For life science research only.
Not for use in diagnostic procedures.

Print Protocol

Print Changes to Previous Version

Cell Proliferation ELISA, BrdU (colorimetric)

Version 16

Content version: January 2013

Colorimetric immunoassay for the quantification of cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis:
A non-radioactive alternative to the [3H]-thymidine incorporation assay

Cat. No. 11 647 229 001 1 kit (1,000 tests)

Store the kit at +2 to +8°C

www.roche-applied-science.com

1. Preface

1.1 Table of contents

Kit contents

Please refer to the following table for the contents of the kit.

Vial	Label	Contents including function
1 red	BrdU labeling reagent	• 1 ml • 1,000× conc. • 10 mM 5-bromo-2'-deoxyuridine in PBS, pH 7.4 • filtered through 0.2 μm pore size membrane
2 red	FixDenat	• 2 × 100 ml • Ready-to-use
3 blue	Anti-BrdU-POD	• Lyophilisate, stabilized • Monoclonal antibody from mouse-mouse hybrid cells (clone BMG 6H8, Fab fragments) conjugated with peroxidase (POD)
4 blue	Antibody dilution solution	• 100 ml • Ready-to-use
5 green	Washing buffer	• 100 ml PBS • 10× conc.
6 black	Substrate solution	• 100 ml TMB (tetramethyl-benzidine) • Ready-to-use

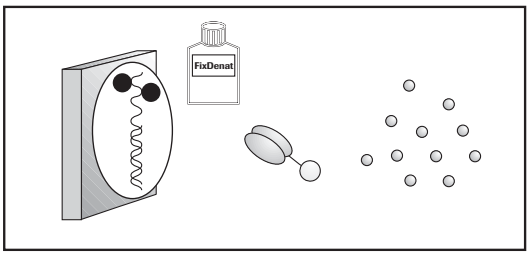
2. Introduction

2.1 Product overview

Test principle

Stage	Description
1	Cells are cultured in the presence of the respective test substances in a 96-well MP at 37°C for a certain period of time (1–5 days, depending on the individual assay system).
2	Subsequently, BrdU is added to the cells and the cells are reincubated (usually 2–24 h). During this labeling period, the pyrimidine analogue BrdU is incorporated in place of thymidine into the DNA of proliferating cells.
3	After removing the culture medium the cells are fixed and the DNA is denatured in one step by adding FixDenat (the denaturation of the DNA is necessary to improve the accessibility of the incorporated BrdU for detection by the antibody).
4	The anti-BrdU-POD binds to the BrdU incorporated in newly synthesized, cellular DNA.
5	The immune complexes are detected by the subsequent substrate reaction.
6	The reaction product is quantified by measuring the absorbance at the respective wavelength using a scanning multiwell spectrophotometer (ELISA reader). The developed color and thereby the absorbance values directly correlate to the amount of DNA synthesis and hereby to the number of proliferating cells in the respective microcultures.

Figure 1 Test principle:



Application

The Cell Proliferation ELISA is designed as a precise, fast and simple colorimetric alternative to quantitate cell proliferation based on the measurement of BrdU incorporation during DNA synthesis in proliferating cells. Thus, the Cell Proliferation ELISA can be used in many different *in vitro* cell systems when cell proliferation has to be determined.

Examples:

- Detection and quantification of cell proliferation induced by growth factors and cytokines
- Determination of the inhibitory or stimulatory effects of various compounds on cell proliferation in environmental and biomedical research and in the food, cosmetic and pharmaceutical industries
- Determination of the immunoreactivity of lymphocytes, stimulated by mitogens or antigens
- Determination of the chemosensitivity of tumor cells to different cytostatic drugs in medical research.

It has been shown that a precise evaluation of cell proliferation could be performed by the measurement of BrdU incorporation in newly synthesized cellular DNA. In addition, there is a good correlation between the Cell Proliferation ELISA using BrdU and the [^{3}H]-thymidine incorporation assay as shown for a variety of murine and human cell systems, including mitogen- and antigen-stimulated lymphocytes and cytokine-induced proliferation of different cell lines (12).

Sample material

- Adherent cells as well as
- suspension cells

cultured in flat-bottomed 96-well MPs (tissue culture grade) with cell concentrations and incubation periods appropriate for the respective assay in an incubator at 37°C, 5% CO_2, 95% humidity.

