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TITLE:

Isolating Human Peripheral Blood Mononuclear Cell for CD4+ T cells from Sézary Syndrome Patients for Transcriptomic Profiling

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KEYWORDS:

cutaneous T-cell lymphoma, sézary syndrome, peripheral blood mononuclear cell, phorbol-12-myrisate13-acetate, A23187, microarray, progression, neoplasm.

SUMMARY:

We present a simple protocol for the isolation of peripheral blood mononuclear cells from whole blood obtained from patients diagnosed with Sézary Syndrome, followed by selection of CD4+ T cells, their stimulation with phorbol12-myristate13-acetate and A23187 ionophore, and preparation of RNA for transcriptomic profiling.

ABSTRACT:

Cutaneous T-cell lymphomas (CTCL) are derived from the transformation and uncontrolled proliferation of mature skin-homing T cells, and mycosis fungoides (MF) and Sézary syndrome (SS) represent the most common subtypes. Despite a number of studies on characterizing gene expression, genetic alterations, and epigenetic abnormalities of CTCL, the molecular pathogenesis of MF/SS remains unclear. MF refers to the more common CTCL with a skin-predominance, and is usually limited to skin, whereas, SS is an aggressive leukemic variant of CTCL with widespread skin involvement and is characterized by neoplastic distribution mainly involving blood, skin, and lymph node. Focusing on clinical practice, the identification of gene expression biomarkers have enormous potential to improve diagnosis and treatment of MF/SS. Indeed, recent transcriptomic studies have identified potential diagnostic biomarkers from differences in gene expression between normal and malignant T cells, which may improve our understanding of SS biology, and reveal potential therapeutic targets. This manuscript describes a detailed reproducible protocol for the isolation of peripheral blood mononuclear cells from fresh whole blood from patients diagnosed with SS, selection of CD4+ memory T cells (CD4+CD45RO+ T cells) subsets, chemical stimulation, and preparation of RNA suitable for transcriptomic profiling to discover novel prognostic molecular markers to gain additional insight

in disease etiology. The stimulation using chemical agonist to activate nuclear regulation provides more specific assessment for pathways important in the dynamic transcription regulation and gene expression and eliminates confounding defects that may arise from upstream signaling defects arising from TCR antigen loss at the cell membrane. The data obtained from comparison of transcriptome of unstimulated to stimulated SS T cells unmasks functional regulatory gene expression defects not evident from analysis of quiescent unstimulated cells. Furthermore, the method outlined from this approach can be adapted for studying T cell gene expression defects in other T cell immune diseases.

INTRODUCTION:

Cutaneous T-cell lymphoma (CTCL), including the most common subtypes mycosis fungoides (MF) and Sézary syndrome (SS), is a heterogeneous group of diseases derived from transformation and uncontrolled proliferation of mature skin-homing T cells[1,2]. The neoplastic T cells have a mature CD4+Cd45RO+, memory phenotype[3], and express skin homing adhesion markers, increasing epidermotropism[4], which manifests as a rash particularly in early disease. The clinical course of MF is often indolent when under routine managed care, but a subset of patients can progress to more advanced disease. In these MF cases, skin lesions grow and thicken into large tumors, and neoplastic T cells may disseminate to lymph nodes and visceral organs. In contrast, SS is a more aggressive, leukemic variant of CTCL[5], characterized by a triad of symptoms: generalized erythroderma (defined as affecting >80% of total body surface area), lymphadenopathy, and presence of more than 1000/mm^3 circulating clonal atypical T cells with cerebriform nuclei, so-called Sézary cells[6,7]. The prognosis for SS patients is significantly worse than MF. SS is rare with an incidence rate of 0.1/100,000, and represents approximately 3% of total CTCL cases[8,9]. CTCL typically presents in older adults with a median age of about 60 years[10]. The incidence rate for CTCL had been increasing and while the cause is unclear, the rate has stabilized since 1998[11,12].

The molecular pathogenesis of SS remains unclear. Genetic, epigenetic, and gene expression studies have produced a wealth of novel data, however there remain inconsistent variable findings, primarily due to the small patients cohorts studied[2], as well as differences in experimental design and control populations[13,14]. Improved genomic and transcriptomic characterization may shed light on both disease mechanisms and previously unexplored therapeutic targets. Therefore, more studies from a larger patient population are needed to better understand this heterogenous malignancy. Biomarker panels that are highly sensitive and specific in one SS cohort have performed less uniformly in other cohorts[15], which represents a serious obstacle in the development of reliable diagnostic and prognostic biomarkers for SS[16]. Ideal diagnostic biomarkers will be consistently and highly over-expressed in malignant T cells, while absent or nearly absent in normal T cells[17]. The discovery of disease-specific biomarkers is important for the advancement of diagnostic and therapeutic protocols for SS.

High quality transcriptomic data for both malignant and normal T cells requires an efficient and reliable approach to sample preparation. Here, we will discuss a detailed yet simple strategy to obtain RNA samples from T cell populations relevant to SS. We will discuss the isolation of peripheral blood mononuclear cells (PBMC) from whole blood, negative magnetic selection of

disease-relevant CD4+CD45RO+ T cell populations, chemical activation to reveal l differences in functional responses, and preparation of RNA for transcriptomic profiling. In the current protocol, chemical activation has been performed using phorbol myristate acetate (PMA) and calcium ionophore (A23187)[18,19], because previous studies have shown defective T-cell receptor signaling in CTCL, and stimulation with PMA/A23187 bypasses the T-cell receptor[20,21]. Also, PMA/A23187 permits a more direct proximal activation of nuclear signals needed for cytokine gene activation. Finally, stimulation of T cells provides an additional level of insight into the regulation of gene expression that could not be gained from resting T cells where dynamic change is absent.

