Improving Reliability and Reproducibility of Real-Time PCR Reactions Sealed With Clear Films

Introduction

Clear adhesive sealers are a convenient sealing option for real-time quantitative PCR because they transmit light effectively and are easy to use. When choosing a sealer, however, it is important to consider its sealing strength as well as its clarity. Many adhesive sealers lack the aggressive adhesion necessary for properly sealing a PCR plate, and therefore can produce inconsistent results.

Improper sealing of reaction wells during thermal cycling is problematic because it can allow the aqueous portion of the sample to evaporate, increasing the concentration of the remaining reagents. This can lead to variability in PCR success, efficiency, and accuracy. Some reactions may fail completely, showing no product, while others may have a nonexponential increase in fluorescence, a late cycle threshold (C_T), or an abnormal melt profile. Such variability complicates the interpretation of results, since it may not be obvious which data are inaccurate.

Many plate sealers marketed for PCR are promoted as being superior due to an appearance of high clarity, without reference to sealing efficiency. The implication is that sealers that appear clearer or thinner would allow greater light transmission and perhaps greater sensitivity or earlier C_T values. But the potential for small improvements in sensitivity must be weighed against the potential loss in accuracy if inadequate sealing leads to questionable results.

We measured reaction variability of Microseal® 'B' seals (catalog #MSB-1001) and two other clear sealers that are marketed for real-time PCR to determine which sealers produced the most desirable results. All seals were tested with and without an optical compression pad (ADR-3296). The Microseal 'B' seals and compression pad are also available in an optical film sealing kit (MSO-1001). Reactions sealed with optical cap strips (TCS-0803) were run for comparison. We also compared the light transmission of all sealers. The results support the view that tight sealing is a more important consideration than apparent clarity for obtaining consistent results in real-time PCR.

Analysis of Reaction Variability

To assess reaction variability, human DNA was used as template in SYBR Green I quantitative PCR reactions. Initial template amount was 10^3 copies; final concentration was 2.0 ng/µl. Twenty-four 20 µl samples were arranged in a checkerboard pattern distributed across six columns and eight rows of a Hard-Shell® 96-well PCR plate with white wells and white shells (HSP-9655). Samples were amplified using a Chromo4™ real-time PCR detection system. All tests were run in triplicate.

Fluorescence traces and melt profiles were examined to assess reaction variability and determine sealing failure rate. Sealing failures were quantified in three steps: First, wells with a cycle threshold (C_T) or melting temperature (T_m) clearly different from the majority of wells were eliminated and counted as failures. Next, the mean C_T and T_m for the remaining wells were calculated. Finally, any wells in which the C_T or T_m differed by more than ±0.75 cycles from the mean were counted as failures. The standard deviation (SD) of the C_T of successful reactions was used as a measure of reaction variability.

When film seals were used without the compression pad, all test samples exhibited some level of sealing failure and greater reaction variability compared to caps (Figure 1; Table 1). Microseal 'B' seals had considerably fewer reaction failures (8/72 reactions in three runs) than film of either competitor A (29/72 reactions) or competitor B (27/72 reactions). Microseal 'B' seals also showed less reaction variability than for competitors A and B (Table 1). In contrast, no sealing failures occurred and reaction variability was considerably lower when optical cap strips were used.

Use of the compression pad further reduced reaction variability and virtually eliminated sealing failures. When the compression pad was used, no more than 1 of 72 reactions failed for any sealer. Reaction variability was improved for all film seals, but all reactions continued to exhibit greater variability than with optical caps (Table 1).

