Antibody-based detection of protein phosphorylation status to track efficacy of novel therapies using nanogram protein quantities from stem cells and cell lines

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ABSTRACT

This protocol describes a highly reproducible antibody-based method providing protein level and phosphorylation status information from nanogram quantities of protein cell lysate. Nanocapillary isoelectric focusing (cIEF) combines with UV activated linking chemistry to detect changes in phosphorylation status. We describe how to detect changes in response to tyrosine kinase inhibitors (TKIs) in the phosphorylation status of the adapter protein CrkL a major substrate of the oncogenic tyrosine kinase BCR/ABL in chronic myeloid leukaemia (CML), using highly enriched CML stem cells and mature cell populations in vitro. This protocol provides a 2.5pg/nL limit of protein detection (<0.2% of a stem cell sample containing $<10^4$ cells). Additional assays are described for pTyr207-CrkL and PTPRC/CD45, developed using this protocol and applied to CML patient samples. This method is high throughput and can act as a screen for in vitro cancer stem cell response to drugs and novel agents.

Summary

This protocol uses a highly sensitive and reproducible antibody-based capillary isoelectric focusing approach to establish protein phosphorylation status from nanogram quantities of protein cell lysate from cell line or primary stem cell material.

Is-protocol-to

Brown S et al. Monocyte-derived dendritic cells from chronic myeloid leukaemia have abnormal maturation and cytoskeletal function that is associated with defective localisation and signalling by normal ABL1 protein. *European Journal of Haematology* DOI: 10.1111/ejh.12306 (2014).

Williamson AJ et al. A specific PTPRC/CD45 phosphorylation event governed by stem cell chemokine CXCL12 regulates primitive hematopoietic cell motility. *Molecular & Cellular Proteomics* 12, 3319-3329 (2013).

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Categories

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KEYWORDS

Chronic myeloid leukaemia, nanogram, primitive cells, imatinib mesylate, dasatinib, phosphorylation, tyrosine kinase inhibitor (TKI), stem cell, capillary isoelectric focusing immunoassay.

INTRODUCTION

Mapping cancer cell signaling pathways using finite clinical material can support new treatment development and further our understanding of cancer biology. Assessing signaling cascades using conventional proteomic technologies often necessitate protein quantities in excess of that which is available, especially when considering human biopsy material or primitive stem cells derived from human or murine sources.

The development of protein capillary isoelectric focusing coupled to antibody-based detection has proven to be both extremely sensitive and frugal in terms of demands on finite samples. The original work by O'Neil and colleagues[1] used a prototype cIEF system to demonstrate sensitivity down to 25 cells (human prostate cancer cell line LNCaP) for the detection of extracellular signal-related kinases-1 and 2 (ERK1 and ERK2) and their associated phosphorylated states. Different iterations of the technology have subsequently been employed in a range of studies, starting with the Firefly instrument (Protein Simple, formerly Cell Biosciences) in which imatinib induced changes in ERK1 and ERK2 activation status were assessed in human CML cells isolated from total blood, whilst a range of phosphorylation changes in proteins including STAT3 and STAT5 were defined for the K562 CML cell line[2]. Next generation systems (NanoPro 1000, formally CB1000) have been used to study proteins including AKT, 4EBP1, MET, PTPRC/CD45 and CrkL in a number of leukemias, using different primary cell types including murine $CD138^{+}$ isolated from bone marrow (BM), human CD34-selected acute myeloid leukemia (AML) cells, CML-derived CD34+ and dendric cells[3-6].

Many malignant diseases are understood to have an underlying stem cell population[7-9] which this technology is capable of probing. For myeloproliferative neoplasms (MPN) and leukaemias, this stem cell population has been associated with deregulated protein tyrosine kinase (PTK) activity (for review see Mitelman[10]), including FMS-like tyrosine kinase 3 (FLT3) in acute myeloid leukaemia (AML)[11], Janus kinase 2 (JAK2) in erythroid neoplasia[12] and BCR-ABL in chronic myeloid leukaemia (CML)[13,14]. Tyrosine kinase inhibitor (TKI) therapy using imatinib mesylate (IM) has been shown to inhibit PTK activity in CML[15-18]. The use of TKIs has improved clinical outcomes markedly for the majority of patients with chronic phase (CP) CML who achieve sustained cytogenetic and molecular response[19,20]. Nonetheless second and third generation TKIs have been developed in response to BCR-ABL mutations inferring resistance to treatment (for review see O'Hare[21]). Despite continued TKI therapy in CML patients, the minimal effect of TKIs on primitive CML haemopoietic cells results in the persistence of minimal residual disease (MDR) which causes disease relapse upon drug withdrawal[22-27]. Given that CML is a stem-cell-driven disease, any new potentially curative therapies must be tested on primary stem cells, which are only available in finite quantities. Thus, methods to screen drug action on suitably limited stem cell populations are of great interest to the scientific community.

Comparison with other methods

Early comparisons of the effect of novel compounds or drugs on very limited numbers of patient stem cells would be of great value to clinicians, both for stratifying treatment response and devising new therapeutic or curative strategies. Defining these effects in terms of cellular signaling requires the identification of proteins and their state of activation in response to post-translational modifications such as phosphorylation. Numerous approaches can be taken to characterize protein state in biological samples which either employ targeted mass spectrometry (MS) or indirect analysis using antibody-based techniques. MS methods such as selected reaction monitoring (SRM)[28], also known as multiple reaction monitoring (MRM), are highly sensitive capable of quantitatively detecting attomolar concentrations of protein in complex biological samples (for review see Hjelle[29]). Antibody-based techniques, which can detect down to femtomolar protein concentrations, include western blot, ELISA, reverse-phase protein arrays (RPPA) and flow cytometry as well as the cIEF assay described here.

In CML peripheral blood cells, CrkL, a protein phosphorylated on tyrosine 207 via BCR-ABL action,[30] is an essential adapter protein for $p210^{BCR-ABL}$-induced leukaemogenic transformation[31] and has been used as a marker for BCR-ABL status and TKI activity.[32,33] Direct assay of primitive cells in the CML clone is technically challenging, limiting our understanding of the leukaemic stem cell response to targeted therapies. The SRM approach can be applied to the measurement of post translational modifications, although this can be very challenging depending on the site of the modification and the concentration of the modified isoform of the protein. SRM has been used to monitor phophorylation of ERK1 from murine vascular smooth muscle tissue, however to measure the PTM the starting amount of material was 400 ug and immunoprecipitation was required to enrich the protein prior to analysis.[34] This requirement would reduce the utility of an SRM assay for high throughput analysis, whilst the required starting material could be challenging when dealing with scarce primitive stem cells.

Western blot studies have previously been used to detect BCR-ABL expression status; however, the instability of the oncogenic tyrosine kinase upon cellular lysis leads to inaccuracies when estimating oncogenic activity[35,36]. In contrast, detection of activated CrkL by western blot provides an indirect method of determining BCR-ABL inhibition in response to TKI treatment[32]. However, this approach is low-throughput, with limited scope for multiplexed sample analysis and, owing to the requirement for bulk $CD34^+$ cells, is of little use for the analysis of more primitive cells, specifically $CD34^+$ cell subsets in this study.

Flow cytometry can detect activated CrkL in CML $CD34^+$ cells[17], with single-cell profiling used to explore signaling networks in a number of leukemic cell backgrounds (for review see Bendall & Nolan 2012[37]). Multiplex signaling data can be acquired from as few as $1x10^4$ cells, meaning that multiple subsets of cells can be identified within a heterogeneous population[38]. Up to 15 distinct signaling events can be assayed simultaneously in live cells. Interference between fluorescent probes is an inherent issue with flow cytometry, becoming more acute with an increasing number of assayed

parameters in any one experiment (for review see Bendall et al 2012[39]). Nonspecific antibody interactions within the cell can also be problematic. ELISA represents an alternative to these approaches and has been used in previous studies to determine BCR-ABL activity in Philadelphia-positive cells.[40] ELISA is high-throughput and capable of processing large sample numbers, using less material than a Western blot, yet considerably more than flow cytometry.

To enhance the limit of detection for markers in stem cells, we employed technology combining nanocapillary isoelectric focusing (cIEF) with immunoassay, on a new platform (NanoPro™ 1000; Protein Simple, Santa Clara, CA)[1]. This system has been developed on the basis of earlier work which demonstrated the potential for capillary isoelectric focusing to resolve proteins with high sensitivity whilst proving compatible with immunoassay and chemiluminescence detection (for overview see O'Neill[1]). Therefore it is theoretically possible for the user to undertake such studies without NanoPro 1000, however we have not undertaken an assessment of this option. Key advantages include the ability to handle nanoliter sample volumes with reproducible, high resolution detection of protein phosphorylation profiles simultaneously for multiple protein targets on extremely small quantities of human tissue. Multiplexed assay times are short, allowing up to 96 assays to be processed in less than 18 hours. Specifically, we have been able to demonstrate changes in CrkL phosphorylation status using 0.1% of available patient material ($<10^4$ cells per patient). From this sample, it would be possible to probe for up to 64 different protein targets simultaneously. This approach is not without limitations, novel protein targets would require assay development which can be time consuming and all potential assays are dependent upon antibody availability. In addition, cIEF-immunoassay must be combined with alternative techniques such as flow-based cell sorting to probe different cell lineages (as described in this protocol). Nonetheless, the system is compatible with a wide range of tissue types including plasma/serum, resected lung tissue, lung and endometrial biopsy material, sputum and cellular organelles. The ability of this platform to handle such a variety of biological samples enables it to be embedded into laboratory workflows which focus on biomarker validation in blood/biopsy, targeted protein PTM assessment in conjunction with MS approaches and signaling cascade mapping in defined primary cell populations.

Experimental design

This protocol defines how cIEF-immunoassay can be applied to material from cell lines and clinical material. Specifically the chronic myelogenous and acute promyelocytic leukaemic cell lines K562 and HL60 have been used in option A to develop an assay for the protein CrkL which is sensitive enough to be applied to clinical material containing d5000 cells. Although this cell line protocol has principally been used for the development of a stem cell compatible assay here, the method described has been applied to a range of cell lines (i.e. murine Ba/F3 cells, embryonic stem cells, A549 and HEK 293 human cell lines) and protein targets (for an up-to-date list of developed assays please refer to www.scalpl.org) in our hands. This method is fully transferable, and can be used to

develop assays for any protein using murine and human cell line material (other cell line sources will likely be compatible, however we cannot verify this).

Assay development involves a number of steps, each defining an important aspect of the final protocol for a given protein. It is important to note that the majority of these development steps are determined by the protein of interest, specifically these relate to a proteins basal pI, associated isoelectric range (with increasing phosphorylation) and capillary immobilisation chemistry. Acquisition of suitable antibodies for total and phosphorylated entities is essential. Those shown to work with other immunoassays (i.e. western blot, immunohistochemistry) do not necessarily function well in the cIEF environment; however we have had considerable success with antibodies developed as part of the Human Protein Atlas (www.proteinatlas.org) programme. Assay development should incorporate suitable controls including test for secondary antibody cross-reaction with sample minus primary antibody (also test for streptavidin-HRP cross-reaction if using 3-step approach; see **step 2**) together with lambda phosphatase treated samples (see **steps 19-23**) to confirm protein observations are a result of phosphorylation as opposed to other PTMs. It is worth observing that this protocol is non-denaturing, with secondary and tertiary protein structure preserved after isoelectric focusing and immobilisation. Consequently, knowledge of a protein's folding structure can be useful when selecting antibodies for assay development, and a focus on antibodies raised to hydrophilic regions is advisable.

It is essential that the limit of detection for an assay is defined, specifically for those designed to probe finite numbers of stem cells. As a guide, a detection limit of e20pg/nL total protein would not be suitable; typically, 2-8pg/nL total protein is required to probe stem cells, especially when dealing with low-copy-number proteins and proteins that undergo transient post-translational modifications. Once developed, if an assay is compatible with low cell number clinical samples, then it is possible to assess the signaling behavior of a given protein in human and murine cells purified using flow-based sorting (**option B**). Issues surrounding high salt content of sample and cell loss during preparation are addressed in this protocol, allowing for assessment of specific cell linage populations, pertinent in this case for CML therapy, but equally applicable to other disease areas. In the majority of cases, the protein concentration of samples derived from clinical samples (i.e. resected lung tissue) is greatly in excess of the protein requirements for this platform, and the simple protocol used with cell lines can be applied (**option A**). For samples with protein concentrations <0.1mg/mL, then dilution step can be omitted and the approach to sample preparation taken here for stem cells should be employed (see **step 5**). When processing large numbers of clinical samples, a number of controls should be applied including triplicate sample loading, positive control (i.e. cell line; n=1) and a clinical sample pool for inter-cycle and inter-experiment referencing. Additionally, during assay development, controls must be used which assess secondary-antibody and streptavidin-HRP cross reaction with sample and primary antibodies.

