TaqMan®-based quantification of invasive cells in the chick embryo metastasis assay

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The chick embryonic metastasis (CEM) assay is a fast in vivo method to investigate the invasive properties of tumor cells. Until now, most quantification methods were semiquantitative and time-consuming. Here we describe a rapid quantification method using TaqMan® technology to quantify the invaded tumor cells in the chorioallantoic membrane of fertilized eggs. This method is based on specific detection of human ALU sequences. Moreover, it provides high sensitivity over a wide linearity range.

INTRODUCTION

The metastatic cascade involves a series of cellular events that are linked both spatially and temporally (1–4). In order to fully understand the cellular, biochemical, and molecular events that contribute to the complexity of this process, in vitro and in vivo models have been developed. For in vitro studies, the Matrigel™ invasion assay, 3-dimensional collagen assay, and the skin reconstruct invasion assay have been successfully established (5–8). Although very useful tools in metastasis research, these in vitro models do not necessarily reflect the physiological events that facilitate the dissemination of tumor cells (8).

The chick embryonic metastasis (CEM) assay provides some advantages over the conventional in vivo models. The chick embryo assay has a long history as a useful and efficient in vivo model for the study of complex physiological processes such as embryonic development, angiogenesis, and tumor metastasis (9–14). Therefore, the chick embryo provides a model to study either spontanous or experimental metastasis in a shorter time period; 7–9 days compared to 4–10 weeks for typical murine models. A drawback is that the chick embryo is a more heterologous system than the murine models. Quantification of metastasis can be conducted in a variety of methods, including flow cytometry, PCR, and enzymatic and/or morphological culture methods.

Detection and quantification of human material in mixed forensic samples is performed with ALU-PCRs (15). The use of ALU-PCR amplification has been reported to be more sensitive than any other method currently used in forensic laboratories (15,16). The semiquantitative PCR method developed by Kim et al. (2) detects invaded tumor cells in the chorioallantoic membrane (CAM) using ALU-specific oligonucleotides. However, this method has some major drawbacks: the PCR is carried out in the presence of [α-^{32}P]CTP, end point PCR limits the linear range of quantification, and PCR products have to be resolved with sodium dodecyl sulfate (SDS) gels and visualized on phosphorimager or X-ray films. The method is time-consuming and harbors the danger of radioactive contamination. Recently, two groups have established a real-time PCR with SYBR® Green using the known ALU primers (17,18).

We provide a major improvement of that method by using ALU sequences of the specific YB8 subfamily, which allow a rapid and absolute quantification with higher specificity of human material in chicken and murine DNA.

MATERIALS AND METHODS

Cell Lines, DNA Samples, and CEM Assay

The melanoma cell line Mel-Juso [Cell Lines Service (CLS), Heidelberg, Germany] and colon carcinoma cell line RKO (ATCC, Rockville, MD, USA) were kept under logarithmic growth conditions according to the suppliers' recommendations. The CEM assay was performed as previously described (2,17). Fertilized special pathogen-free (SPF) eggs were obtained from Charles River (Sulzfeld, Germany). Genomic DNA from either cell lines or chicken CAM was prepared using either DNAzol® (Molecular Research Center, Cincinnati, OH, USA) or PURGENE® (Gentra Systems, Minneapolis, MN, USA) DNA Isolation kits according to the manufacturers' instructions. Genomic mouse DNA was prepared from pooled livers of Balb/c mice (Charles River) as described earlier. Purified DNA was quantified spectrophotometrically and controlled for its high molecular weight on agarose gels. Genomic DNA from Mel-Juso or RKO was serially diluted to concentrations from 50 ng to 23 pg and evaluated in triplicate. Genome sizes were calculated based on the accessible data of the database at http://www.genomesize.com. Diploid genomes of *Mus musculus*, *Gallus domesticus*, and *Homo sapiens* correspond to 6.5, 2.5, and 7 pg, respectively.

