ELITechGroup S.p.A.
C.so Svizzera, 185
10149 Torino ITALY
Offices: Tel. +39-011 976 19 Fax +39-011 936 76 11
E. mail: emd.support@elitechgroup.com
WEB site: www.elitechgroup.com

t(15;17) oligomix Alert kit
reagent for cDNA "nested" amplification

REF **BANG12-02** CE -20°C

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INTENDED USE

The kit is intended for use in the **detection of the cDNA of the PML-RARA translocation, t(15;17) variants bcr1, bcr2 and bcr3** in the product of the reverse transcription reaction obtained from total RNA extracted from samples of peripheral blood collected in EDTA, medullary blood collected in EDTA and leukocyte suspensions.

An amplification assay may be used to detect the cDNA of the PML-RARA translocation for diagnostic testing or for monitoring over a period of time the presence of this rearrangement, which is found in almost all cases of acute promyelocytic leukaemia (APL).

KIT DESCRIPTION

The kit provides the **bcr1 OligoMIX** reaction mixes for the first and second amplifications of PML-RARA variant bcr1 and variant bcr2, the **bcr3 OligoMIX** reaction mixes for the first and second amplifications of PML-RARA variant bcr3 and the **RARA OligoMIX** reaction mixes for the control amplification of RARA, already **aliquoted into ready-to-use *«monotest»* test tubes**.

The procedure involves three groups of amplification reactions:
- Two consecutive amplification reactions (nested) specific for the PML-RARA translocation variant bcr1 and variant bcr2;
- Two consecutive amplification reactions (nested) specific for the PML-RARA translocation variant bcr3;
- A single-round amplification reaction for the transcript of the RARA gene, used as a sample suitability test.

The amplification reactions are carried out from the product of the reverse transcription obtained from the RNA extracted from the test samples. The presence of the specific products of the second amplification reaction means that the cDNA of the PML-RARA translocation is present in the product of the reverse transcription reaction. The presence of the specific product of the control amplification means that the cDNA of the RARA gene is present in the product of the reverse transcription reaction and hence that the test sample is suitable.

KIT CHARACTERISTICS

The **sensitivity of the reaction** enables detection of approx. **10 target DNA molecules** (**approx. ten cDNA of the PML-RARA translocation**) in 5 µL of the product of the reverse transcription added to the «monotest» test tube.

N.B.: If $10x10^6$ leukocytes are extracted with the **«EXTRAzol»** kit (see paragraph below on accessory kits) and if the reverse transcription reaction is carried out with the **«RT - Kit plus»** (see paragraph below on accessory kits) the sensitivity of the assay is c. 250 cDNA / $1x10^6$ leukocytes.

The kit enables **25 reactions**, including positive and negative controls.

OTHER PRODUCTS REQUIRED

The reagents for RNA extraction from the test samples, for the reverse transcription of RNA, for the positive amplification controls and detection of amplified DNA are **not** included in this kit. To perform these analytical steps the following products, manufactured by ELITechGroup S.p.A., are recommended:

«EXTRAzol» (code EXTR01), RNA extraction kit from cellular samples;

«RT - Kit plus» (code BRK200), kit for reverse transcription of RNA with “random primer”;

«t(15;17) - Positive Control» (code CTRG12), positive amplification control of plasmid DNA;

«DNA polymerase 2U / µL» (codes ER40 ed ER140), thermostable DNA polymerase enzyme for amplification of nucleic acids; the kits provide 125 reactions;

«ELECTROPHORESIS 3» (code EPH03), detection of amplified DNA for electrophoresis on agarose gel.

