Detection of DNA Damage Response Biomarkers in Circulating Tumour Cells using ANGLE's Parsortix® instrument and downstream immunofluorescence assays

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Introduction

Gamma H2AX (γ-H2AX) and Phospho KAP1 (pKAP1) are examples of biomarkers that can be used to identify induction of the DNA damage response (DDR). Monitoring the activation of DDR in cancer cells can be valuable when evaluating effectiveness of DNA damage-inducing therapies. Whilst assessing the DDR status in solid tissue biopsies would be optimal for evaluating treatment response at the primary tumour site, obtaining solid biopsies is invasive, challenging and often non-repeatable. ANGLE has developed a Research Use Only (RUO) workflow that could be used to evaluate DDR biomarker expression in Circulating tumour cells (CTCs), enriched from a liquid biopsy (**Figure 1**), offering minimally invasive, repeatable and real-time assessments of treatment response. In this proof-of-concept study, we aimed to assess the performance of ANGLE's immunofluorescence (IF) assays for the identification of epithelial, mesenchymal and transitioning CTCs and to determine DNA Damage status (targeting either pKAP1 or γ-H2AX) on the identified CTCs, by combining its use with the Parsortix® technology, an epitope-independent microfluidic device that enriches and harvests CTCs from blood based on their size and deformability.

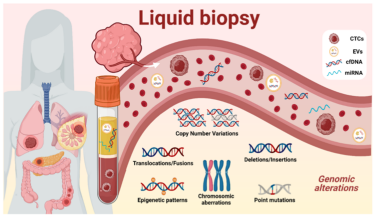


Figure 1. Schematic of biomarkers obtained from liquid biopsy. Figure adapted from Palacin-Aliana I, et al (2021). Clinical Utility of Liquid Biopsy-Based Actionable Mutations Detected via ddPCR. Biomedicines.

For Research Use Only. Not For Use In Diagnostic Procedures.

Workflow

- For analytical verification, blood samples from healthy volunteers were collected into Streck Cell-Free DNA Blood Collection tubes (BCTs) and spiked with DNA Damage-induced H226 or MCF7 cancer cell lines (treated with Etoposide). Samples were processed between 96 and 144 hours post draw, as per workflow in **Figure 2,** and used to assess analytical sensitivity, specificity and linearity.
- Samples from 17 Breast, 5 Prostate and 2 Ovarian cancer patients, on a variety of treatment programmes, were collected into Streck Cell-Free DNA BCTs and processed as shown in **Figure 2**. Blood was collected for up to six draws per donor, with two tubes collected per draw, each processed between 72-144 hours post draw and stained using the ANGLE's IF-based CTC identification assay combined with DDR marker detection (DDR assays).

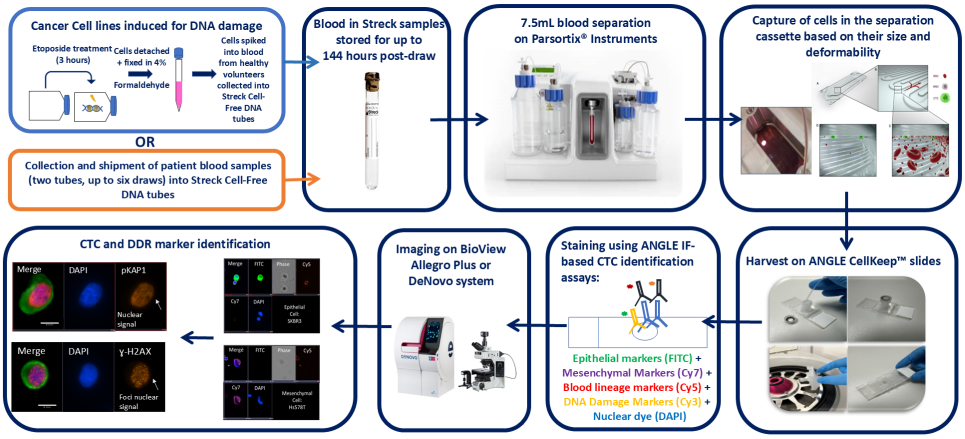
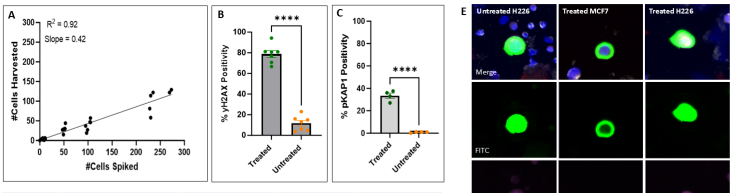


