Glyco-SPOT
DNA Repair Assay kit

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Glyco-SPOT
DNA Repair Assay Kit
LX05001 - 24 wells

For laboratory research use only.
Not for human diagnostic use.

Produced & distributed by

Technology powered by (*)

Fabriqué en France
Made in France

#LX11001
Version: 0117

Table of contents

24 wells
Storage: +4°C
Expiry date: stated on the package

This kit contains:

Designation	Colour of cap	Item #	Quantity per kit	Form
A coated 24 wells glass slide, pre-functionalized with support oligonucleotides	-	LX08001.1 ea	1	-
Mix of duplex-oligonucleotides	Red	LX04001.1 ea	1	Lyophilised
Hybridisation buffer x5 concentrated	Grey	LX07000.24 wells	1	Lyophilised
Excision buffer x5 concentrated	Blue	LX16001.24 well	1	Lyophilised
Wash buffer x5 concentrated	White	LX17001.24 wells	1	Lyophilised
Polysorbate 20 (Tween 20®) 10% diluted	Yellow	LX12001.1 ea	1	Liquid
Instruction booklet	-	LX11001.1 ea	1	-

Each kit contains sufficient reagents for 24 wells.

We strongly recommend to reserve two wells as Control wells with buffer only (22 samples + 2 controls).

Precaution for use

Users are recommended to carefully read all instructions for use before starting work.

Safety precautions

- For research laboratory use only.
- Not for human diagnostic use.
- Do not pipet liquids by mouth.
- Do not use kit components beyond the expiration date.
- Do not eat, drink or smoke in areas where kit reagents are handled.
- Avoid splashing.

The excision buffer contains Dithiotreitol (DTT) and Zinc chloride. Handle these reagents with care, do not throw away in sink.

Principle of the assay

Base Excision Repair (BER) is the predominant repair pathway responsible for removal of small lesions from DNA, like oxidized, alkylated, deaminated bases and abasic sites.

In humans, the mechanism of BER involves the initial action of DNA glycosylases followed by the processing of the resulting abasic site either by the AP-lyase activity of the bifunctional glycosylase or by the apurinic/apyrimidic endonuclease APE1 that incises the DNA strand. BER mechanisms can be induced by oxidative stress and various genotoxic attacks.

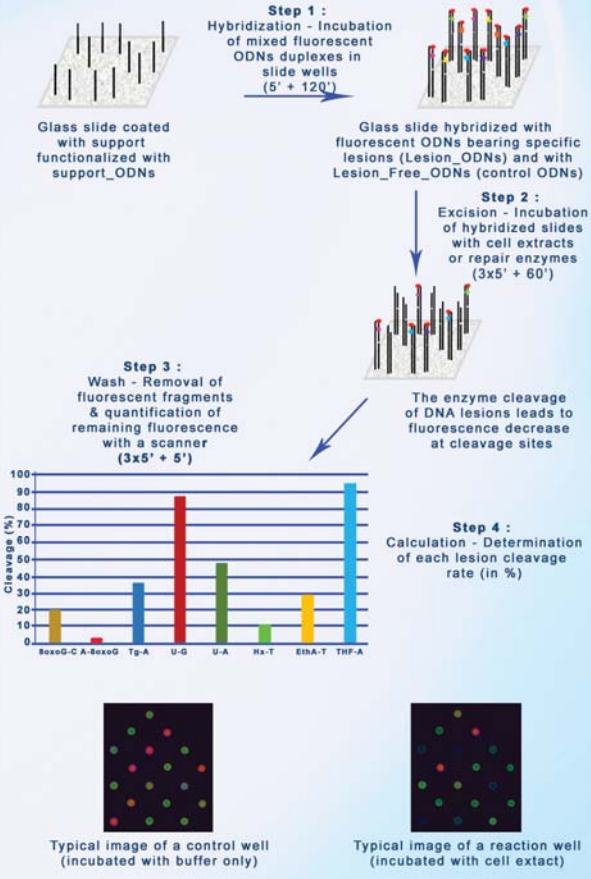
The Glyco-SPOT DNA Repair Assay is a multiplexed oligonucleotide (ODN) cleavage assay developed on support. It is used to monitor simultaneously the cleavage efficiency of several glycosylases/AP endonucleases against a set of emblematic DNA lesions in cell/bacteria/tissue extracts, using fluorescent detection.

