# Multiplexed profiling of RNA and protein expression signatures in individual cells using flow and mass cytometry.

Duckworth AD[1*], Gherardini PF[2,3*], Sykorova M[1], Yasin F[1], Nolan GP[2], Slupsky JR[1§], Kalakonda N[1§]

[*]Equal contribution

[§]Equal contribution

[1]Dept. of Molecular and Clinical Cancer Medicine

Institute of Translational Medicine, University of Liverpool, Liverpool, UK

[2]Department of Microbiology and Immunology

Baxter Laboratory in Stem Cell Biology, Stanford University, Stanford, California, USA

[3]Parker Institute for Cancer Immunotherapy, San Francisco, California, USA

**Correspondence:** Email: nageshk@liverpool.ac.uk

Tel: +44 151 706 4593

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**EDITORIAL SUMMARY** This Protocol describes the Proximity Ligation AssaY for RNA (PLAYR). PLAYR can be used to simultaneously detect at least 27 RNA transcripts using flow or mass cytometry and can be combined with protein detection using conventional antibody staining.

**TWEET** @andy_duckworth *et al* at @livuniITM provides a protocol for Proximity Ligation AssaY for RNA #PLAYR to simultaneously detect RNA and protein in single cells of #CLL using flow cytometry and #CyTOF

**COVER TEASER** Proximity ligation assay for RNA detection

## Abstract

Advances in single cell analysis technologies are providing novel insights into phenotypic and functional heterogeneity within seemingly identical cell populations. RNA within single cells can be analysed by unbiased sequencing protocols or through more targeted approaches using *in situ* hybridisation. The Proximity Ligation AssaY for RNA (PLAYR) approach is a sensitive and high throughput technique that relies on *in situ* and proximal ligation to measure at least 27 specific RNAs by flow or mass cytometry. We provide detailed instructions on combining this technique with antibody-based detection of surface/internal protein allowing simultaneous highly multiplexed profiling of RNA and protein expression at single cell resolution. PLAYR overcomes limitations on multiplexing seen in previous branching DNA-based RNA detection techniques by integration of a transcript-specific oligonucleotide sequence within a rolling circle amplification. This unique transcript-associated sequence can then be detected by heavy metal (for mass cytometry) or fluorophore (for flow cytometry) conjugated complimentary detection oligonucleotides. Included in this protocol is methodology to label oligonucleotides with lanthamide metals for use in mass cytometry. When analysed by mass cytometry, up to 40 variables (with scope for future expansion) can be measured simultaneously. We used the described protocol to demonstrate intraclonal heterogeneity within primary cells from chronic lymphocytic leukaemia patients but it can be adapted to other primary cells or cell lines in suspension. This robust, reliable and reproducible protocol can be completed in 2-3 days and can be paused at several stages for convenience.

## Introduction

Analysis of single cells for their individual complexities in form and function allows greater understanding of biological systems. Through analysis and quantification of gene products at single cell resolution it is becoming evident that despite being genetically identical, there are significant differences in basal and perturbed gene expression even within populations of cells that would otherwise be considered a homogeneous "cell type"[1]. This variance is thought to arise due to the stochastic nature of gene expression and differences in micro-environmental milieu of individual cells. Techniques for profiling and understanding of RNA expression at single cell resolution have rapidly progressed in recent years and the methods broadly fall into two complementary categories: single cell RNA-sequencing (scRNA-seq) or *in situ* hybridisation (ISH).

Over the past 9 years, and since the publication of a landmark article describing an approach for single cell mRNA-seq[2], different methodologies for scRNA-seq have evolved. All methods rely on 3 major steps: 1) Unique nucleotide barcoding and reverse transcription of RNA of each individual cell through either physical isolation[3,4] or probabilistic labelling[5]; 2) amplification of the resulting cDNA; and 3) library preparation for sequencing. Recently this technology has been combined with oligonucleotide-labelled antibodies to allow simultaneous quantification of both protein and RNA within individual cells using DNA sequencing[6,7]. Despite being an extremely promising technology, a number of experimental constraints of scRNA-seq are pertinent: 1) Financial considerations may restrict sequencing depth resulting in a trade-off between the number of cells/samples that are analysed and the sensitivity for detection of transcripts with lower abundance, 2) the technique currently relies on reverse transcription using an oligo-dT primer and therefore analysis is limited to poly-adenylated RNA, 3) the method can be laborious and complicated and take weeks to months to produce informative data. Due to these limitations scRNA-seq may not always be practical for clinical applications.

A complementary method to scRNA-seq is detection and quantitation of RNA at single cell resolution using ISH. This technique measures fewer RNAs that have to be preordained, but the transcripts can be sensitively quantified in a larger number of

cells, within a much shorter timeframe, and with less experimental complexity. Thus, quantification of the resulting signal by cytometry allows hundreds to thousands of cells to be analysed per second. The technology is an adaptation of fluorescent ISH (FISH), and relies on bright signals with high signal to noise ratio. Branching DNA technology also allows measurement of RNA in single cells using flow cytometry[8], and specificity is increased through probe pairs that are required to bind in close proximity on their target RNA strand. Adjacently bound probes can then be detected using sequential binding of predesigned DNA molecules to form repetitive expanding structures of nucleotides that amplify the signal. However, there are currently only 4 non-interfering versions of branching DNA sequences meaning opportunities for multiplexing using this method are limited. This restrictive feature of branching DNA is a significant limiting factor for utility in platforms such as mass cytometry that have higher multiplexing capabilities. An alternative methodology to branching DNA for increasing signal intensity is to use targeted padlock DNA probes and then produce concatenated copies of a resulting circular DNA molecule using rolling circle amplification catalysed by Phi29 DNA polymerase[9]. We have recently developed the Proximity Ligation AssaY for RNA (PLAYR), which provides routine simultaneous measurements for both RNA and proteins at single cell resolution with enhanced multiplexing capabilities for detection in both flow and mass cytometry platforms[10].

Here we provide a detailed protocol that allows analysis of protein expression using antibodies, and simultaneous detection of up to 27 RNA transcripts (multiplexing can be extended beyond this limit). We have now optimised the antibody staining procedure to allow robust and reproducible characterisation of surface and internal antigen expression while maintaining RNA integrity for the PLAYR assay. Although we describe the technique for the analysis of primary peripheral blood mononuclear cells (PBMCs) from chronic lymphocytic leukaemia (CLL) patients, it can be applied to other primary cells or cell lines that are in suspension. The protocol described here can potentially be adapted for the simultaneous measurement of RNA and protein in tissue sections using imaging technologies such as fluorescent microscopy and imaging mass cytometry.

## Overview of the procedure

**PLAYR.** A schematic overview of the critical steps to detect RNA in the PLAYR procedure and the method to combine this with antibody staining is depicted in **Figure 1A**. PLAYR specifically detects transcripts of interest by relying on proximal ligation of predesigned and specific PLAYR probe pairs (**Figure 1B**). Multiple probe pairs are designed to directly bind along each target RNA transcript to produce a robust signal with a high dynamic range of detection (1000 to fewer than 10 transcripts per cell). Cells are fixed and permeabilised prior to incubation with PLAYR probe pairs that are designed to bind adjacently on their target transcripts (Steps 1-17). Two functional sections separated by a linker region of 10 adenine residues make up each probe. The 5' end of the probe binds to its cognate target RNA sequence while the 3' end forms one-half of a template for the binding and circularization of two additional oligonucleotides termed 'insert' and 'backbone'. Only when both PLAYR probes bind to an RNA transcript in proximity can they generate the whole complementary platform for the formation of the nicked circular insert/backbone DNA molecule, which is ligated in a subsequent step (Steps 18-26) (**Figure 1C**). The circular insert/backbone DNA molecule is then amplified, via rolling circle amplification (RCA) using Phi29 DNA polymerase into its complementary concatenated form (Steps 27-28), thus yielding multiple copies of the cognate insert sequence that is detected by metal or fluorescently labelled detection oligonucleotides (Steps 29-32). Any off-target binding of single probes is insufficient to generate a complete circular DNA molecule that is required for RCA. Multiplexing is achieved through use of parallel and unique PLAYR systems (3' probe sequences, insert oligonucleotide sequences, and the detection probe sequences) for each transcript (**Figure 1**). Unique insert sequences introduce a nucleotide barcode within the RCA that can be specifically detected for each transcript. Common substrings between PLAYR insert sequences do not exceed 4bp in length but the latter have similar thermodynamic properties and base composition, allowing formation of unique RCA products with similar efficiencies. Our described insert sequences therefore allow simultaneous quantification of 27 different transcripts. A list of these 27 PLAYR systems including PLAYR probe 3' sequences, inserts, backbone and detection probe sequences are detailed in **Table 1**.

**Combining antibody staining with PLAYR.** Antibody staining has been optimised to alleviate effects on RNA copy numbers (through transcription and degradation) while preserving the surface and intracellular protein integrity for quantification. Staining using metal or fluorescently labelled antibodies that target intracellular antigens is performed alongside the incubation with PLAYR detection probes in the final stage of the assay (Steps 29-32). In contrast, surface antigens are stained in 'unfixed' cells (Steps 5-7), covalently cross-linked to the cell using bis(sulfosuccinimidyl) suberate (BS3), and then fixed using paraformaldehyde (PFA) (Steps 8-10). If used first, PFA will compete with BS3 for protein amine groups reducing the efficiency of cross-linking. As unfixed cells are prone to changes in both transcript levels and protein post-translational modifications, the incubation with antibodies and subsequent BS3 and PFA treatments are performed on ice. We find that signal to noise ratios of the staining antibodies in unfixed cells on ice are similar if not better than those achieved using the standard mass cytometry staining protocol on fixed cells at room temperature (**Figure 2**). Once the cells are fixed they can then be permeabilised in methanol (Steps 11-12) and the PLAYR assay performed with minimal loss of antibody staining. Cells can be stored for several months in methanol at -80$^{o}$C (after step 12) without noticeable effects on RNA integrity or antibody binding.

## Limitations

An inherent drawback of the technique is the paucity of unique heavy metal tags that can be used for detection of specific RNA targets and is currently limited to 40 transcripts. This is due to the chemical properties of the polymer that is used to chelate the metal tags. There are, however, efforts underway to expand the panel of unique tags to assay a larger spectrum of parameters[11].

A further limitation is the requirement for multiple probe pairs to detect a single transcript. Thus, it can be challenging to distinguish different isoforms, if the regions that are specific to each one are not big enough to accommodate multiple probes. In addition, due to constraints on the number of probe-pairs that are able to bind to transcripts of limited length, the detection of small RNAs such as miRNAs is problematic. In the case of miRNAs, the technique may be applicable for detection of

longer pre- or pri-miRNAs as surrogates. More generally, it may be possible to alleviate some of these limitations by using Locked Nucleic Acids (LNAs), which have stronger affinity for their targets[12,13], and as such require less probe pairs to get a robust signal. However, the hybridization conditions for LNAs would have to be significantly modified to achieve good signal to noise ratios.

Although the described PLAYR protocol should work well with most antibodies, there remains the possibility that the signal intensity of some antibodies may be compromised. To be certain, we recommend that the signal strength of each antibody to be tested with and without the PLAYR procedure. From a practical point of view, the protocol does involve many more wash steps than a standard antibody staining protocol, which could result in loss of cells available for final cytometric analysis.

In addition, the issues that surround other single cell RNA technologies (such as specificity, sensitivity, and variable modes of expression) are likely also relevant with PLAYR[14]. Finally, unlike RNA-seq that uses next-generation sequencing, the technique is currently not applicable for discrimination of wildtype and mutated RNA transcripts.

## Experimental Design

An overview of the experimental design workflow is illustrated in **Figure 3**.

