APPLICATION NOTE

Miniaturized PIK3CA Mutational Analysis Utilizing the Labcyte Echo® Liquid Handler

John Lesnick, Linda Orren, and Noël Ruppert

Labcyte Inc.

Abstract

By combining the acoustic liquid transfer of the Labcyte Echo Liquid Handler with real-time PCR mutation analysis, we have demonstrated a highly efficient, low-cost method to detect PIK3CA somatic mutations. The Echo 555 Liquid Handler was used to dispense sub-microliter volumes of reagents from EntroGen's PIK3CA real-time PCR mutation analysis kit for subsequent analysis on the Roche LightCycler® 480 system. The methods detailed here reduce the total volume of reaction from 30 µL to 3 µL.

The miniaturization of the PIK3CA mutational analysis assay results in significantly less input DNA required, allowing conservation of precious and limited samples. Furthermore, miniaturization facilitates the transition of this assay from 96-well microplates to 384-well microplates–a 4-fold increase of throughput. Finally, the analytical sensitivity and limit of detection of this assay technology enables detection of mutant allelic frequency below 1.25% over wild-type background.

INTRODUCTION

Mutations are genetic alterations that are acquired in germ or non-germ (somatic) cells. The mutation can be in the form of an insertion, deletion or a mis-sense or non-sense genetic alteration. These alterations can occur in either coding or non-coding regions. The occurrence of these alterations in DNA sequence can cause various diseases and inherited disorders. The ability to identify acquired mutations not present in normal patient tissue from tumor derived material, including CTC's and DTC's, is important for diagnostic predisposition testing for cancer. Mutational analysis can ultimately inform targeted therapy and affect prognosis through the detection of biomarkers or primary tumor genotypes.

Because the cost to perform a mutational analysis can be prohibitive, many patients are directed to standardized cancer therapies that may be less effective. Typically in these cases mutational analysis will be conducted only after the standardized therapy fails. The level of miniaturization offered by Echo liquid handlers can significantly reduce the costs of mutational analysis to warrant use immediately and provide patients with a more informed treatment decision sooner.

The phosphoinositide-3 kinase (PI3K)/AKT signaling network has been identified as one of the two most commonly mutated pathways in human cancers and the most frequently mutated pathway in breast cancer. Somatic alterations including PIK3CA mutations are the most common genetic alteration of this pathway; 80% or more occur within the helical (E542K, E545K) and kinase (H1047R) domains of p110.

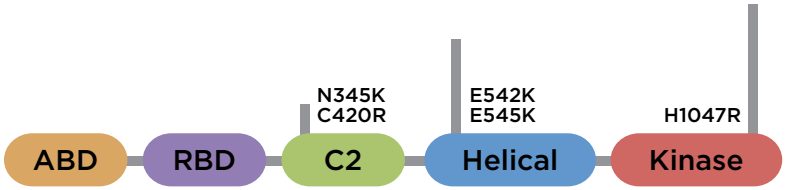


FIGURE 1 ▶

Protein domain structures of PIK3CA with hotspot mutations. The most common mutations in each domain are listed. The heights of the vertical bars indicate relative frequency of mutation in each domain.

Troxell ML (2012) PIK3CA/AKT1 Mutations in Breast Carcinoma: a Comprehensive Review of Experimental and Clinical Studies. J Clin Exp Pathol S1:002. doi: 10.4172/2161-0681

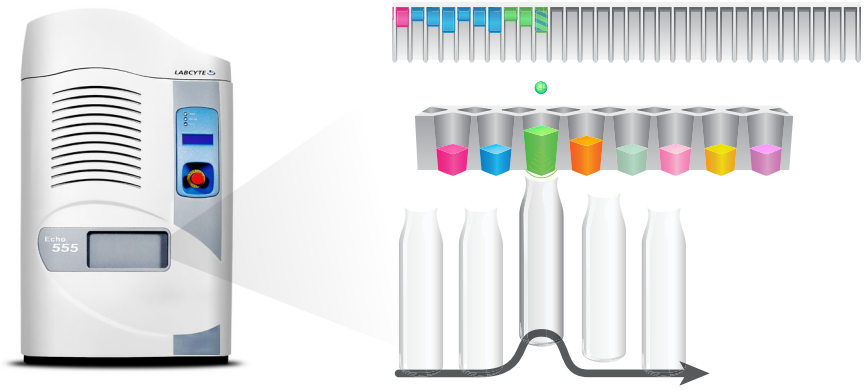


FIGURE 2 ▶

Assay workflow using the Echo 555 Liquid Handler to transfer reagents for a final reaction volume of 3 µL.