Assay time

1.5–3 h depending on the anti-BrdU-POD incubation time chosen excluding the cell culture and labeling period.

Number of tests 1000 tests

Kit storage/stability The unopened kit is stable at +2 to +8°C until the expiration date printed on the label.

Advantage Advantages of the Cell Proliferation ELISA, BrdU, (colorimetric)

Benefit	Feature
Safe	No radioactive isotopes are used.
Accurate	• Results obtained strongly correlate to the number of proliferating cells (see fig. 3, 4). • Low mean variation.
Sensitive	• At least as sensitive as [^{3}H]-thymidine incorporation (see fig. 3, 5, 6).
Fast	• Short assay time: immunoassay can be performed in 1.5–3 h. • The use of a multiwell ELISA reader allows a large number of samples to be processed simultaneously.
Convenient	• No disposal and radiation safety paperwork. • Reagents are provided in stable, optimized form. • No transfer of cells: the entire assay is performed in one and the same MP. • 1 washing and 2 incubation steps only. • The entire immunoassay is performed at +15 to +25°C. • Mild fixation and DNA denaturation preserve cellular morphology and thus allow optical control of the cells during the assay.
Function-tested	Every lot is function-tested on proliferating cells, in comparison to a master lot.

Sensitivity

Depending on the individual cell type used and the incubation time applied for the assay, 0.1–1.0 × 10^4 cells/well are sufficient for most experimental setups with cell lines (fig. 3, 4). 1–40 × 10^4 cells/well should be used when working with primary lymphocytes (fig. 5, 6).

Note: The results revealed from the Cell Proliferation ELISA strongly correlate to the data obtained by the [^{3}H]-thymidine incorporation assay. Increasing the labeling time with BrdU or [^{3}H]-thymidine increases the absorbance and the cpm, respectively. In this assay system 24 h labeling with BrdU results in an increased sensitivity compared to the [^{3}H]-thymidine incorporation assay at low cell concentrations. At higher cell concentration the prolonged labeling time results in absorbance values beyond the measuring range of the ELISA reader.

Figure 2

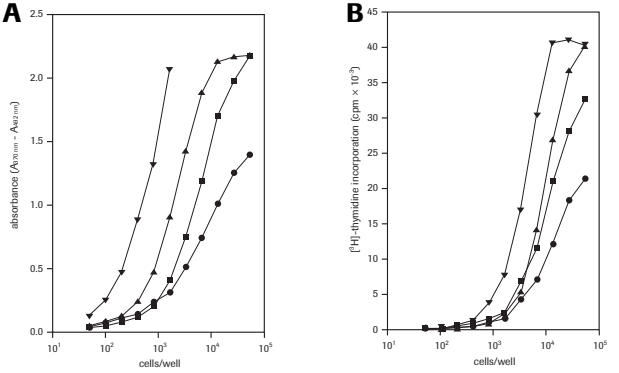


Fig 2: Sensitivity and kinetics of the Cell Proliferation ELISA
L929 cells were titrated in flat-bottomed MTP in 100 µl/well culture medium at the concentrations indicated in the figures. After 24 h of incubation, BrdU (A) or [^{3}H]-thymidine (B) was added and the cells reincubated for additional 2 h (●),
4 h (■), 8 h (▲) and 24 h (▼). BrdU incorporation was determined as described in section 3.2 (Assay procedures). The [^{3}H]-thymidine incorporation assay was performed following a standard protocol.

Measuring range

The immunoassay is designed to fit most of the current proliferation assays. In some cases the absorbance values obtained may be too low or too high. See section 5.1 for adapting the immunoassay to those cell systems.

Specificity

The antibody conjugate reacts with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) and with BrdU incorporated into DNA. For binding to BrdU incorporated into the DNA, the BrdU-labeled DNA has to be denatured. The antibody does not cross-react with any endogenous cellular components such as thymidine, uridine or DNA.

Precision

To determine the intra-assay variance, various cell lines and mitogen-stimulated lymphocytes were titrated in triplicate. For all cell and mitogen concentrations tested, a variance of <10% was established for the absorbance values.

Test interference

With some cell lines, higher cell concentrations (more than 2 × 10^4 cells/well) may lead to increasing absorbance values in the absence of BrdU.

Figure 3

Background values after treatment of cells with mitomycin C and in the absence of BrdU.

