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PCR ELISA (DIG Detection)

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192 reactions

Semi-quantitative detection of DIG-labeled products by a hybridization-based microtiter plate assay

Store at +2 to +8°C

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1. Preface

1.1 Table of contens

1.2 Kit contents

Caution Bottle 3, Denaturation solution, contains NaOH and should be handled with care.

Kit contents Please refer to the following table for the content of this kit.

Bottle/ Cap	Label	Content
1 violet	Control PCR product, digoxigenin-labeled	• DIG-labeled PCR product, lyophilized • sufficient for five control reactions (three dilutions each).
2 violet	Control capture probe, biotin-labeled	• 75 pmol (500 ng), control capture probe, lyophilized • sufficient for five control reactions (three dilutions and one negative control each).
3	Denaturation solution	• 4 × 1.1 ml alkaline denaturation solution, contains NaOH! • ready-to-use
4 blue	Hybridization buffer	• 100 ml solution • ready-to-use • for dilution of the capture probe before hybridization to target DNA (also available as separate product)
5	Washing tablets	5 tablets for preparation of 10 l washing buffer.
6 red	Conjugate dilution buffer	• 100 ml • ready-to-use • for dilution of the reconstituted anti-DIG-POD antibody
7 red	Anti-digoxigenin-POD conjugate (anti-DIG-POD)	• 2 × 250 mU anti-DIG POD • lyophilized
8 green	Substrate buffer	• 125 ml • ready-to-use • for dissolving the ABTS tablets
9	ABTS tablets	20 tablets, (5 mg ABTS Substrate)
10	Microtiter plate (MTP) modules (8 wells)	• 2 foil bags, each containing 12 microtiter plate modules (8 wells each) in a strip frame (total of 192 wells per kit). • The wells of the MTP modules are precoated with streptavidin and postcoated with blocking reagent; shrink wrapped with a desiccant capsule.
11	8 cover foils for microtiter plates	We recommend to cover the MTP modules with the self adhesive cover foils during long incubation steps in order to avoid evaporation.

POD = peroxidase

Additional equipment and reagents required

For detection of DIG labeled PCR products using this kit, you will need the following material in addition:

- Biotin-labeled capture probe specific for the PCR product of the DIG labeling step (see 3.1 Before you begin)
- Shaking incubator (heat controlled) for MTP, or a shaker in a temperature controlled chamber.
- ELISA reader. The green color of the ABTS Substrate can easily be detected by eye, however for quantification, a photometric measurement is required.
- Microtiter plate washer, or multi channel pipettes allow a more convenient method to wash the MTP.

2. Introduction

2.1 Product overview

Test principle

During the DIG detection, a biotin-labeled oligonucleotide probe captures the DIG-labeled PCR products (target DNA).

The probe-PCR product hybrid is immobilized on a streptavidin-coated microtiter plate, and detected with peroxidase-conjugated anti-digoxigenin antibody, and ABTS colorimetric substrate.

Basic steps

DIG-labeled PCR products are detected in a semi-quantitative microtiter plate (MTP) assay. The technique involves the following steps:

Step	Action
1	Production of a biotin-labeled capture probe that specifically recognizes an internal sequence in the amplified target DNA.
2	Hybridization of the biotin-labeled capture probe to the digoxigenin labeled PCR product.
3	Immobilization of the probe-PCR product hybrid on a streptavidin-coated well of the MTP.
4	Removal of unbound, nonspecific amplification products from the sample, by washing the MTP.
5	Visualization of the bound hybrids with an anti-digoxigenin peroxidase conjugate, and the ABTS colorimetric substrate.
6	Analysis of samples with an ELISA plate reader.
7 (optional)	Quantitative determination of the amount of DNA in the unknown sample by comparing unknown samples with control standards of known concentration.

Application

This kit is used for the qualitative or quantitative detection of DIG-labeled PCR products.

Sample material

This kit is used for detection of DIG labeled PCR products, which can be easily prepared using the PCR ELISA (DIG Labeling) Kit*.

Assay time/ Hands on time

The assay takes approx. 4.5 h including 0.5 –1 h hands on time.

Number of tests

The kit is designed for 192 detection reactions (two MTPs), allowing semi-quantitative detection of 50 PCR products.

Quality control

The PCR ELISA is function tested with the described control reactions. All reagents are free of relevant contaminants.

Kit storage/ stability

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

** available from Roche Diagnostics*

2.2 Product characteristics

Sensitivity

DIG detection is approximately 10 –100 fold more sensitive than conventional analysis of PCR products using ethidium bromide stained agarose gels.

10 ng of genomic DNA for a PCR template is usually enough for the detection of single copy sequences in total human genomic DNA.