The assay can be used to screen for DNA repair inhibitors, characterize DNA repair enzymatic signature from samples, check DNA glycosylase specificity, characterize DNA repair inhibitory properties of chemicals (heavy metals …).

The principle of this Oligonucleotide Cleavage Assay (DNA Repair Assay) is summarised below: Oligonucleotides (ODNs) containing specific DNA lesions (Lesion_ODNs) are labelled at their 3'end by a Cy3 dye. They are hybridised onto a support at specific locations in a slide-array format (step 1). Each well also contains Lesion_Free_ODNs (without lesion).

Enzymes or extracts to be characterized are incubated into the wells (step 2). Cleavage of the altered bases by enzymes contained in the extracts leads to the elimination of the fluorescent label after a washing step (step 3).

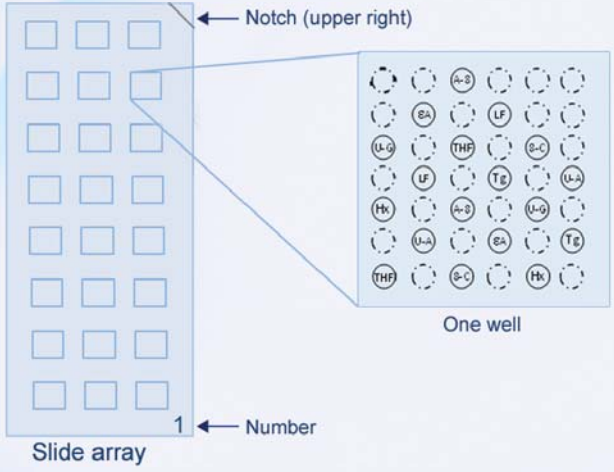
Quantification of the remaining fluorescence at 532 nm using a scanner allows to calculate the percentage of excision of each lesion, after normalization with Lesion_Free_ODN fluorescence and control well fluorescence (well containing buffer without enzymes) (step 4).



The specific DNA lesions present in the duplexed oligonucleotides are as follow (lesion/paired base):

- 8oxoGuanine – Cytosine: 8oxoG-C (8-C)
- Adenine - 8oxoGuanine: A-8oxoG (A-8)
- Thymine glycol – Adenine: Tg-A (Tg)
- Uracil – Guanine: U-G
- Uracil – Adenine: U-A
- Hypoxanthine – Thymine: Hx-T (*Hx*)
- EthenoAdenine - Thymine: EtheA-T (εA)
- THF (AP site) - Adenine: THF-A (THF)
- Lesion_Free duplex: LF

The lesion ODNs and the lesion free ODNs are hybridised in duplicate on the slide array following the pattern below:



The typical enzymes (BER pathway) involved in the repair of these lesions present on the biochip are listed in the table below:

Substrate-paired base	Human	E. coli
8oxoG-C	hOGG1	Fpg
A-8oxoG	hMYH	MutY
Tg-A	NTH1, hNEIL1	Endo III, Nei
U-G	UDG	UNG
U-A	UDG, SMUG1	UNG
Hx-T	MPG (AAG)	AlkA
EthenoA-T	MPG (AAG)	AlkA
AP site (THF)-A	APE1	Endo IV, Endo VIII

Materials and equipment required

In addition to standard laboratory equipment, the following material is required:

For sample preparation

The assay can be conducted with whole cell extracts, nuclear extracts or purified enzymes.
The preparation of cell extracts requires laboratory equipment for cellular biology (i.e. benchtop centrifuges) and equipment for protein quantification.