**PLAYR Probe design.** We recommend the use of 4 or 5 probe pairs for each transcript of interest to ensure a robust and specific signal. The signal generated from these probe pairs is dependent upon the RNA transcript copy number per cell, the efficiency of the probe binding sequences (some probes may fail to bind due to RNA secondary structures) and the duration of the RCA during the assay (see "Protocol optimisation" below). It is also possible to use different number of probe pairs, for different transcripts, according to their expected abundance. This does not constitute a problem for quantitative analysis, because quantification is only relative (as with any other affinity based reagent, e.g. antibodies). An example of how the number of probe pairs impacts PLAYR signal is illustrated in Supplemental figure 4a in the manuscript by Frei et al.[10] .

PLAYR probes can be designed using our open-source R software package called PLAYRDesign[9]. Detailed instructions on how to download and install PLAYRDesign and its dependencies are available at https://github.com/nolanlab/PLAYRDesign. The PLAYRDesign software works by shortlisting candidate probes based upon their thermodynamic properties (using Primer3)[15] and displaying them along the transcript in a graphical interface. Additional graphs are aligned to the target transcript to illustrate BLAST-evaluated sequence similarities with the rest of the transcriptome, as well as a database of repetitive sequences. Non-constitutively spliced exons are also displayed, thus aiding the user in manually choosing the best probe pairs. The user can then select the PLAYR system (i.e. the multiplexing sequences) desired for the 3' end of the transcript probes and the complete probe sequences are generated in a .txt file.

**Handling of samples and reagents.** All enzymes used during the PLAYR assay should be stored at -20$^{o}$C and diluted in reaction buffer immediately before addition to the cells. T4 ligase buffer contains ATP and should therefore be batched to avoid degradation by freeze-thaw cycles.

The procedure involves many more wash steps than a standard flow cytometry staining protocol, and as such cell loss is a concern. We recommend using Polypropylene V-bottom 200 µL PCR strips, or alternatively 1.5 mL Eppendorf tubes. Avoid regular U-bottom Polystyrene FACS tubes, as they will result in significant cell loss. Centrifugation of cells should preferably be performed in a swing bucket centrifuge and the supernatant ideally removed by aspiration (i.e. not by flicking the tubes). The use of small pipette tips during aspiration (e.g. P10) will give greater precision and help to minimise cell loss.

When incubating cells make sure to use an agitation speed such that the cells will not deposit at the bottom during incubation (this is especially critical during amplification with phi29 polymerase). If using PCR strips, use a shaker with a very small orbit to guarantee mixing of the liquids. Tubes subjected to such vigorous agitation should always be spun down before opening to prevent cell loss, as the cells will be on the sides of the tube and on the cap. As cellular RNA can be prone to degradation by both internal and external RNAses, care must be taken to work as efficiently and quickly as possible, particularly when the cells are in any water-based

solution after fixation and before probe hybridisation (e.g. when the cells are remove from methanol prior to hybridisation). All samples should remain on ice during those critical steps to minimise RNAase activity. Once the circular DNA insert/backbone molecule is bound to the probe pairs and ligated, maintaining RNA integrity is less important (unless required for other relevant downstream applications).

**Controls.** The specificity of the PLAYR signal can be verified using cell lines known to reliably express the RNA transcript of interest. Perturbation of target transcripts through cellular stimulation or using RNA knockdown/knockout followed by correlation of mean PLAYR signals with measurements obtained by qPCR can be used to further validate probe pair specificity (**Figure 4A**). Further guidance on examples of controls that may be useful can be found in the publication of Frei AP et al. (Supplementary figures 3 and 4).

Each transcript of interest should be examined individually using all the designed probe pairs for the target RNA. The PLAYR probes for multiple transcripts are then pooled to ensure that the detection of each RNA is not compromised by multiplexing (**Figure 4B**). Reassuringly, we have never encountered cases of interference.

Staining of proteins should be optimised for each antibody panel. Antibodies should be stained according to the manufacturers' recommendations (e.g. on fixed cells at room temperature for 30 mins) and compared with results obtained using the described PLAYR staining protocol. Verification of similar antibody staining patterns and clustering should be ensured using appropriate software, such as bivariant plots and viSNE analysis in Cytobank.

We provide viability staining procedures for both mass and flow cytometry in this protocol. We advise the inclusion of this procedure as cell viability can greatly affect RNA levels detected within cells. For example, see **Figure 1B** cisplatin-194Pt vs ACTB mRNA; dead cells (highest cisplatin levels) are associated with much lower ACTB mRNA.

Further useful controls and troubleshooting steps are listed in **Table 2**.

**Protocol optimisation.** The duration of the RCA can be altered to optimise signal intensity. We have found that 4hrs for mass cytometry and 2hrs for flow cytometry yield comparable and strong signal amplifications. Longer incubation times (16 hrs)

further enhance signal levels but may be detrimental to antibody staining and final cell numbers, and will somewhat increase background as well. In our experience cells that do not generate a detectable signal after 2-4hrs tend not to show enhanced signal even after longer RCA times.

The PLAYR protocol may be optimised further to help preserve efficient antibody binding and, for flow cytometry, fluorophore stability. We have found that the methanol permeabilisation step may be excluded as the Tween20 present within the probe hybridisation buffer is sufficient to permeabilise the cells for RNA detection (**Supplementary Figure 1**). However, exclusion of the methanol step from the protocol means that the cells can no longer be stored at -80$^{o}$C for a prolonged period of time and, as Tween20 does not permeabilise the nuclear membrane, RNA residing within this compartment may not be stained during the assay. Lowering of the temperature during PLAYR probe hybridisation and post-hybridisation wash steps (17 and 22) from 40$^{o}$C to 37$^{o}$C may help further maintain antibody staining. Also, the dilution of the detergent Tween20 used during the probe hybridisation step, and the concentration of SSC during the PLAYR post-hybridisation wash can be modified if needed. Despite the fact that we see no major differences in RNA detection for our transcripts of interest during less stringent conditions (**Supplementary Figure 2**), we advise that all changes in protocol need to be controlled appropriately for your gene(s) of interest (see “Controls” above).

**Considerations specific for use with mass cytometry.** Detection probes can be purchased as 5’ C6-thiol modified oligonucleotides that can then be labelled using commercial Maxpar X8 metal labelling kits (Fluidigm). A detailed protocol for labelling detection probes is described in **BOX1**. Metal-labelled detection probes can be validated by diluting the oligonucleotides in water (e.g. 100pM – 1nM) and running the samples through the mass cytometer to confirm presence of the metal ion. Validation that each PLAYR system (including detection probe) is working with equal efficiently can also be performed using a suspension cell line of your choice to probe a gene of known high abundance. An example using 2-3 probe pairs that target GAPDH transcripts in the B-cell line MAVER1 is shown (**Figure 4C**).

We include in this protocol an optional step for mass cytometry which enables Palladium (Pd)-based barcoding of up to 20 samples so they can be subsequently

pooled together and analysed as one sample [16]. The use of Pd barcoding has been optimised to ensure that it does not interfere with the PLAYR assay and should be performed after probe hybridisation (steps 20-22) as described. Pd barcoding is encouraged as it has multiple benefits including: significantly reducing cell loss during processing, allowing the use of fewer starting number of cells per sample; removing variability in PLAYR and internal antibody staining efficiencies; decreasing reagent use; decreasing instrument measurement time; and reducing sample processing time. *In silico* separation of the samples is performed after data collection using an integrated feature on the CyTOF system control software. Further details are can be found in the Cell-ID 20-Plex Pd Barcoding Kit user guide on the Fluidigm website.

**Considerations for use with flow cytometry.** Care must be taken regarding the choice of fluorophores for surface antigen staining. Alongside the usual concerns regarding spectral overlap, additional attention must be paid to whether fluorophores conjugated to surface staining antibodies will be inactivated during the PLAYR staining procedure. Fluorophores need to withstand chemical treatments (e.g. BS3 crosslinking, PFA treatment and methanol), temperatures, and buffers used during the PLAYR assay. Generally, smaller fluorophores are more resistant to these conditions than protein-based fluorophores which can be denatured by methanol[17]. A list of 10 fluorophores that we have tested for performance under different PLAYR optimisation conditions can be found in **Supplementary Table 2**. However, inactivation is less of a concern for fluorophores that are employed at the end of the protocol (i.e. for PLAYR detection probes and internal antigen staining). The use of PLAYR with advanced multi-parameter flow cytometry may be limited by the number of channels and suitable fluorophores. Please see the section on “Protocol Optimisation” above for other potential ways to increase surface antibody staining efficiency and preservation of fluorophores. As with routine flow cytometry, a negative control sample for PLAYR (e.g. assay performed with probe 1 or 2 only – see **Figure 5A**) is required to correct for background fluorescence. Finally, all incubation steps when using fluorophores should be performed in the dark.

## MATERIALS

**BIOLOGICAL MATERIALS**

- Cultured cell lines of interest. We demonstrate our protocol in this manuscript using the B-cell cell lines MEC1 (DSMZ, cat. no. ACC 497), MAVER1 (ATCC, cat. no. CRL-3008) and the T-cell line HUT78 (ATCC, cat.no. TIB-161) that were authenticated by STR analysis and tested negative for mycoplasma.

**! CAUTION:** Regularly check cell lines to ensure they are authentic and are not infected with mycoplasma.

- Primary cells of interest. We have also demonstrated the protocol use on primary CLL PBMC samples from consenting patients.

**! CAUTION** All experiments involving animal or human samples should adhere to the relevant governmental and institutional ethics guidelines and regulations. CLL cells used for this Protocol were obtained from the peripheral blood taken from patients with informed consent and with the approval of the Liverpool Research Ethics Committees.

**! CRITICAL** Cryo-preserved CLL samples stored in the Liverpool Leukemia Biobank were prepared using standard protocols and stained immediately after thawing.

**REAGENTS**

**(Optional) Surface and internal antibody staining**

**! CRITICAL** Staining with antibody is not required for RNA to be detected by the PLAYR assay. Surface and/or intracellular antibodies should be included if analysis of RNA expression is performed in specific cell types or cell phenotypes within mix populations of cells (e.g. studying B cells within PBMC).

- Metal- (For mass cytometry; Fluidigm)[11] or fluorophore-conjugated (for flow cytometry) antibodies.

**! CRITICAL** Fluorophore-conjugated antibodies used to stain surface antigen need to withstand conditions used during the remaining steps of the protocol (i.e. PFA fixation, MeOH treatment and PLAYR incubations). See the section “Considerations

for use with flow cytometry" for more details. For a list of antibodies used in this Protocol, see Supplementary Table 1.

- Bis(sulfosuccinimidyl) suberate (BS3 crosslinker; Sigma-Aldrich, cat. no. S5799)
- Cell staining buffer (Fluidigm, cat. no. 201068)
- Phosphate-buffered saline (PBS; Gibco, cat. no. 10010015)

**PLAYR**

- 100μM PLAYR probes (Integrated DNA technologies). PLAYR probes can be designed using our open-source R software package called PLAYRDesign[9]. Detailed instructions on how to download and install PLAYRDesign and its dependencies are available at https://github.com/nolanlab/PLAYRDesign (see Experimental Design "PLAYR Probe design"). See **Table 1** for Oligonucleotide sequences for 27 PLAYR systems.
- 100μM 5'-phosphorylated insert and backbone oligonucleotides (Integrated DNA technologies)
- RNAsin Plus (Promega, cat. no. N2611/N2615)

**! CRITICAL** - Other RNAsins may result in suboptimal inhibition and compromise RNA detection.

- 25% (vol/vol) Poly Vinyl Sulfonic acid (PVS) (Polysciences, cat. no. 9002-97-5)
- 200mM Ribo-nucleoside vanadyl complex (RVC) (New England BioLabs, cat. no. S1402S)

**! CRITICAL** - RVC precipitates at low temperatures and oxidises upon repeat freeze thawing. Thaw the bottle of RVC, heat it up to 65$^{o}$C and produce an even suspension of RVC precipitates by gentle agitation before aliquoting. 100uL aliquots can then be produced which should be stored at -20$^{o}$C. Similarly, when using an aliquot, heat it up to 65$^{o}$C with agitation to dissolve any precipitate that has re-formed upon cooling.

