



Research paper

Development of an RNA-based kit for easy generation of TCR-engineered lymphocytes to control T-cell assay performance



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ABSTRACT

Cell-based assays to monitor antigen-specific T-cell responses are characterized by their high complexity and should be conducted under controlled conditions to lower multiple possible sources of assay variation. However, the lack of standard reagents makes it difficult to directly compare results generated in one lab over time and across institutions. Therefore TCR-engineered reference samples (TERS) that contain a defined number of antigen-specific T cells and continuously deliver stable results are urgently needed. We successfully established a simple and robust TERS technology that constitutes a useful tool to overcome this issue for commonly used T-cell immuno-assays. To enable users to generate large-scale TERS, on-site using the most commonly used electroporation (EP) devices, an RNA-based kit approach, providing stable TCR mRNA and an optimized manufacturing protocol were established. In preparation for the release of this immuno-control kit, we established optimal EP conditions on six devices and initiated an extended RNA stability study. Furthermore, we coordinated on-site production of TERS with 4 participants. Finally, a proficiency panel was organized to test the unsupervised production of TERS at different laboratories using the kit approach.

The results obtained show the feasibility and robustness of the kit approach for versatile in-house production of cellular control samples.

1. Introduction

Antigen-specific T cells are key players in the clinical course of cancer (Lu et al., 2014; Robbins et al., 2015; Rizvi et al., 2015), infections (Riou et al., 2014; Ottenhoff and Kaufmann, 2012) and inflammatory diseases (Kleijwegt and Roep, 2013). T-cell immunity also clinically impacts on the efficacy of in vivo gene transfer (Britten et al., 2013), and the success of organ transplantation (Abreu and Roep, 2013). As a consequence, the number of laboratories that assess antigen-specific T-cell responses in biomarker programs accompanying the development of innovative therapies has increased over the years.

Simultaneously, T-cell assays have matured from a technology mainly utilized in the context of basic or exploratory immunology research into a tool to guide drug development and clinical research in patients. This shift of scope has led to increased quality requirements for assay planning, performance and analysis (Britten et al., 2012). In particular, assay harmonization, standardization, validation and/or quality assurance has become the focus of many research efforts (van der Burg et al., 2011; Janetzki and Britten, 2012; Maecker et al., 2012). The importance of implementing suitable “control reagents” to verify protocols, reagents, instrument setup and assay analysis, as well as the need for control samples to continuously monitor assay performance,

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was emphasized by the FOCIS Human Immunophenotyping Consortium in 2010 (Maecker et al., 2010). Our current study introduces a novel kit-based approach to allow for the generation of versatile control reagents by electroporating RNA-encoded TCRs into primary lymphocytes.

The Cancer Immunotherapy Immunoguiding Program (CIP) has recently developed structured research efforts to engineer novel control reagents that contain a defined number of functionally characterized antigen-specific T cells. The group initially used lentiviral gene-transfer to generate TCR-engineered references samples (TERS) (Singh et al., 2013).

Following these proof-of-principle studies, we developed and reported on an alternative method for the generation of TERS, where we replaced lentiviral gene-transfer by RNA electroporation. This modification was introduced to overcome the limitations associated with the need to culture and to sort stably-transduced lymphocytes, and to increase safety measures associated with the production and shipment of TERS (Bidmon et al., 2015). The production of RNA-based TERS also introduced a three-part concept to apply these control reagents to control the performance of immune assays over time. The concept is characterized by (1) standardized and controlled manufacturing, (2) assay-specific cut-off definition, and (3) the regular use of serial TERS aliquots over time. TERS can be easily produced with multiple TCR specificities (MHC-class I and II restrictions), and work across the three most commonly applied immune assays (ELISpot, intracellular cytokine staining and MHC-multimer staining). Most importantly, their regular use could sensitively detect typically observed deviations in cell quality, reagent stability, hardware settings, and analysis of assay results. One shortcoming of the TERS technology is that the generation of a large number of aliquots is manageable, the storage and distribution of TERS batches to laboratories worldwide is associated with high costs and logistical effort as the transport needs uninterrupted temperature control. This fact makes it difficult to scale-up the technology, an essential step which would be required for their broader use.

The current study aims to overcome this limitation and introduces an easy-to-use novel kit-based approach to allow for the generation of versatile control reagents at peripheral sites by electroporation (EP) of RNA-encoded TCRs into primary lymphocytes. The kit is based on the shipment of quality-controlled TCR RNA and a standardized user manual for the manufacturing of TERS at the site of their use. In addition, GFP RNA and a manual are provided for the initial set-up of the lab-specific EP device. Prior TERS application, frozen aliquots of the generated TERS batch are used to define the intensity of the antigen-specific signal and cut-off values reflecting an acceptable signal range for the particular assay. TERS are then ready for continuous use to control the performance of the assay (Fig. 1). In this study, we have generated data showing that the proposed kit-based approach is compatible with most commonly used EP devices. Also stability studies were conducted for the TCR-encoding RNA, which is the critical starting material for the in-house generation of TERS. Finally, a

proficiency panel was performed to confirm that investigators that had never used the kit before can utilize it to generate TERS batches that work in their locally-established assay protocols.

2. Materials and methods

2.1. Optimization of EP settings

Six commonly used devices from 4 vendors were extensively tested over a broad range of settings to optimize device-specific EP conditions for the generation of TERS: ECM830 from BTX/Harvard Apparatus (BTX™/Harvard Apparatus Inc., Holliston, MA), Multiporator from Eppendorf (Eppendorf AG, Hamburg, Germany), Genepulser II and Xcell from Bio-Rad (Bio-Rad, Laboratories Inc. Hercules, CA), Nucleofector II and Amaxa 4D from Lonza (Lonza, Verviers, Belgium).

By Ficoll (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient centrifugation isolated PBMCs from buffy coats obtained from consenting, HLA-A*02-positive donors (Transfusion Center, University Medical Center, Mainz, Germany) were electroporated using GFP-RNA, chimeric NY-ESO-1₁₅₇₋₁₆₈, wild type (wt) human Influenza₅₈₋₆₆ or chimeric tyrosinase₃₆₈₋₃₇₆-specific $\alpha + \beta$ TCR RNA (in-house production; all class I restricted). For the EP using GFP-RNA or the Flu-TCR RNA, 2.0–10.0 μg RNA (per TCR chain) and $1.0\text{--}5.0 \times 10^6$ PBMCs were used. Testing the tumor associated antigen (TAA)-TCRs specific for NY-ESO-1 and tyrosinase (including Flu-TCR only for BTX), EP conditions analog to the TERS production process were used, testing 10.0–75.0 μg RNA (per TCR chain) and $10.0\text{--}60.0 \times 10^6$ PBMCs. Voltages and pulse lengths in the ranges of 200 V–1000 V and 0.05 ms–15 ms/150 μF –1500 μF were tested respectively with square or exponential pulse form and variable number of pulses. The EP volume 20 μl –250 μl differed based on the used EP cuvette: Bio-Rad - 4 mm (Bio-Rad), BTX (BTX/Harvard Apparatus) and Lonza Nucleocuvette Vessel/Stripe (Lonza). Different EP media were tested including X-vivo (Lonza), CTL-Wash supplement medium (CTL, Shaker Heights, OH), OPTI-MEM I (Gibco/Life Technologies, Carlsbad, CA), Bio-Rad-Genepulser EP Buffer (Bio-Rad) and BTXpress High Performance EP Buffer (BTX/Harvard Apparatus), partially in accordance with manufacturer's instructions. The Nucleofector solution and the human T cell Nucleofector solution were included in the EP kit provided by Lonza. The viability of transfected T cells was determined by using the ViaCount Assay (Merck Millipore, Darmstadt, Germany). The cells were stained with the Guava® ViaCount Reagent and analyzed on the Guava® easyCyte 5HT flow cytometry system.