Primer Design and PCR Amplification

Oligonucleotide primers for YB8-ALU-S68 were 5′-GTCAGGAGATCGAGACCATCCT-3′ (position 68–90) and for YB8-ALU-AS244 were 5′-AGTGGCGCAATCTCGGC-3′ (position 244–227). The TaqMan® probe YB8-ALU-167 (Applied Biosystems, Foster City, CA, USA) was 5′-6-FAM-AGCTACTCGGGAGGCTGAGGCAGGA-TAMRA-3′ (position 167–192). The primers and probes were designed using Primer Express® software (Applied Biosystems). As a template for primer design, we used the previously described sequence of the ALU-YB8 subfamily (16,19). Oli-

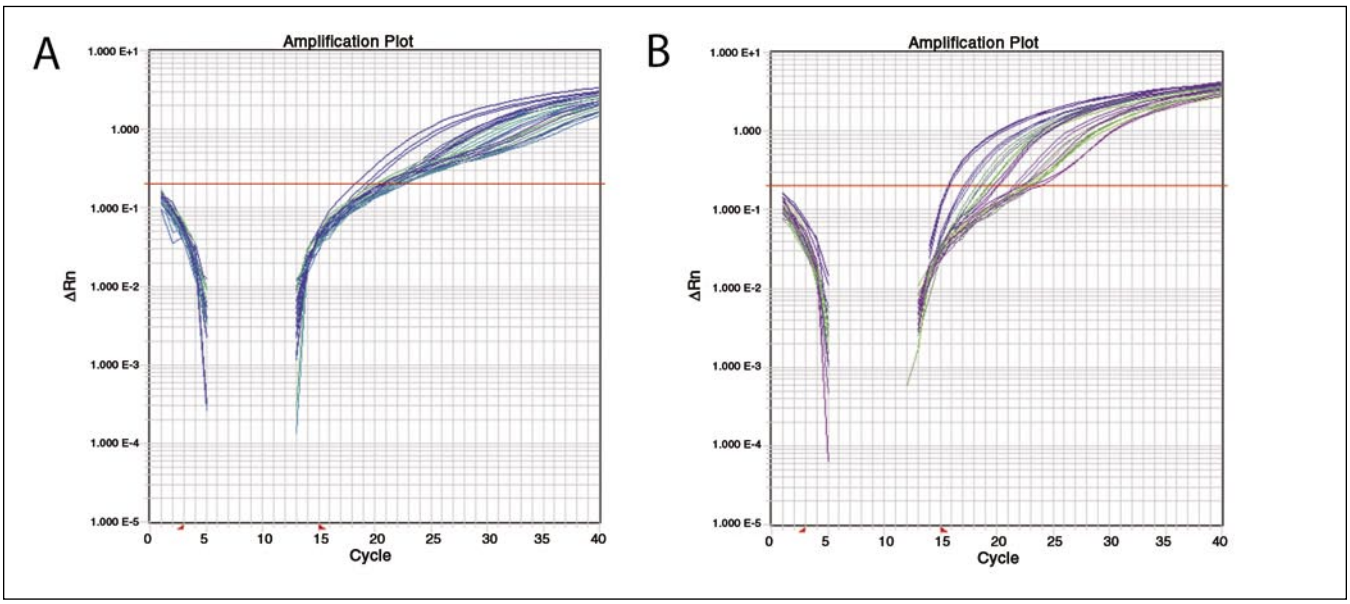

Figure 1. Detection of human ALU sequences with YB8 Alu primers (SYBR Green). Human genomic DNA, equivalent to 1000, 300, 100, 30, 10, 3, 1, and 0.1 cells of the melanoma cell line Mel-Juso, was diluted in (A) 500 or (B) 250 µg chorioallantoic membrane (CAM) DNA and quantified as previously described (15). Detection sensitivity is limited to 1000 cells in panel A and 100 cells in panel B using YB8 Alu primers and SYBR Green. The relative fluorescence (ΔRn) is plotted against the cycle number. The red line indicates the cycle threshold (C_t).

