Oncolipsy PIK3CA kit

Manufacturer**: Pharmassist Ltd**

Instructions for Use (IFU)

PIK3CA kit

Instruction Manual

A real-time qualitative PCR test based on the combination of allele-specific priming, asymmetric PCR, and melting curve analysis for the detection of Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA) mutations.

<24 samples>

-20°C

Pharmassist Ltd, 15 Anthrakorichon Street, Nea Ionia 142 35 Athens, Greece,
Tel.: (0030) 210 65 60 700
website: www.oncolipsy.com

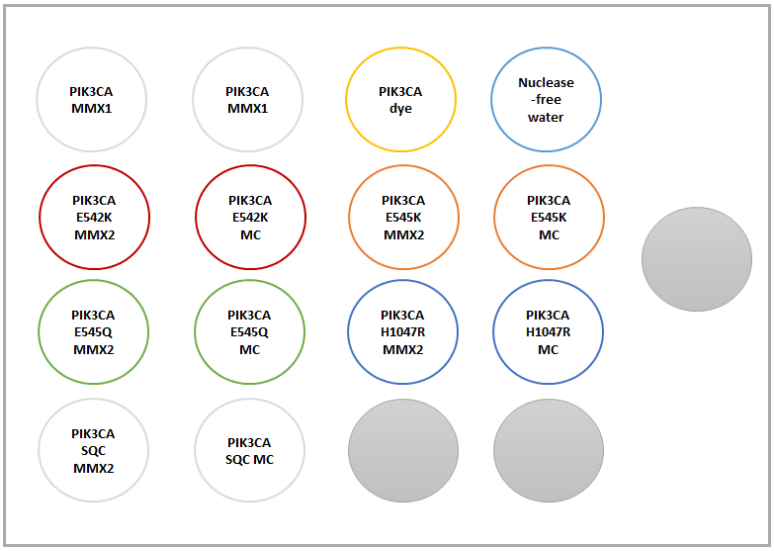
Contents

1. Kit Contents

Each Oncolipsy PIK3CA kit contains 14 vials with different color caps (red, green, blue, orange, white, yellow, light blue, and colourless) including PCR buffer master-mixes, primers/probe master-mixes, dye reagent, positive controls for each biomarker, and nuclease-free water along with a leaflet. The Oncolipsy PIK3CA kit includes the following vials:

N	Kit Contents (Label)	Number of Vials	Cap Color	Volume
1, 2	Oncolipsy PIK3CA MMX1	**2**	**Colorless cap**	1000µL
3	Oncolipsy PIK3CA E542K MMX2	**1**	**Red cap**	34µL
4	Oncolipsy PIK3CA E545K MMX2	**1**	**Orange cap**	34µL
5	Oncolipsy PIK3CA E545Q MMX2	**1**	**Green cap**	34µL
6	Oncolipsy PIK3CA H1047R MMX2	**1**	**Blue cap**	34µL
7	Oncolipsy PIK3CA SQC MMX2	**1**	**White cap**	16µL
8	Oncolipsy PIK3CA E542K MC	**1**	**Red cap**	20µL
9	Oncolipsy PIK3CA E545K MC	**1**	**Orange cap**	20µL
10	Oncolipsy PIK3CA E545Q MC	**1**	**Green cap**	20µL
11	Oncolipsy PIK3CA H1047R MC	**1**	**Blue cap**	20µL
12	Oncolipsy PIK3CA SQC PC	**1**	**White cap**	20µL
13	Oncolipsy dye reagent	**1**	**Yellow cap**	400µL
14	Nuclease-free water	**1**	**Light Blue cap**	2mL

The Oncolipsy PIK3CA kit includes sufficient reagents to analyze 24 samples including PCR positive and negative controls. Four different RT-qPCR test runs may be performed. Each Oncolipsy PIK3CA kit contains 14 labelled vials as indicated in the following schematic visualization:



2. Intended Use

The Oncolipsy PIK3CA kit is a real-time qualitative PCR test that is based on the combination of allele-specific priming, asymmetric PCR, and melting curve analysis for the detection of Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA) mutations. The kit can be applied on DNA samples extracted from circulating tumor cells (CTCs), plasma and formalin-fixed paraffin-embedded (FFPE) tumor tissues samples of metastatic breast cancer patients. Each kit can detect four (4) different mutations in singleplex assays, E542K, E545K, E545Q, H1047R and a 70 base pair region of PIK3CA exon 3 as a sample quality control. The Oncolipsy PIK3CA kit includes sufficient reagents to analyze 24 samples including PCR positive and negative controls. The product is developed to be compatible with Roche LightCycler 480 (Roche Diagnostics) instrumentation. The interpretation of results is performed qualitative using melting curve analysis. The Oncolipsy PIK3CA Kit is to be used by trained personnel in a professional laboratory environment.

3. Introduction

Breast cancer is the most common type of tumor and is the leading cause of cancer-related deaths in the female population worldwide, with a continuous rise in numbers. Despite advancements in the treatment of these patients, metastatic breast cancer (MBC) substantially remains an incurable disease. Two-thirds of breast cancer cases express hormone receptors (HR) and lack HER2 overexpression and/or amplification (1).

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway is commonly deregulated in many human tumors, including breast cancer. Somatic mutations of the PI3K alpha catalytic subunit (PIK3CA) are the most common cause of pathway hyperactivation (2). PIK3CA mutations are associated with many distinct cancers and include hotspot single–amino acid substitutions in the helical (E542K and E545K) or kinase (H1047R) domains. PIK3CA mutations are the most common genetic alterations in the PI3K/AKT/mTOR pathway and can be identified in approximately 20–30 % of all breast cancer cases. However, their incidence varies across disease subtypes (2). Specifically in the case of ER+/HER2- primary and metastatic tumors, PIK3CA mutations have an incidence of 40%.

