IVD

CE

Easy® ALK

User manual – version 2016/02

The Real-Time RT-PCR "**Easy® ALK**" kit enables the detection of the aberrant expression of mRNA encoding the ALK tyrosine kinase domain, in ALK-rearranged Non Small Cell Lung Cancer (NSCLC).

For *in vitro* diagnostic use

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Changes made since the previous version 2015/11:

- Validation of the kit on the Applied Biosystems ABI 7500 instrument.

For further details contact the technical support of the Diatech Pharmacogenetics (support@diatechpharmacogenetics.com).

INTENDED USE

The CE IVD "**Easy® ALK**" kit allows the qualitative detection of the ALK tyrosine-kinase domain over-expression by One Step Real-Time RT-PCR in RNA samples extracted from Non Small Cell Lung Cancer (NSCLC). The test can be performed on total RNA extracted from fresh, frozen or Formalin Fixed Paraffin Embedded (FFPE) tissue.

The "**Easy® ALK**" is validated on the following instruments:

- **CFX96** - Bio-Rad (software v.3.1)
- **ABI 7300** - Applied Biosystems (software v.1.4.1)
- **ABI 7500** - Applied Biosystems (software v.2.0.5)
- **Stratagene Mx3000P**, **Mx3005P** - Agilent Technologies (software v.4.10 build 389)
- **Rotor-Gene Q** - Qiagen (software v. 1.7 - Build 87)
- **Rotor-Gene 6000** - Corbett (software v. 1.7 - Build 87)

PRINCIPLE OF THE ASSAY

The "**Easy® ALK**" selectively amplifies the ALK tyrosine kinase domain RNA to detect the aberrant expression of ALK mRNA, in Non Small Cell Lung Cancer (NSCLC).

The "**Easy® ALK**" multiplex assay allows the simultaneous detection of the ALK RNA and an endogenous control transcript. The detection is achieved using fluorescent probes labelled with FAM (ALK) and HEX (endogenous control).

The amplification of the endogenous control RNA enables to verify the amplification procedure and the quality and quantity of the sample.

KIT CONTENTS

The kit contains sufficient reagents to carry out 24 tests for a maximum of 3 runs (6 samples and two controls per run).

COMP		QUAN	Color	
ALK mix		3 x 20 µl	YELLOW	Mixture of specific primers and probes targeting both ALK tyrosine kinase domain and the endogenous control.
RT Buffer		1 x 400 µl	RED	Solution containing reaction buffer, Mg2+ and dNTP Mixture.
Taq Enzyme		1 x 18 µl	WHITE	Solution containing hot start Taq DNA polimerase.
RT Enzyme		1 x 18 µl	BLACK	Solution containing reverse transcriptase enzyme.
Dye R		1 x 18 µl	BLUE	Inert fluorophore to be used for the amplification on the ABI 7300 instrument.
Dye R II		1 x 18 µl	GREEN	Inert fluorophore to be used for the amplification on the ABI 7500 instrument.
Control RNA	CONTROL +	3 vials	PURPLE	Lyophilized RNA to be used as reverse transcription and amplification control.
WATER	CONTROL -	1 x 1.5 ml		DNase-, RNase-free water to be used exclusively for the preparation of the RT-PCR mix and as negative control.
Water (diluent)		1 x 1.5 ml		DNase-, RNase-free water to be used exclusively as samples and **Control RNA** diluent.

DOCUMENTS AVAILABLE ON-LINE

Easy® ALK User Manual and Material Safety Data Sheet (MSDS) are available at www.diatechpharmacogenetics.com/en/reserved-area.

MATERIALS REQUIRED BUT NOT PROVIDED

RNA extraction

Il kit "**Easy® ALK**" kit does non contain reagents for RNA extraction.

Recommended kit:

"RNeasy FFPE kit" (Qiagen cod.73504)
"NucleoSpin® total RNA FFPE XS" (Macherey Nagel cod.740969.50)

ⓘ In case you employ kits which are different from the recommended ones, it is the user's responsibility to use standardized samples (e.g: VEQ – EQAS quality schemes, Horizon Diagnostics samples) to verify that this does not imply a reduction of the performance of the system under analysis.

Amplification

Real-Time PCR instruments:

- **CFX96** - Bio-Rad (software v.3.1)
- **ABI 7300** - Applied Biosystems (software v.1.4.1)
- **ABI 7500** - Applied Biosystems (software v.2.0.5)
- **Stratagene Mx3000P**, **Mx3005P** - Agilent Technologies (software v.4.10 build 389)
- **Rotor-Gene Q** - Qiagen (software v. 1.7 - Build 87)
- **Rotor-Gene 6000** - Corbett (software v. 1.7 - Build 87)

Detection channels for FAM and HEX fluorescence. Range of environmental temperature: 15-30°C.

Materials:

- 1.5 ml polypropylene twist-lock tubes (DNase-, RNase-, DNA-, PCR inhibitor-free)
- Micropipettes (volumes from 1 to 1.000 µl)
- Sterile filter tips DNase-, RNase-free (volumes from 1 to 1.000 µl)
- 96 well plates and foil or caps compatibles with the thermal-cycler used (check the compatibility in the user manual of the instrument) for instance:
  1. Bio-Rad CFX96: Hard Shell PCR plates 96-well WHT/CLR cod. HSP 9601; MICROSEAL B SEALS cod. MSB 1001
  2. ABI 7300/ABI 7500: MICROAMP OPTICAL 96 WELL RNX PLATE cod. N8010560; OPTICAL ADHESIVE COVERS cod. 4360954
  3. Stratagene Mx3000P/Mx3005P: Optical caps (8x strip) cod. 401425; QPCR 96-Well Plates, Non-Skirted cod. 401333
- DNase-and RNase-free, thin-wall, PCR tubes with flat cap or 0.1 ml tubes in strip, suitable for use on Rotor-Gene, for instance:
  1. Qiagen, cat. no. 981103
  2. LTF-Labortechnik GmbH & Co, no. 102.0170
- Powder-free disposable gloves

STABILITY AND STORAGE

Store all the reagents according to the instructions on the packages, in particular:

- Store all the reagents at -35/-20°C in the original package immediately upon receipt.
- Avoid thawing and re-freezing the reagents more than twice, as this could lead to poor performance.
- Protect all mixes containing probes from light to avoid degradation of the fluorescent dyes.
- If properly stored, the reagents remain stable until the expiration date displayed on the individual label.

SYMBOLS

REF	Catalog number (product code)	CONTROL -	Negative control
LOT	Lot number		Consult the instruction
Σ	Content sufficient for <n> tests		User manual (handbook)
IVD	For *in vitro* diagnostic use		Expiration date
CONT	Content		Storage conditions
COMP	Components		Manufactured by
NUM	Number of aliquots		Important Note
QUAN	Quantity per aliquot	GTIN	Global Trade Item Number
CONTROL +	Positive control		

PRODUCT USE LIMITATIONS

- The "**Easy® ALK**" kit can only be used by specialized personnel, properly instructed and trained.
- It is necessary to operate in compliance with the general guidelines of Good Laboratory Practice (GLP) and the instructions contained in this manual.
- Do not use expired or incorrectly stored reagents.
- The "**Easy® ALK**" kit is designed to be used with the instruments "**Rotor-Gene Q**" (Qiagen), "**Rotor-Gene™ 6000**" (Corbett Research), **CFX96** (Bio-Rad), **ABI 7300**/**ABI 7500** (Applied Biosystems) and **Stratagene Mx3000P**/**Mx3005P** (Agilent Technologies).
- The reliability of the results also depends on the procedures carried out in the pre-amplification stages, including the selection of starting biological speciments, the preservation of the samples and RNA extraction.
- Any diagnostic results generated by this procedure must be interpreted with reference to other clinical or laboratory findings.