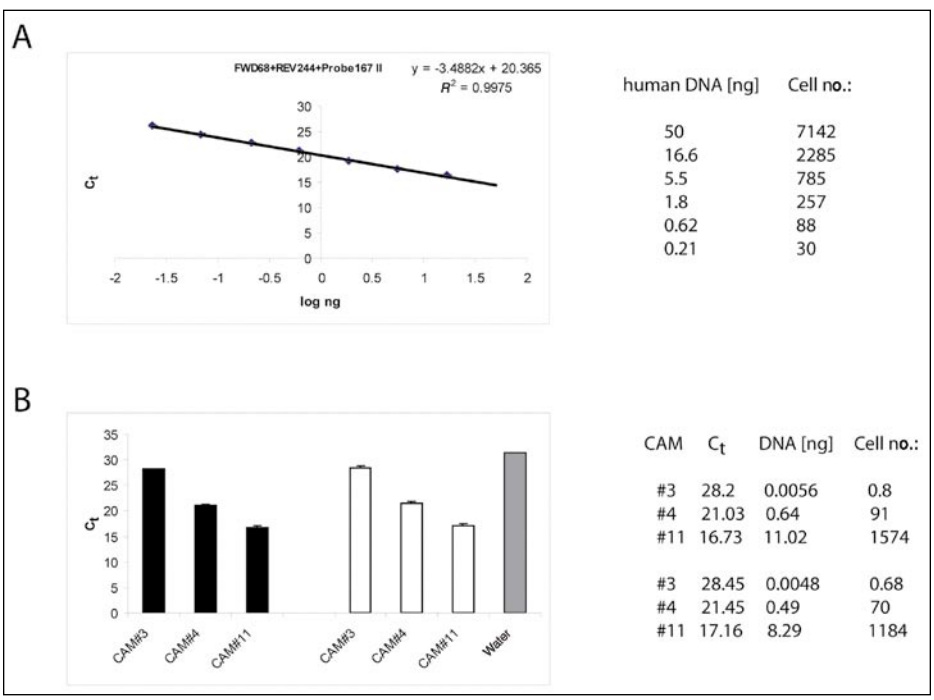

Figure 2. Efficiency and quantitation range of TaqMan-based ALU assay. (A) The effective range for the TaqMan-based ALU assay is shown. Serial dilution of 50 ng to 23 pg Mel-Juso DNA in 560 ng chorioallantoic membrane (CAM) DNA, corresponding to 224,000 cells, is detected through the FAM-167 probe. The fluorescent signal produced by 3-fold dilution series is plotted as the mean of triplicate experiments. (B) Quantification of human RKO colon carcinoma cells in a chick embryonic metastasis (CEM) assay is shown. Invaded RKO cells were detected using either 1.25 µg (500,000 cells; black bars) or 625 ng (260,000 cells; white bars) of total CAM DNA. As a negative control, water was used (gray bar). The R^2 value is at least 99% for the standard curve. Conversion of total DNA to cell numbers is shown in each of the right panels. C_t, cycle threshold; Cell no., cell number.

gonucleotides were purchased from Metabion (Martinsried, Germany). The probe was purchased from Eurogentec (Seraing, Germany), and the TaqMan Universal PCR Master Mix was purchased from Applied Biosystems.

PCR conditions were optimized with regard to concentrations of primers and amount of template DNA. PCRs were carried out in 25 µL using 2× TaqMan Universal PCR buffer (Applied Biosystems), 0.9 µM each oligonucleotide primer, 250 nM TaqMan probe YB8-ALU-167, and either 1.25 µg genomic DNA (CEM assay) or as indicated. Each sample was subjected to an initial denaturation of 95°C for 10 min, followed by 40 amplification cycles of denaturation at 95°C for 15 s and 60°C for 1 min to anneal and extend. Quantitative PCR experiments were performed using an ABI PRISM® 7900HT sequence detection system (Applied Biosystems).

Data Analysis

Data from the replicate DNA standards were exported from ABI PRISM 7000 SDS software (Applied

Biosystems) into a Microsoft® Excel® spreadsheet where the mean value and standard deviation were calculated for each point on the standard curve. Using the Excel trendline option, a line of best fit was plotted with Y-error bars equal to the standard deviation. The Excel chart wizard was used to construct bar graphs with Y-bars equal to one standard deviation.