The cIEF-immunoassay platform utilizes UV-activated linking chemistry (for details of chemistry used see O'Neill[1]), which immobilizes isoelectrically separated proteins to the capillary wall, allowing downstream immunoassay without the need for protein blocking steps. Previous work has shown that the isoelectric point (pI) of a protein shifts to more alkali values with inhibition of phosphorylation[1,41]. Compass software (Protein Simple) is used for every aspect of an assay via three perspectives, "Assay", "Run" and "Analysis". Assay perspective allows the user to design the assay plate and program the NanoPro 1000 system to process samples in a specific order using defined settings (e.g. antibody incubation or UV exposure), allowing flexibility when designing complex experiments. Once an assay has been started, the "Run" perspective gives a real-time display of sample separation on capillary and provides the user with information relating to each stage of the experiment (e.g. sample loading, incubation times, and estimated assay completion time). With data acquisition complete, the "Analysis" perspective provides a range of tools to enable the user to analyse results from capillaries. Given the nature of this system, alternative software is not required, with the exception of total area under curve calculations which require third party software; specifically we use Medcalc (Medcalc Software).

The present technology offers a more sensitive method for assessing protein phosphorylation status, using our workflow (**Fig. 1**). Specifically, when compared to the prototype systems used in earlier studies[1,2] (Firefly, Protein Simple; formaly Cell Biosciences) the NanoPro 1000 has a number of mechanical enhancements including improved robotic z-scheme mapping and liquid handling, a smaller form factor with improved camera and UV attributes. In addition, numerous iterative improvements have been made to consumables chemistry such as the three-step secondary-biotin to streptavidin-HRP detection kit which can be used in place of conventional secondary-HRP antibody, XDR peroxide for improved chemiluminescent detection with low-copy number proteins, second generation ampholyte premixes including acidic pH nesting to prevent sample run-off during isoelectric separation, together with a host of subtle to changed other consumable solutions.

Here, we use cIEF-immunoassay to study the phosphorylation status of the CrkL adapter protein in $CD34^+CD38^+$ and primitive $CD34^+CD38^-$ CML cells. The primary tissue is isolated by flow cytometry using leukapheresis samples, followed by culture in growth factors and TKIs as previously described[42]. A low-ionic-strength buffer wash (20mM Bicine, 250mM Sucrose; pH 7.5) is used prior to lysis with zwitterionic detergent (20mM Bicine; pH 7.6, 0.6% CHAPS). Lysates are combined with cIEF reagents (**see cIEF: general procedure; steps 2-18**) and loaded onto a multiwell plate. The separation gradients have been optimised for the CrkL assay, taking into account the small number of primary cells used in stem cell studies. After isoelectric separation, proteins are immobilised using a UV-sensitive technique that links the proteins to the capillary wall at their native pI. The CrkL assay was developed using control cell lines to determine suitability for clinical sample analysis.

This protocol has been successfully used in two recently published studies involving finite quantities of CML patient material.[5,6] In one of these studies, Brown et al.[6] focused on the defective

function of BCR-ABL expressing mature dendritic cells derived from CML patient monocytes and found that dysregulation of ABL1 protein distribution in CML dendritic cells (CML-DCs) resulted in changes to CrkL (a substrate protein of ABL1), altering the formation of complexes involved in the control of F-actin responses. Specifically, CrkL displayed elevated levels of phosphorylation in CML-DCs when compared with normal DCs [6]. Here, we define and compare the proportional degree of phosphorylation for these two cell types, with particular emphasis on the ABL1-target residue Tyr207 of CrkL, using both anti-CrkL and anti-pTyr207-CrkL antibodies. Analysis with antibodies that detect total protein produces a profile encompassing post-translational modifications (PTM) and isoform expression, which can induce changes in the pI of a protein. Antibodies that can detect specific events, such as phosphorylation, are required to further define a protein profile.

In the other study, proteomic analysis of CXCL12-mediated signaling in hematopoietic progenitor cells identified a novel phosphorylation site on the protein tyrosine phosphatase PTPRC/CD45 at residue Ser962. Phosphorylation at this site was shown to be upregulated in the presence of BCR-ABL in $CD34^+$ cells derived from patients with CML when compared with control samples (from patients without CML), using cIEF with both commercially available and in-house antibodies, respectively.[5] Using the fully optimised PTPRC/CD45 assay developed in our laboratory (for assay development data see **Supplementary Fig.2**), we validated the approach using clinical samples, specifically looking at novel signaling differences between more primitive $CD34^+CD38^-$ CML cells that co-enrich with stem cells and more mature CML $CD34^+CD38^+$ progenitor populations, both in TKI-treated samples and in untreated controls. The quantity of clinical starting material needed for this approach is significantly smaller than those required in previous studies using a cIEF platform[1,2], and this technique also enables a more detailed analysis of protein phosphorylation than was possible with earlier technologies.

MATERIALS

REAGENTS

- Cell Lines of interest. We used K562 and HL60 (kindly provided by Professor Tessa Holyoake, University of Glasgow). We tested the Cell lines using a University of Manchester authentication service employing a Promega Powerplex® 21 system (cat no: DC8902), which examined 21 loci across the genome and compared results to a local database of ATCC references. This can be applied to any ATCC registered cell line.
- Cells derived from clinical samples of interest. We used Human $CD34^+$-enriched cells from fresh leukapheresis samples obtained with written consent from patients with newly diagnosed chronic-phase CML. Normal $CD34^+$ cells were extracted from autologous donors with non-stem-cell disorders. **! CAUTION** All experiments using human clinical material must conform to appropriate national and international regulations (see West of Scotland Research Ethics Committee 4 reference 10/S0704/2).
- RPMI medium 1640 (1X; Gibco by Life Technologies, cat. no. 31870-025)
- Dulbecco's phosphate buffered saline (DPBS), sterile filtered (Sigma-Aldrich, cat. No. D8537) ² **CRITICAL** For use with cultured cell lines.
- Fetal bovine serum, sterile filtered (Sigma-Aldrich, cat. no. F7524)
- L-Glutamine, 200mM (Sigma-Aldrich, cat. no. G7513)
- Penicillin-streptomycin, 10,000U/mL (Gibco by Life Technologies, cat. no. 15140-122)
- Bicine, e99% titration (Sigma-Aldrich, cat. no. B3876)
- CHAPS (Sigma Life Science, cat. no. C9426)
- Protease inhibitor cocktail (Sigma-Aldrich, cat. no. P8340)
- Phosphatase inhibitor cocktail 2 (Sigma-Aldrich, cat. no. P5726)
- Phosphatase inhibitor cocktail 3 (Sigma-Aldrich, cat. no. P0044)
- Benzonase® nuclease HC, 99% purity (Merck-Millipore, cat. no. 71206)
- Sodium orthovanadate, 100mM (Sigma-Aldrich, cat no: S6508) **! CAUTION** Solution preparation involves boiling this reagent in the microwave, with a risk of burning.
- Sodium fluoride, 500mM (Sigma-Aldrich, cat. no. S7920)
- Premix G2, 3-10 separation gradient (Protein Simple, cat. no. 040-968) ² **CRITICAL** Due to the high viscosity of the premix solutions, it is essential that the user employs a reverse pipetting technique to accurately measure the required volume. Failure to do so can lead to inaccuracies in sample preparation and an inadequate amount of premix in the samples under analysis.
- Premix G2, 5-8 (nested) separation gradient (Protein Simple, cat. no. 040-972) ² **CRITICAL** Due to the high viscosity of the premix solutions, it is essential that the user employs a reverse pipetting technique to accurately measure the required volume. Failure to do so can lead to inaccuracies in sample preparation and an inadequate amount of premix in the samples under analysis.
- DMSO inhibitor mix (Protein Simple, cat. no. 040-510)
- Antibody diluent (Protein Simple, cat. no. 040-309)
- pI Standard Ladder 3 (Protein Simple, cat. no. 040-646)
- Wash concentrate (Protein Simple, cat. no. 041-108)
- Peroxide XDR (Protein Simple, cat. no. 041-084)
- Luminol (Protein Simple, cat. no. 040-652)
- Anolyte solution, 0.1M phosphoric acid (Sigma-Aldrich, cat. no. 438081)
- Catholyte solution, 0.1M sodium hydroxide (BDH, cat. no. 102524X)
- Type I ultra-pure laboratory water, 18.2 M&.cm (Elga Process Water). ² **CRITICAL** High-purity water is essential for interassay consistency.
- Amplified Rabbit Secondary Antibody Detection Kit (Protein Simple, cat. no. 041-126)

- Anti-CrkL (C-20) antibody (Santa Cruz, cat. no. sc-319)
- Anti-phospho CrkL (Tyr 207) antibody (Cell Signalling, cat. no. #3181S)
- Anti-PTPRC/CD45 antibody (BD Transduction Laboratories™, cat. no. 610266)
- Anti-pS962 PTPRC/CD45 antibody (Eurogentec, custom antibody)
- Anti-AKT (1+2+3) antibody (Cell Signalling Technology®, cat. no. #9272)
- Imatinib mesylate, STI571 (Selleckchem, cat. no. S1026)
- Dasatinib, monohydrate (Santa Cruz, cat. no. sc-21808)
- Lambda protein phosphatase, 20,000 units (Merck Millipore, cat. no. 14-405)
- Trypan blue solution (w/v), 0.4% (Sigma-Aldrich, cat. no. T8154)
- BIO-RAD Protein Assay Dye Reagent Concentrate (BIO-RAD, cat. no. 500-0006)
- Albumin from bovine serum (Sigma-Aldrich, cat. no. A3059-100G)
- ProtoFlowGel, 30% (w/v) acrylamide: 0.8% (w/v) Bis-acrylamide (Flowgen Bioscience, cat. no. H16996)
- Trizma base (Sigma-Aldrich, cat. no. T1503)
- Glycine (Sigma-Aldrich, cat. no. G8898)
- Hydrochloric acid, 30% (v/v) (BDH, cat. no. 262743N)
- Sodium dodecyl sulphate (BDH, cat. no. 442444H)
- Ammonium persulphate (Sigma-Aldrich, cat. no. A3678)
- N, N, N", N'-Tetramethylethylenediamine bioreagent (Sigma-Aldrich, cat. no. T7024)
- Bromophenol blue sodium salt (Sigma-Aldrich, cat. no. B7021)
- Glycerol (Fisher Scientific, cat. no. BP229)
- 2-mercaptoethanol (Sigma-Aldrich, cat. no. M7154) [2] **CRITICAL** When used with Lamelli buffer, store aliquots at -80ºC to avoid oxidization. Do not freeze-thaw.
- 2-mercaptoethanol, 50mM (Gibco by Life Technologies, cat. no. 31350-101) [2] **CRITICAL** For use with primary cells.
- AnalR NORMAPUR methanol (VWR, cat. no. 20847.307)
- Ponceau S Solution (Sigma-Aldrich, cat. no. P7170)
- Tween-20 (Sigma-Aldrich, cat. no. P1379)
- Nonfat-dried milk powder (Sigma-Aldrich, cat. no. M7409)
- SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, cat. no. 34078)
- Acetic acid, e99.0% purity (Sigma-Aldrich, cat. no. 45740)
- Interleukin 6 (IL-6), recombinant human (Life Technologies, cat. no. PHC0066)
- Granulocyte colony-stimulating factor (G-CSF), recombinant human (Life Technologies, cat. no. PHC2035)
- Granulocyte macrophage colony-stimulating factor (GM-CSF), recombinant human (Life Technologies, cat. no. PHC2015)
- Stem cell factor (SCF) C-Kit ligand, human recombinant (Life Technologies, cat. no. PHC2116)
- Leukaemia inhibitory factor (LIF), recombinant human, 10µg/mL (Merck Millipore, cat. no. LIF1010)
- Macrophage inflammatory protein-1± (MIP-1±), recombinant human (Life Technologies, cat. no. PHC1104)
- BIT (BSA/insulin/transferrin; Stem Cell Technologies, cat. no. 09500)
- Low density lipoprotein, 10mg/ml (Sigma-Aldrich, cat. no. L4646)
- Iscove's modified Dulbecco's media (IMDM; Sigma-Aldrich, cat. no. I3390)
- DNase I, ~2500U/vial (Stem Cell Technologies, cat. no. 07900)
- Magnesium chloride, 1M (Sigma-Aldrich, cat. no. M8266)
- Trisodium citrate (Sigma-Aldrich, cat. no. S1804)
- Albunorm™ 20%, 200g/L (Octapharma) [2] **CRITICAL** Store at room temperature (21°C) prior to use.

