Via Sant'Anna 131/135
61030 Cartoceto PU (IT)
Telephone + 39 (0)721830605
FAX +39 (0)721837154
e-mail:info@diatheva.com
www.diatheva.com

Progerin/Lamin A qPCR Detection Kit

MBK0050

FOR RESEARCH USE ONLY

INTENDED USE

The kit allows the comparative and quantitative detection of progerin expression by Real-Time PCR in cDNA samples and by Real-Time PCR, totally discriminating this specific target from the wild-type form, lamin A.
The content of the kit is sufficient for up to 40 determinations, including two calibration curves for lamin A and progerin (quantitative method) and two further amplification mixes for two reference genes necessary for the normalization of the expression data (comparative method).

INTRODUCTION

Hutchinson-Gilford Progeria Syndrome (HGPS) is a rare and fatal genetic disease characterized by the onset of premature aging in childhood. The majority of cases is sporadic and is caused by a recurrent dominant *de novo* point mutation in *lmna* gene coding for lamin A protein. This mutation constitutively activates a cryptic site that causes the expression of a mutated form of lamin A known as **progerin**.
The possibility to accurately quantify the mutated transcript could give to the researchers the ability to assess the effects of experimental substances *in vitro* administered to HGPS cells on progerin expression; moreover, researchers could also compare the amount of transcript produced in different cell lines or different growth stages and correlate the production of the mutant protein to the wild type and/or to the progression of the disease.
Recent studies have demonstrated the presence of low levels of progerin in cells and tissues from healthy subjects, and its complete absence in immortalized cells. It has been recently shown the existence of a synergistic relationship between progerin expression and progressive telomere damage during cellular senescence in healthy fibroblasts.
While it is well known that progerin production increases with cellular senescence, and thus with the number of cell passages in culture, is yet unclear whether it also correlates with the age of the donor and what is the exact role of progerin in the physiological aging.

PRINCIPLE OF THE ASSAY

The Progerin/Lamin A qPCR Detection Kit allows two possible methods of analysis: quantitative and comparative.
Two positive standard controls for lamin A and progerin are included in the kit to perform the calibration curves needed to derive the copies number of the two target transcripts in the sample.
The primers included in the optimized Lamin A Master Mix recognize a specific cDNA region that is present in the human lamin A coding sequence and absent in human progerin coding sequence. From this amplification reaction originates a PCR product of 179bp.
Optimized Progerin Master Mix includes primers that recognize a specific human cDNA region flanking the deletion of 150 nucleotides that characterized the progerin transcript. From this amplification reaction originates a PCR product of 160bp. Primers design have been accurately designed with the aim of maximizing the ability to discriminate progerin against wild-type lamin A.

Sensibility of the assay

LOQ, Limit Of Quantification: down to 100 lamin A and progerin copies
LOD, Limit Of Detection: down to 10 lamin A and progerin copies

To carry out a comparative analysis of obtained data, each kit includes two further amplification mixes able to amplify short sequences belonging to two genes selected as reference genes (or housekeeping genes).
Reference genes selection was carefully performed on the basis of published data and two genes were chosen that do not have cellular functions linked to each other: beta actin and succinate dehydrogenase subunit A.

However, the operator should validate the use of one or both reference genes for the samples that he intends to analyze before perform the experiment.

Reference genes	Abbr.	Function	Size of PCR product	Melting peaks
Beta actin	ActB	Structural protein of cytoskeleton	285bp	81.2 + 86.8°C
Succinate dehydrogenase subunit A	Sdha	Transfers the electrons in the TCA cycle and respiratory chain	216bp	88.8°C

KIT CONTENTS

Component no.	Reagent	Concentration	Volume
M1	Lamin A Master mix	1X	2 X 1000µl
M2	Progerin Master mix	1X	2 X 1000µl
M3	Beta Actin Master mix	1X	1 X 1000µl
M4	Succinate DH Master mix	1X	1 X 1000µl
C1	Hot Rescue Plus DNA Polymerase	5U/µl	1 X 20µl
C2	Dilution buffer	1X	4 X 2000µl
C3	ROX Solution	-	1 X 100µl
S1	Lamin A Standard	10(8) copies/µl	1X 50µl
S2	Progerin Standard	10(8) copies/µl	1X 50µl

OTHER SUPPLIES REQUIRED

- Disposable latex gloves.
- Precision pipettes.
- Sterile pipette tips.
- Sterile 1.5ml vials and sterile 0.2ml Real-Time PCR vials.
- Vortex.
- Tabletop centrifuge.