- UltraPure™ Salmon Sperm DNA Solution (Life Technologies, cat. no. 15632011)

- 16% (wt/vol) Paraformaldehyde (Electron Microscopy Sciences, cat. no. 15710)

**! CAUTION** – Skin contact should be avoided. Wear appropriate Personal Protective Equipment (PPE).

**! CRITICAL** - Keep the ampoule at 4$^{o}$C in the dark once opened and discard after one week.

- Methanol (Fisher Scientific, cat. no. M/4056/15)

**! CAUTION** – Methanol is toxic. Wear appropriate PPE.

- T4 DNA Ligase (Thermo Scientific, cat. no. EL0011)
- Phi29 DNA polymerase (Thermo Scientific, cat. no. EP0091)
- 10mM dNTPs (Invitrogen, cat. no. 18427013)
- Cell staining buffer (Fluidigm, cat. no. 201068)
- Tween20 (Thermo Scientific, cat. no. BP337-100)
- 10X PBS pH 7.4 (Invitrogen, cat. no. AM9625)
- 20X Saline-Sodium Citrate solution (20X SSC; Fisher Scientific, cat. no. 10418353)

**Additional reagents for mass cytometry**

- 1μM Thiol-Modified C6 S-S detection probe(s) for detection by mass cytometry (see **BOX1** for protocol for metal labelling of detection probes; Integrated DNA technologies). See **Table 1** for Oligonucleotide sequences for 27 PLAYR systems.
- MaxPar® Metal Labeling Kit(s) (Fluidigm)
- Cell-ID Intercalator-Ir (Fluidigm, cat. no. 201192A)
- (Optional) Cell-ID Cisplatin (Fluidigm, cat. no. 201064)

**! CRITICAL** Use cisplatin if identification of cell viability is required.

- (Optional) Cell-ID 20-Plex Pd Barcoding Kit (Fluidigm, cat. no. 201060)

**! CRITICAL** Pd barcoding allows samples to be pooled together into one tube and provides a number of benefits. See the description of Pd barcoding within the Experimental design section for more details.

- Maxpar water (Fluidigm, cat. no. 201069)
- EQ Four Element Calibration Beads (Fluidigm, cat. no. 201078)

### Additional reagents for flow cytometry

- 1µM Fluorophore-conjugated detection probe(s) (resuspended in $ddH_2O$) for detection by flow cytometry (Integrated DNA technologies). See **Table 1** for Oligonucleotide sequences for 27 PLAYR systems.
- (Optional) Live/Dead® Fixable Dead Cell Stain Kits (Invitrogen)

**!CRITICAL** Use Live/Dead® Fixable Dead Cell Stain Kits if identification of cell viability is required.

## EQUIPMENT

### General equipment

- Filter tips
- Polypropylene V-bottom 200 uL PCR strips (Fisher Scientific cat. no. 11849241), or alternatively 1.5 mL Eppendorf tubes (STARLABS, cat. no. S1615-5500)
- Refrigerated microcentrifuge
- Mass cytometer or flow cytometer; we use the Helios (Fluidigm) and Attune NxT (Invitrogen) respectively.

### Antibody staining

- Weighing scale capable of 4 decimal places or more for the weighing of BS3 (Fisherbrand™ Analytical Balance, cat. no. 15315113). Alternatively, pre-weighed BS3 may be purchased (Thermo Scientific, cat. no. 21585).

### PLAYR

- Thermomixer (Eppendorf ThermoMixer® C, cat. no. 5382000031)

### Software

- Fluidigm CyTOF system control software for mass cytometry (www.fluidigm.com/software)
- NxT software (Thermofisher; cat. no. A25554) for the Attune flow cytometer (or equivalent)
- Software appropriate for analysis of multiparametric mass cytometry data files (e.g. Cytobank (https://www.cytobank.org/), Cytosplore (https://www.cytosplore.org/), etc.)
- R software (https://www.r-project.org/) for PLAYR probe design
- PLAYRDesign software to design PLAYR probes for the RNA of interest is available at https://github.com/nolanlab/PLAYRDesign

**REAGENT SETUP**

**10% (vol/vol) Tween20.** Dissolve one part Tween20 to nine parts DEPC-treated dd$H_2O$. Store at room temperature (RT: 15-30°C) for up to 6 months.

**PLAYR oligonucleotides.** PLAYR probes, inserts, and backbone oligonucleotides are delivered dry with purification by standard desalting. Resuspend the oligonucleotides in DEPC-treated ddH2O at a concentration of 100μM, and store them at -20$^{o}$C for up to 3 years. To increase the speed of the protocol, it is recommended that all oligonucleotides are aliquoted before storing at -20$^{o}$C. PLAYR probes with the same insert system that target an individual RNA transcript can be mixed together in equal quantities prior to aliquoting and freezing at -20$^{o}$C. For example, if 4 probe pairs target a unique transcript, then these 8 probes may be mixed together in equal quantities and stored as 8uL aliquots. Each aliquot is enough to make 1mL of hybridization buffer and each aliquot can then be pooled together prior to heating (see PLAYR probe hybridization buffer below).

**PLAYR dilution buffer (PBST).** Take a 9mL aliquot of DEPC-treated dd$H_2O$ and add 1mL of DEPC-treated 10X PBS. Add 100uL of 10% (vol/vol) Tween20 and place on ice. Prepare this buffer fresh at the start of PLAYR assay (step13).

**PLAYR wash buffer (PBSTR).** Add RNAsin Plus to PBST at a final concentration of 4U/mL. Prepare this buffer fresh at the start of PLAYR assay (step13).

**PLAYR wash buffer with extra RNAsin (PBSTR+).** Add RNAsin Plus to PBST to a final concentration of 40U/mL. Prepare this buffer fresh at the start of PLAYR assay (step13).

**PLAYR Probe hybridization buffer.** To simplify and speed up the procedure for preparation of this buffer we recommend aliquoting PLAYR probes, so that each aliquot is enough for 1mL of hybridization buffer (see reagent setup of "PLAYR oligonucleotides" above). We make this buffer at a minimum volume of 1mL, which therefore uses 1 aliquot of each transcript probe set, and 1 aliquot of RVC (enough for 10 samples or less). If more than 10 samples are being stained, then 2mL would be made from 2 aliquots of PLAYR probes and RVC etc. Prepare this buffer fresh at the start of PLAYR assay (step14) using the following procedure:

1. Thaw and pool together the required PLAYR probes and leave at room temperature. For 1mL of hybridization buffer initially heat an aliquot of RVC using a Thermomixer to 65$^{o}$C with vigorous agitation (we use 1400rpm) for 25minutes to dissolve any precipitation (2 aliquots are required for 2mL, etc).
2. While the RVC is incubating at 65$^{o}$C, mix together the following at room temperature for 1mL of hybridization buffer:

| **Reagent** | **Volume** | **Final concentration** |
|---|---|---|
| DEPC-treated dd$H_2O$ | XµL | - |
| DEPC-treated 20X SSC | 50µL | 1X |
| 10% (vol/vol) Tween20 | 100µL | 1% (vol/vol) |
| 25% (vol/vol) PVS | 100µL | 2.5% (vol/vol) |
| Salmon Sperm DNA | 10µL | 100ug/mL |

   The volume XµL of DEPC-treated dd$H_2O$ is calculated as the remaining volume required to produce 1 mL of solution after addition of all other reagents, including RVC, RNAsin, and probes (see Step 5 below).
3. Remove the RVC from the Thermomixer and leave at room temperature to cool while preparing the cells (Step 15 of the main procedure).

   **! CRITICAL** – Do not cool the RVC on ice as this will cause it to precipitate.
4. 10 minutes before addition of the PLAYR hybridization buffer to the cells heat the probe mixture for 5 mins at 90$^{o}$C and then immediately place on ice for 5 mins to avoid denaturing the RNAsin.
5. Immediately before addition of the PLAYR hybridization buffer to the cells, add the following to complete its constituents:

| Reagent | Volume | Final concentration |
|---|---|---|
| 200mM pre-heated RVC | 100µL | 20mM |
| RNAsin Plus | 1µL | 40U/mL |
| Pre-heated PLAYR probes | 1µL of each | 100nM of each |

**PLAYR Post hybridization wash** Dilute DEPC-treated 20X SSC to 4X SSC (1:5 (vol:vol) dilution) in PBST and add RNAsin Plus to a final concentration of 40U/mL. Prepare fresh just before use (step21).

**PLAYR backbone/insert hybridization mix.** Using PBST as the diluent, prepare a solution containing 1X DEPC-treated SSC, 40U/mL RNAsin Plus, 100nM backbone oligonucleotide and 100nM of each insert oligonucleotide. Prepare fresh just before use (step23).

**PLAYR Ligation mix.** Using DEPC-treated dd$H_2O$ as the diluent, prepare a solution containing 1X T4 Ligase Buffer (as supplied by manufacturer), 5U/mL T4 Ligase and 40U/mL RNAsin. Prepare fresh just before use (step25).

**PLAYR Phi29 amplification mix.** Using DEPC-treated dd$H_2O$ as diluent, prepare a solution containing 1X Phi29 Buffer (as supplied by manufacturer), 5U/mL Phi29 DNA polymerase and 200µM dNTP. Prepare fresh just before use (step27).

**PLAYR Mass Cytometry Detection probe buffer.** Use this buffer when performing mass cytometry. Using cell staining buffer as a diluent, prepare a solution containing 1nM of each metal-conjugated detection probe and, if required, appropriate concentration of metal-labelled antibodies against internal antigens. Prepare fresh just before use (step29).

**PLAYR Flow Cytometry Detection probe buffer.** Use this buffer when performing flow cytometry. Using PBST as a diluent, prepare a solution containing 1nM of each fluorophore-conjugated detection probe and, if required, appropriate concentration of fluorophore-labelled antibodies directed against internal antigens. Prepare fresh just before use (step29). For a list of antibodies used in this Protocol, see Supplementary Table 1.

**Cell-ID Intercalator-Ir buffer.** Make a 1:5 dilution of 8% (vol:vol) PFA with PBS to generate a 1.6% (vol:vol) solution and add Cell-ID Intercalator-Ir to a final concentration of 62.5nM (i.e. 1:8000 (vol:vol) of 500μM). Intercalator-Ir buffer should be kept at 4$^{o}$C in the dark and can be stored for up to 1 week.

**PROCEDURE**

**! CRITICAL** See **Fig. 1C** for an overview of the workflow.

**Cell preparation.** ***Timing:*** *6mins*

1. Count the cells and place 3x10$^{6}$ cells into an Eppendorf on ice. In the example described in this Protocol, we use PBMC cells, but the approach can be used with other primary or cultured cell lines of interest.

**! CRITICAL STEP** We encourage a minimum of 3x10$^{6}$ cells per sample, due to cell loss throughout the assay. This gives approximately (depending on pipetting efficiency) 1.5x10$^{6}$ cells at the final wash step to analyze by mass/flow cytometer. Fewer cells per sample may be used for mass cytometry if the experimental plan includes Pd barcoding and pooling of the samples (performed during step 20-22) (see Experimental Design, “Considerations specific for use with mass cytometry”).

**(Optional) Staining to identify dead cells.** ***Timing:*** *15mins*

**! CRITICAL** Use viability staining if identification of cell death is required. See Experimental design – “General considerations” section for more details.
**! CRITICAL** If staining to identify dead cells is not required proceed to Surface Antibody Staining.