RESULTS AND DISCUSSION

In order to explore the detection sensitivity of the published YB8 Alu primers for the CEM assay, we serially diluted genomic DNA of Mel-Juso in either 500 or 250 ng chicken CAM DNA and performed PCR exactly as previously described (Figure 1; Reference 15). We observed that the YB8 Alu primer was unable to specifically detect human DNA when fewer than 1000 cells were used in the 500 ng dilution (Figure 1A). Performing the identical dilution series in 250 ng chicken DNA showed that more specificity was achieved (Figure 1B). However, this result was unsatisfying for our purposes because only 0.1% of the cells invade the system and only 1/100 of prepared DNA (corresponding to 60 pg DNA) is typically used for PCR. Moreover, melting curve analysis and visualization of PCR products on agarose gels have shown that a nonspecific product was amplified even in the absence of human DNA and that this reaction is inhibited in the presence of CAM DNA (data not shown). A major drawback of SYBR Green-based PCR is that a single product must be produced because the dye intercalates in amplified products. Therefore, we set out to establish a TaqMan-based system since the problem of generating background PCR products can be circumvented by a sequence-specific probe. Using the ALU-YB8 sequence as a template, we designed one primer pair and a FAM probe. YB8-ALU-S68 and YB8-ALU-AS244 amplify a 176-bp product to which the FAM probe YB8-ALU-167 specifically hybridizes. After primer concentration optimization, we again serially diluted human DNA in 560 ng CAM and performed

