Version 22-04/24

User's Manual

DIG-Detection PCR ELISA Kit

REF **DEIA8706**

Σ **5 pack**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

This DIG-Detection PCR ELISA, 5-pack kit is used for the qualitative or quantitative detection of DIG-labeled PCR products. It is particularly suitable for the parallel testing of a large number of samples.

Principles of Testing

For the standard protocol of the PCR ELISA, an amplicon incorporates the digoxigenin-labeled nucleotide during the PCR reaction. After thermocycling is complete, the amplicon is denatured and a biotin-labeled oligonucleotide probe hybridizes to the DIG-labeled PCR product (target DNA). This hybrid is immobilized on a streptavidin-coated MTP and detected with peroxidase-conjugated anti-digoxigenin antibody and the colorimetric substrate ABTS.

DIG-labeled PCR products are semi-quantitatively detected in a MTP assay.

1. Production of a biotin-labeled capture probe that specifically recognizes an internal sequence in the amplified target DNA.
2. Denaturation of the amplicon in a microcentrifuge tube.
3. Hybridization of the biotin-labeled capture probe to the digoxigenin-labeled PCR product.
4. Immobilization of the hybrid onto the streptavidin-coated surface of the MTP.
5. Removal of unbound, nonspecific amplification products from the sample by washing the MTP.
6. Visualization of the DIG label with an anti-digoxigenin peroxidase conjugate and the colorimetric substrate ABTS.
7. Analysis of samples with an ELISA plate reader.

Optional: Quantitative determination of the amount of DNA in a sample by comparing unknown samples with control standards of known concentration.

Reagents And Materials Provided

Vial / Bottle	Cap	Label	Function / Description	Content
1a	–	PCR ELISA, DIG-Detection, 5-pack, Denaturation solution for standard protocol	• Ready-to-use alkaline denaturation solution. ⚠ ***Contains NaOH and must be handled with care.*** • For denaturation of DIG-labeled samples in microcentrifuge tubes and immobilization with biotin-labeled capture probe.	1 bottle, 25 ml
1b	–	PCR ELISA, DIG-Detection, 5-pack, Denaturation solution for protocol B	• Ready-to-use alkaline denaturation solution. ⚠ ***Contains NaOH and must be handled with care.*** • For immobilization of biotinylated PCR product, allowing denaturation of the samples directly in the streptavidin-coated microtiter plates.	1 bottle, 100 ml
2	blue	PCR ELISA, DIG-Detection, 5-pack, Hybridization buffer	• Ready-to-use solution. • For the dilution of the capture probe before hybridization to target DNA.	3 bottles, 100 ml each
3	–	PCR ELISA, DIG-Detection, 5-pack, Washing buffer tablets	For the preparation of 10 liters Washing buffer and removal of unbound material.	1 bottle, 5 tablets
4	red	PCR ELISA, DIG-Detection, 5-pack, Conjugate dilution buffer	• Ready-to-use solution. • For the dilution of the reconstituted Anti-DIG-POD antibody.	2 bottles, 100 ml each
5	red	PCR ELISA, DIG-Detection, 5-pack, Anti-DIG-POD (HRP)	• Anti DIG-POD conjugate, lyophilized.	5 vials, 250 mU each
6	green	PCR ELISA, DIG-Detection, 5-pack, ABTS solution	• Clear, colorless ready-to-use solution. ⚠ ***Precipitates or cloudiness in the solution is an indication of instability or deterioration.*** • Colorimetric POD substrate.	2 bottles, 100 ml each
7	foil bag	PCR ELISA, DIG-Detection, 5-pack, Microplate	• Precoated microtiter plate (MTP) wells with streptavidin and post-coated with blocking reagent. • Shrink-wrapped with a desiccant capsule. • 480 wells per kit.	5 foil bags, 12 MTP modules of 8 wells each in a strip frame
8	–	PCR ELISA, DIG-Detection, 5-pack, Self-adhesive Plate Cover Foil	• Prevents evaporation. • Cover the microtiter plate modules with the Cover Foils during long incubation steps.	10 foils

Materials Required But Not Supplied

One of the following products is required:

• PCR ELISA, DIG-Labeling*

• PCR ELISA, DIG-Detection*

For standard protocol

• Water, PCR Grade* or Tris buffer

• Biotin-labeled capture probe specific for the PCR product of the DIG labeling step

• Heat-controlled shaking incubator for MTPs or a shaker in a temperature-controlled chamber or incubator

• ELISA reader

The green color of the substrate ABTS solution can easily be detected by eye, however for quantification, a photometric measurement is required.