MATERIALS PROVIDED IN THE KIT

Component	Description	Quantity	Composition	Labelling
First amplification bcr1 OligoMIX	amplification mixture in **0.2 mL BLUE test tubes**	**25 x 90 µL**	External oligonucleotides, potassium chloride, TRIS base, TRIS hydrochloride, Triton X-100, magnesium chloride, Deoxyribonucleotide triphosphates, Vaseline	-
First amplification bcr3 OligoMIX	amplification mixture in **0.2 mL NEUTRAL test tubes**	**25 x 90 µL**	External oligonucleotides, potassium chloride, TRIS base, TRIS hydrochloride, Triton X-100, magnesium chloride, Deoxyribonucleotide triphosphates, Vaseline	-
RARA OligoMIX for CONTROL amplification	amplification mixture in **0.2 mL YELLOW test tubes**	**25 x 90 µL**	Internal oligonucleotides, potassium chloride, TRIS base, TRIS hydrochloride, Triton X-100, magnesium chloride, Deoxyribonucleotide triphosphates, Vaseline	-
Second amplification bcr1 OligoMIX	amplification mixture in **0.2 mL RED test tubes**	**25 x 94 µL**	External oligonucleotides, potassium chloride, TRIS base, TRIS hydrochloride, Triton X-100, magnesium chloride, Deoxyribonucleotide triphosphates, Vaseline	-
Second amplification bcr3 OligoMIX	amplification mixture in **0.2 mL GREEN test tubes**	**25 x 94 µL**	External oligonucleotides, potassium chloride, TRIS base, TRIS hydrochloride, Triton X-100, magnesium chloride, Deoxyribonucleotide triphosphates, Vaseline	-

MATERIALS REQUIRED BUT NOT PROVIDED IN THE KIT

- Laminar airflow hood.
- Disposable latex gloves or similar material.
- Bench microcentrifuge (12,000 - 14,000 RPM).
- Sterile micropipettes and tips with aerosol filter or positive displacement (0.5-10 µL, 2-20 µL, 5-50 µL).
- Sterile bidistilled water.
- Programmable heater (thermal cycler).

WARNINGS AND PRECAUTIONS

This kit is exclusively for *in vitro* use.

Warnings and general precautions

Handle and dispose of all biological samples as if they were capable of transmitting infective agents. Avoid direct contact with the biological samples. Avoid splashing or spraying. The materials that come into contact with biological samples must be treated with 3% sodium hypochlorite for at least 30 minutes or autoclaved at 121℃ for one hour before disposal.

Handle and dispose of all reagents and all assay materials as if they were capable of transmitting infective agents. Avoid direct contact with the reagents. Avoid splashing or spraying. Waste must be treated and disposed of in compliance with the appropriate safety standards. Disposable combustible materials must be incinerated. Liquid waste containing acids or bases must be neutralised before disposal.

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Wear suitable protective clothing and gloves and protect eyes and face.

Never pipette solutions by mouth.

Do not eat, drink, smoke or apply cosmetic products in the work areas.

Wash hands carefully after handling samples and reagents.

Dispose of leftover reagents and waste in compliance with regulations in force.

Read all the instructions provided with the kit before running the assay.

Follow the instructions provided with the kit while running the assay.

Do not use the kit after the expiry date.

Only use the reagents provided in the kit and those recommended by the manufacturer.

Do not mix reagents from different batches.

Do not use reagents from other manufacturers' kits.

Warnings and precautions for molecular biology

Molecular biology procedures, such as extraction, reverse transcription, amplification and detection of nucleic acids, require qualified staff to prevent the risk of erroneous results, especially due to degradation of the nucleic acids contained in the samples or due to sample contamination by amplification products.

It is necessary to have separate areas for the extraction / preparation of amplification reactions and for the amplification / detection of amplification products. Never introduce an amplification product in the area designed for extraction / preparation of amplification reactions.

It is necessary to have lab coats, gloves and tools which are exclusively employed in the extraction / preparation of amplification reactions and for the amplification / detection of amplification products. Never transfer lab coats, gloves or tools from the area designed for the amplification / detection of amplification products to the area designed for the extraction / preparation of the amplification reactions.

The samples must be exclusively employed for this type of analysis. Samples must be handled under a laminar flow hood. Test tubes containing different samples must never be opened at the same time. Pipettes used to handle samples must be exclusively employed for this specific purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.

Reagents must be handled under a laminar flow hood. The reagents required for amplification must be prepared in such a way that they can be used in a single session. The pipettes employed to handle the reagents must be used exclusively for this purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.

Amplification products must be handled in such a way as to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination. Pipettes used to handle amplification products must be employed exclusively for this specific purpose.

Warnings and precautions specific to components

The test tubes containing **OligoMIX** for the **first, second** and **control amplifications** are disposable and therefore must be used once only in amplification reactions.

