



Promega

Technical Manual

Plexor® qPCR System

INSTRUCTIONS FOR USE OF PRODUCTS A4011 AND A4031.



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Plexor[®] qPCR System

All technical literature is available on the Internet at www.promega.com/tbs
 Please visit the web site to verify that you are using the most current version of this
 Technical Manual. Please contact Promega Technical Services if you have questions on use of
 this system. E-mail techserv@promega.com.

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1. Description

The Plexor[®] qPCR System^(a,b) is a novel real-time PCR system for the quantitation of specific sequences within a DNA sample. The Plexor[®] qPCR System can also be used for genotyping (e.g., single nucleotide polymorphism [SNP] analysis). The sample can be any type of DNA, including genomic DNA, cDNA, mitochondrial DNA or viral DNA. This Technical Manual describes the Plexor[®] qPCR System and provides instructions for reaction setup. Instructions for the use of the

Plexor® System with various real-time PCR instruments and information on data analysis using the Plexor® Analysis Software are provided in separate, instrument-specific Instrument Setup and Data Analysis Technical Manuals.

Before using the Plexor® qPCR System, be sure you have the following:

- **Plexor® System primer pair(s).** The Plexor® qPCR System is designed to work with pairs of PCR primers where one of the primers contains a fluorescent label adjacent to an iso-dC residue at the 5' terminus. The other primer is unlabeled. We recommend using the free, web-based Plexor® Primer Design Software to design primers and match fluorescent reporter choices to your specific real-time instrument and your preferred oligonucleotide manufacturer. For more information, refer to Section 3.B.
- **Instrument-specific manual for instrument setup and data analysis.** These manuals provide details for programming thermal cycling conditions, exporting raw data from the instrument software and data analysis. A complete list of Instrument Setup and Data Analysis Technical Manuals is available at: www.promega.com/plexorresources/. These manuals are available by request from your local Promega Branch Office or Distributor or at: www.promega.com/plexorresources/
- **Plexor® Analysis Software.** The key to the Plexor® technology is the quenching of a fluorescent reporter due to the site-specific incorporation of dabcyI-iso-dGTP. As a result, the fluorescent signal from a Plexor® System reaction decreases as PCR product accumulates. Real-time instrument software can record the quenching data but cannot convert the data into cycle thresholds or melting temperatures. Data analysis is accomplished by exporting the data to the Plexor® Analysis Software. The Plexor® Analysis Software is compatible with data from all supported instruments and is available for free download at: www.promega.com/plexorresources/. The software is also available on CD-ROM; contact your local Promega Branch Office or Distributor, or e-mail: techserv@promega.com. The software is compatible with Windows® 98 and later operating systems.

The Plexor® qPCR System takes advantage of the specific interaction between two modified nucleotides to achieve quantitative PCR analysis (1-3). One of the PCR primers contains a modified nucleotide (iso-dC) linked to a fluorescent label at the 5' end. The second PCR primer is unlabeled. The reaction mix includes deoxynucleotides and iso-dGTP modified with the quencher dabcyI. DabcyI-iso-dGTP is preferentially incorporated opposite the iso-dC residue in the primer. The incorporation of the dabcyI-iso-dGTP at this position results in quenching of the fluorescent dye on the complementary strand and a reduction in fluorescence, which allows quantitation during amplification (Figure 1).

With the Plexor® qPCR System, the accumulation of product is accompanied by a decrease in fluorescence as shown in Figure 2.

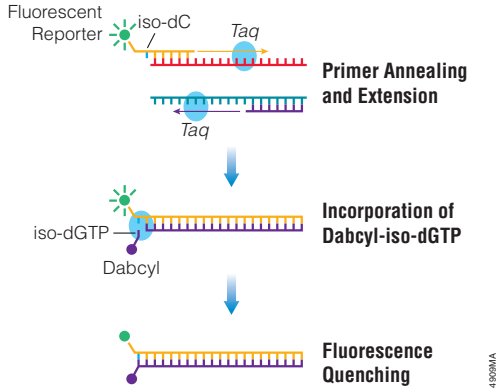


Figure 1. Schematic diagram illustrating the Plexor® System real-time PCR process.

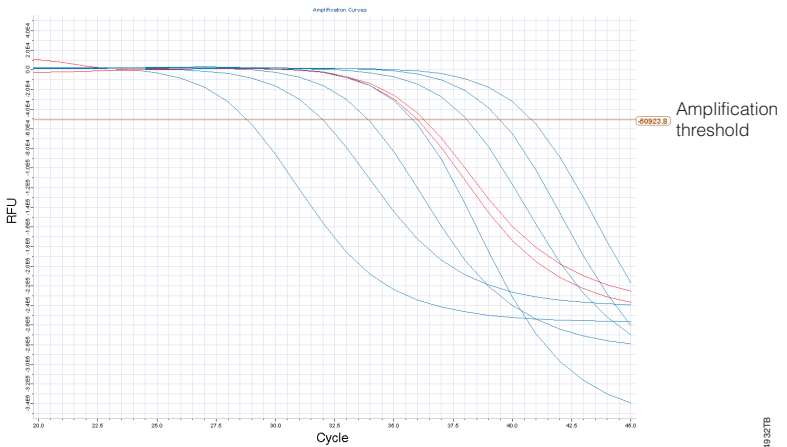


Figure 2. Representative Plexor® qPCR System amplification curve. The amplification curves show the fluorescence (in relative fluorescence units, RFU) at each cycle of the reaction. The amplification threshold is indicated by a horizontal line across the graph. This line is used to determine the cycle threshold (C_t) for the samples.