QUALITY CONTROL

- The "**Easy® ALK**" kit was designed, developed and validated in accordance with D.Lgs n. 332 of 08/09/2000 ("Implementation of Directive 98/79/EC on in vitro diagnostic medical devices") and subsequent legislative changes.
- In accordance with the company system of full quality assurance (certified according to European standards EN ISO 9001 and ISO 13485), to ensure consistent product quality, each batch of "**Easy® ALK**" is subjected to functional quality control according to technical specifications and is released only if it is compliant with the quality control criteria.

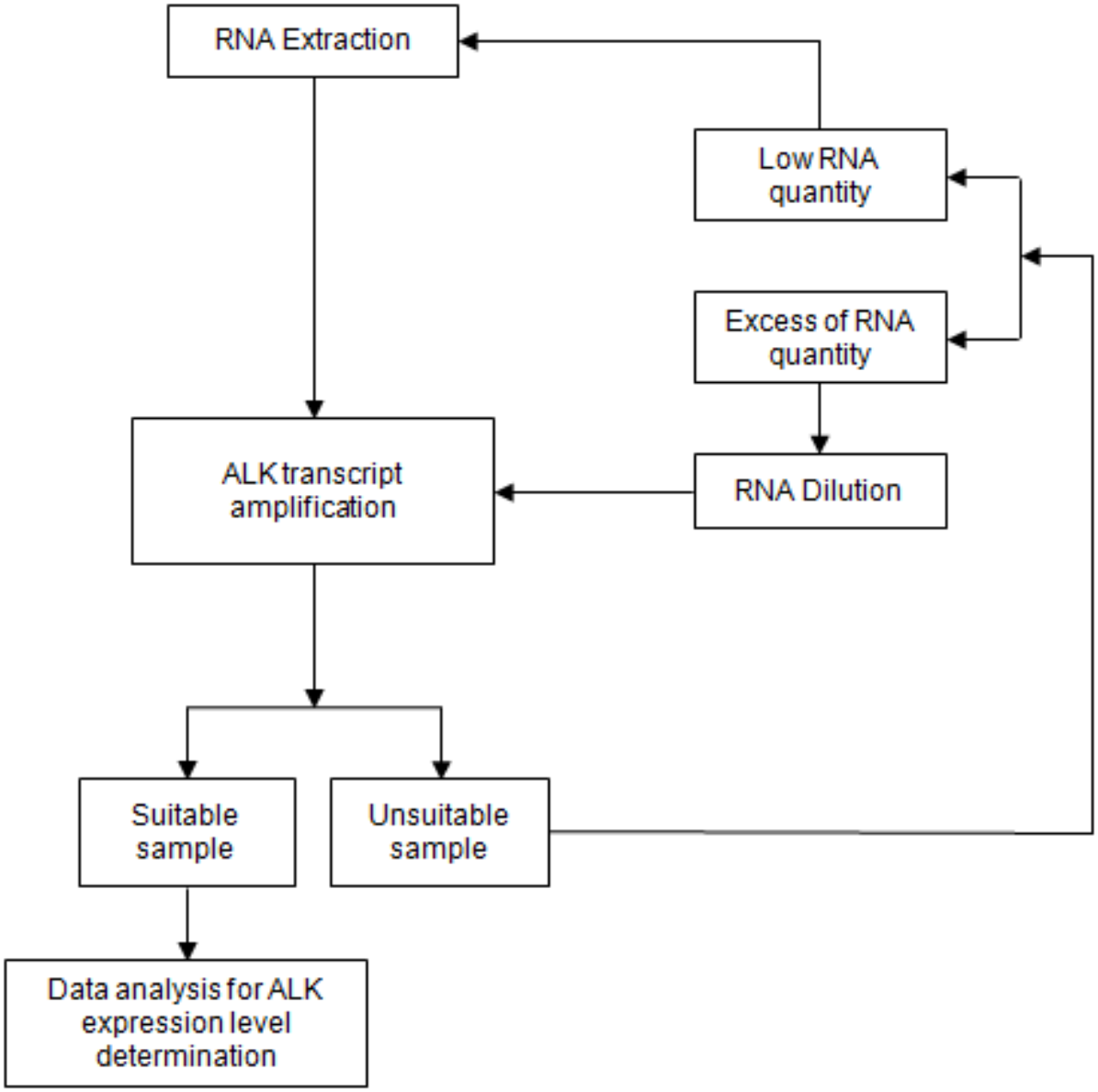
WARNINGS AND PRECAUTIONS

- The kit may only be used by specialist personnel, properly instructed and trained to perform *in vitro* laboratory techniques.
- Handle all samples as potentially infectious material inside a laminar flow hood (class II biological safety cabinet or higher).
- Follow the laboratory safety procedures described in "Biosafety in Microbiological and Biomedical Laboratories" (Richmond, JY and McKinney, RW (eds) - 5th edition (2009) and in the NCCLS (National Committee for Clinical Laboratory Standards) Document M29-T. Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids and Tissue. Tentative guidelines. – Villanova, PA:NCCLS, 1989).
- Do not eat, drink or smoke in the laboratory. When handling biological samples, disposable gloves, gowns and goggles or face masks should be worn to protect against biological agents.
- Constantly check that the gloves are free from contamination by the biological material being treated. If not, replace them immediately to avoid the possibility of cross-contamination between samples and contamination of the workplace. Wash hands thoroughly after handling samples and reagents.
- The Material Safety Data Sheet (MSDS) is available in the reserved area of the web-site Diatech Pharmacogenetics www.diatechpharmacogenetics.com.
- Perform the procedure in accordance with Good Laboratory Practice (GLP) general guidelines.
- It is recommended to ensure that the laboratory work flow proceeds in a unidirectional manner, preparing, if possible, two separate work areas for:
- extraction of nucleic acids;
- amplification reaction;
- Organize the laboratory so that dedicated pipettes, tips and materials are used for each activity.
- Use sterile filter tips. Avoid aerosols.
- Use tubes with twist-lock caps during the extraction of nucleic acids in order to avoid the leakage of the samples and potential contamination.
- During the procedures for nucleic acid extraction and amplification, avoid contamination of reagents with airborne microbes by only opening the reagents within the hood.
- Change the pipette tip before each extraction of reagents and every time you move from one sample to another in any stage of the procedure.
- The precision pipettes used should have an accuracy of within 3% of the set volume.
- Periodically check the calibration status of the dispensing instruments.
- Do not use reagents after the expiration date shown on each container.
- All reagents supplied in the "**Easy® ALK**" kit are intended to be used solely with the other reagents in the same "**Easy® ALK**" kit. Do not substitute or mix reagents in the kit from different batches, in order to maintain optimal performance.
- Only use the **Taq Enzyme, RT Enzyme** e **RT Buffer** that is provided in the kit. Do not substitute them with other kits or with similar reagents from other suppliers.
- Discard unused reagents and the expired kit and waste in accordance with current national laws and local regulations.
- Extraction area: at the end of the procedure, decontaminate the pipettes and the laboratory surfaces on which worked has been carried out, by cleaning with appropriate products (e.g. FD 322, Dürr Dental, Germany) and UV irradiate the work surface of the biological cabinet where the pipettes should be carefully placed after decontamination.
- Amplification area: at the end of the procedure, decontaminate the pipettes and the laboratory surfaces on which work has been carried out, by cleaning with appropriate products to eliminate nucleic acids and amplicons (e.g. "DNA Cleaner" - code DC001, Diatech Pharmacogenetics) and subsequent UV irradiation, if available.
- Avoid contamination of samples and reagents.
- Store reagents and samples separately.
- In order to avoid possible contamination from carry-over, do not open the reaction tubes after amplification.