• MTP washer or multichannel pipettes for convenient washing of MTPs

For protocol B for immobilization of biotinylated PCR products

• Temperature-controlled shaking incubator for microtiter plates or a shaker in a temperature-controlled chamber or incubator

• ELISA reader

The green color of the substrate ABTS solution can easily be detected by eye, however for quantification, a photometric measurement is required.

• MTP washer or multichannel pipettes for convenient washing of MTPs

• DIG-labeled detection probe

• PCR dilution buffer

Storage

When stored at +2 to +8°C, the kit is stable through the expiry date printed on the label.

Vial / Bottle	Cap	Label	Storage
1a	–	Denaturation solution for standard protocol	Store at +2 to +8°C.
1b	–	Denaturation solution for protocol B	
2	blue	Hybridization buffer	
3	–	Washing buffer tablets	
4	red	Conjugate dilution buffer	
5	red	Anti-DIG-POD (HRP)	
6	green	ABTS solution	Store at +2 to +8°C. ⚠ ***Keep protected from light.***
7	foil bag	Microplate	Store at +2 to +8°C.
8	–	Self-adhesive Plate Cover Foil	

Reconstitution And Storage

Content:

Anti-DIG-POD (Vial 5)

Preparation:

Add 250 µl PCR-grade water* to one vial of lyophilizate; mix carefully. Do not vortex.

Let sit 15 minutes at +15 to +25°C.

The lyophilizate should be a clear solution after reconstitution. Any particles within the reconstituted solution are to be considered as deterioration.

Storage and Stability

Store 2 months at +2 to +8°C. Do not freeze.

For use in Anti-DIG-POD working solution.

Reagent Preparation

Before you Begin

Sample Materials

DIG-labeled PCR products

Control Reactions

Controls and sample dilutions

When using this kit for the first time, perform the reaction using the controls described below. For more quantitative results or to determine the linear range for the DIG-labeled probes, use the quantitative controls and the sample dilution series. If standardized reaction conditions for the intended application have already

been established, some of the controls may be skipped and instead, only the analyzed volumes and dilution steps to the observed absorbance readings need to be adjusted.

Preparation of controls

• For the comparison of the DNA sequence of the sample(s) with the capture probe, a DIG-labeled sample, and positive and negative controls are required.

• The determination of a quantitative estimation of the DNA amount in the sample requires a negative control and dilution series of a DIG-labeled sample as well as a positive control.

Standard protocol

Add 10 µl of each sample dilution to a microcentrifuge tube and continue with Step 2, see section, Assay Procedure, Standard protocol.

Sample dilution series

Control	Sample	Dilution
Quantitation A	Sample PCR	1:10 in PCR-grade water*.
Quantitation B	Sample PCR	1:100 in PCR-grade water*.

Negative Control

Control	Sample	For use in determination of...
Negative detection control	PCR-grade water* or Tris buffer.	Background of the DIG-detection procedure.
Negative PCR labeling control	Negative control reaction of the DIG labeling, that is, every reagent of the assay excluding template DNA.	Cross-contamination of the PCR reagents and to measure the background of the DIG-labeling components.

General Considerations

Capture probe

Different capture probes usually yield different sensitivities, even if length and melting temperature of the probes are identical. This is due to secondary structures of the oligonucleotide and the PCR fragment. Secondary structures can inhibit hybridization between capture probe and PCR fragment, resulting in reduction or loss of signal. Analysis by computer programs can help to avoid obvious secondary structures, however, these programs normally do not allow precise predictions about the behavior of a certain probe in the PCR ELISA.

Follow the guidelines in the table to design the most suitable capture probe.

Parameter	Guideline
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. *Longer capture probes, for example, 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 to 50 pmol probe/ml Hybridization solution, depending on 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.* • For an initial experiment, use approximately 7.5 pmol. • Use the Standard protocol and the Denaturation solution (Bottle 1a).
Biotin labeling	Place a biotin label on the oligonucleotide capture probe by one of the following methods: • Incorporate biotin-phosphoramidite during the synthesis of the probe. • Add an amino linker group to the 5′ end during synthesis, and subsequently label with D-biotin-N-hydroxysuccinimide ester. • Enzymatically label the 3′ end of the probe with Biotin-16-ddUTP* and Terminal Transferase*. • Enzymatically tail the 3′ end of the probe with Biotin-16-dUTP* and Terminal Transferase*.