2. Pellet the cells by centrifugation (500xg/3min/4$^{o}$C) and remove the supernatant. Wash the cells once by adding 200µL room temperature PBS, followed by centrifugation and removal of the supernatant.
3. Resuspend cells in either 200µL of a 1:1000 (vol:vol) dilution of Cell-ID Cisplatin (for mass cytometry) or LIVE/DEAD viability dye (for flow cytometry) in room temperature PBS and incubate for 5min (cisplatin) or 10min (LIVE/DEAD dye) at room temperature.

**! CRITICAL STEP** Incubation of cells with viability dye should be done at RT, since cold conditions cause non-specific staining of live cells.

**Surface Antibody Staining.** ***Timing:*** *1hr 15mins*

**! CRITICAL** Surface antibody staining may be omitted and this will not compromise the PLAYR assay. If surface antibody staining is not required, only perform the PBS cell wash in step 7 and proceed to step 10.

4. Wash cells (500xg/3min/4oC) once in 200µL ice cold cell staining buffer.
5. Resuspend cells in ice cold antibody cocktail (for a list of antibodies used in this Protocol, see Supplementary Table 1) in cell staining buffer (100µL/3x10$^{6}$cells). Leave on ice for 30mins, agitating the cells every 10mins.

**! CRITICAL STEP** Cells must remain on ice at all times to avoid further transcription and RNA changes in live cells that may result from binding of surface antibodies.

6. Wash cells (500xg/3min/4$^{o}$C) in 200µL ice cold cell staining buffer.
7. Wash cells (500xg/3min/4$^{o}$C) in 200µL ice cold PBS.
8. Resuspend cells in 50µL of 5mM BS3 crosslinker in ice cold PBS – mix immediately and leave on ice for 30mins, agitating the cells every 10mins.

**! CRITICAL STEP** The NHS-ester moiety within BS3 readily hydrolyses upon contact with water. Warm the vial to room temperature to avoid moisture formation upon opening and weigh fresh powder for each experiment/timepoint. Dissolve the powder immediately before addition to the cells to avoid loss of reactivity

9. Pellet cells (500xg/3min/4$^{o}$C) and remove supernatant.

**Cell Fixation and permeabilisation.** ***Timing:*** *25mins*

10. Resuspend cells in ice cold 1.6% (wt/vol) PFA at a maximum cell concentration of $2x10^6$ cells/mL. Leave on ice for 10 minutes.

**! CRITICAL STEP** – PFA fixation should be performed at room temperature if no surface antibody staining and BS3 cross-linking was performed.

11. Spin-down the cells (500xg/3min/4$^o$C), remove the supernatant, loosen the pellet by flicking the tube and add ice-cold methanol dropwise, while vortexing (300uL/$3x10^6$cells)

**! CRITICAL STEP** - Methanol can cause clumping of cells if they are not in a single cell suspension

12. Incubate for 10 minutes on ice.

**! CRITICAL STEP** - Cells in methanol should always be kept on ice during handling.

**! PAUSE POINT** - Once placed in methanol the cells can be stored at -80$^o$C for an extended period of time (several months) without RNA degradation.

**PLAYR and intracellular antibody staining.** ***Timing*** *7-8hrs for flow cytometry or 9-10hrs for mass cytometry with frequent incubations steps*

13. Prepare PBST, PBSTR and PBSTR+ buffers (see Reagent Setup) and place on ice.
14. Prepare PLAYR probe hybridization buffer (refer to Reagent Setup for a detailed guide).
15. Spin-down the cells (800xg/3min/4$^o$C) which are suspended in methanol and wash (800xg/3min/4$^o$C) with 200µL ice cold PBSTR+ buffer.

**! CRITICAL STEP** Methanol has a higher density at -80$^o$C and pelleting of cells by centrifugation may be inhibited; warm the tube on ice for 5mins if stored at -80$^o$C before centrifugation.

**! CRITICAL STEP** Once cells are removed from ice-cold methanol, RNA degradation can set in and hence speed in sample preparation during this stage is essential to maintain RNA integrity and PLAYR signal.

16. Resuspend cells immediately in 100µL room temperature PLAYR probe hybridization buffer and place on the Thermomixer at 40$^{o}$C.

**! CRITICAL STEP** Due to the small volumes used, the failure to remove all PBSTR + wash supernatant before addition of probe hybridization buffer can affect assay efficiency.

17. Incubate cells for a 1hr (this can be extended if signal is weak) at 40$^{o}$C with vigorous agitation (we use 1400rpm).
18. (Optional) When analysing using mass cytometry, remove an aliquot of PBST from the ice while incubating the cells, and place at room temperature to perform cell Pd barcoding (step 20).

**! CRITICAL STEP** Only perform this step if you are Pd barcoding for mass cytometry.

19. Wash cells once (800xg/3min/4$^{o}$C) with ice cold PBSTR.
20. (Optional) When analysing using mass cytometry, perform Pd barcoding at room temperature in PBSTR+ buffer (add RNAsin Plus to room temperature PBST from step 16). We recommend resuspending the cells with 99µL of room temperature PBSTR+ and then adding 1µL of the appropriate Pd barcode and incubate for 10mins.

**! CRITICAL STEP** Only perform this step if you are using Pd barcoding for mass cytometry. Pd barcoding allows samples to be pooled together into one tube which provides a number of benefits. See Experimental design – General considerations for more details.

21. Wash cells twice (800xg/3min/4$^{o}$C) in 200µL ice cold PBSTR and prepare PLAYR post hybridization wash buffer (see Reagent Setup) and place on ice.
22. Resuspend cells in 100µL ice cold post probe hybridization buffer and incubate for 20mins at 40$^{o}$C with vigorous agitation (we use 1400rpm). Pd-barcoded cells for mass cytometry may be pooled together at this stage **!**

**CRITICAL STEP** Remember to increase the volume of buffer if using increased number of cells per sample.

23. Wash cells twice (800xg/3min/4$^{o}$C) in 200µL ice cold PBSTR and prepare PLAYR backbone/insert hybridization mix (see Reagent Setup) on ice.
24. Resuspend cells in 100µL ice cold PLAYR backbone/insert hybridization mix and incubate at 37$^{o}$C for 30mins under agitation (we use 1100rpm).
25. Wash cells twice (800xg/3min/4$^{o}$C) in 200µL ice cold PBSTR and prepare PLAYR Ligation mix (see Reagent Setup) on ice.
26. Resuspend cells in 50µL of ice cold PLAYR Ligation mix and incubate at 21$^{o}$C for 30mins with agitation (we use 1100rpm).
27. Wash cells twice (800xg/3min/4$^{o}$C) in 200µL ice cold PBSTR and prepare PLAYR Phi29 amplification mix (see Reagent Setup) on ice.
28. Resuspend cells in ice cold PLAYR Phi29 amplification mix and incubate at 30$^{o}$C with agitation for ≥ 2hrs (flow cytometry) or ≥ 4hrs (mass cytometry). Longer incubations (up to overnight) may increase signal intensity. We typically agitate the cells at 1100rpm. In any case, make sure that you use a speed such that the cells do not settle at the bottom of the tubes, as this will decrease the amplification efficiency).
29. Wash cells twice (800xg/3min/4$^{o}$C) in 200µL ice cold cell staining buffer (mass cytometry) or ice cold PBSTR (flow cytometry), and prepare PLAYR mass cytometry detection probe buffer or PLAYR flow cytometry detection probe buffer on ice. Intracellular antibodies can also be added to this buffer as described in the Reagent Setup section.
30. Resuspend cells in 100µL of the appropriate ice cold detection probe buffer and incubate at 37$^{o}$C for 30mins with agitation (we use 1100rpm).
31. Wash cells twice (800xg/3min/4$^{o}$C) in ice cold cell staining buffer (for mass cytometry) or twice in ice cold PBSTR (for flow cytometry).

**! CRITICAL STEP** Do not use PBSTR to wash cells for mass cytometry after detection probe hybridisation as it will lead to signal loss.

32. For flow cytometry, resuspend cells in ice cold PBS and analyse immediately using laboratory specific protocols. For mass cytometry, resuspend cells in Cell-ID Intercalator-Ir buffer, incubate for 1hr at room temperature or for up to 2 weeks at 4$^{o}$C and proceed to Step 33.

**? TROUBLESHOOTING**

**! PAUSE POINT** Samples for mass cytometry can be stored at 4°C for up to two weeks in Cell-ID Intercalator-Ir buffer with minimal loss of signal.

**(Optional) Preparation of samples for mass cytometry analysis. *Timing*** *20mins*

**CRITICAL** Steps 33-37 describe how to prepare the samples for mass cytometry.

33. To prepare the sample for the mass cytometer, wash the cells once (800xg/3min/4°C) with 1mL cell staining buffer then once (800xg/3min/4°C) with 1mL Maxpar water.
34. Resuspend cells in 1mL Maxpar water, filter cells through a 30µM filter and count cells.
35. Adjust the concentration of cells to a maximum of $5x10^5$cells/mL in Maxpar water.
36. Add EQ Four Element Calibration Beads 1:10 (vol:vol), mix by vortexing and then analyse using the mass cytometer. Details on instrument and panel-template setup can be found at www.fluidigm.com/productsupport/cytof-helios-support-hub
37. Analyse data using linked software (e.g. cytobank).

**? TROUBLESHOOTING**

**TIMING**

Step 1, Cell preparation: 6mins

(Optional) Steps 2-4, Staining to identify dead cells: 25mins

Steps 5-9, Surface antibody staining: 1hr 15mins

Step 10-12, Cell fixation and permeabilization: 25mins

Steps 13-32, PLAYR and internal antibody staining: 7-8hrs for flow cytometry or 9-10hrs for mass cytometry

(Optional) Steps 33-36, Preparation of samples for mass cytometry: 20mins

Step 37, Analysis of mass cytometry data: 2-6 hours

BOX1 – Metal labelling of detection probe oligonucleotides: 7hrs with an overnight incubation step

## TROUBLESHOOTING

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

## ANTICIPATED RESULTS

This protocol provides a detailed and step-by-step guide to multiplexed and simultaneous detection of RNA and antigen expression using the PLAYR technique combined with antibody labelling for up to 40 variables. The methodology described allows the use of the technique for either flow or mass cytometric applications.

**Assay optimisation.** We recommend optimising PLAYR and antibody assays separately before the two procedures are combined (**Figure 3**). We have optimised the PLAYR assay by studying control genes with high copy numbers in a cell line to confirm assay efficiency. See **Figure 5A** for an example comparing signal intensity achieved in primary CLL cells vs. the cell line MEC1. It is important to appreciate that expression of “classical” control genes may not be uniform within cell populations, particularly in primary cells. For example, we observed high unimodal expression of ACTB, RPL27 and GAPDH in cell lines, but GAPDH exhibited a multimodal pattern of expression in primary CLL cells (**Figure 5A top right panel**). Upon confirming that each PLAYR detection probe was correctly labelled and working with similar efficiencies on our positive control gene (**Figure 4C**), we applied the assay to other transcripts of interest. To confirm that PLAYR signals were specific for each transcript, we performed a time course analysis of RNA expression changes, known to occur on *in vitro* PMA and Ionomycin stimulation of MAVER1 cells and correlated PLAYR mean signal intensities with qPCR quantification (**Figure 4A and Suppl. Fig 2**). When optimised the pattern of expression of each individual transcript examined on its own or when multiplexed should be consistent (**Figure 4B**).

In the top two row of panels in Figure 2, we plot CD4 expression (y-axis) vs CD335, CD161, CD45, CD14, CD278, CD3, CD194, and CD5 (x-axes)). As demonstrated, detection of antigens (cell identity) should not be compromised by additional steps required for the PLAYR assay (second row of panels in Figure 2) when compared with standard antibody staining of cells (top row panels). Similarly, the additional steps for antibody staining of cells should not affect the performance of the PLAYR assay.