PROTOCOL:

This research protocol was approved by the Institutional Review Board (IRB) of the University of Arkansas for Medical Sciences (UAMS, Little Rock, AR) The microarray data presented in this study was done on the samples recruited under a research protocol approved by the IRB of Henry Ford Hospital (Detroit, MI). Human cells are potentially infectious. Therefore, the experiments are performed strictly in accordance with the required precautions and procedures discussed as occupational safety and health administration (OSHA) and personal protection equipment (PPE).

1. Isolation of PBMCs from whole blood

1.1. Collect all the materials needed from **Table 1** and bring them to room temperature (RT). Warm RP10F to 37 °C. Adjust centrifuge to RT. Except for centrifugations and cell counting, perform all the steps using viable cells in a biological safety cabinet.

1.2. Obtain blood in five 10 mL tubes (desired amount) containing anticoagulant. Store the whole blood at ambient temperature (18–24 °C). Label 50 mL separation tubes with the human research subject number for the blood sample to be processed.

1.3. Transfer 10–15 mL of blood into each separation tube(s) with the matching subject number. Dilute the blood at least 2 fold with Hank's balanced salt solution (HBSS). Do not exceed 35 mL of diluted blood per tube.

1.4. Carefully and slowly underlay the blood with ~13 mL of density medium. Watch through the transparent density medium at the bottom of the tube, and stop pipetting when the pipette is almost empty (to prevent bubble release). Carefully remove the pipette to avoid mixing the blood and density medium layers.

1.5. Carefully transfer the filled separation tubes to the centrifuge without disturbing the layers.

1.6. Centrifuge at 500 x *g* for 30 min with the centrifuge brake off (deceleration set to zero).

NOTE: If the centrifuge only display rpm, consult rotor specifications to estimate the rpm equivalent for 500 x *g*.

1.7. Carefully remove separation tubes from the centrifuge without disturbing the layers. Observe the buffy coat, which has been formed in between the density medium and plasma layers.

1.8. Pipette from the top to remove and discard most of the upper plasma fraction, so that 10 mL remains above the buffy coat. Carefully and slowly collect the buffy coat. Transfer the buffy coats from two separation tubes into a new pre-labeled and sterile 50 mL tube, as shown in **Figure 1**.

1.9. Dilute the PBMCs at least 2x with HBSS, bringing the volume in each new tube up to 50 mL. Remember to switch the centrifuge brake to full. Pellet PBMCs by centrifugation at 400 x *g* for 10 min. Remove the supernatant as much as possible and tap the bottom of the tube to loosen the pellet.

1.10. To lyse residual red blood cells (RBC), resuspend each cell pellet in 1–2 mL of ammonium-chloride-potassium (ACK) lysis buffer per 10 mL original blood volume. Incubate for exactly 5 min. Promptly stop lysis with an equal or greater volume of HBSS and adjust volume to 50 mL. Centrifuge at 400 x *g* for 10 min.

1.11. Remove the supernatant and tap the bottom of tube to loosen the cell pellet. Pool cells from the same donor. Bring volume up to 50 mL with HBSS. Centrifuge at 400 x *g* for 10 min.

1.12. Remove the supernatant and tap the bottom of tube to loosen the cell pellet. Resuspend cells in 10 mL of warm RP10F medium, and take an aliquot for viable cell counting using trypan blue.

1.13. Calculate the total cell number in each sample using a hemocytometer.

2. Purification of CD4+CD45RO+ T cells from PBMCs

NOTE: Purification of CD4+CD45RO+ T cells from PBMCs is done by using commercially available magnetic separation (see **Table of materials**) with minor modifications. It is preferred to follow the kit manual for incubation time as each commercial kit has their own instructions.

2.1. Wash the desired quantity of PBMCs in 10 mL of selection buffer. Centrifuge at 400 x *g* for 10 min. Remove the supernatant and tap the bottom of the tube to loosen the pellet.

2.2. Dilute PBMCs to 5 x 10^7 cells/mL in selection buffer and transfer them to a 5 mL of polystyrene round-bottom tube (12 x 75 mm).

2.3. Add 50 µL of antibody cocktail per 1 mL of sample, and mix gently. Incubate at room temperature for 5 min.

2.4. Immediately before use, vortex magnetic particles for 30 s on high speed. Add 50 µL of magnetic particles per 1 mL of sample to the tube containing PBMCs, and mix gently.

2.5. Bring the volume up to 2.5 mL with selection buffer and mix gently. Place the tube (without lid) into the magnet, and incubate at RT for 2.5 min.

2.6. Pick up the magnet, and in a continuous motion, invert the magnet and tube to pour the enriched cell suspension into a new sterile tube.

2.7. To increase recovery, add 2.5 mL of selection buffer to the tube remaining in the magnet, without disturbing the immobilized beads. Keep in the magnet for another 2.5 min, and repeat step 2.6 to recover additional cells.

2.8. Take an aliquot for viable cell counting using trypan blue. Calculate the total cell number using a hemocytometer or cell counter.

2.9. Confirm the purity by flow cytometry (**Figure 2**, **Figure 3**).

3. Chemical activation

3.1. Adjust CD4+CD45RO+ T cells to 5 x 10^6 cells/mL with warm RP10F medium, and distribute cells into culture dishes of the desired size. Rest cells in a humidified 37 °C 5% CO_2 incubator overnight.

3.2. Pellet rested cells by centrifugation at 400 x *g* for 10 min. Remove the supernatant and tap the bottom of tube to loosen the cell pellet.

3.3. Adjust cell concentration to 5 x 10^6 cells/mL with warm RP10F medium, and distribute 0.5–1 x 10^7 cells into each of three sterile, screw-cap tubes.

NOTE: If sufficient cells are available, duplicate stimulations or additional time points may be included in the experimental design.

