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An improved CPRG colorimetric ligand-receptor signal transduction assay based on beta-galactosidase activity in mammalian BWZ-reporter cells

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Abstract

Introduction: Reporter cells expressing a chimeric receptor that activates a reporter can be used for screening ligand-mediated signal transduction. In this study, we used reporter cells harboring an NFAT/lacZ construct that express β-galactosidase when the chimeric receptor is stimulated. A colorimetric β-galactosidase substrate, chlorophenol-red β-D-galactopyranoside (CPRG), was used to detect enzymatic activity. Sub-optimal conditions have unfortunately extensively been reported with such reporter-based β-galactosidase assays. Here, we aimed to improve the CPRG-based colorimetric assay such that receptor ligands could be effectively screened with reporter cells.

Methods: After stimulation of reporter cells, we determined β-galactosidase activity by absorbance measurement of β-galactosidase-dependent CPRG hydrolysis. We systematically examined each component in a standard lysis buffer most commonly reported for this type of reporter cells. Furthermore, we evaluated literature in the field.

Results: An increased CPRG substrate concentration combined with a different detergent, Saponin, and an optimal wavelength recording markedly increased the sensitivity for the detection of β-galactosidase activity (≈4-fold increase). Moreover, the improved protocol resulted in increased linear time-dependent recording of enzymatic activity once cells had been lysed, and a more stable and reproducible assay to detect a ligand-stimulus with the reporter cells. The optimal time length of exposure to a stimulus was ligand-dependent.

Discussion: In conclusion, we provide an improved protocol with an optimized lysis buffer that gives up to a six-fold higher and more robust specific signal when NFAT/lacZ-based receptor-expressing reporter cells are exposed to a stimulus.
Keywords: methods; reporter cells; receptor stimulation; BWZ.36; β-galactosidase; chlorophenol-red-β-D-galactopyranoside; CPRG.

Introduction

The β-galactosidase enzyme has been studied extensively since the mid-1900s. It is encoded by the lacZ gene, and its enzymatic activity was used in pioneering studies of genetic regulation in enteric bacteria (Pardee, Jacob, & Monod, 1959). It has found use as a reporter of gene expression in a wide range of organisms and assay formats. The protein functions as a homotetramer catalyzing the hydrolysis of an aglycone from the glycosyl group on a glycoside (Tenu, Viratelle, Garnier, & Yon, 1971). While the natural substrate is lactose, the reactions allow for colorimetric absorbance assays in which other substrates are hydrolyzed by β-galactosidase and converted into colored, fluorescent, or luminescent products. A well-known colorimetric substrate is ortho-nitrophenyl-β-D-galactopyranoside (ONPG). An alternative and more sensitive colorimetric substrate, chlorophenol-red-β-D-galactopyranoside (CPRG), makes it possible to detect lower levels of β-galactosidase activity (Eustice, Feldman, Colberg-Poley, Buckey, & Neubauer, 1991). In the present paper, we have used CPRG as a substrate to detect β-galactosidase expression and activity in mammalian reporter cells.

The murine reporter cells used in this study originate from a cell line produced by White and colleagues in 1989 (White et al., 1989). Three mutant cell lines were made from the murine thymoma BW5147 cell line: cells devoid of either the endogenous T-cell receptor chain (TCR) α or chain β, or both the α- and β-chain (White et al., 1989). The BWZ.36 cell line used in our studies was generated by transfecting α β BW5147 cells with a DNA construct harboring a nuclear factor for activated T cells (NFAT) promoter
coupled to *lacZ* (Sanderson & Shastri, 1994). When used as reporter cells, they express a chimeric antigen receptor. As a response to a receptor stimulus, the *NFAT* promotor is activated by an increased intracellular Ca\(^{2+}\) concentration such that β-galactosidase is expressed. The reporter cells were used in an antigen-presenting assay with CPRG colorimetry, and the referred study (Sanderson & Shastri, 1994) forms the basis for our literature review and method optimization, and will henceforth be called “the original report”.

By extensively reviewing the 277 publications citing the original report by Sanderson and Shastri (Sanderson & Shastri, 1994), we found a general lack of information about the lysis buffer composition used for the CPRG assay with reporter cells. The originally described lysis buffer recipe (Sanderson & Shastri, 1994) was used in most of the publications that do report the buffer composition. The enzymatic activity of β-galactosidase depends on pH, temperature (Roth & Huber, 1994; Tenu et al., 1971), on monovalent and divalent ions (Hill & Huber, 1971; Huber, Parfett, Woulfe-Flanagan, & Thompson, 1979; Juers, Matthews, & Huber, 2012; Tenu, Viratelle, & Yon, 1972) as well as on substrate concentration. The enzyme might also be inhibited by components of the lysis buffer. When testing the original CPRG lysis buffer recipe (Sanderson & Shastri, 1994), a non-linear time-dependent enzymatic reaction was observed, indicating sub-optimal conditions for β-galactosidase or cell lysis (unpublished observations). We found it essential to establish a modified protocol of this much-used reporter assay such that the readout related to a linear time-dependent enzyme reaction. Moreover, we found it critical to optimize the cell lysis buffer composition when monitoring β-galactosidase enzymatic activity. In the present study, we generated a clearly improved reporter cell assay by systematically evaluating and changing lysis buffer components. We discovered that there is an optimal time of stimulation which depends on the ligand and therefore, rather than relying on the standard protocol, this parameter needs be empirically determined.
Materials and methods