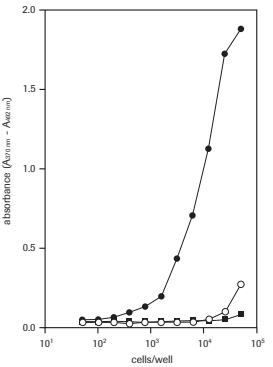


Fig. 3: Background values after treatment of cells with mitomycin C and in the absence of BrdU

A549 cells were incubated at different concentrations in 100 µl/well culture medium with (■) or without (●, ○) mitomycin C (5 µg/ml). After 24 h of incubation, BrdU was added (■, ●) to the cell culture. In the respective background controls BrdU was omitted (○). The cells were reincubated for additional 2 h and the immunoassay was done as described in section 3.2 (Assay procedures).

Determination of cell proliferation

Traditionally, cell proliferation *in vitro* is determined by counting cells directly,
- by the determination of the mitotic index or,
- in the case of hematopoietic cells, by performing a clonogenic assay.

All these assays are labor-intensive and therefore not practical for evaluating large numbers of samples.

Indirect measurement of cell proliferation

Alternatively, as an indirect measure of viable cell number, the overall metabolic activity in a cell population may be determined. Tetrazolium salts like MTT, XTT or WST-1 are metabolized by NAD-dependent dehydrogenase activity to form a colored reaction product. In these assays the amount of dye formed directly correlates to the number of viable cells. These assays are performed in a 96-well microplate (MP) and the results are easily quantified with a standard ELISA reader, allowing the processing of large sample numbers. However such assays, which measure the number of metabolically active cells, would fail when, for example, a small number of proliferating cells are masked by an overwhelming majority of non-proliferating cells (*e.g.*, antigen-specific stimulation of lymphocytes); or when DNA synthesis is induced in an arrested cell population without any change in cell number or cell viability (*e.g.*, short-term measurement of growth factor activity on 3T3 or AKR-2B cells).

Measurement of DNA synthesis with [^{3}H]-thymidine

Since cellular proliferation requires the replication of cellular DNA, the monitoring of DNA synthesis is another indirect parameter of cell proliferation as well as being suitable for the study of the regulation of DNA synthesis itself. DNA synthesis has been the most common measure of mitosis and cell proliferation, and [^{3}H]-thymidine has traditionally been used to label the DNA of mitotically active cells.

Disadvantages of the [^{3}H]-thymidine incorporation assay are:
- the necessity of radioisotopes;
- the inherent handling and disposal problems;
- the requirement of specialized and expensive equipment like a cell harvester and scintillation counter and the hazard caused by the handling of toxic scintillation fluids.

These problems have led to the pursuit of non-radioactive replacements for this assay.

Non-radioactive measurement of DNA synthesis

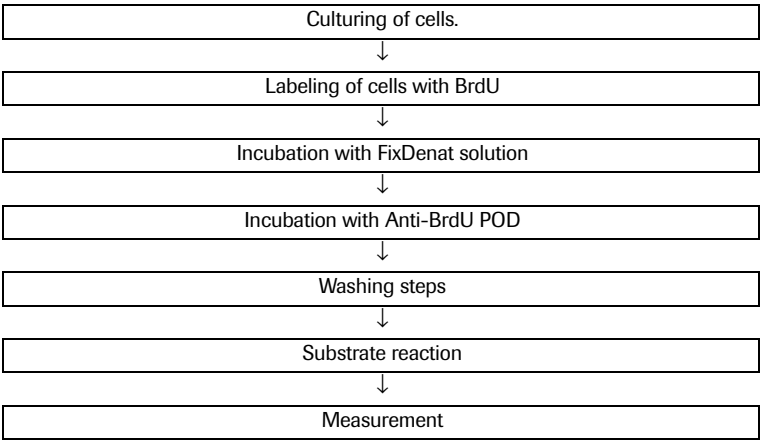
An important development has been the replacement of [^{3}H]-thymidine by 5-bromo-2'-deoxyuridine (BrdU). This technique is based on the incorporation of the pyrimidine analogue BrdU instead of thymidine into the DNA of proliferating cells. After its incorporation into DNA, BrdU is detected by immunoassay. Several monoclonal antibodies which are highly specific for BrdU have been described (1-6). Originally, immunohisto-chemical detection of cells during the S-phase and quantification of cell proliferation has been done by microscopic or flow cytometric analysis of the cell samples. Although very informative, these techniques do not allow a high sample throughput in routine cell proliferation analysis. In 1985, Porstmann et al. first described an enzyme immunoassay for the assessment of cell proliferation by quantification of BrdU incorporation into DNA (7). Subsequently, this method was varied and optimized by several laboratories (8–11).