Hybridization step

In standard assays, an incubation time of 3 hours will increase extinction by 50% compared to an incubation time of 1 hour. Longer incubation times up to overnight are possible, but do not increase the signal

significantly. The incubation time can also be decreased to yield lower absorbance readings.

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

Working Solution

Kit working solutions

The immunoreagent and washing solutions should be clear and colorless. Precipitates or cloudiness in the reagent solutions are indications of instability or deterioration.

Standard protocol

Working solution	Preparation	Storage and Stability	For use in...
Hybridization solution	• Add the biotin-labeled capture probe to the ready-to-use Hybridization buffer (Bottle 2). For an initial experiment, use a final concentration of 7.5 pmol/ml capture probe, corresponding to approximately 50 ng/ml of a 20mer oligonucleotide. • Add a maximum of 50 µl probe volume to 1 ml of the Hybridization buffer. • Bring to +15 to +25°C before use. ⚠ ***Avoid foaming; do not freeze.***	Store 2 weeks at +2 to +8°C. ⚠ ***Prepare fresh before use.***	Step 3
Washing solution	Dissolve one Washing buffer tablet (Bottle 3) in 2 liters of PCR-grade water.	Store 6 weeks at +2 to +8°C.	Steps 5 and 7
Anti-DIG-POD	• Dilute 1 volume of the reconstituted Anti-DIG-POD conjugate with 99 volumes of Conjugate dilution buffer (Bottle 4) at least 1 hour prior to beginning the experiment. • Bring to +15 to +25°C before use. ⚠ ***Avoid foaming; do not freeze.*** ⚠ ***Keep protected from light.***	Store 2 weeks at +2 to +8°C. ⚠ ***Prepare fresh before use.***	Step 6

Working solution	Preparation	Storage and Stability	For use in...
PCR dilution buffer	PBS*/Tween 20* (0.2%, v/v), pH 7.8	–	Steps 1 and 2
Hybridization solution	Add the DIG labeled detection probe to1 ml Hybridization buffer (Bottle 2) to a final concentration of approximately 50 ng/ml. ⚠ ***Avoid foaming; do not freeze.***	Store 2 weeks at +2 to +8°C. ⚠ ***Prepare fresh before use.***	Step 6
Washing solution	Dissolve one Washing buffer tablet (Bottle 3) in 2 liters of PCR-grade water.	Store 6 weeks at +2 to +8°C.	Steps 3, 5, 7, and 9
Anti-DIG-POD	• Dilute 1 volume of the reconstituted Anti-DIG-POD conjugate with 99 volumes of Conjugate dilution buffer (Bottle 4) at least 1 hour prior to beginning the experiment. • Bring to +15 to +25°C before use. ⚠ ***Avoid foaming; do not freeze.*** ⚠ ***Keep protected from light.***	Store 2 weeks at +2 to +8°C. ⚠ ***Prepare fresh before use.***	Step 8

Assay Procedure

Standard protocol

Ensure that all kit reagents are at +15 to +25°C and use only the MTP supplied with the kit.

1. For a single-well detection, pipette 1 to 30 µl of DIG-labeled sample into a sterile microcentrifuge tube.

Use 5 µl sample for a first qualitative result.

2. Add 20 µl Denaturation solution (Bottle 1a) to each reaction tube, including controls.

– For a reagent blank, add 20 µl Denaturation solution (Bottle 1a) into an empty microcentrifuge tube.

– Mix, centrifuge briefly, and incubate for 10 minutes at +15 to +25°C.

3. Add Hybridization solution to a final volume of 250 µl.

– Mix by vortexing.

4. Pipette a 200 µl aliquot from each microcentrifuge tube into a well of a MTP strip.

– Incubate the strips on a MTP shaker for 1 to 3 hours at +37 to +55°C.

Incubate 3 hours at +37°C for a first result. The chosen temperature should allow hybridization of the probe with the required stringency.

5. Carefully remove the solution by aspirating the buffer.

– Wash 3 to 5 times with 250 µl Washing solution.

– After the last wash step, aspirate the wells, invert the MTP modules, and tap the modules on a dry, lint-free absorbent cloth.

6. Add 200 µl of Anti-DIG-POD working solution, and incubate for 30 minutes at +37°C on a MTP shaker.
7. Remove the solution by aspirating the buffer.

– Wash 3 to 5 times with 250 µl Washing solution.

– After the last wash step, aspirate the wells, invert the MTP modules, and tap the modules on a dry, lint-free absorbent cloth.

8. Add 200 µl ABTS substrate solution (Bottle 6).

– Incubate in the dark for 20 to 30 minutes at +37°C on a MTP shaker until color becomes visible in all wells.