- Protein quantification kit (commercial kits)
- Spectrophotometer UV-visible
- Distilled or deionised water

For the assay

- Biochip/slide array scanner for fluorescence (with Cy3 filters) (i.e. Innoscan 710/900, Innopsys)
- Hybridisation cassette + silicon gasket 1x24 wells or 4x24 wells (i.e. AHC1X24 or AHC4X24, Arrayit®) available from Bertin Pharma (ref. #19001.1 ea)
- Thermo-microplate shaker with lid (to keep the slides in the dark) (i.e. PHMP/PHMP-4 Grant Instruments)
- Benchtop centrifuge for microtubes (1.5 and 2 mL tubes)
- Precision micropipettes (2 to 1000 µL) and multichannel pipet (optional)
- Microtubes
- Distilled or deionised water

- Tubes 15 and 50 mL
- Adhesive plastic sheets (to cover the microarray during incubation (optional))

Sample preparation

Please read carefully before you start

Preamble

Extract quality is a crucial point for the success of the DNA repair reactions conducted with the Glyco-SPOT DNA Repair Assay.
Please ensure to follow the recommendations below:

- We recommend to work with nuclear extract vs whole cell extracts, although repair proteins are present in cytoplasm and nucleus, to get a more specific reaction (no dilution of the proteins of interest). Indeed, proteins present in the nucleus reflect more accurately the repair systems at a given moment.

- We recommend to use, among the numerous described methods, a fast protocol for nuclear or whole cell extract preparation that yields better results than long and extensive protocols. Examples of protocols are presented in the section "standard protocols for extract preparation".

- We recommend to quantify protein content in the extracts and use all samples at the same concentration for the DNA repair experiments as to allow the comparison between samples and avoid bias.

Extract preparation from Cell culture

When the extracts are prepared from cells treated with potentially toxic compounds (like chemicals, drugs, ionizing radiations, ultra-violet radiations, etc), one must check that cell viability remains high (i.e. >80%).

Apoptotic/dying cells contain nucleases that would interfere with the excision reaction and lead to non-specific degradation of the ODNs present on the biochip.

We recommend to conduct cell viability assays to verify this point prior preparing the cell pellets for the DNA repair experiments.

Ideally, the cells are collected at 80 - 90% confluence. Cells can be used fresh or frozen (cryopreserved).

If frozen cells are used:
Methods used for cell cryopreservation must ensure high cell viability (i.e. >90%). Several cryopreservation medium (as calf serum + 10% DMSO) can be used. The cell pellet must be stored at -80°C or in liquid nitrogen.

DMSO is toxic for cells. Consequently before extract preparation, the cells must be quickly thawed, diluted in culture medium, and centrifuged to eliminate the supernatant. Then the cell pellet must be rinsed twice with cold PBS.

Dried cell pellets give little repair activities. Results obtained with extracts prepared from dried cell pellets, even stored at -80°C, are not reliable.

If fresh cells are used:
Prepare the cell extracts as quickly as possible after cell collection. Work at +4°C.

For cell collection, we recommend, if required, the mildest possible trypsin treatment followed by a neutralisation with cell culture medium +10% serum.

The cells must be counted.
At least 3.5 million of cells are required for nuclear extract preparation. Our suggested protocols are given for a maximum of 5 million of cells.
One million of cells is enough for whole cell extract preparation. The volume of the lysis buffer must be adjusted according to the number of cells in the pellet.

Extract preparation from tissues

Whole cell extracts can be prepared after homogenisation of fresh tissues. If enough material is available, cells can be dissociated from fresh tissues, after incubation of the tissue piece with collagenase/DNase in appropriate medium. Then the cells might be frozen in cryopreservation medium or processed for extract preparation.

Note: Extracts prepared from stored dried frozen tissues may have inactive DNA repair activities.

DNA repair activities according to cell/tissue type

- Excision activities are detectable in all sorts of cells/tissues. Note that some glycosylase/AP endonuclease activities are dominant over the others and that some activities might need to be induced to be measurable.

- DNA Repair activities obtained from lymphocytes are particularly fragile. Lymphocytes must be isolated and processed quickly and handled at +4°C.

Extract storage conditions

Once ready, quickly freeze the extracts as small aliquots (10 µL) and store them at -80°C or in liquid nitrogen. Preserve 2 µL for the protein concentration determination.