3. Procedures and required material

3.1 Before you begin

Flow chart

Please find a quick overview about the assay procedure.



Additional equipment and reagents required

To perform assays with this ELISA Kit, you will need the following equipment for the sample preparation and the ELISA Assay:

- 37°C incubator
- Centrifuge with rotor for MP (for suspension cells only)
- ELISA-reader (for MP) with 370 nm filter (without stop solution) or 450 nm filter (with stop solution). The reference wavelength should be 492 nm (without stop solution) and 690 nm (with stop solution)
- Microscope
- Hemacytometer
- Multichannel pipettor (10 µl and 100 µl)
- Sterile pipette tips
- Flat-bottomed 96-well MP, tissue culture grade
- If the peroxidase reaction is to be stopped, 1 M H_2SO_4 is required (25 µl/well; = 2.5 ml/100 tests).

Note: All other reagents necessary to perform 1000 tests are included in the kit.

Preparation of kit working solutions

Please refer to the following table for the preparation of the working solutions.

Solution	Preparation	Storage/ stability	Use
BrdU labeling solution	Dilute BrdU labeling reagent (bottle 1) 1:100 with sterile culture medium (resulting concentration: 100 µM BrdU). For one 96-well MP, 1 ml BrdU labeling solution is required if the cells were cultured in 100 µl/well (10 µl/well) and 2 ml BrdU labeling solution is required if the cells were cultured in 200 µl/well (20 µl/well)	The undiluted BrdU labeling reagent (1000 ×): At +2 to +8°C for several months protected from light. The diluted BrdU labeling reagent: At +2 to +8°C stable for several weeks. Store protected from light. For long-term storage it is recommended to store the BrdU labeling solution in aliquots at −15 to −25°C.	Labeling of cells
Anti-BrdU-POD stock solution	Dissolve Anti-BrdU-POD (bottle 3) in 1.1 ml double dist. water for 10 min and mix thoroughly.	At +2 to +8°C for several months. For long-term storage it is recommended to store the solution in aliquots at −15 to −25°C.	Stock for the preparation of the Anti-BrdU-POD working solution
Anti-BrdU-POD working solution	Dilute Anti-BrdU-POD stock solution 1:100 with antibody dilution solution (bottle 4). For one 96-well MP dilute 100 µl Anti-BrdU-POD stock solution in 10 ml antibody dilution solution (bottle 4).	Prepare shortly before use ! Do not store!	Binding of the POD labeled anti-BrdU antibody.
Washing solution	Dilute Washing buffer concentrate (bottle 5) 1:10 with double dist. water. For one 96-well MP dilute 10 ml Washing buffer concentrate (bottle 5) with 90 ml double dist. water.	At +2 to +8°C for several weeks	For the removal of unbound antibodies

Controls

Blank	Has to be performed in each experimental setup. The blank provides information about the unspecific binding of BrdU and anti-BrdU-POD conjugate to the MP. The absorbance value obtained in this control should not exceed 0.1 absorbance and has to be subtracted from all other values.
Background control	This is facultative and has only be performed once with the respective cell system. It provides information about the unspecific binding of the anti-BrdU-POD conjugate to the cells in the absence of BrdU. The absorbance value obtained in this control should not exceed 0.1 absorbance. This control may significantly increase with some cell lines using high cell concentrations (more than 2×10^4 cells/100 µl).

Overview of the controls

Well contents	Blank	Background control
Culture medium	100 µl	–
Cells	–	100 µl
BrdU	10 µl	–
Anti-BrdU-POD	100 µl	100 µl