Literature review

The original report by Sanderson & Shastri (Sanderson & Shastri, 1994) in 1994 describes the use of β-galactosidase-expressing αβ BW5147 reporter cells with a CPRG colorimetric assay. The 277 publications that have cited this report (according to Web of Knowledge, www.webofknowledge.com) until the time of writing (July 2017) were systematically evaluated. Publications were omitted from the literature review if they did not use reporter cells that were based on the β-galactosidase-expressing αβ BW5147 cells. Furthermore, publications were excluded if they were review articles, they were not written in English, or they did not use CPRG as a β-galactosidase substrate when performing the β-galactosidase reporter assay. After exclusions, a total of 158 publications were included in the detailed CPRG method analysis. An overview of the 277 publications is found in Supplementary Table 1.

Materials and Reagents

Transparent Maxisorp 96-well plates (#442404), Roswell Park Memorial Institute (RPMI) 1640 medium (#61870-044), ionomycin (#I-24222), ethylenediamine tetra acetic acid (EDTA, #15575-020), 2-mercaptoethanol (#31350-010), and Trypan Blue (#T10282) were all acquired from Thermo Fisher Scientific (Waltham, MA, USA). Phorbol myristate acetate (PMA, #P1585), CPRG (#59767), fetal bovine serum (FBS, #F7524), magnesium chloride hexahydrate (#M9272), potassium chloride (#P5405), sodium phosphate dibasic dihydrate (#30412), sodium chloride (#71376), IGEPAL CA-630 (Nonidet P-40 substitution, #I8896), and Tergitol-type NP-40 (#NP40S) were all bought from Sigma-Aldrich (St. Louis, MO, USA). An unconjugated, polyclonal rabbit anti-goat antibody (#ab97101), and a polyclonal goat
antibody against mouse Triggering Receptor Expressed on Myeloid cells 2 (anti-mTREM2, #ab95470) were both from Abcam (Cambridge, UK). Saponin (#S58255) was bought from Merck Millipore (Darmstadt, Germany). Molecular biology grade 2-mercaptoethanol (#A1108) was from AppliChem (Darmstadt, Germany). Electran 1,4-Dithiothreitol (Cleland’s reagent, DTT, #443853B) was from VWR (Radnor, PA, USA). Human apolipoprotein E isoform ε3 (ApoE ε3; #4696) was from BioVision (Milpitas, CA, USA).

Three specialized phosphate-buffered saline (PBS) buffers with pH7.3 were used in the assay: “regular PBS” (10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, 2.7 mM KCl, 137 mM NaCl), “S-PBS” (10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, 0.27 mM KCl, 13.7 mM NaCl), and “Hi-S-PBS” (60.7 mM Na$_2$HPO$_4$, 13.2 mM KH$_2$PO$_4$, 0.27 mM KCl, 13.7 mM NaCl).

**Cell culture**

All cells were grown in RPMI 1640 medium supplemented with 1 % (v/v) penicillin/streptomycin, 5 % (v/v) FBS, 50 μM EDTA, and 50 μM 2-mercaptoethanol (cRPMI used as abbreviation) in a cell incubator at +37 °C with 5 % CO$_2$. The BWZ.36 cells were based on an immortalized T lymphocyte murine cell line (Sanderson & Shastri, 1994; White et al., 1989) constitutively expressing NFAT/lacZ DNA. Naïve BWZ.36 cells (kindly provided by Dr. Nilabh Shastri, UC Berkeley, CA, USA) and BWZ.36 transfected with murine TREM2-CD8-CD3ε pcDNA4 construct (BWZ-mTREM2) (Daws et al., 2003) were used in the analyses.