The Labcyte Echo Liquid Handlers revolutionize liquid transfer by using acoustic energy to transfer samples and reagents. Echo systems have an acoustic transducer that emits low energy sound waves that propel droplets from a source plate to an inverted destination plate above. Droplets are retained in the destination plate by electrostatics and surface tension. Transfer with Echo liquid handlers is completely touchless—no tips, nozzles, or physical material contacts the sample as it moves from a source microplate to destination plate, array or other type of labware. The elimination of tips when using the Echo Liquid Handler provides additional cost savings and eliminates waste, carry-over effects and cross-contamination. The Echo 555 Liquid Handler can transfer in 2.5 nL increments to allow miniaturization with unmatched accuracy and precision.

MATERIALS

• **PIK3CA Mutation Analysis Kit**	EntroGen # PI3K-RT48
• **PI3Kα E542K DNA Reference Standard**	Horizon # HD688
• **PI3Kα E545K DNA Reference Standard**	Horizon # HD689
• **PI3Kα H1047R DNA Reference Standard**	Horizon # HD690
• **PI3Kα Wild Type DNA Reference Standard**	Horizon # HD691
• **384-well Echo Qualified Polypropylene Source Plate**	Labcyte # P-05525
• **384-well White LC480 PCR Plate**	Roche # 04729749001
• **ISOLATE II Genomic DNA Kit**	Bioline # BIO-52066
• **MCF-7 Positive Control (E545K) Cell Line**	ATCC # HTB-22
• **PC-3 Negative Control Cell Line**	ATCC # CRL-1435
• **Genomic DNA ScreenTape**	Agilent # 5067-5365
• **Genomic DNA Reagents**	Agilent # 5067-5366

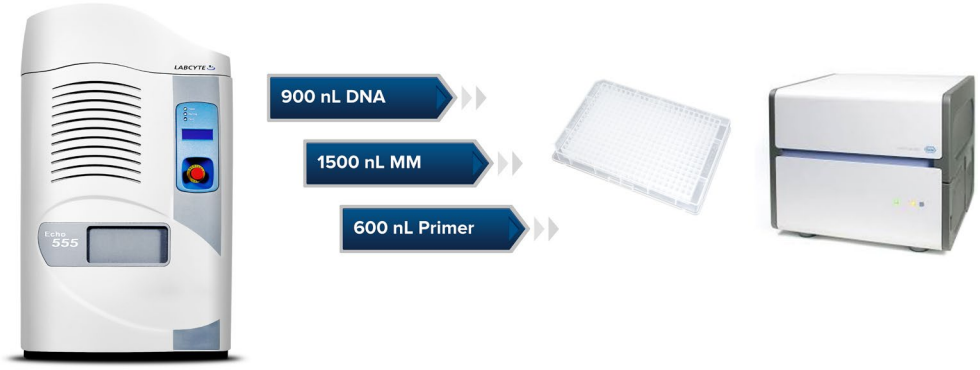
METHODS

Mutational analysis experiments were performed using the Echo 555 Liquid Handler to create 3 µL reactions composed of 900 nL of DNA template, 1.5 µL of Entrogen Mutation Detection Reaction Mix (2X), and 600 nL of mutation-specific primers. qPCR was performed on the Roche LightCycler® 480 in 384-well microplates with the following conditions: 95°C for 10 minutes; 45 cycles: 95°C for 10 seconds and 60°C for 30 seconds. All samples were run in triplicate. CT values were obtained from the real-time PCR data collection software provided with the instrument. In the software, Dual Color Hydrolysis Probe / UPL Probe with FAM (460-510) and VIC (533-580) detection format was used with the auto-baseline option selected. The data was analyzed using the: "Abs Quant/2nd derivative Max" criteria for all samples. The mutation status of each sample was determined by the endogenous control (VIC) amplification plot and the positive control (FAM) amplification plot. A sample was identified as positive for the mutation evaluated if the VIC CT value was greater than or equal to25 and the FAM CT value was less than or equal to 38. A sample was identified as negative for the mutation evaluated if the VIC CT value was less than or equal to 31 and FAM CT value was greater than or equal to 38.01 or absent altogether.