OligoMIX for the **first, second** and **control amplifications** carries the following safety warnings (S):

S 23-25. Do not breathe gas/fumes/vapour/spray. Avoid contact with eyes.

SAMPLES AND CONTROLS

Samples

The material used with this kit must consist of the product of the reverse transcription reaction (**cDNA**) obtained from the RNA extracted from biological samples.

The reverse transcription system of the RNA must use random primers to trigger the polymerization reaction.

When using cellular biological samples, such as whole blood, it is recommended to check the total quantity of RNA extract that is released into the reverse transcription reaction, in order to prevent the appearance of non-specific products and the possibility of inhibition in the next amplification reaction.

The maximum final concentration of total RNA in the reverse transcription reaction should be c.40 ng / µL. For example, the maximum quantity of total RNA that can be released into the reverse transcription reaction with a total final volume of 25 µL is c.1 µg.

The system for RNA extraction from the starting sample must provide RNA that is suitable for reverse transcription and amplification reactions.

It is advisable to set up several aliquots of the samples that are to be stored frozen in order to prevent repeated cycles of freezing and thawing.

Amplification controls

It is absolutely mandatory to validate each amplification session with a positive control reaction and a negative control reaction.

For the negative control, use sterile bidistilled water (not supplied with kit) added to the reaction in place of the cDNA extracted from the sample.

For the positive control, use the cDNA extracted from a sample that has already tested positive, or **«t(15;17) - Positive control».**

Quality controls

It is recommended to validate the whole analysis procedure of each extraction and amplification session by processing an already been tested negative and positive sample or a calibrated reference material.

PROCEDURE

Setting the thermal cycle

Before starting the session it is important to do the following:

- Make sure that the thermal block of the programmable heater (thermal cycler, T.C.) is compatible with the format of the ***«monotest»*** **test tubes** supplied with the kit (0.2 mL test tubes);
- Check the instrument documentation for how to regulate the temperature of the thermal cycler during the thermal cycle;
- When possible, select **temperature regulation directly on the thermal block** (e.g. thermal cycler Hybaid™ or Eppendorf™): do not regulate temperature via the test tube-probe or with simulation software;
- Where this is not possible and for GeneAmp® PCR System **9600, 2400, 9700** or **2700** thermal - cyclers (Applied Biosystems™) use the preset temperature regulation system;
- Set the parameters of the thermal cycle on the thermal cycler as shown in the following table.

First amplification thermal cycle

Number of cycles	Temperature	Timing
1 cycle	**95° C**	4 min.
35 cycles	**95° C**	1 min.
	65° C	1 min.
	72° C	1 min.
1 cycle	**72° C**	5 min.

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Second amplification thermal cycle

Number of cycles	Temperature	Timing
1 cycle	**95° C**	2 min.
30 cycles	**95° C**	1 min.
	65° C	1 min.
	72° C	1 min.
1 cycle	**72° C**	5 min.

Preparation of the first amplification

Before starting the session it is important to do the following:

- Thaw the test samples and the positive control, centrifuge for 5 seconds to bring the contents to the bottom and keep in ice.
- Thaw a **blue (bcr1)** ***«monotest»*** **test tube** for each sample that is to be analysed, one for the negative control and one for the positive control; centrifuge for 5 seconds to bring the contents to the bottom and mark clearly using a permanent marker pen and keep in ice.
- Thaw a **neutral (bcr3)** ***«monotest»*** **test tube** for each sample that is to be analysed, one for the negative control and one for the positive control; centrifuge for 5 seconds to bring the contents to the bottom and mark clearly using a permanent marker pen and keep in ice.
- Thaw a **yellow (RARA control)** ***«monotest»*** **test tube** for each sample that is to be analysed and one for the negative control; centrifuge for 5 seconds to bring the contents to the bottom and mark clearly using a permanent marker pen and keep in ice.
- Dilute the **«DNA pol. 2U / µL»** enzyme with sterile bidistilled water (not supplied with the kit) as described in the following table. Prepare enough diluted enzyme for all the reactions (including controls) plus one extra. The diluted enzyme **cannot** be stored.