Figure 2. Schematic representation of the assay workflow. In analytical samples (light blue square), cultured cancer cell lines expressing Epithelial and/or Mesenchymal markers were induced for DNA damage, fixed with 4% Formaldehyde and spiked into healthy volunteer blood samples collected in Streck Cell-Free DNA tubes. For patient-derived samples (orange square), two tubes of 7.5 mL of peripheral blood were collected into Streck Cell-Free DNA tubes from 17 breast, 5 prostate and 2 ovarian cancer patients for up to six draws per donor. Blood was stored up to 144 hours from collection before processing. Blood samples were processed on a Parsortix® instrument, a microfluidic device capable of capturing and harvesting CTCs from bodily fluids based on cell size and lack of deformability. Harvested CTCs were cytospun onto ANGLE's CellKeep™ slides. Slides were stained using ANGLE's IF-based CTC identification assay combined with DDR markers (γ-H2AX and pKAP1) and CTCs were defined as epithelial (FITC+, Cy7-, Cy5-, DAPI+), mesenchymal (FITC-, Cy7+, Cy5-, DAPI+), or EMT transitioning (FITC+, Cy7+, Cy5-, DAPI+). CTCs were then investigated for the presence of DNA damage signal. γ-H2AX positivity was identified mostly by the presence of distinct nuclear foci and, occasionally, diffuse nuclear signal, while pKAP1 positivity was identified by the presence of a distinct diffuse nuclear signal. Stained slides were imaged using a BioView Allegro Plus system or the BioView DeNovo system, a platform equipped with artificial intelligence for automated imaging, CTC candidate identification and reporting.

Analytical Results

- **Linearity** was established by plotting the number of stained and harvested Etoposide-treated H226 cells against the number of spiked cells. A linear relationship between the number of harvested and stained cells was confirmed, with R^2= 0.92, slope = 0.42 (**Figure 3A**) over the range of 0-250 cells.
- For both γ-H2AX and pKAP1 markers, a statistically significant increase (*p <0.0001*) in DNA damage was observed in the positive/treated cell model vs the negative/untreated cell model **(Figure 3B,C)**.
- **Analytical Sensitivity** and **Specificity**, referring to the percentage of harvested cells known to express/not express a marker that had a mean fluorescence intensity (MFI) above/below the established thresholds for that marker, respectively, were all ≥ 90% in both DDR assays **(Figure 3D, E).**



D		Epithelial Markers	Mesenchymal Markers	Blood lineage Markers
γ-H2AX Assay	Analytical Sensitivity	100.00	90.46	99.65
	Analytical Specificity	94.13	93.34	92.75
pKAP1 Assay	Analytical Sensitivity	99.65	99.19	100.00
	Analytical Specificity	99.64	94.21	99.21

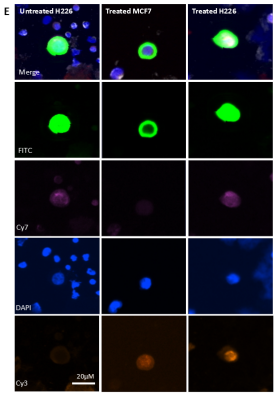
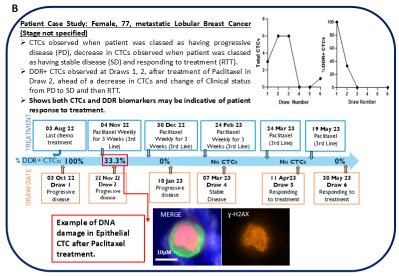


Figure 3. Analytical performance of the IF CTC identification assay combined with detection of γ-H2AX or pKAP1. **(A)** Dot plot shows the number of cancer cells spiked (x-axis) vs the number of cancer cells harvested (y-axis) across spiking levels for Etoposide treated fixed H226 cells harvested onto CellKeep Slides. The trendline equation and R^2 value are included for the first order polynomials. Histograms show the mean ± SEM of the mean percentage of **(B)** γ-H2AX positive cancer cells and **(C)** pKAP1 positive cancer cells in respective positive and negative cell models (Unpaired t test, *p**** <0.0001)*. **(D)** Summary table shows the analytical sensitivity (proportion of cells known to express the marker(s) of interest which were positive) and specificity (proportion of cells known to not express the markers of interest which were marker negative in the assay) of both DDR assays. **(E)** Representative images of untreated H226 cell (**left**) and treated (**right**) H226 cell positive for pKAP1 (FITC+, Cy7+, Cy5-, DAPI+, Cy3+/-) cells and treated (**middle**) MCF7 cell positive for γ-H2AX (FITC+, Cy7-, Cy5-, DAPI+, Cy3+) cells and PBMCs. Epithelial markers (FITC) in green, Mesenchymal markers (Cy7) in magenta, Blood lineage markers (Cy5) in white (in merge), Nuclear dye (DAPI) in blue and DDR markers (Cy3) in orange.