Cellular gDNA was isolated from MCF-7 and PC-3 cells following the Bioline Isolate II Genomic DNA Kit. The quantity and quality of the DNA samples was analyzed using the Agilent 2200 TapeStation.

FIGURE 3 ▶

Assay workflow using the Echo 555 Liquid Handler to transfer reagents for a final reaction volume of 3 µL.



Experiment 1

Reduction of Sample Requirement

If the sample size can be reduced without compromising the validity of results then the cost to test samples will be reduced. This is important whether you have many tests to run, limitations due to scarcity of sample or very costly samples. To evaluate the impact of reducing sample input the Echo 555 Liquid Handler was used to transfer variable amounts of control DNA, supplied with the kit from Entrogen, to the destination plate. The control DNA was backfilled with water to a volume of 900 nL. To this, 600 nL of E545Q primers and 1.5 µL of master mix was added to reach a final volume of 3 µL. The amounts of DNA transferred were; 10 ng, 8 ng, 6 ng, 4 ng, 2 ng, 1 ng and 0.5 ng.

Results

TABLE 1 ▶

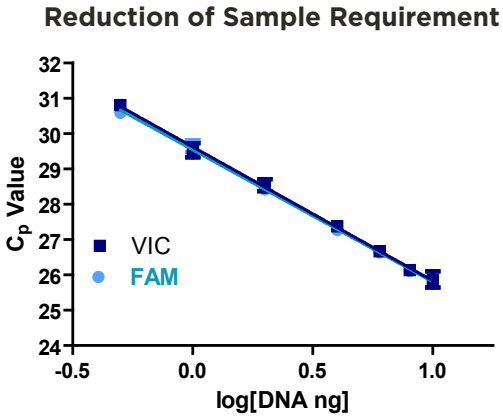
Comparison of Cp values from varying concentrations of control DNA in miniaturized reactions.

	VIC Cp		FAM Cp	
	Average	StDev	Average	StDev
10 ng	25.87	0.23	25.83	0.15
8 ng	26.14	0.03	26.11	0.02
6 ng	26.67	0.05	26.64	0.04
4 ng	27.38	0.06	27.27	0.03
2 ng	28.54	0.17	28.42	0.13
1 ng	29.53	0.2	29.65	0.17
0.5 ng	30.81	0.13	30.59	0.07

A sample is positive for the mutation evaluated when VIC Cp is greater than or equal to 25 and FAM Cp is less than or equal to 38. The results shown in the table above demonstrate the ability to detect the E545Q mutation in control DNA. A robust positive signal is observed at 1 ng of DNA. This demonstrates the ability to reduce sample requirement with the Echo Liquid Handler.

FIGURE 4 ▶

VIC is plotted in dark blue with an R2 = 0.999 and slope of -3.74. FAM is plotted in light blue with an R2 = 0.999 and slope of -3.79.



The results show very good linearity for both the VIC and FAM readouts with R2 values of 0.999 and a slope of 3.79 on a logarithmic scale. This demonstrates the precision and accuracy of transfers performed by the Echo Liquid Handler.

Experiment 2

Limit of Detection (LOD) Evaluation

It is important to determine the threshold of an assay and subsequently, the lowest concentration of a target reliably distinguished from a blank sample. This is even more imported for the detection of CTC's in blood or point mutations in a genome. To assess the LOD for a miniaturized PIK3CA mutational analysis assay the Echo 555 Liquid Handler was used to transfer variable amounts of control mutant DNA and control wildtype (WT) DNA for a range of percent mutant / WT DNA from 50% to 0.5% in 900 nL for 10ng, 3ng and 1 ng total DNA in triplicate. The LOD for E542K (2A), E545K (2B) and H1047R (2C) was evaluated.

Results

TABLE 2 ▶

Table comparing Cp values for 10 ng, 3 ng and 1 ng input amounts of control DNA at varying proportions of WT and mutant E542K.