A range of experiments were performed (detailed described in the results' part and summarized in Table 1) to identify the optimal EP settings to achieve maximum TCR expression and maximum viability of transfected T cells. This initial strategy enabled us to define rough parameters by testing large ranges of voltages and pulse lengths resulting in detectable signals and acceptable cell viabilities (see read out method below). The second step was to further optimize the rough

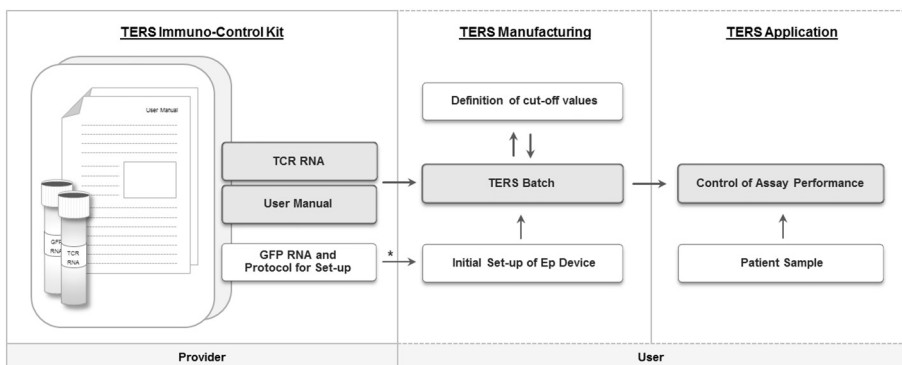


Fig. 1. Concept and design of the TERS Immuno-Control Kit. The provided kit contains an antigen-specific, quality-controlled TCR RNA and a device-specific user manual for the manufacturing of one TERS batch (highlighted in grey). The generated TERS batch is then to be tested to define the batch and assay specific signal including the cut-off values. The TERS aliquots can be repetitively applied as assay performance control in parallel to the test material or the patient samples. Additionally, a GFP RNA and a protocol for the initial set-up of the lab-specific EP device will be provided to support the fine-tuning of optimal EP parameters on the locally available EP device prior TERS generation. This optimization step would have to be performed only

once when starting using the new technology (indicated by “*”).

Table 1
Overview of the optimization process of 6 commonly used electroporation (EP) devices.

EP device	BTX/Harvard Apparatus ECM830	Eppendorf Multiporator	Bio-Rad GenePulser II	Bio-Rad GenePulser Xcell	Lonza Nucleofector II	Lonza Amaxa 4D
Voltages tested [V]	200; 300; 400; 500	300; 400; 500; 600; 700; 800; 1000	400; 450; 500	270; 300; 350; 400; 450; 500; 1000	n.a.	n.a.
Pulse form	Square	Exponential	Exponential	Exponential or square	n.a.	n.a.
Pulse number	1; 2; 3; 4	1; 2; 3; 4	1	1; 2	n.a.	n.a.
Multiple pulsing	Variable time interval: 100 ms–300 ms	Fixed time interval: 1 min	n.a.	Fixed time interval: 1 ms	n.a.	n.a.
Pulse length [μs/ms]	0.5 ms; 1 ms; 2 ms; 3 ms; 5 ms; 6 ms; 8 ms; 10 ms; 15 ms	50 μs; 150 μs; 250 μs; 300 μs; 400 μs; 500 μs	250 μF	150 μF; 250 μF; 350 μF; 450 μF; 500 μF; 960 μF; 1000 μF; 1500 μF	n.a.	n.a.
capacitor [μF]				12 ms; 10 ms; 8 ms; 5 ms; 3 ms; 1 ms; 0.5 ms		
EP Buffer	X-vivo; CTL-Wash supplement medium; OPTI-MEM I; Bio-Rad-Gene Pulser EP buffer; BTXpress high perf. EP buffer	Hypoosmolar buffer; Isoosmolar buffer	X-vivo; OPTI-MEM I; Bio-Rad-Gene Pulser EP buffer	OPTI-MEM I; Bio-Rad-Gene Pulser EP buffer	Human T cell nucleofector solution	Nucleofector solution
Volume [μl]	250	250	250	250	100	20
EP cuvette	Bio-Rad; BTX: 4 mm	Eppendorf: 4 mm	Bio-Rad: 4 mm	Bio-Rad: 4 mm	Lonza Nucleocuvette™ Vessel	Lonza Nucleocuvette™ Strip
Defined Program	Freely Programmable	Freely Programmable	Freely Programmable	Freely Programmable	U-014; V024; T-020; T023	FF-115; EO-115; FL-115; FF-113
Cell number [× 10 ⁶ cells]	60; 30; 20; 6.0	20; 5.0	20	20; 5.0	20; 10; 5.0	1.0
RNA amount [μg]	75; 50; 25; 10	30; 10	30	30; 10	30; 15; 7.5	5.0; 2.5
RNA Specificity	GFP; NY-ESO TCR	GFP; NY-ESO TCR	NY-ESO TCR	GFP; NY-ESO TCR	NY-ESO TCR	GFP; NY-ESO TCR

All EP settings (left column) tested are listed. The selection of the material is mainly device-specific, based on the instructions of the provider. The settings and ranges of parameters were chosen based on the properties of each specific device. In some experiments unconventional settings or additional material was tested based on initial results obtained with each single device.

parameters to reach the maximum achievable increase in transfection efficiency and viability. In a third step, single parameters within the defined parameter ranges were fine-tuned. Finally, the optimized settings were used to test the EP on high number of cells and large amount of RNA including the use of TAA-specific TCR RNA for the TERS production process. This optimization strategy was adapted specifically to each device based on the manufacturer's recommended material (buffers, cuvettes), device-specific limits of settings (max./min. voltage, pulse form: exponential (pulse length: μ s or ms) or square (capacitance: μ F), number of pulses) and defined pulse forms.

2.2. RNA stability study

In this study, we used a chimeric TCR construct with a murinized constant domain and a human variable domain of the HLA-A*02-restricted TCR specific for the epitope NY-ESO-1₁₅₇₋₁₆₅ (SLLMWITQC). The original human wild-type construct was kindly provided by T. Schumacher (The Netherlands Cancer Institute, Amsterdam, The Netherlands). A large-scale TCR RNA α and β batch was manufactured, based on “fed-batch” T7 transcription and followed by immuno-magnetic bead separation using DynaMag™ magnets (Invitrogen/Life Technologies) in combination with Dynabeads® MyOne™ Carboxylic Acid (Invitrogen). The purified and qualified RNA was aliquoted in 10 μ g aliquots for quality control testing and determination of RNA concentration, and also in larger aliquots containing 35 μ g RNA per chain for in vitro testing. From each RNA concentration several aliquots were stored at -80°C , at $2-8^{\circ}\text{C}$ and at RT to assess degradation profiles. Single 10 μ g RNA aliquots of each storage condition were used after 0, 3, 6, 12, 18 and 24 months of storage to test RNA integrity using the Agilent RNA 6000 Nano Assay (Agilent Technologies, Inc., Santa Clara, CA) and the Agilent 2100 Bioanalyzer System (Agilent Technologies, Inc.). In parallel, the RNA concentration of each aliquot was tested using the NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE). In addition, the TCR surface expression on CD8⁺ T cells was tested after applying these TCR RNA's for the EP of PBMCs using the ECM 830 Square Wave Electroporation System (BTX/Harvard Apparatus) at the same temperature and storage time points.