Protocol

Please refer to the following table.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Culture cells together with various dilutions of test substance (e.g., mitogens, growth factors, cytokines, cytostatic drugs) in a 96-well MP (tissue culture grade, flat bottom) in a final volume of 100 µl/well in a humidified atmosphere at 37°C.
Note: The incubation period of the cell cultures depends on the particular experimental approach and on the cell type used for the assay. For most experimental setups, an incubation time of 24 to 120 h is appropriate.</td></tr>
<tr><td>2</td><td>Add 10 µl/well BrdU labeling solution if the cells were cultured in 100 µl/well (final concentration:10 µM BrdU) and reincubate the cells for additional 2 to 24 h at 37°C (if the cells were cultured in 200 µl/well, add 20 µl/well BrdU labeling solution).
Note: For most applications, a 2 h labeling time is adequate. Prolongation of the incubation time will increase the amount of BrdU incorporated into cellular DNA and thus lead to increased absorbance values and sensitivity (fig. 3, 5).</td></tr>
<tr><td>3</td><td>Removal of labeling medium:
<table>
<tr><td>For adherent cells</td><td>Remove labeling medium by tapping off or suction.</td></tr>
<tr><td>For suspension cells</td><td>• Centrifuge the MP at 300 g for 10 min and remove the labeling medium by flicking off or aspiration by pipetting.
• Dry cells using a hair-dryer for about 15 min or, alternatively, at 60°C for 1 h.</td></tr>
</table></td></tr>
<tr><td>4</td><td>The assay can be interrupted after the labeling process.
<table>
<tr><th>IF you want...</th><th>THEN...</th></tr>
<tr><td>to stop</td><td>after removal of the labeling medium and drying of the labeled cells the dry cells can be stored for up to one week at +2 to +8°C.</td></tr>
<tr><td>to go ahead</td><td>continue with step 5.</td></tr>
</table></td></tr>
<tr><td>5</td><td>• Add 200 µl/well FixDenat (bottle 2) to the cells.
• Incubate for 30 min at +15 to +25°C.</td></tr>
<tr><td>6</td><td>• Remove FixDenat solution thoroughly by flicking off and tapping.</td></tr>
<tr><td>7</td><td>• Add 100 µl/ well anti-BrdU-POD working solution
• Incubate for approx. 90 min at +15 to +25°C.
Note: Alternatively, this incubation period can be varied between 30–120 min, depending on individual requirements (see section 5.1).</td></tr>
<tr><td>8</td><td>Remove antibody conjugate by flicking off and rinse wells three times with 200 µl-300 µl/well Washing solution (PBS, 1×).</td></tr>
<tr><td>9</td><td>• Remove Washing solution by tapping.
• Add 100 µl/well Substrate solution.
• Incubate at +15 to +25°C until color development is sufficient for photometric detection (5-30 min).</td></tr>
</table>

Measurement

Without stop solution	Measure the absorbance of the samples in an ELISA reader at 370 nm (reference wavelength: approx. 492 nm). ***Note***: Not stopping the substrate reaction allows repeated measurement at various time points (*e.g.*, 5, 10 and 20 min). Thus, the optimal time for the substrate reaction for the respective cell system can be determined.
With stop solution	• Add 25 µl 1 M H_2SO_4 to each well and incubate the MP for approx. 1 min on the shaker at 300 rpm (or mix thoroughly). • Measure the absorbance of the samples in an ELISA reader at 450 nm (reference wavelength: 690 nm). ***Note***: Measurement has to be carried out within 5 min after adding the stop solution.

3.2.1 Example 1: Measurement of the proliferation of mitogen-activated, human peripheral blood lymphocytes (PBLs)

Protocol

The following protocol describes the measurement of the proliferation of mitogen-activated, human peripheral blood lymphocytes (PBLs) (13).

Note: To study the proliferation of lymphocytes, the cells are stimulated *e.g.*, with growth factors, cytokines or mitogens. The increase in cell numbers can (in special cases) lead to cluster formation of the lymphocytes: cells from the same progenitor stick together and form aggregates in the culture plate. This effect may disturb the antibody recognition of the ELISA system and thereby result in an underestimation of the response. To avoid signal variation: carefully resuspend the cells after the BrdU-labeling period and before removing the culture medium by pipetting. This will enable the equal accessibility of each cell for the antibody recognizing the BrdU-label.

Step	Action
1	Titrate mitogen (PHA) in the appropriate culture medium in sterile 96-well MPs by serial dilutions (*e.g.*, 1:3) to obtain a final volume of 50 µl/well.
2	For the determination of spontaneous proliferation add 50 µl culture medium without mitogen into triplicate wells.
3	Determine the blank by adding 100 µl culture medium into triplicate wells.
4	Isolate PBLs from human peripheral blood by density gradient centrifugation, wash cells in culture medium and dilute in culture medium to 1×10^6 cells/ml.
5	Add 50 µl of this cell suspension into each well except the wells required for the blank.
6	Incubate the cells in an incubator (37°C, 5% CO_2, 90% humidity) for 48 h.
7	Add BrdU labeling reagent and reincubate for 2 to 24 h.
8	Removal of labeling medium. Continue with section 3.2 step 3.