If amplifying plasmid DNA or other less complex DNA templates, femtogram or picogram amounts of DNA can usually be detected.

Specificity

The high specificity of DIG detection allows you to distinguish between specific and nonspecific amplification products. This is due to the hybridization step with the capture probe. The specificity of this hybridization follows the standard rules of hybridization assays.

Stringent hybridization in the DIG detection depends on the length and the GC content of the capture probe and the incubation temperature. Temperatures up to +55°C are compatible with DIG-detection. Under these conditions, and with short capture probes (18 – 20mers), identification of a single mismatch base in the capture oligonucleotide is possible. Therefore, you can also analyze mutations in template DNAs with DIG detection.

Advantages

Benefit	Feature
High specificity	The capture probe detects specific sequences in the PCR product, even a point mutation.
Flexibility	The capture probe can be used to screen various types of mutations, pathogens, or microorganisms.
High sensitivity	Detect single copy genes in genomic DNA templates with the combination of PCR and ELISA.

3. Procedures and required materials

3.1 Before you begin

Capture probe

Different capture probes yield different sensitivities, even if length and melting temperatures of the probes are identical. This is due to secondary structures of the oligonucleotide and the PCR fragment.

Secondary structures can inhibit hybridization between the capture probe and PCR fragment resulting in loss of signal. Analysis by computer programs can help avoid obvious secondary structures, but it is normally not powerful enough to allow precise predictions about the behavior of a certain probe in the PCR ELISA.

The following table will help you to design the most suitable capture probe for your test:

Parameter	Recommendation
Sequence	Choose a capture probe that is complementary to an internal sequence of the target DNA (PCR product).
Length	Use oligonucleotides from 17 to 40 nucleotides long. ***Note:*** Longer capture probes (*e.g.*, plasmids) will require optimization of the PCR ELISA procedure.
Amount	The optimal amount of capture probe needed for a single PCR ELISA sample ranges from 1–50 pmol, depending upon the ability of the probe to hybridize to target DNA under the PCR ELISA reaction conditions. The optimal amount of capture probe must be determined empirically.
Biotin labeling	Place a biotin label at either end of the oligonucleotide probe by one of the following methods:

Method	Product
Add an amino linker group and D-biotin-N-hydroxysuccinimide ester to the 5´-end	• D-biotin-N-hydroxy-succinimide ester
Enzymatically label the 3´-end with biotin-16-ddUTP ***or*** biotin-16-dUTP and terminal transferase	• Biotin-16-ddUTP* • Biotin-16-dUTP* • Terminal transferase*

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Controls and sample dilutions

If you are using this kit for the first time, perform the reaction using the controls described in section 3.3 (Prior to the procedure).

Sensitivity of sample detection

The sensitivity of detection can be adjusted by using up to 60 µl of PCR product, or by using appropriate dilutions of the PCR product.

Analyze different amounts of the PCR product if you are using new PCR conditions (*e.g.*, new template or primers, different amounts of template DNA), or if you are using a new capture probe. This is because the signal intensity can vary over a wide range, depending on the amount of captured hybrid.

Hybridization step

In standard assays an incubation time of three hours will increase extinction by 50% compared to an incubation time of 1 hour. Longer incubation times (*e.g.*, overnight) are possible, but do not increase the signal significantly.

By using a shaking incubator, you will more than double the signal, in comparison to incubations without shaking.

3.2 Preparation of working solutions

Caution

Bottle 3, Denaturation solution, contains NaOH and should be handled with care.

Reconstitution of lyophilizates

Before preparing the kit working solutions, it is necessary to reconstitute the following lyophilizates:

Vial	Reconstitution	Stability of solution	Use
Control PCR product, (vial 1)	• Reconstitute the lyophilizate in 100 µl double dist. water, centrifuge briefly. • Incubate at +15 to +25°C for 30 min to allow full reconstitution of the DNA.	Stable at −15 to −25°C until the expiration date printed on the label	Positive control
Control capture probe, (vial 2)	• Reconstitute the lyophilizate in 100 µl double dist. water, centrifuge briefly. • Incubate at +15 to +25°C for 30 min to allow full reconstitution of the DNA.	• Stable at −15 to −25°C until the expiration date printed on the label	Hybridization solution for the control Capture probe
Anti-digoxigenin-POD-conjugate (anti-DIG-POD), (vial 7)	Reconstitute the lyophilizate of 1 vial in 250 µl double dist. water and let sit 15 min at +15 to +25°C. DO NOT VORTEX!	2 months at +2 to +8°C DO NOT FREEZE!	Anti-DIG-POD working solution

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Kit working solutions

In addition to the ready-to-use solutions supplied with this kit, you will have to prepare the kit working solutions described in the following table.