**Cell stimulation and CPRG colorimetry assay**

We have previously found that human ApoE is an agonist to murine and human TREM2 (Jendresen, Årskog, Daws, & Nilsson, 2017), and therefore used ApoE ε3 to stimulate the reporter cells in this study.
Transparent Maxisorp 96-well plates were coated with polyclonal anti-goat antibody (2.5 µg/mL in S-PBS) or recombinant ApoE ε3 (15 nM or 300 nM in S-PBS) for 1 hour at +37 °C in a sterile environment. Coating with S-PBS was used as a negative control. The coating solutions were aspirated and wells were washed once with S-PBS at room temperature (RT). PMA (3.3 nM) was added to the BWZ-cells before transferring to the coated Maxisorp plate at a final density of 2.4x10⁵ cells/well in 200 µL cRPMI. To a proportion of the cells, goat anti-mTREM2 antibody (2 µg/mL) was added before the cells were transferred to wells coated with anti-goat antibody. Cells treated only with PMA served as a negative treatment control. The cells were incubated on the plate for 4 hours at +37 °C with 5 % CO₂ unless otherwise stated. The cell viability was tested with Trypan Blue and an Invitrogen Countess automated cell counter (Thermo Fisher Scientific). The viability was always >90% when used in reporter assays.

Before lysis, cells were carefully washed once with Hi-S-PBS at RT. The lysis buffer consisted of Hi-S-PBS or regular PBS supplemented with CPRG, MgCl₂, a detergent (NP-40, Nonidet P-40, or Saponin), and a reducing agent (DTT or 2-mercaptoethanol). Cell-free lysis buffer served as background control. The lysis buffer was freshly prepared before cell lysis in order to avoid losing activity of the reducing agents and detergents. The lysis buffer was added to all wells and absorbance was read immediately after adding the lysis buffer (“time point 0”). Absorbance was monitored for another 20 hours.

Based on spectral analyses, specific absorbance was measured at 570 nm, while lysis buffer background was measured at 700 nm with a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA) or with an EnVision2104 Multilabel Reader (#2104-0010; Perkin-Elmer Inc., Waltham, MA, USA). The plate was tightly sealed with plastic adherent as to avoid evaporation and contamination, and incubated at +37 °C on a VWR rocking platform shaker (40 rpm) between measurements. All experiments were repeated to a total of three independent experiments performed on different days, and all measurements were done in triplicates. Since all comparisons were planned before experiments were
performed, two-way ANOVAs were made and planned comparisons were corrected for multiple comparisons using the *a priori* Bonferroni method. In this, the $\alpha$-level was set at 0.05 and statistical level defined individually in each experiment dependent on the number of comparisons made as indicated in the figure legends (figures 3, 4, 5, and 6, and supplementary figures 1 and 2). A two-way ANOVA with Dunnett’s multiple comparison test was used to evaluate the effect of stimulation time in Figure 7. Time-dependent linearity of enzymatic recordings was tested with linear regression analysis (figures 2 and 6 and supplementary figures 3 and 4). In figures with data analyzed by two-way ANOVA and multiple comparisons, the stated $p$-values are from multiple comparisons. All reported $p$-values are two-tailed.

A bullet point overview of a suggested experimental protocol is reported in Supporting Information.

**Results**

**Wavelengths for optimal measurements of absorbance**

Almost two thirds of the investigated publications (n=95 out of 158) measured absorbance at 595 nm when monitoring the $\beta$-galactosidase activity, as was also done in the original report by Sanderson and Shastri (Sanderson & Shastri, 1994). Only 30 out of 158 publications measured absorbance at 560-570 nm. Three papers reported absorbance measurement at 540 or 550 nm, while one paper measured at 610 nm. In some papers (n=21 out of 158) there was no information or reference to the wavelength used.

We performed spectral analyses with two different plate readers and compared the optimized lysis buffer described in this paper (3 mM CPRG, 0.2 % Saponin, 5 mM DTT, and 20 mM MgCl$_2$ in Hi-S-PBS) with the most commonly used, which was described in the original report (Sanderson & Shastri, 1994); hereafter referred to as the “standard lysis buffer” (0.15 mM CPRG, 0.125 % Nonidet P-40, 100 mM 2-
mercaptoethanol, and 9 mM MgCl$_2$ in PBS). Using a SpectraMax spectrophotometer, detection of a specific signal was optimal at 560-570 nm for both the optimized lysis buffer (Fig. 1A) and the standard lysis buffer (Fig. 1B), while the peak was slightly right-shifted using EnVision (Fig. 1C). For absorbance measurements in this study, we have used the SpectraMax spectrophotometer. As reference backgrounds, most papers measured absorbance above 635 nm (n=79 out 96 studies reporting background measurement). We subtracted a reference background at 700 nm. The optimal wavelength should be determined for the conditions used in each laboratory.