Results

The results revealed from the cell proliferation ELISA strongly correlate to the data obtained by the [^{3}H]-thymidine incorporation assay. Increasing the labeling time with BrdU or [^{3}H]-thymidine up to 8 h increases the absorbance and the cpm, respectively.

A prolongation of the labeling period from 8 h to 24 h increases the absorbance values obtained in the immunoassay but reduces the cpm measured by the radioactive assay.

Please see Figure 5 section 4.

3.2.2 Example 2: Measurement of the proliferation of allogeneic-stimulated human PBLs (mixed lymphocyte reaction, MLR)

Controls

The following controls are required for the determination of the spontaneous proliferation of responder and stimulator cells in a one- and two-way MLR:

One way MLR:	
Stimulator control	Provides information about the BrdU incorporation of the mitomycin C treated stimulator cells.
Responder control I	Provides information about the spontaneous proliferation of the responder cells.
Responder control II	High values in this control indicate potential autoreactivity.
Two way MLR:	
Syngeneic control I	Provides information about the spontaneous proliferation of the first responder cell population at the cell concentration used in the assay.
Syngeneic control II	Provides information about the spontaneous proliferation of the second responder cell population at the cell concentration used in the assay.

Table 2

Pipetting scheme for the following protocol step 5.

Sample number (see Fig. 6)	Sample	Donor A	Donor B	Donor A (Mit. C treated)	Donor B (Mit. C treated)	Culture medium
1	Stimulator control	–	–	–	100 µl	100 µl
2	Responder control I	100 µl	–	–	–	100 µl
3	Responder control II	100 µl	–	100 µl		–
4	One way MLR	100 µl	–	–	100 µl	–
5	Syngeneic control I	200 µl	–	–	–	–
6	Syngeneic control II	–	200 µl	–	–	–
7	Two way MLR	100 µl	100 µl	–	–	–

Protocol

The following protocol describes the measurement of the proliferation of alloge-

neic-stimulated human PBLs (mixed lymphocyte reaction, MLR) (13)

Step	Action
1	Isolate PBLs from the blood of both donors by density gradient centrifugation, wash cells in culture medium and dilute in culture medium to 1×10^6 cells/ml.
2	Incubate an aliquot of allogeneic stimulator cells and syngeneic PBLs (for control) with mitomycin C (final concentration 25 µg/ml, Cat. No. 10 107 409 001) in an incubator (37°C, 5% CO_2, 90% humidity) for 30 min. ***Note***: Protect mitomycin C from light. Discard if precipitate is present.
3	Wash mitomycin C treated cells at least three times in culture medium to remove free mitomycin C.
4	Adjust cell concentration of all cell populations to 1×10^6 cell/ml.
5	Pipette cell suspensions in a flat bottomed MP, according to the scheme shown in table 2.
6	Incubate the cells in an incubator (37°C, 5% CO_2, 90% humidity) for 5 days.
7	Add BrdU labeling reagent and reincubate for 24 h.
8	Proceed as described in section 3.2 step 3.

Results

The results revealed from the cell proliferation ELISA strongly correlate to the data obtained by the [^{3}H]-thymidine incorporation assay.

Please see figure 6 section 4.

Figure 4 Measurement of the proliferation of mitogen-activated, human peripheral blood lymphocytes (PBLs).

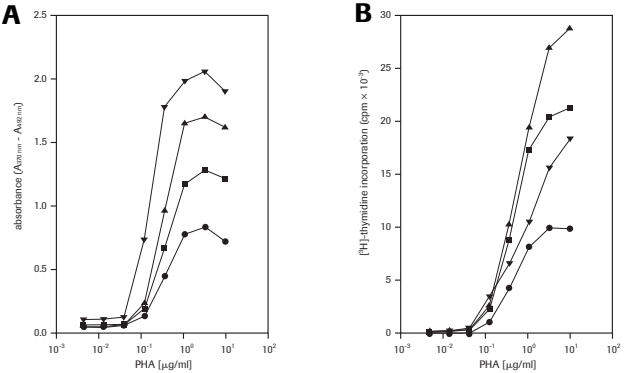
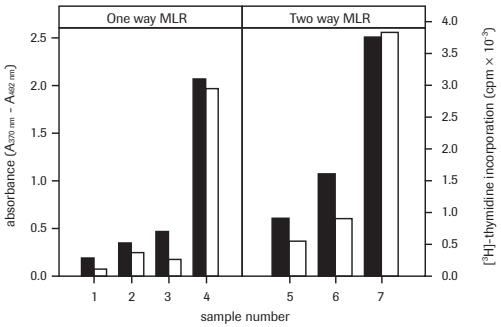


