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ProductInformation

PCR Plate Detection Kit

Product Code **PPD-1**
Storage Temperature 2-8 °C

TECHNICAL BULLETIN

Product Description

The PCR† Plate Detection Kit is intended for the solid-phase capture and sequence-specific detection of PCR products in an easy to use and automatable format utilizing streptavidin-coated strip-well microplates. This detection format offers several advantages over gel-based detection, including increased sensitivity and specificity, as well as simplified, objective data analysis. PCR amplification is carried out with one 5' biotinylated primer and one unlabeled primer. Amplified PCR products are immobilized in streptavidin-coated wells and hybridized to a fluorescein-labeled oligonucleotide probe. The presence of bound probe is detected with an anti-fluorescein-HRP (peroxidase) conjugate and the colorimetric peroxidase substrate TMB (3,3',5,5'-tetramethylbenzidine). This kit contains all the reagents (except sequence specific primers and probes) required for 480 detection reactions. The assay can be completed in 2.5 hours.

Kit Components

- SigmaScreen™ Streptavidin-coated 8x12 Strip-well Plates, Product Code M 3433, 5 each (96 wells per plate in 8-well strips)
- Dilution Buffer, Product Code D 0813 , 250 ml
- Denaturation Solution, Product Code D 4063, 100 ml
- PlateHyb™ Hybridization Buffer, Product Code. H 4909, 200 ml
- Anti-Fluorescein-HRP conjugate, 500X stock, Product Code F 6050, 4 units
- TMB Liquid Substrate System, Product Code T 8665, 2 x 100 ml
- Stop Solution, Product Code S 4686, 100 ml
- Phosphate Buffered Saline with Tween 20® (PBS–T) Wash Dry Packs, Product Code P 3563, 10 each
- Sealing film for 96-well multiwell plates, Product Code Z36,967-5, 15 each

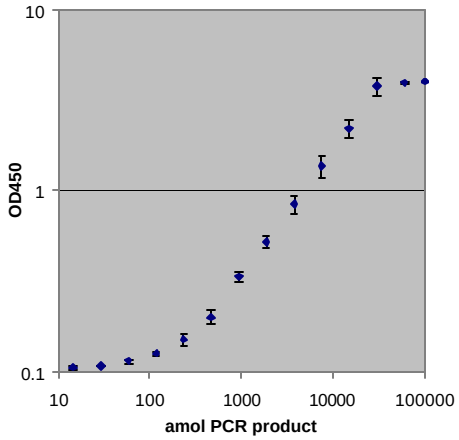
Reagents and equipment required but not provided

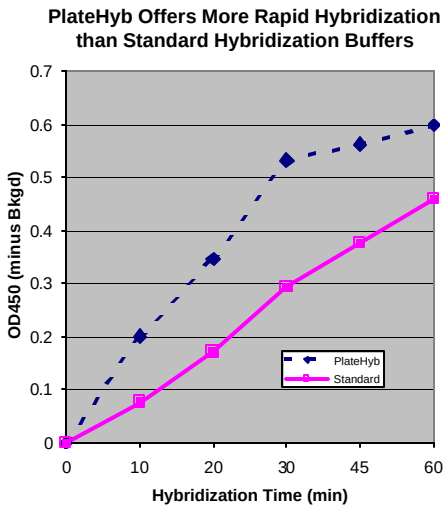
- Sequence specific primers and probes
- PCR reagents
- Target DNA to be amplified
- Hybridization oven(s)
- Microtiter plate reader and wash station
- Multichannel pipettors and reservoirs
- Deionized formamide (if desired)

The PCR Plate Detection Kit is:

Rapid - takes 2.5 hr total time (30-45 min hands-on); PlateHyb Hybridization Buffer reduces time required for efficient hybridization
Specific – probe provides an additional level of sequence-specificity; PlateHyb Hybridization Buffer improves genotyping of single base mutations
Sensitive – typically 10-100X more sensitive than gel electrophoresis
Semi-quantitative – discrimination between PCR product levels over a 2-2.5 log range of concentration
Objective - allows for simplified data analysis with user defined cut-offs

The PCR Plate Detection Kit Offers Sensitive, Semi-quantitative Detection of PCR Products





Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability
All reagents should be stored at 2-8 ºC. Under proper storage conditions, kit reagents are stable for 1 year from date of receipt.