Number of reactions	DNA pol. 2U / µL	Water	Total volume
4	2.0 µL	18.0 µL	20 µL
5	2.5 µL	22.5 µL	25 µL
6	3.0 µL	27.0 µL	30 µL
7	3.5 µL	31.5 µL	35 µL
8	4.0 µL	36.0 µL	40 µL
9	4.5 µL	40.5 µL	45 µL
10	5.0 µL	45.0 µL	50 µL
11	5.5 µL	49.5 µL	55 µL
12	6.0 µL	54.0 µL	60 µL
13	6.5 µL	58.5 µL	65 µL
14	7.0 µL	63.0 µL	70 µL
15	7.5 µL	67.5 µL	75 µL
16	8.0 µL	72.0 µL	80 µL
17	8.5 µL	76.5 µL	85 µL
18	9.0 µL	81.0 µL	90 µL
19	9.5 µL	85.5 µL	95 µL
20	10.0 µL	90.0 µL	100 µL
21	10.5 µL	94.5 µL	105 µL

1. Add **5 µL** of diluted thermostable DNA polymerase (1 U) to each **blue, neutral and yellow *«monotest»* test tube**.
2. Add **5 µL** of cDNA extract to each **blue, neutral** and **yellow *«monotest»* test tube**. Proceed in the same way for the negative and positive controls.
3. Transfer the **blue, neutral and yellow *«monotest»* test tubes** to the thermal cycler and start the thermal cycle of the first amplification.

Preparation of the second amplification

Before starting the session it is important to do the following:
- Thaw the same number of **red (bcr1) «*monotest*» tubes** as blue ones; centrifuge the tubes for 5 seconds to bring the contents to the bottom and mark clearly using a permanent marker pen and keep in ice.
- Thaw the same number of **green (bcr3) «*monotest*» tubes** as neutral ones; centrifuge the tubes for 5 seconds to bring the contents to the bottom and mark clearly using a permanent marker pen and keep in ice.
- Dilute the **«DNA pol. 2U / µL»** enzyme with sterile bidistilled water (not supplied with the kit) to a final concentration of **0.2 U/µL** as described previously.

4. Add **5 µL** of diluted thermostable DNA polymerase (1 U) to each **red and green «*monotest*» test tube**.
5. Add **1 µL** of the product of the first amplification to each **red (bcr1) «*monotest*» test tube** from the corresponding **blue (bcr1) *«monotest»* test tube**.
6. Add **1 µL** of the product of the first amplification to each **green (bcr3) «*monotest*» test tube** from the corresponding **neutral (bcr3) «monotest» test tube**.

N.B.: The product of the first amplification reaction can contaminate subsequent assays. Isolate the residual product of the first amplification reaction and change gloves at the end of this phase of the procedure.

7. Transfer the **red and green *«monotest»* test tubes** to the thermal cycler and start the thermal cycle of the second amplification.

N.B.: The reaction product may be stored at -20°C for a maximum of one month.

Detection of the specific amplification product

The specific product of the second amplification can be detected and identified by electrophoretic separation as follows:
- Using a 2% agarose gel with 1 µg / mL ethidium bromide in 1x TBE buffer (89 mM TRIS, 89 mM boric acid, 2 mM EDTA disodium);
- Using a 4% agarose gel with 1 µg / mL ethidium bromide in 1x TAE buffer (40 mM TRIS, 20 mM acetic acid, 1 mM EDTA disodium), as in the products in the **«ELECTROPHORESIS»** series.

The **specific products of the second bcr1 amplification** measure:	**214 bp (PML-RARA bcr1)**
	from 50 to 200 bp (PML-RARA bcr2)
The **specific products of the second bcr3 amplification** measure:	**289 bp (PML-RARA bcr3)**
The **specific product of the RARA control amplification** measures**:**	**175 bp (RARA)**

N.B.: The specific amplification product of the cDNA of the PML-RARA translocation variant bcr2 may have different measurements in different positive sample owing to the variability of the breakpoint on hexon 6 of the PML gene.

N.B.: The product of the second amplification reaction can contaminate subsequent assays. Isolate the residual product of the second amplification and product waste in the detection phase.