3.4. Stimulate cells in tubes 2 and 3 with PMA and A23187. Add PMA to 25 ng/mL and A23187 to 500 ng/mL and mix gently. Add an equal volume of dimethyl sulfoxide (DMSO) to the cells in tube 1 that will serve as a vehicle (control). For instance, if using 1 µL of PMA and 1 µL of A23187, then add 2 µL of DMSO to the vehicle tube. Keep the final concentration of DMSO below 0.5% in all tubes.

3.5. Loosen the caps on the tubes, and return cells to the 37 °C 5% CO_2 incubator for 2 h (tube 2) and 6 h (tubes 1 and 3). At the indicated time, centrifuge cells at 500 x *g* for 10 min.

3.6. Prior to lysis, discard as much of the supernatant as possible, without disturbing the cell pellet.

3.7. Promptly lyse the cells, as directed by the as directed by the commercially available RNA isolation kit (see **Table of Materials**). Proceed with RNA isolation, or freeze the lysate at -80 °C to process later with additional samples.

NOTE: RNA purity and integrity may be verified using a microcapillary electrophoresis.

3.8. Optional: To completely remove all traces of DNA from the purified RNA sample, use RNA clean up kit (see **Table of Materials**), according to manufacturer's instructions.

REPRESENTATIVE RESULTS:

This protocol includes procedures for the isolation of PBMCs from SS blood, purification of CD4+CD45RO+ T cells by negative selection and stimulation of purified T cells, and isolation of total RNA for transcriptomic profiling. **Figure 1** describes the process of PBMC isolation from whole blood. Please note that the total yield of SS PBMCs will vary with starting blood volume and circulating tumor burden of each patient. In our laboratory, the average yield of SS PBMCs was 4.6×10^6 cells/mL of whole blood ($1.85 \times 10^6 – 3.25 \times 10^7$ cells/mL for 7 SS). The mean viability of isolated PBMCs was 95–99%. **Figure 2** shows high purity and viability of selected CD4+CD45RO+ memory T cells. The yield of CD4+CD45RO+ T cells from SS PBMCs was 75% (75.6% – 84%), compared to 15.9% (3% – 30%) from normal donors (ND) PBMCs obtained from leukoreduction system (LRS) chambers. The viability and purity of CD4+CD45RO+ T cells obtained by this negative selection protocol has been consistently high (**Figure 3**).

We previously combined the activation protocol above with microarrays to study the functional changes in the transcriptomes of both SS and ND T cells, and have demonstrated that SS memory T cells and SS PBMCs poorly express cytokine and other immune response genes compared to cells from ND T cells and PBMCs[19,22,23]. **Figure 4** shows the robust activation of several cytokine genes including *IL4*, *IL 10*, *IL13* and *IL22* in ND T cells, but not in SS T cells. This gene expression defects in functional gene expression in SS T cells have since been confirmed by other groups[24]. In addition, many genes not normally expressed in ND T cells are highly expressed in SS T cells, both at rest and following stimulation (**Figure 4**). These include the previously described SS biomarker genes *DNM3, PLS3*, *TOX* and *TWIST1*[25-27], as well as *ANK1* and *SGCE*, which were first reported by our group. These positive biomarkers are highly and mostly expressed in SS, but not ND, and avoid technical pitfalls associated with negative biomarkers.

FIGURE AND TABLE LEGENDS:

Figure 1: PBMC isolation from whole blood.

Figure 2: Negative selection of CD4+ memory T cells from isolated PBMCs.

Figure 3: Purity of CD4+CD45RO+ T cells was confirmed by flow cytometry. Lymphocytes were gated by light scatter (**A**), live lymphocytes excluded eFluor780 viability dye (**B**), and (**C**) represents non-selected normal donors (ND). Negative selection resulted in nearly pure populations of CD45RO+ T cells in ND (**D**) and SS patients (**E**).

Figure 4: Differential gene expression in resting and activated CD4+CD45RO+ memory T cells from SS and ND. Gene expression z-score is represented by a color scale from red (high expression) to green (low expression). Colored bars at the top of the heat map represents cell treatments: mock/vehicle treated (red), 2 h stimulated (blue), and 6 h stimulated (yellow). Several SS biomarker genes are highly expressed and cytokine genes are poorly expressed in SS T cells compared to ND T cells.

Table 1: Reagents.

DISCUSSION:

Several ways of isolating PBMCs have been developed, and each has their own advantages and limitations[28]. We routinely collect up to 50 mL of blood in five 10 mL tubes containing anticoagulant. The volume of the blood for PBMC isolation depends on several factors such as health and age of the research subject and also on phlebotomist expertise. A critical procedural step in the protocol is the formation of the step gradient. Poor layering may result in partial or complete failure of PBMCs to sediment at the interface. We prefer the under-layering method described here, as it is easy to start the bottom layer. To completely dispense all of the density medium below the blood, it is critical to use a pipet aid with no air leaks. Contamination of the PBMC fraction by undesired cell types can be minimized by careful and consistent collection of the buffy coat, which should be performed in the same way for each isolation. If PBMCs will not be further fractionated, collecting different amounts of the density gradiesnt and plasma layers between isolations should be avoided. RBC lysis is performed to minimize the potential impact of contaminating RBC- and reticulocyte-derived RNA on downstream gene expression analyses. Hypotonic lysis will be inhibited by excess isotonic was buffer.