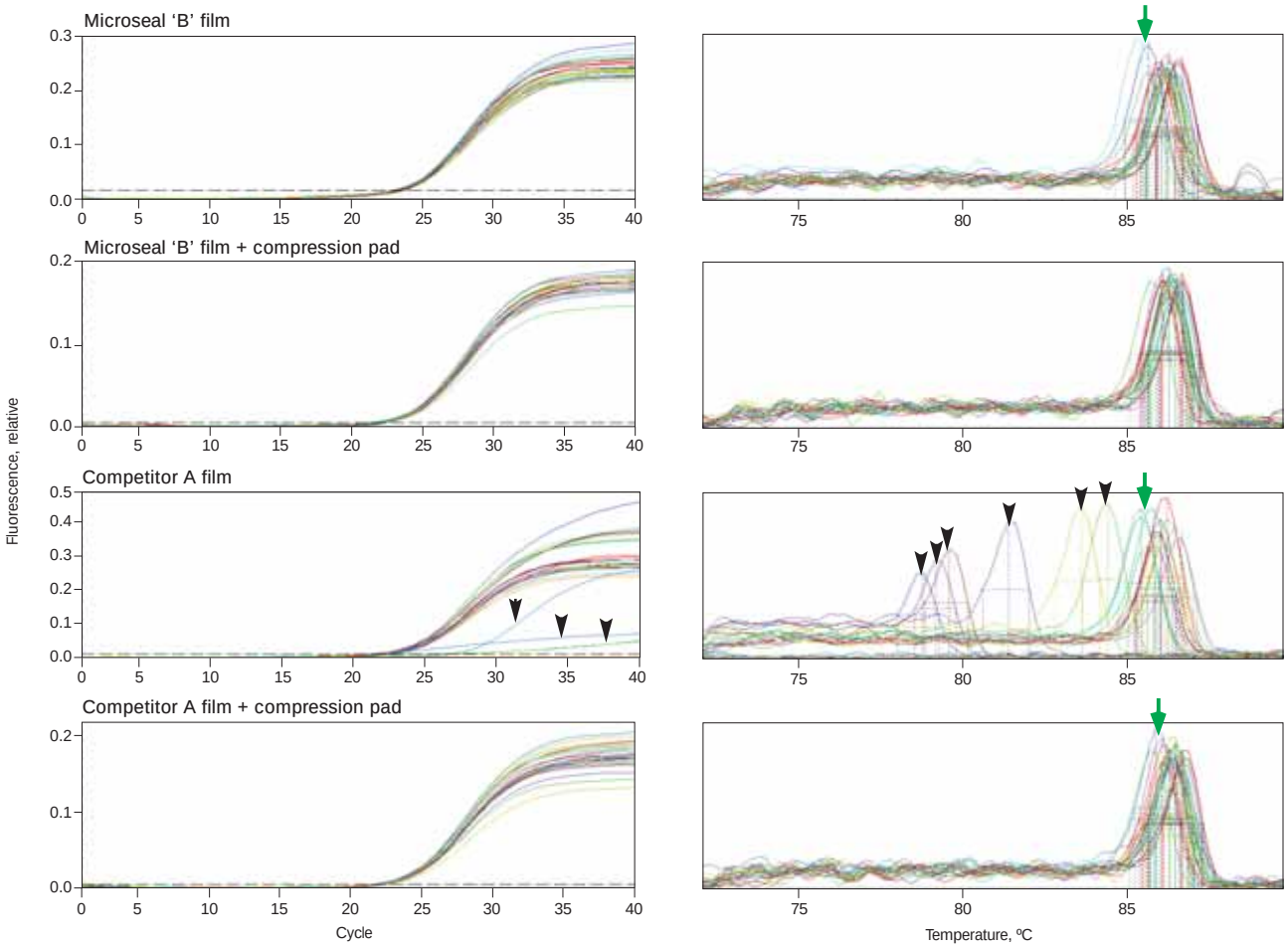

Fig. 1. Comparison of real-time PCR results with various plate film sealers. Left panels, amplification profiles. With the competitor's seals, there were reaction failures (arrowheads) when the pad was not used. Right panels, melting profiles. With the competitor's seals, there were spurious melt peaks (arrowheads) when the pad was not used. In addition, increased variability in the melting temperature (green arrows) was consistently observed with the competitor's seals (both with and without pads), but not with Microseal 'B' film plus a pad. Data shown are the best of three runs performed with each test condition.

Analysis of Light Transmission

To measure light transmission, fluorescence was measured for each sealer in each of the four channels of the Chromo4 real-time system. Different experimental plates, containing different channel-specific reporter dyes (FAM, HEX, ROX, and Cy5) attached to a 15-base poly(T) oligonucleotide, were prepared for each channel. Reaction components were assembled and 20 µl volumes were pipetted into each well of Hard-Shell 96-well PCR plates with white wells and white shells.

Each plate was read 5 times at 25°C, first without a sealer and then with each of the sealers. After each series of reads, the sealer was removed and another sealer was placed on the plate. For each sealer, raw fluorescence measurements (without background subtraction) from five reads of all 96 wells were averaged for each channel, and well-to-well variability was calculated. Light transmission for each sealer was then calculated by dividing the raw signal obtained with the sealer by the raw signal obtained with no sealer, and was expressed as a percentage.

Table 1. Reaction successes and failures for three film seals with or without a compression pad.

