Improved Indicators for Assessing the Reliability of Detection and Quantification by Kinetic PCR, *Richie Soong*[1*] *and András Ladányi*[2] ([1] Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL 35294; [2] Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA 02115; * address correspondence to this author at: Department of Pharmacology and Toxicology, University of Alabama at Birmingham, 1824 6th Avenue South, WTI 608B, Birmingham, AL 35294, e-mail Richie.Soong@ccc.uab.edu)

Because of their low detection limits, PCR and the adapted kinetic (real-time quantitative) PCR have been used extensively for the detection and quantification of nucleic acids *(1–3)*. However, at the detection limits of an assay, mathematical models *(4–6)* and experimental evidence *(7–12)* have shown that nucleic acids are detected inconsistently and quantified imprecisely. With this potential unreliability, it is essential that indicators be used to identify the concentration at which analysis is occurring to ensure the accuracy of results. Conventional PCR indicators, however, currently provide only an indirect assessment of the concentration at which analysis occurs. The total nucleic acid content of a sample, commonly used to define the adequacy of sample loading, does not precisely reflect specific gene concentrations. Reference (control or housekeeping) genes, qualitative detection of which is often used to validate assay and sample integrity, are often present at concentrations different from those of the target genes of interest *(13)*. In kinetic PCR, the inclusion of fluorescent probes allows the monitoring of reaction kinetics, which enables the measurement of a crossing point (CP), or cycle threshold, at a partial cycle number at which fluorescence becomes detectable above background signal *(14)*. CP values are linearly proportional to gene copy number in an inverse logarithmic relationship *(14, 15)*, and this correlation is central to algorithms for determining gene quantity *(16, 17)*. In this study, we hypothesized that CP values might be more accurate indicators of the reliability of an analysis than conventional indicators because of their direct relationship with gene concentration. The results of this study provide experimental evidence to support this hypothesis; we also describe models for use of CP values as indicators for assessing the reliability of analysis.

To test our hypothesis, we simulated the analysis of low concentrations of the putative colorectal micrometastasis marker cytokeratin 20 (CK20) *(16)* and the reference genes porphobilinogen deaminase (PBGD) and β_2-microglobulin (β2M) and compared the capabilities of conventional indicators (total nucleic acid content and qualitative reference gene detection) and CP values to identify samples with unreliable analysis.

We prepared, by serial dilution, four sample series comprising 10 000, 1000, 100, 10, and 1 plasmid DNA copy for CK20, PBGD, and β2M *(16)* and 20 000, 2000, 200, 20, and 2 pg of SK29-Mel-1 RNA *(18)*. Five replicates of each concentration were analyzed for CK20, PBGD, and β2M by kinetic reverse transcription-PCR on the LightCycler instrument according to the protocol in the LightCycler CK20 Quantification Kit (cat. no. 3118835). A reverse transcription reaction was performed for each SK29-Mel-1 RNA replicate, amounting to 25 reverse transcription reactions. The plasmid DNA and SK29-Mel-1 cDNA series for each gene were analyzed in separate PCR runs, amounting to six individual runs of 25 samples each. Detection mixtures for CK20 and PBGD were obtained from the LightCycler CK20 Quantification Kit, and the detection mixture for β2M was obtained from the LightCycler β2M Housekeeping Gene Set (cat. no. 3146081). CP values were determined by the LightCycler Analysis Software (Ver. 3.5), using the data-driven second-derivative maximum function. All reagents and instruments were from Roche Diagnostics unless otherwise specified.

The replicate detection frequency, mean CP values, and their SDs for each gene in each sample are provided in the Data Supplement that accompanies the online version of this Technical Brief (http://www.clinchem.org/content/vol49/issue6/). In accordance with previous findings *(7–12)*, in samples with high concentrations, gene detection was consistent, with high CP reproducibility and linearity (R^2 >0.999) between concentrations and CP values, whereas in those with low concentrations, genes were detected inconsistently or not detected, CP reproducibility was decreased, and linearity was lost.