Liquid biopsy has a high potential to significantly change the therapeutic strategy in cancer patients (3). Detection, enumeration, and molecular characterization of circulating tumor cells (CTCs) and analysis of circulating tumor DNA (ctDNA) provide useful information regarding the individual molecular profile of each patient in real time, before and after treatment (4). The field of liquid biopsy applications is growing exponentially, including molecular target identification, prognosis assessment, diagnosis of recurrence, monitoring of response to treatment, and monitoring of tumor genomic profiles over time (4). Most importantly, blood-based tests are very challenging and highly important in cases where tumor biopsies are not accessible (5).

CTCs as viable cells circulating in peripheral blood can provide real-time information on the metastatic spread and reveal active and possibly targetable signaling networks, while ctDNA can give specific information on the presence or absence of specific alterations deriving from the tumor, indicating therapy response or resistance (6). CTC detection and enumeration are

associated with progression-free survival (PFS) and overall survival (OS) in metastatic (4) and early breast cancer (7). However, CTCs are highly heterogeneous, and there is a lack of a unique marker for their isolation and identification, since there are not well-defined universal surface targets among all malignant cell types (4). PIK3CA mutational status has already been studied in CTCs and plasma-ctDNA for breast cancer patients (8-14).

4. Kit principle

The Oncolipsy PIK3CA Kit provides all detection reagents required to detect four *PIK3CA* mutations including E542K, E545K, E545Q and H1047R. The Kit is based on a combination of allele-specific priming, asymmetric PCR, and melting curve analysis for the detection of *PIK3CA* mutations. All primers and probes have been designed with attention to avoiding amplification of a pseudogene on chromosome 22 that has >95% homology to exon 9 of PIK3CA. The interpretation of results is performed by melting curve analysis using a melting probe which provides a different melting temperature for its binding to the mutant allele as compared to its binding to the wild type allele.

4.1. DNA extraction from plasma

DNA is extracted from 2ml plasma samples derived from 10ml peripheral blood collected in K2EDTA blood collection tubes using the QIAamp DSP Circulating Nucleic Acid Kit (Qiagen, cat. No. 61504), according to manufacturer's instructions. In last step, purified ctDNA must be eluted in 30 µl Buffer AVE (elution buffer).

4.2. CTC enrichment and DNA extraction

CTCs is isolated using immunomagnetic BerEP4-coated Dynabeads® (CELLection™ Epithelial Enrich; Invitrogen) according to manufacturer's instructions to enrich for epithelial cells. DNA from CTCs is extracted using trizol reagent.

4.3. DNA extraction from tumor tissues

DNA is extracted from FFPEs using the QIAamp DSP DNA FFPE Tissue Kit (Qiagen, cat No. 60404), according to manufacturer's instructions.

4.4. Real-Time PCR Instruments

The Oncolipsy PIK3CA kit was developed and validated to be used with the Roche® LightCycler 480 (Roche Diagnostics) instrument.

5. Identification of Material & Devices Required but Not Provided

5.1. Reagents Devices

a. Equipment

- Laminar Flow Hood with ultraviolet (UV) lamp
- Centrifuge
- Vortex
- White Roche® LightCycler 480 Multiwell plate 96 or strips
- Adjustable pipettes
- Pipette tips with filters
- Disposable gloves

• 0.5ml PCR microcentrifuge tubes for Section 6.1. extraction and Section 6.2. Master Mix preparation.

b. Real-Time PCR Machine

• Appropriate Real-Time PCR instrument (please refer to Section 4.4. "Real-Time PCR Instruments").

6. Protocol

6.1. Sample preparation

Most typically, samples are DNA extracted from CTCs and/or plasma derived from peripheral blood and formalin-fixed paraffin embedded (FFPE) tissue sections (30 µm).

6.1.1. ctDNA extraction from plasma

For plasma-ctDNA analysis, 10 mL peripheral blood in EDTA from metastatic patients is required. However, the first 5 ml of blood should be discarded to avoid contamination of skin epithelial cells. Peripheral blood should be collected into K_2EDTA tubes (BD Vacutainer, Plymouth, UK) and mixed immediately after blood draw by inverting gently, maintained at room temperature (RT) and processed preferably within 3h. Plasma is isolated by centrifugation at 530 g for 10 min at room temperature. Once isolated, plasma samples are further centrifuged twice at 2000 g for 10 min, before transferring into clean 2-mL tubes and freezing at -70 °C until further processing. The QIAamp DSP Circulating Nucleic Acid Kit (Qiagen, cat. No. 61504) is recommended to isolate ctDNA from 2mL of plasma according to manufacturer's instructions. Purified ctDNA must be eluted in 30µl elution buffer (provided in the QIAamp DSP Circulating Nucleic Acid Kit).

6.1.2. DNA extraction from tumor tissues

DNA is extracted by formalin-fixed paraffin embedded (FFPEs) using the CE-IVD kit QIAamp DNA FFPE Tissue Kit (Qiagen, cat No. 60404), according to manufacturer's instructions. Purified DNA must be eluted in 30µl elution buffer (provided in the QIAamp DNA FFPE Tissue Kit). Each DNA sample should be diluted up to 50ng/µl.