STORAGE

All kit components must be kept at a constant temperature of -20°C.
The repeated freezing and thawing of components M1, M2, M3 and M4 and of the standards S1 and S2 and the light exposure of the components M1, M2, M3 and M4 can reduce the sensibility of the test. **It is strongly recommended to aliquot these components and to store them in the dark.**

GENERAL PRECAUTIONS

The operator should always pay attention to:

- MAINTAIN STRICTLY SEPARATE WORKING AREAS FOR DNA EXTRACTION AND PCR SET-UP;
- use pipette tips with filter;
- store positive material (specimens, controls and amplicons) separately from all other reagents and, if possible, add it to the reaction mix in a facility separated space;
- thaw all components and samples at room temperature before starting an assay;
- work on ice;
- Do not use any reagent after the expiration date indicated on the label;
- Wear gloves during all procedures.

PROTOCOL

1. cDNA preparation

The quality of RNA and cDNA is fundamental for the success of the test. The presence of inhibitors in the cDNA can significantly reduce the limit of detection of the targets in the samples. The use of reliable and validated methods of reverse transcription is strongly recommended. However, the kit offers the possibility to execute an inhibition control reaction for each extracted sample, which highlights the presence of any inhibiting substances that would distort the results.

2. Execution of a quantitative analysis

It is necessary to include a calibration curve for lamin A and one for progerin. Both curves must comprise at least three dilution points of the respective supplied standard, but it's strongly recommended to use five points (from S-a, containing 10(6) copies per reaction, to S-e, containing 10(2) copies per reaction, see table 1 below), for a better accuracy and reliability of the results. Finally, it is suggested to perform at least two replicates for each sample (NTC, standards, cDNAs). During the preparation of the reaction mixes is appropriate to keep the two standards on ice.

A. Reagents preparation – Before use, wait until all reagents are completely thawed. Then mix thoroughly and spin down all reagents, with the exception of Hot Rescue Plus DNA Polymerase.

B. Standards preparation – Use the following scheme for making the dilutions of both standards included in the kit:

Starting concentration (Copies/μl)	Volume to be taken (μl)	Volume of dilution buffer (μl)	Final volume (μl)	STANDARD CONCENTRATION (Copies/μl)	STANDARD ABBR.
Stock: 10(8)	10	90	100	10(7)	-
10(7)	10	90	100	10(6)	S-a
10(6)	10	90	100	10(5)	S-b
10(5)	10	90	100	10(4)	S-c
10(4)	10	90	100	10(3)	S-d
10(3)	10	90	100	10(2)	S-e
10(2)	10	90	100	10	-

NOTE: It is essential to mix well the intermediate dilutions for **at least 30 seconds** and then spin down before carrying out the subsequent dilution.
Avoid storing the already diluted standards after use. To ensure maximum reliability and accuracy of results, it is necessary to make **fresh dilutions** from the provided stocks before each new run.

The two calibration curves, in addition to allowing the absolute quantification of the two target transcripts, also serve as positive amplification control, to verify that each reagent properly works.

C. Preparation of two amplification mixes – Before the first use of the provided Master Mixes, only for instruments that require a passive reference dye (see below), it is necessary to add the ROX solution:

- **Low ROX**: ABI Prism® 7500, 7500 Fast.
- **High ROX:** ABI Prism® 7000,7300, 7700, 7900, 7900HT, StepOne, StepOne Plus.