Further isolation of T cell subset is important for molecular studies. Here we described subsequent CD4+CD45RO+ T cells selection by negative selection to remove undesired cell types. Negative selection relies on antibodies recognizing specific cell surface markers for all undesired cells. Antibody coated cells are then removed by magnetic beads. This selection protocol removes unwanted cells while allowing untouched and unstimulated target cells, which is essential in studying gene activation, to flow freely pass the magnetic beads. However, care must be taken to avoid cell clumps, which will occlude the flow past the beads, and reduce the final purity of selected CD4+ CD45RO+ T cells. Ethylenediaminetetraacetic acid (EDTA) present in the selection buffer minimizes cell clumping. Yield of T cells depends on factors such as initial volume of the blood, patients variable such as the treatment being administered to the patient and disease stage at the time of sample collection. Treatment given to the patients may also affect the cell viability. In addition, sample collection before any procedure such as photopheresis also have positive impact on CD4+CD45RO+ T cells purity. We have observed that sample collection after photopheresis treatment procedure has negative impact on CD4+CD45RO+ T cells yield.

Neoplastic T cell clones from SS patients most frequently express surface markers consistent with a mature, memory CD4 T cell phenotype[29,30]. However, phenotypic plasticity has been occasionally observed with respect to surface markers including CD4, CD45RO, CD45RA, CD7 and/or CD26[31]. Previous studies have also shown the heterogeneity in CD45RO and CD45RA expression among SS patients[29]. Moreover, this heterogeneity is not significant as shown in the literature where majority of SS cases are still CD45RO+. Roelens et al.[31] also showed that SS may exhibit interindividual and intraindividual heterogeneity with mixed population of naïve (TN), TCM, transitional memory (TTM), effector memory (TEM), and terminal effector memory (TEMRA) subsets. However, their results clearly show that majority of the SS cells has TCM phenotype. We focused our study on the surface immunophenotype of the most common SS patients, and confirmed phenotype by flow cytometry. In planning studies of T cell subsets in patients, it is important to consider phenotypic heterogeneity of the disease being studied, and purification strategy may therefore be adjusted as needed to obtain the desired T cell population for analysis.

There are several ways to stimulate T cells and PBMCs to examine functional gene expression. We prefer chemical activation (PMA + A23187 ionophore), since we are interested in gene regulation in the nucleus. Chemical activation is a best option for this purpose because it acts as a broad activator and is more uniformed compared to antigen specific stimulation. PMA is a small organic compound that diffuses through the cell membrane into the cytoplasm, and directly activates protein kinase C. A23187 allows calcium to pass through membranes. These compounds bypass surface receptors, and together mimic the effects of T cell receptor ligation with co-stimulation mediated by CD28. The chemicals activate several intracellular signaling pathways, resulting in nuclear transcription factor activation and upregulation of cytokines in genes that are accessible to transcription activation. Although, chemical activation and CD3CD28 ligation produce strikingly similar global gene expression profiles in normal cells[32], chemical activation with PMA + A23187 is a good choice since SS T cells can lose expression of surface receptors including TCR components components[33]. Chong et al.[22] compared the activation of cytokine gene between PMA/A23187 to anti-CD3 and anti-CD28 antibodies in PBMCs from normal, early MF/CTCL, and late MF/CTCL patients. They report that PMA/A23187 caused more rapid and intense activation of the *IL-2* gene as compared to anti-CD3/CD28 stimulation. Additionally, they showed that the slower activation kinetics with anti-CD3/CD28 antibodies is potentially from cross-linking and membrane signaling necessary for stimulation. Furthermore, trends in expression of cytokines among the different cell populations studied were preserved with PMA/A23187. Since, we are interested in gene expression activation, chemical stimulation is an ideal approach because it acts as a broad activator and is more consistent compared to antigen specific stimulation. CD3/CD28 ligation is ideal to investigate pathways important in membrane based signal transduction. In addition, chemical activation is less expensive and does not require special equipment. In the current study, PMA + A23187 significantly activated cytokine genes in ND but not SS T cells, suggesting that SS T cells have functional nuclear deficiencies downstream of the TCR.

In summary, this protocol provides phenotypically pure T cells from precious patient-derived blood, and a method for assessing genome-wide changes in functional gene expression. We demonstrate that transcriptomic profiling of SS T cells compared to normal CD45+RO+ T cells reveal profound differences in gene activation in fresh human T cells from patients with CTCL. These studies will aid the development of diagnostic biomarkers and therapeutic strategies targeting novel markers in CTCL. In addition, this strategy and protocol in studying primary human T cells may be valuable in adapting to studies of other T cell mediated diseases.

Ethical Disclosure:
This research protocol was approved by the Institutional Review Board (IRB) of the University of Arkansas for Medical Sciences (UAMS, Little Rock, AR) The microarray data presented in this study was done on the samples recruited under a research protocol approved by the IRB of Henry Ford Hospital (Detroit, MI).