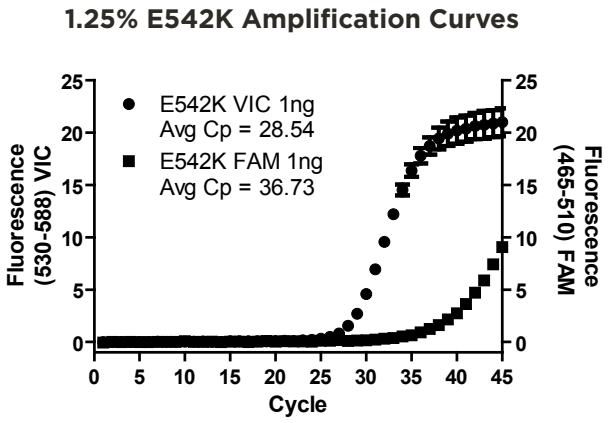
Positive Detection for E542K at 1.25% and 1 ng DNA Input

% E542K		10 ng Input		3 ng Input		1 ng Input	
		Average Cp	StDev Cp	Average Cp	StDev Cp	Average Cp	StDev Cp
50.00	VIC	24.26	0.3	26.22	0.0	28.07	0.1
	FAM	26.85	1.8	27.93	0.2	29.73	0.0
10.00	VIC	24.37	0.1	26.46	0.2	28.30	0.2
	FAM	28.79	0.2	30.29	0.4	32.08	0.1
5.00	VIC	24.42	0.2	26.46	0.2	28.66	0.1
	FAM	33.85	0.9	35.05	0.7	35.41	0.3
2.5	VIC	24.42	0.2	26.46	0.2	28.66	0.1
	FAM	36.83	0.9	38.36	1.0	36.73	0.4
1.25	VIC	24.66	0.0	26.70	0.1	28.54	0.1
	FAM	36.83	0.9	38.36	1.0	36.73	0.4
1.00	VIC	24.56	0.1	26.51	0.0	28.64	0.1
	FAM	36.86	0.5	38.72	1.9	37.80	1.9
0.50	VIC	24.53	0.2	26.66	0.1	28.56	0.1
	FAM	38.41	0.5	39.57	0.9	40.00	0.0

Control WT PI3Kα DNA supplied in the kit from Entrogen was mixed with a PI3Kα E542K DNA Reference Standard from Horizon Discovery to create DNA samples with variable percentages of mutant DNA in a WT background. A sample is positive for the mutation evaluated where the VIC Cp value is greater than or equal to 25 and the FAM Cp value is less than or equal to 38. The results shown in the table above demonstrate the ability to reliably detect 1% of E542K mutant DNA in 1 ng of sample DNA.

FIGURE 5 ▶

Amplification Plot for 1.25% E542K of 1ng DNA



The results for a representative amplification curve of 1 ng DNA comprised of 1.25% E542K mutant DNA demonstrate a positive result. The VIC readout is plotted in solid circle symbols against the left hand Y-axis and the FAM readout is plotted in solid squares data against the right hand Y-axis. Both datasets plot Cp over cycle. The VIC Cp value of 28.54 and FAM Cp value of 36.73 allow a positive determination.

TABLE 3 ▶

Table comparing Cp values for 10 ng, 3 ng and 1 ng input amounts of control DNA at varying proportions of WT and mutant E545K.

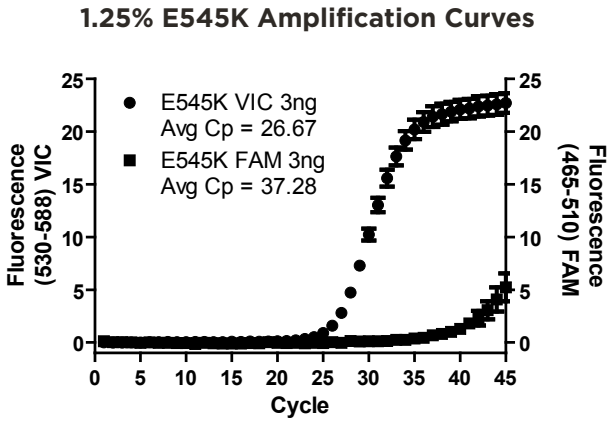
Positive Detection for E542K at 1.25% and 3 ng DNA Input

% E542K		10 ng Input		3 ng Input		1 ng Input	
		Average Cp	StDev Cp	Average Cp	StDev Cp	Average Cp	StDev Cp
50.00	VIC	24.32	0.6	26.66	0.1	28.75	0.1
	FAM	25.60	0.6	27.57	0.3	29.50	0.2
10.00	VIC	24.78	0.2	26.72	0.0	28.70	0.1
	FAM	28.34	0.8	29.94	0.3	31.63	0.2
5.00	VIC	24.81	0.2	26.79	0.0	28.68	0.0
	FAM	30.18	0.5	31.60	0.7	33.44	0.4
2.5	VIC	24.58	0.1	26.79	0.0	28.51	0.1
	FAM	32.09	0.8	32.67	0.5	33.99	0.2
1.25	VIC	24.84	0.1	26.63	0.1	28.47	0.0
	FAM	37.86	1.9	36.41	0.7	37.68	1.7
1.00	VIC	24.54	0.1	26.67	0.1	28.52	0.1
	FAM	37.06	0.2	37.28	0.6	38.48	1.6
0.50	VIC	24.41	0.3	26.67	0.0	28.38	0.1
	FAM	39.04	1.2	38.94	0.8	39.47	1.1

Control WT PI3Kα DNA supplied in the kit from Entrogen was mixed with a PI3Kα E545K DNA Reference Standard from Horizon Discovery to create DNA samples with variable percentages of mutant DNA in a WT background. A sample is positive for the mutation evaluated where the VIC Cp value is greater than or equal to 25 and the FAM Cp value is less than or equal to 38. The results shown in the table above demonstrate the ability to detect as low as 1% E545K mutant DNA in 1 ng of sample DNA.

FIGURE 6 ▶

Amplification Plot for 1.25% E542K of 1ng DNA



The results for a representative amplification curve of 3 ng DNA comprised of 1.25% E545K mutant DNA demonstrate a positive result. The VIC readout is plotted in solid circle symbols against the left hand Y-axis and the FAM readout is plotted in solid squares data against the right hand Y-axis. Both datasets plot Cp over cycle values. The VIC Cp value of 26.67 and FAM Cp value of 37.28 allow a positive determination.

TABLE 4 ▶

Table comparing Cp values for 10 ng, 3 ng and 1 ng input amounts of control DNA at varying proportions of WT and mutant H1047R.

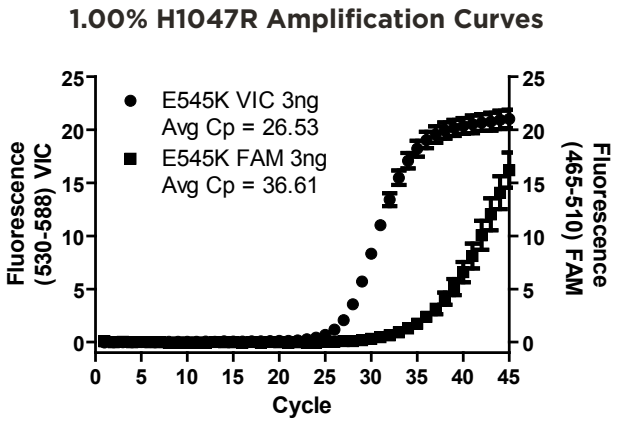
Positive Detection for H1047R at 1.00% and 3 ng DNA Input

% H1047R		10 ng Input		3 ng Input		1 ng Input	
		Average Cp	StDev Cp	Average Cp	StDev Cp	Average Cp	StDev Cp
50.00	VIC	29.03	0.3	26.28	0.1	20.62	10.1
	FAM	30.32	0.8	27.83	0.1	28.96	
10.00	VIC	24.64	0.1	26.67	0.1	28.26	0.3
	FAM	36.13	1.5	30.69	0.4	32.04	0.6
5.00	VIC	24.69	0.1	26.74	0.1	28.30	0.2
	FAM	37.29	0.8	32.25	0.2	34.06	0.6
2.5	VIC	24.48	0.2	26.63	0.1	28.34	0.0
	FAM	36.07	0.1	34.70	0.6	36.11	1.9
1.25	VIC	24.45	0.4	26.72	0.1	28.20	0.1
	FAM	38.69	1.4	36.94	0.5	40.00	0.1
1.00	VIC	26.25	0.6	26.53	0.0	28.41	0.1
	FAM	33.28	1.1	36.61	0.2	37.81	1.9
0.50	VIC	26.73	1.2	26.51	0.1	28.26	0.4
	FAM	35.71	2.6	39.22	0.7	39.49	1.0