Fig. 4: PBLs were isolated and cultured in microtiter plates for 48 h as described in section 3.2.1. Subsequently, BrdU (A) or [^{3}H]-thymidine (B) was added and cells reincubated for additional 2 h (●), 4 h (■), 8 h (▲) and 24 h (▼). BrdU incorporation was determined as described in section 3.2 (Assay procedures).
The [^{3}H]-thymidine incorporation assay was performed following a standard protocol.

Figure 5 Measurement of the proliferation of allogeneic-stimulated human PBLs (mixed lymphocyte reaction, MLR)

Human PBLs were isolated, aliquots were treated with mitomycin C and seeded in MP as described in section 3.2. After 5 days of incubation, BrdU (closed columns) or [^{3}H]-thymidine (open columns) was added and the cells were reincubated for additional 24 h. Subsequently the immunoassay was done as described in section 3.2 (Assay procedures). The [^{3}H]-thymidine incorporation assay was performed following a standard protocol (legend for sample number see Table 2).



5. Appendix

5.1 Trouble shooting

Since the various cell culture systems greatly differ in cell number, proliferating activity of the cells and incubation periods, most problems will occur because of too high or too low absorbance values.

The opportunities to adapt the assay conditions to those test systems are shown in the following table.

Problem	Recommendation
Too low absorbance values	• Increase cell number or incubation time (section 3.2 step 1) • Increase labeling period with BrdU to 24 h (section 3.2. step 2) • Increase incubation time with FixDenat to 60 min (section 3.2 step 5) • Increase concentration of Anti-BrdU-POD conjugate 2-fold to 4-fold (section 3.2 step 6) • Increase incubation time with antibody-conjugate to 2 h and/or incubate the MP at 37°C (section 3.2 step 6) • Increase incubation time with Substrate solution to 30 min (section 3.2 step 9)
Too high absorbance values	• Decrease cell number or incubation time (section 3.2 step 1) • Decrease labeling period with BrdU to 2 h (section 3.2 step 2) • Decrease incubation time with FixDenat to 15 min (section 3.2, step 5) • Decrease incubation time with Substrate solution to 5 min (section 3.2, step 9)
Too high variations of absorbance values	Increase in cell numbers of lymphocytes following stimulation can (in special cases) lead to cluster formation of the lymphocytes: Cells from the same progenitor stick together and form aggregates in the culture plate. This effect may disturb the antibody recognition of the ELISA system and thereby result in an underestimation of the response. To avoid signal variation: carefully resuspend the cells after the BrdU-labeling period and before removing the culture medium by pipetting. This will enable the equal accessibility of each cell for the antibody recognizing the BrdU-label.
High background control	• Some cell lines show an increase in unspecific binding of the antibody conjugate at high cell concentrations (more than 2×10^4 cells/well). Reduction of the cell concentration will avoid this background. • After removing FixDenat (section 3.2 step 6), add 200 µl/well Blocking buffer (*e.g.*, Blocking Reagent for ELISA, Cat. No. 11 112 589 001 or for nucleic acid hybridization (Cat. No. 11 096 176 001) and incubate for 30 min at +15 to +25°C. Remove blocking solution by tapping and add Anti-BrdU-POD working solution (section 3.2 step 7).

1 Gratzner, H. G. (1982) *Science* **218**, 474-475.
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13 Current Protocols in Immunology 1, chapter 7.10. 1 (Coligan, J. E. et al., eds.) John -Wiley & Sons, New York

5.3 Related products

This table only shows a selection of the most important products related to the product described in this package insert.