- All reagents contained in the kit, exept **Control RNA,** are ready-to-use and don't need to be diluted. The reagent dilution may result in a loss of performance.
- Carefully read this User Manual.
- Check that the version of this User Manual corresponds to the one described on the "**Easy® ALK**" kit box label.
- The right to contest the kit before the expiration date becomes void if the product is used in violation of GLP guidelines and the manufacturer's recommendations.
- The registered names and trademarks indicated in this document are to be considered protected by law, even when not explicitly stated.

ANALYTICAL PROCEDURE

the analytical protocol is based on the following steps:



RNA EXTRACTION

ⓘ Perform this step in the area dedicated to nucleic acid isolation and dilution, using dedicated materials and instruments.

- The "**Easy® ALK**" kit does not include the reagents for RNA extraction.
- The quantity of biological material required for the RNA extraction depends on protocols.
- Refer also to the extraction kit manual for selection and treatment of FFPE slides.
- Commercial kits working with silica filters are recommended. Avoid methods that use phenol or without RNA purification.
- Perform the RNA extraction following the instructions of the extraction kit in use.
- If the extraction protocol involves the use of wash buffers containing ethanol, it is advisable to perform a further centrifugation before final elution to remove any possible traces of ethanol. This will prevent inhibition of the reaction by the ethanol.
- After the extraction, proceed immediately with the quali-quantitative evaluation of the RNA and the amplification reaction, or store the extracted RNA at ≤-78°C, divided into aliquots in order to maintain the experimental conditions constant in case of repetition.
- Just as an indication, for non-paraffin embedded samples, the recommended RNA amount is 5-250 ng/reaction; for paraffin embedded samples, the recommended RNA amount is 50-1500 ng/reaction.

ⓘ As absorbance reading cannot distinguish between fragmented and not fragmented RNA and therefore it can overestimate the concentration of template, RNA assessment should be based on the endogenous control. RNA assessment based on the endogenous control may differ from spectrophotometric quantification.

ⓘ The "**Easy® ALK**" assay amplifies short RNA sequences. However heavily fragmented RNA can generate no amplification product.

ⓘ The "**Easy® ALK**" assay selectively amplifies target RNA without amplifing potential contaminant genomic DNA.

CONTROL RNA RESUSPENSION

ⓘ Resuspend the lyohilized **Control RNA** in the area dedicated to the RNA isolation and dilution.

- Spin down the **Control RNA** before opening the tube.
- Add 20 µl of **Water (diluent)** to the lyophilized **Control RNA**, vortex for 10-15 seconds and spin down briefly.

ⓘ Resuspend the lyohilized **Control RNA** using **Water (diluent)**. Do not use other diluents.

ⓘ Place the resuspended **Control RNA** on ice or in a PCR-cooler during reaction setup.

ⓘ Resuspended **Control RNA** can be stored up to three months at -78°C. Always report the resuspension date on the tube.

ⓘ Do not freeze and thaw the resuspended **Control RNA** more than once.

AMPLIFICATION

General recommendations for amplification *(valid for all instruments)*

ⓘ Perform this step in the area dedicated to amplification mixes preparation, using dedicated materials and instruments. Before starting, decontaminate pipettes, benches and wood and possibly radiate with UV light for at least 30 minutes.

- Switch on the instrument and the software at least 20-30 minutes before starting the reaction to allow the heating of the lamps where necessary.
- Thaw all necessary reagents, except **Taq Enzyme** and **RT Enzyme**, before use. Thoroughly mix the reagents in a vortex, or inverting each tube ten times, and spin them briefly before use.
- Gently spin down **Taq Enzyme** and **RT Enzyme** prior to pipetting. Avoid generating bubbles. Do not vortex.
- Place **Taq Enzyme** and **RT Enzyme** on ice or in a PCR-cooler during the amplification reaction setup. Store the reagents at -20°C immediately after use.
- Prepare and mark an appropriate number of tubes or wells of the plate to use.

INSTRUMENT SETUP

Rotor-Gene Q, Rotor-Gene 6000

- Follow the instructions reported in the user manual of the instrument to set up the following fluorescence acquisition channels and thermal profile:
  - "Green": source 470 nm – detector 510 nm – "Gain Optimisation" 60°C Before 1st acquisition, "Tube Position": position 1 corresponding to the negative control (**WATER**), "Target Sample Range" 20-30 Fl
  - "Yellow": source 530 nm – detector 555 nm – "Gain Optimisation" 60°C Before 1st acquisition, "Tube Position": position 1 corresponding to the negative control (**WATER**), "Target Sample Range" 20-30 Fl

Thermal profile	
Hold	42°C per 5 minutes
Hold	95°C per 10 seconds
40 cycles	95°C per 5 seconds / 60°C per 30 seconds (acquire fluorescence in channels "Green", "Green 2", "Yellow", "Yellow 2")

- Reaction volume: 25 µl.

Stratagene Mx3000P, Mx3005P

- Select "New" – "Quantitative PCR (Multiple standards)", then "OK".
- Check for the presence of the message "Lamp – Warm-up".
- Follow the instructions reported in the user manual of the instrument to set up the following fluorescence acquisition channels and thermal profile:

- "FAM": source 492 nm – detector 516 nm – Filter gain factor x8
- "HEX": source 535 nm – detector 555 nm – Filter gain factor x2

Thermal profile	
Hold	42°C per 5 minutes
Hold	95°C per 10 seconds
40 cycles	95°C per 5 seconds / 60°C per 30 seconds (acquire fluorescence in channels "FAM" e "HEX" setting up END 1)

- Reaction volume: 25 µl.

CFX96

- Follow the instructions reported in the user manual of the instrument to set up the following fluorescence acquisition channels and thermal profile:

Thermal profile	
Step	
1	42°C per 5 minutes
2	95°C per 10 seconds
3	95°C per 5 seconds
4	60°C per 30 seconds (Plate read – All Channels)
5	GOTO 3 39 more times

- Reaction volume: 25 µl.

ⓘ Select the options "All Channels" to acquire the signal in both FAM and HEX.

ABI 7300

- Select "Create new document" – On Assay: "Standard Curve (Absolute Quantitation)", then "Next".
- Follow the instructions reported in the user manual of the instrument to set up the following fluorescence acquisition channels and thermal profile:

Thermal profile	
Hold	42°C per 5 minutes
Hold	95°C per 10 seconds
40 cicli	95°C per 5 seconds / 60°C per 30 seconds (acquire fluorescence in channels FAM, JOE; Quencer – None; Passive Reference Dye - ROX)

- Reaction volume: 25 µl.

ABI 7500

- Select “New Experiment” then “7500 (96 Wells)”, “Quantitation – Standard Curve”, “TaqMan® Reagents”, “Standard (~2 hours to complete a run)”.
- Follow the instructions reported in the user manual of the instrument to set up the following fluorescence acquisition channels and thermal profile:

Thermal profile	
Hold	42°C for 5 minutes
Hold	95°C for10 seconds
40 cicli	95°C for 5 seconds / 60°C for 30 seconds (acquire fluorescence in channels FAM, JOE; Quencer – None; Passive Reference Dye - ROX)

- Reaction volume: 25 µl.

