordant positive mutations detected in plasma and FFPE colon samples with the Oncomine Colon cfDNA Assay are highlighted in green in Figure 5. Mutations detected only in the FFPE tissue samples are highlighted in purple, and mutations detected only in plasma are highlighted in blue. Calls for different mutations in the same gene that were detected in both plasma and FFPE tissue samples are highlighted in orange. These calls were considered partially concordant.

Table 2. Detailed concordance summary for FFPE tissue and plasma samples.

Sample	AA change	Gene	Allelic frequency in FFPE tissue (NGS data)	Allelic frequency in plasma (NGS data)	Allelic frequency in FFPE tissue (dPCR data)	Allelic frequency in plasma (dPCR data)
Colon 1	p.V600E	*BRAF*	12.69	13.59	7.99	10.02
	p.R273C	*TP53*	2.33	0.59		
	p.G244D	*TP53*	–	8.35		
Colon 2	p.R213Q	*TP53*	29.30	–		
	p.R361H	*SMAD4*	32.66	–		
Colon 3	p.R1114Ter	*APC*	17.89	–		
	p.E1309fs	*APC*	35.57	0.50		
	p.G244D	*TP53*	51.88	–		
	p.R273H	*TP53*	0.57	–	<LOD	
	p.G245S	*TP53*	0.40	–	<LOD	
Colon 4	p.E1309fs	*APC*	31.78	–		
	p.G12D	*KRAS*	26.41	–	13.27	
Colon 5	p.E1379Ter	*APC*	58.60	0.16		
	pG245S	*TP53*	46.88	0.05	38.97	<LOD
Colon 6	p.G12V	*KRAS*	34.06	0.09	29.10	<LOD
Colon 7	p.E545K	*PIK3CA*	4.63	–	<LOD	
	p.Q546K	*PIK3CA*	11.45	–		
	p.R479Q	*FBXW7*	15.08	0.34		
	p.Q1291Ter	*APC*	16.41	0.09		
Colon 8	p.R248W	*TP53*	50.15	0.054		
	p.G12V	*KRAS*	38.15	–		
Colon 9	p.R465C	*FBXW7*	–	0.053		
	p.H179Y	*TP53*	–	0.051		
	p.R175H	*TP53*	–	0.13		
	p.R201H	*GNAS*	–	1.46		
Colon 10	p.G13D	*KRAS*	23.73	0.28		
	p.R248W	*TP53*	38.67	0.35		
	p.Q61K	*NRAS*	–	0.064		
	p.E1306Ter	*APC*	–	0.13		
	p.R273L	*TP53*	–	0.066		
	p.R213L	*TP53*	–	0.066		
	p.R175L	*TP53*	–	0.052		
	p.G510V	*SMAD4*	–	0.055		
Breast 1	p.E542K	*PIK3CA*	3.12	–	<LOD	
	p.H1047R	*PIK3CA*	1.51	–	1.48	
Breast 2	No mutations detected					
Breast 3	p.H1047R	*PIK3CA*	52.09	–	<LOD	
Breast 4	p.C238Y	*TP53*	–	0.088		
	p.H1047R	*PIK3CA*	20.91	–		
Breast 5	p.H1047R	*PIK3CA*	44.25	–		
Breast 6	p.R273H	*TP53*	–	0.30		
	p.L194R	*TP53*	–	0.19		
	p.C176Y	*TP53*	40.32	–		
Lung 1	NA	*NKX2-1*	NA	NA		
Lung 2	p.Gly12Cys	*KRAS*	8.99	–	7.90	