Note: Thawed aliquots should never be frozen again and must be discarded since this will cause loss of repair activities.

Checking list for good results with Glyco-SPOT DNA Repair Assay.

Parameter	Recommendations	Check
Cell confluence	80 % - 90%	
Cell viability	>80%	
Cell trypsinization	Mild	
	Trypsin neutralization	
Cell number	>3.5 10^5 up to 5 10^6 for nuclear extracts	
Cell pellet freezing	Stored in cryopreservation medium	
	(serum + 10 % DMSO)	
	Stored at -80°C or liquid nitrogen	
Cell pellet transportation	Exclusively on dry ice	

Commercial purified repair enzymes can also be assayed, follow the supplier instructions of use.

Reagent preparation

- All solutions must be kept on ice during procedure
- ODNs mix and hybridised slide must be kept in the dark during procedure

The slide array is provided ready to use. The oligonucleotide mix (ODNs mix) is lyophilised. All buffers are lyophilised and 5 times concentrated (hybridisation, excision and wash buffers).

Hybridisation buffer

Reconstitute extemporaneously the vial content (x1 final concentration) by adding 5 mL of distilled water in the vial. Allow it to stand for 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

Oligonucleotide mix

After a short centrifugation (30" at 300 g in a microcentrifuge), reconstitute extemporaneously the content of the microtube in three steps:

- by adding first 280 µL of chilled distilled water. Allow it to stand on ice for 15 minutes. Resuspend by gently flicking the tube.
- Add then 1 mL of chilled hybridisation buffer in the microtube, mix by flicking gently with finger. Once solubilisation is complete (15 minutes) transfer in a chilled 15 mL tube.

> Rinse the microtube with 960 µL of cold hybridisation buffer and add it into the 15 mL tube content. Allow it to stand on ice until use (15 minutes) and homogenise by gentle inversion.

The final volume of oligonucleotide mix is then 2240 µL.

Note 1: Open the microtube with care because of the volatility of the lyophilisate.
Note 2: The suspension step of the ODNs mix is particularly critical in terms of good solubilisation of the powder. **Do not pipette** the ODNs mix suspended in water, rather dissolve it by gently flicking the tube.
Make sure not to warm up the tube during this step and to respect the suspension time.

▷ Excision buffer

Reconstitute extemporaneously the vial content (x1 final concentration) in two steps as follows:

> Add 10 mL of distilled water and allow it to stand on ice until complete dissolution (10' minimum).
> Then transfer in a sterile conical tube (50 mL). Rinse the first vial with 15 mL of water to complete the volume to 25 mL. Transfer the solution to the 50 mL tube and then mix thoroughly by gentle inversion. Allow it to stand on ice until use (10' minimum).

Sometimes insoluble particles can appear after excision buffer dissolution. In this case, filter the solution with a 0.45 µm -syringe filter. This step will not affect the performance of the test.

▷ Wash buffer

Reconstitute extemporaneously the vial content (x1 final concentration) in two steps as follows:

> Add 5 mL of distilled water and allow it to stand on ice until complete dissolution (10' minimum).
> Then transfer the solution in a sterile conical tube (50 mL). Rinse the first vial with 15 mL of water to complete the volume to 20 mL. Transfer the solution to the 50 mL tube and then mix thoroughly by gentle inversion.

▷ Tween 20 10 % dilution

Add 200 µL of this solution to the reconstituted wash buffer (final concentration 0.1%), then mix gently to avoid formation of foam.

Assay procedure

- **Please read the entire procedure, come steps have to be anticipated.**
- **All solutions must be kept on ice during procedure**
- **ODNs mix and hybridised slide must be kept in the dark during procedure**
- **Be careful not to make bubbles when transferring solutions into the wells.**
- **Gasket not properly seated may lead to leaks.**
- **Do not use refrozen cell extract**

ODNs hybridisation step

- Prepare the hybridisation buffer as indicated in the reagent preparation section.
- Open the slide packet and position the slide in the hybridisation cassette. The notch on the glass must be positioned in the upper right. Then position correctly the silicon gasket and close the upper part of the cassette with screws.
- Resuspend the ODNs mix as indicated in the previous section.
- During the suspension of the ODNs mix, rinse each well with 80 µL of hybridisation buffer and incubate for 5 minutes at room temperature under shaking at 700 rpm.
- Then discard the buffer by pipetting.
- Once dissolution of the ODNs mix is completed, distribute 80 µL of the ODNs mix per well.

