Human Y chromosome quantification assay

Quantification of the Human Y chromosome by real-time PCR
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Kit contents

- Human Y chromosome primer/probe mix (150 reactions, BROWN)
- Positive control genomic DNA template (for standard curve, RED)
- Internal extraction control primer/probe mix (150 reactions, BROWN)
- Internal extraction control DNA (150 reactions, BLUE)
- RNase/DNase free water (WHITE) for resuspension of primer/probe mixes.
- Template preparation buffer (YELLOW) for resuspension of internal control template, positive control template and standard curve preparation.

Reagents and equipment to be supplied by user

- Real-time PCR Instrument
- DNA extraction kit
  This kit is recommended for use with genesig® EASY DNA/RNA Extraction kit. However, it is designed to work well with all processes that yield high quality DNA with minimal PCR inhibitors.
- Precision®PLUS, Precision®FAST or oasig™ 2X qPCR Master Mix
  This kit is designed to work well with all commercially available master mixes. However, we recommend the use of Primerdesign PrecisionPLUS, PrecisionFAST or oasig 2X qPCR Master Mix.
- Pipettors and Tips
- Vortex and centrifuge
- Thin walled 0.2 ml PCR reaction tubes
Kit storage and stability
This kit is stable at room temperature but should be stored at -20°C on arrival. Primerdesign does not recommend using the kit after the expiry date stated on the pack. Once the lyophilised components have been resuspended, unnecessary repeated freeze/thawing should be avoided. The kit is stable for six 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.

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 test sample with RNase/DNase free water.

Licensing agreement and limitations of use
PCR is covered by several patents owned by Hoffman-Roche Inc and Hoffman-LaRoche, Ltd. Purchase of Primerdesign kits does not include or provide licence with respect to any patents owned by Hoffman-La Roche or others.

Primerdesign satisfaction guarantee
Primerdesign takes pride in the quality of all our products. Should this product fail to perform satisfactorily when used according to the protocols in this manual, Primerdesign will replace the item free of charge.

Quality control
As part of our ISO9001 and ISO13485 quality assurance systems, all Primerdesign products are monitored to ensure the highest levels of performance and reliability.
Notices and disclaimers

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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 licenses 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 U.S. Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc, and by U.S. Patent 5,538,848, owned by The Perkin-Elmer Corporation. The purchase of Biosearch Technologies products does not, either expressly or by implication, provide a license to use this or other patented technology. Licensing information can be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404 or the Licensing Department at Roche Molecular Systems Inc., 1145 Atlantic Avenue, Alameda, CA 94501.

The purchase of the Primerdesign™ reagents cannot be construed as an authorization or implicit license to practice PCR under any patents held by Hoffmann-LaRoche Inc or others.

Trademarks

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oasig™ is a trademark of Primerdesign Ltd.
genesig® is a registered trademark of Primerdesign Ltd.
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Principles of the test

Introduction
This kit provides reagents for the detection and quantification of human Y chromosome. The primers detect a region of the Y chromosome in the TSPY1 gene that is not present in the X chromosome. The amplicon is present at multiple copies within the Y chromosome which increases the sensitivity for detecting very small amounts of genomic DNA and even degraded DNA.

The kit contains a number of additional components to avoid both false positive and false negative test results.

Real-time PCR
When resuspended, this kit provides primers that have been tested for priming specificity and amplification efficiency at optimal concentrations. qPCR is a very sensitive technology and it is not recommended to use more or less than the specified amount of primer and probe in each reaction. However, final reaction volumes between 15\(\mu\)l and 50\(\mu\)l are often successful and may be tested at the user’s discretion. Unfortunately, Primerdesign is not able to provide technical support for protocols other than those provided.

Positive control
For the purpose of DNA quantification, and as a positive control for the PCR set up, the kit contains positive control genomic DNA. This can be used to generate a standard curve of Y chromosome concentration. Alternatively, the positive control can be used at a single dilution for a qualitative analysis of the samples. Each time the kit is used, at least one positive control reaction must be included on the run. A positive result indicates that the primers and probes for quantification of the Y chromosome are working on that particular run. 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.

Negative control
To confirm absence of contamination, a negative control reaction should be included every time the kit is used. For this reaction, the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated. If a positive result is obtained the results should be ignored and the test samples repeated. Possible sources or contamination should first be explored and removed.
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 genomic DNA and can be detected as a positive control for the extraction process. Successful co-purification and qPCR amplification of the control DNA also indicates that PCR inhibitors are not present at a high concentration.

A separate primer/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 detection of the Y chromosome target DNA even when present at low copy number. The internal control is detected through the VIC channel and gives a Cq value of 28+/-3.
Bench-side 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 cabinet. Filter tips are recommended for all pipetting steps. The positive control template is a significant contamination risk and should therefore be pipetted after negative control and sample wells.