**Insights into genotypic and phenotypic heterogeneity at single cell resolution.** The main advantage of the combined analysis of antigen and RNA expression using the technique is the potential to address genotypic and phenotypic variations at single cell resolution. Our study of primary CLL cells (**Figure 5**) is an example of how the technique can provide novel insights into intraclonal heterogeneity. CLL cells from a single patient were resolved into newly emerged vs old quiescent cells (**Figures 5D**) based on the intensity of CXCR4 and CD5 surface antigen expression[18]. It is clear that there is considerable variation of Ki67 expression **(Figure 5E)** and CD83, JUND and TXNIP mRNAs as measured by PLAYR **(Figure 5F)**. This was not apparent in previously reported studies performed on whole cell populations[17,19-21]. Additional computational analyses allow clustering of seemingly identical neoplastic CLL cells into distinct phenotypic and genotypic subpopulations **(Figures 5G and H)** with a potential for variant behaviour and therapeutic responses. Hence, the combined and multiplexed analysis of antigen and RNA is a useful tool to monitor and track CLL disease evolution, biology, and heterogeneity. Relevant questions regarding heterogeneity in other cellular contexts can be addressed using our protocol.

**BOX1 – Metal labelling of detection probe oligonucleotides**

**Reagents**

- Ice cold 100% Ethanol
- Ice cold 75% Ethanol
- 3M Sodium Acetate, pH 5.2 (Thermo Scientific, cat. no. R1181)
- Bond-Breaker™ TCEP Solution, Neutral pH ((Thermo Scientific, cat. no. 77720)
- Maxpar® X8 Antibody labelling Kit (Fluidigm)

**! CRITICAL** The choice of metal for each PLAYR detection probe depends upon your panel design and PLAYR insert system used.

- Detection probe carrying a 5'-Thiol-Modifier C6 S-S (IDT)

**Equipment**

- Nanodrop spectrophotometer (Thermo Scientific, cat. no. ND-2000)
- Centrifugal filter with 30kDa MWCO (EMD Millipore, Cat. No. UFC503024)
- Centrifugal filter with 3kDa MWCO (EMD Millipore, Cat. No. UFC500396)

**Procedure.** ***Timing:*** *7hrs with an overnight incubation step*

1. Resuspend the detection probes carrying a 5'-Thiol-Modifier C6 S-S at 250µM in dd$H_2O$. Calculate or make a note of the starting number of nmoles for each detection probe as this will be required during calculations in step 15.
2. Add TCEP to a final concentration of 50mM and incubate at room temperature for 30mins.
3. Increase the volume if required to a minimum of 200µL using dd$H_2O$ and add 0.1 volumes 3M Sodium acetate and 3 volumes pure ethanol. Vortex to mix. Precipitate detection probe at -20$^{o}$C overnight.
   **! PAUSE POINT** – Alternatively, PLAYR DNA detection probes can be stored at -80$^{o}$C in 75% ethanol for up to 6 months without degradation before being processed.
4. From the metal labelling kit, spin polymer tube (4000xg/30sec/RT) to ensure all reagent is at the bottom.

5. Resuspend polymer in 95µL L-Buffer and then add 5µL of lanthamide metal solution, mix by pipetting.
6. Incubate polymer-metal mix at 37$^{o}$C for 30mins.
7. While the polymer-metal mix is incubating, centrifuge the detection probe oligonucleotide from step 3 at 14000xg/4$^{o}$C/30mins.
8. Add 200µL of L-Buffer to a 3kDa filter.
9. After incubation, add the metal loaded polymer from step 6 into the filter containing the L-Buffer.
10. Centrifuge metal conjugated polymer at 12000xg/RT/25mins.
11. Wash detection probe pellet from the centrifugation in step 7 twice in ice cold 75% ethanol centrifuging at 14000xg/4$^{o}$C/10mins.
12. Add 300µL of C-Buffer to the 3kDa filter from step10 and centrifuge at 12000xg/RT/30mins – leave at room temperature while completing detection probe purification.
13. After final wash of detection probe in step 11, remove as much ethanol as possible (a brief centrifuge step (14000xg/4$^{o}$C/30s) may help to pool residual ethanol for removal) and air dry the pellet (tube can be placed at 40$^{o}$C on a heat block to decrease drying time).

**! CRITICAL STEP** Failure to fully dry the pellet and remove all ethanol can affect downstream applications. Pellet is dry when it turns from opaque/white to bright white in colour.

14. Assuming a yield of 80%, resuspend the dried pellet in C buffer (supplied in the metal labelling kit) to a concentration of 1nmol/25µL. For example, a starting quantity of 10nmoles would give an assumed yield of 8nmoles and would therefore be resuspended in 200µL of C buffer.
15. Measure the reduced detection probe concentration from step 14 by absorption at 260nm on a nanodrop (use C buffer as a blank).
16. Using the concentration from step 15, calculate the volume required for 2nmol of detection oligonucleotide (MW=6789g/mol) and transfer this to a fresh eppendorf.
17. Transfer the entire metal loaded polymer from the 3kDa filter into the eppendorf containing the 2nmol oligonucleotide.

18. Increase the volume of the mixture to 100µL using C-Buffer and mix by pipetting.
19. Incubate at room temperature for 2hrs.
20. Add TCEP to a final concentration of 5mM, immediately mix by vortexing and incubate at room temperature for 30mins.
21. Add 400µL dd$H_2O$ into a 30kDa filter.
22. Transfer the metal-polymer-detection probe mixture into the 30kDa filter containing the dd$H_2O$ and centrifuge at 14000xg/RT/12mins.
23. Discard flow through, add 500µL dd$H_2O$ to filter and centrifuge at 14000xg/RT/12mins.
24. Repeat step 23.
25. Invert filter into a clean collection tube and centrifuge at 1000xg/RT/2min.
26. Wash the filter with 250uL of dd$H_2O$ and elute into the same collection tube by inverting the filter and centrifuging at 1000xg/RT/2min.
27. Measure the oligonucleotide concentration by nanodrop using dd$H_2O$ as a blank.
28. Adjust the volume of dd$H_2O$ to give a final concentration of 1µM metal-labelled detection probe (assuming 6789ng/mL = 1µM).
29. Aliquot and store the metal-labelled detection probe at -20$^{o}$C.

**! CRITICAL STEP** Confirm efficient detection probe labelling by targeting a control gene which is known to have high copy number. For example, we have confirmed efficient detection probe labelling by using the cell line MAVER1 stained with PLAYR probe pairs containing the appropriate 3' sequences all targeting the same loci on GAPDH mRNA (Figure 4C).

**? TROUBLESHOOTING**

- **END OF BOX 1 -**

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## Author contributions

A.D., M.S. and F.Y. performed the experiments. A.D. and P.F.G collaborated on assay development and optimisation. A.D., PFG, GPN, JRS, NK contributed to critical analysis of data and manuscript preparation.

## Competing interests

G.P.N. had a personal financial interest in Fluidigm, the manufacturer of the mass cytometer used in this study, for the duration of this project. The remaining authors have no competing interests.

## Data availability and patient derived material

The data that support the findings of this study are available from the authors on reasonable request.

## RELATED LINKS

Key reference(s) using this protocol:

**1.** Frei, A. P. et al. Nat Methods 13, 269-275 (2016). https://www.nature.com/articles/nmeth.3742

## Figure legends

### Figure 1. Schematic representation of the PLAYR assay

**(A)** Schematic overview of PLAYR (Stages 1 - 8) for simultaneous staining of surface/intracellular proteins and mRNAs. The steps in the procedure are indicated for each stage. Initial staining of surface proteins is performed on ice in unfixed cells (stage 1). Bound antibodies are covalently cross-linked to the cells using the chemical agent BS3 (stage 2), and cells can then be fixed with paraformaldehyde and permeabilised using methanol (stage 3). Once suspended in methanol, cells may be stored at -80$^{o}$C for up to several months without loss of RNA integrity or surface antibody staining (Pause Point). Stages 4 to 8 illustrate the strategy for RNA detection using PLAYR. Intracellular antibody staining is performed during incubation of the PLAYR detection probes in stage 8. **(B)** Overview of the PLAYR probe design with different sequence components colour coded. Sequences with N vary depending upon their target (RNA-binding sequence) and the PLAYR system (insert-binding sequence) used. **(C)** Diagram illustrates the interaction of different oligonucleotide sequences during the PLAYR assay. 5' probe sequences bind to their target RNA (top). The insert and backbone oligonucleotides subsequently bind to the 3' end of probe 1 and 2 (bottom). Colour coding of probe components is the same as in (B).

### Figure 2. Comparison of surface antigen staining in the PLAYR assay to a standard protocol for a panel of 27 antibodies

Bivariate dot plots, coloured for event density, showing peripheral blood mononuclear cells simultaneously analysed for 27 surface proteins and measured by mass cytometry. Cells were either fixed first and then stained with antibody at room temperature (Standard staining protocol; top rows in each set of 9 panels) or antibody staining for use with PLAYR that includes BS3 crosslinking, fixation and permeabilisation steps (Staining for PLAYR; bottom rows in each set of 9 panels). For list of antibodies see Supplementary Table 1.

### Figure 3. Schematic of experimental design workflow for PLAYR

Workflow showing: *in silico* experimental design (orange); acquisition of labelled detection probes either for mass (blue) or flow (green) cytometry; control

experiments for PLAYR RNA detection and antibody staining (pink); data collection and analysis of single-cell RNA expression in samples of interest (purple).

**Figure 4. Examples of control experiments for PLAYR**

**(A)** The B-cell line MAVER1, treated for up to 6hrs with 100nM PMA + 1μM ionomycin, was examined by PLAYR (mass cytometry) and qPCR for expression changes in 9 RNAs. Each dot represents the expression of the transcript at specific time points measured by qPCR (x-axis) and PLAYR (y-axis); Linear regression is represented by the blue line with grey shading indicating 95% confidence intervals. *A.U. - Arbitrary Units*. **(B)** Nine variably expressed mRNAs were examined (individually (rows 1-9) or multiplexed (row 10)) using the PLAYR assay, labelled with transcript specific detection probes (for mass cytometry) within primary leukemic cells from a CLL patient. **(C)** Validation of PLAYR system efficiency (including detection probe labelling) using 3 (top row) or 2 (bottom row) probe pairs targeting GAPDH mRNA in the MAVER1 B-cell line.

**Figure 5. Use of PLAYR for analysis of surface/internal protein and RNA expression in primary CLL cells**

**(A**) Quantification of three “housekeeping” mRNAs using PLAYR analysed by flow cytometry in primary peripheral blood mononuclear cells of CLL patients or the MEC1 cell line. Histograms are representative of 3 CLL samples and 3 MEC1 biological replicates. *(ATTO647 - Alexa-Fluor 647 equivalent from IDT technologies; For definition of Probes 1 and 2 see Fig. 1).* **(B)** Flow cytometric quantification of ACTB mRNA (using PLAYR) in a mixed sample containing HUT78 T-cell line and the MAVER1 B-cell line. Singlet cells are gated using FSC and SSC (top 2 panels); The cell type was determined using CD5 and CD19 surface antigen expression (bottom left panel; purple gate - $CD19^{-}CD5^{++}$ HUT78; blue gate - $CD19^{+}CD5^{+}$ MAVER1); The shaded histograms in the bottom right panel show ACTB mRNA expression within the gated HUT78 and MAVER1 cells. Unshaded histograms depict ‘probe 2 only’ negative controls. **(C)** Gating strategy for mass cytometric detection of live singlet CLL cells and T-cells in human peripheral blood samples. **(D)** Mass cytometry data showing gating strategy to identify cells that have remained in the blood for an extended period of time, termed old quiescent (OQ; $CD5^{dim}CXCR4^{bright}$), and cells that have recently exited the tissue microenvironment, termed newly emerged (NE;

$CD5^{bright}CXCR4^{dim}$)[18]. **(E)** Percentages of CLL cells expressing the proliferative marker Ki67 within OQ and NE subpopulations of 18 CLL samples. Results are comparable to previous findings that used flow cytometry[18]. Error bars represent one standard deviation from the mean. **(F)** Characterisation of intraclonal heterogeneity in expression of prognostic mRNAs[19] (top row; CD83, JUND, TXNIP) and surface antigens (bottom row; CD21, CD22 and CD27) within cells resolved for CD5 and CXCR4 surface expression in a representative CLL sample. **(G)** viSNE analysis of the data in (F) based upon 9 CLL surface antigens (CD5, CD19, CD21, CD22, CD27, CXCR4, IgD and IgM). Top panel indicates event density coloured by temperature (high - red; low - blue) and the corresponding bottom panel highlights Ki67+ cells (red dots) within the viSNE contour plot. **(H)** viSNE plot from (G) with additional colour-coding for either mRNA (top row) or surface antigen expression (bottom row) to resolve expression for multiple parameters.