2.3. Quantification of TCR expression

The in vitro testing was performed by the EP of freshly isolated PBMCs with the TCR RNA on the basis of the optimized standard procedure for the generation of TERS, also using 20×10^6 PBMCs and 35 μ g TCR RNA of α and β , which was described in detail in our previous publication (Bidmon et al., 2015). After over-night culture, the TCR-transfected cells were harvested to check the recovery and viability of cells using Erythrosin B (Sigma-Aldrich, Saint Louis, MO). 1×10^6 cells were used for flow cytometry to detect TCR surface expression on CD8⁺ T cells using MHC-peptide dextramer. NY-ESO-1₁₅₇₋₁₆₅ APC dextramer (Immudex, Copenhagen, Denmark) staining was performed based on the staining protocol described in Bidmon et al., 2015. Deviating from this protocol, the viability staining was performed prior to dextramer staining using Fixable Viability Dye eFluor506 (65–0866, eBioscience, San Diego, CA). Additional antibodies including CD8 Brilliant Violet 421 (clone RPA-T4, BD Biosciences, San Jose, CA) and CD4 APC-Cy7 (clone: SK3, BD Biosciences) were applied after dextramer staining. The samples were acquired on the Canto II (BD Biosciences) and the FCS files were analyzed with FlowJo software version 7.6.5. (TreeStar, San Carlos, CA). The gating was as follows: singlets (FSC-H vs. FSC-W), living cells (FSC vs. Live/Dead), lymphocytes (FSC vs. SSC), CD8⁺ (CD4⁺ vs. CD8⁺), % Dext⁺ of CD8⁺ (CD8⁺ vs. Dext⁺).

2.4. Proficiency panel

Six experienced labs from four European countries (Germany, UK,

Denmark, The Netherlands) participated in a proficiency panel. For the first part, each participant received sets of 2 vials of a centrally manufactured TERS, each containing 5×10^6 cells shipped on dry ice. The first vial of each set contained cells from a sub-batch with a low frequency of NY-ESO-1₁₅₇₋₁₆₅-specific T cells (TERS#33 1:100 dilution), which was generated by transfecting PBMCs from a HLA-A*0201 positive healthy donor with a chimeric NY-ESO-1₁₅₇₋₁₆₅ TCR specific for the SLLMWITQC epitope. The second vial contained cells from a sub-batch lacking NY-ESO-1-specific T cells (TERS#33 negative fraction). The task of the first part was to thaw the two vials of one set of the centrally provided TERS and to stain the two samples with the two provided dextramers: A*0201 NY-ESO-1 (SLLMWITQV)-APC and A*0201 Negative control (irrelevant)-APC in one experiment (Test 1, T1). Then we asked the participants to repeat the experiment using the second set of vials on a different date (Test 2, T2), to show reproducibility of the results generated with the TERS. The labs were allowed to use their locally established staining protocol for the experiments with two mandatory restrictions: use 5 μ l of dextramer and incubate for 10 min at RT and the staining cocktail should include at least one CD8 antibody, one CD4 antibody and a dead cell dye. All labs were allowed to add additional markers and to perform the analysis of data depending on their local preferences.

The second part, implemented the TERS kit approach for the production of the TERS at peripheral sites, as depicted under user part in Fig. 1. Two tasks (Initial set-up of EP device (2A) & Manufacturing of TERS (2B)) had to be performed. For part 2A, each participant received GFP-RNA and a protocol to set up tailored EP parameters on the lab-specific EP device prior to TERS manufacturing. For part 2B, including the production of the internal TERS batch, chimeric NY-ESO-1₁₅₇₋₁₆₅ D1-capped TCR RNA was provided. All participants also received two HLA-peptide dextramers: an irrelevant dextramer (Negative control dextramer) and a specific NY-ESO-1₁₅₇₋₁₆₅ dextramer.

Part 2A aimed at establishing and fine tuning the EP settings, such as voltages (V), pulse length (ms) or capacitance (μ F) and the number of pulses on the locally available, lab-specific EP device, based on the provided optimization protocol using GFP-RNA and the recommended EP parameters that were pre-tested on an identical EP device by the central lab. The aim was to achieve maximal TCR-expression levels ($> 80\%$ GFP⁺ in CD8⁺) with maximal viability of transfected CD8⁺ T cells ($> 90\%$). The task of part 2B was to use the device-specific, optimized EP settings and the provided standard protocol for the manufacturing of TERS to generate and test locally-produced TAA-specific TERS. For this purpose, centers used freshly isolated, A*0201-positive PBMCs, transfected with the provided A*0201-restricted, NY-ESO-1₁₅₇₋₁₆₅-specific TCR RNA (SLLMWITQC) and mixed them with untouched PBMCs (negative fraction) of the same donor in a pre-defined ratio of 1:100 NY-ESO-1⁺ of CD8⁺. Aliquots of the produced TERS batch and of the negative fraction were frozen and stored in liquid nitrogen. One aliquot of the 1:100 TERS batch and the negative fraction represent one set of the internal TERS. The two samples of one set were tested in dextramer staining experiments on two independent time points (T1 and T2) using the provided dextramers (detailed testing procedure was described above for the central TERS).

Finally, participants had to complete a questionnaire containing basic information regarding the cell quality, the staining and analyzing procedure, as well as a report form showing the resulting dot plots and the number of lymphocytes, CD8⁺, and CD8⁺ Dext⁺ cells as well as the % of Dext⁺ within the CD8⁺ population.

3. Results

3.1. Utility of several EP devices for TERS manufacturing

It is known that different EP devices, even the same type from the same company may perform differently and thus it is required to optimize the EP parameters for each locally available device. A wide range

of different EP devices are available on the market and used worldwide. Therefore a survey asking peers for their in-house EP devices was performed leading to the identification of six most commonly used devices from 4 vendors: Harvard Apparatus - BTX ECM830, Eppendorf - Multiporator, Bio-Rad - Genepulser II and Xcell and Lonza - Nucleofector II and Amaxa 4D. To assess whether commonly used EP devices are suitable for the generation of TERS and to narrow down the range of settings under which TERS with optimal properties may be manufactured, a series of tests were conducted to pre-define EP settings on six commonly used devices with the aim to achieve maximum TCR expression levels (benchmark of > 80% TCR⁺ of CD8⁺) and maximum viability of transfected CD8⁺ T cells (benchmark > 90% viability).

First, we extensively tested the ECM 830 from BTX/Harvard Apparatus (Table 1; 1st column). We applied broad range of voltages, pulse times and pulse numbers (fixed square pulse form) with variable time intervals. Additionally, different EP buffers and cuvettes were used. We tested a range of cell numbers and RNA amounts in a matrix. A detailed overview of selected optimization results is shown in Supplementary Fig. 1A. A voltage of 500 V and one pulse with a length of 3 ms using the BTX EP buffer yielded high transfection efficiencies (TEs) of up to 97.3% antigen-specific CD8⁺ T cells with high viability up to 96.0% (Fig. 2; 1st column).