- Dulbecco's PBS, Mg^{2+}/Ca^{+} Free (Gibco by Life Technologies, cat. no. 14190-169) [2] **CRITICAL** For use with primary cells.
- Anti-human CD34-APC (Becton Dickinson, cat. no. 555824)[42]
- Anti-human CD38-FITC (Becton Dickinson, cat. no. 555821)[42]
- Dimethyl sulfoxide (DMSO), ACS spectrophotometric grade, e99.9% (Sigma-Aldrich, cat. no. 154938)
- Rely+On™ Virkon® powder (DuPont™)

EQUIPMENT

- FACSAria Cell Sorting System (Becton Dickinson)
- Capillaries-Charge Separation for Peggy/NanoPro 1000 (Protein Simple, cat. no. CBS700) [2] **CRITICAL** Capillaries are light and moisture sensitive; they should be stored in a dark dry place at room temperature.
- Sponge Pack for Peggy/NanoPro 1000 (Protein Simple, cat. no. 041-528)
- NanoPro 1000 Simple Western Charge-based Assay System (Protein Simple)
- Compass software, version 1.8.2 (Protein Simple). This software is used to setup, run and analyse data generated using the Peggy System.
- Medcalc, version 13.3.3 (Medcal Software). This software is used to calculate total area under curve values for data generated by Compass.
- Purelab Ultra Laboratory Water Purification System (Elga Process Water)
- Eppendorf safe-lock tubes, 0.5mL, (Eppendorf, cat. no. 0030121023)
- Eppendorf safe-lock tubes, 1.5mL, (Eppendorf, cat. no. 0030120086)
- Plastic tips for P10, P200 and P1000 pipettes
- Terumo® 60cc luer lock tip syringe without needle, graduation 1cc, 60cc total (Terumo medical products, cat. no. SS-60L)
- Millex® syringe filter unit, sterile, 25mm diameter, 0.2µm pore size (Merck Millipore, cat. no. SLFG025LS)
- Microfuge, 5415D (Eppendorf)
- Centrifuge, SL16R (Thermo Scientific)
- Vortex PV-1 (Grant-Bio)
- Microscope, DMIL (Leica)
- Water bath (Grant Instruments)
- Corning® flask, 25cm^2 cell culture flask, angled neck, 0.2µm vented Cap (Corning Inc, cat. no. 3056)
- Corning® flask, 75cm^2 cell culture flask, canted neck, 0.2µm vented cap (Corning Inc, cat. no. 430641)
- Adam automatic cell counter (NanoEnTek Inc, cat. no. ADAM-MC)
- Adam AccuChip^{4X} Kit (NanoEnTek Inc, cat. no. AD4K-200)
- Double Cell Neubauer Haemocytometer Counting Chamber, 0.1mm; 1/400 mm^2 (Hawksley, cat. no. AC2000)
- Haemocytometer cover glass, 20mm x 26mm (Fisher Scientific, cat. no. 12352168)
- Finnpipette® Novus 10-100µL single-channel electronic pipette (Thermo Scientific, cat. no. 4620040)
- Pipette controller (Starlab, cat. no. E4866-0021)
- Costar® 5mL Stripette (Corning Inc, cat. no. 4487)
- Costar® 10mL Stripette (Corning Inc, cat. no. 4488)
- Costar® 25mL Stripette (Corning Inc, cat. no. 4489)
- SafeFlow 1.2 microbiological safety cabinet, class II-B3 (Bioair Instruments, IT)
- Heracell 240i CO_2 incubator (Thermo Scientific, cat. no. 51026331)

- Corning® CentriStar™ 15mL centrifuge tube (Corning Inc, cat. no. 430791)
- Corning® CentriStar™ 50mL centrifuge tube (Corning Inc, cat. no. 430829)
- Microtitre plate, 96 well/flat bottom, non-sterile (Sterilin, cat. no. 611F96)
- Multiskan Ascent plate reader (Lab Systems Inc, USA)
- Amersham™ Hybond ECL nitrocellulose blotting membrane (GE Healthcare Life Sciences, cat. no. RPD203D)
- Hoefer power pack (EPS2A/200)
- Stuart magnetic stirrer (UC151)
- Spinba® magnetic stirring fleas (Sigma-Aldrich)
- Sterile Duran® laboratory bottles 100, 500 and 1000mL sizes
- pH meter (MP220) with an Inlab® Expert Pro pH probe (Metter Toledo)
- Stuart roller mixer (SRT2)
- Mini-PROTEAN® Tetra Cell (Biorad, cat. no. 165-8001)
- Chemidoc™ XRS System (Biorad)
- Quantity One 1-D Analysis Software, version 4.6.0 (Biorad)

REAGENT SETUP

- **Bicine/CHAPS buffer and sample diluent (20mM Bicine, 0.6% CHAPS (w/v); pH 7.6)** Prepare a solution containing 326mg Bicine and 600µL CHAPS and make up to 100mL with Purelab ultra-pure water. Adjust the pH to 7.6 using a magnetic stirrer and pH meter. Store at 4ºC for up to 2 months.
- **Bicine/CHAPS lysis buffer** For 1ml of buffer, combine 957µL Bicine/CHAPS buffer, 3 µL Benzonase® Nuclease, 10 µL Protease Inhibitor Cocktail, 10 µL Phosphatase Inhibitor Cocktail 2, 10 µL Phosphatase Inhibitor Cocktail 3, 10 µL 100mM Sodium Orthovanadate and 1 µL 500mM Sodium Fluoride. When lysing cell lines, 50µL buffer is required for every $1x10^6$ cells. Primary cells will require <10µL for every $1x10^6$ cells and the user should be cautious not to over dilute the lysate by using too large a volume of lysis buffer. Add the 10µL of lysis buffer 2µL at a time until the primary cell pellet begins to dissolve. Make lysis buffer just prior to use and keep on ice for a maximum of 2 hours.
- **Sample diluant/DMSO inhibitor mix solution (SSD)** Mix 10µL of DMSO inhibitor mix with 490µL of sample diluent. Vortex and store at 4ºC for up to 6 months.
- **Anolyte solution (0.1M H_3PO_4)** Take 6.9mL Phosphoric Acid and make to 1 litre with Purelab ultra-pure water. Check pH with pHydrion insta-check paper. Store at 4ºC for up to 6 months. **!** **CAUTION** take care when decanting phosphoric acid as it is very corrosive. Ensure correct personal protective equipment (PPE) are used and decant using pipette controller.
- **Catholyte solution (0.1M NaOH)** Dissolve 4g sodium hydroxide pellets and make to 1 litre with Purelab ultra-pure water. Check pH with pHydrion insta-check paper. Store at 4ºC for up to 6 months.
- **Tris-HCl resolving solution (1.5M; pH 8.8)** Dissolve 18.2g Trisma-base in 30mL Purelab ultra-pure water and set pH with HCl using pH meter and magnetic stirrer. Make to 100mL and store at room temperature for up to 6 months.
- **Tris-HCl stacking solution (0.5M; pH 6.8)** Dissolve 6.1g Trisma in 30mL Purelab ultra-pure water and set pH with HCl using pH meter and magnetic stirrer. Make to 100mL and store at room temperature for up to 6 months.
- **SDS stock solution** Dissolve 10g sodium dodecyl sulphate in 50mL Purelab ultra-pure water using a magnetic stirrer. Make to 100mL store at room temperature for up to 2 months. **!** **CAUTION** Toxic if inhaled; dispense detergent in fume hood.
- **Ammonium persulphate solution** Mix 500mg ammonium sulphate in 4.5mL Purelab ultra-pure water and vortex to dissolve. Freshly prepare solution before use.

- **Bromophenol blue stock solution (1%, w/v)** Dissolve 100mg bromophenol blue sodium salt into 2mL Purelab ultra-pure water and vortex to mix. Make up to 10mL final concentration and store at room temperature for up to 6 months.
- **Reservoir buffer stock solution (25mM Tris, 142mM Glycine)** Make x10 stock solution by adding 30.33g Trisma to 144g Glycine and making up to 500mL with Purelab ultra-pure water until dissolved. Make solution to a final concentration of 1000mL. Store at room temperature for up to 6 months.
- **Reservoir buffer/SDS 0.05% (w/v) solution** Take 100mL of reservoir buffer stock solution, add 1mL SDS stock solution and make up to 1000mL with Purelab ultra-pure water. Freshly prepare buffer before use.
- **Western transfer buffer** Dilute 90mL reservoir buffer stock solution 1:10 with Purelab ultra-pure water (add 100mL methanol for use with proteins of <40kDa). Freshly prepare buffer before use.
- **Lamelli Stock Sample Buffer x5 (60mM Tris-HCl, pH 6.8; 2% SDS (w/v); 10% Glycerol (v/v); 5% 2- mercaptoethanol (v/v); 0.01% Bromophenol blue(w/v))** Mix 4mL 1.5M Tris-HCl stacking solution, 10mL Glycerol, 5mL 2-mercaptoethanol and 1mL Bromophenol blue stock solution. Vortex until bully mixed and aliquot into suitable volumes. Store at -20ºC for up to 1 year.
- **PBS-Tween solution** Add 2.5mL Tween-20 to 500mL of Dulbeccos PBS solution and mix using a magnetic stirrer. Store at room temperature for up to 3 months.
- **NFDM solution 5% (w/v)** Used for membrane blocking and secondary antibody incubation. Mix 2.5g nonfat dried milk and dilute in 25mL PBS-Tween solution using a magnetic stirrer. Make to 50mL final volume. Store at 4ºC for up to 3 days.
- **NFDM solution 1% (w/v)** Used for primary antibody incubation. Mix 0.5g nonfat dried milk and dilute in 25mL PBS-Tween solution using a magnetic stirrer. Make to 50mL final volume. Store at 4ºC for up to 3 days.
- **ECL solution** Combine SuperSignal® West Pico Chemiluminescent Substrate A and B in a 1:1 volume to make 2mL final volume. Use immediately.
- **Acetic acid (100mM) solution** Dilute 5.75µL acetic acid (>99.0%) to 1mL with Purelab ultra-pure water and store at room temperature for up to 6 months. Filter sterilise using 0.2µ sterile filter before use.
- **PBS/Tween (0.02%, v/v) solution** Dilute 200µL Tween-20 into 99.8mL DPBS. Filter sterilise using 0.2µ sterile filter before use. Store at room temperature for up to 5 days.
- **Magnesium chloride (1M) stock** Dissolve 952.1mg $MgCl_2$ in 5mL Purelab ultra-pure water using a magnetic stirrer. Make to 10mL final volume. Filter sterilise using 0.2µ sterile filter before use. Store at 4ºC for up to 6 months.
- **Trisodium citrate (0.155M)** Dissolve 45.59g in 500mL Purelab ultra-pure water using a magnetic stirrer. Make to 1000mL final volume. Filter sterilise using 0.2µ sterile filter water before use. Store at 4ºC for up to 6 months.
- **Base serum-free medium (SFM)** Make in a sterile Duran® bottle in a Class II hood. For a 125mL stock, combine 97.25mL IMDM, 25mL BIT, 1.25mL 200mM L- Glutamine, 1.25mL 10,000U/mL Penicillin-Streptomycin, 250µL 50mM 2-mercaptoethanol, and 500µL 10mg/mL Low density lipoprotein while mixing the solution with a magnetic stirrer. Filter sterilise using 0.2µ sterile filter water prior to storage at 4ºC for up to 1 week.
- **SFM plus growth factors** SCF 0.2ng/ml, G-CSF 1ng/ml, GM-CSF 0.2ng/ml, IL-6 1ng/ml, LIF 0.05ng/ml, MIP-± 0.2ng/ml. Once prepared, store at 4ºC and use within 2 weeks.
- **IL-6 stock** Supplied as 25µg lyophilized, carrier-free powder. To regain full activity, IL-6 requires reconstitution in 250µL of 100mM acetic acid to 0.1mg/mL. Make 20µL aliquots and store at -20ºC until needed. For working concentration, prepare fresh. Dilute stock 1:20 with DPBS to 0.005mg/mL. Use 2µL per 10mL SFM to give a final concentration of 1ng/mL. <CRITICAL> Prepare the reagent under sterile conditions using a Class II microbiological safety cabinet. Do not store it in a frost-free freezer.