Proceed by completing each 1ml Master Mixes with the addition of ROX solution according to the following scheme:

	1ml Master Mix (1 Vial)
Low ROX	1μl
High ROX	12μl

The final volume of each reaction is 25μl. Count the number of samples that must be prepared for each mix, including at least one NTC (no template control) for each used amplification mix. Calculate the volumes of the necessary components using the amounts listed below valid for 1 sample (consider one or two additional reactions to cover pipetting losses):

	N°1 reaction
Progerin/Lamin A 1X Master Mix	23.875
Hot-Rescue Plus DNA Polymerase	0.125
Template*	1
TOTAL	**25**

*The template consists of:
- Water or supplied dilution buffer, for negative control NTC;
- Fresh serial dilutions of standards;
- cDNA (eventually pre-diluted).

After transferring the appropriate volumes of 1X Lamin A Master Mix and Hot Rescue Plus DNA Polymerase in a sterile 1,5ml tube, mix thoroughly and centrifuge briefly. Repeat with Progerin 1X Master Mix. Aliquot 24μl of the just completed amplification mixes in each PCR tube or well.

D. Samples preparation - Add 1μl of the supplied dilution buffer (or sterile water) in the tubes containing the NTC and close them well, to avoid any cross-contamination during the subsequent phase of DNA addiction.
Add the cDNA that has to be tested or standard dilutions in the remaining tubes.
Proceed as specified in point 4, PCR Run.

3. Execution of a comparative analysis

It is necessary to select one cDNA concentration and to use it as template for the amplification with the two master mixes (lamin A and progerin). For a proper data normalization, the same cDNA concentration has to be simultaneously used as template for at least one of the two supplied master mixes for the amplification of reference genes.
Anyhow, it is recommended to experimentally determine the most appropriate cDNA concentration, which can vary depending on the RNA extraction and reverse transcription methods, as well as on the nature of the analysed sample.
Finally, it is suggested to perform at least two replicates for each sample (NTC e cDNA).

A. Reagents preparation – Before use, wait until all reagents are completely thawed. Then mix thoroughly and spin down all reagents, with the exception of Hot Rescue Plus DNA Polymerase.
B. Three/four master mixes preparation - The final volume of each reaction is 25μl. Count the

number and the identity of the reference genes that have to be considered. Count the number of samples that must be prepared for each mix, including at least one NTC (no template control) for each used amplification mix. Make sure that the appropriated quantity of ROX Solution has been added to each mix as indicated in step C.
Calculate the volumes of the necessary components using the amount listed below and valid for 1 sample (consider one or two additional reactions to cover pipetting losses):

	N°1 reaction
Lamin A/Progerin/ActB/Sdha 1X Master Mix	23.875
Hot-Rescue Plus DNA Polymerase	0.125
Template*	1
TOTAL	**25**

*The template consists of:
- Water or dilution buffer, for negative control NTC;
- Appropriate standard dilution as positive control;
- cDNA (eventually pre-diluted).

After transferring the appropriate volumes of 1X Lamin A Master Mix and Hot Rescue Plus DNA Polymerase in a sterile 1,5ml tube, mix thoroughly and centrifuge briefly. Repeat for supplied progerin, beta actin and succinate dehydrogenase 1X master mixes.

C. Samples preparation - Aliquot 24μl of the appropriate amplification mix in each PCR tube or well. Add 1μl of the supplied dilution buffer (or sterile water) in the tubes containing the NTC and close them well, to avoid any cross-contamination during the subsequent phase of DNA addiction. Add the cDNA that has to be tested in the remaining tubes or wells (or standard dilution for the positive control). Proceed as specified in point 4, PCR Run.

4. PCR Run Progerin/Lamin A qPCR Detection Kit has been optimized with Rotor-gene 6000 thermalcycler (Qiagen) and successfully tested with ABI PRISM 7500 thermalcycler (Applied Biosystems).