6.1.3. DNA extraction from CTCs

- For CTC analysis, 10 mL peripheral blood in EDTA from metastatic patients is required. However, the first 5 ml of blood should be discarded to avoid contamination of skin epithelial cells. Peripheral blood should be collected into K2EDTA tubes (BD Vacutainer, Plymouth, UK) and mixed immediately after blood draw by inverting gently, maintained at room temperature (RT) and processed preferably within 3 h, to CTC isolation.

- CTCs enrichment can be performed using immunomagnetic BerEP4-coated Dynabeads® (CELLection™ Epithelial Enrich; Invitrogen) according to manufacturer's instructions to enrich for epithelial cells. DNA is extracted from CTCs using trizol reagent (the extraction protocol is provided upon request).

6.2. PCR Preparation

Before starting: Thaw all reagents and RNase-free water. Vortex, centrifuge quickly, and store on ice.

6.2.1. Preparation of Oncolipsy PIK3CA SQC PCR master mix

1. Prepare the Oncolipsy *PIK3CA* SQC MMX2 as shown in Table 1 according to the number of samples. Note that Nuclease-free water as negative control, and the positive mutant control must always be included in each experiment run. For example, in case that one clinical sample (number of samples) should be analyzed, the user should prepare the Oncolipsy PIK3CA SQC MMX2 reagent for four reactions (number of reactions). The addition of an extra reaction is added to avoid eventual volume deviations due to pipetting. Therefore, 1.6µL of PIK3CA SQC MMX2 and 6.4µL water should be mixed in order to make a final volume of 8µL.

Table 1: Volumes of Oncolipsy PIK3CA SQC MMX2 reagent are based on number of samples and reactions.

Number of samples	Number of reactions	Volume of Oncolipsy PIK3CA SQC MMX2	Volume of nuclease-free water	Final Volume
1	4	1.6	6.4	8
2	5	2.0	8.0	10
3	6	2.4	9.6	12
4	7	2.8	11.2	14
5	8	3.2	12.8	16
6	9	3.6	14.4	18
7	10	4.0	16.0	20

2. In the above PCR tube containing Oncolipsy PIK3CA SQC MMX2, add 10µL of Oncolipsy PIK3CA MMX1, 2µL of Oncolipsy PIK3CA dye and 4µL of nuclease-free water per reaction. Then vortex and collect liquid at the bottom of the tube before use. Volumes of reagents needed for Oncolipsy PIK3CA PCR master mix are presented in Table 2.

Table 2: Preparation of Oncolipsy PIK3CA SQC PCR mastermix

Component	Volume/reaction (µL)
Oncolipsy PIK3CA MMX1	10
Oncolipsy PIK3CA SQC MMX2	2
Oncolipsy PIK3CA dye	2
Nuclease-free water	4
Total volume	**18**

3. Dispense 18 µL of the Oncolipsy PIK3CA SQC PCR Master Mix into each reaction well of the microwell plate or strip. Do not allow the pipettor tip to touch the plate outside the well.
4. Pipette 2 µL of nuclease-free water into PIK3CA negative control (NC) of the microwell plate or strip; mix well using pipette to aspirate and dispense within the well a minimum of two times.
5. Pipette 2 µL of Oncolipsy PIK3CA SQC PC into well of the microwell plate or strip; mix well using pipette to aspirate and dispense within the well a minimum of two times.
6. Using new pipettor tips for each DNA sample, add 2 µL of the first DNA sample to well of the microwell plate or strip; mix each well using a pipette to aspirate and dispense within the well a minimum of two times. Repeat this procedure for all DNA samples.
7. Cover the microwell plate or strip with sealing film or caps, respectively.
8. Confirm that all liquid is collected at the bottom of each well before starting PCR.
9. Place the plate or strip in the instrument in the instrument and start the run according to the protocol below.

6.2.2. Preparation of Oncolipsy *PIK3CA* E542K PCR master mix

1. Prepare the Oncolipsy *PIK3CA* E542K MMX2 as shown in Table 3 according to the number of samples. Note that Nuclease-free water as negative control, and the positive mutant control must always be included in each experiment run. For example, in case that one clinical sample (number of samples) should be analyzed, the user should prepare the Oncolipsy PIK3CA E542K MMX2 reagent for four reactions (number of reactions). The addition of an extra reaction is added to avoid eventual volume deviations due to pipetting. Therefore, 3.36µL of PIK3CA E542K MMX2 and 4.64µL water should be mixed in order to make a final volume of 8µL.

Table 3: Volumes of Oncolipsy PIK3CA E542K MMX2 reagent are based on number of samples and reactions.

Number of samples	Number of reactions	Volume of Oncolipsy PIK3CA E542K MMX2	Volume of nuclease-free water	Final Volume
1	4	3.36	4.64	8
2	5	4.2	5.8	10
3	6	5.04	6.96	12
4	7	5.88	8.12	14
5	8	6.72	9.28	16
6	9	7.56	10.44	18
7	10	8.4	11.6	20

2. In the above PCR tube containing Oncolipsy PIK3CA E542K MMX2, add 10µL of Oncolipsy PIK3CA MMX1, 2µL of Oncolipsy PIK3CA dye and 4µL of nuclease-free water per reaction. Then vortex and collect liquid at the bottom of the tube before use. Volumes of reagents needed for Oncolipsy PIK3CA PCR master mix are presented in Table 4.