DETERMINATION OF THE ALK EXPRESSION LEVEL

ⓘ The kit content is optimized to analyze six clinical samples and two controls (**Control RNA** e **WATER**) in each run.

ⓘ Please pay attention to the samples dispensation order, tubes/wells identification, tubes or plate loading on the instrument and to patient data entered in the software.

ⓘ Each run must include at least one amplification negative control (**WATER**) and one amplification positive control (**Control RNA**).

Mx3000P/3005P, ABI 7300, ABI 7500, CFX96 (RT005 sample grid A)

	1	2	3	4	5	6	7	8	9	10	11	12
A	WATER											
B	Control RNA											
C	RNA1											
D	RNA2											
E	RNA3											
F	RNA4											
G	RNA5											
H	RNA6											

ALK mix

Rotor-Gene (RT005 sample grid B)

1	• WATER	9	•	17	•	25	•	33	•	41	•	49	•	57	•	65	•
2	• Control RNA	10	•	18	•	26	•	34	•	42	•	50	•	58	•	66	•
3	• RNA1	11	•	19	•	27	•	35	•	43	•	51	•	59	•	67	•
4	• RNA2	12	•	20	•	28	•	36	•	44	•	52	•	60	•	68	•
5	• RNA3	13	•	21	•	29	•	37	•	45	•	53	•	61	•	69	•
6	• RNA4	14	•	22	•	30	•	38	•	46	•	54	•	62	•	70	•
7	• RNA5	15	•	23	•	31	•	39	•	47	•	55	•	63	•	71	•
8	• RNA6	16	•	24	•	32	•	40	•	48	•	56	•	64	•	72	•

ⓘ For each sample and control prepare an amplification mixture (**RT-Amp-Mix**), according to the following table, where N is the total number of samples and controls to be tested:

Rotor-Gene, Stratagene, CFX96		
RT-Amp Mix	**Reagent volume for 1 reaction (µl)**	**Reagent volume for N reactions +1 (µl)**
RT Buffer	12.5	
Taq Enzyme	0.5	
RT Enzyme	0.5	
WATER CONTROL -	4.5	
ALK mix	2	
Total Volume	20	

ABI 7300		
RT-Amp Mix	**Reagent volume for 1 reaction (µl)**	**Reagent volume for N reactions +1 (µl)**
RT Buffer	12.5	
Taq Enzyme	0.5	
RT Enzyme	0.5	
Dye R	0.5	
WATER CONTROL -	4	
ALK mix	2	
Total Volume	20	

ABI 7500		
RT-Amp Mix	**Reagent volume for 1 reaction (µl)**	**Reagent volume for N reactions +1 (µl)**
RT Buffer	12.5	
Taq Enzyme	0.5	
RT Enzyme	0.5	
Dye R II	0.5	
WATER CONTROL -	4	
ALK mix	2	
Total Volume	20	

- Mix the **RT-Amp-Mix** thoroughly by repeated pipetting or rapid vortexing, then centrifuge briefly.
- Pipette 20 µl of THE **RT-Amp-Mix** in all the tubes/wells previously marked.
  Add to the respective tubes/wells:

negative cotrol	5 µl **WATER**	CONTROL -
samples	5 µl RNA	
Positive control	5 µl **Control RNA**	CONTROL +

- Reaction volume: 25 µl.
- Briefly centrifuge the plate.
- Check that the thermal profile is setted up correctly and start the run.

ⓘ Before starting the run, please pay attention to the plate orientation (well A1 on the upper left position) or to the Rotor-Gene 0.1ml strips of tubes orientation (mark the first tube of each strip).

ⓘ Proceed with the analysis following the instructions of the section “Data analysis”.

DATA ANALYSIS

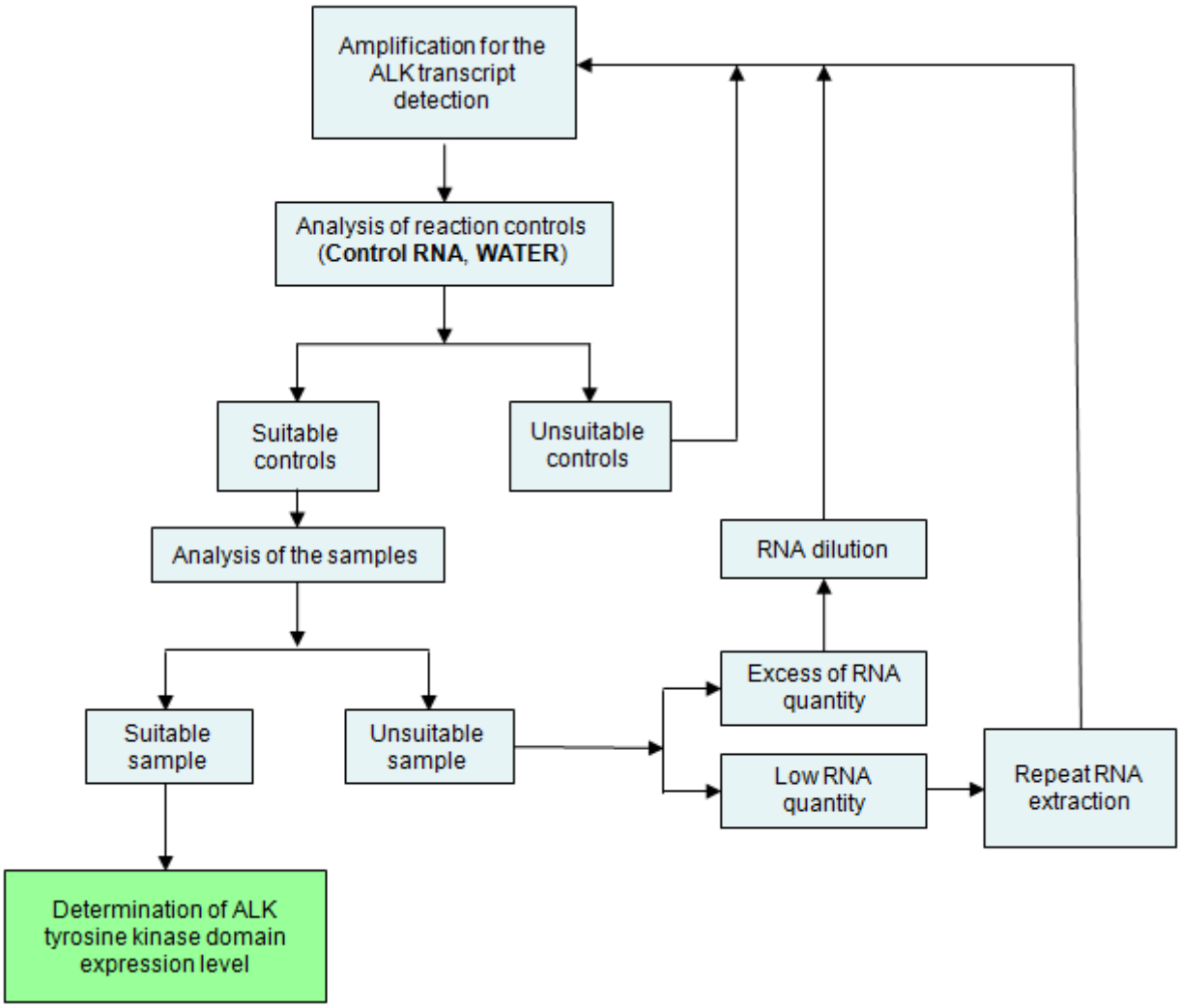
General reccomandations pertain to all the instruments

- Analyze first the negative control **WATER** and the positive control **Control RNA.** If they are in the range of expected values, proceed with the analysis of the samples, otherwise the session should be considered invalid and the results of the samples should be rejected.