Working solution	Preparation	Stability of solution	For use in
Hybridization solution for your PCR sample	• Add your biotin-labeled capture probe to the ready-to-use hybridization buffer (vial 4) to a final conc. of 7.5 pmol/ml capture probe (corresponds to about 50 ng/ml of a 20mer oligonucleotide). • Add a maximum of 50 µl probe volume to 1 ml of the hybridization buffer. • Allow to come to +15 to +25°C before use. ***Note:*** Avoid foaming! Do not freeze!	2 weeks at +2 to +8°C, but we recommend to prepare it fresh before use	step 3
Hybridization solution for the control capture probe	Add 10 µl of the reconstituted control capture probe to 1 ml Hybridization buffer (vial 4). ***Note:*** Avoid foaming! Do not freeze!	2 weeks at +2 to +8°C but we recommend to prepare it fresh before use	step 3
Washing solution	Dissolve one Washing tablet (bottle 5) in 2 l of double distilled water.	6 weeks at +2 to +8°C	• step 5 • step 7
Anti-DIG-POD working solution	• Dilute 1 volume of the reconstituted Anti-DIG-POD conjugate with 99 volumes of Conjugate dilution buffer (vial 6) at least one hour prior to beginning the experiment. • Allow to come to +15 to +25°C before use. ***Note:*** Avoid foaming! Store away from light! Do not freeze!	2 weeks at +2 to +8°C but we recommend to prepare it fresh before use	step 6
ABTS substrate solution	Dissolve one ABTS tablet (bottle 9) in 5 ml Substrate buffer (bottle 8). Allow to come to +15 to +25°C before use. ***Note:*** Store protected from light!	1 week at +2 to +8°C	step 8

Reagent stability

- All lyophilizates are clear solutions after reconstitution. Any particles that remain in solution should be considered a contaminant.
- The immunoreagent, substrate solution, and washing buffer are clear and colorless. Precipitates or cloudiness in the reagent solutions should be considered as indications of instability or deterioration.

3.3 Procedure

Prior to the procedure

If you want to get quantitative results or if you want to analyze the linear range of your DIG-labeled PCR product, dilute your PCR sample and the Control PCR product as described in the table below. The experienced user, who has already established standardized reaction conditions, will adjust the analyzed volumes and dilution steps to the results obtained in a previous experiment.

We recommend strongly to run a positive and a negative control with each test.

Sample/Control	Recommendation
Your PCR sample	Dilute your PCR sample • 1:10, and • 1:100 with double dist. water.
Positive control	Use the Control PCR product supplied with this kit. Dilute the reconstituted Control PCR product • 1:10, and • 1:100 with double distilled water
Negative control	Use double distilled water instead of a PCR product.

Working solutions and MTPs

Before starting with the assay, please ensure that

- the kit reagents are at +15 to +25°C, and that
- the microtiter plate included with the kit **must** be used for the assay.

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Procedure Please refer to the following table to perform the PCR ELISA.

Step	Action	Volume/ tube or well	Time/ Temperature
1	For a single well detection pipet into 1.5 ml reaction tubes either • PCR sample, or • reconstituted **Control PCR product**, or • double distilled water as a negative control. ***Notes***: • Use 5 µl PCR sample for a first qualitative result.	1–30 µl 5 µl 5 µl	
2	• Add **Denaturation solution** (bottle 3) to each reaction tube (including controls). • Mix, centrifuge briefly, and incubate.	20 µl	10 min at +15 to +25°C
3	Add **Hybridization solution** for your **PCR sample** and for the **Control PCR product**, and mix by vortexing.	fill up to 250 µl	
4	• Pipet into a well of an MTP strip. • Incubate the strips on an MTP shaker. ***Note:*** Use 3 h at +37°C for the Control PCR product that is supplied with the kit. The incubation temperature depends on length and sequence of the capture oligonucleotide. The chosen temperature allows hybridization of the probe with the required stringency.	200 µl	1 h to 3 h at +37 to +55°C
5	• Remove the solution by aspiration. • Wash 3–5 times with **Washing solution**. • After the last wash step, empty the wells, invert the MTP modules, and tap the modules on a dry, lint-free, absorbent cloth.	3–5 × 250 µl	
6	Add **Anti-DIG-POD working solution**, and incubate in the dark on an MTP shaker.	200 µl	30 min at +37°C
7	• Remove the solution by aspiration. • Wash with **Washing solution** 3–5 times. • After the last wash step, empty the wells, invert the MTP modules, and tap the modules on a dry, lint-free, absorbent cloth.	3–5 × 250 µl	

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8	• Add ABTS **substrate solution**. • Incubate in the dark on an MTP shaker.	200 µl	30 min at +37°C
9	For the photometric measurement • read the absorbance of each well at 405 nm (reference wavelength 492 nm). • Then subtract the extinction of the negative control from the absorbance of each sample.		