Table 4: Preparation of Oncolipsy PIK3CA E542K PCR mastermix

Component	Volume/reaction (µL)
Oncolipsy PIK3CA MMX1	10
Oncolipsy PIK3CA E542K MMX2	2
Oncolipsy PIK3CA dye	2
Nuclease-free water	4
Total volume	**18**

3. Dispense 18 µL of the Oncolipsy PIK3CA E542K PCR Master Mix into each reaction well of the microwell plate or strip. Do not allow the pipettor tip to touch the plate outside the well. into each reaction well of the microwell plate or strip. Do not allow the pipettor tip to touch the plate outside the well.
4. Pipette 2 µL of nuclease-free water into PIK3CA negative control (NC) of the microwell plate or strip; mix well using pipette to aspirate and dispense within the well a minimum of two times.

5. Pipette 2 µL of Oncolipsy PIK3CA E542K MC into well of the microwell plate or strip; mix well using pipette to aspirate and dispense within the well a minimum of two times.
6. Using new pipettor tips for each DNA sample, add 2 µL of the first DNA sample to well of the microwell plate or strip; mix each well using a pipette to aspirate and dispense within the well a minimum of two times. Repeat this procedure for all DNA samples.
7. Cover the microwell plate or strip with sealing film or caps, respectively.
8. Confirm that all liquid is collected at the bottom of each well before starting PCR.
9. Place the plate or strip in the instrument in the instrument and start the run according to the protocol below.

6.2.3. Preparation of Oncolipsy PIK3CA E545K PCR master mix

1. Prepare the Oncolipsy *PIK3CA* E545K MMX2 as shown in Table 5 according to the number of samples. Note that Nuclease-free water as negative control, and the positive mutant control must always be included in each experiment run. For example, in case that one clinical sample (number of samples) should be analyzed, the user should prepare the Oncolipsy PIK3CA E545K MMX2 reagent for four reactions (number of reactions). The addition of an extra reaction is added to avoid eventual volume deviations due to pipetting. Therefore, 3.36µL of PIK3CA E545K MMX2 and 4.64µL water should be mixed in order to make a final volume of 8µL.

Table 5: Volumes of Oncolipsy PIK3CA E545K MMX2 reagent are based on number of samples and reactions.

Number of samples	Number of reactions	Volume of Oncolipsy PIK3CA E545K MMX2	Volume of nuclease-free water	Final Volume
1	4	3.36	4.64	8
2	5	4.2	5.8	10
3	6	5.04	6.96	12
4	7	5.88	8.12	14
5	8	6.72	9.28	16
6	9	7.56	10.44	18
7	10	8.4	11.6	20

2. In the above PCR tube containing Oncolipsy PIK3CA E545K MMX2, add 10µL of Oncolipsy PIK3CA MMX1, 2µL of Oncolipsy PIK3CA dye and 4µL of nuclease-free water per reaction. Then vortex and collect liquid at the bottom of the tube before use. Volumes of reagents needed for Oncolipsy PIK3CA PCR master mix are presented in Table 6.

Table 6: Preparation of Oncolipsy PIK3CA E545K PCR mastermix

Component	Volume/reaction (µL)
Oncolipsy PIK3CA MMX1	10
Oncolipsy PIK3CA E545K MMX2	2
Oncolipsy PIK3CA dye	2
Nuclease-free water	4
Total volume	**18**

3. Dispense 18 µL of the Oncolipsy PIK3CA E545K PCR Master Mix into each reaction well of the microwell plate or strip. Do not allow the pipettor tip to touch the plate outside the well.
4. Pipette 2 µL of nuclease-free water into PIK3CA negative control (NC) of the microwell plate or strip; mix well using pipette to aspirate and dispense within the well a minimum of two times.
5. Pipette 2 µL of Oncolipsy PIK3CA E545K MC into well of the microwell plate or strip; mix well using pipette to aspirate and dispense within the well a minimum of two times.
6. Using new pipettor tips for each DNA sample, add 2 µL of the first DNA sample to well of the microwell plate or strip; mix each well using a pipette to aspirate and dispense within the well a minimum of two times. Repeat this procedure for all DNA samples.
7. Cover the microwell plate or strip with sealing film or caps, respectively.
8. Confirm that all liquid is collected at the bottom of each well before starting PCR.
9. Place the plate or strip in the instrument in the instrument and start the run according to the protocol below.

6.2.4. Preparation of Oncolipsy PIK3CA E545Q PCR master mix

1. Prepare the Oncolipsy *PIK3CA* E545Q MMX2 as shown in Table 7 according to the number of samples. Note that Nuclease-free water as negative control, and the positive mutant control must always be included in each experiment run. For example, in case that one clinical sample (number of samples) should be analyzed, the user should prepare the Oncolipsy PIK3CA E545Q MMX2 reagent for four reactions (number of reactions). The addition of an extra reaction is added to avoid eventual volume deviations due to pipetting. Therefore, 3.36µL of PIK3CA E545Q MMX2 and 4.64µL water should be mixed in order to make a final volume of 8µL.

Table 7: Volumes of Oncolipsy PIK3CA E545Q MMX2 reagent are based on number of samples and reactions.

Number of samples	Number of reactions	Volume of Oncolipsy PIK3CA E545Q MMX2	Volume of nuclease-free water	Final Volume
1	4	3.36	4.64	8
2	5	4.2	5.8	10
3	6	5.04	6.96	12
4	7	5.88	8.12	14
5	8	6.72	9.28	16
6	9	7.56	10.44	18
7	10	8.4	11.6	20

2. In the above PCR tube containing Oncolipsy PIK3CA E545Q MMX2, add 10µL of Oncolipsy PIK3CA MMX1, 2µL of Oncolipsy PIK3CA dye and 4µL of nuclease-free water per reaction. Then vortex and collect liquid at the bottom of the tube before use. Volumes of reagents needed for Oncolipsy PIK3CA PCR master mix are presented in Table 8.

