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PRIMER DESIGN

Product Name: genesig 2G Multidye Advanced kit handbook (DNA)
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Primerdesign™ Ltd

Handbook for genesig® 2G Multidye Advanced kits (DNA targets)

150 tests

For general laboratory and research use only

1. Advanced Kit contents (see product datasheet for definitive details)

Multiplex primer/probe mix (150 reactions BROWN cap)
FAM and Cy5 labelled.

Internal extraction control primer/probe mix (150 reactions BROWN cap)
VIC labelled.

Endogenous Control primer/probe mix (150 reactions BROWN cap)
FAM labelled.

Multiplex positive control template (for standard curve RED cap)

Internal extraction control DNA (150 reactions BLUE cap)

RNase/DNase-free water (WHITE cap)
for resuspension of primer/probe mixes.

Template preparation buffer (YELLOW cap)
for resuspension of internal control template, positive control template and standard curve preparation.

2. Reagents and equipment to be supplied by the user

Real-time PCR Instrument

Extraction kit
This kit is recommended for use with the genesig Easy DNA/RNA extraction kit. However, it is designed to work well with all processes that yield high-quality RNA and DNA with minimal PCR inhibitors.

oasig™ lyophilised or Precision®PLUS 2X qPCR Master Mix.
This kit is intended for use with oasig or PrecisionPLUS2X qPCR Master Mix.

Pipettors and tips
Vortex and centrifuge
Thin-walled PCR reaction tubes/plates appropriate to instrument used

3. Kit storage and stability

This kit is stable at room temperature but should be stored at -20ºC on arrival. Once the lyophilised components have been resuspended, they should not be exposed to temperatures above -20°C for longer than 30 minutes at a time and can only be freeze/thawed a maximum of four times. The kit is stable for 18 months from the date of resuspension under these circumstances.

If a standard curve dilution series is prepared, this can be stored frozen for an extended period. If you see any degradation in this serial dilution, a fresh standard curve can be prepared from the positive control. Primerdesign does not recommend using the kit after the expiry date stated on the pack.

4. Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and DNA integrity (an internal PCR control is supplied to test for non-specific PCR inhibitors). Always run at least one negative control with the samples. To prepare a negative control, replace the template DNA sample with RNase/DNase-free water.

5. Dynamic range of test

Under optimal PCR conditions, genesig target detection kits have very high priming efficiencies of >90% and can detect less than 100 copies of the target template.

6. Notices and disclaimers

This product is developed, designed and sold for research purposes only. It is not intended for human diagnostic or drug purposes or to be administered to humans unless clearly expressed for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. During the warranty period, Primerdesign genesig detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation of the general GLP guidelines and the manufacturer's recommendations, the right to claim under guarantee is expired. PCR is a proprietary technology covered by several US and foreign patents. These patents are owned by Roche Molecular Systems Inc. and have been sub-licensed by PE Corporation in certain fields. Depending on your specific application, you may need a license from Roche or PE to practice PCR. Additional information on purchasing licences to practice the PCR process may be obtained by contacting the Director of Licensing at Roche Molecular Systems, 1145 Atlantic Avenue, Alameda, CA 94501 or Applied Biosystems business group of the Applera Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404. In addition, the 5' nuclease assay and other homogeneous amplification methods used in connection with the PCR process may be covered by US Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc, and by US Patent 5,538,848, owned by The Perkin-Elmer Corporation.

7. Trademarks

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The PCR process is covered by US Patents 4,683,195, and 4,683,202 and foreign equivalents owned by Hoffmann-La Roche AG. BI, ABI PRISM® GeneAmp® and MicroAmp® have registered trademarks of the Applera Genomics (Applied Biosystems Corporation). BIOMEK® is a registered trademark of Beckman Instruments, Inc.; iCycler™ is a registered trademark of Bio-Rad Laboratories, Rotor-Gene is a trademark of Corbett Research. LightCycler™ is a registered trademark of the Idaho Technology Inc. GeneAmp®, TaqMan® and AmpliTaqGold® are registered trademarks of Roche Molecular Systems, Inc., The purchase of the Primerdesign™ reagents cannot be construed as an authorisation or implicit license to practice PCR under any patents held by Hoffmann-LaRoche Inc.

8. Principles of the test

Real-time PCR

The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridise to the target DNA. A fluorogenic probe is included in the same reaction mixture, which consists of a DNA probe labelled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved, and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of qPCR platforms.