Control WT PI3Kα DNA supplied in the kit from Entrogen was mixed with PI3Kα H1047R DNA Reference Standard from Horizon to create sample DNA with variable percentages of mutant DNA in a WT background. A sample is positive for the mutation evaluated where VIC Cp is >=25 and FAM Cp is <=38. The results shown in the table above demonstrate the ability to detect as low as 1% H1047R mutant DNA in 3 ng of sample DNA.

FIGURE 7 ▶

Amplification Plot for 1.00% H1047R of 3ng DNA



Control WT PI3Kα DNA supplied in the kit from Entrogen was mixed with PI3Kα H1047R DNA Reference Standard from Horizon to create sample DNA with variable percentages of mutant DNA in a WT background. A sample is positive for the mutation evaluated where VIC Cp is >=25 and FAM Cp is <=38. The results shown in the table above demonstrate the ability to detect as low as 1% H1047R mutant DNA in 3 ng of sample DNA.

Experiment 3 (A–B)

Evaluation of Cellular Control Experiments

To further illustrate the application specific relevance of the methods described here, gDNA from control cell lines to be profiled were tested for mutational status. Positive cellular control MCF7 (3A) and negative cellular control PC-3 (3B) gDNA was prepared with the ISOLATE II genomic DNA Kit from Bioline. 10 ng and 3 ng were tested in quadruplicate in EntroGen's PIK3CA Mutation Analysis Kit at a 3 µL total volume reaction.

Results

TABLE 5 ▶

Mutation Status for Positive Cellular Control MCF-7

Mutation Status for Positive Cellular Control MCF-7

MCF-7		10 ng Input		3 ng Input	
		Average Cp	StDev Cp	Average Cp	StDev Cp
E542K	VIC	26.02	0.1	28.00	0.1
	FAM	40.00		40.00	
E545K	VIC	25.89	0.1	27.90	0.1
	FAM	25.31	0.1	27.10	0.0
E545Q	VIC	26.07	0.1	28.05	0.1
	FAM	40.00		40.00	
H1047R	VIC	26.13	0.1	28.09	0.1
	FAM	40.00		40.00	
H1047L	VIC	26.11	0.1	28.07	0.1
	FAM	40.00		40.00	0

The MCF-7 cell line is known to harbor the E545K mutation. DNA was purified from this cell line and tested in the miniaturized reaction using the Echo liquid handler. The mutational status was tested for E542K, E545K, E545Q, H1047R and H1047L. The purified MCF-7 was positive for E545K at both 10 ng and 3 ng of DNA and negative, did not indicate false positives, for all other mutations tested.