Table 8: Preparation of Oncolipsy PIK3CA E545Q PCR mastermix

Component	Volume/reaction (µL)
Oncolipsy PIK3CA MMX1	10
Oncolipsy PIK3CA E545Q MMX2	2
Oncolipsy PIK3CA dye	2
Nuclease-free water	4
Total volume	**18**

3. Dispense 18 µL of the Oncolipsy PIK3CA E545Q PCR Master Mix into each reaction well of the microwell plate or strip. Do not allow the pipettor tip to touch the plate outside the well.
4. Pipette 2 µL of nuclease-free water into PIK3CA negative control (NC) of the microwell plate or strip; mix well using pipette to aspirate and dispense within the well a minimum of two times.
5. Pipette 2 µL of Oncolipsy PIK3CA E545Q MC into well of the microwell plate or strip; mix well using pipette to aspirate and dispense within the well a minimum of two times.

6. Using new pipettor tips for each DNA sample, add 2 µL of the first DNA sample to well of the microwell plate or strip; mix each well using a pipette to aspirate and dispense within the well a minimum of two times. Repeat this procedure for all DNA samples.
7. Cover the microwell plate or strip with sealing film or caps, respectively.
8. Confirm that all liquid is collected at the bottom of each well before starting PCR.
9. Place the plate or strip in the instrument in the instrument and start the run according to the protocol below.

6.2.5. Preparation of Oncolipsy PIK3CA H1047R PCR master mix

1. Prepare the Oncolipsy *PIK3CA* H1047R MMX2 as shown in Table 9 according to the number of samples. Note that Nuclease-free water as negative control, and the positive mutant control must always be included in each experiment run. For example, in case that one clinical sample (number of samples) should be analyzed, the user should prepare the Oncolipsy PIK3CA H1047R MMX2 reagent for four reactions (number of reactions). The addition of an extra reaction is added to avoid eventual volume deviations due to pipetting. Therefore, 3.36µL of PIK3CA H1047R MMX2 and 4.64µL water should be mixed in order to make a final volume of 8µL.

Table 9: Volumes of Oncolipsy PIK3CA H1047R MMX2 reagent are based on number of samples and reactions.

Number of samples	Number of reactions	Volume of Oncolipsy PIK3CA H1047R MMX2	Volume of nuclease-free water	Final Volume
1	4	3.36	4.64	8
2	5	4.2	5.8	10
3	6	5.04	6.96	12
4	7	5.88	8.12	14
5	8	6.72	9.28	16
6	9	7.56	10.44	18
7	10	8.4	11.6	20

2. In the above PCR tube containing Oncolipsy PIK3CA H1047R MMX2, add 10µL of Oncolipsy PIK3CA MMX1, 2µL of Oncolipsy PIK3CA dye and 4µL of nuclease-free water per reaction. Then vortex and collect liquid at the bottom of the tube before use. Volumes of reagents needed for Oncolipsy PIK3CA PCR master mix are presented in Table 10.

Table 10: Preparation of Oncolipsy PIK3CA H1047R PCR mastermix

Component	Volume/reaction (µL)
Oncolipsy PIK3CA MMX1	10
Oncolipsy PIK3CA H1047R MMX2	2
Oncolipsy PIK3CA dye	2
Nuclease-free water	4
Total volume	**18**

3. Dispense 18 µL of the Oncolipsy PIK3CA H1047R PCR Master Mix into each reaction well of the microwell plate or strip. Do not allow the pipettor tip to touch the plate outside the well.
4. Pipette 2 µL of nuclease-free water into PIK3CA negative control (NC) of the microwell plate or strip; mix well using pipette to aspirate and dispense within the well a minimum of two times.
5. Pipette 2 µL of Oncolipsy PIK3CA H1047R MC into well of the microwell plate or strip; mix well using pipette to aspirate and dispense within the well a minimum of two times.
6. Using new pipettor tips for each DNA sample, add 2 µL of the first DNA sample to well of the microwell plate or strip; mix each well using a pipette to aspirate and dispense within the well a minimum of two times. Repeat this procedure for all DNA samples.
7. Cover the microwell plate or strip with sealing film or caps, respectively.
8. Confirm that all liquid is collected at the bottom of each well before starting PCR.
9. Place the plate or strip in the instrument in the instrument and start the run according to the protocol below.

6.3. Programming the Real-Time PCR Instrument

Enter the following PCR cycling program for PIK3CA SQC:

Step	Temperature	Acquisition mode	Time	Ramp Rate	Acquisitions (per °C)	Cycles
Initial Denaturation	95°C	none	2 min	4.40 °C/s		1
Denaturation	95° C	none	5 sec	4.40 °C/s		45
Annealing	63° C	none	10 sec	2.20 °C/s		
Extention	72° C	**single**	10 sec	4.40 °C/s		
Melting	55° C	none	30 sec	2.20 °C/s		1
	95° C	**continuous**	0 sec	0.11 °C/s	5	

	40° C	none	20 sec	2.20 °C/s		

Enter the following PCR cycling program for PIK3CA E542K:

Step	Temperature	Acquisition mode	Time	Ramp Rate	Acquisitions (per °C)	Cycles
Initial Denaturation	95°C	none	2 min	4.40 °C/s		1
Denaturation	95° C	none	1 sec	4.40 °C/s		70
Annealing	63° C	none	4 sec	2.20 °C/s		
	72° C	**single**	1 sec	4.40 °C/s		
Melting	50° C	none	1 min	2.20 °C/s		1
	95° C	**continuous**	0 sec	0.11 °C/s	5	
	40° C	none	30 sec	2.20 °C/s		

Enter the following PCR cycling program for PIK3CA E545K:

Step	Temperature	Acquisition mode	Time	Ramp Rate	Acquisitions (per °C)	Cycles
Initial Denaturation	95°C	none	2 min	4.40 °C/s		1
Denaturation	95° C	none	1 sec	4.40 °C/s		70
Annealing	62° C	none	4 sec	2.20 °C/s		
	72° C	**single**	1 sec	4.40 °C/s		
Melting	50° C	none	1 min	2.20 °C/s		1
	95° C	**continuous**	0 sec	0.11 °C/s	5	
	40° C	none	30 sec	2.20 °C/s		

Enter the following PCR cycling program for PIK3CA E545Q:

Step	Temperature	Acquisition mode	Time	Ramp Rate	Acquisitions (per °C)	Cycles
Initial Denaturation	95°C	none	2 min	4.40 °C/s		1
Denaturation	95° C	none	1 sec	4.40 °C/s		80
Annealing	62° C	none	4 sec	2.20 °C/s		
	72° C	**single**	1 sec	4.40 °C/s		
Melting	45° C	none	1 min	2.20 °C/s		1
	95° C	**continuous**	0 sec	0.11 °C/s	5	
	40° C	none	30 sec	2.20 °C/s		

Enter the following PCR cycling program for PIK3CA H1047R:

Step	Temperature	Acquisition mode	Time	Ramp Rate	Acquisitions (per °C)	Cycles
Initial Denaturation	95°C	none	2 min	4.40 °C/s		1
Denaturation	95° C	none	1 sec	4.40 °C/s		70
Annealing	62° C	none	4 sec	2.20 °C/s		
Extention	72° C	**single**	1 sec	4.40 °C/s		
Melting	53° C	none	1 min	2.20 °C/s		1
	95° C	**continuous**	0 sec	0.11 °C/s	5	
	40° C	none	30 sec	2.20 °C/s		

NOTE: Please select the following detection format SYBR Green I/ HRM Dye for the Roche® LightCycler 480.

7. Results

Note: A test run in order to be valid should meet the following criteria:

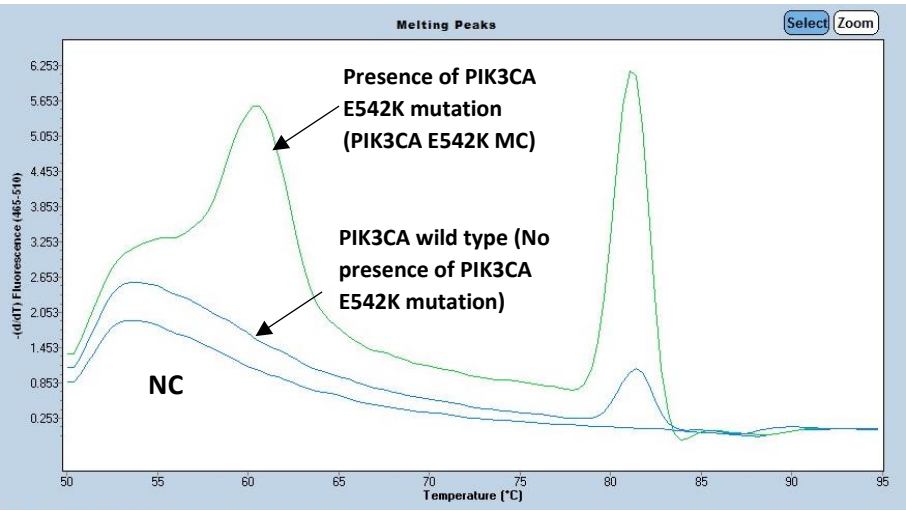
- In NC no signal should be detected
- The PIK3CA E542K, E545K & H1047R MC Cq value is ≤55. The PIK3CA E545Q MC Cq value is ≤65.
- The PIK3CA SQC PC Cq value is ≤23. The Cq values of clinical samples for the PIK3CA SQC assay must be under 38.

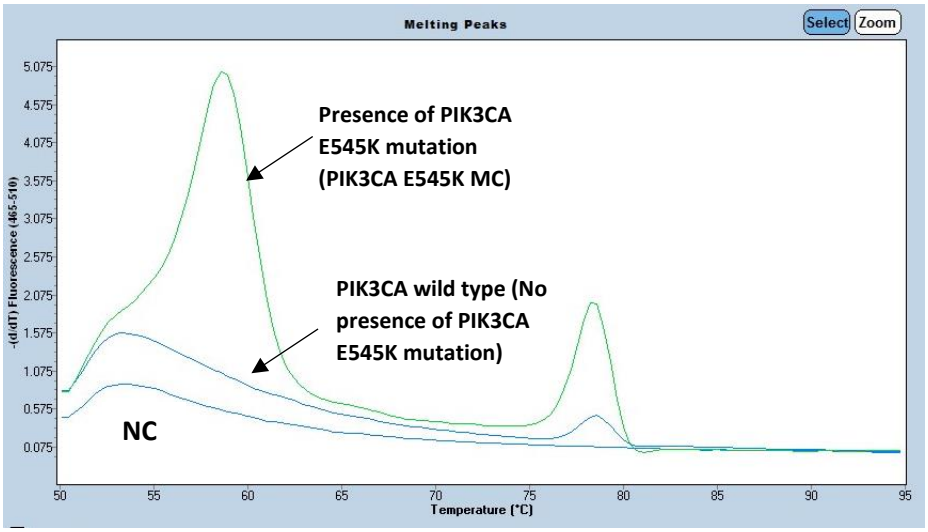
- In the melting curve analysis, positive controls shall present two peaks in two different temperature conditions:
  - The PIK3CA E542K MC melting peak shall be detected at 59-62^{0}C.
  - The PIK3CA E545K MC melting peak shall be detected at 58-60^{0}C.
  - The PIK3CA E545Q MC melting peak shall be detected at 54-56^{0}C.
  - The PIK3CA H1047R MC melting peak shall be detected at 60.5-62.5^{0}C.