- Incubate the hybridisation cassette in Thermo-microplate shaker with lid at +37°C under shaking at 700 rpm during 2 hours.
- Then discard the solution by pipetting and keep on ice until use.

Excision step – incubation with samples

Please read carefully before beginning sample incubation
It is strongly advised to run samples in duplicate.
Additionally two wells must be kept for incubation with excision buffer only (control wells containing no extract). The data resulting from the quantification of the control well will be required to calculate the excision rates of the different DNA lesions.

- Equilibrate the wells with reconstituted excision buffer (see previous section) 3 x 5 minutes at room temperature, 190 µL/well.
- During the incubation with the ODNs mix, prepare the cell extracts dilutions at the chosen concentrations with chilled excision buffer. The extracts must be thawed at the last moment. The dilution of the extracts / enzymes solutions must be performed rapidly on ice. Typical range of protein concentration used is 10-50 µg/mL (typically 20 µg/mL; see sample preparation section for evaluation of the protein concentration).
- Rapidly transfer the 80 µL of the extract dilutions in the wells (chilled hybridised slide + holder) and incubate at +37°C under shaking (700 rpm) between 10 minutes to 2 hours (a typical digestion time is 60 minutes).
- Cover with an adhesive plastic film (optional)
- Discard the extracts by pipetting

Washing step

- Wash 3 x 5 minutes with190 µL/well of reconstituted washing buffer + tween 20 (see previous section), keep in the dark.
- Discard the solution by pipetting
- Disassembled the slide holder
- Put the slide in a closed 50 mL tube (or in a microscope slide rotor) and centrifuge the slide 2 minutes at 500 g at room temperature.
- Dry at +30°C during 5 minutes in the dark.

Advice: Gaskets might become porous upon extensive use and retain fluorescence. In case fluorescent smears appear on the biochip, it is advisable to wash the gasket for 2 hours with a mild detergent after each experiment. Then thoroughly rinse with deionised water and incubate for the night in deionised water. Dry before use.

Fluorescence measurement

- Read the spot fluorescence (spot total intensity) with a scanner (laser excitation at 532 nm). The gain should be set up so as not have saturated signals of the ODNs in the control wells.

Note: The hybridised slide arrays can be stored at room temperature in the dark.

Data analysis

Calculate the mean fluorescence and the standard deviation of each Lesion_ODN and of the Lesion_Free_ODN in each experimental condition and for the control wells (with buffer only). Four values are used: 2 duplicate spots per well and 2 replicates per experimental conditions.

Then to calculate the percentage of excision of the lesions: The mean fluorescence of the Lesion_ODNs and of the Lesion_Free_ODN of the control wells are taken as reference and set up to 100.

For each experimental condition, the excision rate of each lesion is calculated as a percentage of fluorescence of the corresponding ODN in the control wells.

In addition, each well contains a Lesion_Free_ODN, which serves to check for any unspecific degradation activity. For calculation of final Lesion_ODN cleavage percentage, a correcting factor is applied that takes into account the possible Lesion_Free_ODN degradation.

Consequently the final Lesion_ODN cleavage percentage is 100 x (1 – percentage of fluorescence of each Lesion_ODN/percentage of fluorescence of Lesion_Free_ODN).

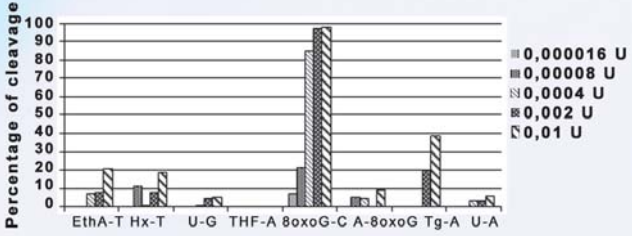
Lesion ODNs cleavage rate < 5% cannot be accurately measured using the Glyco-SPOT DNA Repair Assay kit.
The cleavage rate of the Lesion_Free_ODN should not exceed 15%. Higher digestion rates could reveal high nuclease levels in the extracts.