- It is necessary to verify that the Ct values obtained are generated from a real amplification reaction (sigmoidal fluorescense curve) and not from an artifact (linear fluorescence curve), checking the normalized fluorescence graphs.

- Only samples with a Ct value that falls into the HEX/Yellow channel acceptability range are suitable and can be analyzed for the ALK tyrosine kinase domain expression.

Proceed with the analysis as indicated by the following scheme:



Determination of the ALK tyrosine kinase domain expression level

Rotor-Gene

- At the end of the run, click Analysis and select Quantitation.
- Highlight Dynamic tube and Slope correct for all the channels.
- Set Threshold 0.04 for the Green and Yellow channels.

ⓘ If it is not possible to perform the analysis in the Green and/or Yellow channel because the fluorescence level is too high, see Troubleshooting section.

1. Analysis of the reaction controls

ALK mix	Green	Yellow	Results
WATER	Ct > 35	Ct > 35	Proceed with analysis of the samples.
	Ct ≤ 35	Ct ≤ 35	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Control RNA	25 ≤ Ct ≤ 29	23 ≤ Ct ≤ 27	Proceed with analysis of the samples.
	Ct < 25 or Ct > 29	Ct < 23 or Ct > 27	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).

2. Analysis of sample suitability

ALK mix	Green	Yellow	Results
RNA samples	Any value	22 ≤ Ct ≤ 31	Suitable sample. Proceed with the mutation analysis.
	Any value	Ct < 22	Excess of RNA. Samples must be diluted with **Water (diluent)** so that Ct fall in the ranges indicated above. Consider that the dilution 1:2 of the RNA increases the Ct of 1 unit.
	Any value	Ct > 31	Suboptimal amount of starting RNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: ▪ If it is possible, amplify a RNA volume > 5µl (max 9µl) or proceed with a new RNA extraction to obtain an higher concentration of template and/or a RNA of higher quality. ▪ If the presence of inhibitors is suspected, dilute the sample with **Water (diluent)**. Consider that the dilution reduces the presence of inhibitor, but also decreases the concentration of the target RNA.

3. Analysis of the ALK tyrosine kinase domain expression level

ⓘ Only samples suitable as defined at point 2 can be analyzed for ALK expression level.

- Compare ΔΔCt values of the samples with those reported in the following table. The specified values are in the range and include extremes. The ΔΔCt values should be calculated with the following formula, taking care that the Ct value in Green and the equivalent for Yellow belong to the same sample:

$$\Delta Ct \text{ ALK (Sample)} = Ct \text{ Yellow (Sample)} - Ct \text{ Green (Sample)}$$

$$\Delta Ct \text{ ALK (}\textbf{Control RNA}\text{)} = Ct \text{ Yellow (}\textbf{Control RNA}\text{)} - Ct \text{ Green (}\textbf{Control RNA}\text{)}$$

$$\Delta\Delta Ct \text{ ALK (Sample)} = \Delta Ct \text{ ALK (Sample)} - \Delta Ct \text{ ALK (}\textbf{Control RNA}\text{)}$$

Assay	Amplification of endogenous control (Yellow)	Ct Green	ΔΔCt	Results
ALK mix	OK (Suitable sample)	No Ct	\	Negative sample or beneath the LOD[1]
		Any value	< 1.5	
		Any value	≥ 1.5	Positive (aberrant ALK expression)
	NO (Unsuitable sample)	No Ct	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
		Any value	\	

Note
1. LOD = Limit Of Detection
\ ΔΔCt not computable

Stratagene Mx3000P, Mx3005P

- At the end of the run click Set-up, Plate Setup, and enter the sample names.
- In the section Analysis, Analysis Selection/Setup, deselect the function Amplification-based threshold and select only the reactions controls **WATER** and **Control RNA**.
- In the section Analysis, Results, click on the "lock icon" in the box Threshold Fluorescence (in this way the automatically selected values cannot be modified).
- Go back to the section Analysis, Analysis Selection/Setup, and select all the samples.
- In the section Analysis, Results, select Text report to see the results in both channels with their respective threshold and Ct values.
- Click Save.

1. Analysis of reaction control

ALK mix	FAM	HEX	Results
WATER	Ct > 35	Ct > 35	Proceed with analysis of the samples.
	Ct ≤ 35	Ct ≤ 35	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Control RNA	28 ≤ Ct ≤ 32	25 ≤ Ct ≤ 29	Proceed with analysis of the samples.
	Ct < 28 or Ct > 32	Ct < 25 or Ct > 29	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).

2. Analysis of sample suitability

ALK mix	FAM	HEX [1]	Results
RNA samples	Any value	(x-3) ≤ Ct ≤ (x+6)	Suitable sample. Proceed with the mutation analysis.
	Any value	Ct < (x-3)	Excess of RNA. Samples must be diluted with **Water (diluent)** so that Ct fall in the ranges indicated above. Consider that the dilution 1:2 of the RNA increases the Ct of 1 unit.
	Any value	Ct > (x+6)	Suboptimal amount of starting RNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: ▪ If it is possible, amplify a RNA volume > 5µl (max 9µl) or proceed with a new RNA extraction to obtain an higher concentration of template and/or a RNA of higher quality. ▪ If the presence of inhibitors is suspected, dilute the sample with **Water (diluent)**. Consider that the dilution reduces the presence of inhibitor, but also decreases the concentration of the target RNA.
1. x = Ct **Control RNA**			

3 Analysis of the ALK tyrosine kinase domain expression level

ⓘ Only samples suitable as defined at point 2 can be analyzed for ALK expression level.

- Compare ΔΔCt values of the samples with those reported in the following table. The specified values are in the range and include extremes. The ΔΔCt values should be calculated with the following formula, taking care that the Ct value in FAM and the equivalent for HEX belong to the same sample:

$$\Delta Ct \text{ ALK (Sample)} = Ct \text{ HEX (Sample)} - Ct \text{ FAM (Sample)}$$

$$\Delta Ct \text{ ALK (}\textbf{Control RNA}\text{)} = Ct \text{ HEX (}\textbf{Control RNA}\text{)} - Ct \text{ FAM (}\textbf{Control RNA}\text{)}$$

$$\Delta\Delta Ct \text{ ALK (Sample)} = \Delta Ct \text{ ALK (Sample)} - \Delta Ct \text{ ALK (}\textbf{Control RNA}\text{)}$$

Assay	Amplification of endogenous control (HEX)	Ct FAM	ΔΔCt	Results
ALK mix	OK (Suitable sample)	No Ct	\	Negative sample or beneath the LOD[1]
		Any value	< 1.8	
		Any value	≥ 1.8	Positive (aberrant ALK expression)
	NO (Unsuitable sample)	No Ct	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
		Any value	\	

Note
1. LOD = Limit Of Detection
\ ΔΔCt not computable

CFX96

- At the end of the run, click Plate Setup – View/Edit Plate, select the wells used, pick the FAM and HEX fluorophores, enter samples name and exclude all empty wells from analysis.
- In the section Settings set up the following analysis criteria as default:
  - Cq determination mode: Single Threshold
  - Baseline settings: Baseline Subtracted Curve fit
  - Analysis Mode: Fluorophore
  - Baseline Threshold: Auto Calculated for every channel
- In the page Quantification data, the results are displayed for both channels with the corresponding values of Cq.
- Click Save.