Subsequently, a similar test matrix was used for the Multiporator from Eppendorf (Table 1; 2nd column). But pulse length (limited to 500 μs) with fixed square pulse form and the EP buffers differed from that of the BTX system, precluding the simple transfer of the settings that had been established on the ECM830. The voltages using the Multiporator were increased up to 1000 V. Initial tests were performed using low amounts of GFP-RNA (10 μg) with 5 × 10⁶ PBMCs, whereas the defined optimal cell number of 20 × 10⁶ and RNA amount of 30 μg of each TCR-chain was used for final tests. Based on manufacturer's recommendations the provided EP cuvettes and buffer system were tested for our applications (Supplementary Fig. 1B). The optimal

parameters for TERS generation were 700 V, one pulse of 500 μs using the hypo-osmolar EP buffer (optimum 1), resulting in 89.7% Dext⁺ of CD8⁺ and 94.6% viable cells (Fig. 2; 2nd column). When voltage was changed to 800 V and the isoosmolar EP buffer was used (optimum 2), the TE was 88.1% Dext⁺ of CD8⁺ T cells and 97.4% viable cells (Supplementary Fig. 1B).

The results of the Genepulser II from Bio-Rad are shown in Table 1 (3rd column). We applied 3 different voltages (400 V, 450 V and 500 V), one capacitance of 250 μF (based on our experiences with this device) with fixed exponential pulse form and compared 3 media: X-vivo, OPTI-MEM I and Bio-Rad-Genepulser EP Buffer (Supplementary Fig. 1C). A maximum TE of 78.3% with a high viability of 92.0% of transfected T cells was achieved with 400 V, one pulse of 250 μF in X-vivo medium (Fig. 2; 3rd column).

The Genepulser Xcell from Bio-Rad allowed to set a large variety of parameters regarding voltage, pulse forms: exponential (capacitance) or square (pulse length) and EP buffers that could be tested (Table 1; 4th column). A large series of tests were run based on our findings with the previous devices and published data, as detailed in Supplementary Fig. 1D. Three different EP settings led to a maximum TE > 90% and maximum viability of T cells > 90% that were finally set to 400 V, 10 ms, 1 pulse (optimum 1) or 1000 V, 0.5 ms, 1 pulse (optimum 2) or 450 V, 3 ms, 2 pulse (optimum 3) using square pulse form and OPTI-MEM I to achieve > 90% cell viability and > 90% antigen-specific CD8⁺ T cells for all three settings (Fig. 2; 4th column and Supplementary Fig. 1D).

The two EP systems Nucleofector II and Amaxa 4D, provided by Lonza, did not allow a flexible, user-driven choice of parameters. Therefore, only a limited optimization series could be performed (Table 1; 5th and 6th column), applying defined buffers and EP cuvettes that restricted EP volume and thus the number of cells to be transfected. Pre-defined, encoded programs were recommended and specified by the manufacturer (Supplementary Fig. 1E, F). The performed

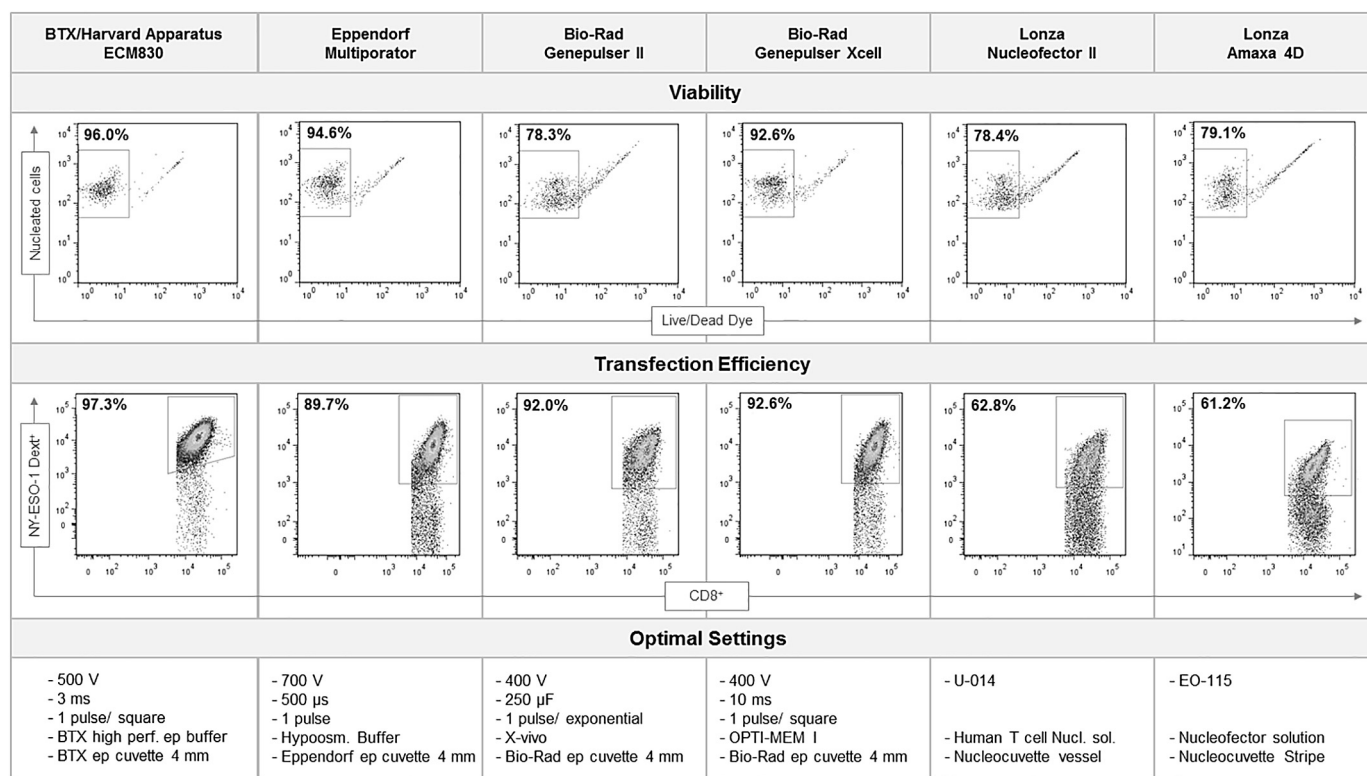


Fig. 2. Optimized EP settings for 6 commonly used EP devices. By the use of optimized EP settings (lower part) for each of the tested EP devices a high viability > 90% of transfected T cells (upper part) and a high transfection efficiency > 90% NY-ESO-1⁺ of CD8⁺ cells (middle part) was achieved for almost each EP device. Lower viabilities of 78–79% and transfection efficiencies of 61–62% NY-ESO-1⁺ of CD8⁺ were reached for the Lonza devices.

experiments resulted in TEs of < 63% and viabilities of ~80% using 2.5 µg of the TCR RNAs, adapted to the recommended number of cells of 1×10^6 PBMCs to be used for one EP. With GFP RNA a maximum TE of 90% (Fig. 2; 5th and 6th column) was reached. In preparation for the production of TERS, a reduced TE can be compensated by spiking higher numbers of TCR-transfected T cells into non-transfected cells in order to obtain the desired frequency of antigen-specific T cells. The limited number of cells that can be electroporated will make the procedure more complex than with other EP systems.