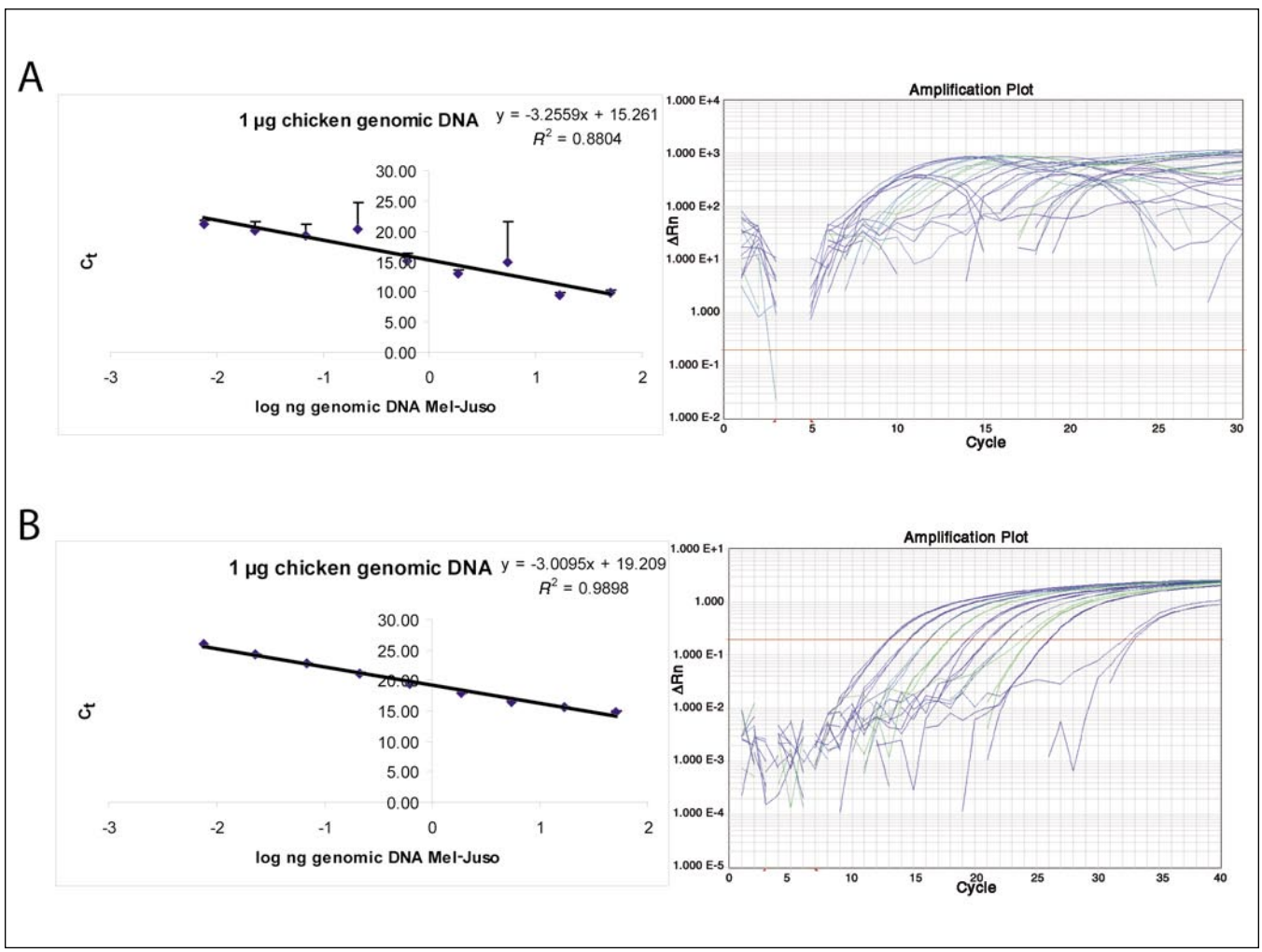

Figure 3. Comparison of SYBR Green PCR with TaqMan-based ALU assay. (A) The effective range for the SYBR Green PCR ALU assay is shown. Human genomic DNA, equivalent to 1000, 300, 100, 30, 10, 3, 1, and 0.1 cells of the melanoma cell line Mel-Juso, was diluted in 1 µg chorioallantoic membrane (CAM) DNA and either quantified as previously described (17) or according to our new system. The detection sensitivity is limited to 100 cells in panel A and 1/10 cell in panel B using YB8 Alu primers and SYBR Green. The relative fluorescence (ΔRn) is plotted against the cycle number. The red line indicates the cycle threshold (C_t).

PCR. We observed a robust signal, indicating that even 10 cells could be detected (Figure 2A).

To test our PCR conditions and detection sensitivity in a CEM assay, we inoculated fertilized SPF eggs with 500,000 cells of the invasive colon carcinoma cell line RKO. We prepared the genomic DNA and used either 1.25 µg or 625 ng of total CAM DNA as a template (Figure 2B). In parallel, we diluted genomic RKO DNA in 1.25 µg CAM DNA and used this set of dilutions as standards (data not shown). As expected, we detected robust products, showing that our primer pair in conjunction with the probe produced a highly specific product. Even the high amount of CAM DNA did not inhibit PCR. Then, we sought to compare the sensitivity of the previously established SYBR Green-based PCR (17) with our primers and probe set (Figure 3). Therefore, we again diluted Mel-Juso genomic DNA in CAM DNA and performed the reactions exactly as described earlier. We observed that the published primers were not sensitive enough to yield a robust signal without affecting PCR dynamics with 1 µg CAM DNA (Figure 3A), but using 100 ng of CAM DNA significantly improved the detection sensitivity and PCR dynamics (data not shown). However, again YB8-ALU-S68, YB8-ALU-AS244, and the FAM probe YB8-ALU-167 produced a specific product within 1 µg of CAM DNA, which allowed us to specifically detect as little as 1/10 of the human genome. Finally, we addressed the question of whether the primers could detect human ALU sequences in a high murine DNA background since most in vivo metastasis models are performed in mice. Therefore, we serially diluted Mel-Juso DNA in murine genomic DNA and performed the reaction. We observed that either 1 or 2 µg mouse DNA, equivalent to 154,000 and 308,000 cells, respectively, allowed us to quantify 1/10 human genome or 0.1 cells (Figure 4, A and B, lower panel). The PCR dynamic was unaffected with the high content of rodent material (Figure 4, A and B, upper panel).

Although a real-time PCR for the CEM assay has recently been established (17,18), our improvement harbors major advantages: we combine the specificity of the YB8-ALU subfamily with TaqMan technology, thereby significantly reducing background products. Moreover, our primers allow