Typical data

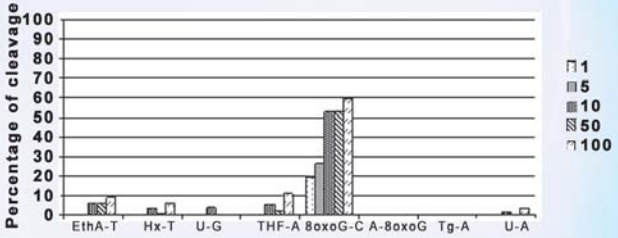
Glycosylases were from New England Biolabs (recombinant enzymes). Recommended buffers were used.
(http://www.neb.com/nebecomm/tech_reference/modifying_enzymes/dna_repair_enzymes.asp)
Incubation for 30 min at +37°C with the indicated amount of enzymes.
Results are expressed as percentage of cleavage of each Lesion_ ODN.
They result from calculations using the fluorescence intensity of 4 spots (2 wells, duplicate spots).
Typical Pearson correlation coefficient between 2 independent experiments is 0.92-0.99.

Bifunctional glycosylases

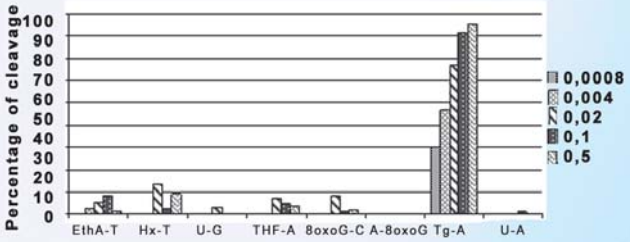
Excision of lesions by Fpg (U/well)



Excision of lesions by hOGG1 (U/well)

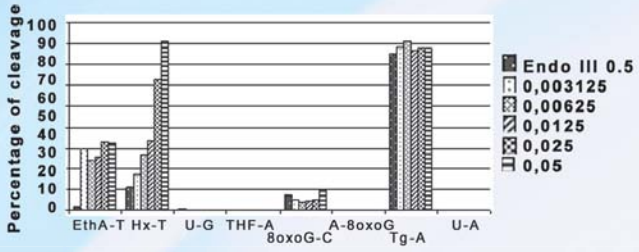


Excision of lesions by Nth (U/well)

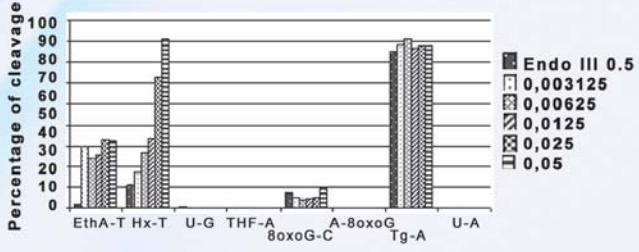


Monofunctional glycosylases used with Endo III to cleave the residual abasic site

Excision of lesions by UNG (U/well**) + Endo III (**0.5 U/well**)**



Excision of lesions by hAAG (U/well) **+ Endo III (**0.5 U/well**)**



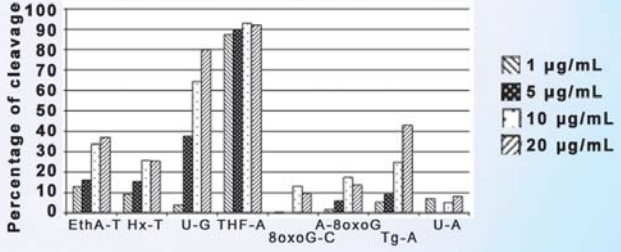
Standard protocols for extract preparation

Suggested protocols for the preparation of nuclear extracts, whole cell extracts and from specific cell type (i.e. lymphocytes, bacteria) are at your disposal on our website (www.bioreagent.bertinpharma.com), follow the link on the logo SPOT LX.