1. Analysis of reaction controls

ALK mix	FAM	HEX	Results
WATER	Cq > 35	Cq > 35	Proceed with analysis of the samples.
	Cq ≤ 35	Cq ≤ 35	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Control RNA	25 ≤ Cq ≤ 29	23 ≤ Cq ≤ 27	Proceed with analysis of the samples.
	Cq < 25 or Cq > 29	Cq < 23 or Cq > 27	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).

2. Analysis of sample suitability

ALK mix	FAM	HEX [1]	Results
RNA samples	Any value	(x-4) ≤ Cq ≤ (x+6)	Suitable sample. Proceed with the mutation analysis.
	Any value	Cq < (x-4)	Excess of RNA. Samples must be diluted with **Water (diluent)** so that Cq fall in the ranges indicated above. Consider that the dilution 1:2 of the RNA increases the Cq of 1 unit.
	Any value	Cq > (x+6)	Suboptimal amount of starting RNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: ▪ If it is possible, amplify a RNA volume > 5µl (max 9µl) or proceed with a new RNA extraction to obtain an higher concentration of template and/or a RNA of higher quality. ▪ If the presence of inhibitors is suspected, dilute the sample with **Water (diluent)**. Consider that the dilution reduces the presence of inhibitor, but also decreases the concentration of the target RNA.
1. x = Cq **Control RNA**			

3. Analysis of the ALK tyrosine kinase domain expression level

ⓘ Only samples suitable as defined at point 2 can be analyzed for ALK expression level.

- Compare ΔΔCq values of the samples with those reported in the following table. The specified values are in the range and include extremes. The ΔΔCq values should be calculated with the following formula, taking care that the Cq value in FAM and the equivalent for HEX belong to the same sample:

$$\Delta Cq \text{ ALK (Sample)} = Cq \text{ HEX (Sample)} - Cq \text{ FAM (Sample)}$$

$$\Delta Cq \text{ ALK (**Control RNA**)} = Cq \text{ HEX (**Control RNA**)} - Cq \text{ FAM (**Control RNA**)}$$

$$\Delta\Delta Cq \text{ ALK (Sample)} = \Delta Cq \text{ ALK (Sample)} - \Delta Cq \text{ ALK (**Control RNA**)}$$

Assay	Amplification of endogenous control (HEX)	Ct FAM	ΔΔCq	Results
ALK mix	OK (Suitable sample)	No Cq	\	Negative sample or beneath the LOD[1]
		Any value	< 1.5	
		Any value	≥ 1.5	Positive (aberrant ALK expression)
	NO (Unsuitable sample)	No Cq	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
		Any value	\	

Note
1. LOD = Limit Of Detection
\ ΔΔCq not computable

ABI 7300

- At the end of the run, click Results – Plate, enter samples name and omit all empty wells.
- In the page Results – Amplification plot select all the samples and controls.
- Select Detector – All.
- In Analysis settings select Auto Ct then Analyze.
- Select Manual Ct, Manual baseline Start cycle 3, End cycle 15 then Analyze.
- In the page Report Ct values are visualized for both channels.
- Click Save.

1. Analysis of reaction controls

ALK mix	FAM	JOE	Results
WATER	Ct > 35	Ct > 35	Proceed with analysis of the samples.
	Ct ≤ 35	Ct ≤ 35	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Control RNA	25 ≤ Ct ≤ 29	23 ≤ Ct ≤ 27	Proceed with analysis of the samples.
	Ct < 25 or Ct > 29	Ct < 23 or Ct > 27	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).

2. Analysis of sample suitability

ALK mix	FAM	JOE [1]	Results
RNA samples	Any value	(x-3) ≤ Ct ≤ (x+6)	Suitable sample. Proceed with the mutation analysis.
	Any value	Ct < (x-3)	Excess of RNA. Samples must be diluted with **Water (diluent)** so that Ct fall in the ranges indicated above. Consider that the dilution 1:2 of the RNA increases the Ct of 1 unit.
	Any value	Ct > (x+6)	Suboptimal amount of starting RNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: ▪ If it is possible, amplify a RNA volume > 5µl (max 9µl) or proceed with a new RNA extraction to obtain an higher concentration of template and/or a RNA of higher quality. ▪ If the presence of inhibitors is suspected, dilute the sample with **Water (diluent)**. Consider that the dilution reduces the presence of inhibitor, but also decreases the concentration of the target RNA.
1. x = Ct **Control RNA**			

3. Analysis of the ALK tyrosine kinase domain expression level

ⓘ Only samples suitable as defined at point 2 can be analyzed for ALK expression level.

- Compare ΔΔCt values of the samples with those reported in the following table. The specified values are in the range and include extremes. The ΔΔCt values should be calculated with the following formula, taking care that the Ct value in FAM and the equivalent for HEX belong to the same sample:

$$\Delta Ct \text{ ALK (Sample)} = Ct \text{ JOE (Sample)} - Ct \text{ FAM (Sample)}$$

$$\Delta Ct \text{ ALK (}\textbf{Control RNA}\text{)} = Ct \text{ JOE (}\textbf{Control RNA}\text{)} - Ct \text{ FAM (}\textbf{Control RNA}\text{)}$$

$$\Delta\Delta Ct \text{ ALK (Sample)} = \Delta Ct \text{ ALK (Sample)} - \Delta Ct \text{ ALK (}\textbf{Control RNA}\text{)}$$

Assay	Amplificazione endogenous control (JOE)	Ct FAM	ΔΔCt	Results
ALK mix	OK (Suitable sample)	No Cq	\	Negative sample or beneath the LOD[1]
		Any value	< 1.5	
		Any value	≥ 1.5	Positive (aberrant ALK expression)
	NO (Unsuitable sample)	No Cq	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
		Any value	\	

Note
1. LOD = Limit Of Detection
\ ΔΔCt not computable

ABI 7500

- At the end of the run, click Setup – Plate setup – Assign Targets and Samples and enter samples name.
- In the section Analysis – Amplification plot in the page View Plate layout select all the samples and controls and omit all empty wells.
- Select Reanalyse.
- In Analysis settings, for each channel, deselect Use Default Settings, Automatic Threshold and Automatic Baseline.
- Click on Apply Analysis Settings and then Reanalyse.
- In the page View Well Table Ct values are visualized for both channels.
- Click Save.

1. Analysis of reaction controls

ALK mix	FAM	JOE	Results
WATER	Ct > 35	Ct > 35	Proceed with analysis of the samples.
	Ct ≤ 35	Ct ≤ 35	Possible contamination: it is not possible to analyze the samples (see Troubleshooting).
Control RNA	26 ≤ Ct ≤ 31	24 ≤ Ct ≤ 28	Proceed with analysis of the samples.
	Ct < 26 or Ct > 31	Ct < 24 or Ct > 28	Possible error in the set up of the reaction/run: it is not possible to analyze the samples (see Troubleshooting).