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| System | 3’ Probe1 | 3’ Probe2 | Insert | Detection Probe |
|---|---|---|---|---|
| 1 | AAAAAAAAAACTCAGTCGTGACACTCTT | AAAAAAAAAAGACGCTAATATCGTGACC | P-ACGACTGAGTTTGGTCACGAT | Z-ACGACTGAGTTTGGTCACGAT |
| 2 | AAAAAAAAAATATCGTCCGGACACTCTT | AAAAAAAAAAGACGCTAATCTTCGAGAC | P-CGGACGATATTTGTCTCGAAG | Z-CGGACGATATTTGTCTCGAAG |
| 3 | AAAAAAAAAAGATTCCTCGGACACTCTT | AAAAAAAAAAGACGCTAATCTGCCAATG | P-CGAGGAATCTTTCATTGGCAG | Z-CGAGGAATCTTTCATTGGCAG |
| 4 | AAAAAAAAAACTACCTTGGGACACTCTT | AAAAAAAAAAGACGCTAATCAGGCTACT | P-CCAAGGTAGTTTAGTAGCCTG | Z-CCAAGGTAGTTTAGTAGCCTG |
| 5 | AAAAAAAAAACTCTTCGAGGACACTCTT | AAAAAAAAAAGACGCTAATCACCAGTTG | P-CTCGAAGAGTTTCAACTGGTG | Z-CTCGAAGAGTTTCAACTGGTG |
| 6 | AAAAAAAAAACTTAGCCTGGACACTCTT | AAAAAAAAAAGACGCTAATCCAGACTGT | P-CAGGCTAAGTTTACAGTCTGG | Z-CAGGCTAAGTTTACAGTCTGG |
| 7 | AAAAAAAAAACCGCTTATGGACACTCTT | AAAAAAAAAAGACGCTAATCTACATGGC | P-CATAAGCGGTTTGCCATGTAG | Z-CATAAGCGGTTTGCCATGTAG |
| 8 | AAAAAAAAAACTCGATCTGGACACTCTT | AAAAAAAAAAGACGCTAATCAACCTGGT | P-CAGATCGAGTTTACCAGGTTG | Z-CAGATCGAGTTTACCAGGTTG |
| 9 | AAAAAAAAAATACGTTCCGGACACTCTT | AAAAAAAAAAGACGCTAATCATCCTGAG | P-CGGAACGTATTTCTCAGGATG | Z-CGGAACGTATTTCTCAGGATG |
| 10 | AAAAAAAAAACTGCTCATGGACACTCTT | AAAAAAAAAAGACGCTAATCGCAAGTCT | P-CATGAGCAGTTTAGACTTGCG | Z-CATGAGCAGTTTAGACTTGCG |
| 11 | AAAAAAAAAATGACTCTCGGACACTCTT | AAAAAAAAAAGACGCTAATCTCGGAATC | P-CGAGAGTCATTTGATTCCGAG | Z-CGAGAGTCATTTGATTCCGAG |
| 12 | AAAAAAAAAACTGTCTACGGACACTCTT | AAAAAAAAAAGACGCTAATCACAGTGTC | P-CGTAGACAGTTTGACACTGTG | Z-CGTAGACAGTTTGACACTGTG |
| 13 | AAAAAAAAAATTCTCCAGGGACACTCTT | AAAAAAAAAAGACGCTAATCTCAATCGG | P-CCTGGAGAATTTCCGATTGAG | Z-CCTGGAGAATTTCCGATTGAG |
| 14 | AAAAAAAAAACACTTGTCGGACACTCTT | AAAAAAAAAAGACGCTAATCAGATGCCT | P-CGACAAGTGTTTAGGCATCTG | Z-CGACAAGTGTTTAGGCATCTG |
| 15 | AAAAAAAAAACTTCTGCAGGACACTCTT | AAAAAAAAAAGACGCTAATCCAGGATCT | P-CTGCAGAAGTTTAGATCCTGG | Z-CTGCAGAAGTTTAGATCCTGG |
| 16 | AAAAAAAAAATCTATCCGGGACACTCTT | AAAAAAAAAAGACGCTAATCTGTAGACC | P-CCGGATAGATTTGGTCTACAG | Z-CCGGATAGATTTGGTCTACAG |
| 17 | AAAAAAAAAACGCATCTTGGACACTCTT | AAAAAAAAAAGACGCTAATCTGGCACAT | P-CAAGATGCGTTTATGTGCCAG | Z-CAAGATGCGTTTATGTGCCAG |
| 18 | AAAAAAAAAATCTCACGTGGACACTCTT | AAAAAAAAAAGACGCTAATCCTCGAATG | P-CACGTGAGATTTCATTCGAGG | Z-CACGTGAGATTTCATTCGAGG |
| 19 | AAAAAAAAAATCGCTACTGGACACTCTT | AAAAAAAAAAGACGCTAATCGCCATGAT | P-CAGTAGCGATTTATCATGGCG | Z-CAGTAGCGATTTATCATGGCG |
| 20 | AAAAAAAAAATACGCTCTGGACACTCTT | AAAAAAAAAAGACGCTAATCACACTTGG | P-CAGAGCGTATTTCCAAGTGTG | Z-CAGAGCGTATTTCCAAGTGTG |
| 21 | AAAAAAAAAACGTCTTACGGACACTCTT | AAAAAAAAAAGACGCTAATCATGCGACT | P-CGTAAGACGTTTAGTCGCATG | Z-CGTAAGACGTTTAGTCGCATG |
| 22 | AAAAAAAAAACCATTCGTGGACACTCTT | AAAAAAAAAAGACGCTAATCATCAGCGT | P-CACGAATGGTTTACGCTGATG | Z-CACGAATGGTTTACGCTGATG |
| 23 | AAAAAAAAAACCTAGTTCGGACACTCTT | AAAAAAAAAAGACGCTAATCTACCTAGG | P-CGAACTAGGTTTCCTAGGTAG | Z-CGAACTAGGTTTCCTAGGTAG |
| 24 | AAAAAAAAAACTCCGATTGGACACTCTT | AAAAAAAAAAGACGCTAATCAACGCGTT | P-CAATCGGAGTTTAACGCGTTG | Z-CAATCGGAGTTTAACGCGTTG |
| 25 | AAAAAAAAAATTCGCACTGGACACTCTT | AAAAAAAAAAGACGCTAATCAATTCCGG | P-CAGTGCGAATTTCCGGAATTG | Z-CAGTGCGAATTTCCGGAATTG |
| 26 | AAAAAAAAAATCCTTCAGGGACACTCTT | AAAAAAAAAAGACGCTAATCCGCTAAGT | P-CCTGAAGGATTTACTTAGCGG | Z-CCTGAAGGATTTACTTAGCGG |
| 27 | AAAAAAAAAACGTTACTCGGACACTCTT | AAAAAAAAAAGACGCTAATCTTAAGCGC | P-CGAGTAACGTTTGCGCTTAAG | Z-CGAGTAACGTTTGCGCTTAAG |
| **Universal Backbone:** P-ATTAGCGTCCAGTGAATGCGAGTCCGTCTAGGAGAGTAGTACAGCAGCCGTCAAGAGTGTC | | | | |

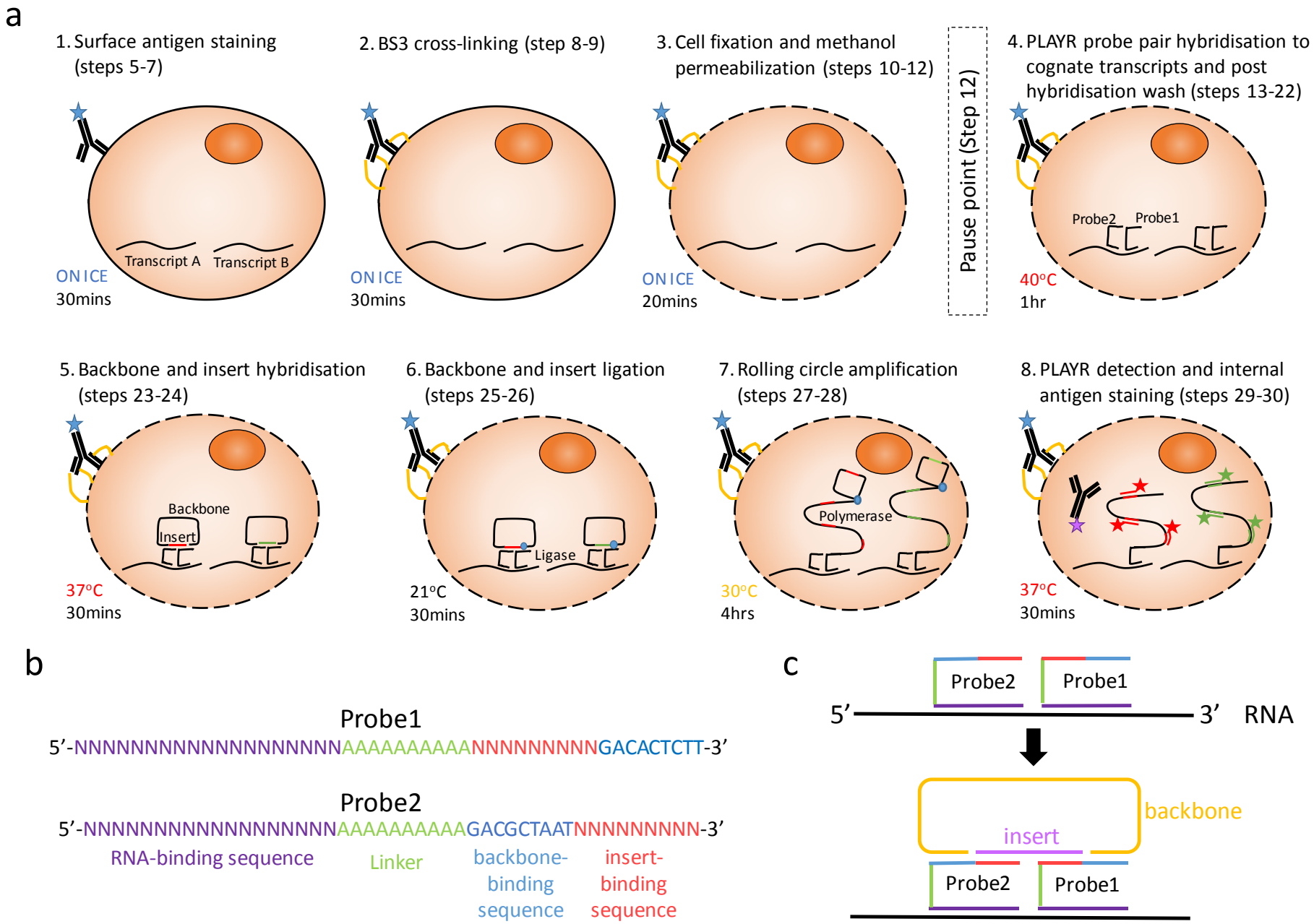
**Table 1 – Oligonucleotide sequences for 27 PLAYR systems**

