Optimisation of circulating biomarkers of cell death for routine clinical use

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Background: M30™ and M65™ enzyme-linked immunosorbent assays detect circulating cytokeratin 18 fragments released during caspase-dependent or total cell death, respectively, and have potential as biomarkers in epithelial cancers. While these assays have been validated, their robustness for routine clinical use is unknown.

Patients and methods: M30 and M65 were measured in matched serum and plasma samples from 31 lung cancer patients and 18 controls.

Results: Time allowable between sample acquisition and processing is critical for assays in clinical use. A 4-h delay in processing at room temperature increased M30 (P < 0.0001), an effect minimised by incubation on ice. M30 and M65 in serum were resistant to processing variations including delays. Serum and plasma measurements correlated well although M30 but not M65 was lower in serum (P < 0.0005). Less variation between duplicate assays was observed in serum. Prolonged storage (−80°C) led to increased M30 (12%, 6 months; 34%, 1 year). Sample dilution in the supplied assay diluent proved non-linear, whereas dilution in donor serum or porcine plasma restored linearity up to a ratio of 1 : 6.

Conclusion: We present recommendations that improve the reliability of these assays for clinical use and recommend serum as the preferred matrix with data more resistant to variations in collection.

Key words: apoptosis, cell death biomarkers, clinical utility, M30, M65, small-cell lung cancer

Cytokeratin 18 (CK18) is a member of the intermediate filament family of cytoskeletal proteins and is widely expressed in epithelial and endothelial cells but not in haemopoietic cells [11]. During apoptosis, caspasces cleave cytokeratins leading to collapse of the cytoskeleton and subsequent formation of apoptotic bodies [12]. The M30 apoptosense enzyme-linked immunosorbent assay (ELISA) detects a neoepitope specific to apoptosis caused by caspase cleavage of CK18 at aspartate 396 (CK18asp396). The M65 ELISA measures total soluble CK18 (both caspase cleaved and intact forms) which are released during apoptotic and non-apoptotic forms of cell death [12]. These assays may provide clinically useful information in the management of patients with epithelial cancers [13–15].

These assays were validated in-house as part of a biomarker package to support a phase 1 trial of an antiapoptotic therapy in our laboratory [5, 6]. Plasma samples collected for accompanying pharmacokinetic studies were used for M30/ M65 assay validation. Other studies have measured CK18 products in serum (thought to be equivalent to plasma [16]). Serum is often recommended by assay manufacturers as a cleaner matrix for analysis but is inappropriate for analytes that are generated or consumed during clotting [17, 18]. The method of blood collection can affect assays for circulating biomarkers (including cytokeratins [19]) and subsequent analyte stability in storage [20]. Therefore, one goal of the current study was to determine the most appropriate matrix (i.e. plasma versus serum) for these assays for future studies.

Given the nature of the clinical environment, a critical issue with the analysis of blood-borne biomarkers is variations in sample collection processing and storage. Strict sample acquisition protocols minimise this potential problem in specialist units undertaking early phase clinical trials. This rigor...
is, however, difficult to implement in standard clinical practice or in higher throughput later phase trials. Failure to demonstrate that these assays are robust enough to accommodate inevitable discrepancies in sample acquisition would restrict their future clinical utility.

Therefore, the overall objectives of this study were to optimise the M30 and M65 assays for future clinical use, using blood samples obtained from patients with small-cell lung cancer (SCLC) being treated in a busy clinic at the Christie Hospital (Manchester, UK).

**patients and methods**

The study was carried out according to the Declaration of Helsinki and International Conference for Harmonization of Good Clinical Practice guidelines. Blood samples were collected from patients with SCLC treated with platinum- and etoposide-based chemotherapy at the Christie Hospital, who had given written informed consent for the study according to an ethically approved protocol. Control samples were collected from healthy volunteers at AstraZeneca, Macclesfield, UK.

Blood samples were collected using standard phlebotomy techniques with the vacutainer system (BD Franklin Lakes, NJ). Plasma samples were collected in tubes containing lithium–heparin and centrifuged at 1000 g for 10 min. Serum samples were collected in tubes containing a silica clot activator and centrifuged at 2000 g for 10 min. The impact of delay between sample collection and processing was compared in matched samples with the primary sample maintained at room temperature (RT) and processed within 30 min of collection. Analysis of the samples for M30 and M65 was carried out as previously described [5, 6].

Sample dilution was carried out using diluent supplied with the assays (Peviva, Bromma, Sweden), pooled donor serum or pig plasma (Cambridge Biosciences, Cambridge, Cambridgeshire, UK, and BioSera, Ringmer East Sussex, UK). Assessment of linearity in dilution was carried out using patient samples that had been previously assayed and found to have readings in the upper 50% of the dynamic range of the tests (500–1000 and 1000–2000 U/I for M30 and M65, respectively).

Comparison of 6-month stability at −80°C in plasma and serum was undertaken by repeat analysis of matched samples from SCLC patients. Assessment of 1-year stability at −80°C was determined by repeat analysis of samples from patients with testicular cancer treated in the University Medical Center Groningen, The Netherlands, known to have values throughout the range of the assays.

**statistics**

**statistical methods.** All statistical tests were carried out using STATA (version 9.2, College Station, CA). Significance was taken at $P < 0.05$ throughout. As the distributions of all variables were skewed (Kolmogorov–Smirnov test), data were log transformed. Geometric mean differences and their 95% confidence intervals (CIs) were calculated. Pearson’s coefficients ($r_p$) and 95% CIs were calculated. Bland–Altman plots were constructed [21], and differences quantified by estimating the bias (i.e., the mean difference) and the limits within which most differences lie (i.e., the limits of agreement). Differences in variances were tested statistically using Pitman’s test [21]. Comparison of samples requiring reanalysis was carried out using the $\chi^2$ test.

**results**

**influence of time from sample collection to processing on M30 and M65 measurement**

Matched blood samples from patients with SCLC ($n = 13$) were processed to obtain plasma either immediately or following incubation at RT for 4 h. Delays in processing resulted in significantly higher M30 readings (mean $+49.4\%$, $P < 0.0001$) but only minimal differences in M65 measurements were seen (mean $+12.2\%$, $P < 0.05$).

To test the hypothesis that an increase in M30 (CK18$^{asp396}$) accompanied by stable M65 (full-length CK18) is explained by ongoing proteolysis of circulating CK18 complexes, rather than further cell death within the sample, we assessed the effect of sample cooling before processing. Cooling should reduce enzyme-mediated alterations in signal; therefore, matched samples were taken from SCLC patients ($n = 10$); one sample was processed immediately while three other samples were incubated for 1, 2 and 4 h on ice before centrifugation. This eliminated the increase in M30 seen with delayed processing at RT (see Table 1). Similar results were seen with 2-h storage in

![](image)

<table>
<thead>
<tr>
<th>Effect of variation in sample processing</th>
<th>M30</th>
<th>M65</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-h incubation at RT (plasma) ($n = 13$)</td>
<td>49% (27% to 72%), $P &lt; 0.0001$</td>
<td>12% (7% to 27%), $P &lt; 0.05$</td>
</tr>
<tr>
<td>1-h incubation on ice (plasma) ($n = 10$)</td>
<td>2% (−15% to 19%)</td>
<td>2% (−10% to 13%)</td>
</tr>
<tr>
<td>2-h incubation on ice (plasma) ($n = 10$)</td>
<td>5% (−7% to −17%)</td>
<td>−2 (−25% to 20%)</td>
</tr>
<tr>
<td>4-h incubation on ice (plasma) ($n = 13$)</td>
<td>−4% (−19% to 11%)</td>
<td>6% (−3% to 15%)</td>
</tr>
<tr>
<td>2-h incubation at 4°C in cooler (plasma) ($n = 8$)</td>
<td>7% (−13% to 27%)</td>
<td>8% (−13% to 28%)</td>
</tr>
<tr>
<td>2-h incubation at RT (serum) ($n = 13$)</td>
<td>2% (−7% to 11%)</td>
<td>10% (5% to 15%)</td>
</tr>
<tr>
<td>Silica versus gel clot activator (serum) ($n = 13$)</td>
<td>3% (−8% to 14%)</td>
<td>−7% (−14% to 0%)</td>
</tr>
<tr>
<td>Effect of different matrix</td>
<td>M30</td>
<td>M65</td>
</tr>
<tr>
<td>Plasma versus serum ($n = 49$)</td>
<td>16% (8% to 24.0%), $P &lt; 0.0001$</td>
<td>0% (−6% to 6%)</td>
</tr>
<tr>
<td>Effect of dilution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilution 1 : 3 assay diluent ($n = 22$)</td>
<td>92% (65% to 117%)</td>
<td>83% (63% to 103%)</td>
</tr>
<tr>
<td>Dilution 1 : 6 assay diluent ($n = 22$)</td>
<td>115% (92% to 139%)</td>
<td>115% (92% to 139%)</td>
</tr>
<tr>
<td>Dilution 1 : 3 porcine plasma ($n = 22$)</td>
<td>−4% (−10% to 2%)</td>
<td>7% (3% to 14%)</td>
</tr>
<tr>
<td>Dilution 1 : 6 porcine plasma ($n = 22$)</td>
<td>6% (−0.6% to 3.6%)</td>
<td>7% (−1% to 16%)</td>
</tr>
<tr>
<td>Dilution 1 : 3 donor serum ($n = 22$)</td>
<td>−4% (12% to 4%)</td>
<td>12% (3% to 21%)</td>
</tr>
<tr>
<td>Dilution 1 : 6 donor serum ($n = 22$)</td>
<td>−10% (−15% to 5%)</td>
<td>17% (9% to 25%)</td>
</tr>
<tr>
<td>Dilution in different batches of porcine plasma ($n = 12$)</td>
<td>5% (−2% to 12%)</td>
<td>2% (−2% to 6%)</td>
</tr>
</tbody>
</table>

Results expressed as % change from baseline sample (95% confidence intervals). RT, room temperature.
A bench-top cooler set at 4°C (Nalgene, Hereford, UK) \( (n = 8) \) which is more feasible for clinical use (see Table 1).

A similar assessment of the impact of time between venesection and processing on M30 and M65 measurement in serum was carried out. In contrast to the studies in plasma, no differences were seen with 2-h incubation at RT in M30 (mean difference 2%) or M65 (mean difference 10%) or with different clot activators (gel versus silica) [see Table 1 \( (n = 13) \)].

**direct comparison of plasma versus serum for measurement of M30 and M65**

Matched plasma and serum samples from both healthy controls \( (n = 18) \) and patients with SCLC \( (n = 31) \) were analysed using the M30 and M65 assays. Plasma M30 levels were \( 16 \pm 4\% \) (95% CI 8–24.0%) higher than the measurements in serum (geometric mean M30 values; plasma 302 U/l, serum 240 U/l, \( P < 0.0001 \)). There was, however, good correlation \( (R^2 = 0.7868) \) between plasma and serum M30 measurements. There was no difference between plasma and serum M65 levels (geometric mean M65 plasma 427 U/l and serum 417 U/l). Again there was good correlation between plasma and serum M65 measurements \( (R^2 = 0.8955) \). Bland–Altman analysis of these data sets showed no bias in M30 but a minor bias in M65 results with higher values seen in plasma than in serum in upper ranges of the assay (Figure 1 and Table 1).

**comparison of long-term stability at –80°C of M30 and M65 antigens in serum and plasma**

Long-term stability (–80°C for 6 months) was similar in plasma and serum; 23 matched samples showed a \( -14\% \) (95% CI –28 to –2%) change in M30 in plasma with a 12% (95% CI 4%–21%) increase in serum. A slight reduction in M65 was seen in both plasma \( [-6.4\% (95\% CI –10 to –14\%)] \) and in serum \( [-3.1\% (95\% CI –7\% to 1\%)] \). Serum samples \( (n = 29) \) analysed from 10 patients with testicular cancers were reanalysed following a year in storage at –80°C. Trends were similar to those observed for 6-month storage with

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**Figure 1.** Correlation of M30 (A) and M65 (B) and Bland–Altman analysis of M30 (C) and M65 (D) in plasma and serum in matched samples.
an increase in the M30 [+34% (95% CI +24 to +44%)] and a slight reduction in M65 reading [−12% (95% CI −18% to −6%)] (see Figure 2).

**effect of sample dilution on M30 and M65 measurement**

The M30 and M65 assays, as with all ELISAs, have a defined dynamic range (25–1000 U/l and 25–2000 U/l, respectively). This limited range poses problems for the analysis of clinical samples with elevated readings, outside the defined linear range. The problem is resolved typically by diluting the sample to bring the reading back within this range, then multiplying by the dilution factor to generate the ‘true’ reading. This process is only valid if the dilution process per se does not affect linearity of the assay readout. We observed that a number of samples from patients with cancer were above the upper limit and set out to optimise the dilution process.

Dilution of samples (n = 18) in diluent supplied with the kit [16] resulted in a consistent artefactual rise in readings of both M30 and M65 (Table 1 and Figure 3A). This finding was consistent between different patients and when carried out by different analysts. Dilution of the recombinant CK18 standard in kit diluent, however, was linear.

This non-linearity in diluted patient samples, not seen with the recombinant protein, indicated that the buffer used affected the CK18 protein complex detected in the clinical samples leading to the artificial increases in readout. Dilution in 0.9% saline and 1% bovine albumin caused similar aberrant increases to the assay diluent. Porcine plasma is a closer surrogate matrix to human plasma and when analysed alone gave low and consistent readings for both M30 (118 ± 3 U/l) and M65 (122 ± 5 U/l). Dilution of samples (n = 22) with porcine plasma restored linearity at least up to a dilution of one in six and gave similar results to dilution in donor serum (Table 1 and Figure 3).

Dilution of the highest concentration recombinant standard from the assays (1000 U/l in the M30 and 2000 U/l in the M65 assay) and two patient samples with similar values demonstrated linear and parallel curves in porcine plasma over the dynamic range of the assays (dilutions of 3 : 1–1 : 13) (Figure 3). The potentially problematic issue of batch-to-batch variability was assessed using porcine plasma supplied by two different companies which when used to dilute patient samples gave favourably similar results (see Table 1).

**conclusions**

We conducted a rigorous assessment of the M30 and M65 assays to optimise their performance for high-throughput routine clinical use. Our results demonstrate that the M30 and M65 assays are more reliable in serum compared with plasma and dilution in porcine plasma can overcome the difficulty in assessing high, out of range, values.

Time to processing is a critical variable to consider for optimisation of biomarker assays for routine clinical use. Caspase-cleaved CK18asp396 fragment (M30) rises with delay in processing but this can be prevented by incubation at 4°C. The reason for the observed rise is not clear. Proteases such as caspases may cause further cleavage of CK18 ex vivo. No increase in caspase-cleaved CK18 was reported, however, when patient serum was incubated with recombinant caspase-3 [23] indicating that non-caspase-mediated proteolysis or modification of CK18-containing complexes may be responsible.

Serum levels of M30 were lower than in plasma and this should be considered in comparison of studies. Measurements in plasma and serum correlated well, however, indicating

![Figure 2](image2.png) **Figure 2.** The stability of M30 and M65 stored at −80°C in plasma and serum relative to value following immediate assay (asterisk indicates data previously reported on long-term stability (9–26 months) in plasma at −80°C [22]).

![Figure 3](image3.png) **Figure 3.** (A) The impact of dilution in different mediums on M30 and M65 readings. (B) Assessment of parallelism of dilution of recombinant CK18 standard and patient samples in porcine plasma in the M30 assay. (AD = assay diluent; PP = porcine plasma; DS = donor serum; rCK18 = recombinant CK18; Pt = patient)
that studies using either matrix are valid provided the same matrix is used throughout. Our data demonstrated less variation with serum consistent with the suggestion that it is a ‘cleaner’ biological matrix. No stability data have been published for these assays in serum; however, long-term stability was similar to plasma and comparable to previously reported data [22]. Consequently, we recommend that for large trials particularly those involving multiple centres, serum should be the matrix of choice for studies incorporating M30 and M65 assays.

Given the limited dynamic range of ELISA-based assays, it is important to establish that sample dilution will not influence assay results, particularly where high, out of range, levels are observed, as in the present study. The phosphate buffer-based solution used as the ‘0’ standard was originally recommended as diluent for these samples [16] led to an artificial, non-linear increase in M30 and M65 measurements in both plasma and serum.

Dilution of the recombinant CK18 standard in the kit diluent did not affect the signal, making interference and technical problems with the dilution or assay performance unlikely. Fragments of CK18 are known to circulate in complexes with other cytokeratins in the circulation [22] and it is plausible that non-physiological diluents cause conformational changes or disassociation of CK18-containing complexes, potentially revealing additional antigen/antibody-binding sites. The same phenomenon has been reported for other protein biomarkers [24] but not for other cytokeratin assays [25, 26].

Our data on the kit diluent have contributed to revision of the assay data sheets to recommend donor serum as the optimum diluent [27]. There are, however, logistical and ethical challenges to obtaining a consistent source of donor serum. We found that porcine plasma had lower levels of CK18 products than human donor serum and was as robust for diluting samples and restoring linearity. Reasonable volumes can be obtained from one animal to accommodate long-term consistent use within a laboratory. Dilution of samples in porcine plasma restores linearity to both assays at least up to a dilution of 1 : 6; the highest dilution factor was tested and the highest required for all patient samples studied to date. Furthermore, batch variation did not adversely affect our data. This study underlines the importance of reporting the precise protocols used for sample preparation in clinical trials utilising serological biomarkers, given the impact they can have on assay performance. The demonstration of a significant difference between assay performance using patient samples and the recombinant protein highlights the potential problems of biomarker qualification using a recombinant standard. Clinical samples should be used for this purpose where possible.

In conclusion, the results of this study will enable the M30 apoptosense and M65 assays to be used reliably and routinely in a standard busy clinical setting and if applied consistently will enable meaningful comparison of data between trials. These assays have considerable potential as biomarkers of cell death in a range of diseases including cancer, sepsis and liver disease. The findings reported will assist in ensuring that these assays can be developed appropriately to contribute to clinical decision making.

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