Sealer	Success Rate (% of Wells)	C_T Spread (SD of Successful Reactions)	Total # of Failed Wells	# Failed With Low T_m	# Failed With Early C_T	# Failed With Late C_T
Microseal 'B' seal	89	0.48	8	6	0	6
Microseal 'B' seal + pad	99	0.39	1	0	0	1
Competitor A	60	0.67	29	29	8	6
Competitor A + pad	100	0.57	0	0	0	0
Competitor B	63	0.77	27	18	2	19
Competitor B + pad	99	0.51	1	0	1	0
Optical cap strips	100	0.25	0	0	0	0

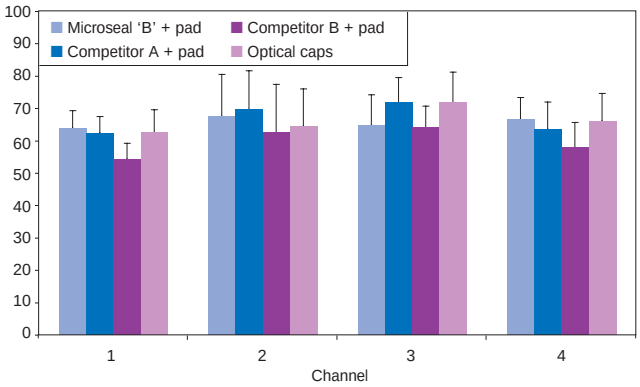

Fig. 2. Effect of using a compression pad. Results using three commercially available films to seal a plate were compared to those with optical caps. Values shown are average ± SD of the percentage of the signal obtained from the four channels of the Chromo4 system with unsealed plates.

Despite obvious differences in thickness, apparent clarity, or both among the film seals, light transmission at the four wavelengths measured was very similar. The fluorescent signals measured for the three seals fell within 10% of each other, and no sealer exhibited problematic signals. When the compression pad was added, transmission was reduced about 30% — to a level similar to transmission through optical caps (Figure 2).

Summary and Recommendations

The results of this study demonstrate that effective sealing is important for achieving low variability and a high success rate in quantitative PCR. Although some suppliers emphasize the clear appearance of their optical sealing films, we found no significant difference in light transmission among the different sealers, indicating that such differences are not responsible for differences in success rate. The sealing methods that produced the lowest variability were optical caps, followed by sealing films with a compression pad.

Our major findings were, first, that Microseal 'B' seals provide the most consistent sealing of the three adhesive seals tested. Second, addition of a compression pad to uniformly distribute the pressure of the heated lid across the sample holder greatly improved the sealing of all films but reduced light transmission slightly, to a level comparable to that of optical caps. Finally, the variability of reactions sealed with adhesive seals was greater than that achieved with optical caps, even when the compression pad was used.

These findings indicate that the choice of sealer should be dictated by the specific needs of an experiment. If obtaining uniform results from all wells is essential, optical caps are preferable to any adhesive seal. On the other hand, if the need for high sensitivity outweighs the need for optimal consistency, use of Microseal 'B' seals without a compression pad may be desirable. When ease of sealing is a greater concern than optimal uniformity, we recommend using Microseal 'B' seals with a compression pad. Although the compression pad slightly reduced light transmission, this effect did not significantly alter the C_T values obtained.

Ordering Information

Catalog #	Description
MSO-1001	Optical Film Sealing Kit, for 96-well plates, includes optical compression pad, 100 Microseal 'B' clear adhesive seals
MSB-1001	Microseal 'B' Clear Adhesive Seals, 100
ADR-3296	Optical Compression Pad, for improved film sealing of 96-well plates in real-time systems
TCS-0803	Optical Flat 8-Cap Strips, for 0.2 ml tubes and plates, ultraclear, 120
HSP-9655	Hard-Shell 96-Well PCR Plates, white well, white shell, rigid two-component design, 50

This tech note was current as of the date of writing (2004) and not necessary the date this version (Rev A, 2005) was published.

Bio-Rad Laboratories, Inc.

Life Science Group

Web site www.bio-rad.com **USA** (800) 4BIORAD **Australia** 02 9914 2800 **Austria** (01)-877 89 01 **Belgium** 09-385 55 11 **Brazil** 55 21 2527 3454
Canada (905) 712-2771 **China** (86 21) 6426 0808 **Czech Republic** + 420 2 41 43 05 32 **Denmark** 44 52 10 00 **Finland** 09 804 22 00
France 01 47 95 69 65 **Germany** 089 318 84-0 **Greece** 30 210 777 4396 **Hong Kong** (852) 2789 3300 **Hungary** 36 1 455 8800
India (91-124)-2398112/3/4, 5018111, 6450092/93 **Israel** 03 951 4127 **Italy** 39 02 216091 **Japan** 03-5811-6270 **Korea** 82-2-3473-4460
Latin America 305-894-5950 **Mexico** 55-52-00-05-20 **The Netherlands** 0318-540666 **New Zealand** 64 9 415 2280 **Norway** 23 38 41 30
Poland + 48 22 331 99 99 **Portugal** 351-21-472-7700 **Russia** 7 095 721 1404 **Singapore** 65-64153188 **South Africa** 00 27 11 4428508
Spain 34 91 590 52 00 **Sweden** 08 555 12700 **Switzerland** 061 717 95 55 **Taiwan** (886 2) 2578 7189/2578 7241 **United Kingdom** 020 8328 2000

Bulletin 5266 US/EG Rev A 05-0610 0805 Sig 1204