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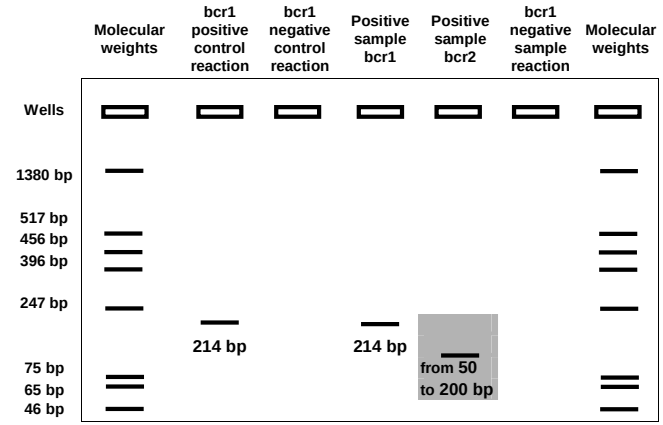
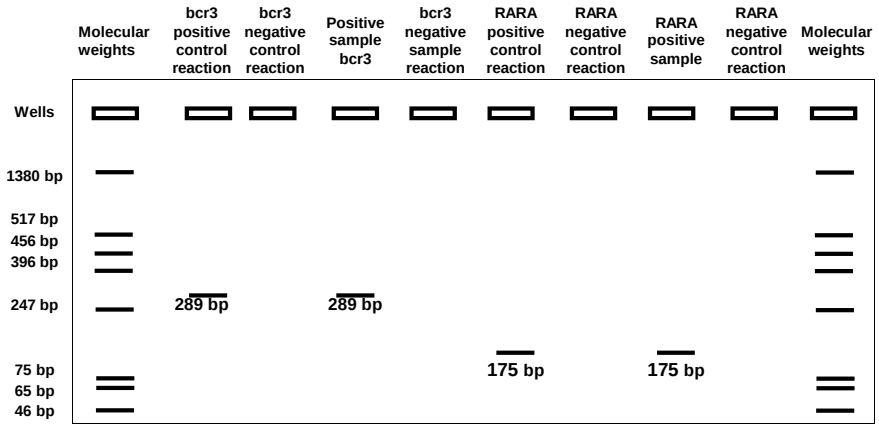
By way of example, below is a diagram representing the results of the electrophoretic separation of an amplification session for the bcr1 and bcr2 variants, in which samples with the different electrophoretic profiles were analysed.



N.B.: The specific amplification product of the cDNA of the PML-RARA translocation variant bcr2 may show different migration to gel in the different positive sample owing to the variability of the breakpoint on hexon 6 of the PML gene.

By way of example, below is a diagram representing the results of the electrophoretic separation of an amplification session for the bcr3 variant and the RARA control in which samples with the different electrophoretic profiles were analysed.



It is absolutely mandatory to validate the identity of the product of the second amplification of a sample by comparing its migration to gel with the migration of a molecular weight marker, and with the migration of the product of the second amplification of the positive control.

The presence of non-specific products of different dimensions from the specific products of the second amplification of a sample has no significance for the detection of the cDNA of the PML-RARA translocation, variants, bcr1, bcr2, bcr3 and the cDNA of the RARA gene.

General validation of the amplification session

The results of the negative and positive control reactions are used to validate the amplification session as shown in the following tables:

Amplification of the negative control	Amplification
Specific product ABSENT	**correct**

Amplification of the positive control	Amplification
PRESENT specific product	**correct**

If the specific amplification product is present in the negative control reaction, this means that problems have occurred during the amplification phase (contamination), which may cause false positives, the assay must be repeated from the amplification phase.

If the specific amplification product is absent from the positive control reaction, this means that problems have occurred during the amplification phase (inefficient or absent amplification), which may cause false negatives; the assay must be repeated from the amplification phase.

Interpreting the results

The results of the reactions in the test samples are used to evaluate the presence of the cDNA of the PML-RARA translocation as described in the following table:

Amplification of the sample	Assay result	cDNA PML-RARA
Specific product ABSENT	**negative**	**ABSENT**
PRESENT specific product	**positive**	**PRESENT**

Before final validation of a test sample as **"PML-RARA cDNA ABSENT"** it is necessary to analyse the result obtained with the control amplification for the cDNA of the RARA gene in the reaction for that sample.