When we assessed analysis reliability, using conventional indicators, there was little correlation between the occurrence of an unreliable analysis and the sample nucleic acid content or among the three genes. Detection was inconsistent or absent in samples containing 200, 20, or 2 pg of CK20 RNA and samples containing 2 pg of PBGD RNA, whereas β2M was detected consistently in all samples. With increasing dilution, CP reproducibility markedly decreased for CK20, PBGD, and β2M in samples containing 2000, 20, and 2 pg of RNA, respectively. Linearity was lost (R^2 <0.999) in the same samples in which RNA was inconsistently detected, i.e., samples containing 200 and 2 pg for CK20 and PBGD, respectively. Hence, for this samples series, nucleic acid content or qualitative reference gene detection provided a poor indication of the reliability of detection and quantification. The correlation between the occurrence of unreliable analysis and copy numbers among the genes was better. Inconsistent detection and loss of linearity occurred in samples with single copies of CK20 and PBGD. Reduced CP reproducibility occurred in samples with 10 copies for all three genes, highlighting that the inadequacy of conventional indicators was likely attributable to their lack of direct correlation with specific gene quantities.

Assessing CP values as potential indicators showed that inconsistent detection (Fig. 1A), poor CP reproducibility (Fig. 1B), and loss of linearity between CP values and concentration (Fig. 1C) occurred within specific ranges of CP values. Importantly, these correlations were independent of the gene or the nucleic acid type being analyzed, suggesting that CP values could be useful indicators for reliability of analysis of other genes and samples of unknown concentration.

To define models for the use of CP values as indicators, we considered that four zones of PCR reliability indicating consistent detection with high CP reproducibility (white zone), poor CP reproducibility (gray1), inconsistent detection and loss of linearity (gray2), and unlikely detection (black) could be mapped from the coincidence of the different types of PCR reliability with CP values. Using the lowest mean CP value for poor CP reproducibility, we defined the white/gray1 boundary at CP 33.06. We chose the CP value of 37.01, corresponding to the first observation of inconsistent detection, as the gray1/gray2 boundary and the theoretical CP value at which CK20 in 20 pg of RNA should have been detected to indicate the gray2/black boundary. Extrapolating the CP difference of 3.73 between the mean CP values for 20 000 and 2000 pg of CK20 RNA, we determined that detection of CK20 in 20 pg of RNA should have occurred around CP 42.80 [31.61 + (3.73 × 3)]. Thus, using this model, we could determine the reliability of detection or quantification of a test sample by its CP value and its corresponding zone.

Apart from defining the reliability of a result, indicators also identify the adequacy of samples to provide a reliable result, particularly when detection is likely to be inconsistent. To define a model for this, we observed that the difference in CP values between two genes (ΔCP) remained relatively constant, independent of sample con-