The Plexor® qPCR System allows the use of a melt curve or dissociation curve to determine the melting temperature (T_m) of the products following amplification (Figure 3). This is useful in assessing the specificity of the reaction. It is also important when the Plexor® qPCR System is used for genotyping.

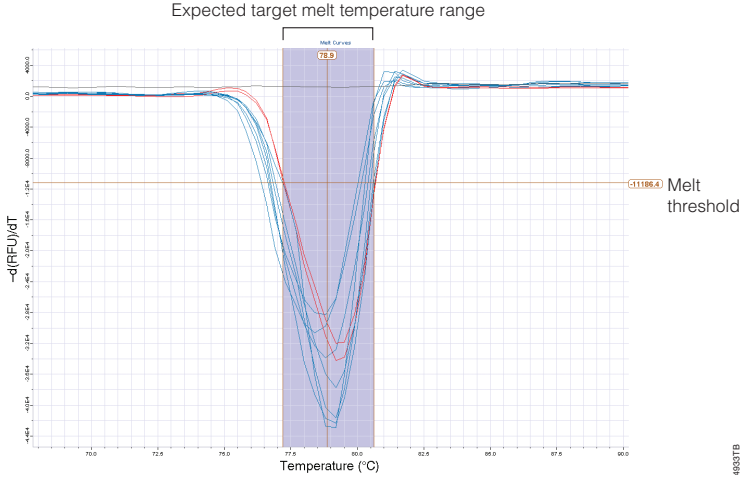


Figure 3. Representative Plexor® System melt curve. Melting temperature is empirically determined by plotting the change in fluorescence with temperature ($-\Delta RFU/\Delta T$) versus temperature and determining the temperature at which the greatest rate of change in fluorescence occurs.

2. Product Components and Storage Conditions

Product	Size	Cat. #
Plexor® qPCR System	200 reactions	A4011
	200 reactions	A4031*

For Research Use Only. Not for use in diagnostic procedures. *This catalog number only available in Europe or through Distributors supported by Promega European Branch Offices. Includes:

- 2 × 1.25ml Plexor® Master Mix, 2X
- 25µl Plexor® qPCR Control, 5X
- 3 × 10ml MOPS/EDTA Buffer
- 2 × 1.25ml Nuclease-Free Water

Storage Conditions: Store all components at -20°C.

Items Available Separately

Product	Cat.#
Plexor® Analysis Software	A4071

Not for Medical Diagnostic Use. This CD-ROM contains the Plexor® Analysis Software and instructions for use of the software. This software can also be downloaded at:

www.promega.com/plexorresources/

Product	Size	Cat.#
MOPS/EDTA Buffer	3 × 10ml	Y5101

3. General Considerations

1. We recommend using designated work areas and pipettes for pre- and postamplification steps to minimize the potential for cross-contamination between samples and prevent carryover of nucleic acid from one experiment to the next.
2. Wear gloves and change them often.
3. Do not open reaction tubes after amplification is complete. Opening the tubes increases the risk of contaminating subsequent reactions with the amplified product.
4. Prevent contamination by using aerosol-resistant pipette tips.
5. Always include a no-template control (NTC) reaction to detect contamination.
6. Always dilute primers in MOPS/EDTA Buffer to maintain the integrity of the iso-dC-containing primers. **Do not** dilute the primers in water.

3.A. Assay Design

The Plexor® qPCR System reactions are similar to standard two-primer amplification reactions except that one of the two amplification primers contains the modified nucleotide, iso-dC, adjacent to the 5' fluorescent label, and the dNTP mix contains dabcyl-iso-dGTP. The Plexor® qPCR System reactions can be performed on a variety of real-time PCR instruments. A complete list of available instrument-specific Technical Manuals is available at: www.promega.com/plexorresources/



It is possible to design assays to quantify multiple targets within the same reaction. In these multiplex reactions, use a different fluorophore for each target. When analyzing the results from multiplex reactions, you may observe a small deflection in the Melt Curve plot in addition to the melt curve from the expected product. Color-to-color cross-talk is the most likely source of this nonspecific signal if this deflection has approximately the same T_m as the product observed in another channel. The ability to detect fluorescent dyes varies with instrument and instrument filter type.