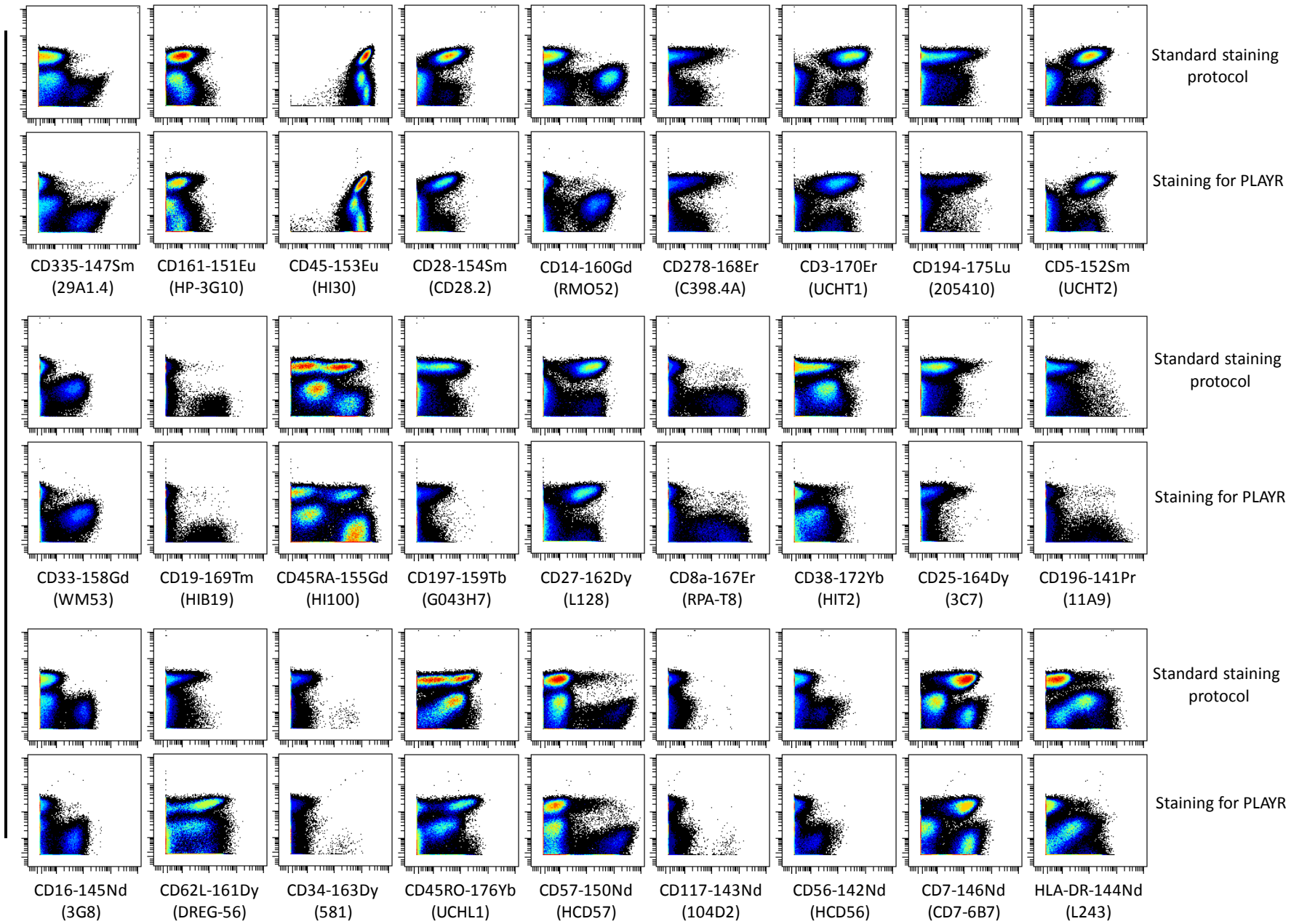
P, 5’-phosphorylated; Z, 5’-C6-thiol modification for mass cytometry or fluorophore modification for flow cytometry.

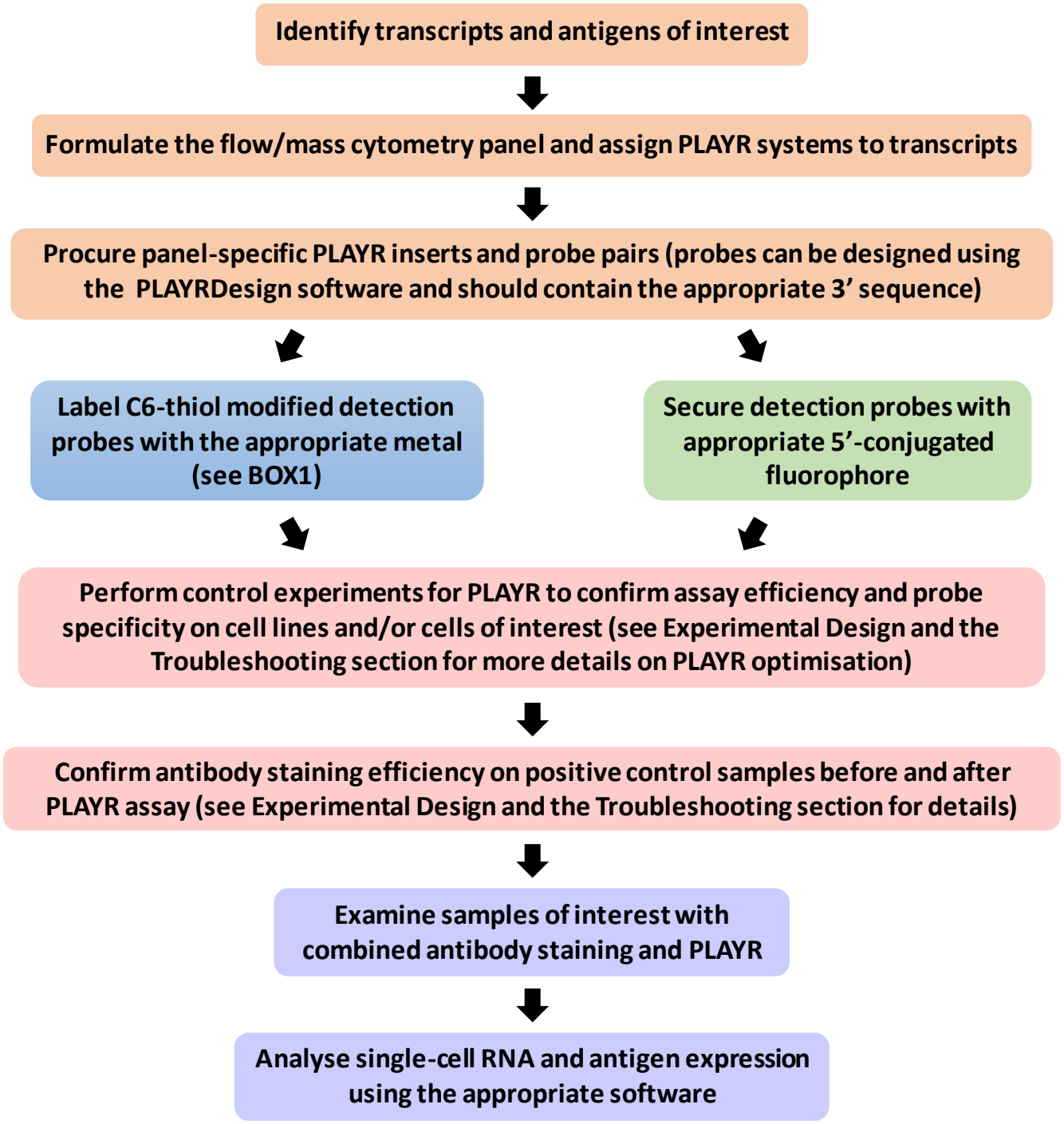
| Step | Problem | Possible Cause | Suggested Solution |
|---|---|---|---|
| 32,36 and BOX1 step30 | Little or no PLAYR signal | RNA is low in cells of interest | Use appropriate positive control cell line |
| | | | Increase number of probe pairs |
| | | | Increase RCA time in step 28 |
| | | RNA degradation by release of internal RNases | Confirm correct production of probe hybridisation buffer |
| | | | Work fast after cell permeabolization with MeOH |
| | | | Keep cells cold at all times during wash steps |
| | | Detection probe isn't labelled efficiently | Run titrated detection probe through the mass cytometer to confirm metal-labelling |
| | | Insert or backbone oligonucleotides are not 5'-phosphorylated | Phosphorylate oligonucleotides with PNK |
| | | Inefficient ligase or Phi29 enzyme activity | Remove all wash buffer from the sample before addition of the enzyme reaction solution |
| | | | Confirm efficiency of the PLAYR system using highly expressed RNA transcript |
| | | | Use new batch of enzymes |
| | Weak antibody staining | Antigen expression is low in target cells | Confirm staining in positive control cells |
| | | Low antibody concentrations | Increase antibody concentrations |
| | | BS3 cross-linker has hydrolysed before contact with cells | Use fresh BS3 cross-linker dissolved just before use |
| | | | Warm tube containing BS3 to RT prior to opening to avoid condensation |
| | | Weak antibody cross-linking and PLAYR conditions are too harsh for the antibody clone | Increase BS3 concentration |
| | | | Wash cells in icPBS to remove all non-cellular proteins from solution |
| | | | Reduce stringency of the PLAYR assay (see protocol optimisation) |
| | High background PLAYR signals | Non-specific probes | Confirm specificity of the probe pairs in silico |
| | | | Test each probe pair individually |
| | | | Increase stringency of the PLAYR assay (temperature, post hybridisation wash, etc) |
| | | Concentration of detection probe is too high | Confirm using a negative control sample such as performing the assay when no ligase enzyme is added to the ligase reaction |
| | | | Titrate detection probe |

| | | | |
|---|---|---|---|
| | High background staining from antibody | Antibody concentrations are too high | Titrate antibody concentrations |
| | Not enough cells for analysis | Not enough cells at the start of the assay | Use a minimum of $3x10^6$ primary leucocytes per sample and increase in necessary |
| | | Loss of cells during assay | Improve pipetting efficiency |
| | | | During repeat washes, leave small quantities of supernatant to avoid the pellet |
| | | | Use swing rotors if not already doing so |