FIGURE 8 ▶

Amplification Plot for E545K mutation detection of MCF-7 gDNA

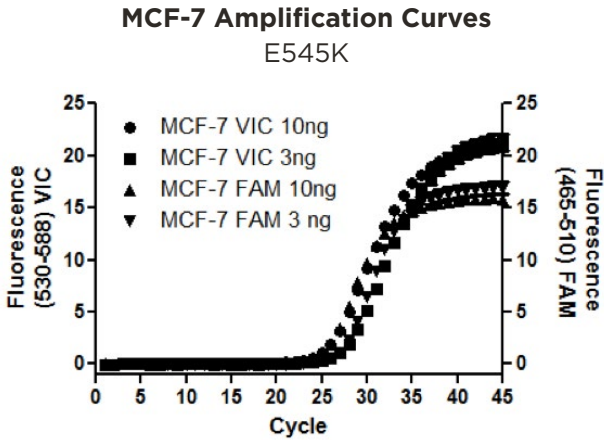


TABLE 6 ▶

Mutation Status for Negative Cellular Control PC-3

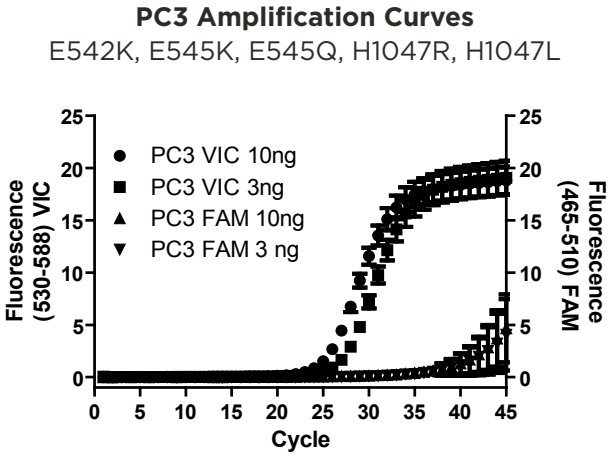
Mutation Status for Negative Cellular Control PC-3

PC-3		10 ng Input		3 ng Input	
		Average Cp	StDev Cp	Average Cp	StDev Cp
E542K	VIC	25.23	0.1	27.06	0.1
	FAM	40.00		40.00	
E545K	VIC	25.21	0.1	27.09	0.1
	FAM	40.00		40.00	
E545Q	VIC	25.27	0.0	27.04	0.1
	FAM	40.00		40.00	
H1047R	VIC	25.25	0.0	27.21	0.1
	FAM	40.00		40.00	
H1047L	VIC	25.31	0.0	27.22	0.1
	FAM	40.00		40.00	

The PC-3 cell line is known to be PIK3Ca wild-type. DNA was purified from this cell line and tested in the miniaturized reaction using the Echo liquid handler. The mutational status was tested for E542K, E545K, E545Q, H1047R and H1047L. The purified PC-3 DNA was negative, did not indicate false positives, for all mutations tested.

FIGURE 9 ▶

Amplification Plot for mutation detection of PC-3 gDNA



The results for a representative amplification curve of 10 ng DNA and 3 ng DNA purified from PC-3 cells demonstrates a negative mutational status for the sample tested. The VIC readout is against the left hand Y-axis with solid square or circle symbols and the FAM readout is plotted against the right hand Y-axis with solid triangles. Both datasets plot Cp over cycle. The VIC Cp of between 26 and 31 with a FAM Cp of >38 confirm a negative determination.

DISCUSSION

The combination of precise and accurate low volume Echo liquid handling, robust EntroGen reagents, and high sensitivity Roche LightCycler real-time PCR amplification and detection instrument provides a robust assay platform and high quality data for mutational analysis.

The sample reduction experiment demonstrates with control DNA that the method reduces the sample input requirement from 10 ng to 0.5 ng in the miniaturized assay. The total assay volume is reduced from 30 µL to 3 µL to reduce cost of reagents per sample. The higher density format, 384-well versus 96-well, can increase throughput by processing four times the wells per qPCR detection step. Quality is not compromised by the miniaturized assay protocol and reduced sample volume. The sensitivity of the assay remains high as indicated by the LOD experiments. The sensitivity is shown for 1.25% E542K at 1 ng input, 1.25% E545K at 3 ng input and 1.00% H1047R at 3 ng input material. The method is further validated with mutational status detection from gDNA isolated from control cell lines; the MCF-7 cell line was confirmed to be E545K positive and the PC-3 cell line was confirmed to be negative for mutants evaluated. We have demonstrated a highly efficient, low-cost assay platform to detect PIK3CA somatic mutations.

Echo 500-series Liquid Handers

- **Volume reduction**
- **Unsurpassed accuracy and precision**
- **Any source well to any destination well**
- **Intra-plate flexibility**

Application Areas of Interest

- **Cancer research**
- **Genomic research**
- **Translational medicine**
- **Target validation**
- **Synthetic biology**

LABCYTE™
The Future of Science is Sound

LABCYTE INC.
1190 Borregas Avenue
Sunnyvale, CA 94089, USA
Toll-free: +1 877 742-6548
Fax: +1 408 747-2010

SALES

North America	+1 408 747-2000	info-us@labcyte.com
Europe	+353 1 6791464	info-europe@labcyte.com
Japan	+61 39018 5780	info-japan@labcyte.com
Asia Pacific	+61 39018 5780	info-asiapacific@labcyte.com
Other	+1 408 747-2000	info-us@labcyte.com

APP-PI3K-1.0
MAR 2016