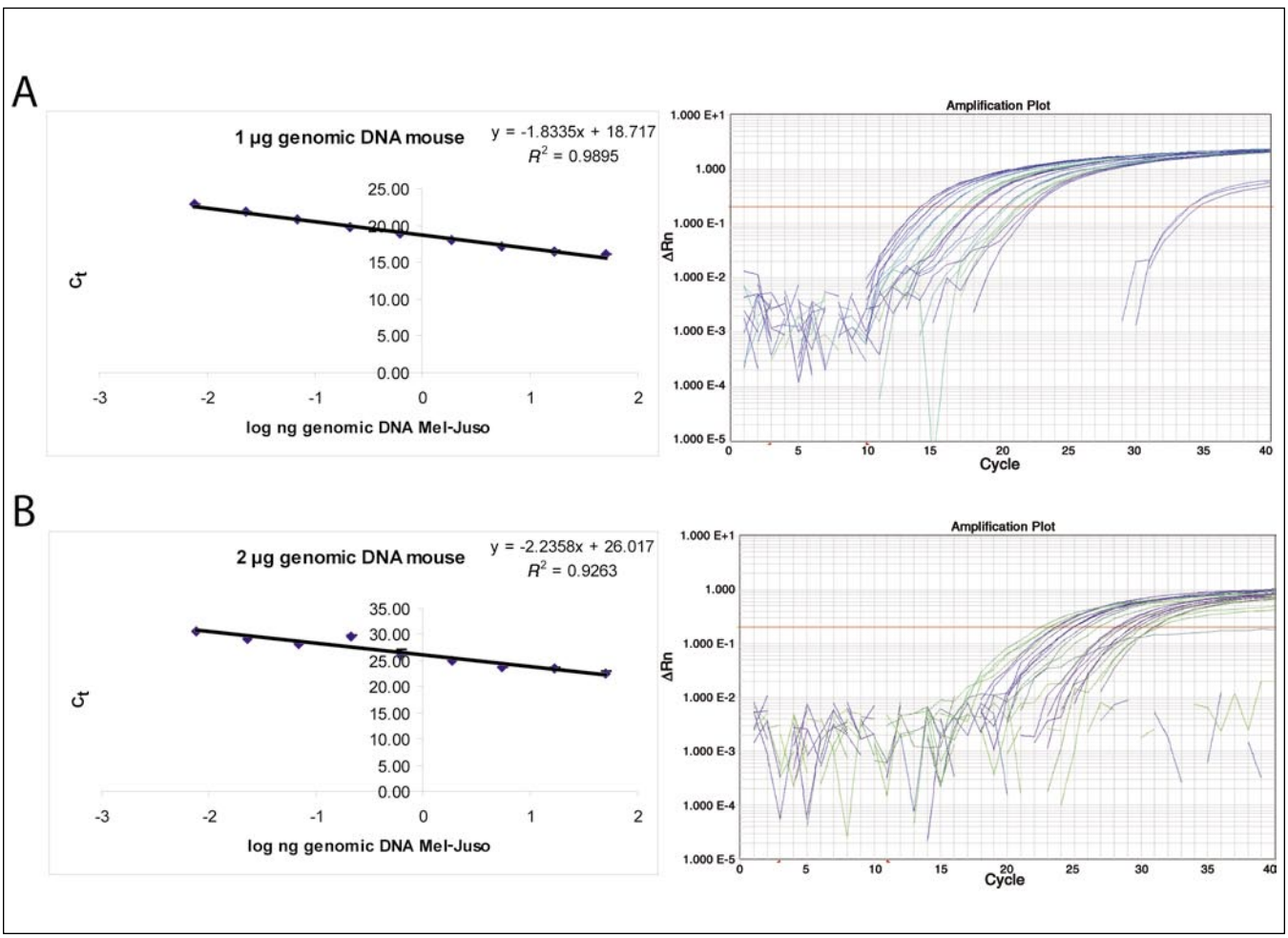

Figure 4. Efficiency and quantitation range of TaqMan-based ALU assay. The effective range for the TaqMan-based ALU assay is shown. Human genomic DNA, equivalent to 1000, 300, 100, 30, 10, 3, 1, and 0.1 cells of the melanoma cell line Mel-Juso, was diluted in 1 or 2 µg liver mouse (Balb/c) DNA and quantified as previously described (16). Detection sensitivity is limited to 1/10 cell using YB8 Alu primers. The relative fluorescence (ΔRn) is plotted against the cycle number. The red line indicates the cycle threshold (C_t). The R^2 value is at least 99% and 92% for the standard curve of panels A and B, respectively.

researchers to quantify metastasized human cells in murine xenografts without the labor-intense immunohistochemistry effort. Additionally, the improvement of the detection of invaded cells into CAM and rodent backgrounds save researchers using the CEM assay or the mouse model as a tool at least one day. The preparation of DNA, setting up PCR, and data analysis can all be done in 1 day. The conventional semiquantitative method takes 2 days: running the nondenaturing DNA gel, drying the gel, and exposing it to phosphorimager plates takes at least 2 days before a result can be seen. In summary, the primers and probe produce a specific product, which is not inhibited in vast excess of CAM or rodent DNA.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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