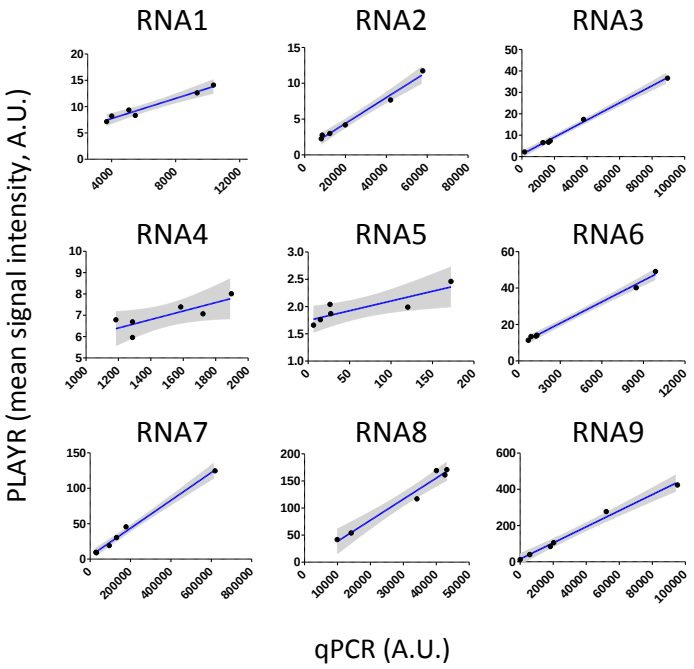
**Table 2 – Troubleshooting**


a
1. Surface antigen staining (steps 5-7)
Transcript A
Transcript B
ON ICE
30mins
2. BS3 cross-linking (step 8-9)
ON ICE
30mins
3. Cell fixation and methanol permeabilization (steps 10-12)
ON ICE
20mins
Pause point (Step 12)
4. PLAYR probe pair hybridisation to cognate transcripts and post hybridisation wash (steps 13-22)
Probe2
Probe1
40°C
1hr
5. Backbone and insert hybridisation (steps 23-24)
Backbone
Insert
37°C
30mins
6. Backbone and insert ligation (steps 25-26)
Ligase
21°C
30mins
7. Rolling circle amplification (steps 27-28)
Polymerase
30°C
4hrs
8. PLAYR detection and internal antigen staining (steps 29-30)
37°C
30mins
b
Probe1
5'-NNNNNNNNNNNNNNNNNNNNAAAAAAAAAANNNNNNNNNGACACTCTT-3'
Probe2
5'-NNNNNNNNNNNNNNNNNNNNAAAAAAAAAAGACGCTAATNNNNNNNNN-3'
RNA-binding sequence
Linker
backbone-binding sequence
insert-binding sequence
c
Probe2
Probe1
5'
3'
RNA
backbone
insert
Probe2
Probe1

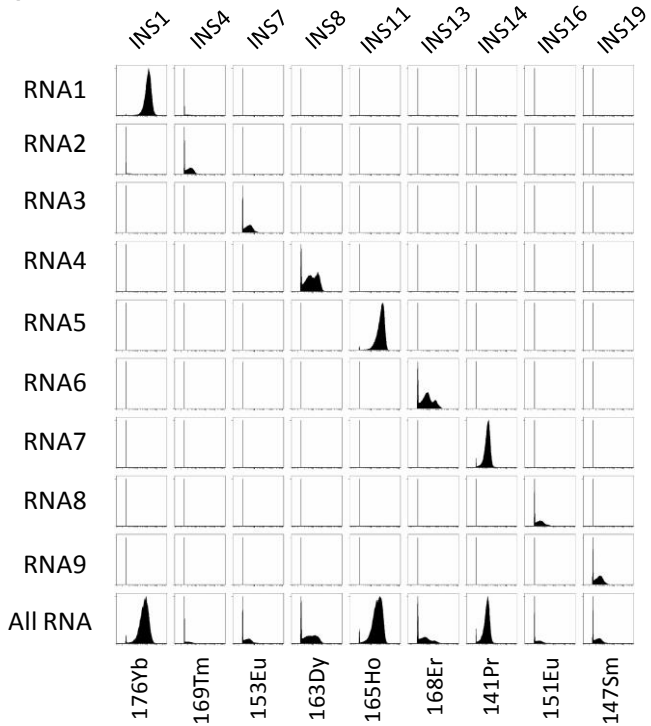

CD4-148Nd (RPA-T4)
Standard staining protocol
Staining for PLAYR
CD335-147Sm (29A1.4)
CD161-151Eu (HP-3G10)
CD45-153Eu (HI30)
CD28-154Sm (CD28.2)
CD14-160Gd (RMO52)
CD278-168Er (C398.4A)
CD3-170Er (UCHT1)
CD194-175Lu (205410)
CD5-152Sm (UCHT2)
CD33-158Gd (WM53)
CD19-169Tm (HIB19)
CD45RA-155Gd (HI100)
CD197-159Tb (G043H7)
CD27-162Dy (L128)
CD8a-167Er (RPA-T8)
CD38-172Yb (HIT2)
CD25-164Dy (3C7)
CD196-141Pr (11A9)
CD16-145Nd (3G8)
CD62L-161Dy (DREG-56)
CD34-163Dy (581)
CD45RO-176Yb (UCHL1)
CD57-150Nd (HCD57)
CD117-143Nd (104D2)
CD56-142Nd (HCD56)
CD7-146Nd (CD7-6B7)
HLA-DR-144Nd (L243)


Identify transcripts and antigens of interest
Formulate the flow/mass cytometry panel and assign PLAYR systems to transcripts
Procure panel-specific PLAYR inserts and probe pairs (probes can be designed using the PLAYRDesign software and should contain the appropriate 3' sequence)
Label C6-thiol modified detection probes with the appropriate metal (see BOX1)
Secure detection probes with appropriate 5'-conjugated fluorophore
Perform control experiments for PLAYR to confirm assay efficiency and probe specificity on cell lines and/or cells of interest (see Experimental Design and the Troubleshooting section for more details on PLAYR optimisation)
Confirm antibody staining efficiency on positive control samples before and after PLAYR assay (see Experimental Design and the Troubleshooting section for details)
Examine samples of interest with combined antibody staining and PLAYR
Analyse single-cell RNA and antigen expression using the appropriate software

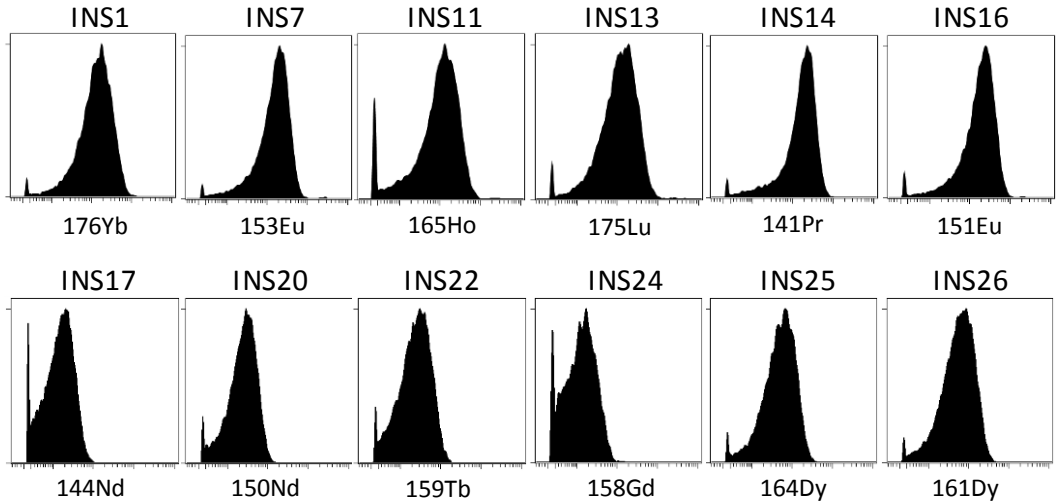
a

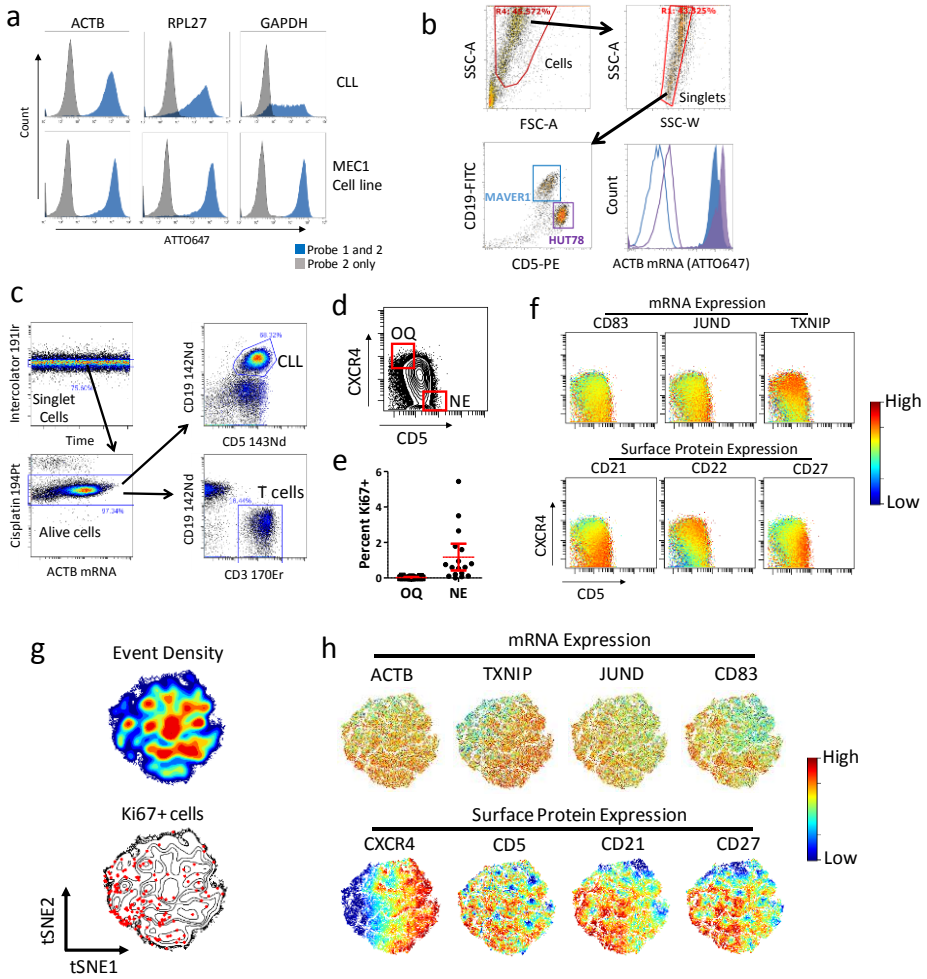

RNA1
RNA2
RNA3
RNA4
RNA5
RNA6
RNA7
RNA8
RNA9
PLAYR (mean signal intensity, A.U.)
qPCR (A.U.)

b


INS1
INS4
INS7
INS8
INS11
INS13
INS14
INS16
INS19
RNA1
RNA2
RNA3
RNA4
RNA5
RNA6
RNA7
RNA8
RNA9
All RNA
176Yb
169Tm
153Eu
163Dy
165Ho
168Er
141Pr
151Eu
147Sm

c


INS1
INS7
INS11
INS13
INS14
INS16
176Yb
153Eu
165Ho
175Lu
141Pr
151Eu
3 pairs of GAPDH detection probes
INS17
INS20
INS22
INS24
INS25
INS26
144Nd
150Nd
159Tb
158Gd
164Dy
161Dy
2 pairs of GAPDH detection probes


a
ACTB
RPL27
GAPDH
CLL
MEC1
Cell line
Count
ATTO647
Probe 1 and 2
Probe 2 only
b
SSC-A
Cells
FSC-A
Singlets
SSC-W
CD19-FITC
MAVER1
HUT78
CD5-PE
Count
ACTB mRNA (ATTO647)
c
Intercolator 191Ir
Singlet Cells
Time
CD19 142Nd
CLL
CD5 143Nd
Cisplatin 194Pt
Alive cells
ACTB mRNA
T cells
CD3 170Er
d
OQ
NE
CXCR4
CD5
e
Percent Ki67+
OQ
NE
f
mRNA Expression
CD83
JUND
TXNIP
High
Low
Surface Protein Expression
CD21
CD22
CD27
CXCR4
CD5
g
Event Density
Ki67+ cells
tSNE2
tSNE1
h
mRNA Expression
ACTB
TXNIP
JUND
CD83
High
Low
Surface Protein Expression
CXCR4
CD5
CD21
CD27