Program the PCR thermal cycler with the following parameters and start the run:

	Temperature	Time	Cycle no.
Initial denaturation	95°C	5 minutes	1
Denaturation	95°C	15 seconds	40
Annealing	62°C	30 seconds	
Extension with fluorescence acquisition	72°C	30 seconds	
Dissociation curve (from 62 to 99°C, default parameters)			

ANALYSIS OF RESULTS

The analysis of results must be made according to the analysis software provided by the instrument's manufacturer. If the data analysis automatically carried out by the software would not be satisfactory, threshold values could be manually set for the calculation of threshold cycles (C_T). As a general guideline, it is suggested to evaluate the C_T together with the quality of each curve: curves derived from a good amplification have low initial fluorescence and a regular sigmoidal trend. Replicates of each point should show trends, curves and C_T values very similar; also, in the logarithmic representation should be clearly recognizable a phase of linear growth (good reaction efficiency index) followed by a plateau (flattening, due to the progressive loss of amplification efficiency).
Finally, any kind of amplification should never be observed within the 40 cycles in all negative controls (NTC). Sometimes primer dimers may form causing a higher background and leading to a generation of CT<40 for NTC samples. The presence of primer dimers can be easily identified in the dissociation curve by the additional peak(s). The primer dimer peaks are usually seen at lower melting temperatures.

1. Quantitative analysis of results

- S-a standard for lamin A containing 10(6) copies/reaction must show a C_T value of 18 ± 2 cycles, when threshold is less than 0,05;
- S-a standard for progerin containing 10(6) copies/reaction must show a C_T value of 18 ± 2 cycles, when threshold is less than 0,05;
- Starting from the C_T values for the standards and the corresponding known target quantities, it is possible to obtain by interpolation two calibration curves which have to show R^2 values close to 1 and efficiencies between 90 and 100%.
- The estimated quantities of progerin and lamin A transcripts in the sample is carried out by extrapolating from the equation of the respective calibration curve the number of detected copies of the target. The obtained data must be eventually corrected for any dilution factor of analyzed cDNA.
- It is advisable that the C_T values for the cDNA samples fall in the range of linearity of the calibration curve for the relative standard. If the C_T value for a sample is lower than the one corresponding to the maximum dilution of the standard or greater than the one corresponding to the minimum dilution of the standard, it is recommended to repeat the test after the modification of the tested cDNA concentration.

NOTE: If the sample amplification, which acts as a positive internal control, proves to be critical or null, PCR inhibitors could be present in the tested cDNA. It is recommended to repeat the analysis after a further dilution of the used cDNA or to repeat RNA extraction and reverse transcription.

2. Comparative analysis of results

• Optimal C_T values for lamin A, progerin, beta actin and succinate dehydrogenase subunit A on a cDNA sample ranged between 10 and 30. Data are reliable if the amplifications of selected reference genes do not indicate the presence of inhibitors.

• In the absence of PCR inhibitors, is then possible to estimate the relative amount of progerin transcript in a tested cDNA sample, considering the difference between progerin and lamin A C_T values, both normalized to the selected reference gene C_T value, according to $\Delta\Delta C_T$ method. It's well known that the better the cDNA sample quality is – in terms of performance, integrity and amplificability – the more accurate will be the quantification.