Protocol Overview
Basic Steps for specific detection of PCR product (one amplification primer biotin-labeled at 5' end):

1. Biotinylated PCR product diluted in Dilution Buffer in microplate well and incubated to allow binding.
2. Denaturation Solution added to denature PCR product. Plate washed to remove non-biotinylated PCR product strand.
3. Fluorescein-labeled probe (complementary to biotinylated strand of PCR product) added to well and hybridized. Plate washed to remove unbound probe.
4. Anti-Fluorescein-HRP conjugate added to well and incubated to allow enzyme-labeling of specifically bound probe. Plate washed to remove unbound conjugate.
5. Bound HRP label visualized with the colorimetric substrate TMB.
6. Stop solution added and signal read photometrically in colorimetric plate reader.

Primer / Probe Selection
Primers and probes should be chosen that are specific for the target of interest, utilizing one of several available primer selection software packages. Primer/probe sequences should be chosen to contain minimal secondary structure and minimal primer/primer and primer/probe interactions. Such interactions can typically be avoided through the use of commercially available primer/probe design programs. As a general rule of thumb, probes should be 14-30 nucleotides long and should contain 6-16 GC bonds. **The probe should be designed such that it is complementary to the biotinylated PCR product strand.**

Hybridization Conditions
The optimal hybridization temperature for a standard probe (25 nucleotides long, containing 12-14 GC bonds) is typically near 50 °C. Alternatively, formamide can be added to the hybridization mix at a final concentration of 20% and hybridization can be carried out at 37 °C. For shorter probes and for products with low secondary structure, optimal results can be achieved at 37 °C without formamide. Do not carry out hybridizations containing formamide at temperatures above 40 °C, as performance will be degraded. We have found that amplicons produced with dUTP tend to exhibit increased secondary structure, increasing the need for either increased hybridization temperatures or the addition of formamide to the hybridization mixture. For standard hybridization reactions, a probe concentration of approximately 0.2 pmol per ml should be utilized. The appropriate probe concentration for allele-specific hybridization will vary depending upon the characteristics of the probe.

Preparing for a PCR Plate Detection experiment
Before beginning the experimental protocol, bring kit components to room temperature. PBS–T wash buffer can be prepared by resuspending one PBS–T dry pack in 1 liter of molecular biology grade water (W 4502 or equivalent). Dilution of probe can be done at this time if desired. A working solution of anti-Fluorescein-HRP conjugate may be prepared by diluting the conjugate stock solution 500-fold in PBS–T wash buffer (i.e. 2 µl diluted with 998 µl PBS–T wash buffer).

Experiments should be set up to contain control samples run in parallel that ensure that non-specific signal is not being generated by binding of probe or conjugate to the microplate wells. These controls can include samples containing:
1. no PCR product
2. no fluorescein-labeled probe
3. a non-specific fluorescein-labeled probe
4. no anti-fluorescein-HRP conjugate.

If semi-quantitation is desired, a series of calibrator PCR products should be incorporated into the detection protocol.