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

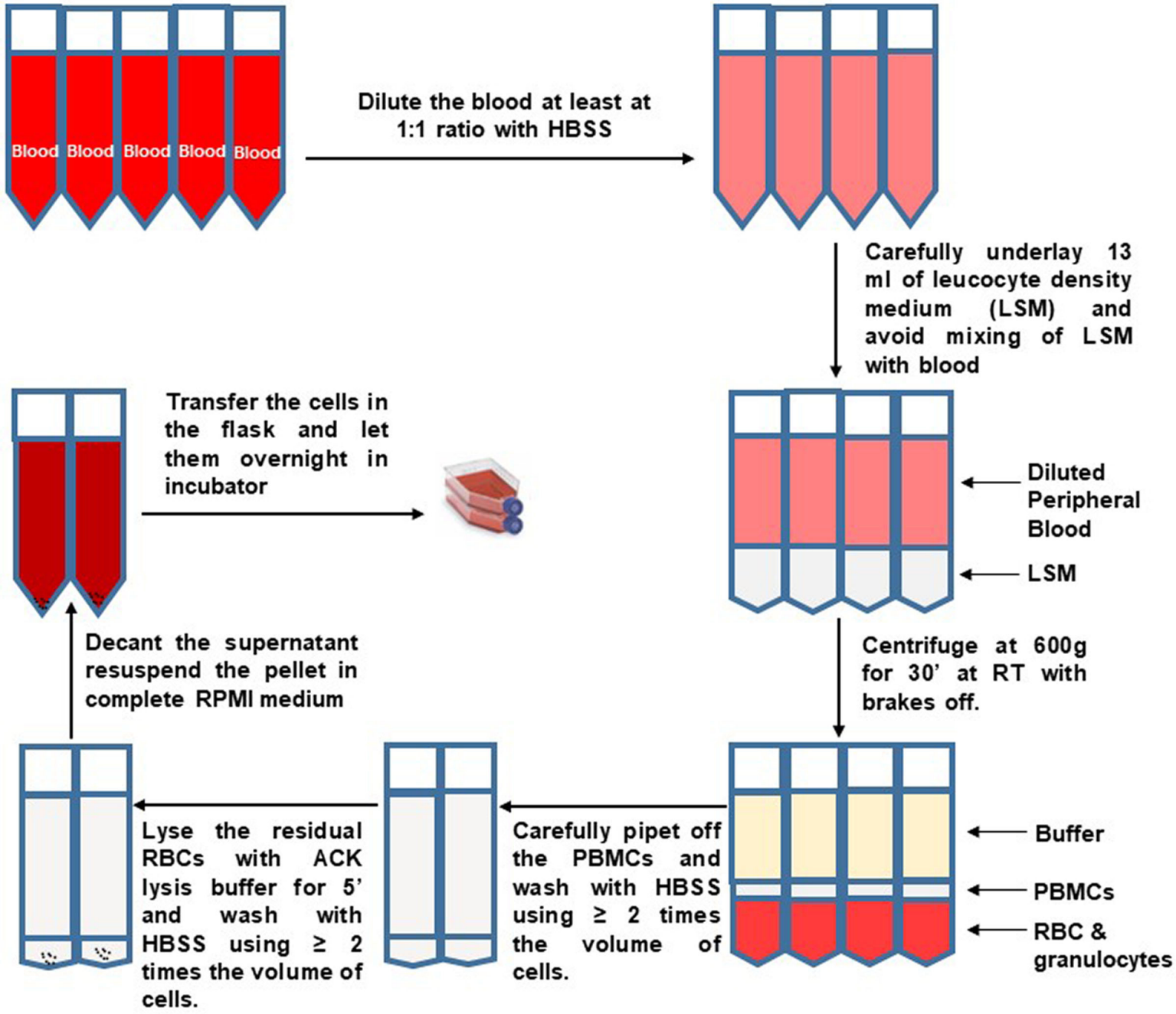

Figure 2

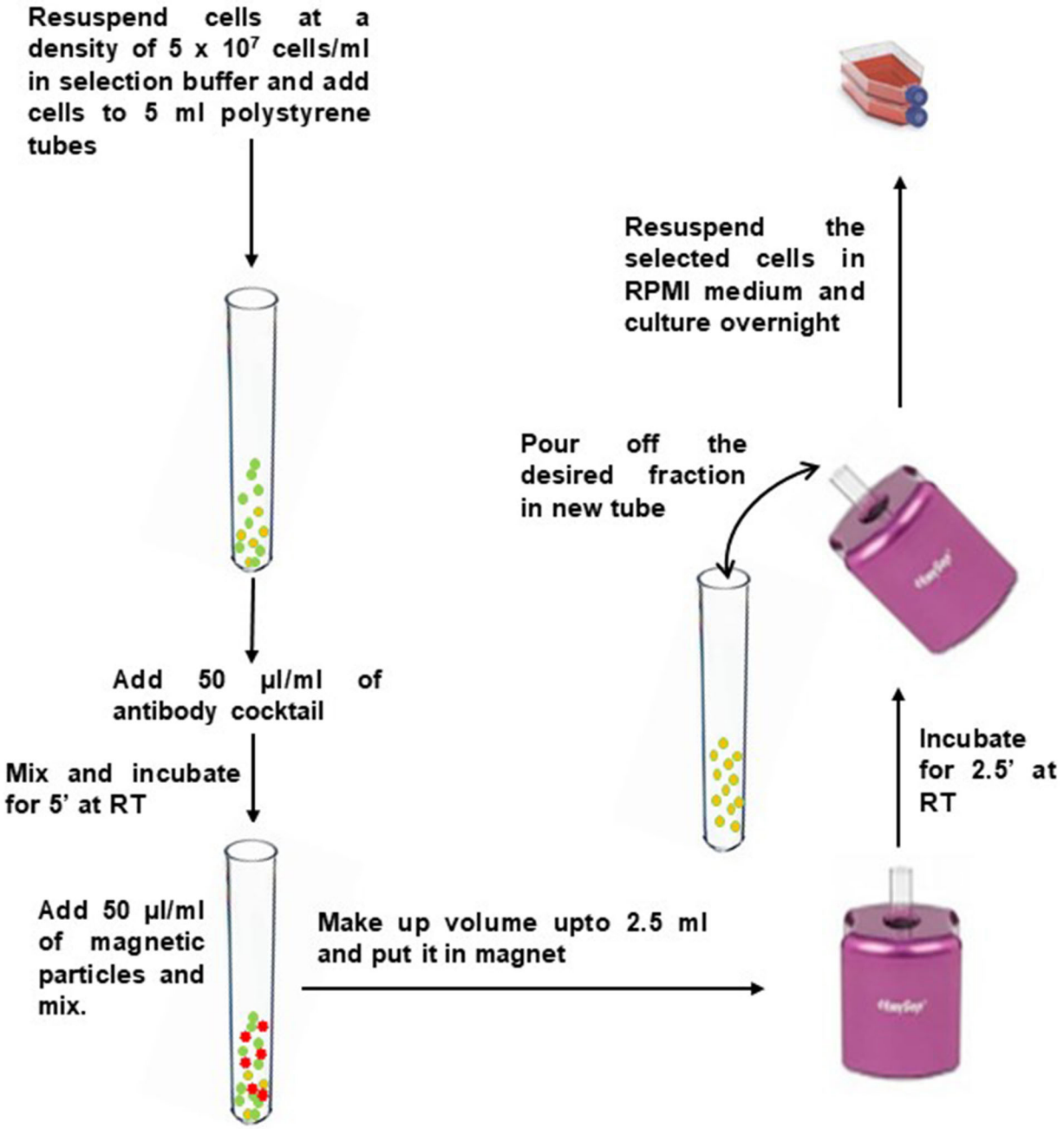

Figure 3