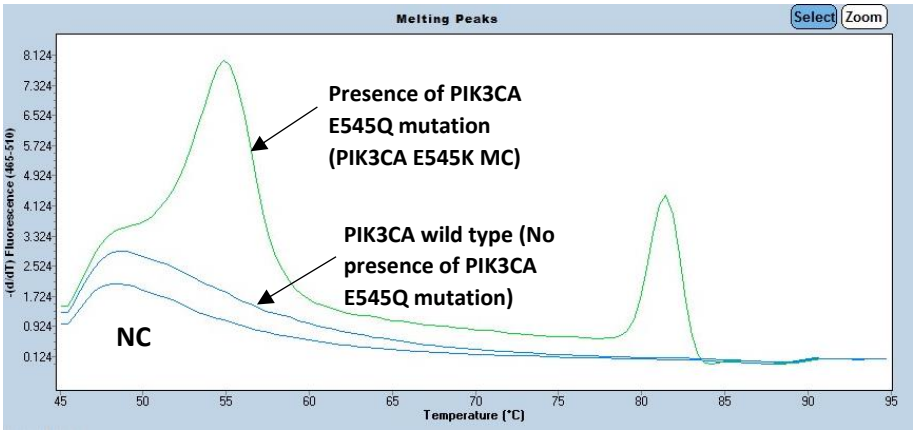
8. Interpretation of results

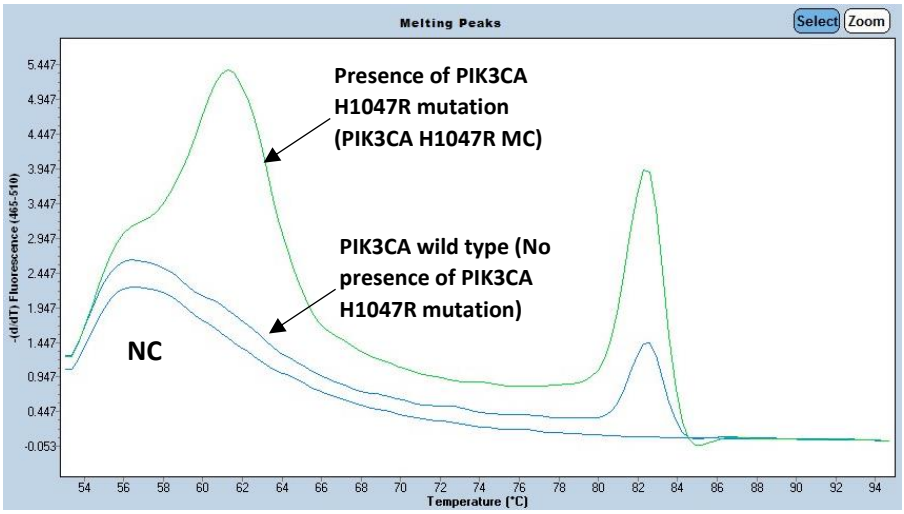
The quality of the clinical samples is evaluated by PIK3CA SQC assay. Only the samples with Cq value **under 38** will be analyzed for the four PIK3CA mutations. In the melting curve analysis, a positive sample will present two peaks in two different temperature conditions. The presence of each PIK3CA mutation is detected by the presence of the first melting peak curve which is depicted in lower temperature condition according to the above acceptance criteria.

Please see below examples for each *PIK3CA* mutation including positive control containing the presence of mutation, negative control and wild type control.




Melting Peaks
Select
Zoom
-(d/dT) Fluorescence (465-510)
Temperature (°C)
Presence of PIK3CA
E545K mutation
(PIK3CA E545K MC)
PIK3CA wild type (No
presence of PIK3CA
E545K mutation)
NC


Melting Peaks
Select
Zoom
-(d/dT) Fluorescence (465-510)
Temperature (°C)
Presence of PIK3CA
E545Q mutation
(PIK3CA E545K MC)
PIK3CA wild type (No
presence of PIK3CA
E545Q mutation)
NC


Melting Peaks
Select
Zoom
-(d/dT) Fluorescence (465-510)
Temperature (°C)
Presence of PIK3CA
H1047R mutation
(PIK3CA H1047R MC)
PIK3CA wild type (No
presence of PIK3CA
H1047R mutation)
NC

9. Analytical Performance Evaluation

Analytical Sensitivity-Limit of Detection

To assess performance of the Oncolipsy PIK3CA kit in the absence of template and to ensure that a blank sample does not generate an analytical signal that might indicate a low concentration of mutation, blank solutions with no template specimens as negative controls were evaluated. The Limit of Blank was determined to be zero for all mutations.