2. Analysis of sample suitability

ALK mix	FAM	JOE [1]	Results
RNA samples	Any value	(x-3) ≤ Ct ≤ (x+6)	Suitable sample. Proceed with the mutation analysis.
	Any value	Ct < (x-3)	Excess of RNA. Samples must be diluted with **Water (diluent)** so that Ct fall in the ranges indicated above. Consider that the dilution 1:2 of the RNA increases the Ct of 1 unit.
	Any value	Ct > (x+6)	Suboptimal amount of starting RNA or PCR inhibition; YOU CAN NOT CONTINUE WITH THE ANALYSIS OF MUTATION: ▪ If it is possible, amplify a RNA volume > 5µl (max 9µl) or proceed with a new RNA extraction to obtain an higher concentration of template and/or a RNA of higher quality. ▪ If the presence of inhibitors is suspected, dilute the sample with **Water (diluent)**. Consider that the dilution reduces the presence of inhibitor, but also decreases the concentration of the target RNA.
1. x = Ct **Control RNA**			

3. Analysis of the ALK tyrosine kinase domain expression level

ⓘ Only samples suitable as defined at point 2 can be analyzed for ALK expression level.

- Compare ΔΔCt values of the samples with those reported in the following table. The specified values are in the range and include extremes. The ΔΔCt values should be calculated with the following formula, taking care that the Ct value in FAM and the equivalent for HEX belong to the same sample:

ΔCt ALK (Sample) = Ct JOE (Sample) – Ct FAM (Sample)

ΔCt ALK (**Control RNA**) = Ct JOE (**Control RNA**) – Ct FAM (**Control RNA**)

ΔΔCt ALK (Sample) = ΔCt ALK (Sample) – ΔCt ALK (**Control RNA**)

Assay	**Amplificazione endogenous control (HEX)**	**Ct FAM**	**ΔΔCt**	**Results**
ALK mix	OK (Suitable sample)	No Cq	\	Negative sample or beneath the LOD[1]
		Any value	< 2.0	
		Any value	≥ 2.0	Positive (aberrant ALK expression)
	NO (Unsuitable sample)	No Cq	\	Failed: not sufficient template/PCR inhibition / mistake during samples dispensation
		Any value	\	

Note
1. LOD = Limit Of Detection
\ ΔΔCt not computable

TROUBLESHOOTING

Problem	Possible reason	Recommendation
Low or absent amplification signal in the channel "Green/FAM" and/or in the channel "Yellow/HEX/JOE" for both **Control RNA** and samples.	Incorrect selection of the fluorescence acquisition channels.	Check the fluorescence acquisition channels and repeat amplification with the settings described in this manual.
	Incorrect selection of the Gain on the Rotor-Gene/Stratagene Mx.	Repeat amplification of Rotor-Gene/Stratagene Mx setting the gain properly, as described in this manual.
	Incorrect setting of the thermal-profile.	Check the temperature profile and repeat amplification with the settings described in this manual.
	Incorrect dispensation or manipulation of the reagents.	▪ Vortex **ALK mix** and **RT Buffer** and briefly spin down before use. ▪ Place **Taq Enzyme** and **RT Enzyme** on ice or on a PCR-cooler during reaction setup. Do not vortex to avoid formation of bubbles.
	Reagents improperly stored or expired.	▪ Protect all mixes from light. ▪ Store all reagents at -35/-20°C and avoid thawing and refreezing more than twice. ▪ Do not store the mixes containing primers and probes at +2/+8°C for more than 5 hours. ▪ Do not store the resuspended **Control RNA** at -78°C for more than 3 months. ▪ Avoid thawing and refreezing resuspended **Control RNA** more than once. ▪ Do not use expyred reagents.
Low or absent amplification signal in the channel "Yellow/HEX/JOE" or in both "Yellow/HEX/JOE" and "Green/FAM" for samples but expected results for **Control RNA**.	Insufficient amount of starting RNA and/or presence of PCR inhibitors.	▪ Check the quantity and quality of the extracted RNA and, if appropriate, repeate the extraction faithfully following the instructions of the extraction kit. ▪ If the extraction protocol involves the use of washing buffers containing ethanol, it is advisable to carry out a further centrifugation prior to final elution to remove any possible trace of alcohol. ▪ If it is assumed that the amount of starting RNA is insufficient, repeat the reaction amplifying > 5ul RNA (max volume 9 ul), reducing the corresponding volume of **WATER** in the **Amp-Mix**. Otherwise repeat the RNA extraction by reducing the volume of elution. ▪ If you suspect the presence of inhibitors, repeat the amplification diluting sample 1:5 or 1:10 with **Water (diluent)**.
	Incorrect or no dispensation of the samples.	▪ Repeat the amplification dispensing the correct volume of RNA and including positive and negative controls.
Low or absent amplification signal in the channel "Yellow/HEX/JOE" and "Green/FAM" only for **Control RNA**.	Degratation of **Control RNA**.	▪ Resuspend lyophilized **Control RNA** with **WATER (diluent)**, as reported in this user manual. ▪ After resuspension, place **Control RNA** on ice or in a PCR-cooler during amplification reaction setup. ▪ Store the rersuspended **Control RNA** at -78°C as recommended. ▪ Do not store the resuspended **Control RNA** at -78°C for more than 3 months . ▪ Repeat amplification reaction using a properly stored **Control RNA** tube.
	Incorrect of failure in the dispensation of the **Control RNA**.	▪ Repeat the amplification by pipetting the appropriate volume of **Control RNA**. ▪ Mark unequivocally samples and controls reaction tubes.
	Incorrect samples names set-up in the software.	▪ Check samples names setup.
The negative control **WATER**, shows an amplification signal in both FAM/Green and HEX/Yellow/JOE channels.	Contamination.	▪ The results should be rejected and samples must be reamplified using new reagents. ▪ Prepare RT-Amp Mix in a dedicated area. Carefully decontaminate benches, pipettes and instruments.
	Incorrect dispensation of the samples.	▪ Repeat the amplification paying attention to the sample order when loading the RNA and **Control RNA** samples.
	Incorrect samples names set-up in the software.	▪ Check samples names setup.

Fluorescence intensity variable.	Cutaneous fat on the reaction tube.	▪ Wear gloves.
If the problems persist despite the implementation of the recommendations given and for any other questions or problems, please contact technical support of Diatech Pharmacogenetics: ▪ e-mail support@diatechpharmacogenetics.com ▪ telephone +39 0731 213243 ▪ fax +39 0731 213239		

PERFORMANCE VALIDATION

The performance validation has been performed using all the reagents supplied with the Kit "**Easy® ALK**".

The experiments have been performed according to the instructions reported in this user manual on the following real-time instruments:

- **CFX96** - Bio-Rad (con software v. 3.1)
- **ABI 7300**- Applied Biosystems (con software v. 1.4.1)
- **ABI 7500** - Applied Biosystems (software v.2.0.5)
- **Stratagene Mx3000P**, **Mx3005P** - Agilent Technologies (con software v. 4.10 - Build 389)
- **Rotor-Gene Q** - Qiagen (con software v. 1.7 - Build 87)
- **Rotor-Gene 6000** - Corbett (con software v. 1.7 - Build 87)

Clinical specificity

In order to evaluate the kit specificity RNA samples isolated from FFPE tumor tissue have been tested. Samples were suitable in terms of starting RNA amount and have been already analyzed through fluorescence in situ hybridization (FISH) and/or immunohistochemistry (IHC).