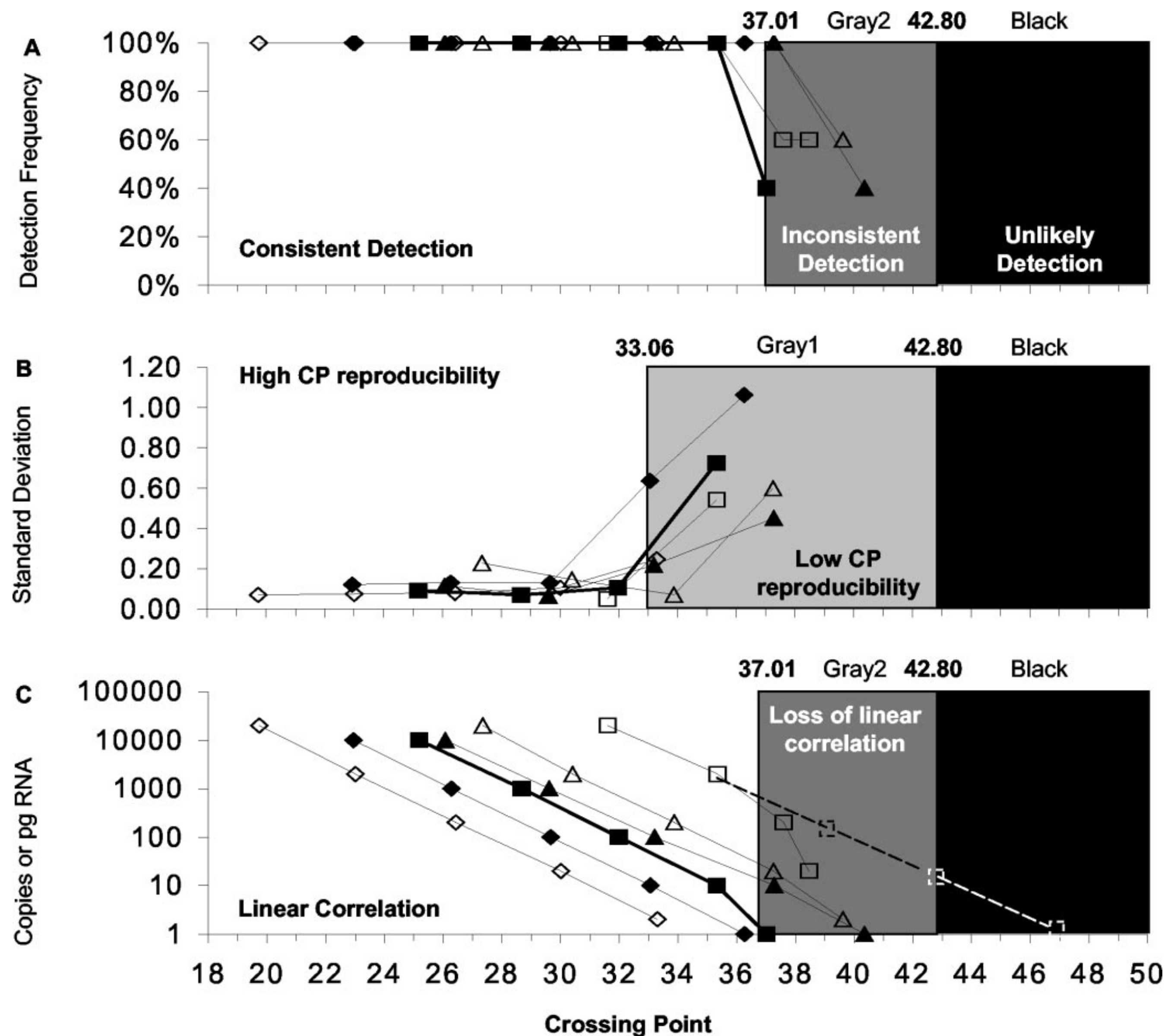

Fig. 1. Correlation between mean CP values and detection frequency (*A*), SD of the CP (*B*), and sample concentration (*C*) for the detection of CK20 (*squares*), PBGD (*triangles*), and β2M (*diamonds*) in plasmid DNA (*filled symbols*) and SK29-Mel RNA (*open symbols*).

Zones of PCR reliability are indicated with the estimated CP values for white/gray1 (*33.06*), white/gray2 (*37.01*), and gray/black (*42.80*) boundaries. Extrapolation of the expected CP values for the detection of CK20 in SK29-Mel is also displayed (*dashed line* and *dashed open squares*).

centration (Fig. 1C), although this was valid only for reliably analyzed samples. We surmised that addition of a reference gene CP to an expected ΔCP(target − reference) would enable the CP of the target gene, and hence its reliability of analysis from its detection zone, to be estimated. At a ΔCP(CK20 − β2M) of 11.88, based on the analysis of 20 000 pg of RNA, this model accurately predicted the reliability of CK20 detection for each respective β2M CP and sample concentration (Table 1). Using this model, we could thus determine the likely adequacy of a sample to provide reliable analysis for a given target gene from the CP for a reference gene and knowledge of an expected ΔCP(target − reference).