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

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

<table>
<thead>
<tr>
<th>Component – resuspend in water</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-PCR pack</td>
<td></td>
</tr>
<tr>
<td>Y chromosome primer/probe mix (BROWN)</td>
<td>165μl</td>
</tr>
<tr>
<td>Internal extraction control primer/probe mix (BROWN)</td>
<td>165μl</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Component – resuspend in template preparation buffer</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-PCR heat-sealed foil</td>
<td></td>
</tr>
<tr>
<td>Positive control template (RED) *</td>
<td>100μl</td>
</tr>
<tr>
<td>Pre-PCR heat sealed foil</td>
<td></td>
</tr>
<tr>
<td>Internal extraction control DNA (BLUE)</td>
<td>600μl</td>
</tr>
</tbody>
</table>

* This component contains a high concentration of genomic DNA and is a significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.
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 lysis buffer.

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

1. Add 4μl of internal extraction control DNA (BLUE) to each sample in DNA lysis/extraction buffer.

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

qPCR detection protocol

1. Prepare the Y chromosome quantification mix according to table below:

<table>
<thead>
<tr>
<th>Component</th>
<th>1 reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrecisionPLUS, PrecisionFAST or oasig Master Mix</td>
<td>10μl</td>
</tr>
<tr>
<td>Y chromosome primer/probe mix (BROWN)</td>
<td>1μl</td>
</tr>
<tr>
<td>Internal extraction control primer/probe mix (BROWN)</td>
<td>1μl</td>
</tr>
<tr>
<td>RNase/DNase free water (WHITE)</td>
<td>3μl</td>
</tr>
<tr>
<td><strong>Final volume</strong></td>
<td><strong>15μl</strong></td>
</tr>
</tbody>
</table>

2. Pipette 15μl of this mix into each well according to your qPCR experimental plate set up.

3. Prepare sample DNA templates for each of your samples (suggested concentration 5ng/µl) in RNase/DNase free water.
   If the concentration of DNA is not known, then dilute your DNA samples 1:20 (10μl of sample DNA and 190μl of water).

4. Pipette 5μl of diluted 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 20μl.
5. Preparation of 1:4 standard curve dilution series

1) Pipette 90μl of template preparation buffer into 5 tubes and label 2 – 6.
2) Pipette 30μl of positive control template (RED) into tube 2.
3) Vortex thoroughly.
4) Change pipette tip and pipette 30μl from tube 2 into tube 3.
5) Vortex thoroughly.

Repeat steps 4 and 5 to complete the dilution series

<table>
<thead>
<tr>
<th>Standard curve</th>
<th>DNA conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1 Positive control (RED)</td>
<td>5000 pg/μl</td>
</tr>
<tr>
<td>Tube 2</td>
<td>1250 pg/μl</td>
</tr>
<tr>
<td>Tube 3</td>
<td>312 pg/μl</td>
</tr>
<tr>
<td>Tube 4</td>
<td>78 pg/μl</td>
</tr>
<tr>
<td>Tube 5</td>
<td>19 pg/μl</td>
</tr>
<tr>
<td>Tube 6</td>
<td>5 pg/μl</td>
</tr>
</tbody>
</table>

6. Pipette 5μl of standard curve dilution templates into each well, according to your experimental plate set up.
The final volume in each well is 20μl.
qPCR amplification protocol

Please select the correct cycling protocol for the master mix that you are using.

Amplification conditions using PrecisionPLUS or oasig 2X qPCR Master Mix

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activation</td>
<td>2min</td>
<td>95°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>10s</td>
<td>95°C</td>
</tr>
<tr>
<td>DATA COLLECTION*</td>
<td>60s</td>
<td>60°C</td>
</tr>
</tbody>
</table>

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

Amplification conditions using PrecisionFAST 2X qPCR Master Mix

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activation</td>
<td>2min</td>
<td>95°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>5s</td>
<td>95°C</td>
</tr>
<tr>
<td>DATA COLLECTION*</td>
<td>20s</td>
<td>60°C</td>
</tr>
</tbody>
</table>

*Fluorogenic data should be collected during this step through the FAM and VIC channels.
Interpretation of results

For qualitative analysis, the table below can be used to interpret the data

<table>
<thead>
<tr>
<th>Y chromosome DNA</th>
<th>Internal control</th>
<th>Negative control</th>
<th>Positive control</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>Exp fail</td>
</tr>
<tr>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>Exp fail</td>
</tr>
</tbody>
</table>

Internal PCR control

When used according to the above protocols and assuming a 100% extraction efficiency, a Cq value of 28 is expected. However, this can 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 gene with a high 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.