- **G-CSF stock** Supplied as 25µg lyophilized, carrier-free powder. To regain full activity, G-CSF requires reconstitution in 250µL of Purelab ultra-pure water to 0.1mg/mL. Make 20µL aliquots and store at -20ºC until needed. For working concentration, prepare fresh. Dilute stock 1:50 with DPBS to 0.002mg/mL. Use 5µL per 10mL SFM to give a final concentration of 1ng/mL. <CRITICAL> Prepare the reagent under sterile conditions using a Class II microbiological safety cabinet. Do not store it in a frost-free freezer.
- **GM-CSF stock** Supplied as 10µg lyophilized, carrier-free powder. To regain full activity, GM-CSF requires reconstitution in 100µL of Purelab ultra-pure water to 0.1mg/mL. Make 10µL aliquots and store at -20ºC until needed. For working concentration, prepare fresh. Dilute stock 1:1000 with DPBS to 0.0001mg/mL. Use 20µL per 10mL SFM to give a final concentration of 0.2ng/mL. <CRITICAL> Prepare the reagent under sterile conditions using a Class II microbiological safety cabinet. Do not store it in a frost-free freezer.
- **SCF stock** Supplied as 25µg lyophilized, carrier-free powder. To regain full activity, SCF requires reconstitution in 250µL of Purelab ultra-pure water to 0.1mg/mL. Make 20µL aliquots and store at -20ºC until needed. For working concentration, prepare fresh. Dilute stock 1:200 with DPBS to 0.0005mg/mL. Use 4µL per 10mL SFM to give final concentration of 0.2ng/mL. <CRITICAL> Prepare the reagent under sterile conditions using a Class II microbiological safety cabinet. Do not store it in a frost-free freezer.
- **MIP-1-alpha stock** Supplied as 10µg lyophilized, carrier-free powder. To regain full activity, MIP-1 alpha requires reconstitution in 100µL of Purelab ultra-pure water to 0.1mg/mL. Make 10µL aliquots and store at -20ºC until needed. Perform under sterile conditions using a Class II microbiological safety cabinet. For working concentration, prepare fresh. Dilute stock 1:1000 with DPBS to 0.0001mg/mL. Use 20µL per 10mL SFM to give a final concentration of 0.2ng/mL. <CRITICAL> Prepare the reagent under sterile conditions using a Class II microbiological safety cabinet. Do not store it in a frost-free freezer.
- **LIF stock** Supplied as 10µg/mL solution. Dilute 1:100 in DPBS/Tween solution to a final concentration of 0.1µg/mL. Perform under sterile conditions using a Class II microbiological safety cabinet. Store at 4ºC for up to 1 month.
- **Physiological low-growth factor cocktail (PGF)** To 10mL SFM medium, add working concentrations of growth factors SCF, G-CSF, GM-CSF, IL-6, LIF and MIP-1± (as detailed in the associated text for each stock solution). Perform under sterile conditions using a Class II microbiological safety cabinet. Once prepared, store at 4ºC and use within 2 weeks.
- **DAMP solution** From stock solutions, combine 2mL DNase I, 1.25mL $MgCl_2$, 53mL Trisodium Citrate, 25mL Albunorm™ (20%) and make to 500mL with DPBS. Perform under sterile conditions using a Class II microbiological safety cabinet. Store in 50mL aliquots at 4ºC for up to 6 months. Warm to 37.5ºC before use.
- **Imatinib mesylate stock solution (10mM)** Supplied as 100mg lyophilized powder. Perform under sterile conditions using a Class II microbiological safety cabinet. Dissolve in 900µL DMSO with vortex, giving a bulk stock concentration of 169.58mM, which should be stored at -20ºC until needed. Take 58.97µL of this solution and dilute to 1mL with DPBS (giving 10mM stock). Aliquot and store at -20ºC. For a final concentration of 5µM, use 5µL 10mM stock for every 10mL cultured cells ($2x10^5$ cells/mL).
- **Dasatinib stock solution (150µM)** Supplied as 10mg lyophilized powder. Perform under sterile conditions using a Class II microbiological safety cabinet. Dissolve in 5mL DMSO with vortex, make to 10mL final bulk stock volume (1.976mM), and store at -20ºC until needed. Take 75.9µL and dilute to 1mL with DPBS (giving 150µM stock). Aliquot and store at -20ºC. For a final concentration of 150nM, use 10µL 150µM stock for every 10mL cultured cells ($2x10^5$ cells/mL).
- **RPMI base media (10%FBS (v/v); 20mM L-Glutamine; 36U/mL Pen-Strep)** To 500mL RPMI 1640 medium, add 50mL FBS, 2mL penicillin-streptomycin (10,000U/mL) and 5mL 200mM L-Glutamine. Perform under sterile conditions using a Class II microbiological safety cabinet. Store at 4ºC for up to 2 weeks. Warm to 37.5ºC prior to use.

- **K562 and HL60 cells** Store K562 and HL60 cells in liquid nitrogen in 1mL 90% FBS and 10% DMSO at densities of $3x10^6$ and $5x10^6$ per ampule, respectively.
- **Rely+On™ Virkon® solution (1% w/v)** With 10g Virkon powder make to 1L using luke-warm water in a fume hood. Store at RT for up to 6 months.

PROCEDURE

Sample preparation

1) There are two options for preparing samples, the first (**option A**) focuses specifically on approaches to preparing cell line material for analysis and can be used independently of the second (**option B**) which defines a method specific for primary stem cell material.

OPTION A – Cell Lines Ï TIMING Steps i-xii, 1-2 weeks, depending upon cell recovery time. Steps xii-xix, 8h.

i. *Sample preparation of controls (K562 & HL60 cell lines): thawing and passaging.* Prior to thawing of cells, prepare base media by adding 50mL FBS, 5mL 200mM L-Glutamine and 2mL Penicillin-Streptomycin to 500mL RPMI media (total volume of 557mL). Mix by shaking and place in a water bath that has been pre-warmed to 37.5ºC for 30m.

ii. Dispense 8mL RPMI base media in a 15mL centrifuge tube using a pipette controller and 10mL stripette.

iii. Thaw cell ampules in water bath at 37.5ºC. Transfer to RPMI base media prepared in **option A; step i**. ² **CRITICAL STEP** the cell stock should be part-frozen at this point. As soon as the frozen material is dislodged by melted buffer, transfer contents to the pre-warmed media.

iv. Wash ampule with by adding 1mL RPMI base media into tube, and quickly pipetting three times (5s each), ensuring the tube walls are fully washed. Remove media and add to the centrifuge tube. This allows for a 1:10 dilution of the frozen cell medium to displace the DMSO.

v. Replace screw cap and invert mixture. Centrifuge at 450g at room temperature for 3m to pellet.

vi. Discard the supernatant in liquid virkon (1% w/v) and resuspend the pellet in 10mL RPMI base media Place in a 25cm^2 cell culture flask and incubate overnight using standard conditions of 37ºC, 5% CO_2 and a humidified atmosphere.

vii. Assess cells with a microscope at x10 magnification to visually determine cell recovery. At this point, there should be some dead cells, but a significant number of viable cells should be observed (>70% viable).**?TROUBLESHOOTING**

viii. Transfer cell culture from flask to a 15mL centrifuge tube. Take 2 x 50µL aliquots for cell count. ² **CRITICAL STEP** Before decanting cell culture, gently tap sides of flask first to dislodge cells that have adhered to the bottom of the flask and gently shake culture.

ix. Pellet sample by centrifuging at 450g for 3m to remove dead cells & debris. Discard supernantant in liquid virkon (1% w/v). Resuspend cell pellet in 10mL pre-warmed RPMI base media and transfer to fresh 25cm^2 cell culture flask. Incubate overnight under standard conditions.

x. Count cells. We use an ADAM automatic cell counter for large experiments requiring fast cell counting, however Trypan Blue exclusion is perfectly adequate. To count using an ADAM automatic cell counter, add 50µL of solution T and 50µL solution N into two separate 0.5mL Eppendorf tubes. Next, displace and cells that have adhered to the cell culture flask as in **step viii,** take two 50µL aliquots of cell culture, adding one to each of the Eppendorf tubes containing ADAM solutions. Mix gently by pipetting and place 20µL from each aliquot into either

channel T or channel N (depending on which solution is used) of an Adam AccuChip™. Place the chip in the cell counter and start the count. The value given will determine the percentage of cells that recover post-thaw over 24 h (typically >50%). [2] **CRITICAL STEP** check cell viability and number weekly using trypan blue to ensure accuracy of Adam automatic cell counter.

xi. Continue to culture cells for a further 24h, and then repeat **steps viii and ix**. Determine the cell number per mL as in **step x** and calculate the volume of cultured cells required for passaging. Cells should be seeded at a concentration of $1x10^5$ cells/mL. For example, 1.67ml of culture at a concentration of $6x10^5$ cell/mL should be added to 8.33ml of fresh pre-warmed RPMI base media to make a final volume of 10mL (see calculation below).

Volume of culture required = ($1x10^5$ cells/mL / $6x10^5$ cells/mL) x 10mL

i. = 1.67mL

Amount of fresh pre-warmed RPMI base media required

ii. = 10ml (total final volume) - 1.67mL culture
iii. = 8.33mL

xii. Place passaged culture in incubator for 48h. [2] **CRITICAL STEP** Experiments should be undertaken using cells between passages 5 and 40. Cells harvested for storage should be split <20 times.

xiii. *Sample preparation of controls (K562 & HL60 cell lines): TKI treatment, harvesting and lysis.* Repeat **option A, steps xvii-x** and then seed cells at $2x10^5$ cells/mL($2x10^6$ total cell number in 10mL pre-warmed RPMI base media; cultured in a $25cm^2$ cell culture flask). Incubate cells for 1h using standard conditions (37°C, 5% CO_2, 95% relative humidity) prior to proceeding to treatment (next step).

xiv. Incubate cells using standard conditions (see **step xiii**), with treatment of interest, for an appropriate time period. We treat with TKIs at the following drug titrations for 5h using standard conditions (see **step xiii**):

Titration	Stock Dilution (in 10mL media)
No drug control (NDC)	N/A
IM – 1.0µM	1.0µL of 10mM stock solution
IM – 2.5µM	2.5µL of 10mM stock solution
IM – 5.0µM	5.0µL of 10mM stock solution
DAS – 10nM	0.67µL of 150µM stock solution
DAS – 150nM	10.0µL of 150µM stock solution

[2] **CRITICAL STEP** Proceed to next step 4h into the treatment.

xv. After 4h treatment, prepare Bicine/CHAPS lysis buffer (50µL lysis buffer for every $1x10^6$ cells) and store on ice until required (up to a maximum of 4h).

xvi. After the 5h total drug treatment, repeat **steps viii – x**. Re-suspend pellet in 10mL DPBS to wash off excess media. Pellet again and re-suspend in 1mL DPBS, transfer to 1.5mL microcentrifuge tube and repellet using a bench top microfuge at 1500g for 3m. Discard supernatant in liquid virkon (1% w/v) under a fume hood, and invert tubes on tissue paper to dry for 20s.

xvii. Add the required volume of lysis buffer to each cell pellet (50µL lysis buffer to every $1x10^6$ cells). Vortex until the pellet is suspended. Incubate on ice for 15m. Sonicate for 10s, vortex for 10s and return to ice for 15m. Precool the bench top microfuge to 4°C. Repeat vortex and incubation twice more. [2] **CRITICAL STEP** visually inspect the sample; the buffer should appear cloudy due to CHAPS dissolution on ice, whereas the solution should appear clear. **?TROUBLESHOOTING**

xviii. Centrifuge in the pre-chilled bench top microfuge for 15min at 4ºC and 18,188g. Pellet should be very small and compact. If the pellet is similar in size to the original cell pellet, lysis is incomplete. Pellet should be resuspended and the volume of lysis buffer increase by 50µL. Repeat until pellet has diminished. Remove the supernatant and place in a fresh 0.5mL microtube. Discard the pellet.

xix. Determine the protein concentration of the supernatant using a suitable assay (i.e. Bradford assay). Aliquot samples at a protein concentration of 2mg/mL and store at -80ºC until required. <PAUSE POINT> Aliquots can be stored for several years at this temperature if unthawed. Thawed samples should be used and discarded.

OPTION B

Sample preparation of primary material (clinical cells) Ï TIMING 8h.

i. To isolate the $CD34^+CD38^-$ population, thaw cells in DAMP solution and stain simultaneously with anti-human CD34-APC and anti-human CD38-FITC antibodies. Sort cells into $CD34^+CD38^-$ and $CD34^+CD38^+$subpopulations using a BD FACSAria Cell-Sorting System.[42]

ii. Culture sorted sells overnight in SFM plus growth factors.

iii. Treat cells for 24h with a TKI as previously described[32] in **option A; step xiv** using final concentrations of 150nM DAS and 5µM IM.

iv. After 23h, prepare 1mL Bicine/CHAPS lysis buffer and store on ice until required or a maximum of 3h.

v. Pre-chill the bench top microfuge to 4ºC. ² **CRITICAL STEP** To minimize changes in protein phosphorylation status, ensure samples are kept at 4ºC up until the point of lysis.

vi. Stain cells in a 1:1 mixture of trypan blue solution and cultured cells (100µL total volume). Briefly vortex and take 10µL of stained cells to count using a double-cell Neubauer haemocytometer counting chamber (0.1mm; 1/400 mm^2).

vii. Use cells counts to calculate the volume of solution containing 10,000 cells and aliquot into 1.5mL Eppendorf tubes.

viii. Centrifuge at 200 x g for 10m in a pre-chilled microfuge (4ºC) and discard supernatant in liquid virkon (1% w/v). ² **CRITICAL STEP** be careful not to disturb the cell pellet. If it is difficult to see, make a note of the Eppendorf tube orientation whilst in the centrifuge and ensure supernatant is carefully removed from the opposite side of the tube to the direction of centrifugal force.

ix. Resuspend and wash cells in 1mL low ionic buffer (i.e. Sucrose/Bicine) to remove excess salt. ² **CRITICAL STEP** buffer salt concentrations used with SFM plus PGF exceed the maximum salt tolerance of the cIEF platform (150mM) which results in poor protein separation in capillary (see **supplementary video 1**). **?TROUBLESHOOTING**

x. Centrifuge washed cells at 200 x g for 10m and discard supernatant in liquid virkon (1% w/v). Invert tubes on tissue paper to remove excess liquid and dry for 20s. ² **CRITICAL STEP** it is essential that liquid carryover is avoided at this point as excess wash buffer will reduce lysis efficiency and dilute final sample concentration. **?TROUBLESHOOTING**

xi. Immediately add 5µL lysis buffer, gently vortex for 10s and incubate on ice for 15m. Sonicate for 10s, gently vortex for 10s and return to ice for a further 15m. Repeat gentle vortex and incubation twice more.

xii. Centrifuge in pre-chilled bench top microfuge for 15m at 4ºC and 10,000 x g. Remove supernatant and place in fresh 0.5mL microtube. Discard pellet. **PAUSE POINT** samples can be snap frozen and stored at -80ºC until required (>1 year).

cIEF: general procedure Ï TIMING 3h, with subsequent overnight assay.