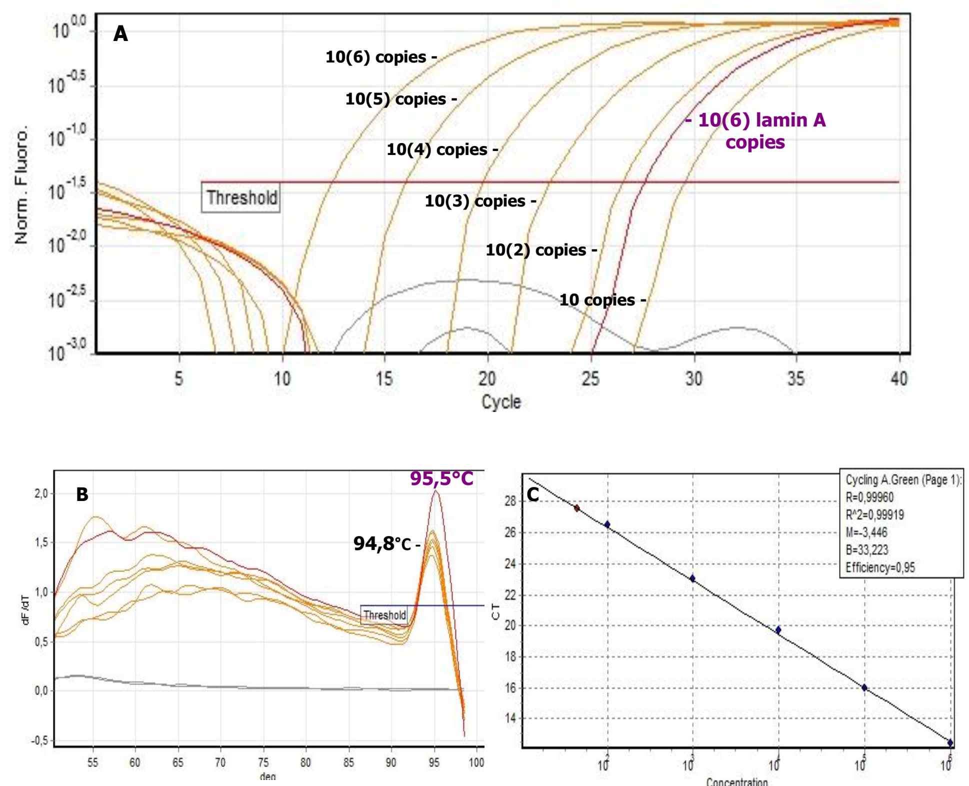
• If there is no amplification in all replicates for a cDNA sample and the progerin standard amplification used as positive amplification control has the correct C_T value, it can be deduced that the expression of progerin is absent or undetectable (<10 copies).

NOTE: If the reference genes amplification that also act as positive amplification controls proves to be null or critical, PCR inhibitors could be present in the tested cDNA. It is recommended to repeat the analysis after a further dilution of the used cDNA or to repeat RNA extraction and reverse transcription.

TROUBLESHOOTING

Observation	Possible cause	Suggested solutions
No amplification	Missing components (e.g. template or DNA Polymerase)	Check the assembly of the reaction
	Missing or incorrect essential step in the cycler protocol	Check the cycler protocol
	Poor quality template	cDNA preparation should be carried out according to manufacturer suggestions and with validated methods of reverse transcription.
	Insufficient starting template	Increase starting template and repeat the assay.
	Degraded reagents	Store reagents at -20°C in the dark. Avoid multiple freeze-thaw cycles.
	Pipetting mistake	Check pipetting and repeat the assay.
Poor amplification, not optimal Ct values for standards, low efficency and/or R^2 value	Poor quality template	cDNA preparation should be carried out according to manufacturer suggestions and with validated methods of reverse transcription.
	Standard dilution mistake	Check the used dilution volumes and repeat the assay starting from new and freshly made standard dilutions.
	Pipetting mistake	Check pipetting and repeat the assay.
	Degraded reagents	Check the storage conditions of the reagents. Repeat the assay. Store reagents at -20°C in the dark. Avoid multiple freeze-thaw cycles.

IMAGES



Standard curve for progerin fragment based on amplification of 10-fold serial dilutions of the included standard. A, *quantitation analysis* using six serial dilution of progerin standard and using 10(6) copies of lamin A (violet curve); **B**, *melting curve:* presence of a unique peak at 94,8°C for progerin standard samples and of a different peak at 95,5°C due to low lamin A amplification. **C,** The Ct is plotted against the log of the initial quantity of template for each dilution. This graph allows to calcolate the efficiency and the R^2 value for progerin standard curve: 95% and 0.999, respectively.

REFERENCES

Available on library section: **http://www.diatheva.com/library.htm**