Results from Patient Samples

- 76% of Breast, 60% of Prostate and 100% of Ovarian cancer donors showed ≥ 1 CTC, in at least one draw, with a CTC range of 1-53 in positive samples (**Figure 4A**).
- The majority of CTCs identified across all cancer types were mesenchymal only (11/13 Breast, 2/3 Prostate and 2/2 Ovarian cancer patients), with one Breast cancer patient (1/13) and one Prostate cancer patient (1/3) showing both epithelial and mesenchymal CTCs.
- DDR marker positivity (either γ-H2AX or pKAP1) was identified in at least one CTC across all cancer types (≥1 DDR+ CTCs identified in 4/13 Breast, 1/3 Prostate and 1/2 Ovarian cancer patients).
- A case study from the Breast cancer cohort (**Figure 4B**) demonstrates the potential clinical utility of assessing CTCs and DDR+ CTCs evolution over time. DDR+ CTCs were identified ahead of the patient being classed as responding to treatment. A decrease in CTCs was identified once the patient stabilised and responded to treatment.
- Examples of CTCs identified in cancer patient samples are shown in **Figure 4C** and **D.**

A	Breast	Prostate	Ovarian
N (Donors)	17	5	2
N (Samples)	93	17	6
N Donors with ≥1 CTC	13 (76.4%)	3 (60.0%)	2 (100.0%)
N Samples with ≥1 CTC	28 (30.1%)	6 (35.3%)	3 (50.0%)
N CTC+ donors with ≥1 CTC Cluster	9 (69.2%)	3 (100.0%)	1 (50.0%)
N CTC+ samples with ≥1 CTC Cluster	18 (64.3%)	5 (83.3%)	2 (66.7%)
CTC range	1-52	1-21	1-53
CTC mean	9	8	29
CTC median	5	6	34
Donors with ≥1 Epithelial only CTCs	1 (7.7%)	0 (0.0%)	0 (0.0%)
Donors with ≥1 Epithelial and Mesenchymal CTCs	1 (7.7%)	1 (33.3%)	0 (0.0%)
Donors with ≥1 EMT CTCs	0 (0.0%)	0 (0.0%)	0 (0.0%)
Donors with ≥1 Mesenchymal only CTCs	11 (84.6%)	2 (66.7%)	2 (100.0%)
Donors with ≥ 1 CTC with DNA damage (N and %)	4 (30.7%)	1 (33.3%)	1 (50.0%)
Samples with ≥ 1 CTC with DNA damage (N and %)	6 (21.4%)	1 (16.7%)	1 (33.3%)



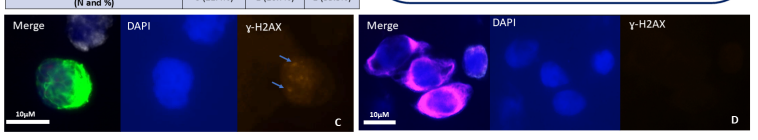


Figure 4. CTC identification and phenotyping in metastatic breast, prostate and ovarian cancer patient samples. **(A)** Table showing number (N) of donors and samples, percentage of CTC+ donors and samples (≥1 CTC), percentage of CTC cluster+ donors and samples (≥1 CTC cluster), mean, median, range of total CTCs and different CTC phenotypes by donor, percentage of donors with ≥1 DDR+ CTC over the CTC+ donors and draws. **(B)** Case study of one breast cancer patient with changes in CTC numbers and DDR+ expression in CTCs identified across draws and treatment. Representative images from patient samples of **(C)** an epithelial CTC from a prostate cancer patient with γ-H2AX foci signal and **(D)** a mesenchymal cluster of CTCs and one white blood cell (right) from a breast cancer patient negative for DNA damage markers (images taken using 60× objective lens). DDR signal indicated by blue arrows. Epithelial markers (FITC) in green, Mesenchymal markers (Cy7) in magenta, Blood lineage markers (Cy5) in white, Nuclear dye (DAPI) in blue and DDR markers (Cy3) in orange.

Conclusions

- Analytical verification demonstrated that ANGLE's DDR IF assays produce linear data, with high analytical sensitivity and analytical specificity for epithelial, mesenchymal, blood lineage and DDR markers.
- ANGLE's DDR assays identified CTCs in samples from breast, prostate and ovarian cancer patient cohorts, with ≥1 CTC observed in 76%, 60% and 100% of patients, respectively. DDR markers were detected in CTCs from all cancer cohorts, on a variety of treatment programmes.
- This study demonstrated the possibility of using ANGLE's DDR workflow to monitor both number of CTCs, and DNA damage in CTCs over time. Applied in a clinical setting, this workflow can potentially allow for minimally invasive monitoring of DNA damage inducing therapies targeting cancer cells.