In summary, a set of optimized EP settings for 6 commonly used EP devices was established that lead to high cell viability (> 90%) and high TCR RNA transfection efficiency (> 60% of CD8⁺ T cells) (Fig. 2). These data sets are now available to guide the fine-tuning of settings on the lab-specific device to effectively generate high quality TERS.

3.2. RNA stability and shelf-life

We conducted an extended TCR RNA stability test, testing 30 µg aliquots of a chimeric HLA-A*0201-restricted TCR RNA specific for NY-ESO-1₁₅₇₋₁₆₅ (α- and β-chain) stored at -80 °C, 2–8 °C (accelerated instability), and RT (forced instability) to assess the degree of degradation of TCR RNA over time. At different time points RNA integrity was assessed using the Bioanalyzer and functionally by transfection of the RNA into freshly isolated PBMCs and subsequent analysis of TCR cell surface-expression levels using dextramer staining.

The results in Fig. 3 show a high stability of NY-ESO-1₁₅₇₋₁₆₅ TCR RNA up to 24 months of storage at -80 °C. The integrity ranged from 85% to 92%, reflecting the high stability of the RNA molecules over time. The final detected signal of 91% antigen-specific CD8⁺ T cells is slightly reduced after 24 months compared to the signal intensity detected at the very beginning using freshly produced TCR RNA (95% antigen-specific CD8⁺ T cells, left dot-plot). The RNA stability was significantly reduced after 12 and 24 months of storage at 4 °C showing integrity of 79% and 72%, respectively. Still, the TCR RNA retained high functionality as shown by an only slightly decreased TE of 87% after 24 months. These are similar levels that are comparable to the TE

of 95% obtained with the fresh RNA. In contrast the stability of the TCR RNA stored at RT was significantly decreased (integrity 70%) after 2 months of storage (TE was not tested; data not shown) which was an expected finding. In summary, the stability data from this study will allow guaranteeing TCR RNA stability at -80 °C and 2–8 °C for at least 24 months of storage. The data from this study will allow assigning appropriate shelf-life to the TCR RNA to the future immuno-control kit, facilitating the shipment conditions at 2–8 °C and defining the handling and storage of the RNA upon receipt.

3.3. TERS kit approach testing in a workshop

We conducted a workshop to test the TERS production process with non-experienced investigators under the developer's guidance. Four investigators from Leiden (The Netherlands), Southampton (UK) and two labs from Tuebingen (Germany), all experienced in T-cell assays, participated. The aim was to generate a HLA-A*0201-restricted TERS specific for the NY-ESO-1₁₅₇₋₁₆₅ SLLMWITQC epitope. The participants were divided into two groups (#1 and #2) and generated two different TERS batches (#1 and #2) from freshly isolated PBMCs from the same donor. The groups generated their own TERS batch using the in-house available EP device (BTX ECM830) following the centrally provided production manual stating a defined number of PBMCs and applying the same TCR RNA. The TCR-transfected T cells of each group were cultured at 37 °C as two independent positive fractions, whereas the untouched PBMCs of the donor kept as one negative fraction at RT for both groups. After overnight culture, the TCR⁺ T cells and the negative fraction were analyzed via NY-ESO-1₍₁₅₇₋₁₆₅₎ dextramer staining to determine the TE and the background signal. In parallel, the viability of both fractions was tested. Supplementary Fig. 2 summarizes these results. The TCR-transfected T cells of both positive fractions showed a high viability of 94% (#1) and 93% (#2), and TEs of 79.0% (#1) and 66.0% (#2) NY-ESO-1₍₁₅₇₋₁₆₅₎-specific CD8⁺ T cells. The highly viable T cells (91%) of the negative fraction displayed a background of 0.03% NY-ESO-1₍₁₅₇₋₁₆₅₎-specific CD8⁺ T cells. Each group prepared diluted TERS batches and defined their final NY-ESO-1-batch-specific signal

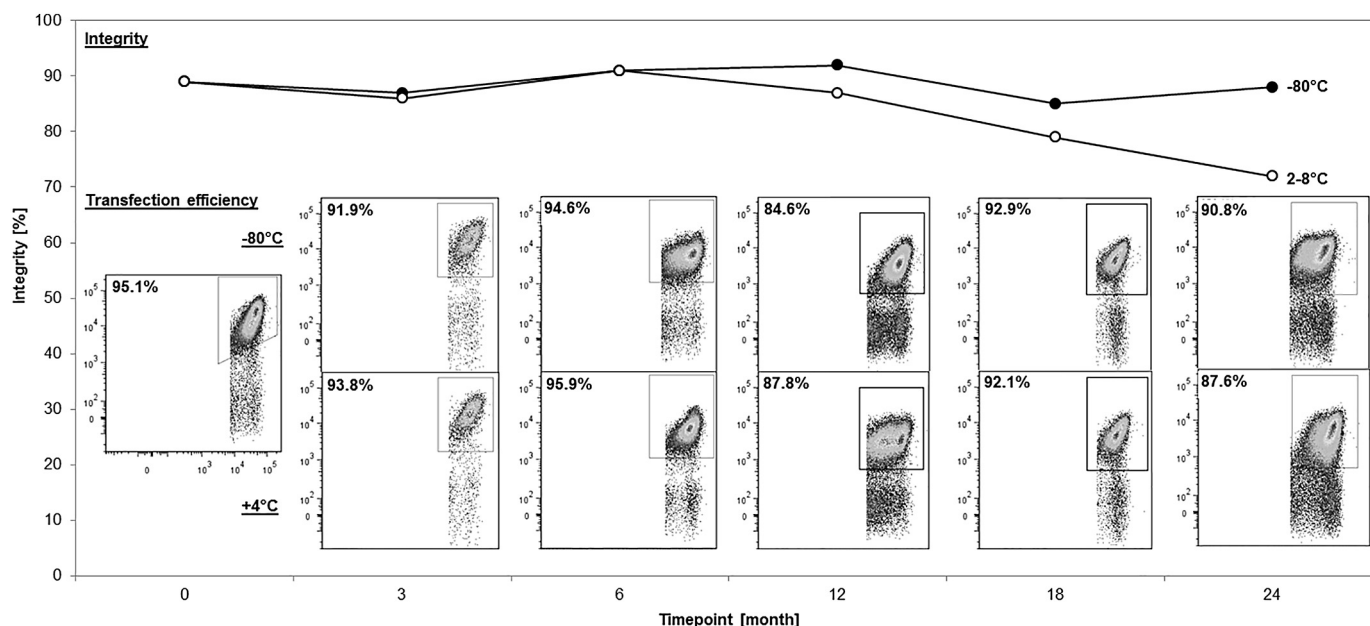


Fig. 3. RNA stability study to prove RNA half-life at different storage conditions over a period of time. The stability of the NY-ESO-1 TCR RNA, stored at -80 °C (closed circle) or at 2–8 °C (open circle), was proved by the determination of the integrity [%] shown on the y-axis in the diagram and depicted as line-graph in the upper part of the figure. The tests were performed on independent time points after 0, 3, 6, 12, 18 and 24 months (x-axis of the diagram) of storage. The NY-ESO-1 TCR expression on CD8⁺ T cells is depicted as dot plots (NY-ESO-1 dextramer staining on the y-axis and CD8 on the x-axis) and integrated in the graph (lower part of the figure) and visually linked to each data point of integrity measurements. The corresponding transfection efficiency [% NY-ESO-1⁺ of CD8⁺] for each time point is shown in the boxes on the upper left of each dot plot.

aiming a moderate signal of ~0.5% (#1) and a higher signal of ~1% (#2). Measured by flow cytometry, both TERS dilutions show high viability of 86.0% (#1) and 88.0% (#2) and a NY-ESO-1₍₁₅₇₋₁₆₅₎-specific signal of 0.67% (#1) and 0.90% (#2) CD8⁺ T cells.