3.B. Primer-Design Software

Promega has developed a web-based, primer-design software for use with the Plexor® qPCR System. The Plexor® Primer Design Software designs primers for monoplex and multiplex qPCR reactions and genotyping assays using the parameters described in Sections 7.A and 7.B. We strongly recommend using this software, which can be found at: www.promega.com/plexorresources/

The software also assists in selecting the appropriate fluorescent labels for primers used in single and multiplex qPCR assays and will match fluorescent reporter choices to your specific real-time instrument and preferred oligonucleotide manufacturer. Fluorescently labeled oligonucleotides for use as primers with the Plexor® qPCR System assays must be ordered with an iso-dC residue adjacent to the 5' fluorescent label. A number of oligonucleotide suppliers have been licensed to provide these iso-dC-containing primers. Convenient links to these suppliers' web sites are included within the Plexor® Primer Design Software.

4. Quantitation Protocol

Prior to reaction assembly, review the appropriate Technical Manual for the particular instrument to be used. A complete list of Instrument Setup and Data Analysis Technical Manuals can be found at:

www.promega.com/plexorresources/

When using the Plexor® qPCR System for the first time, we recommend programming the thermal cycling conditions and checking that the instrument is compatible and configured for the dyes used before assembling the reactions, so the reactions are not kept on ice for prolonged periods of time before thermal cycling. Once you are familiar with the programming process, the instrument can be programmed after reaction assembly.

Materials to Be Supplied By the User

(Solution compositions are provided in Section 7.E.)

- standard reference template of known DNA concentration
- sterile, aerosol-resistant pipette tips
- pipettes dedicated to pre-amplification work
- iso-dC-containing, fluorescently labeled primer and unlabeled primer
- crushed ice
- real-time PCR instrument and related consumables, such as the appropriate capillary, cuvette or PCR plate and plate covers
- the appropriate Instrument Setup and Data Analysis Technical Manual for the real-time PCR instrument used
- Plexor® Analysis Software

4.A. Primer Preparation

Primers containing iso-dC must be stored in a buffer with a pH greater than 7.0. Use the supplied MOPS/EDTA Buffer to resuspend and dilute the primers.



Iso-dC-containing primers are sensitive to pH and must be resuspended and diluted in MOPS/EDTA Buffer to maintain primer integrity. Do not use water to resuspend or dilute primers or prepare primer mixes. The fluorescently labeled primer is light-sensitive and must be stored in the dark.

Prepare 25X primer stocks using the MOPS/EDTA Buffer. For most assays, the 25X primer concentration will be 5 μ M (for a final concentration of 200nM for each primer). Primers can be prepared and stored as individual primers or as primer mixes. Multiplex assays may require optimization of primer concentrations. We have used 1X primer concentrations in the range of 50–400nM successfully.

4.B. Template Preparation

Sample DNA Templates

In general, less than 100ng of template should be used per reaction. When using cDNA as a template, no purification is typically required following the reverse transcription reaction. However, we recommend diluting cDNA templates at least 1:10 prior to amplification.

Dilute the sample DNA templates in MOPS/EDTA Buffer to an appropriate concentration, so 5 μ l of template is added per reaction.

Standard Reference Template for the Standard Curve

Use a DNA template of known concentration as the standard reference template. A standard reference template with any unit of concentration or amount can be used to generate the standard curve. In general, copy number or mass is used. However, other units that are appropriate for your experiment, such as plaque forming units or dilution factors from a known stock, can be used.

Serial dilutions of this standard reference template are amplified, and the results are used to generate a standard curve and determine the concentrations of unknown samples. The same primers that are used to amplify your samples must be used to amplify the standard reference template. We recommend performing duplicate or triplicate amplification reactions with each dilution of the standard reference template. Instructions for preparing serial dilutions of the standard reference template are provided in Section 7.C. An example of a plate layout for the standard curve is shown in Figure 4.

Dilute the standard reference template in MOPS/EDTA Buffer. Prepare the dilution series, so 5µl of DNA template is added to each reaction. For example, to add 1,000 copies per reaction, dilute the standard reference template to 1,000 copies/5µl or 200 copies/µl.

Note: The use of fewer than ten copies of target may produce inconsistent results due to the random distribution inherent in dilutions of samples with low copy numbers.

	1	2	3	4	5	6	7	8	9	10	11	12
A	water	water	water									
B	10 ⁰	10 ⁰	10 ⁰									
C	10 ¹	10 ¹	10 ¹									
D	10 ²	10 ²	10 ²									
E	10 ³	10 ³	10 ³									
F	10 ⁴	10 ⁴	10 ⁴									
G	10 ⁵	10 ⁵	10 ⁵									
H	10 ⁶	10 ⁶	10 ⁶									

Figure 4. Schematic diagram showing distribution of the diluted standard reference template in a 96-well PCR plate. Row A. No-template control. Row B. 1 copy of target sequence. Row C. 10 copies. Row D. 100 copies. Row E. 1,000 copies. Row F. 10,000 copies. Row G. 100,000 copies. Row H. 1,000,000 copies.