9. For the photometric measurement, read the absorbance of each well at 405 nm (reference wavelength 492 nm).

– Subtract the extinction of the reagent blank from the absorbance of each sample.

Extinction of the negative controls should be <0.1 after subtraction of reagent blank.

Protocol B for immobilization of biotinylated PCR products

Ensure that all kit reagents are at +15 to +25°C and use only the MTP supplied with the kit.

1. Dilute an aliquot of the PCR product directly with PCR dilution buffer up to a volume of 120 µl.

– Mix the solution thoroughly and centrifuge briefly.

2. Distribute 5 µl of the pre-diluted PCR product per well of the streptavidin-coated MTP each containing 160 µl of PCR dilution buffer.

– Incubate the MTP for 1 hour at +42°C on an MTP shaker.

As a reagent blank, add PCR dilution buffer into an empty well.

3. Carefully remove the solution by aspirating the buffer.

– Wash each well 4 times with 250 µl Washing solution.

4. Carefully remove the solution by aspirating the buffer.

– Add 175 µl Denaturation solution (Bottle 1b).

– Incubate plates for 3 minutes at +15 to +25°C on a MTP shaker.

5. Wash each well 4 times with 250 µl Washing solution.
6. Carefully remove the solution by aspirating the buffer.

– Add 175 µl Hybridization solution.

– Incubate MTP for 1 hour at +42°C on a MTP shaker.

7. Wash each well 4 times with 250 µl Washing solution.

8. Carefully remove the solution by aspirating the buffer.

– Add 175 µl of Anti-DIG-POD working solution.

– Incubate for 30 minutes at +37°C on a MTP shaker.

9. Wash each well 4 times with 250 µl Washing solution.

10. Carefully remove the solution by aspirating the buffer.

– Add 175 µl ABTS solution (Bottle 6).

– Incubate in the dark for 20 to 30 minutes at +37°C on a MTP shaker until color becomes visible in all wells.

11. For the photometric measurement, read the absorbance of each well at 405 nm (reference wavelength 492 nm).

– Subtract the extinction of the reagent blank from the absorbance of each sample.

Sensitivity

The sensitivity of the PCR ELISA technology is generally about 10 to 100-fold above that of the conventional analysis of PCR products in ethidium bromide stained agarose gels.

• 10 ng of genomic DNA as 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

PCR ELISA achieves a high specificity by utilizing capture probes to specifically bind to sequences on the PCR product. A single-copy gene can be detected in genomic DNA templates, or even a single base change in the sequence (point mutation).

The high specificity of DIG detection allows to distinguish between specific and nonspecific amplification products 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 to 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.

Precautions

• The Denaturation solutions (Bottles 1a and 1b) contain NaOH which is an irritant and must be handled with care.

• Always wear gloves and follow standard safety precautions.

Laboratory procedures

• Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer

of potential pathogens in the sample material varies, the operator must optimize pathogen inactivation by the Lysis / Binding Buffer or take appropriate measures, according to local safety regulations.

• Do not eat, drink or smoke in the laboratory work area.

• Do not pipette by mouth.

• Wear protective disposable gloves, laboratory coats and eye protection, when handling samples and kit reagents.

• Wash hands thoroughly after handling samples and reagents.

Waste handling

• Discard unused reagents and waste in accordance with country, federal, state, and local regulations.

Troubleshooting

Observation	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 use.
	Poor quality water negatively influences the test.	Always use PCR-grade water* for reconstitution and preparation of the working solutions. *Avoid microbial contaminants.*
	ABTS substrate or container used to aliquot substrate is contaminated with oxidative substances.	Do not pipette directly from the substrate bottle. *Check the bottle for contamination.*
Questionable readings	Use of unsuitable filters in the MTP reader.	Check the filters in the MTP reader for the correct wavelength. Read the absorbance at 405 nm (reference wavelength 492 nm).
Drift	Unequal distribution of temperature in the wells.	Ensure that all reagents are at +15 to +25°C prior to assay, and use the recommended incubation times and temperatures.
Poor precision	Turbidity or particles in samples or solutions.	Centrifuge sample to pellet particles.
		Mix samples well before pipetting.
	Carryover between samples and standards.	Change pipette tips after each pipetting step.
	Unequal volumes added to the wells.	Check pipette function and recalibrate if necessary.
	Washing was incomplete.	Carefully wash wells to fully remove previous buffers.
	Insufficient mixing of reagents during incubation.	Use a plate shaker to ensure adequate mixing and even temperatures.