The limit of detection (LoD) for each PIK3CA mutation assay was evaluated using serial dilutions of synthetic mutant oligonucleotides (gene-blocks) in synthetic wild-type strands generating different ratios of 50%, 10%, 1%, and 0.5% for each PIK3CA mutation at three different batches. These dilutions were verified for their mutation status rating using commercially available ddPCR assays (where it was available). The results showed that the Oncolipsy PIK3CA kit could reliably detect the mutation rate of 0.5% for all mutations tested. For the analytical sensitivity, commercially available reference standards (cfDNA, 20ng/ul) at specific mutant allele frequencies (12.5% for E542K, E545K, H1047R, 5%, 1% and 0.1% for E545K, 1% for E542K and 0.8% for H1047R) were also analyzed. Their mutation status was evaluated by ddPCR according to the supplier providing the relevant certificate of quality control. The Oncolipsy *PIK3CA* kit could successfully detect the samples with allele frequency 12.5% for all mutations tested as well the 1% for E545K and E542K and 0.8% for H1047R.

Analytical Specificity

A wild-type (WT) cfDNA certified reference standard was also analyzed for all PIK3CA mutations. This WT standard had a concentration of 20ng/µl and its mutation status (allele frequency 0%) was evaluated by ddPCR according to the supplier providing the relevant certificate of quality control. The data from all batches for each PIK3CA mutation met the acceptance criteria, as none non-specific result was demonstrated. More specifically, the mutations were not detected at WT sample for none of the measurements.

Precision

Precision of the Oncolipsy PIK3CA Kit (Reproducibility, Repeatability) was evaluated using serial dilutions mutant vs WT alleles, corresponding to 50%, 10% and 1% and 0% respectively. The mutation status and percentage of dilutions (50%, 10% and 1%) used for these experiments were known prior to their analysis and were analyzed by commercially available ddPCR assays (where it was applicable). As for WT sample a reference standard was used (20ng/µl cfDNA) which mutation status (allele frequency 0%) was evaluated by ddPCR according to the supplier providing the relevant certificate of quality control. Repeatability was performed by analyzing the WT samples and positive controls in triplicate for the three batches and for each mutation. As for reproducibility, the above samples were analyzed in three replicates for three different days. The data showed that all the mutation assays are characterized by high reproducibility and repeatability for all the mutations tested.

10. Shipping, Storage & Handling Conditions

The Oncolipsy PIK3CA kit is shipped in a styrofoam box, which contains dry ice and must be stored in a constant-temperature freezer at -20°C upon arrival.

Oncolipsy PIK3CA kit is stable until the stated expiration date when stored under the specified storage conditions. Reagents can be stored in their original packaging at –20°C for 18 months, until the stated expiry date shown on the packaging.

The components must not be used beyond the expiration date shown on the packaging. Repeated thawing and freezing should be avoided. It is highly recommended to aliquoting all reagents after their first opening.

If the protective kit packaging is damaged upon receipt, please contact Pharmassist for instructions. The components must not be used beyond the expiration date.

11. Warning and Precautions

11.1. Handling and Procedural Requirements

• Always wear disposable gloves when handling kit components.

• Use separated working areas for specimen preparation, reaction set up and amplification.

• Supplies and equipment should be separated in each work area and not moved between them.

• When mixing reagents by pipetting up and down this should be done with a volume roughly equal to 50% of the total component volume.

11.2. Precautions

To reduce risk of contamination:

- DNA extraction, and preparation of the Real-Time PCR steps should be performed in separate rooms.
- Preparation of the PCR mixture should be done in a PCR-hood.
- In every extraction step during the whole procedure, filter tips, RNAse-DNAse-free reaction vials and calibrated precision pipettes dedicated for each step should be used.
- Mutant positive controls should be opened and processed away from test samples and kit components to avoid cross-contamination.
- Before each procedure, PCR hood and the acquired equipment (filter tips, reaction vials, pipettes etc.) should be disinfected with UV for 20 minutes. The entire hood work surface, as well as pipettes, should be clean using 10% bleach prior to UV.
- Use DNase/RNase free plasticware and pipettes reserved for DNA/RNA work to avoid the cross-contamination from shared equipment.
- Throughout the whole procedure use DNase/RNase free filter tips to avoid further contamination.
- Do not mix vials from different kits, even if they are produced in the same lot.
- Aliquoting all kit's reagents after their first opening.

12. Limitations of Use

- The Oncolipsy PIK3CA kit has been validated for use with plasma and tumor tissue samples run on the LightCycler 480 Real-Time PCR instrument.

- The procedures in this handbook must be followed, as described. Any deviations may result in assay failure or cause erroneous results.
- Good laboratory practice is required to ensure the performance of the kit, with care required to prevent contamination of the kit components. Components should be monitored for contamination and any components thought to have become contaminated should be discarded as standard laboratory waste in a sealed pouch or zip-lock plastic bag.
- All samples should be handled as if they are infectious following proper biosafety precautions.
- Interpretation of results must account for the possibility of false negative and false positive results.
- False negative results may be caused by:
  - Pre-analytical errors, eg unsuitable sample collection, handling and/or storage of samples.
  - Failure to follow procedures in this handbook.
  - Use of unauthorised extraction kit or PCR platform.
- False positive results may be caused by:
  - PCR contamination
- All results should be interpreted by a health care professional in the context of patient medical history and clinical symptoms.

13. Explanation of Symbols

Symbol	Explanation
	Contains sufficient for <n> samples
	In vitro diagnostic use
	In vitro Diagnostic Medical Device
	Batch Code
	Limit of Temperature
	Manufacturer

14. References

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