The result obtained for the cDNA of the RARA gene is used to perform the suitability test on the sample (external quality control), as shown in the following table:

Amplification of the sample	Assay result	RARA cDNA	Sample
Specific product ABSENT	**negative**	**PRESENT**	**SUITABLE**
PRESENT specific product	**positive**	**ABSENT**	**NOT SUITABLE**

If the result of the RARA amplification control is **negative**, the cDNA of the RARA gene is **ABSENT** and the sample is **UNSUITABLE;** it means that problems occurred during amplification (inefficient or absent amplification), during the reverse transcription phase (inefficient or absent reverse transcription) or during extraction (RNA absent or inhibitors present). The sample is a **false negative** and the assay must be repeated beginning with extraction of a new sample.

REFERENCES

J.J.M. van Dongen et al. (1999) *Leukemia* 211/99 b 13 (1): 1901 - 1928

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TROUBLESHOOTING

Specific amplification product present in the negative control reaction

Possible causes	Solutions
Error displacing the cDNA	Open one test tube at a time; avoid spilling the contents of the test tube; always change tips.
Error displacing the first amplification product	Open one test tube at a time; avoid spilling the contents of the test tube; always change tips.
Contamination of the reagents prepared for the session	Take great care when diluting and displacing the enzyme; always change tips.
Contamination of the sterile water for enzyme dilution.	Use a new aliquot of sterile water.
Contamination of the enzyme stock.	Use a new aliquot of enzyme.
Contamination of the extraction/preparation area for amplification reactions	Clean surfaces and instruments using aqueous detergents, wash lab coats, replace tubes and tips in use.

Specific amplification product absent in the Positive Control reaction

Possible causes	Solutions
Enzyme too diluted or error displacing enzyme.	Check the dilution calculations; take great care when displacing the enzyme and mix properly.
Error displacing the positive control.	Take great care when displacing the positive control.
Degradation of the positive control.	Use a new aliquot of positive control.
Thermal cycle setting error.	Check the thermal cycle settings on the thermal cycler.
Error displacing the first amplification product	Carefully remove and dispense the product of the first amplification into the second amplification.
Error displacing the second amplification product in the gel.	Take care when loading the product of the second amplification in the gel.

Non-specific amplification products present in the sample reactions

Possible causes	Solutions
Enzyme too concentrated.	Check the calculations of the enzyme dilution.
Preparation times of the first amplification reaction are too long.	Keep the test tubes to which cDNA has already been added in ice until transferred to the thermal cycler.
Thermal cycle setting error.	Check the thermal cycle settings on the thermal cycler.
Excess cDNA in the reaction	Assess the concentration of RNA in the reverse transcription reaction; do not exceed the concentration of 40 ng / µL (1 µg of RNA in a reaction in the final 25 µL)

Specific amplification product absent in the ABL sample control reaction

Possible causes	Solutions
Heparin present in the whole blood sample	The blood sample must use EDTA or citrate as anticoagulants.
Error storing blood sample	Blood must be treated for RNA extraction within a few hours and must not be frozen.
Error during extraction	Check extraction operations. Take care when preparing and performing extraction and follow instructions closely.
Degradation of extracted sample	RNA must be extracted with materials and reagents containing no RNAses. RNA extract must be stored at –20°C or at –80°C.
Error during reverse transcription	Check reverse transcription operations. Take care when preparing and performing reverse transcription; follow instructions closely.
Error during amplification	See suggestions in paragraph “Specific amplification product absent in the positive control reaction”

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SYMBOLS

REF — Catalogue number.


Upper temperature limit.

LOT — Batch code.


Use by (last day of month).

IVD — *In vitro* diagnostic medical device.


In keeping with the requirements of European Directive 98\79\EC for *in vitro* diagnostic medical devices.


Contents sufficient for "N" tests.

CONT — Contents.


Please refer to the instructions for use.


Manufacturer.

The purchase of this product allows the purchaser to use it for amplification of nucleic acid sequences providing human *in vitro* diagnostic services. This right is granted only if this product is used in association with ELITechGroup S.p.A. licensed products for "Positive Control" and for detection.
No general patent or other license of any kind other then this specific right of use from purchase is granted hereby.