For the purposes of brevity and clarity, we have used a selected sample series to demonstrate the concepts of this study. In exploring the wider application of our models, we have observed similar trends with other genes, sample sources, and detection formats (TaqMan probes), using the same assay system (results not shown). However, analyses on other assay systems and instruments have indicated that these systems differ in their detection limits and hence the positioning of the zonal CP boundaries. Nevertheless, the concept of zones of reliability and the capabilities of CP values to indicate these zones remained. These results have suggested a wider application for our models, but they also indicate that with different assay systems, preliminary determination of the boundaries is required. This would require only analysis of a replicated sample series serially diluted to the assay detection limit, similar to that of this study.

An important element of our models is the derivation of an expected ΔCP(target − reference). Our experience indicates that this value can be determined with use of cell lines or pilot samples representative of the samples to be analyzed at a high concentration (results not shown). Alternatively, it could be determined mathematically. In this context, using boundaries defined in this study and assuming ideal product doubling with each PCR cycle, a ΔCP(target − reference) of 4 would make it conditional for samples to have reference gene CP values <29.07 (33.06 − 4) to ensure that a 16-fold (2^4) difference between two genes is quantified reliably. However, it is also important to recognize that the constancy of ΔCP(target − reference) is dependent on similarities in the PCR efficiencies between two genes. Using methods described previously *(16)*, we determined that the efficiencies of the CK20, PBGD, and β2M PCRs for RNA samples in the linear range were 93%, 100%, and 98%, respectively. By linear regression analysis, these efficiencies were not significantly different from each other, suggesting that constancy of ΔCP(target − reference) for a given sample type could be assumed. Clearly however, depending on the predictive precision required, it may be prudent to ensure equivalence in efficiency before making this assumption.

In summary, lack of reproducibility among laboratories and lack of adequate guidelines for assay standardization *(11, 19–21)* continue to hinder the clinical application of many new assays. One problem has been that, with the improved detection limits and dynamic ranges of assays, there has been little revision of the indicators used *(22)*. This study demonstrates that CP values may provide an assessment of PCR reliability that is superior to conventional indicators, and we devised models for their implementation. Because CP values and ΔCP(target − reference) are already integral elements in kinetic PCR quantification *(16, 17)*, use of these parameters provides direct and quantitative indicators that require no additional preparation. These measures potentially provide for more intuitive selection of samples and interpretation of results, but this promise awaits further validation and application in future studies.

Table 1. Estimation of CK20 CP and its reliability of detection based on β2M CP and ΔCP(CK20 − β2M).

β2M CP (Sample)	Estimated CK20 CP[a] (Expected feature)	Observed mean CK20 CP (Observed feature)
19.73 (20 000 pg)	31.61 (Consistent CP/detection)	31.61 (Consistent CP/detection)
23.00 (2000 pg)	34.88 (Poor CP reproducibility)	35.34 (Poor CP reproducibility)
26.43 (200 pg)	38.31 (Inconsistent detection)	37.59 (Inconsistent detection)
30.01 (20 pg)	41.89 (Inconsistent detection)	38.46 (Inconsistent detection)
33.29 (2 pg)	45.17 (Unlikely detection)	ND[b] (No detection)

[a] Estimated CK20 CP = β2M CP + 11.88 [ΔCP(CK20 − β2M) for 20 000 pg of SK29-Mel-1]. The expected feature was determined from the PCR reliability zones defined in Fig. 1.

[b] ND, not determinable.

References

1. Freeman WM, Walker SJ, Vrana KE. Quantitative RT-PCR: pitfalls and potential. Biotechniques 1999;26:112–22,124–5.
2. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000;25: 169–93.
3. Bernard PS, Wittwer CT. Real-time PCR technology for cancer diagnostics. Clin Chem 2002;48:1178–85.
4. Stenman J, Orpana A. Accuracy in amplification. Nat Biotechnol 2001;19: 1011–2.
5. Peccoud J, Jacob C. Theoretical uncertainty of measurements using quantitative polymerase chain reaction. Biophys J 1996;71:101–8.
6. Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, et al. Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Res 1996;24:3189–94.
7. Karrer EE, Lincoln JE, Hogenhout S, Bennett AB, Bostock RM, Martineau B, et al. In situ isolation of mRNA from individual plant cells: creation of cell-specific cDNA libraries. Proc Natl Acad Sci U S A 1995;92:3814–8.
8. Morrison TB, Weis JJ, Wittwer CT. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 1998;24:954–8,960,962.
9. Schmitt Y. Performance characteristics of quantification assays for human immunodeficiency virus type 1 RNA. J Clin Virol 2001;20:31–3.
10. Cavrois M, Wain-Hobson S, Wattel E. Stochastic events in the amplification of HTLV-I integration sites by linker-mediated PCR. Res Virol 1995;146: 179–84.
11. Jung R, Ahmad-Nejad P, Wimmer M, Gerhard M, Wagener C, Neumaier M. Quality management and influential factors for the detection of single metastatic cancer cells by reverse transcriptase polymerase chain reaction. Eur J Clin Chem Clin Biochem 1997;35:3–10.
12. Melo JV, Yan XH, Diamond J, Lin F, Cross NC, Goldman JM. Reverse transcription/polymerase chain reaction (RT/PCR) amplification of very small numbers of transcripts: the risk in misinterpreting negative results. Leukemia 1996;10:1217–21.

13. Lion T. Current recommendations for positive controls in RT-PCR assays. Leukemia 2001;15:1033–7.
14. Higuchi R, Fockler C, Dollinger G, Watson R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology 1993;11:1026–30.
15. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res 1996;6:986–94.
16. Soong R, Beyser K, Basten O, Kalbe A, Rueschoff J, Tabiti K. Quantitative reverse transcription-polymerase chain reaction detection of cytokeratin 20 in noncolorectal lymph nodes. Clin Cancer Res 2001;7:3423–9.
17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C_T)$ method. Methods 2001;25:402–8.
18. Wolfel T, Hauer M, Klehmann E, Brichard V, Ackermann B, Knuth A, et al. Analysis of antigens recognized on human melanoma cells by A2-restricted cytolytic T lymphocytes (CTL). Int J Cancer 1993;55:237–44.
19. Zaaijer HL, Cuypers HT, Reesink HW, Winkel IN, Gerken G, Lelie PN. Reliability of polymerase chain reaction for detection of hepatitis C virus. Lancet 1993;341:722–4.
20. Noordhoek GT, van Embden JD, Kolk AH. Reliability of nucleic acid amplification for detection of Mycobacterium tuberculosis: an international collaborative quality control study among 30 laboratories. J Clin Microbiol 1996;34:2522–5.
21. Keilholz U, Willhauck M, Rimoldi D, Brasseur F, Dummer W, Rass K, et al. Reliability of reverse transcription-polymerase chain reaction (RT-PCR)-based assays for the detection of circulating tumour cells: a quality-assurance initiative of the EORTC Melanoma Cooperative Group. Eur J Cancer 1998;34:750–3.
22. Pritzker KP. Cancer biomarkers: easier said than done. Clin Chem 2002;48:1147–50.