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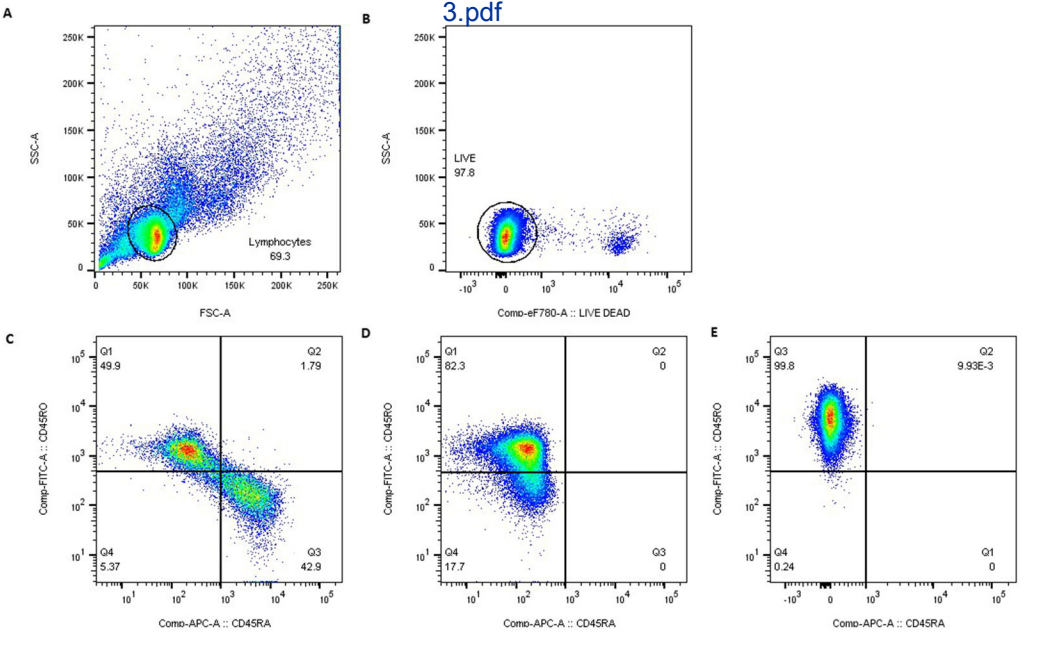

Figure 4

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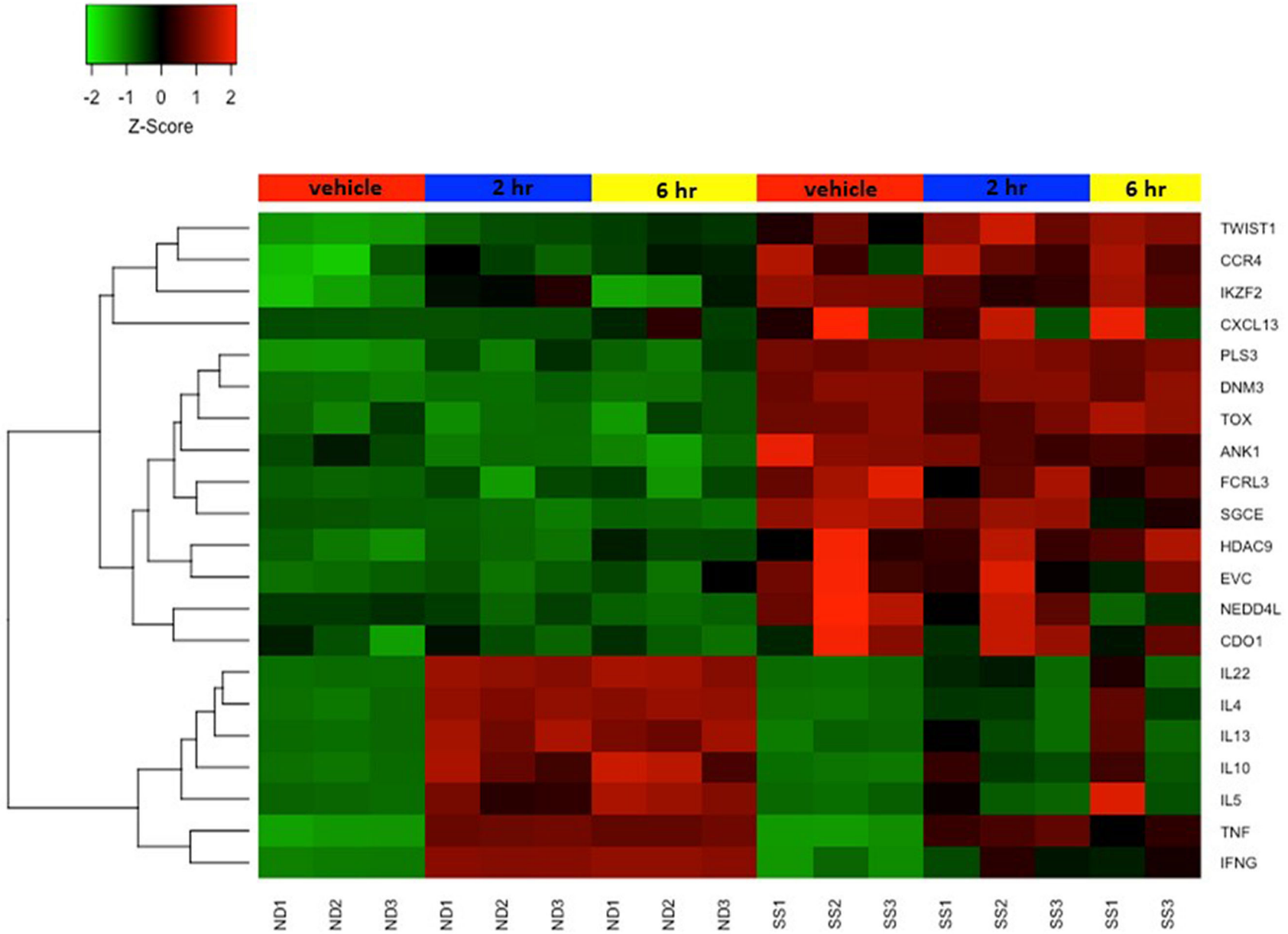