Positive control

For copy number determination and as a positive control for the PCR set-up, the kit contains a positive control template. This can be used to generate a standard curve of the target copy number / Cq value. Alternatively, the positive control can be used at a single dilution where full quantitative analysis of the samples is not required. Each time the kit is used, at least one positive control reaction must be included in the run. A positive result indicates that the primers and probes for detecting the target gene worked properly in that particular experimental scenario. If a negative result is obtained, the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component, which would lead to false-positive results. This can be achieved by handling this component in a post-PCR environment. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

Negative control

To validate any positive findings, a negative control reaction should be included every time the kit is used. For this reaction, RNase/DNase-free water should be used instead of the template. A negative result indicates that the reagents have not become contaminated while setting up the run.

Internal DNA extraction control

When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis buffer. This control DNA is then co-purified with the sample DNA and can be detected as a positive control for the extraction process. Successful co-purification and qPCR for the control DNA also indicate that PCR inhibitors are not present at a high concentration.

A separate primer and probe mix are supplied with this kit to detect the exogenous DNA using qPCR. The

primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control DNA does not interfere with the detection of the target DNA even when present at a low copy number. The Internal control is detected through the VIC channel and gives a Cq value of 28+/-3.

Endogenous control

To confirm the extraction of a valid biological template, a primer and probe mix is included to detect an endogenous gene. Detection of the endogenous control is through the FAM channel, and it is NOT, therefore, possible to perform a multiplex with the target primers. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

9. Resuspension protocol

To minimise the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally, this would be a designated PCR lab or PCR cabinet. Filter tips are recommended for all pipetting steps.

1. Pulse-spin each tube in a centrifuge before opening.
This will ensure that the lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

2. Resuspend the primer/probe mixes in the RNase/DNase-free water supplied, according to the table below:
To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in water	Volume
Multiplex primer/probe mix (**BROWN** cap)	165 µl
Internal extraction control primer/probe mix (**BROWN** cap)	165 µl
Endogenous control primer/probe mix (**BROWN** cap)	165 µl

3. Resuspend the internal control template and positive control template in the template preparation buffer supplied, according to the table below:
To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in template preparation buffer	Volume
Internal extraction control DNA (**BLUE** cap)	600 µl
Positive Control Template (**RED** cap) *	500 µl

* This component contains a high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

10. DNA extraction

The internal extraction control DNA can be added either to the DNA lysis/extraction buffer or to the DNA sample once it has been resuspended in the lysis buffer.

DO NOT add the internal extraction control DNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal.

1. Add 4 µl of the Internal extraction control DNA (BLUE cap) to each sample in DNA lysis/extraction buffer per sample.

2. Complete DNA extraction according to the manufacturer's protocols.

11. qPCR detection protocol

1. For each DNA sample, prepare a reaction mix according to the table below:
Include sufficient reactions for positive and negative controls.

Component	Volume
oasig or PrecisionPLUS 2X qPCR Master Mix	10 µl
Multiplex primer/probe mix (**BROWN** cap)	1 µl
Internal extraction control primer/probe mix (**BROWN** cap)	1 µl
RNase/DNase-free water (WHITE cap)	3 µl
Final Volume	**15 µl**

2. For each DNA sample, prepare an endogenous control reaction according to the table below (Optional):
This control reaction will provide useful information regarding the quality of the biological sample.

Component	Volume
oasig or PrecisionPLUS 2X qPCR Master Mix	10 µl
Endogenous control primer/probe mix (**BROWN** cap)	1 µl
RNase/DNase-free water (WHITE cap)	4 µl
Final Volume	**15 µl**

3. Pipette 15 µl of each mix into individual wells according to your qPCR experimental plate set-up.

4. Prepare sample DNA templates for each of your samples.

5. Pipette 5 µl of DNA template into each well, according to your experimental plate set-up.
For negative control wells, use 5µl of RNase/DNase-free water. The final volume in each well is 20 µl.