2. Design a suitable assay template. We use the “Assay Perspective” in Compass (**Fig. 2**) which allows for selection of sample number and well locations together with primary and secondary

antibodies (2-step experiment); or primary, secondary and tertiary antibodies (3-step experiment using biotin conjugated secondary antibody coupled to streptavidin conjugated HRP for increased sensitivity). In both 2-step and 3-step experiments, chemiluminescent reagent location and well number is also defined at this stage. Once an assay plate has been designed, the system must be programmed to allow the automated robotics to collect the samples, antibodies and chemiluminescent reagent in the correct order.

3. Make up Sample Diluent/DMSO (SSD) inhibitor mix solution stock as required. Keep on ice when in use.

4. Make up G2 Premix/Ladder stock (PLS; see tables below for volumes defined for up to 4 cycles). When preparing the stock, always round-up to the nearest whole-number volume of ladder to be used. Make up a minimum volume of 50ml PLS stock as accurate pipetting of <1µL ladder is difficult to achieve. For samples undergoing >4 cycles, double the volumes involved. [2] **CRITICAL STEP** A reverse pipetting technique is essential at this point. The Premix solution is extremely viscous and difficult to pipette; to ensure the correct volume is used, displace the solution 3 times from tip prior to reverse pipetting. [2] **CRITICAL STEP** Keep at room temperature to minimize viscosity.

Stock	Ampholyte Range (pH)	Standard Ladder
Broad - Standard	3 – 10	Standard Ladder 1 (pI 4, 4.9, 6.0, 6.4 & 7.3)
Narrow – Low	4 – 7	Standard Ladder 2 (pI 4.2, 4.9, 6.0, 6.4 & 7.0)
Narrow – Mid	5 – 8	Standard Ladder 3 (pI 4.9, 6.0, 6.4, 7.0 & 7.3)
Narrow – Nested	5 – 8 (2 – 4 plug, nested)	Standard Ladder 3 (pI 4.9, 6.0, 6.4, 7.0 & 7.3)
Narrow – Focused	5 – 6	Standard Ladder 4 (pI 4.9, 5.5 & 6.0)
Broad – Modified	High pI protein; 3 – 10 (broad)	Extended (custom) Ladder (L1 + pI 8.4 & 9.7)
Focused high pH	High pI protein; 5 – 8 (80%)/ 3 – 10 (20%)	Extended (custom) Ladder (L1 + pI 8.4 & 9.7)

Wells	Total Volume (Sample, SDD & PLS)	Sample & SDD	PLS	PLS (30% Excess Required)		
				Premix (µL)	Ladder (µL)	Total (µL)
1	10	2.5	7.5			
2	20	5.0	15.0			
3	30	7.5	22.5	49.0	1.0	50.0
4	40	10.0	30.0			
5	50	12.5	37.5			
6	60	15.0	45.0	57.5	1.0	58.5
7	70	17.5	52.5	66.3	2.0	68.3
8	80	20.0	60.0	76.0	2.0	78.0
9	90	22.5	67.5	86.8	2.0	87.8
10	100	25.0	75.0	95.5	2.0	97.5
11	110	27.5	82.5	105.3	2.0	107.3
12	120	30.0	90.0	116.0	2.0	117.0

5. Dilute 2µg/µL sample stock 1:5 with SSD, using volumes defined in table above (**step 5**). Vortex briefly to mix and keep on ice.[2] **CRITICAL STEP** for finite cell numbers (<5,000 cells), mix lysate 1:1 with SSD to avoid overdilution.

6. Combine sample from **step 5** with the appropriate PLS volume and vortex to mix. [2] **CRITICAL STEP** vortex sample for a minimum of 20s, ensuring a vortex forms in the sample solution. A universal faint pink color should be observed after this. **?TROUBLESHOOTING**

7. Load 8µL per well immediately and keep on ice.

8. Dilute primary antibodies using antibody diluent (see Box 2). Vortex briefly and load 8µL antibody per well for samples undergoing d4 cycles (double load volume for samples undergoing >4 cycles). **?TROUBLESHOOTING**

9. Dilute secondary biotin antibodies and tertiary streptavidin-conjugated HRP antibodies 1:100 with antibody diluent. Vortex briefly and load 8µL antibody per well for samples undergoing d4 cycles (double load volume for >4 cycles). [2] **CRITICAL STEP** the biotin-streptavidin system must be used in conjunction with a 3-step assay (see **step 2** for details) **?TROUBLESHOOTING**

10. Prepare 150µL Peroxide XDR and Luminol in a 1:1 ratio and load 8µL per well for samples undergoing 4-cycles (double load volume for >4 cycles) [2] **CRITICAL STEP** keep chemiluminescent reagent at least 2 rows away from streptavidin-conjugated HRP (normal position is row J; see **Fig. 2**) CRITICAL STEP

11. Once the samples and reagents are loaded onto the plate, replace lid and centrifuge at 1000 x g for 10m. [2] **CRITICAL STEP** After plate centrifugation, visually inspect wells to ensure no bubbles are present in sample wells. Use a spare capillary to rupture them if any exist. Air bubbles trapped inside a capillary will prevent sample separation (breaks electrical current). **TROUBLESHOOTING**

12. Replace ultra-pure water reservoir and empty waste reservoir (decontaminate as required). Open Compass and run a cleanup cycle (Instrument>Cleanup>OK). Clean up takes approximately 10m to complete. [2] **CRITICAL STEP** ensure all capillaries have been removed from reagents tray prior to running cleanup as the system will move any unused capillaries to waste which is unnecessary (opened, unused capillaries can be placed in dark moisture free storage for later use).

13. Once cleanup cycle has completed, select “Assay Perspective” in Compass and open assay file. Check each cycle is picking up the correct sample and antibody combinations before clicking START. After pressing start, Compass will prompt you to save settings if any unsaved changes have been made to the assay file. It will not be possible to proceed without saving.

14. Follow onscreen instructions, which should prompt you to replace water & empty waste (top up water if **step 9** has been performed) and replace sponge.

15. Replace or top-up wash concentrate, anolyte and catholyte buffers in reagents tray. Load capillaries. **CRITICAL STEP** reagent buffers can be toped up over a 48h period; however, they should be regularly replaced and the containers washed in ultrapure water. **? TROUBLESHOOTING**

16. Place plate onto cooled sample tray **CRITICAL STEP** ensure lid is on, as system will not run without a plate lid.

17. Select suitable location to save data after the run has completed and click Start. Compass will automatically switch to Run Perspective and provide an estimated time of completion together with a pictorial breakdown of each cycle and live camera feed of the separation, including thefluorescence ladder. **? TROUBLESHOOTING**

18. Upon completion, Compass will create a data file containing the original assay file, run summery information & video together with the results data. Compass will automatically switch to “Analysis Perspective” at this point in preparation for data analysis.

Assay Controls – Sample phosphatase treatment plus secondary antibody and streptavidin cross reaction Ï TIMING 3h, with overnight assay using NanoPro 1000.

[2] **CRITICAL STEP** The following section describes methods for assay controls to confirm phosphorylate peaks using lambda phosphatase together with cross-reaction controls for secondary antibodies and/or tertiary streptavidin-HRP depending upon which approach is taken (2-step or 3-step; see **step 2**).

19. Thaw the Lambda Phosphatase Kit on ice

20. In a fresh 0.5mL microtube, dilute 1.25µL 1M DTT with 25µL 10X reaction buffer

21. Transfer 8µL lysate (2mg/mL) into a fresh 0.5mL microtube and add 1µL of lambda phosphatase together with 1µL of diluted DTT made in **step 32**. Vortex briefly to mix and pulse centrifuge (10s at 16,100g). Assessing both K562 and HL60 samples treated with TKI provides a detailed analysis of the phosphorylation profile for CrkL, both in the presence and absence of oncogenic tyrosine kinase activity.

22. Incubate sample at 37ºC in a water bath for 60m.

23. Remove and place on ice.

24. Process samples as described in **cIEF – general procedure; steps 2-18** using the assay plate design shown in **Supplementary Fig 1.** Dephosphorylation and antibody controls are applied as follows:

Sample	Primary Antibody	Secondary-Biotin	Streptavidin-HRP
Bicin/CHAPS + PLS	☑	☑ ☑	☑ ☑

	- -	-	☑
Sample +PLS	☑ - -	☑ ☑ -	☑ ☑ ☑
Lambda Sample + PLS	☑ - -	☑ ☑ -	☑ ☑ ☑
Lambda Only + PLS	☑ - -	☑ ☑ -	☑ ☑ ☑

cIEF – Assay development Ï TIMING variable (expect 1 – 8 weeks)

² **CRITICAL STEP** Development of a fully optimized protocol for a new assay is essential to ensure reproducible peak detection with a linear range of protein and antibody concentration. To facilitate this UV binding efficiency must be determined along with suitable pI and CID ranges.

25. Select a cell line known to undergo TK-mediated phosphorylation (positive control) and another cell line known to definitely not undergo TK-mediated phosphorylation (negative control). Cell pellets should be acquired and lysates prepared as in **option A; steps viii-x and xvi-xix**. For CrkL, we use lysates from K562 and HL60 cell lines as positive and negative controls, respectively.

26. Determine the predicted isoelectric point for the target protein using a suitable proteomics database. For CrkL, the basal isoelectric point is 6.26, although high phosphorylation shift to pI 3.57 is possible (www.phosphosite.org[43])

27. Use default assay settings with a simple assay plate design (**Fig. 2**) together with a broad PLS (see **Table 1**) to ensure detection of protein peaks that might occur outside of the predicted pI range **?TROUBLESHOOTING**

28. Use Compass software to determine the extreme isoelectric points for phosphorylated and non-phosphorylated protein peaks. ² **CRITICAL STEP** with this information, a suitable ampholyte range can be determined, together with a corresponding standards ladder (see **step 4**). For CrkL, use a nested pI 5-8 premix coupled with ladder 3.**?TROUBLESHOOTING**

29. As the immobilisation period can vary between proteins and must be titrated to maximise signal intensity, perform a UV titration assay between 80s and 110s with measurements taken at 10s intervals, keeping all other parameters as default.

30. Export relevant profiles (**Fig. 3a**) as text format (Compass>Analysis Perspective>File>Export Spectra>Text Format) and analyse area under the curve (AUC) for each profile using an appropriate software package, such as the Medcalc Software package (www.medcalc.org). Spectrum reproducibility has been validated (n=3) and AUC calculated to determine optimal UV exposure (100s; AUC 218.2±3.5), giving a 1.6 fold increase in signal over default instrument settings (**Fig.3a insert**).

31. Using the pI range and immobilisation period determined in **step 28**, perform primary antibody titration against the default protein concentration (0.1mg/mL), and probe for 240m at the following antibody dilutions: 1:10, 1:25, 1:50, 1:100, 1:200, and 1:400 using antibody diluent solution (**Fig. 3b**). A non-specific peak can be detected at pI 6.8 with antibody dilutions <1:50 Optimal peak profile detection (pI 4.5-7) is usually achieved with the 1:100 primary antibody dilution at 240m, which is a sufficient incubation period for low-signal material**?TROUBLESHOOTING**

32. Detect chemiluminescence over exposure limits using 0.4mg/mL protein concentration (optimised pI, immobilisation and antibody settings applied). This concentration of protein will be sufficient to cause overexposure in the majority of cases and should not be protein dependent. Quantifiable signal is detectable within 10-240s of CID within a range of pI 4.5-6.0 for CrkL (this range will be protein dependent), meaning that CID duration can be increased for clinical samples with weaker observed signals. Overexposure can be seen within the range pI 4.5-5.5 after 240 secs of CID (**Fig. 3c**).**?TROUBLESHOOTING**

33. Under optimised conditions, determine protein limit of detection (LOD) with an on-capillary protein concentration ranging from 16µg to 0.0156µg. ² **CRITICAL STEP** Different CID periods can be used to either minimise signal burn out or maximise protein detection.

34. Calculate AUC, as in **step 26**. For a serial dilution of K562 lysates, as little as 1.6ng total protein on capillary can be used to reliably detect CrkL (**Fig. 3d**). This value is equivalent to 0.005% of the starting material ($1x10^6$ K562 cells) per capillary (R^2= 0.9896), with a linear range of detection up to 32ng total protein. Inter- and intra- cycle reproducibility is high with a coefficient of variation (CV) of 2.59 and 6.07 respectively. [2] **CRITICAL STEP** LOD is assay specific and is dependent on the function of the protein target together with the antibody used. For example, total-AKT has a LOD of 10ng total protein, which is insufficient for the analysis of patient samples containing $<10^4$ cells. A minimum of 4.8ng LOD is required for analysis of these sample types.

TIMING

Thawing and passaging K562 & HL60 cell lines (**option A; steps i-xiii**): 1-2 weeks, depending upon cell recovery time.
TKI treatment, harvesting and lysis of K562 & HL60 cells (**option A; steps xiv-xix**): 8h.
Sample preparation of primary material (**option B; steps i-xii**): 8h.
cIEF general procedure (**steps 3-18**): 3h, with subsequent overnight assay on NanoPro 1000 (~4-15h*).
cIEF assay development (**steps 26-34**): variable depending on target protein: 1 – 8 weeks
Sample phosphatase treatment plus secondary antibody and streptavidin cross reaction (**steps 20-24**): 3h, with overnight assay on NanoPro 1000 (~4-15h*).

* Overnight assay using the NanoPro 1000 will take between 4h and 15h. The precise duration will mainly depend on the number of samples processed and the setting used for antibody incubation although other settings such as U.V. exposure will have a small effect on duration. For example, an assay processing 4 samples in triplicate (12 sample wells in total; assume the samples reflect necessary controls) probed with primary antibody for 120m and subsequently processed using a 3-step assay (see **step 2**) and 10 CID exposures would take approximately 4h to complete, after the plate has been setup by the user. Increasing the number of samples and/or the antibody incubation period will increase the time taken for an assay. However, it is worth noting that the NanoPro 1000 runs samples in sets of 12, known as 1 cycle. The system is capable of running cycles simultaneously, so running 24 samples takes ~5h rather than 8h.

?TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

Table 2: Troubleshooting

Step	Problem	Possible Reason	Solution
Option A; step viii	Poor recovery	Age of Cryo-stored cells	With large numbers of dead cells, centrifuge the culture as described in option A; step vi, resuspend in 3mL fresh media and transfer to a 6-well plate to recover. Ensure the unused wells are filled with ample ultra-pure water to avoid the culture drying out (use gaps between wells if necessary). Allow to recover for 24-48h.
Option A; step xviii	Cell lysis – Large granules in solution	Higher density of cells in pellet	Apply 10µL additional lysis buffer, incubate for 10m on ice and check for granules. If dissipating, leave for longer. If there is no change, repeat addition.
Option B; step x	Random ladder shift upon cIEF separation	High salt (>150mM) See **Supplementary Video**	Wash cell pellets in non-ionic buffer prior to lysis. If samples are lysed, consider buffer exchange.
Option B; step xi	Liquid carryover	SFM+PGF not removed effectively after pelleting	In this case, dilution with SSD is not possible. Lyse samples as normal. Add DMSO Inhibitor solution directly to PLS in a 1:50 ratio. Then use PLS-DMSO in a 1:3 ratio with lysate as normal.
7	Capillary fails to completely fill	Incomplete mixing of sample with PLS	Ensure sample-PLS solution is vortexed for a minimum of 20s prior to loading onto the plate.
8	Lack of signal	Antibody dilution not appropriate	Check antibody has been added at the correct dilution. Ensure suitable secondary antibody has been used. Check protein is present in sample with spot-blot or western.
9	Lack of signal	Tertiary probe not matched to secondary protein	Make sure secondary-biotin antibodies are matched to streptavidin-HRP tertiary probe in the assay file. Alternatively, it is possible to use secondary-conjugated HRP with a 2-step assay.
11	Bubble effect	Air bubbles in sample-PLS	After plate centrifugation, visually inspect sample wells to ensure no air bubbles are present. Use a spare capillary to rupture them if any exist.
14	Salt crystals in reagent containers	Increased salt concentration due to prolonger buffer container top up & evaporation	Replace buffers every 48h.
15	Separation errors (<10m)	Air bubbles introduced during mixing	See points 36 and 40 above.
15	Separation errors (>20m)	Salt compression	Non-ionic buffer wash prior to lysis. Can be unavoidable with clinical material.
25	High pI protein/peak	Natural protein pI	Proteins that focus above pI 7.3 cannot be mapped accurately using standard ladders. Attempts to use this ladders are justified as a first step; however, should high pI peaks be observed then a modified ladder should be used with a Premix Ladder stock containing 5% TEMED, which blocks the capillaries alkaline end, preventing the sample from running off above pI 8.5.
27	No peaks detected (noise)	Various causes, including poor lysate quality or lack of	Check for poor lysate quality or lack of target protein in cell line with SDS-PAGE; check for ineffective antibody

		target protein in cell line, and ineffective antibody, poor antibody sensitivity	with spot-blots; check for poor antibody sensitivity by increasing primary antibody concentration (up to 1:50), incubation period (up to 480m) and exposure time (up to 1940s).
30	Artefacts	Aberrant/non-linear peaks at higher antibody/lysate concentrations	Reduce protein concentration, increase antibody dilution or reduce CID.
31	Profile burnout	Chemiluminescence substrate depletion	Reduce protein concentration, increase antibody dilution or reduce CID.

ANTICIPATED RESULTS

Therapeutic use of TKIs has been effective for patients with several leukaemias[44-48]. To determine the effect of PTK inhibition on CrkL phosphorylation and demonstrate the TKI inhibitor assessment potential of the approach, leukaemogenic PTK expressing cell lines were treated with IM and DAS (5 and 0.15µM respectively) for 5h. Treatment with either IM or DAS caused significant increase in peaks above pI 5.9, characteristic of the HL60 control (**Fig. 4a**).

Even with a well characterized assay such as this, problems can present themselves that cause data analysis difficulties. Examples can include spectra mismatching caused by poor ladder detection by compass (Supplementary Fig 3a), resulting in spectra that are biologically comparable (as in the case of Fig. 4a) or identical technical replicates not aligning as they should. This can be easily remedied by manually assigning the ladder values for the mismatched capillaries, instead of relying on compass automated detection (Supplementary Fig 3b). The user can save considerable time and effort by manually checking that automated ladder associations are correct, prior to undertaking detailed analysis of spectral data. Weak, noisy or distorted spectra generated from carefully prepared cell line material can occur in older samples that have undergone freeze-thaw cycles. cIEF is particularly sensitive to protein instability, especially as phosphorylation events can be unstable during freezing and thawing (personal observation). Good laboratory practice can minimize these events, ensuring multiple aliquots of each new sample are prepared in suitable volumes and stored at -80ºC until required.

Lambda phosphatase treatment of K562 and HL60 samples ±TKI, indicated that peaks detected below pI 5.9 were phosphorylated forms of CrkL (**Supplementary Fig 1**). The CrkL protein has been observed with a total of 28 PTMs identified in MS experiments (see www.phosphosite.org), however only phosphorylated tyrosine 207 (pTyr207) has been validated (using a range of methods including western blot, site-directed mutatgenesis and flow cytometry[30,49-54]). Tyrosine 207 phosphorylation has been shown to be the principle phosphorylation event related to BCR-ABL activity[30]. To assess the contribution of pTyr207 in the profile observed for total-CrkL we probed un-treated K562 samples with both anti-CrkL and anti-pTyr207 CrkL antibodies (**Fig. 4b**). The resulting profiles overlay perfectly, suggesting that the highly acidic peaks observed in the total-CrkL profile are

indeed a result of Tyr207 phosphorylation, in keeping with the use of CrkL proteins phosphorylation status as a surrogate marker for BCR-ABL kinase activity[30,55,56]. The effect of TKI on the pTyr207 profile demonstrates the superior capacity for DAS to inhibit BCR-ABL, and by direct association, CrkL phosphorylation (**Fig. 4b**). Lambda phosphatase treatment of samples used in Fig.4c show a significant reduction in acidic peaks, with the anti-Tyr207 antibody failing to detect peaks above pI 6 which are attributed to unphosphorylated CrkL species (**Fig. 4c**). The assay development for pCrkL Tyr207, using the protocol defined here, demonstrated LOD of 0.2ng total protein (R^2=0.9966) with a linear range of detection up to 32ng total protein (**Fig. 4d**). Reproducibility for the assay is excellent with inter-cycle CV=9.027 and intra-cycle CV=9.27. It is worth noting that phospho-specific antibodies can generate spectra with 5- to 10- fold less intensity than the equivalent total-protein, although this is not always the case. Direct comparison of spectra can be difficult when this occurs, however modification of CID will aid in normalization of spectral intensities. The user must provide an adequate range of camera exposures during assay design to accommodate for these antibody effects. Differences in spectra intensity do not affect percentage peak contribution analysis, however total AUC will be. Treatment with IM or DAS reduced the population of p-CrkL peaks between pI 4.5-5.8, with a corresponding increase in peaks above pI 5.9 (**Fig. 5a**), when probed with total-CrkL antibody. Potent DAS-mediated changes in the region of pI 5.5 could be seen when compared to IM (**Fig. 5a**). These differential DAS-mediated changes were also observed in the limited quantities of clinical CML $CD34^+CD38^+$ committed progenitor cells available for analysis (**Fig. 5b**), and again in primitive CML $CD34^+CD38^-$ cells (**Fig. 5c**). Given the nature of these samples, the cIEF system is push to operational limits in detecting changes in small numbers of stem cells. Poorly defined, noisy spectra can occur in clinical samples, whilst salt contamination seriously effects protein separation (compare **Supplementary videos 1 and 2**). The protocol outlined here is designed to minimize the frequency of these events, however, on occasion, clinical samples can be of poor quality and yield little usable data despite the best efforts of those undertaking the research. To determine the value of the approach in quantification of differential drug effects AUC for individual peaks were plotted. Analysis of mapped peaks for K562 cells showed a stark contrast between treated and untreated samples (**Fig. 5d**). Both TKI's induced marked decreases in pI distribution indicative of phosphorylation changes compared to control, however DAS had an effect at pI 5.45 (**Fig. 5d-blue circle**) that can be clearly

seen. Furthermore, this DAS-specific effect extends to peaks detected between pI 4.8-5.4 (**Fig. 5d-blue line**). In primary cells, peak contribution analysis displayed a similar differential effect in both $CD34^+CD38^+$ and $CD34^+CD38^-$ cells treated with IM and DAS (**Fig. 5e/f-black triangle/dash**).

Thus drug effect kinetics and dose response on limited cells populations can be achieved with this protocol in murine and human cell lines and primary human stem cells. The ability of cIEF-immunoassay technology to provide highly resolved, reproducible data on protein phosphorylation events at the nanogram level has previously been demonstrated[1,2]. Here we describe how to use the next generation cIEF-immunoassay platform, which has multichannel capacity, to probe pI protein profiles related to protein phosphorylation. This can be used to investigate LOD from a complex biological sample as a function of observed area under the curve type measurements. This provides relative quantification of observed AUC as a function of total protein, which cannot be determined using a statistical definition of LOD based on recombinant protein detection[2]. In the example application we present here, profiles of a key marker for oncogenic PTK activity in response to TKI treatment in CML cells per capillary were achieved, allowing 0.1% of sample containing <10,000 cells to be processed per assay, building on earlier work involving the protein tyrosine phosphatase PTPRC/CD45, in which we used this protocol to assay the levels of pS962 PTPRC/CD45 in CML and normal $CD34^+$ patient samples. Assay development for PTPRC/CD45 was again developed in accordance with our protocol (**Supplementary Fig 2**) displaying LOD down to 1.6ng total protein with inter- and intra assay CV of 7.82 and 4.41 respectively.

Our work has enabled differential effects of inhibitors on CrkL to be recorded in a format that lends itself to high throughput use on extremely low availability cell populations from clinical trials or patients at presentation (for drug screening prior to decisions on appropriate therapy). We have demonstrated the system's ability to directly target proteins of interest in very small patient samples ($<10^4$ cells), without the need for larger quantities of starting material, recombinant protein expression (to define LOD), or validation in cell lines and the inference for further use is clear. The procedure we have used in relation to primary patient material is well documented for the study of TKI effects, and as such applies to a wider audience given the likelihood of access to biobanked primary cell material. This

builds on earlier published work using CML patient material[5,6], and makes a case for focusing this technology toward personalised medicine, for use assessing patient stratification (in selecting suitable TKI treatment) or potentially monitoring development of drug resistance in patients undergoing chemotherapy for CML. Nonetheless users need to be aware of the technologies limitations. The presence of reproducibly detectable changes in protein profiles can support patient stratification (perhaps in terms of treatment), however such complex spectra, whilst being very informative, also poses complex biochemical questions relating to the origin of observed peaks.

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AUTHOR CONTRIBUTIONS

ADW and TLH devised the study. MAO performed experimental and study design and wrote the manuscript with ADW. AP contributed to study design and instrument setup. FP and MS prepared clinical material. AW provided Src inhibitor. All authors contributed experimental methods and reviewed the manuscript.

Competing financial interest

The authors declare that they have no competing financial interests.

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FIGURE LEGENDS

Fig. 1. Workflow from patient sample collection to data analysis. Algorithm for the process of sample acquisition, TKI treatment, sorting and lysis (blue section) prior to application to the cIEF platform (orange section), which is split into sections detailing experimental design, separation, capillary registration and analysis. System Experimental Design; using the NanaPro 1000, sample, primary antibody, secondary biotin antibody, tertiary streptavidin-HRP antibody, and chemiluminescence should be loaded into wells A, B, C, D, and J, respectively. Up to eight rows of sample can be loaded (A-H) followed by antibodies and chemiluminescence making use of the 384-well plate format. Separation, immobilization and exposure; after the system starts the NanoPro 1000 automatically load capillaries with sample and initiate isoelectric protein separation over 40m. At this point proteins are fixed to the capillary wall during brief UV exposure prior to washing, antibody probing and chemiluminescence detection. Data analysis; chemiluninescent and fluorescent signitures detected using a CCD camera are converted into electropherogram form.

Fig. 2. Assay plate design. The different aspects of assay plate design. **a**, 384 well plate layout defines the locations for sample, antibody and chemilumiescence reagent with colour coded rows. The rows can be positioned anywhere on the plate, although row blocks (12 wells) must be placed between columns 1-12 or 13-24, not centrally (i.e. 6-18). Chemilumenescence reagent must be located 2 rows from HRP-conjugated antibody. **b**, Compass automatically creates a template corresponding to the layout, which allows the user to populate individual wells or whole rows with relevant sample, antibody or reagent information. This is used later for assay analysis. **c**, Anticipated results for high and low phosphorylation status (specifically n5-8/L3) for CrkL using K562 and HL60 cell line models respectively.

Fig. 3. Assay development process for CrkL antibody. The assay development process considers factors such as UV immobilisation, antibody sensitivity, dynamic range and limit of detection. ***a*****,** UV titration profiles for CrkL isoforms using K562 lysate (0.1µg/mL) probed with CrkL antibody (1:50 dilution; 480m incubation; 240s CIE) after 80s (red), 90s (blue), 100s (green) and 110s (orange) UV exposure. Insert bar-chart shows mean AUC (n=3) with standard error for UV exposure time points. ***b*****,** CrkL antibody sensitivity titration against K562 cells (0.1µg/mL; 240s CIE) with spectrum represented atop capillary image for each dilution factor. ***c*****,** Dynamic range shows CIE overexposure effects

probing K562 lysate (0.4µg/mL) with CrkL antibody (1:100) represented as spectra & capillary CCD image at given time points (s). ***d*****,** limit of detection for CrkL assay probed using optimised conditions (100s UVB; 240m incubation; 1:100 antibody dilution) against K562 cell lysates with a protein titration of 80, 40, 20, 12, 4, 1 & 0.5pg/nL total lysate shown.

Figure 4: Assay development process for pTyr207-CrkL antibody. The assay development process considers the antibody limit of detection, sensitivity to TKI and lambda phosphatase together with comparison to total-CrkL antibody. **a,** Comparison of total-CrkL (blue) and pTyr207-CrkL (red) antibody profiles using untreated K562 lysate (0.1µg/mL) probed with suitable antibody (1:50 dilution; 480m incubation; 240s CIE). **b,** pTyr207-CrkL antibody sensitivity to TKI changes in K562 cells (0.1µg/mL; 240s CIE), untreated control (black), 150nM DAS (red) and 5µM IM (blue). **c,** Lambda phosphatase effect on untreated K562 cells probed with total-CrkL (black) and pTyr207-CrkL (red) antibodies. **d,** limit of detection for pTyr207-CrkL assay probed using optimised conditions (100s UVB; 240m incubation; 1:100 antibody dilution) against K562 cell lysates with a protein titration of 80, 40, 20, 12, 4, 1 & 0.5pg/nL total lysate shown. All assays used 1:50 antibody dilution; 480m primary incubation; 240s CIE; 0.1µg/mL total protein unless otherwise stated.

Fig. 5. Chemiluminescence data for DAS and IM drug treatments displaying phosphorylation profile shifts from control. Chemiluminescence data for DAS and IM drug treatments displaying phosphorylation profile shifts from control. **a**, CrkL spectra from the human CML blast crisis K562 cell line (treated for 5h with drug); blue circle and line denote DAS-mediated effect. **b**, spectra from patient-derived CML $CD34^{+}CD38^{+}$ committed progenitor cells (treated for 24 hr with drug). Black triangle and dashed line denote DAS-mediated effect. **c**, spectra from clinical CML $CD34^{+}CD38^{-}$ primitive progenitor cells (treated for 24 hr with drug). Black triangle and dashed line denote DAS-mediated effect. Histograms representing percentage peak contribution analysis for CrkL protein profiles from cell lines K562 (d; treated for 5 hrs, S.E.M n=3 biological repeat), clinical $CD34^{+}CD38^{+}$ (e; treated for 24 hrs, S.E.M n=3 patient sample) and clinical $CD34^{+}CD38^{-}$ cells (f; treated for 24 hrs, S.E.M n=3 patient sample). DAS-mediated effect on K562 cells (blue circle) was pI 5.45. DAS-mediated peak changes (blue bar) were pI 5.34-4.99. DAS-mediated effects on clinical samples (black triangle/dotted line) were pI 5.58-5.45.

Supplementary Fig.1. Lambda phosphatase treatment of HL60 and K562 cell lysates. Assay plate design and results created using Compass software assay and analysis perspectives respectively. **a,** layout of samples, antibodies and reagents in 384-well plate format **b,** detailed description of samples and antibodies applied to plate designed in (a). **c,** representative differences in total CrkL spectra resulting from lambda phosphatase (»p) treatment, K562-NDC (black), K562-NDC-»p (green), K562-IM 5µM-»p (blue) and K562-DAS 150nM-»p (red).

Supplementary Fig.2. CD45 assay development. Representative trace for total-CD45 (black) and pCD45 (red). **b**, LOD curve for CD45. **c**, UV titration for CD45 using total antibody, error bars S.E.M (n=3 technical replicate).

Supplementary Fig.3. Spectra mismatching and poor ladder detection. Assay data showing total CrkL antibody probed against K562 cell lysate with no TKI reatment. Spectra shown represent technical replicates from the same sample. **a,** mismatched data example; correct standard ladder pI associations applied to the black trace, and incorrect associations applied to the blue trace. Red dotted lines indicate matching peaks and the extent of peak offset in the blue trace as a result of poor ladder detection. **b,** manual alteration of this error results in perfect alignment.

Supplementary Video 1. Poor separation; sample salt content >150mM. Video showing the effect on protein separation with cIEF when using clinical samples prepared in a high ionic strength buffer (n=4 biological samples, loaded in triplicate).

Supplementary Video 2. Good separation; sample salt content <150mM. Video showing the effect on protein separation with cIEF when using clinical samples prepared in a low ionic strength buffer (n=4 biological samples, loaded in triplicate).

Table 1 – Default assay settings

Parameter		Setting	Assay Development
Lysate protein concentration (mg/mL)		0.1 [36ng on capillary]	☑
Sample loading duration (s)		25	-
Separation (s)		40	-
Separation power (µW)		21000	-
Standards exposure (s)		1	-
Immobilisation period (UV exposure (s))		80	☑
Immobilisation Wash	Number	2	-
	Load period (s)	20	-
	Soak period (s)	150	-
Primary antibody	Load period (s)	2	-
	Incubation (m)	240	☑
Primary antibody wash	Number	2	-
	Load period (s)	20	-
	Soak period (s)	150	-
Secondary antibody	Load period (s)	2	-
	Incubation (m)	60	-
Secondary antibody wash	Number	2	-
	Load period (s)	20	-
	Soak period (s)	150	-
Tertiary antibody	Load period (s)	2	-
	Incubation (m)	10	-
Tertiary antibody wash	Number	2	-
	Load period (s)	20	-
	Soak period (s)	150	-
Chemiluminescence Detection (CID)	Load period (s)	2	-
	Exposure number	7	☑
	Exposure period (s)	30, 60, 120, 240, 480, 720, 960	☑

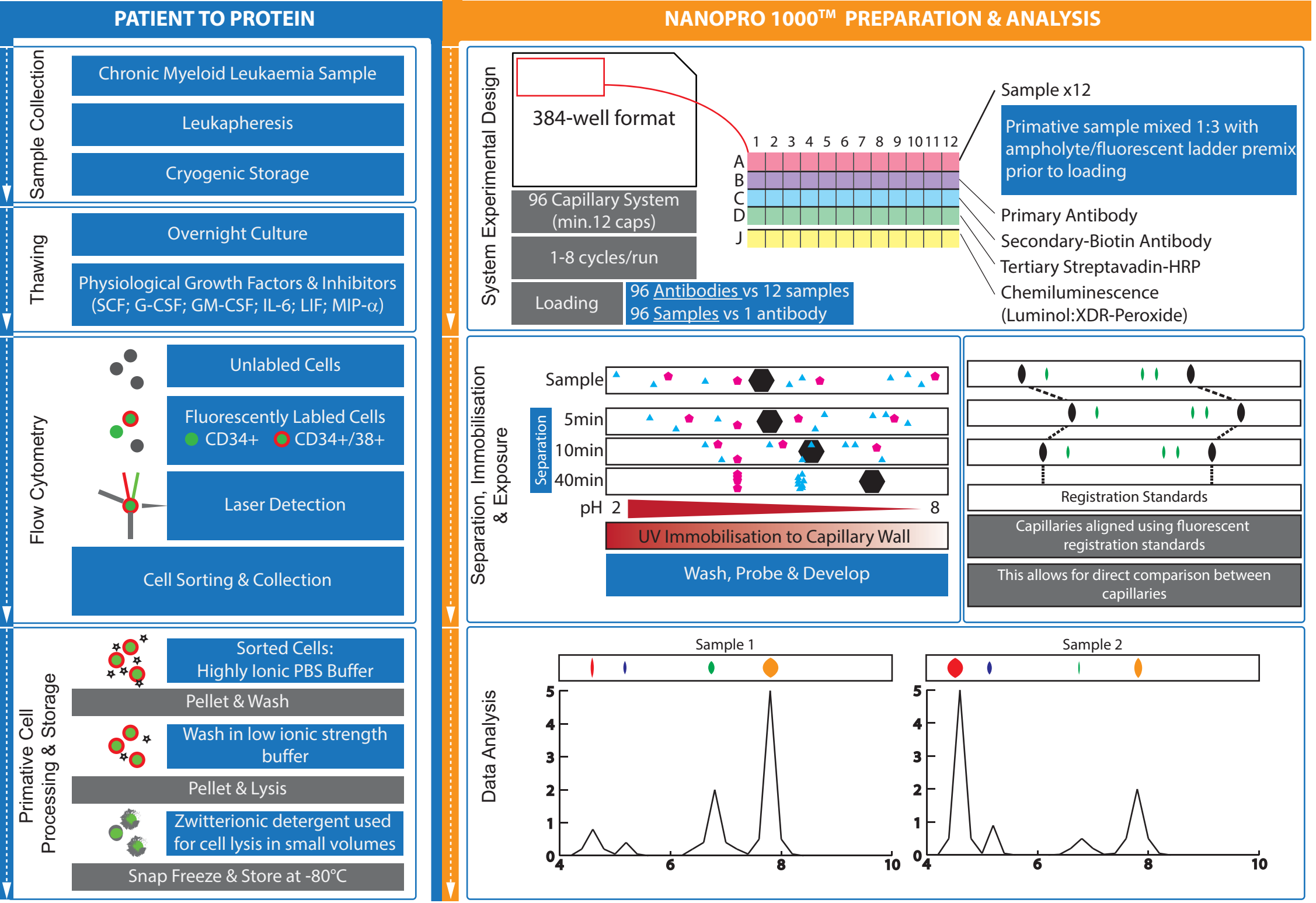
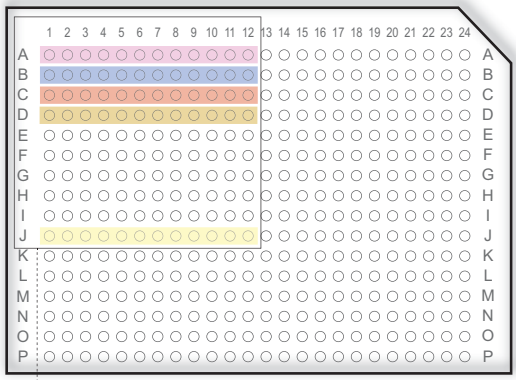
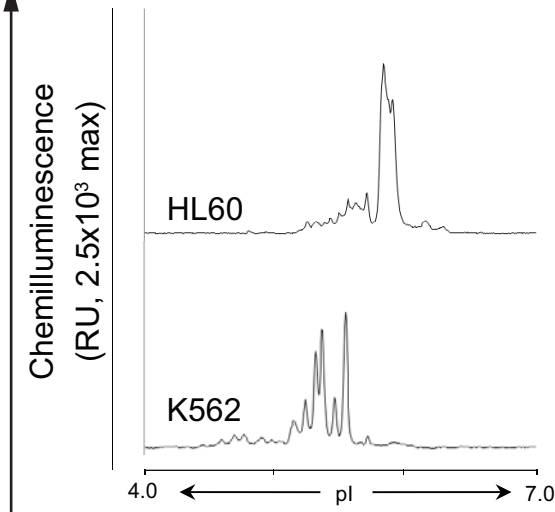


Figure 1: Workflow from patient sample collection to data analysis.



a, 384 well plate layout

c, Anticipated result



b, Sample, antibody & chemiluminescence loading template

	1	2	3	4	5	6	7	8	9	10	11	12
A	HIGH PHOSPHORYLATION (n5-8/L3) 0.1mg/mL Total Protein			HIGH PHOSPHORYLATION (3-10/L1) 0.1mg/mL Total Protein			LOW PHOSPHORYLATION (n5-8/L3) 0.1mg/mL Total Protein			LOW PHOSPHORYLATION (3-10/L1) 0.1mg/mL Total Protein		
B	Primary Antibody 1:50 Dilution											
C	Secondary-Biotin Antibody 1:100 Dilution											
D	Tertiary Streptavadin-HRP Antibody 1:100 Dilution											
J	Chemiluminescence Detection 1:1 (Luminol : XDR Peroxide)											

Figure 2: Assay Plate Design.

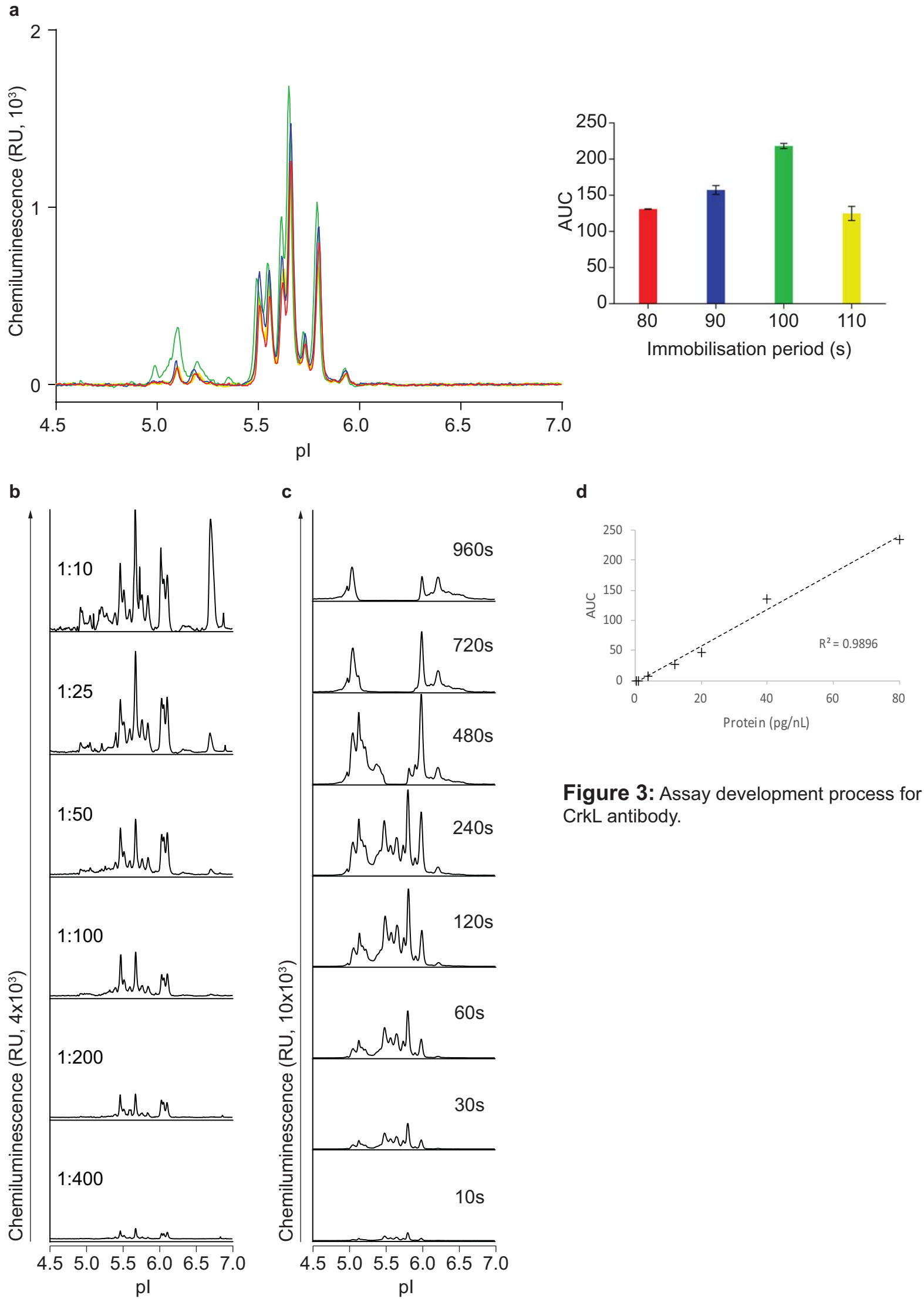


Figure 3: Assay development process for CrkL antibody.

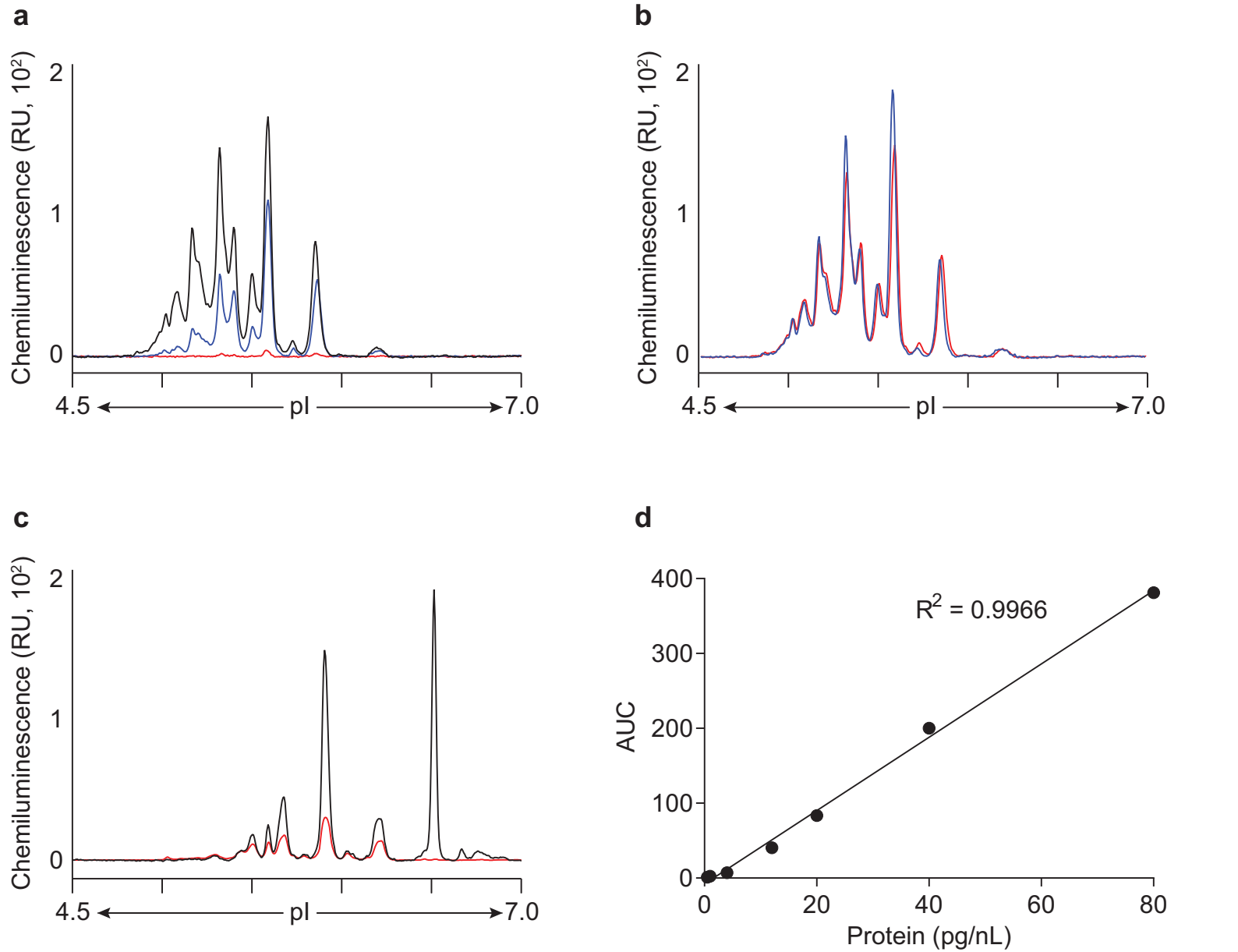


Figure 4: Assay development for pTyr207-CrkL antibody

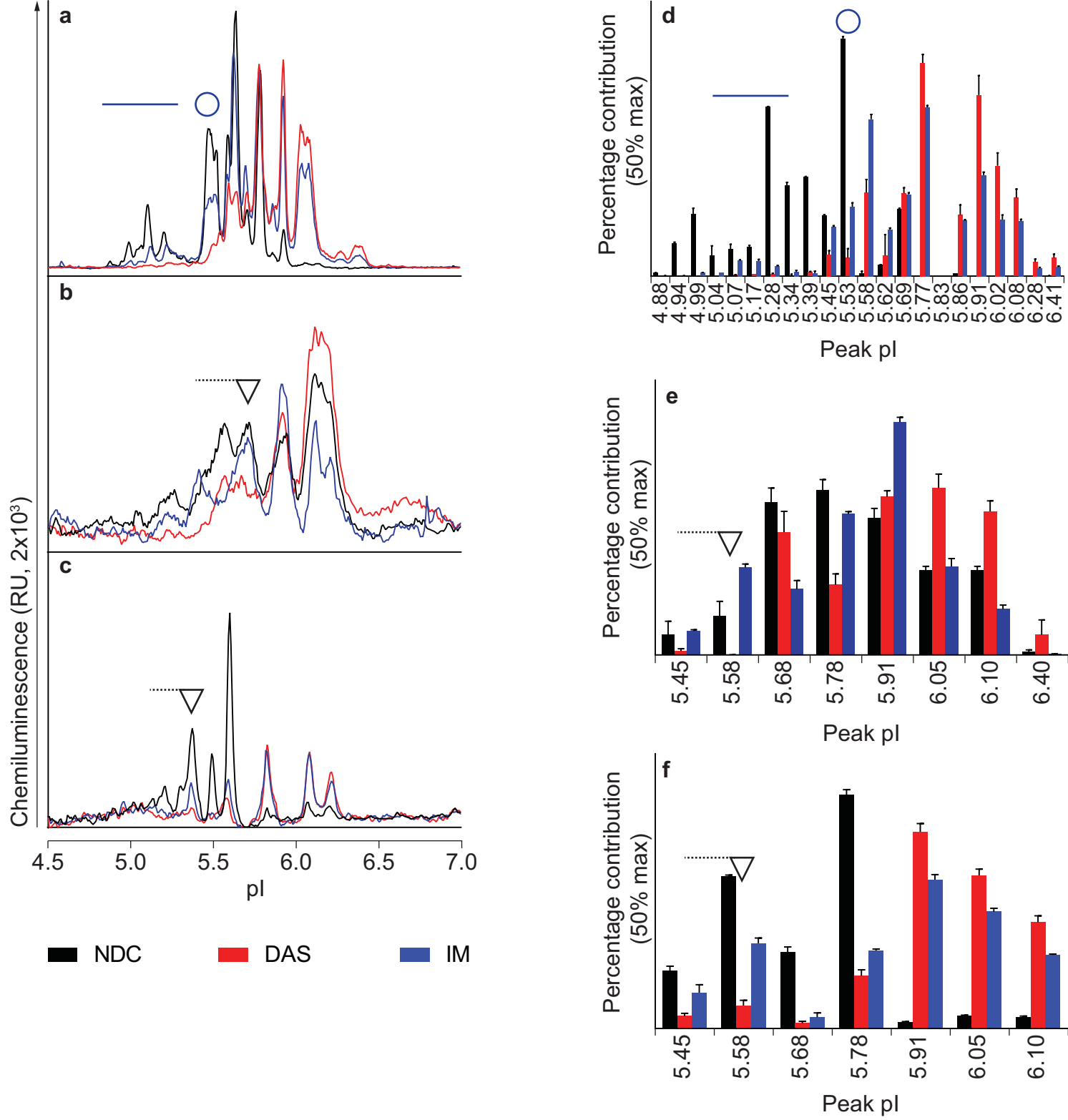


Figure 5: Chemiluminescence data for DAS and IM drug treatments displaying phosphorylation profile shifts from control