4. Results of the control reactions

Control PCR product (264 bp)

The Control PCR product (vial 1) is prepared with plasmid DNA as a template in the DIG labeling, using the same primers as in the control assay of the PCR ELISA (DIG Labeling).

Since the plasmid contains the cDNA of the tPA gene, and the genomic sequence amplified by our control primers contains a small intron of 111 bases, it is possible to differentiate between the two control PCR products by gel electrophoresis.

Control	**Extinction range**
Control PCR product	1.0 to 2.0 after 3 h hybridization at +37°C with shaking, and a 30 min substrate reaction at +37°C.
Negative control	$<$0.1

5. Appendix

5.1 Troubleshooting

Problem	Possible Cause	Recommendation
Unexpected color development	Inadequate incubation time and temperature	Ensure that incubation intervals are correct and that all reagents are at +15 to +25°C before using.
	Poor quality water influences the test negatively	Always use double distilled water for reconstitution and when preparing the working solutions! Avoid microbial contaminants.
	Substrate or vial used to aliquot substrate contaminated with oxidative substances.	• **DO NOT** pipet directly from the substrate bottle. • Check the vial for contamination.
Questionable readings	Use of unsuitable filters in the MTP reader.	Check the filters in your MTP reader for the correct wavelength.
Drift	Unequal distribution of temperature in the wells.	Ensure that all reagents achieve +15 to +25°C prior to assay, and use the recommended incubation times and temperatures.
Poor precision	Turbidity, particles or high lipid content in sample.	• Centrifuge sample to pellet particles. • Mix sample well before pipetting.
	Carryover between samples/standards.	Change pipette tips after each pipetting step.
	Unequal volumes added to the wells.	Check pipette function and recalibrate if necessary.
	Inadequate aspiration of fluids.	Fluid should not remain in the wells after aspiration.
	Washing was incomplete.	Ensure that the automatic washer is working properly.
	Unequal mixing of reagents during incubation.	Use a plate shaker to ensure adequate mixing.

5.2 Ordering Information

Product	Pack size	Cat. No.
PCR ELISA (DIG-Labeling)	50 reactions	11 636 120 910
PCR ELISA (DIG Detection), 5-Pack	480 reactions	11 965 409 910
Hybridization Buffer	100 ml	11 717 472 001

Associated Reagents

Product	Pack size	Cat. No.
Biotin dUTP	50 nmol (50 µl)	11 093 070 910
Biotin ddUTP	25 nmol (25 µl)	11 427 598 910
Terminal Transferase, recombinant	8,000 U for 20 Tailing or 3'End labeling reactions 24,000 U for 60 Tailing or 3'End labeling reactions	03 333 566 001 03 333 574 001
Streptavidin-coated MTPs	5 plates	11 664 778 001

5.3 Changes to previous version

Editorial changes.

5.4 Trademarks

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6. Quick reference procedure

Procedure

Step	Action	Volume/ tube or well	Time/ Temperature
1	For a single well detection pipet into 1.5 ml reaction tubes either • PCR sample, or • reconstituted **Control PCR product**, or • double distilled water as a negative control. ***Notes*:** • Use 5 µl PCR sample for a first qualitative result.	1–30 µl 5 µl 5 µl	
2	• Add **Denaturation solution (bottle 3)** to each reaction tube (including controls). • Mix, centrifuge briefly, and incubate.	20 µl	10 min at +15 to +25°C
3	Add **Hybridization solution for your PCR sample** and for the **Control PCR product**, and mix by vortexing.	fill up to 250 µl	
4	• Pipet into a well of an MTP strip. • Incubate the strips on an MTP shaker.	200 µl	1 h to 3 h at +37 to +55°C
5	• Remove the solution by aspiration. • Wash 3–5 times with **Washing solution**. • After the last wash step, empty the wells, invert the MTP modules, and tap the modules on a dry, lint-free, absorbent cloth.	3–5 x 250 µl	
6	Add **Anti-DIG-POD working solution**, and incubate in the dark on an MTP shaker.	200 µl	30 min at +37°C
7	• Remove the solution by aspiration. • Wash with **Washing solution** 3-5 times. • After the last wash step, empty the wells, invert the MTP modules, and tap the modules on a dry, lint-free, absorbent cloth.	3–5 x 250 µl	
8	• Add **ABTS substrate solution.** • Incubate in the dark on an MTP shaker.	200 µl	30 min at +37°C
9	For the photometric measurement • read the absorbance of each well at 405 nm (reference wavelength 492 nm). • Then subtract the extinction of the negative control from the absorbance of each sample.		

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