Assay validation

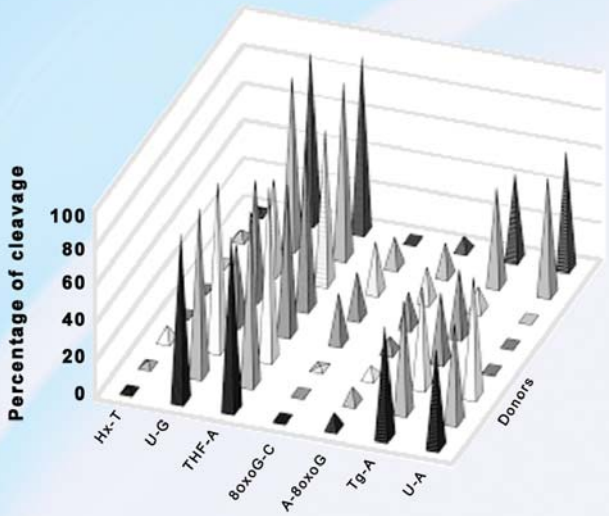
Typical dose-response obtained with PBMCs nuclear extracts after 120 min of repair reaction at +37°C.

PBMCs nuclear extracts



Typical DNA Repair Signature obtained with series of nuclear extracts prepared from human skin fibroblasts. Repair reaction was conducted for 60 minutes at +37°C.

Human Fibroblasts Nuclear Extracts



Assay trouble shooting

> **No fluorescent signal is observed**
The slides were scanned at the wrong wavelength: control scanner set-up at 532 nm (Cy3 dye).
Slides were put upside down in the scanner
The fluorescent duplexes could not get hybridised on the support or they were washed out: check the hybridisation buffer and the washing buffer.

Glyco-SPOT DNA repair assay slide arrays should never been washed with water.

> **Very low fluorescent signal is observed** in the reaction wells compared to the Control wells
Check extract quality: extracts containing high nuclease levels would digest the ODNs present on the Biochip, leading to fluorescent disappearance at all spot locations.

> **High cleavage rate of the Lesion-Free-ODN**
Higher digestion rates reveal high nuclease levels in the extracts. Thus check viability of the cells (must be >20%).
The cleavage rate of the Lesion-Free-ODN should not exceed 15%.

> **Heterogeneous results / high variability in the results**
Check that gasket and screws are properly sealed: there might be leaks on the Biochip device and transfer of liquid from one well to the other

> **No excision of the lesions is observed**

- No repair activity present on the Biochip: wrong excision buffer was used,
- extract was forgotten,
- error was made during preparation and dilution of the extract,
- poor extract quality and/or poor storage conditions.

> **Fluorescent smears on the Biochip**

- This can happen when cells/tissues are incubated in the presence of high concentrations of fluorescent/colored compounds or when experiments are conducted after *in vitro* incubation of the extracts with fluorescent/colored compounds.

These are a few examples of trouble shooting that may occur. If you need further explanation, Bertin Pharma will be happy to assist you. Feel free to contact our technical support staff by phone (+33 (0)139 306 036), fax (+33 (0)139 306 299) or E-mail (bioreagent@bertinpharma.com), and be sure to indicate the batch number of the kit (see outside the box).

(*) This technology named "Method for detecting and characterizing activity of proteins involved in lesion and DNA repair" is patented under #WO200190408 by the CEA-UJF and is licensed to LXRepair.

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Glyco-SPOT booklet

Bertin Pharma, over the last decades, has been developing and marketing over **100 biomarker assays**, pre-analyticial products, kits, antibodies and biochemicals thanks to its **innovative work in research and development**.
Our core areas are orientated to inflammation, oxidative injury, endocrinology, diabetes, obesity, hypertension, neurodegenerative diseases, HIV, prion diseases, pharmacokinetics and metabolism.

Bertin Pharma is active worlwide either with direct sales or through our qualified and trained international distribution network from the United States to Japan.

We are able to provide you with **local technical support** to use at ease our products.

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