For further information please access our web-site address at:
http://roche-applied-science.com

or Special Interest Site Apoptosis:
http://roche-applied-science.com/apoptosis

Parameter	Detection by	Products	Cat. No.
BrdU labeling of proliferating cells	*In situ* assay	• BrdU Labeling and Detection Kit I • BrdU Labeling and Detection Kit II • BrdU Labeling and Detection Kit III • In Situ Cell Proliferation Kit, FLUOS	11 296 736 001 11 299 964 001 11 444 611 001 11 810 740 001
	ELISA	• Cell Proliferation ELISA, BrdU (colorimetric) • Cell Proliferation ELISA, BrdU (chemiluminescent)	11 647 229 001 11 669 915 001
	Single reagents for *in situ* assays and ELISA applications	• Anti-BrdU* formalin grade • Anti-BrdU -Fluorescein, formalin grade • Anti-BrdU -Peroxidase, Fab fragments, formalin grade • FixDenat	11 170 376 001 11 202 693 001 11 585 860 001 11 758 764 001
Measurement of metabolic activity	Quantification in microtiterplate	• Cell Proliferation Kit I (MTT) • Cell Proliferation Kit II (XTT) • Cell Proliferation Reagent WST-1	11 465 007 001 11 465 015 001 11 644 807 001

5.4 Changes to Previous Version

Editorial changes

Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

6. Quick reference protocol for the ELISA assay

Procedure Please refer to the following table.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Culture cells together with various dilutions of test substance (e.g., mitogens, growth factors, cytokines, cytostatic drugs) in a 96-well MP (tissue culture grade, flat bottom) in a final volume of 100 µl/well in a humidified atmosphere at 37°C.
Note: The incubation period of the cell cultures depends on the particular experimental approach and on the cell type used for the assay. For most experimental set-ups, an incubation time of 24 to 120 h is appropriate.</td></tr>
<tr><td>2</td><td>Add 10 µl/well BrdU labeling solution if the cells were cultured in 100 µl/well (final concentration:10 µM BrdU) and reincubate the cells for additional 2 to 24 h at 37°C (if the cells were cultured in 200 µl/well, add 20 µl/well BrdU labeling solution).
Note: For most applications, a 2 h labeling time is adequate. Prolongation of the incubation time will increase the amount of BrdU incorporated into cellular DNA and thus lead to increased absorbance values and sensitivity (fig. 3, 5).</td></tr>
<tr><td>3</td><td>Removal of labeling medium:
<table>
<tr><td>For adherent cells</td><td>Remove labeling medium by tapping off or suction.</td></tr>
<tr><td>For suspension cells</td><td>• Centrifuge the MP at 300 g for 10 min and remove the labeling medium by flicking off or suction using a canulla.
• Dry cells using a hair-dryer for about 15 min or, alternatively, at 60°C for 1 h.</td></tr>
</table></td></tr>
<tr><td>4</td><td>The assay can be interrupted after the labeling process.
<table>
<tr><th>IF you want...</th><th>THEN...</th></tr>
<tr><td>to stop</td><td>after removal of the labeling medium and drying of the labeled cells the dry cells can be stored for up to one week at +2 to +8°C.</td></tr>
<tr><td>to go ahead</td><td>continue with step 5</td></tr>
</table></td></tr>
<tr><td>5</td><td>• Add 200 µl/well FixDenat (bottle 2) to the cells.
• Incubate for 30 min at +15 to +25°C.</td></tr>
<tr><td>6</td><td>• Remove FixDenat solution thoroughly by flicking off and tapping.</td></tr>
<tr><td>7</td><td>• Add 100 µl/ well anti-BrdU-POD working solution
• Incubate for approx. 90 min at +15 to +25°C.
Note: Alternatively, this incubation period can be varied between 30–120 min, depending on individual requirements (see section 5.1).</td></tr>
<tr><td>8</td><td>Remove antibody conjugate by flicking off and rinse wells three times with 200 µl-300 µl/well Washing solution (PBS, 1×).</td></tr>
<tr><td>9</td><td>• Remove Washing solution by tapping.
• Add 100 µl/well Substrate solution.
• Incubate at +15 to +25°C until color development is sufficient for photometric detection (5-30 min).</td></tr>
<tr><td>10</td><td>Without stop solution:
Measure the absorbance at 370 nm (reference wavelength: approx. 492 nm).
With stop solution:
• Add 25 µl 1 M H_2SO_4 to each well and incubate the MP for approx. 1 min on the shaker at 300 rpm (or mix thoroughly).
• Measure the absorbance at 450 nm (reference wavelength: 690 nm).</td></tr>
</table>

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