3.4. TERS kit approach in a proficiency panel testing

In this proficiency panel (CIMT Immunoguiding Program (CIP) panel, CIP_ID17_2013_RSKITMUL/E) we tested how the TERS kit approach performs at peripheral sites. For the first part of the proficiency panel, a centrally manufactured TERS was provided as a “positive” control ensuring control of how a pre-manufactured TERS performs if tested in the hands of different investigators using different staining protocols. In the second part of the proficiency panel all participants generated their own TERS, making use of locally available EP devices and their optimized and fine-tuned EP settings established by using GFP RNA. Both the centrally provided as well as the peripherally manufactured TERS were tested in parallel at two independent time points to assess the reproducibility of the results. 6 labs (including the 4 labs having participated to the workshop) participated to this proficiency panel.

First, the participants used the provided TERS in their locally-established staining protocols at two independent time points (T1 and T2). The overview of the results (Supplementary Fig. 3), presenting dot plots of the 100-fold diluted TERS (T1) stained with the NY-ESO-1₍₁₅₇₋₁₆₅₎-specific dextramer (1st row) or the irrelevant dextramer (2nd row), and the percentage of NY-ESO-1₍₁₅₇₋₁₆₅₎-specific CD8⁺ T cells on both time points (T1 and T2). The participants successfully detected clearly clustered dextramer-positive populations with robust and highly comparable NY-ESO-1₍₁₅₇₋₁₆₅₎-specific CD8⁺ T cell signals of 0.55–0.76% (T1) and 0.5–0.8% (T2), indicated by a low intra-lab variation (CV < 11.6%). When focusing on the results generated across institutions, we found a high concordance of reported frequencies characterized by a low CV of 12.3% (T1) and 17.0% (T2). The low non-specific binding of irrelevant dextramers is indicated by dextramer-positive CD8⁺ T cells with frequencies below 0.06% (mean ≤ 0.02%). These data confirmed that there is a high concordance of results generated within and across institutions irrespective of multi-user diversity, as published previously for centrally manufactured TERS (Bidmon et al., 2015).

Considering the manufacturing of internal TERS, each lab electroporated 20×10^6 cells of freshly isolated PBMCs from a HLA-A*0201-positive buffy coat donor, based on the optimized lab-specific EP settings (Supplementary Fig. 4). The cells were cultured at 37 °C in the incubator as positive fraction and the non-transfected T cells were stored at RT as negative fraction (based on the optimization experiments for the standardized manufacturing of TERS described in Bidmon et al., 2015). On the next day, both fractions were tested for viability and the frequency of NY-ESO-1₍₁₅₇₋₁₆₅₎-specific CD8⁺ T cells (Supplementary Fig. 4). The fractions of lab 01 and lab 14, using the ECM830 (BTX/Harvard) Apparatus (450 V, 8 ms, 1 pulse and 500 V, 3 ms, 1 pulse) displayed > 90% viability and TEs > 92% in the positive fraction. Three labs (labs 08; 09; 11) used the Genepulser II from Bio-Rad applying EP settings (400/450 V, 250 μF, 1 pulse), very close to the recommended settings. Their fractions showed > 90% viability and a TE of ~83% for lab 08 and 09, and of ~67% for lab 11. Finally, the Genepulser Xcell from Bio-Rad was used by Lab 50 (450 V, 5 ms, 2 pulse) resulting in a viability of 71% of transfected T cells (97% for the negative fraction) and a TE of 54%.

Altogether, the background of 0.03–0.29% was low, as detected by staining of the negative fraction with the specific dextramer, and the average cell viability was 87% for the 100-fold diluted TERS batch and the negative fraction (data not shown).

The overview of the results from all laboratories (Fig. 4), showing dot plots of the NY-ESO-1₍₁₅₇₋₁₆₅₎-specific locally produced TERS stained with the specific (upper row) and the irrelevant (lower row)

dextramer. Thus, all 6 labs were able to generate TERS without supervision using the provided TCR RNA and the manufacturing protocol. The clustering of events differed across labs, e.g. ID11 showed a more spread population than the other labs, which is based on different staining protocols and analyzing methods. Differences of the frequencies across labs between 0.4% and 1.5% (T1) and 0.3% and 1.3% (T2) NY-ESO-1⁺ of CD8⁺ were detected. The mean NY-ESO-1-specific response of 0.98% (T1 + T2) is highly comparable to the 1.0% goal frequency. The differences between independent batches were not an unexpected finding. But this variation across labs is irrelevant, as the variation across aliquots of one in-house produced batch is low. The reproducibility of the NY-ESO-1-specific signal was assessed by the staining on two independent time points (T1 and T2). Each lab detected robust NY-ESO-1-specific responses at both time points that were highly comparable, indicated by a low inter-assay (intra-batch) variation. 2 of 6 labs showed a variation of 10% and 20%, whereas 4 of 6 labs generated results with a variation < 10%.

The unspecific background of self-made TERS, as detected by the staining with an irrelevant dextramer was low (< 0.07%) in all tests.

These results demonstrate that the TERS technology is a robust system applicable to various staining protocols and multi-user diversity.

4. Discussion

The recently introduced TERS technology is an easy to use and robust tool to generate reference samples for commonly applied T cell assays (Bidmon et al., 2015). Here we show that a kit-based approach, providing stable RNA, that can be manufactured in large quantities and be shipped at 2–8 °C, rather than electroporated TCR-engineered cells now allows each laboratory to manufacture their own TERS. With this kit we provide a novel tool for the generation of reference samples for T cell assays. Alternative technical solutions proposed in the past utilized cryopreserved TILs, Jurkat cells and PBMCs from healthy donors or cancer patients with pre-existing T cell responses against recall antigens to be used to control T cell assay performance (Xu et al., 2008). Also lyophilized, pre-labeled PBMC samples (Stebbins et al., 2015; Wang et al., 2015) or specially processed and cryopreserved PBMCs (Garcia et al., 2014) were provided to be used as standard controls. So far none of the available alternatives had the same favorable properties as TCR-engineered PBMCs that best mimic assay signals from endogenous T cells. In addition to the superior function, the TERS kit offers advantages compared to the previously described concepts. Most importantly, the transport costs for the shipment of RNA instead of cell material are considerably lower, which supports scalability and broad adoption. An additional advantage is that all investigators can control the size of the antigen-specific signal (low, medium and high frequency responses can be achieved) at will to generate a reference sample that is tailored to the specific user requirements.