Not tested	Not detected	True positive concordance

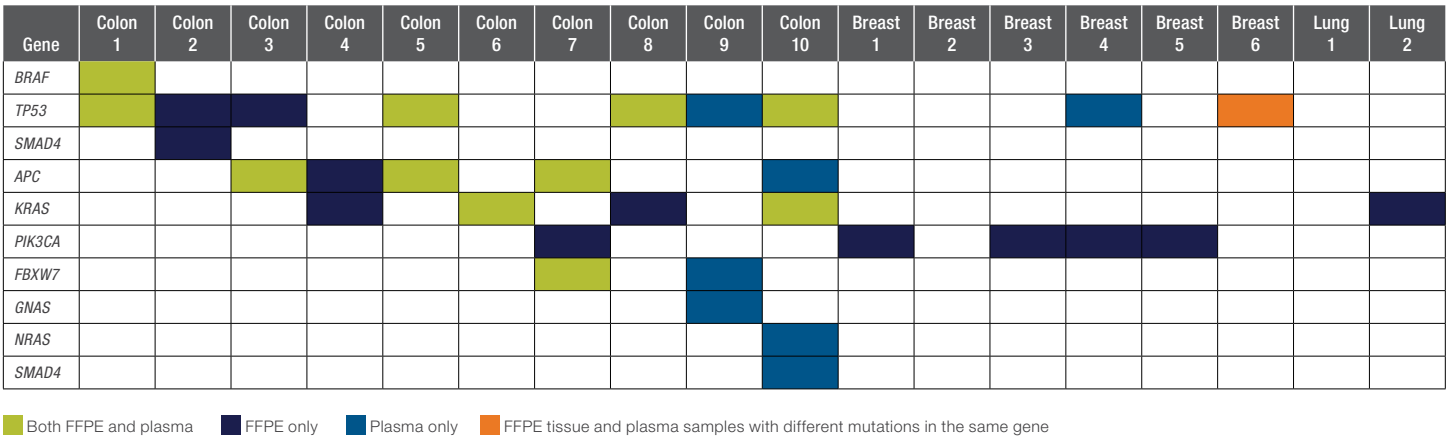

Figure 5. Concordance heatmap for FFPE tissue and plasma samples. The sequencing libraries were prepared using the Oncomine Lung Cell-Free Total Nucleic Acid Research Assay.

Conclusion

The findings of our study confirmed that data obtained from FFPE tissue and plasma samples were complementary. Although concordance was low when allelic frequencies were low in plasma, the same mutations were detected in the matched FFPE tissue samples. The sensitivity for detecting mutations in nucleic acid isolated from plasma would presumably be higher if the sample volume exceeded 4 mL. Sequencing revealed that analyzing nucleic acid from matched plasma and FFPE tissue samples provided more complete information than analyzing nucleic acid from only one sample type (Table 3), because alterations not detected in one sample type were detected in the other. Concordance between sample types will vary depending on the samples, the stage of cancer, and the treatment status of the patient. For example, biopsies for certain types of cancer will contain more detectable cell-free nucleic acid. Understanding the source material and optimizing sample preparation and mutation detection methods are important first steps for developing molecular profiling tools.

Table 3. Composite NGS data for cell-free nucleic acid (cfNA) and nucleic acid isolated from FFPE tissue samples.

Total number of mutations detected in cfNA	25
Total number of mutations detected in FFPE tissue samples	29
Total number of mutations detected	44
Mean allelic frequency in cfNA	1.08% (0.05% to 13.59%)
Mean allelic frequency in FFPE tissue samples	23.7% (0.15% to 58.60%)
Number of unique cfDNA mutations not detected in FFPE tissue samples	15
Number of unique FFPE tissue mutations not detected in cfNA	19
Number of true positive concordant mutations*	10 (22.70%)

* Same mutation detected in the same gene in both sample types.

Ordering information

Product	Cat. No.
MagMAX FFPE DNA/RNA Ultra Kit	A31881
AutoLys M Tubes and Caps	A38738
MagMAX Cell-Free DNA Isolation Kit	A29319
MagMAX Cell-Free Total Nucleic Acid Isolation Kit	A36716
KingFisher Flex Purification System, KingFisher with 24 Deep-Well Head	5400640
KingFisher Flex Purification System, KingFisher with 96 Deep-Well Head	5400630
KingFisher Presto Purification System with 24 DW Head	5400840
KingFisher Presto Purification System with 96 DW Head	5400830
Oncomine Lung Cell-Free Total Nucleic Acid Research Assay	A35864
Oncomine Breast cfDNA Research Assay v2	A35865
Oncomine Colon cfDNA Assay	A31182
QuantStudio Absolute Q Digital PCR System, desktop	A52864

References

1. Chae YK, Davis AA, Carneiro BA et al. (2016) Concordance between genomic alterations assessed by next-generation sequencing in tumor tissue or circulating cell-free DNA. *Oncotarget* 7(40):65364-65373.
2. Kuderer NM, Burton KA, Blau S et al. (2017) Comparison of 2 commercially available next-generation sequencing platforms in oncology. *JAMA Oncol* 3(7):996-998.

Authors

Angie Cheng, Hannah Saunders, Lillie Manley, Michelle Leija

Thermo Fisher Scientific, Austin, Texas, USA

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