Reagents

Density Medium
HBSS
RP10F
ACK lysis buffer
Selection buffer
phorbol12-myristate13-acetate (PMA)
A23187 ionophore

Lymphocyte Separation Medium, Ficoll-Hypaque, or equivalent density medium with density = 1.077-1.080g/ml at 20^{o}C
1x Hank's balanced salt solution, 4.2 mM $NaHCO_3$, 10 mM HEPES, pH 7.2
RPMI 1640 medium, 10 % heat inactivated fetal bovine serum (FBS), 1x penicillin-streptomycin
155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, No need to adjust pH. It should be ~ 7.3.
1x HBSS, 2% FBS, 2 mM EDTA
50 µg/ml in DMSO
500 µg/ml in DMSO

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 ml microcentrifuge tubes	Fisher Scientific	02-681-320	
10 ml Disposable Plastic Pipette	Thermo Scientific	170356	
1000 ul Pipet tips	VWR	10017-038	
15 ml Conical Tubes	Corning	352196	
25 ml Disposable Plastic Pipette	Thermo Scientific	170357	
5 ml Disposable Plastic Pipette	Thermo Scientific	170355	
50 ml Conical Tubes	Thermo Scientific	339652	
A23187 ionophore	Fisher Scientific	BP595	
Centrifuge	Thermo Scientific	75004381	
DMSO	Sigma	D2650-5x5ml	
EasySep Human Memory CD4+ T cell Enrichment Kit	StemCell	19157	
FBS	GIBCO	16140-071	
HBSS	GIBCO	14185-052	
HEPES	Fisher Bioreagents	BP310-500	
KHCO3	Fisher Bioreagents	P184-500	
Lymphocyte Separation Medium	Corning	25-072-CV	
Na2EDTA	ACROS	10378-23-1	
NaHCO3	Fisher Bioreagents	S233-500	
NH4Cl	Fisher Bioreagents	A661-500	
Penicillin-streptomycin solution	GIBCO	15140122	
phorbol12-myristate13-acetate (PMA)	Sigma	P-8139	
Pipet-Lite LTS Pipette L-1000XLS+	RAININ	17014382	
Pipet-Lite LTS Pipette L-10XLS+	RAININ	17014388	
Pipet-Lite LTS Pipette L-200XLS+	RAININ	17014391	
Pipet-Lite LTS Pipette L-20XLS+	RAININ	17014392	
RNA Clean & Concentrator-5	Zymo Research	R1013	
Rneasy Plus Mini Kit	Qiagen	74136	
RPMI 1640	GIBCO	31800-022	
T-25 Flask	Thermo Scientific	2024-10	

- We sincerely thank the reviewers for their time and valuable suggestions that helped us to improve the manuscript.

- We also made some additional edits beyond those requested by the reviewers to correct mistakes or formatting inconsistencies, which we did not notice before the first submission. For example, Figure 3 has been modified.

- We have replaced the reference number 15 (Kohnken, R. et al. Sézary Syndrome: Clinical and Biological Aspects. Current Hematological Malignancy Reports. 11, 468-479 (2016)), which is now Reference no. 17 with Dulmage, B. et al. The biomarker landscape in mycosis fungoides and Sézary syndrome. Experimental Dermatology. 26 (8), 668- 676 (2017).

- All the changes are tracked.

Editorial Comments:

- Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
  - ✓ The manuscript was thoroughly read to assure no spelling and grammatical errors.

- Protocol Language: Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible. 1) Lines 84-90 should be a note. Please renumber your steps accordingly.
  - ✓ All the text in the protocol section has been written in the imperative tense, as needed. Some of the notes, which cannot be written in the imperative tense, have been moved to discussion section between lines 336-339, 347-349 and 358-359. Notes have been provided where it is needed in the protocol. Part of the lines 84-90 have been moved to under heading "Protocol" between lines 115-117. Line 88-90 have been written as a step 1.1 and 1.2.

- Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please ensure all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
  - ✓ Every required details has been written as a step in the protocol so that it can easily be replicated.

- Protocol Numbering: There must be a one-line space between each protocol step.
  - ✓ Protocol numbering has been updated and one-line space has been inserted between each protocol step.

- Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
    - ✓ 2.5 pages including the headings and spaces have been highlighted.

    1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
        - ✓ All the relevant details required to perform the step have been highlighted and the necessary sub-steps are also included in the highlighted steps for filming.

    2) Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
        - ✓ Shorter protocol steps have been merged with not more than 4 sentences per step.

    2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
        - ✓ Highlighted steps form a logical flow and are in continuation to their previous steps.

    3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
        - ✓ Complete sentences including sub-headings and spaces have been highlighted.