4.C. Control Reactions

No-Template Control: Include a no-template control (NTC) reaction for each set of reactions. Add 5µl of MOPS/EDTA Buffer to these reactions in place of the template DNA. There should be no amplification product detected in the NTC reaction. Amplification in the NTC reaction indicates nonspecific amplification or the presence of contaminating DNA.

Positive Control: Include a positive control reaction to verify that reagents and instrumentation perform consistently. The Plexor® qPCR Control, 5X, contains a pair of primers and a synthetic template. One of the primers has an iso-dC residue adjacent to a 5' fluorescein (FAM) label. Product should be detected in the FAM channel during amplification of the positive control reaction. The absence of product formation in the positive control reaction indicates a problem with the Plexor® System reagents, reaction assembly or the real-time PCR instrument. The positive control reaction should have a cycle threshold in the range of 25–35 cycles.

The positive control reaction can also be used to detect amplification inhibitors in the DNA template. To test for inhibitors, add the DNA sample in question to the positive control reaction. Decrease the amount of Nuclease-Free Water, so the final reaction volume is 25µl (20µl final volume if using LightCycler® 20µl capillaries). A decrease in product formation in the positive control reaction or a large increase in the C_t value (>5 cycles) for the positive control reaction indicates the presence of amplification inhibitors in the DNA sample.

4.D. Reaction Setup

1. Thaw the Plexor® Master Mix and primers on ice.
2. Briefly vortex the Plexor® Master Mix and primers to mix. Store on ice.
3. Prepare the reaction mix by combining the Plexor® Master Mix and primers as indicated in Table 1. The reaction mix will be used to amplify the samples and standard reference DNA templates, and to perform the NTC reaction.

Prepare sufficient reaction mix for the desired number of reactions on ice.

Note: Multiplex assays can be performed by adding additional primer pairs specific for each target.

Table 1. Preparation of 25µl Reaction Mix for Quantitation Assays. Note:

Reactions using 20µl LightCycler® capillaries should be scaled down from 25µl reactions to 20µl reactions.

Component	Volume (Per Reaction)
Plexor® Master Mix, 2X	12.5µl
25X (5µM) primer pair ^{1,2}	1.0µl
Nuclease-Free Water	to a final volume of 20.0µl

¹Individual primers can be used. Add 1.0µl of each primer at 5µM.

²For assays where more than one pair of primers will be used (i.e., multiplex assays), add 1.0µl of each primer pair.

- Vortex briefly to mix.
- Assays with sample DNA templates:** Add 20µl of the reaction mix prepared in Step 3 (Table 1) to each of the appropriate cuvettes or wells of an optical-grade PCR plate on ice (15µl to LightCycler® 20µl capillary). Add 5µl of the sample DNA template to the appropriate well, cuvette or capillary.

Assays for the standard curve: Add 20µl of the reaction mix prepared in Step 3 (Table 1) to each of the appropriate cuvettes or wells of an optical-grade PCR plate on ice (15µl to LightCycler® 20µl capillary). Add 5µl of the appropriate dilution of the standard reference template to each well, cuvette or capillary.

No-template control reactions: Add 20µl of the reaction mix prepared in Step 3 (Table 1) to each of the appropriate cuvette or well of an optical-grade PCR plate on ice (15µl to LightCycler® 20µl capillary). Add 5µl of MOPS/EDTA Buffer to each well, cuvette or capillary.

- Assemble the positive control reaction as indicated in Table 2. Assemble the reaction on ice.

Table 2. Assembling the Positive Control Reaction (25µl). Note: Reactions using 20µl LightCycler® capillaries should be scaled down from 25µl reactions to 20µl reactions.

Component	Volume (Per Reaction)
Plexor® Master Mix, 2X	12.5µl
Nuclease-Free Water	7.5µl
Plexor® qPCR Control, 5X	5.0µl

- Vortex the reactions briefly to mix. Transfer the positive control reaction to the appropriate capillary, cuvette or well of an optical-grade PCR plate on ice.

4.E. Real-Time Analysis

After the amplification reactions have been assembled, prepare the capillaries, cuvettes or plates as described in the appropriate Instrument Setup and Data Analysis Technical Manual for the real-time PCR instrument used (these manuals are available at: www.promega.com/plexorresources/).

More information about cycling conditions for specific real-time instruments and instructions on data export for the Plexor® Analysis Software are included in the specific Instrument Setup and Data Analysis Technical Manual for the real-time PCR instrument used.