Detailed Protocol
1. Pipette 90-100 µl Dilution Buffer into an adequate number of microplate wells. Employing RedTaq™ in the PCR mixture makes it easy to confirm this addition step and doe not interfere with the assay.
2. Add 1-10 µl appropriate PCR product to microplate wells and incubate 30 min at 37 °C.
3. Add 100 µl Denaturation Solution by pipetting up and down. Incubate 10 min at room temperature.
4. Wash microplate wells 3-5X with 300 µl PBS–T
5. Add 200 µl PlateHyb Hybridization Buffer containing 0.2 pmol of the appropriate fluorescein-labeled probe (complementary to biotinylated strand of PCR product) to well and hybridize 30-45 min at 37-50 °C.
6. Wash microplate wells 5X with 300 µl PBS–T.
7. Add 150 µl diluted anti-fluorescein-HRP conjugate to well and incubate 30-45 min at 37°C.
8. Wash microplate wells 5X with 300 µl PBS–T.
9. Add 100 µl colorimetric substrate TMB and incubate at room temp for 5-30 min.
10. Add 50 µl stop solution and detect signal in a colorimetric plate reader (set at 450 nm).

Data Analysis / Data Reduction
Major benefits offered by the PCR Plate Detection Kit are:
- assay background is easily subtracted,
- objective criteria for data acceptance can be generated
- positive and negative assay cut-off values can be set.
- good signal differentiation between PCR product levels over a 2.5-log dynamic range and the ability to differentiate allelic variants through differences in hybridization efficiency.

Related Products
- REDTaq™ DNA Polymerase, Product Code D 4309
- JumpStart™ REDTaq™ ReadyMix™ PCR Reaction Mix with $MgCl_2$, Product Code P 0982
- Enhanced Avian First Strand Synthesis Kit, Product Code STR1
- Deoxynucleotide Mix, 10 mM each, Product Code D 7295
- Custom Oligonucleotides. Sigma Genosys offers a complete line of custom oligonucleotides for your research needs. For more information:
  Call 800-234-5DNA or
  visit our website at www.sigma-aldrich.com.

[†]The PCR process is covered by patents owned by Hoffman-LaRoche, Inc.

Troubleshooting Guide

Problem	Cause	Solution
No Signal	No PCR product made	When initially testing a capture-based detection system, it is prudent to ensure that the appropriate product has been made. This is typically confirmed by gel electrophoresis.
	Probe does not recognize biotinylated product strand	Confirm that the fluorescein-labeled probe is complementary to the biotinylated strand of PCR product that is synthesized. If this is not the case, resynthesize the appropriate probe.
	Biotin-labeling of primer or fluorescein-labeling did not occur	The efficient addition of these labels can typically be confirmed on a 8-20% denaturing acrylamide gel by the presence of a single band (as visualized by UV shadowing) migrating somewhat more slowly than would be expected for an unlabeled oligonucleotide of the same size. If this is not the case, have primer and/or probe re-synthesized .
Low Signal	Probe unable to bind due to hybridization conditions	Decrease stringency of hybridization by reducing hybridization temperature and/or by utilizing hybridization buffer that does not contain added formamide.
	Probe does not bind efficiently due to secondary structure in PCR product	Increase stringency of hybridization reaction, either by increasing temperature or by adding up to 20% formamide (v/v) to hybridization mixture.
	Probe is not completely complementary to PCR product	Confirm that the sequence utilized is correct, either by comparing to other published sequences or by sequencing PCR product.
Background Signal	Probe utilized is non-specifically hybridizing to biotin-labeled primers and/or PCR side products	Ensure that primers and their complements are not predicted to form secondary structure with the fluorescein-labeled probe (if primer-dimer is synthesized, the biotin-labeled product strand will contain sequence complementary to the non-biotinylated primer). This non-specific hybridization can often be eliminated by increasing the hybridization temperature or by adding up to 20% formamide to the hybridization reaction.
	Inadequate removal of unbound reagents from wells	Ensure that plate washer is adequately removing liquid from wells. If necessary increase the number of wash cycles utilized.
	TMB substrate is contaminated with peroxidase or other oxidative substances	Check the vial to ensure that substrate is not contaminated. DO NOT pipette directly from the substrate bottle
Poor precision	Unequal addition of sample or detection reagents	Check pipetting volumes and recalibrate if necessary.
	Inadequate incubation times	Ensure that the incubation times utilized are correct.