In this study, we showed that the advanced kit-based TERS approach is compatible with commonly used EP devices, which makes it a broadly applicable technology (Fig. 2). The initial optimization studies were performed by adapting EP settings specifically to each device and allowed to narrow down the choice of settings for participants in the proficiency panel. The EP systems from Harvard Apparatus, Eppendorf and Bio-Rad offered a wide range of optimization strategies, giving the possibility to freely combine all required parameters and finally leading to perfectly optimized EP settings resulting in maximum TEs (> 80%) and maximum viabilities (> 90%). Whereas the Lonza EP systems offered less flexibility by only providing defined programs with concealed parameter sets to be used with manufacture-specified kit components and pre-defined procedures finally showing reduced TEs and viabilities. Additionally, the limited number of cells that can be electroporated in one reaction will make the procedure more complex than with other EP systems. Within the TERS production process a reduced TE of the TCR which may result from the use of suboptimal EP conditions or from inefficient RNA transfer on a given day, or due to donor variation can

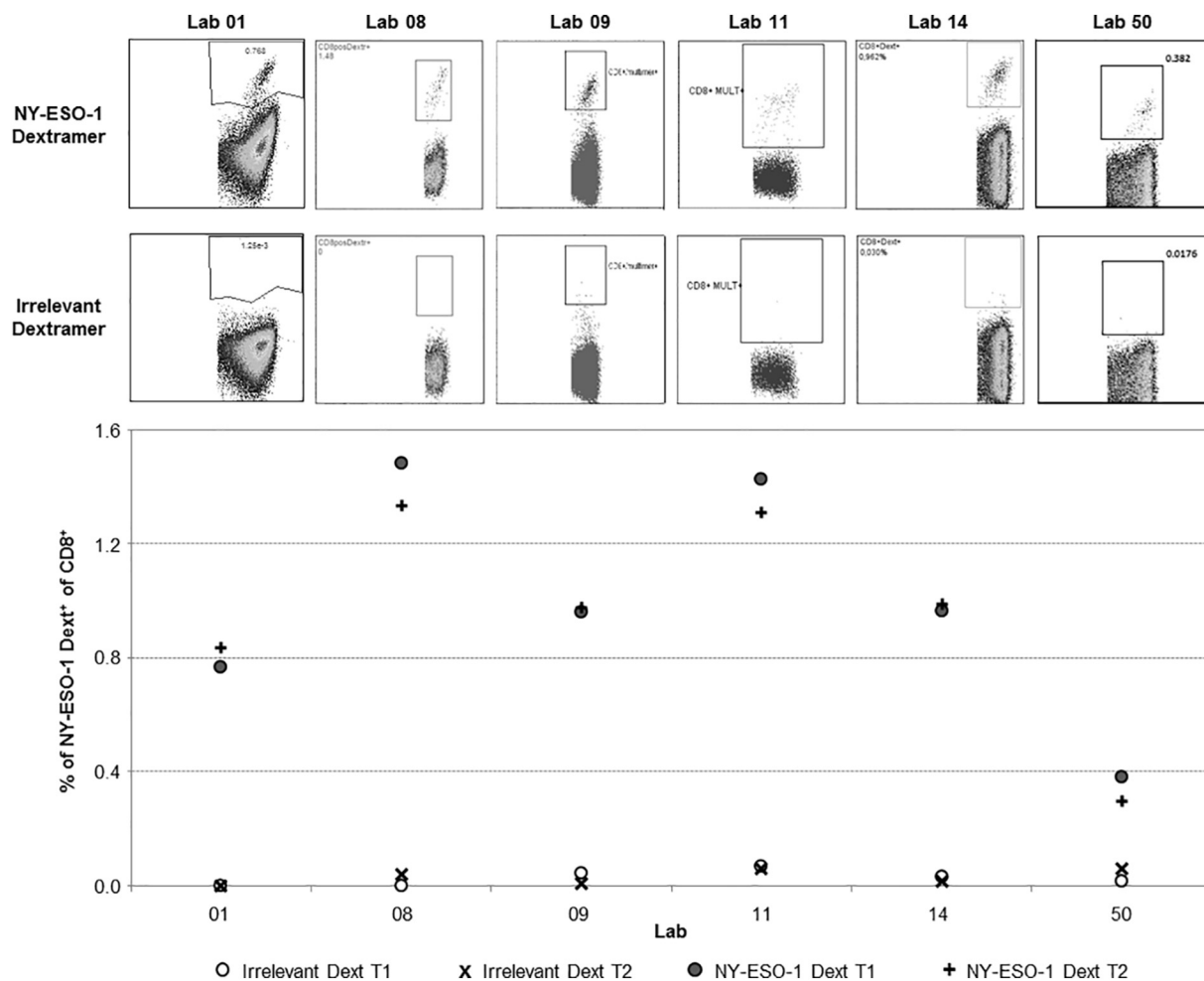


Fig. 4. Proficiency Panel - Multi-user testing of the TERS kit approach (part 2B): 6 participating labs used their own EP device and the lab- and device-specific, optimized EP settings to generate TERS in a defined dilution of 1:100 with an expected signal of ~1% NY-ESO-1 Dext⁺ of CD8⁺, using the provided kit components: the NY-ESO-1 TCR RNA, the EP-device-specific manufacturing protocol and dextramers. The upper part of the figure shows dot plots of the staining results of one time point (T1) using the specific NY-ESO-1 dextramer and an irrelevant dextramer. The diagram in the lower part of the figure summarizes all results. The x-axis shows the unique ID of each participating lab. The y-axis shows the percentage of NY-ESO-1 Dext⁺ of CD8⁺ T cells, as reported by the participants after staining of the TERS batch at two independent time points (T1 and T2). Each symbol represents the result from one independent dextramer staining. The experiments were performed by staining with an irrelevant dextramer at T1 (open circles) and T2 (indicated by “x”), with the specific NY-ESO-1 dextramer at T1 (closed circles) and T2 (indicated by “+”).

easily be compensated by spiking higher numbers of TCR-transfected T cells into non-transfected cells in order to obtain the desired frequency of antigen-specific T cells within the final TERS batch.

We showed that the genetically and chemically engineered RNA is characterized by its high stability over a long period of time (Fig. 3). The generated data showed that the RNA as the key element of the kit is a highly robust reagent that is perfectly suited for the shipment even at 2–8 °C which is easy and cost effective. Long-time storage up to 12–24 months did not have a significant effect on RNA stability and functionality. Even slightly reduced integrities or loss of RNA concentration that we observed at the end of the monitoring period did not cause a significant decrease of the TE. This finding reflects the robustness of the standardized method based on RNA transfection by EP and the specifically engineered RNA constructs. The results of the proficiency panel demonstrate that the TERS technology provided with the kit format is transferrable to a variety of labs, and works in the hands of different experienced investigators and across multiple protocols (Fig. 4). The differences of the final antigen-specific frequencies between independent batches, especially across labs were an expected finding. Variations of the manufacturing process are mainly driven by the resulting TCR transfection levels, as well as on the precision of cell

counts and the spike-in process. This variation across labs is acceptable, as the variation across aliquots of each batch is not affected. Even the batch-to-batch variation within one lab is acceptable because each batch is newly validated for its signal intensity and assay-dependent signal variation (definition of assay- and batch-specific cut-off values).

The envisioned, RNA-based kit can contain quality-controlled TCR RNA specific for selected viral or tumor-associated antigens. A detailed manual would describe the manufacturing process in general and provides step-by-step instructions for EP and cell processing. The same kit could also contain a protocol for the initial set-up of each user's EP device as well as the required GFP RNA for the establishment, testing and potential optimization of EP settings (Fig. 1). Based on TCR RNA as critical starting material all investigators can control the number of spiked TCR⁺ T cells over a range of frequencies and define the intensity of the antigen-specific signal, the number of frozen cells per aliquot, and the number of aliquots per batch. Each investigator is free to adapt parts of the production process to local preferences and conditions and is able to use own protocols for the isolation of PBMCs, freezing and thawing of cells, MHC-multimer staining, or functional read-outs. Initially, the batch-specific signal size and the acceptable signal range need to be defined (definition of cut-off values) for the lab-specific

assay protocols and the experimental setup. In the application phase, the pre-tested TERS aliquots can be subsequently used to control assay performance in a variety of ways, such as prior to assessing a series of test samples (e.g. patient samples) as well as after the last test sample has been run or in each experiment, on each day, or if required, for each test item depending on the user's preferences.

TERS can be used to control and to detect technical sources of assay variation as presented in a range of studies and proficiency panels for most of the commonly used T-cell assays such HLA-peptide multimer staining (Britten et al., 2009), intracellular cytokine flow cytometry (Jaimes et al., 2011; Welters et al., 2012) and ELISPOT assay (Chudley et al., 2014; Janetzki and Britten, 2012). Furthermore, TERS can be used for the validation and the day to day quality control of one T-cell assay. Controlled assay performance makes it also possible to compare results across centers leading to the desired harmonization of T cell assays (van der Burg et al., 2011). The development of an easy to use and easy to scale kit-based approach will allow the broad use of the TERS technology enabling the labs to continuously produce TERS in-house and will help to generate documented evidence of the validity of assay-results generated throughout their biomarker programs and thus may become a valuable tool to enhance the development of innovative immune-therapies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jim.2018.04.007>.

References

- Abreu, J.R.F., Roep, B.O., 2013. Immune monitoring of islet and pancreas transplant recipients. *Curr. Diabetes Rep.* (5), 704–712.
- Bidmon, N., Attig, S., Rae, R., Schröder, H., Omokoko, T.A., et al., 2015. Generation of

- TCR-engineered T cells and their use to control the performance of T cell assays. *J. Immunol.* (12), 6177–6189.
- Britten, C.M., Janetzki, S., Ben-Porat, L., Clay, T.M., Kalos, M., et al., 2009. Harmonization guidelines for HLA-peptide multimer assays derived from results of a large scale international proficiency panel of the cancer Vaccine Consortium. *Cancer Immunol. Immunother.* (10), 1701–1713.
- Britten, C.M., Janetzki, S., Butterfield, L.H., Ferrari, G., Gouttefangeas, C., et al., 2012. T cell assays and MIATA: the essential minimum for maximum impact. *Immunity* (1), 1–2.
- Britten, C.M., Walter, S., Janetzki, S., 2013. Immunological monitoring to rationally guide AAV gene therapy. *Front. Immunol.* 4, 273.
- Chudley, L., McCann, K.J., Coleman, A., Cazaly, A.M., Bidmon, N., et al., 2014. Harmonisation of short-term in vitro culture for the expansion of antigen-specific CD8(+) T cells with detection by ELISPOT and HLA-multimer staining. *Cancer Immunol. Immunother.* (11), 1199–1211.
- van der Burg, S.H., Kalos, M., Gouttefangeas, C., Janetzki, S., Ottensmeier, C., et al., 2011. Harmonization of immune biomarker assays for clinical studies. *Sci. Transl. Med.* (108), 108ps44.
- Garcia, A., Keinonen, S., Sanchez, A.M., Ferrari, G., Denny, T.N., et al., 2014. Leukopak PBMC sample processing for preparing quality control material to support proficiency testing programs. *J. Immunol. Methods* 409, 99–106.
- Jaimes, M.C., Maecker, H.T., Yan, M., Maino, V.C., Hanley, M.B., et al., 2011. Quality assurance of intracellular cytokine staining assays: analysis of multiple rounds of proficiency testing. *J. Immunol. Methods* (2), 143–157.
- Janetzki, S., Britten, C.M., 2012. The impact of harmonization on ELISPOT assay performance. *Methods Mol. Biol.* 792, 25–36.
- Kleijwegt, F.S., Roep, B.O., 2013. Infectious tolerance as candidate therapy for type 1 diabetes: transfer of immunoregulatory properties from human regulatory T cells to other T cells and proinflammatory dendritic cells. *Crit. Rev. Immunol.* (5), 415–434.
- Lu, Y.-C., Yao, X., Crystal, J.S., Li, Y.F., El-Gamil, M., et al., 2014. Efficient identification of mutated cancer antigens recognized by T cells associated with durable tumor regressions. *Clin. Cancer Res.* (13), 3401–3410.
- Maecker, H.T., McCoy, J.P., Amos, M., Elliott, J., Gaigalas, A., et al., 2010. A model for harmonizing flow cytometry in clinical trials. *Nat. Immunol.* 11, 975–978.
- Maecker, H.T., McCoy, J.P., Nussenblatt, R., 2012. Standardizing immunophenotyping for the Human Immunology Project. *Nat. Rev. Immunol.* (3), 191–200.
- Ottenhoff, T.H.M., Kaufmann, S.H.E., 2012. Vaccines against tuberculosis: where are we and where do we need to go? *PLoS Pathog.*(5), e1002607.
- Riou, C., Burgers, W.A., Mlisana, K., Koup, R.A., Roederer, M., et al., 2014. Differential impact of magnitude, polyfunctional capacity, and specificity of HIV-specific CD8+ T cell responses on HIV set point. *J. Virol.* (3), 1819–1824.
- Rizvi, N.A., Hellmann, M.D., Snyder, A., Kvistborg, P., Makarov, V., et al., 2015. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 6230, 124–128.
- Robbins, P.F., Kassim, S.H., Tran, T.L.N., Crystal, J.S., Morgan, R.A., et al., 2015. A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. *Clin. Cancer Res.* (5), 1019–1027.
- Singh, S.K., Tummers, B., Schumacher, T.N., Gomez, R., Franken, K.L.M.C., et al., 2013. The development of standard samples with a defined number of antigen-specific T cells to harmonize T cell assays: a proof-of-principle study. *Cancer Immunol. Immunother.* (3), 489–501.
- Stebbing, R., Wang, L., Sutherland, J., Kammel, M., Gaigalas, A.K., et al., 2015. Quantification of cells with specific phenotypes I: determination of CD4+ cell count per microliter in reconstituted lyophilized human PBMC prelabeled with anti-CD4 FITC antibody. *Cytometry A* 3, 244–253.
- Wang, L., Stebbings, R., Gaigalas, A.K., Sutherland, J., Kammel, M., et al., 2015. Quantification of cells with specific phenotypes II: determination of CD4 expression level on reconstituted lyophilized human PBMC labelled with anti-CD4 FITC antibody. *Cytometry A* 3, 254–261.
- Welters, M.J., Gouttefangeas, C., Ramwadhoebe, T.H., Letsch, A., Ottensmeier, C.H., et al., 2012. Harmonization of the intracellular cytokine staining assay. *Cancer Immunol. Immunother.* (7), 967–978.
- Xu, Y., Theobald, V., Sung, C., DePalma, K., Atwater, L., et al., 2008. Validation of a HLA-A2 tetramer flow cytometric method, IFN γ real time RT-PCR, and IFN γ ELISPOT for detection of immunologic response to gp100 and MelanA/MART-1 in melanoma patients. *J. Transl. Med.* 6, 61.