5. Genotyping Assays

5.A. Assay Design

The Plexor® qPCR System can be used for genotyping assays to distinguish between two alternative bases at a specific site in a known DNA sequence. For genotyping, the Plexor® qPCR System uses three primers in an allele-specific PCR. There are two genotyping primers. Each of the genotyping primers contain one of the alternative bases near the 3' end. These two genotyping primers both contain an iso-dC residue but are labeled with different fluorescent labels, so they can be differentiated. The third primer is the anchor primer. When used in a reaction with either genotyping primer, the anchor primer will amplify the target sequence. The anchor primer is unlabeled.

The design of the genotyping primers enhances amplification specificity. The two genotyping primers have an annealing temperature lower than the anchor primer. Sequence that is not complementary to the template is added to the 5' end of each genotyping primer. This makes the melting temperatures of the genotyping primers similar to that of the anchor primer after the first cycle of amplification is complete (Figure 5). The noncomplementary sequence added to the 5' ends of each genotyping primer must be different.

The annealing temperature for the first round of amplification is 50°C to allow the primers to anneal and be extended. Subsequent rounds are performed using an annealing temperature of 60°C, which is the melting temperature (T_m) of the primer pair including the noncomplementary bases. During the subsequent rounds of amplification, performed at the higher annealing temperature, the target formed in the first round is amplified.

Following thermal cycling, a melt curve determination is performed for all samples. To assign a genotype, examine the fluorescent signal in the melt curve for reactions with each of the labeled genotyping primers. A change in fluorescence at the correct melt temperature indicates product formation, allowing you to determine which genotyping primer was complementary to the target sequence and therefore the sequence at the polymorphic site. A change in fluorescent signal for both dyes indicates product formation with both genotyping primers, indicating the DNA sample is heterozygous at that locus.

When designing your assays, be sure to include the appropriate control reactions containing templates characterized as homozygous allele 1, heterozygous or homozygous allele 2, as well as a no-template control reaction and a positive control reaction (see Section 6.C).

Note: Optimization of conditions for genotyping assays may be required. Changes to the amount of input DNA, cycle number or annealing temperature may result in improved specificity.

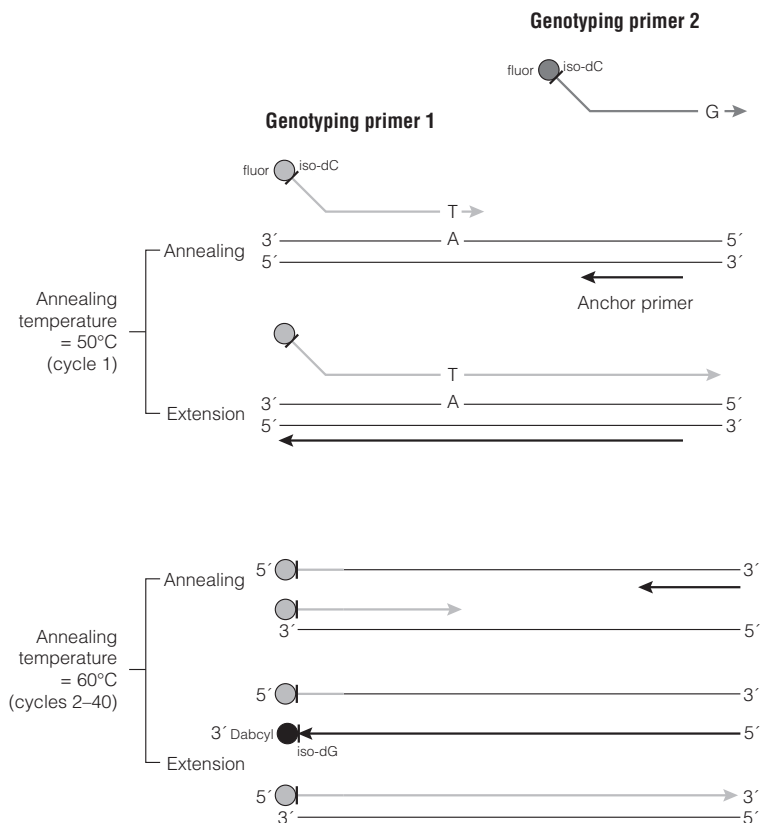


Figure 5. Schematic of a genotyping experiment using the Plexor® qPCR System.

5.B. Primer Design for Genotyping Assays

Promega has developed a web-based primer-design program for use with the Plexor® qPCR System. We strongly recommend using this software, which can be found at: www.promega.com/plexorresources/. This Plexor® Primer Design Software designs primers for genotyping using the parameters described in Section 7.B.

Fluorescently labeled oligonucleotides for use as primers in Plexor® qPCR System assays must be ordered with an iso-dC residue adjacent to a 5' fluorescent label. A number of oligonucleotide suppliers have been licensed to provide these iso-dC-containing primers. Convenient links to these suppliers' web sites are included within the Plexor® Primer Design Software.

6. Genotyping Protocol

Prior to reaction assembly, review the appropriate instrument-specific Technical Manual for the particular instrument used to detect the amplified products. A complete list of Instrument Setup and Data Analysis Technical Manuals can be found at: www.promega.com/plexorresources/

When using the Plexor® qPCR System for the first time, we recommend programming the thermal cycling conditions and checking that the instrument is compatible and configured for the dyes used before assembling the reactions, so the reactions are not kept on ice for prolonged periods of time before thermal cycling. Once you are familiar with the programming process, the instrument can be programmed after reaction assembly.

Materials to Be Supplied By the User


(Solution compositions are provided in Section 7.E.)


- control templates characterized as homozygous allele 1, heterozygous or homozygous allele 2
- sterile, aerosol-resistant pipette tips
- pipettes dedicated to pre-amplification work
- genotyping and anchor primers
- crushed ice
- real-time PCR instrument and related consumables, such as the appropriate capillary, cuvette or PCR plate and plate covers
- the appropriate Instrument Setup and Data Analysis Technical Manual for the real-time PCR instrument used
- Plexor® Analysis Software

6.A. Primer Preparation

Primers containing iso-dC must be stored in a buffer with a pH greater than 7.0. Use the supplied MOPS/EDTA Buffer to resuspend and dilute the primers.

Prepare 25X primer stocks using the MOPS/EDTA Buffer. For most assays, the 25X concentration of each of the genotyping primers will be 5µM (for a final concentration of 200nM) and the 25X concentration of the anchor primer will be 10µM (for a final concentration of 400nM). Primers can be prepared and stored as individual primers or as primer mixes (5µM of each labeled primer and 10µM of the anchor primer).

 The concentration of the unlabeled anchor primer should be equal to the combined concentration of the labeled primers. Since there are two labeled genotyping primers, this is twice the concentration of each labeled primer (i.e., 10µM at 25X, 400nM at 1X).

 Iso-dC-containing primers are sensitive to pH and must be resuspended and diluted in MOPS/EDTA Buffer to maintain primer integrity. **Do not use water to resuspend or dilute primers or prepare primer mixes.** Fluorescently labeled primers are light-sensitive and must be stored in the dark.

6.B. Template Preparation

Standard curves are not required for genotyping assays, so serial dilution of a standard reference template of known concentration is not necessary.

Dilute the genomic DNA template in MOPS/EDTA Buffer to an appropriate concentration, so 5 μ l of template is added per reaction.

For example, to add 10ng of genomic DNA to each reaction, dilute the DNA to a concentration of 2ng/ μ l (10ng/5 μ l).

Note: Use 10-100ng of genomic DNA per reaction.

6.C. Control Reactions

Include control reactions that contain templates characterized as homozygous allele 1, heterozygous or homozygous allele 2.

Include a no-template control (NTC) reaction for each set of reactions. Add 5 μ l of MOPS/EDTA Buffer to these reactions in place of the template DNA. There should be no amplification product detected in the NTC reaction. Amplification in the NTC reaction indicates nonspecific amplification or the presence of contaminants.

Include a positive control reaction to verify that the reagents and instrument perform consistently. The Plexor[®] qPCR Control, 5X, contains a pair of primers and a synthetic template. One of the primers has an iso-dC residue adjacent to a 5' fluorescein (FAM) label. Product should be detected in the FAM channel during amplification of the positive control reaction. The absence of product formation in the positive control reaction indicates a problem with the Plexor[®] System reagents, reaction assembly or the real-time PCR instrument. The positive control reaction should have a cycle threshold in the range of 25-35 cycles.

The positive control reaction can be used to detect amplification inhibitors in the DNA template. To test for inhibitors, add the DNA sample in question to the positive control reaction. Decrease the amount of Nuclease-Free Water, so the final reaction volume is 25 μ l (20 μ l when using LightCycler[®] 20 μ l capillaries). A decrease in product formation or a large increase in the C_t value (>5 cycles) for the positive control reaction indicates the presence of amplification inhibitors in the DNA sample.

6.D. Reaction Setup

Optimization of conditions for genotyping assays may be required. Changes to the amount of input DNA, cycle number or annealing temperature may result in improved specificity.

1. Thaw the Plexor® Master Mix and primers on ice.
2. Briefly vortex the Plexor® Master Mix and primers to mix. Store on ice.
3. Prepare the reaction mix by combining the Plexor® Master Mix and primers as indicated in Table 3. The reaction mix will be used to amplify the sample DNA templates and to perform the NTC reaction.

Prepare sufficient reaction mix for the desired number of reactions on ice.

Table 3. Preparation of 25µl Reaction Mix for Genotyping Assays. Note:

Reactions using 20µl LightCycler® capillaries should be scaled down from 25µl reactions to 20µl reactions.

Component	Volume (Per Reaction)
Plexor® Master Mix, 2X	12.5µl
25X primer mix (5µM each genotyping primer and 10µM anchor primer) ¹	1.0µl
Nuclease-Free Water	to a final volume of 20.0µl

¹When using a primer mix, be sure that the final concentration of the anchor primer (10µM) is twice that of each genotyping primer (5µM). These instructions assume a mix of all three primers, but individual primers can be used.

4. Vortex the reaction mix briefly to mix.
5. **Assays with sample DNA templates:** Add 20µl of the reaction mix prepared in Step 3 (Table 3) to each of the appropriate cuvettes or wells of an optical-grade PCR plate on ice (15µl to LightCycler® 20µl capillary). Add 5µl of the DNA template to the appropriate well, cuvette or capillary.

No-template control reactions: Add 20µl of the reaction mix prepared in Step 3 (Table 3) to each of the appropriate cuvette or well of an optical-grade PCR plate on ice (15µl to LightCycler® 20µl capillary). Add 5µl of MOPS/EDTA Buffer to each well, cuvette or capillary.

6. Assemble the positive control reaction mix by combining the reagents as indicated in Table 4. Assemble the reaction on ice.

Table 4. Assembling the Positive Control Reaction. Note: Reactions using 20µl LightCycler® capillaries should be scaled down from 25µl reactions to 20µl reactions.

Component	Volume (Per Reaction)
Plexor® Master Mix, 2X	12.5µl
Nuclease-Free Water	7.5µl
Plexor® qPCR Control, 5X	5.0µl

7. Vortex the positive control reaction briefly to mix. Transfer the positive control reaction to the appropriate capillary, cuvette or well of an optical-grade PCR plate on ice.

6.E. Real-Time Analysis

After the amplification reactions have been assembled, prepare the capillaries, cuvettes or plates as described in the appropriate Instrument Setup and Data Analysis Technical Manual for the real-time PCR instrument used (these manuals are available at: www.promega.com/plexorresources/).

More information about cycling conditions for specific real-time instruments and instructions on data export for the Plexor® Analysis Software are included in the specific Instrument Setup and Data Analysis Technical Manual for the real-time PCR instrument used.

7. Appendix

7.A. Primer-Design Parameters for Quantitative PCR

The web-based Plexor® Primer Design Software, which is described in Section 3.B, uses the following parameters to design primers for use with the Plexor® qPCR System.

1. The primers amplify a region of target sequence that is a minimum of 40bp in size and a maximum of 150bp. The optimal size is 100bp.
2. The primers have a T_m of 60–70°C. The optimal T_m is 65°C.

Note: The software uses T_m -calculating algorithms that are not available elsewhere, so the melting temperature calculated with this software may not be identical to melting temperatures calculated by other means.

3. The primers are typically 20–35 bases in length.
4. The primers must not contain 4 or more consecutive G residues or 4 or more consecutive C residues.
5. The primers must not contain 10 or more consecutive A residues or 10 or more consecutive T residues.
6. The primers do not contain a GC clamp. A GC clamp is defined as 3 G or C residues at the 3' end of the primer.
7. One of the primers must contain a 5' fluorescent label attached to an iso-dC residue. The chosen fluorescent label depends upon the detection capabilities of the real-time PCR instrument. More information on the compatibility of fluorescent labels and real-time PCR instruments is available at: www.promega.com/plexorresources/

A number of oligonucleotide suppliers have been licensed to provide these iso-dC-containing primers. Convenient links to these suppliers' web sites are available at: www.promega.com/plexorresources/

7.B. Primer-Design Parameters for Genotyping Assays

The web-based Plexor® Primer Design Software, which is described in Section 3.B, uses the following parameters to design genotyping primers for use with the Plexor® qPCR System. The software also designs the anchor primer.

1. The two genotyping primers differentiate between the possible alleles for the polymorphism of interest (genotyping primer #1 and genotyping primer #2). The primers will differ in sequence at the polymorphic site. The polymorphism will be positioned 1 or 2 bases upstream of the 3' terminal nucleotide.
2. The primers amplify a region of the target sequence that is 40–120bp in length. The genotyping primers have a T_m of 48–52°C.

Note: The software uses T_m -calculating algorithms that are not available elsewhere, so the melting temperature calculated with this software may not be identical to melting temperatures calculated by other means.

3. The Plexor® Primer Design Software automatically adds a short, random sequence to the 5' end of each genotyping primer to increase the T_m to approximately 60°C. This additional sequence is not complementary to the target and is different for the two genotyping primers.
4. Each genotyping primer contains an iso-dC residue and a fluorescent label at the 5'-end. The two genotyping primers are labeled with different fluorescent dyes that are compatible with the detection capabilities of the real-time PCR instrument. More information about compatibility of fluorescent labels and real-time PCR instruments, is available at:
www.promega.com/plexorresources/

A number of oligonucleotide suppliers have been licensed to provide these iso-dC-containing primers. Convenient links to these suppliers' web sites are available at: **www.promega.com/plexorresources/**

5. When used with either genotyping primer, the anchor primer amplifies the target sequence. This primer has a T_m of 58–62°C, is approximately 18–27bp in length and has no 3' self-complementary regions.

7.C. Serial Dilution of a Standard Reference Template

A standard reference template with any units of concentration or amount can be used to generate the standard curve. In general, copy number or mass is used, but other units that are appropriate for your experiment can be used, such as plaque forming units or dilution factors from a known stock.

1. Thaw and vortex a standard reference template of known concentration.
2. Prepare serial dilutions of the standard reference template. Be sure to change pipette tips between dilutions.

An example of a tenfold dilution series starting with a standard reference template at a concentration of 10^7 copies/ μ l is given below.

- a. For a tenfold dilution series, label microcentrifuge tubes as 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 and 10^0 .
- b. Add 90 μ l of water to each tube.
- c. Add 10 μ l of the standard reference template to the first tube (10^6) and vortex to mix.
- d. Transfer 10 μ l from the 10^6 tube to the next tube in the series (10^5) and vortex to mix.
- e. Transfer 10 μ l from the 10^5 tube to the next tube in the series (10^4) and vortex to mix.
- f. Continue this dilution series with the tubes labeled 10^3 , 10^2 , 10^1 and 10^0 .

7.D. References

1. Sherrill, C.B. *et al.* (2004) Nucleic acid analysis using an expanded genetic alphabet to quench fluorescence. *J. Am. Chem. Soc.* **126**, 4550-6.
2. Johnson, S.C. *et al.* (2004) A third base pair for the polymerase chain reaction: Inserting isoC and isoG. *Nucl. Acids Res.* **32**, 1937-41.
3. Moser, M.J. and Prudent, J.R. (2003) Enzymatic repair of an expanded genetic information system. *Nucl. Acids Res.* **31**, 5048-53.

7.E. Composition of Buffers and Solutions

1M MOPS (pH 7.5)

209.3g MOPS free acid (Sigma
Cat.# M1254)
100ml 5M sodium hydroxide

Dissolve MOPS free acid in 750ml of autoclaved, deionized water. Adjust to pH 7.5 with sodium hydroxide. Bring the volume to 1 liter with autoclaved deionized water.

MOPS/EDTA Buffer (1mM MOPS, 0.1mM EDTA)

1.0ml 1M MOPS (pH 7.5)
0.2ml 0.5M EDTA (pH 8.0)
998.8ml autoclaved, deionized water

7.F. Related Products

Genomic DNA Purification, Manual Systems

Product	Size	Cat. #
Wizard® SV Genomic DNA Purification System	50 preps	A2360
	250 preps	A2361
Vacuum Adapters	20 each	A1331
Wizard® Genomic DNA Purification Kit*	100 isolations	A1120
	500 isolations	A1125

*For Laboratory Use.

Genomic DNA Purification, Manual or Automated Systems

Product	Size	Cat. #
Wizard® SV 96 Genomic DNA Purification System	1 x 96 preps	A2370
	4 x 96 preps	A2371
Vac-Man® 96 Vacuum Manifold	1 each	A2291
Wizard® Magnetic 96 DNA Plant System	2 x 96 preps	FF3760
	4 x 96 preps	FF3761
MagnaBot® 96 Magnetic Separation Device	1 each	V8151
MagnaBot® Spacer	1 each	V8381

Genomic DNA Purification, Automated Systems

Product	Size	Cat. #
MagneSil® ONE, Fixed Yield Blood Genomic System*	1 x 96 preps	MD1370
MagneSil® Blood Genomic, Max Yield System*	1 x 96 preps	MD1360
Deep Well MagnaBot® 96 Magnetic Separation Device*	1 each	V3031
MagnaBot® Spacer, 1/8 inch	1 each	V8581
MagneSil® Genomic, Large Volume System*	8 preps	A4080
	48 preps	A4082
	96 preps	A4085

*For Laboratory Use.

RNA Purification, Manual Systems

Product	Size	Cat. #
SV Total RNA Isolation System	10 preps	Z3101
	50 preps	Z3100
	250 preps	Z3105
RNAgents® Total RNA Isolation System	Scalable	Z5110

*For Laboratory Use.

RNA Purification, Manual or Automated System

Product	Size	Cat. #
SV 96 Total RNA Isolation System	1 x 96 preps	Z3500
	5 x 96 preps	Z3505

*For Laboratory Use.

RNA Purification, Automated System

Product	Size	Cat. #
MagneSil® Total RNA mini-Isolation System	4 plate	Z3351

*For Laboratory Use.

cDNA Synthesis System

Product	Size	Cat. #
ImProm-II™ Reverse Transcription System	100 reactions	A3800

*For Laboratory Use.

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^(b)The PCR process, which is the subject of European Pat. Nos. 201,184 and 200,362 owned by Hoffmann-LaRoche*, is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process. Use of this product is recommended for persons that either have a license to perform PCR or are not required to obtain a license.

*The above primary European Pat. Nos. 201,184 and 200,362 will expire on March 28, 2006. In the U.S., the patents covering the foundational PCR process expired on March 29, 2005.

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