6. If a standard curve is included for quantitative analysis, prepare a reaction mix according to the table below:

Component	Volume
oasig or PrecisionPLUS 2X qPCR Master Mix	10 µl
Multiplex primer/probe mix (**BROWN** cap)	1 µl
RNase/DNase-free water (WHITE cap)	4 µl
Final Volume	**15 µl**

7. Preparation of standard curve dilution series.

a) Pipette 90 µl of template preparation buffer into 5 tubes and label 2-6
b) Pipette 10 µl of Positive Control Template (**RED** cap) into tube 2
c) Vortex thoroughly
d) Change pipette tip and pipette 10 µl from tube 2 into tube 3
e) Vortex thoroughly

Repeat steps d) and e) to complete the dilution series

Standard Curve	Copy Number
Tube 1 Positive control (**RED** cap)	2 x 10^5 per µl
Tube 2	2 x 10^4 per µl
Tube 3	2 x 10^3 per µl
Tube 4	2 x 10^2 per µl
Tube 5	20 per µl
Tube 6	2 per µl

8. Pipette 5 µl of the standard template into each well for the standard curve according to your experimental plate set-up.
The final volume in each well is 20 µl.

12. qPCR amplification protocol

Amplification conditions using oasig or PrecisionPLUS 2X qPCR Master Mix.

	Step	Time	Temp
	Enzyme activation	2 min	95°C
Cycling x50	Denaturation	10 s	95°C
	DATA COLLECTION *	60 s	60°C

* Fluorogenic data should be collected during this step through the FAM, Cy5 and VIC channels

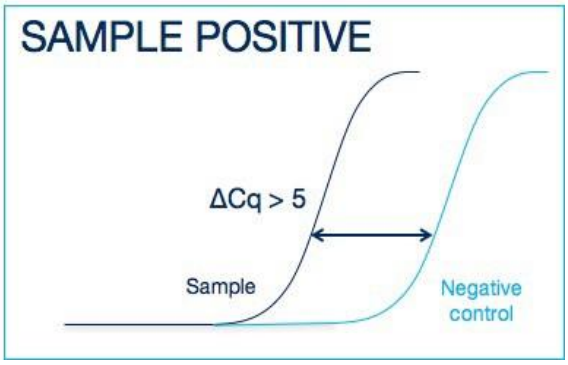
13. Acceptance criteria

Before interpreting sample results, it is necessary to verify the success of the run. If the following criteria are not satisfied, then testing needs to be repeated:

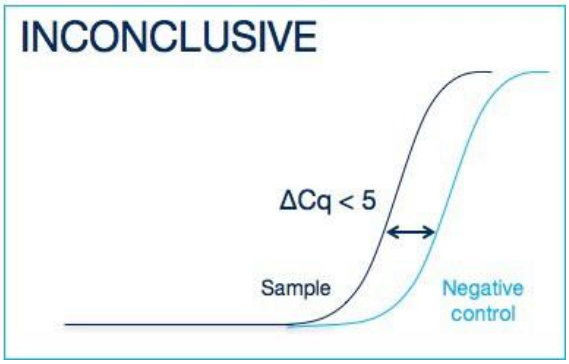
- The positive control template (**RED** cap) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.
- The negative control is expected to be clear from amplification.

Positive control	Negative control	Interpretation
-	**+ / -**	EXPERIMENT FAILED
+	**≤ 35**	EXPERIMENT FAILED due to test contamination
+	**> 35**	*

*Where the test sample is positive and the negative control is positive with a Cq > 35, the sample must be reinterpreted based on the relative signal strength of the two results:



If the sample amplifies > 5 Cq earlier than the negative control, then the sample should be reinterpreted (via the table above) with the negative control verified as negative.



If the sample amplifies < 5 Cq earlier than the negative control, then the positive sample result is invalidated, and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.

13. Interpretation of results

Target (FAM/Cy5)	Internal control (VIC)	Interpretation
≤ 30	**+ / -**	Positive quantitative result* calculate copy number
> 30	**+**	Positive quantitative result* calculate copy number
> 30	**-**	Positive qualitative result DO NOT REPORT THE COPY NUMBER as this may be due to poor sample extraction
-	**+**	Negative result
-	**-**	Sample preparation failed

* The standard curve efficiency should be between 90 and 110% (if this criterion is not satisfied, the test results can still be considered qualitative).

Internal PCR control

The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of DNA added to the PCR reaction and the individual machine settings. Cq values of 28±3 are within the normal range. When amplifying a target sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Endogenous control

The signal obtained from the endogenous control primer and probe set will vary according to the amount of biological material present in a given sample. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample.