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

Nicole Bidmon\textsuperscript{a,d}, Sonja Kind\textsuperscript{b}, Marij J.P. Welters\textsuperscript{b}, Deborah Joseph-Pietras\textsuperscript{c}, Karoline Laske\textsuperscript{d}, Dominik Maurer\textsuperscript{e}, Sine Reker Hadrup\textsuperscript{f}, Gerty Schreibelt\textsuperscript{g}, Richard Rae\textsuperscript{a}, Ugur Sahin\textsuperscript{a,h,i}, Cécile Gouttefangeas\textsuperscript{d}, Cedrik M. Britten\textsuperscript{a,1}, Sjoerd H. van der Burg\textsuperscript{b,n}

\textsuperscript{a} Translational Oncology at the University Medical Center of the Johannes-Gutenberg University Mainz (TRON gGmbH), Freiligrathstraße 12, Mainz 55131, Germany
\textsuperscript{b} Department of Medical Oncology, Leiden University Medical Center, Albinusdreef 2, Leiden, ZA 2333, The Netherlands
\textsuperscript{c} ECMC, Cancer Sciences Unit, Faculty of Medicine, University of Southampton, Tremona Road, Southampton SO16 6YD, United Kingdom
\textsuperscript{d} Department of Immunology, University of Tuebingen, Auf der Morgenstelle 15, Tuebingen 72076, Germany
\textsuperscript{e} Immatics biotechnologies GmbH, Paul-Ehrlich-Str. 15, Tuebingen 72076, Germany
\textsuperscript{f} Laboratory of Hematology, University Hospital Herlev, Ringvej 75, Herlev DK-2730, Denmark
\textsuperscript{g} Dept. of Tumor Immunology, Rudbod university medical center, Rudbod Institute for Molecular Life Sciences, P.O. Box 9101, Nijmegen, HB 6500, The Netherlands
\textsuperscript{h} University Medical Center of the Johannes Gutenberg-University Mainz, Langenbeckstrasse 1, Mainz D-55131, Germany
\textsuperscript{i} BioNTech AG, An der Goldgrube 12, 55131 Mainz, Germany

\textsuperscript{1} Current address: Immuno-Oncology & Combinations Discovery and Performance Unit, Oncology R&D, GlaxoSmithKline, Stevenage, SG1 2NY, United Kingdom.

\textsuperscript{n} Corresponding author.

E-mail address: shvdburg@lumc.nl (S.H. van der Burg).

\textsuperscript{1} Current address: Immuno-Oncology & Combinations Discovery and Performance Unit, Oncology R&D, GlaxoSmithKline, Stevenage, SG1 2NY, United Kingdom.

ARTICLE INFO

Keywords: Immuno-monitoring
Immunoguiding
T-cell assays
Performance
Validation
Standardization

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 Kauffman, 2012) and inflammatory diseases (Kleijwegt and Roep, 2013). T-cell immunity also clinically impacts on the development of innovative therapies and has increased over the years. 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,
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 Amazax 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 influenza A8-66, or chimeric tyrosinase +368-376-specific α + β 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 1.0–5.0 × 10⁶ 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 10.0–60.0 × 10⁶ PBMCs. Voltages and pulse lengths in the ranges of 200 V–1000 V and 0.05 ms–15 ms/150 μl–1500 μl 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 “*” or “**”).
Table 1
Overview of the optimization process of 6 commonly used electroporation (EP) devices.

<table>
<thead>
<tr>
<th>EP device</th>
<th>BTX/Harvard Apparatus ECM830</th>
<th>Eppendorf Multiporator</th>
<th>Bio-Rad Gene pulser II</th>
<th>Bio-Rad Gene pulser Xcell</th>
<th>Lonza Nucleofector II</th>
<th>Lonza Amaza 4D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltages tested [V]</td>
<td>200; 300; 400; 500</td>
<td>300; 400; 500; 600; 700; 800; 1000</td>
<td>400; 450; 500</td>
<td>270; 300; 350; 400; 450; 500; 1000</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Pulse form</td>
<td>Square</td>
<td>Exponential</td>
<td>Exponential</td>
<td>Exponential or square</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Pulse number</td>
<td>1; 2; 3; 4</td>
<td>1; 2; 3; 4</td>
<td>1</td>
<td>1; 2</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Multiple pulsing</td>
<td>Variable time interval: 100 ms–300 ms</td>
<td>Fixed time interval: 1 min</td>
<td>Fixed time interval: 1 ms</td>
<td>Fixed time interval: 1 ms</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Pulse length [μs]</td>
<td>0.5 ms; 1 ms; 2 ms; 3 ms; 5 ms; 6 ms; 8 ms; 10 ms</td>
<td>50 μs; 150 μs; 250 μs</td>
<td>500 μs; 1000 μs</td>
<td>12 ms; 10 ms; 8 ms; 5 ms; 3 ms; 1 ms; 0.5 ms</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Volume [μl]</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>EP cuvette</td>
<td>Bio-Rad; BTX: 4 mm</td>
<td>Eppendorf: 4 mm</td>
<td>Bio-Rad: 4 mm</td>
<td>Bio-Rad: 4 mm</td>
<td>Lonza Nucleocuvette™ Vessel</td>
<td>Lonza Nucleocuvette™ Strip</td>
</tr>
<tr>
<td>Defined Program</td>
<td>Freely Programmable</td>
<td>Freely Programmable</td>
<td>Freely Programmable</td>
<td>Freely Programmable</td>
<td>U-014; V024; T-020; T023</td>
<td>Fl-115; EO-115; FL-115; FF-113</td>
</tr>
<tr>
<td>Cell number [×10⁶ cells]</td>
<td>60; 30; 20; 6.0</td>
<td>20; 5.0</td>
<td>20</td>
<td>20; 5.0</td>
<td>20; 10; 5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>RNA amount [μg]</td>
<td>75; 50; 25; 10</td>
<td>30; 10</td>
<td>30</td>
<td>30; 10</td>
<td>30; 15; 7.5</td>
<td>5.0; 2.5</td>
</tr>
<tr>
<td>RNA Specificity</td>
<td>GFP; NY-ISO TCR</td>
<td>GFP; NY-ISO TCR</td>
<td>NY-ISO TCR</td>
<td>GFP; NY-ISO TCR</td>
<td>NY-ISO TCR</td>
<td>GFP; NY-ISO TCR</td>
</tr>
</tbody>
</table>

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: μs or ms) or square (capacitance: μF), 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 (SLLMWWTBQ). 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 μg aliquots for quality control testing and determination of RNA concentration, and also in larger aliquots containing 35 μg per chain for in vitro testing. From each RNA concentration several aliquots were stored at –80 °C, at 2–8 °C and at RT to assess degradation profiles. Single 10 μg 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 × 10⁶ PBMCs and 35 μg 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 × 10⁶ cells were used for flow cytometry to detect TCR surface expression on CD8⁺ T cells after 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 (μF) 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 (SLLMWWTBQ) 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 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⁺ and 94.6% viable cells (Fig. 2; 2nd column). When voltage was changed to 800 V and the isosmolar 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

<table>
<thead>
<tr>
<th>BTX/Harvard Apparatus ECM830</th>
<th>Eppendorf Multiporator</th>
<th>Bio-Rad Genepulser II</th>
<th>Bio-Rad Genepulser Xcell</th>
<th>Lonza Nucleofector II</th>
<th>Lonza Amaxa 4D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleated cells</td>
<td>96.0%</td>
<td>94.6%</td>
<td>78.3%</td>
<td>92.6%</td>
<td>79.4%</td>
</tr>
<tr>
<td>Live/Dead Dye</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transfection Efficiency</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NY-ESO-1 Dext⁺</td>
<td>97.3%</td>
<td>89.7%</td>
<td>92.0%</td>
<td>92.6%</td>
<td>62.8%</td>
</tr>
<tr>
<td>CD8⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>61.2%</td>
</tr>
</tbody>
</table>

![Fig. 2. Optimized EP settings for 6 commonly used EP devices. 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.](image-url)
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 \(1 \times 10^6\) 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 ECMB30) 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 (#1) 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.](image-url)
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_REKTMUTL/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 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 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 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 cells on both time points (T1 and T2).

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, prelabeled PBMC samples (Stebbings 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 TEIs (>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 manufacturer-specified kit components and pre-defined procedures finally showing reduced TEIs 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...
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.

Acknowledgements

Many thanks to all participants of the workshop and the proficiency panel, namely S. R. Hadrup and P. thor Stratcn (Laboratory of Hematology, University Hospital Herlev, Herlev, Denmark), M.J.P. Welters and J. J. van Ham (Dept. of Clinical Oncology, Leiden University Medical Center, Leiden, The Netherlands), G. Schreibelt and T. Duiveman-de Boer (Dept. of Tumor Immunology, Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands), D. Joseph-Pietras and K. McCann (EMC, Southampton General Hospital, Southampton, England), K. Laske and C. Gouttefangeas (Dept. of Immunology, University of Tubingen, Tubingen, Germany), D. Paduch and D. Maurer (Immatics biotechnologies GmbH, Tubingen, Germany).

We further thank Dr. T. Schumacher for providing the wild-type NY-ESO-1 157-165-specific TCR and Immudex (Copenhagen, Denmark) for the dextramers reagents.

This project was funded by the Wallace Coulter Foundation (Florida, USA).

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


