Validation of the Plexor™ Primer Design System

Katharine Hoffmann, Benjamin Krenke, Cynthia Sprecher, and Douglas Storts
Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, USA
Abstract #240 (PCR NOS Session 36)

1. Abstract

The Plexor™ Primer Design System was used to design primers for twenty different human mRNA targets suggested by a collaborator. Each primer pair was designed to span an intron, minimizing the potential of generating an amplification product from genomic DNA. A BLAST search was performed against the NCBI database to verify the primers were specific for the mRNA target of interest. All primer pairs were designed for use in duplex reactions targeting a 'housekeeping' gene (GAPDH). Quantitative RT-PCR was performed with the Plexor™ Two-Step qRT-PCR System. We will present data demonstrating the results of the validation study.

2. Introduction

The Plexor™ qPCR and qRT-PCR Systems[a-b] are multiplex-capable, real-time amplification systems that use novel base-pair chemistry (1–4). Each target is measured directly during the amplification process, as opposed to using a secondary reaction to detect product accumulation (e.g., probe cleavage).

The Plexor™ Systems work by measuring a reduction in fluorescent signal during amplification. Amplification uses only two primers, one of which contains both a 5'-fluorescent tag and an adjacent modified base. As amplification proceeds, fluorescence is reduced by site-specific incorporation of a fluorescent quencher, which is attached to a modified nucleotide inserted opposite the complementary modified base. The quencher is in close proximity to a fluorescent dye, resulting in a reduction in the fluorescent signal. After PCR, a melt analysis can be performed to expedite troubleshooting during assay development and provide an internal control. The system also includes a proprietary reagent to minimize primer-dimer formation.

3. Primer Design

The Plexor™ Primer Design Software is a freely available, web-based program (www.promega.com/plexorresources/) that designs primers for monoplex and multiplex qPCR. The software was used to design primers for twenty different human mRNA targets suggested by a collaborator. Each primer pair was designed to span an intron, minimizing the potential of generating an amplification product from genomic DNA. A BLAST search was performed against the NCBI GenBank database to verify the primers were specific for the mRNA target of interest. All primer pairs were designed for use in duplex reactions targeting a 'housekeeping' gene (glyceraldehyde-3-phosphotransferase; GAPDH). The GAPDH primer set, labeled with CAL Fluor® Red 610, was obtained from Biosearch Technologies (Cat. # PLX-3002-1). The other mRNA-specific primers were synthesized by Biosearch Technologies and labeled with FAM™.

4. Quantitative, Real-Time RT-PCR

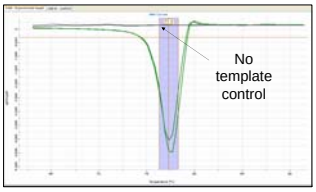
Human total RNA (Stratagene Universal Human Reference RNA) was converted to cDNA as directed in the *Plexor™ Two-Step qRT-PCR System Technical Manual* (available at www.promega.com/plexorresources/). The qPCR reactions were assembled with cDNA generated from 10ng of total RNA. Amplification was performed in 25µl reactions using 200nM of target-specific primers, except for the GAPDH primers, which were used at 100nM. Amplification was performed for 40 cycles as recommended in the *Plexor™ Two-Step qRT-PCR System Technical Manual*. Data were collected on an Applied Biosystems 7500 Real-Time PCR System and analyzed using the Plexor™ Analysis Software (also available at www.promega.com/plexorresources/).

5. Ideal Assays

Nineteen of twenty duplex assays produced useable data at the first design, without optimization. Fourteen assays were ideal, yielding quantitative results over multiple log dilutions of cDNA (data not shown), a single amplification product (as evidenced by a discrete melt curve) and no background amplification in the no-template control reactions. The melt curve data for one of these assays, retinoblastoma, clearly demonstrates a single, well-defined melt curve. Comparable melt curves (not shown) were observed for the other targets in this group of assays.

Cycle threshold (C_t) values from duplex assays producing no nonspecific amplification in duplex with GAPDH. (No-template controls did not generate a detectable, nonspecific amplification product in 40 cycles).

Accession Number	Name	Target C_t (Avg)	GAPDH C_t (Avg)	ΔC_t
NM_000321	Retinoblastoma RB1	26.97	16.96	10.01
NM_000942	Cyclophilin B	24.67	17.11	7.56
NM_001237	Cyclin A2	26.42	17.13	9.28
NM_001838	Chemokine receptor 7	34.82	16.86	17.96
NM_001949	E2F transcription factor 3	28.77	17.08	11.69
NM_002853	RAD1 homolog, transcript variant 1	28.19	17.59	10.60
NM_002895	Retinoblastoma-like 1 RBL1 transcript variant 1	29.85	16.77	13.08
NM_004091	E2F transcription factor 2	30.19	16.61	13.58
NM_004526	MCM2 mini-chromosome maintenance deficient 2	26.51	16.74	9.77
NM_004701	Cyclin B2	27.11	17.09	10.02
NM_005611	Retinoblastoma-like 2 RBL2	28.70	16.61	12.09
NM_005914	MCM4 mini-chromosome maintenance deficient 4	25.83	16.94	8.89
NM_031966	Cyclin B1	24.67	17.44	7.23
NM_033031	Cyclin B3	34.57	17.35	17.22



Melt curve of retinoblastoma product. Data is presented for the FAM™ channel.

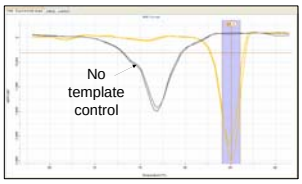
6. Usable Assays

Three designs produced sharp melt curves, but also yielded aberrant products in the no-template control reactions. An example of the results observed for this group of assays is represented by the lamin A/C transcript variant 1 experiment. Although a discrete melt curve attributable to the lamin A/C amplicon was observed, some nonspecific amplification occurred in the no-template control reactions during the late cycles.

Cycle threshold (C_t) values from assays generating sharp melt curves, but some background amplification in duplex with GAPDH.

Accession Number	Name	Target C_t (Avg)	GAPDH C_t (Avg)	ΔC_t	Target NTC* (Avg)	GAPDH NTC* (Avg)
NM_003914	Cyclin A1	33.78	16.92	16.86	37.16	35.32
NM_004935	Cyclin-dependent kinase 5	26.51	17.04	9.47	34.55	>40
NM_170707	Lamin A/C (LMNA), transcript variant 1	25.49	17.15	8.33	32.32	>40

*NTC = No-template Controls.



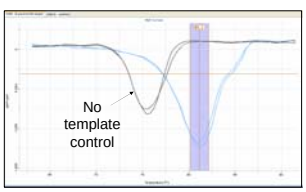
Melt curve of lamin A/C amplification product. Data is presented for the FAM™ channel.

Two designs were target specific, but had broader melt curves than other assays. An example of the results observed for this group of assays is evident in the MCM3 mini-chromosome maintenance deficient 3 experiment. The amplicon yields a broad melt curve and an amplification artifact in the no-template control reaction. The artifact can clearly be distinguished from the desired product based upon the melt temperature. The assays that produced broad melt curves may be improved by primer redesign, but may be the result of a heterogeneous target population due to internal polymorphism.

Cycle threshold (C_t) values from assays generating broad melt curves and varying levels of nonspecific amplification.

Accession Number	Name	Target C_t (Avg)	GAPDH C_t (Avg)	ΔC_t	Target NTC* (Avg)	GAPDH NTC* (Avg)
NM_001799	Cyclin-dependent kinase 7	27.44	16.79	10.65	>40	>40
NM_002388	MCM3 mini-chromosome maintenance deficient 3	25.80	17.33	8.47	38.62	36.21

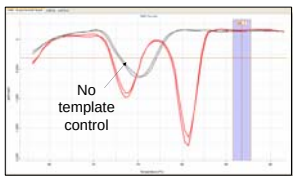
*NTC = No-template Controls.



Melt curve of MCM3 amplification product. Data is presented for the FAM™ channel.

7. Unacceptable Assay

The assay for cyclin-dependent kinase 3 did not yield usable data. Two different products, as evidenced by two discrete melt curves, were observed in the experiment. The presence of two discrete products may be attributed to transcript variants, or primers annealing to another RNA target. In addition, a nonspecific amplification product was observed in the no-template control reaction. This assay clearly requires redesign.



Melt Curve of Cyclin-dependent kinase 3 amplification product. Data is presented for the FAM™ channel.

8. Conclusion

The Plexor™ Primer Design System software, when used in conjunction with the Plexor™ qRT-PCR System reagents, offers a robust method to design multiplex, real-time amplification assays. The data show that 70% success for ideal assays and 95% for useable assays can be achieved using this software.

9. References

1. Johnson, S.C. et al. (2004) *Nucl. Acids Res.* **32**, 1937–41.
2. Frackman, S. et al. (2005) *Promega Notes* **90**, 2–4.
3. Sherrill, C.B. et al. (2004) *J. Am. Chem. Soc.* **126**, 4550–6.
4. Moser, M.J. et al. (2005) *Antimicrob. Agents Chemother.* **49**, 3334–40.