Results are listed in the table below:

Rotor-Gene 6000/Q				Stratagene Mx3000P/3005P				CFX96				ABI 7300				ABI 7500			
No. of samples	FISH	IHC	Easy® ALK	No. of samples	FISH	IHC	Easy® ALK	No. of samples	FISH	IHC	Easy® ALK	No. of samples	FISH	IHC	Easy® ALK	No. of samples	FISH	IHC	Easy® ALK
9	+	+	+	\	\	\	\	\	\	\	\	\	\	\	\	1	+	+	+
13	+	\	+	8	+	\	+	8	+	\	+	8	+	\	+	10	+	\	+
4	+	\	-	4	+	\	-	4	+	\	-	4	+	\	-	\	\	\	\
1	+	-	-	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
1	-	+	+	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
27	-	\	-	13	-	\	-	17	-	\	-	17	-	\	-	21	-	\	-
17	-	-	-	\	\	\	\	\	\	\	\	\	\	\	\	1	-	-	-

A comparison between "**Easy® ALK**" and a commercial RT-PCR assay has been performed for samples already tested with both FISH and IHC. Results are listed in the table below:

Rotor-Gene 6000/Q						
N° samples tested	RT005 Easy® ALK			RT-PCR test			Concordance RT005/RT-PCR
	Suitable samples[1]	FISH/RT005	IHC/RT005	Suitable samples[1]	FISH/RT-PCR	IHC/RT-PCR	
33	33/33 (100%)	29/33 (88%)	25/25 (100%)	31/33 (94%)	25/31 (71%)	22/24 (92%)	28/31 (90%)
1. Suitable samples in terms of input RNA, as defined in the user manual of the kit in use.							

Numerous studies reveal a partial discordance between FISH, IHC and real-time results caused by differences in terms of sensibility and specifity between the different techniques but also due to unknown biological events.

"**Easy® ALK**" results show a concordance rate with FISH and IHC results similar to data reported in the literature. See table below:

Study	RT-PCR target	FISH	IHC	Concordance FISH/RT-PCR	Concordance FISH/IHC	Concordance RT-PCR /IHC
RT005 Easy® ALK	ALK Tyrosine Kinase Domain (TKD)	ALK dual color FISH probe	α-ALK (D5F3)	92%	93%	100%
Soda et al. 2012	ALK Fusion breakpoint	ALK dual color FISH probe	α-ALK (5A4)	99%	99%	100%
Wallander et al. 2012	EML4-ALK v1-v3a/b Fusion breakpoint	ALK dual color FISH probe	α-ALK (CD246)	98%	94%	96%
Sholl et al. 2013	\	ALK dual color FISH probe	α-ALK (5A4)	\	98%	\
Wang et al. 2014	ALK Fusion breakpoint	ALK dual color FISH probe	α-ALK (D5F3)	89%	98%	\
Gruber et al. 2014	ALK 3'/5' Ratio	ALK dual color FISH probe	α-ALK (D5F3)	94%	98%	98%
Ilie et al 2015	\	ALK dual color FISH probe	α-ALK (D5F3)	\	96%	\

Analitical sensitivity

In order to evaluate "**Easy® ALK**" analitycal sensitivity the following samples have been tested in duplicate in at least three independent runs:

1) Human RNA ALK wild-type at 250, 100, 50, 25, 10 e 2.5 ng/reaction (qPCR Human Reference Total RNA cod. 636690, Clontech).
2) RNA entracte from Horizon Diagnostics standard EML4-ALK v1 containing 25% of translocated cells at 25, 2.5, 1.25 ng/reaction.

All samples have been quantified by "XPOSE™" spectrophotometer (Trinean).

Limit of detection has been defined as the lowest input RNA quantity, used for the amplification, that generates the correct results for all samples and replicates tested.

The analitical sensitivity of the kit is 2.5 ng/reaction.

Reproducibility

System reproducibility (*inter-assay* variability) has been evaluated analyzing Horizon Diagnostics EML4-ALK v1 standard harbouring 50% and 25% of translocated cells at the concentration of 25 and 2.5 ng/reaction. Each sample has been tested in duplicate in at least three independent experiments performed by two different oparators in different days.
Results, expressed as median ΔΔCt value ± standard deviation, are reproducible for all instruments tested.

	***Inter-assay* variability ($\Delta\Delta Ct\ ALK_{mean}$ ± SD)**				
Sample	**Rotor-Gene 6000/Q**	**Stratagene Mx3000P/3005P**	**CFX96**	**ABI 7300**	**ABI 7500**
Horizon Diagnostics EML4-ALK v1 50%	3.7 ± 0.3	4.0 ± 0.3	3.4 ± 0.1	3.6 ± 0.2	4.4 ± 0.1
Horizon Diagnostics EML4-ALK v1 25%	2.8 ± 0.2	3.1 ± 0.2	2.4 ± 0.1	2.6 ± 0.3	3.2 ± 0.3

Repeatability

System repeatability (*intra-assay* variability) has been evaluated analyzing Horizon Diagnostics EML4-ALK v1 standard harbouring 25% of translocated cells at the concentration of 25 and 2.5 ng/reaction. Each sample has been tested in replicate in three independent experiments.
Results, expressed as median ΔΔCt value ± standard deviation, are reproducible for all instruments tested.

	Intra-assay variability ($\Delta\Delta Ct\ ALK_{mean}$ ± SD)														
Sample	**Rotor-Gene 6000/Q**			**Stratagene Mx3000P/3005P**			**CFX96**			**ABI 7300**			**ABI 7500**		
	RUN1	RUN2	RUN3	RUN1	RUN2	RUN3	RUN1	RUN2	RUN3	RUN1	RUN2	RUN3	RUN1	RUN2	RUN3
Horizon Diagnostics EML4-ALK v1 25%	2.5±0.1	2.8±0.1	2.7±0.1	3.4±0.2	3.2±0.2	3.2±0.3	2.5±0.2	2.3±0.1	2.4±0.2	2.7±0.1	2.8±0.3	2.5±0.1	3.0 ± 0.2	3.3 ± 0.2	3.2 ± 0.4

Robustness

Lot to lot consistency
Different batches of RT Buffer, Taq Enzyme and RT Enzyme have been tested with the same panel of samples. Results from the different lots are comparable.
Two different batches of primers and probes have been tested with the same panel of samples. Results from the different lots are comparable.

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RT005 sample grid A – Easy® ALK

DATE		PLATE NAME	

	1	2	3	4	5	6	7	8	9	10	11	12
A	WATER											
B	Control RNA											
C												
D												
E												
F												
G												
H												

ALK mix

INSTRUMENT n.				USER MANUAL VERSION	
CODE		LOT		EXPIRY DATE	
NOTES					
OPERATOR				SIGN	

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RT005 sample grid B – Easy® ALK

DATE		EXPERIMENT NAME	

ALK mix								
1 • WATER	9 •	17 •	25 •	33 •	41 •	49 •	57 •	65 •
2 • Control RNA	10 •	18 •	26 •	34 •	42 •	50 •	58 •	66 •
3 •	11 •	19 •	27 •	35 •	43 •	51 •	59 •	67 •
4 •	12 •	20 •	28 •	36 •	44 •	52 •	60 •	68 •
5 •	13 •	21 •	29 •	37 •	45 •	53 •	61 •	69 •
6 •	14 •	22 •	30 •	38 •	46 •	54 •	62 •	70 •
7 •	15 •	23 •	31 •	39 •	47 •	55 •	63 •	71 •
8 •	16 •	24 •	32 •	40 •	48 •	56 •	64 •	72 •

INSTRUMENT n.				USER MANUALVERSION	
CODE		LOT		EXPIRY DATE	
NOTES					
OPERATOR				SIGN	

OPERATOR NOTES

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