Comparison of the Biomedica NT-proBNP Enzyme Immunoassay and the Roche NT-proBNP Chemiluminescence Immunoassay: Implications for the Prediction of Symptomatic and Asymptomatic Structural Heart Disease, *Thomas Mueller,[1] Alfons Gegenhuber,[2] Werner Poelz,[3] and Meinhard Haltmayer[1*]* [[1] Department of Laboratory Medicine and [2] Department of Internal Medicine (Division of Cardiology), Konventhospital Barmherzige Brueder Linz, A-4021 Linz, Austria; [3] Department of Applied System Sciences and Statistics, University of Linz, A-4040 Linz, Austria; * author for correspondence: fax 43-732-7897-2299, e-mail meinhard.haltmayer@bblinz.at]

The amino-terminal fragment of the B-type natriuretic peptide prohormone (NT-proBNP) is a marker for functional cardiac impairment and is increased in heart disease with or without symptoms of heart failure (HF) *(1)*. There are indications that currently used assays for NT-proBNP may differ in their cross-reactivity with circulating NT-proBNP split products and may also be affected by breakdown products of NT-proBNP produced after blood collection *(2)*. A newer generation assay, a commercially available competitive enzyme immunoassay (EIA) for NT-proBNP (Biomedica Gruppe) *(3)* that does not require sample pretreatment, has been used in various methodologic and clinical studies *(4–6)*, but noncompetitive immunoassays may offer advantages of better speed, sensitivity, precision, and possibly specificity over competitive immunoassays *(7)*. Recently, a noncompetitive immunoassay for NT-proBNP has been developed *(8)*. A fully automated version of this assay (Roche Diagnostics) has now been cleared by the US Food and Drug Administration. Our aim was to compare the Biomedica and Roche NT-proBNP assays, addressing whether the predictive values of both assays are similar with respect to structural heart disease with or without symptoms of HF.

The present study, carried out at the Division of Internal Medicine, St. John of God Hospital (Linz, Austria), was approved by the local ethics committee in accordance to the Helsinki Declaration. We prospectively recruited 157 consecutive patients admitted for extensive cardiac evaluation (including performance of bicycle ergometry) and 23 consecutive patients with symptomatic HF admitted for inpatient treatment; all participants gave written informed consent. Study participants were classified according to the American College of Cardiology/American Heart Association guidelines for the evaluation and management of chronic HF in the adults *(9)* to one of the four following categories: (*a*) healthy individuals (n = 42); (*b*) patients at high risk for developing HF but without structural disorders of the heart (HF stage A; n = 39); (*c*) patients with structural disorders of the heart but without symptoms of HF (HF stage B; n = 56); and (*d*) patients with past or current symptoms of HF associated with underlying structural heart disease (HF stage C; n = 43). None of the study participants belonged to HF stage D according to the above guidelines (patients with end stage disease requiring specialized treatment strategies). This classification was done by one experienced cardiologist (one of the investigators) blinded to the NT-proBNP results and was based on detailed anamnesis with evaluation of the patients' medical reports, appropriate risk factor assessment, physical examination, 12-lead electrocardiography, chest radiography, two-dimensional echocardiography coupled with Doppler flow studies, bicycle ergometry (in the 157 consecutive patients admitted for extensive cardiac evaluation), and an ongoing assessment of the patients' clinical status. Normal echocardiographic findings (i.e., individuals without structural disorders of the heart) were defined by a left ventricular end-diastolic diameter <56 mm without left ventricular hypertrophy or without wall motion abnormities, a right ventricular systolic pressure <35 mmHg, and a left ventricular ejection fraction >60%. No attempt was made to define diastolic HF. Drug therapy (i.e., angiotensin-converting enzyme inhibitors, beta-blockers, digitalis, and diuretics) was recorded at the day of blood collection and was modified in the sequel. The demographic and clinical characteristics of the study participants are listed in Table 1.

Blood for measurement of NT-proBNP concentrations was collected by venipuncture in Vacuette® polyethylene terephthalate glycol clot activator tubes (Greiner Bio-One) after an overnight fast, with the study participants in supine position for at least 20 min. Serum was separated for each assay. Roche NT-proBNP assays were done within 4 h after specimen collection on a Roche Elecsys 2010 analyzer. Samples for the Biomedica NT-proBNP assay were stored at −70 °C until analysis (up to 12 weeks of storage). Both assays were performed according to the manufacturers' recommendations.

The Biomedica assay is a competitive EIA designed to