    4) Notes cannot be filmed and should be excluded from highlighting.
        - ✓ Most of the notes have removed from the protocol steps. There is only one note remaining in the protocol, which is not highlighted.

- Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
    - ✓ The suggested change has been made. Discussion has been modified based on the critical steps, limitations, modifications and troubleshootings. Changes can been seen between line 336-339,347-349,356-365and 392-405.

- Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the

mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are vacutainer, EasySepTM, e RNeasy Plus Mini kit (Qiagen, (Zymo Research). 1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

✓ All the commercial language has been modified to generic names of the materials/reagents, as per suggestion.

- If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

  ✓ Figures and tables in the manuscript are original.

Comments from Peer-Reviewers:

Reviewer #1:

General comments: The manuscript describes in detail a useful to isolate and study malignant T cells from Sezary Syndrome patients. This protocol would be used by scientists since it provides adequate information and is very targeted, useful and specific for clinician scientists, who wish to study this deadly disease.

Specific comments:

1. Throughout the protocol and in the tables please spell out all acronyms at least once e.g., HBBS -- Hanks' balanced salt solution; Affymetrix HG (human Genome), ACK, EDTA, DMSO, RT -- room temperature; PMA -- phorbol myristate acetate, ND -- normal donor cells, etc. Please do this for every abbreviation or chemical name used.

   ✓ Thank you for alerting us to this oversight. The suggested edits were made.

2. Please specify "T" cells every time you are referring to CD4+ o CD4+CD45RO+ cells. Please specify "T" cells throughout the manuscript/figures.

   ✓ Thank you for the suggestion. Necessary changes have been made.

3. The authors clarify that many T cells loose classic T cell markers including CD3 -- please in the discussion elaborate that this is the reason why CD3+ CD28+ Dynabeads -- may not be optimal to activate Sezary cells.

- ✓ Thank you for the comment. The discussion section has been updated and the required changes have been made between line 383-405.

4. Please specify that another name for A23187 is Ionomycin.
   - ✓ Thank you for the comment. A23187 is an ionophore but is not Ionomycin. Ionomycin and A23187 (Calcimycin) are two different compounds with formula weight 747.06 and 523.6, respectively that can both increase intracellular calcium.

5. Please change 37oC Co2 --> 37 oC 5% CO2 throughout the paper.
   - ✓ Thank you for highlighting this oversight. It has been corrected.

6. in 3.15 should be 10 ml "of" selection media.
   - ✓ The suggested edit was made.

Reviewer #2:
General Comments: This is a methodological paper describing in details a method of Sézary cells purification and activation, although it is not novel. Isolation of PBMCs from whole blood and CD4/CD45RO cells purification are widely used in many laboratories. Also the method of T cell activation has been already 40 years ago by Pick et al: "Intracellular mediation of lymphokine action: mimicry of migration inhibitory factor (MIF) action by phorbol myristate acetate (PMA) and the ionophore A23187." Ann N Y Acad Sci. 1979;332:378-94. The first author Wong used this method in his paper "Induced Sézary Syndrome PBMCs poorly express immuneresponse genes up-regulated in stimulated memory T cells. J Dermatol Sci. 2010 October ; 60(1): 8-20. Those papers should been cited in the current typescript.

- ✓ Thank you for the comments. Suggested references have been added to the manuscript (Reference: 18, 19).

Specific comments:
Rows: 28-40. The abstract consists almost exclusively of the introduction part, and only the lasts sentence reports the current invention. Since the same information is repeated in the introduction it should be removed from the abstract and substituted by the description of the new method.

- ✓ We thank the reviewer for this helpful suggestion. The suggested changes have been made and the abstract has been modified.

Reviewer #3:
Specific comments:
Major:

1. The original technique may be of intestest for researchers who want to perform either functional or molecular analyses of Sezary syndrome cells (SC) such as genomic profiling. However as evidenced by several authours (Roelens et al for example); SC may exhibit interindividual or intraindividual heterogeneity or plasticity. Gating and selecting SC cells on CD4 and CD45RO will only be suitable for memory cells and therefore not adapted for other SC displaying a CD45RA or CD45ROneg or low phenotype. This should be definitively addressed. The authors could indicate how many SS cases were indeed found

CD45RA or CD45RO positive in their experience and refer to the fact that SC cells phenotype and maturation stage seems to be heterogeneous.

- ✓ We appreciate reviewer's comment. It is reported in several studies that SS cells do have heterogeneity and express CD45RA and CD45RO population or both. However, same studies have shown that the TCM is the characteristic feature of SS cells with rare SS population that is CD45RA+. Based on this, the discussion section has been modified (Line 367-379; Reference: 29-31).

2. In the Roelens paper (Blood 2017) up to 70% of SC displayed a CD45RO negative phenotype (should be discussed page 3 line 75).
   - ✓ Thank you for the comments. We reviewed the mentioned paper and the modification has been added to the discussion (Line 372-375; Reference: 31).

Minor:

1. Line 139 to 141: It could be important to check by flow cytometry that the protocol permits to recover SC with the original CD4+CD45RO+ phenotype. Did the authors check this point and also how many living cells they obtain as indicated by Trypan blue line 161.
   - ✓ We thank reviewer for his concern. However, it is mentioned in the protocol to confirm the purity with flow cytometer as we have done (Line 248; Figure 3).

2. Line 178 and below: What is the rationale for the chemical stimulation by PMA ? If the authors want to characterize SC expression profile they may introduce important bias in stimulating the proliferation of cells that may be at a relative quiescent stage. As discussed line 256 costimulation by antiCD3/antiCD28 seems more appropriate and could have been compared.
   - ✓ Thank you for the nice comment. We are more interested in gene activity in the nucleus. Therefore, chemical activation is a best option. However, the reason for not using costimulation by antiCD3/antiCD28 in the present study is mentioned in the discussion section (Line 391-405) (Reference: 18-21).