![Fig. 1: Spectral absorbance analysis of optimized and standard lysis buffer.](image)

**A:** Absorbance spectrum after an ApoE ε3 stimulus and the optimized lysis buffer (3 mM CPRG, 20 mM MgCl$_2$, 5 mM DTT, and 0.2 % Saponin in Hi-S-PBS). **B:** Absorbance spectrum after the same stimulus with ApoE ε3 and the standard lysis buffer (0.15 mM CPRG, 9 mM MgCl$_2$, 100 mM 2-mercaptoethanol, and 0.125 % Nonidet P-40 in PBS). **C:** Absorbance spectrum after an ApoE ε3 stimulus and the optimized lysis buffer (3 mM CPRG, 20 mM MgCl$_2$, 5 mM DTT, and 0.2 % Saponin in Hi-S-PBS) measured with a separate spectrophotometer (EnVision). The optimal spectra were comparable between the two lysis buffers, and it was best to measure the absorbance at 560-570 nm. By measuring the unspecific signal at 700 nm the background was somewhat reduced. The wavelengths (in nm) are shown on the x-axis. OD: optical density is shown on the y-axis. The data shown was obtained 24 hours after cell lysis.

**Lysis buffer components in published protocols**

In the original report, the authors used a CPRG-based assay for detecting β-galactosidase expression and activity from stimulated reporter cells. Out of the 277 papers citing the original report (Sanderson &
Shastri, 1994), 158 papers were selected for further methodological assessment as described in the Methods section. By extensively reviewing the literature, we found that the large majority of the 158 papers used the originally reported protocol (Sanderson & Shastri, 1994): 0.15 mM CPRG, 100 mM 2-mercaptoethanol, 9 mM MgCl$_2$, and 0.125 % Nonidet P-40. Nonidet P-40 is an obsolete name which has been replaced with IGEPLA CA-630 (Sigma-Aldrich). For consistency with previous publications, however, we have used the name Nonidet P-40. Unfortunately, Nonidet P-40 (octyl phenoxypolyethoxyethanol) can be abbreviated inaccurately as NP-40. However, there is a distinct detergent called NP-40 (nonyl phenoxypolyethoxyethanol). As a consequence of the confusion on the name NP-40, and a general lack of product numbers in the investigated publications, it is likely that some papers have used NP-40 but written Nonidet P-40, or vice-versa. Table 1 shows an overview of lysis buffer components reported in the literature. A total of 45 out of the 158 publications using CPRG did not provide information about lysis buffer composition, nor did they refer to methods used in other publications.

Table 1: Overview of lysis buffer components used in 158 papers studied that describe the methodology

<table>
<thead>
<tr>
<th>Buffer component</th>
<th>Buffer component concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-mercaptoethanol</td>
<td>10 µM (n=1) 100 µM (n=7) 100 mM (n=83)</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>1 mM (n=3) 9 mM (n=99)</td>
</tr>
<tr>
<td>Nonidet P-40 (% v/v)</td>
<td>0.125 % (n=68) 0.5 % (n=5)</td>
</tr>
<tr>
<td>NP-40 (% v/v)</td>
<td>0.1 % (n=3) 0.125 % (n=29) 0.15 % (n=1) 0.5 % (n=1)</td>
</tr>
<tr>
<td>CPRG</td>
<td>100 µM (n=1) 120 µM (n=2) 150 µM (n=100) 170 µM (n=1) 300 µM (n=3) 30 mM (n=2)</td>
</tr>
</tbody>
</table>

A total of 277 publications were analyzed and of these, 158 used the colorimetric CPRG assay with the β-galactosidase-expressing αβBW5147 reporter cells. Nonidet P-40 and IPEGAL CA-630 are considered to be the same product (Sigma-Aldrich). Reports using either of these products are referred to as Nonidet P-40 in the table. Nonidet P-40 should not be confused with NP-40. The name NP-40 has likely, but unfortunately, been used for both detergents. “n” refers to the number of publications that used the specified concentration of a buffer component.
As β-galactosidase activity is affected by the surrounding environment, we found it essential to ensure that the conditions were optimal for the enzymatic activity. As enzyme activity should be linear with time when collecting data, effects of each component in the original lysis buffer was tested (CPRG substrate, reducing agent, detergent, as well as phosphates and ions). This was done to ensure that 1) the chosen component at the concentration used gave equal or higher specific signals than the component did in the original report, and 2) the chosen component at the concentration used gave linear time-dependent increasing signal when adding the CPRG substrate-containing lysis buffer to the reporter cells.

In our experiments, we used an altered PBS with six-fold increased phosphate concentration, two-fold increased Mg$^{2+}$ concentration, and three-fold reduced Cl$^{-}$ concentration. This buffer composition was chosen since previous studies have shown that these ions affect β-galactosidase activity (Crescimbeni, Nolan, Clop, Marín, & Perillo, 2010; Hill & Huber, 1971; Juers et al., 2000, 2009, 2012; Roth & Huber, 1996; Sutendra, Wong, Fraser, & Huber, 2007; Tenu et al., 1972; Wutor, Togo, & Pletschke, 2007). However, these lysis buffer modifications of ion concentrations did not affect the results (Supplementary figure 1).

A Saponin-containing lysis buffer gives a more sensitive monitoring of β-galactosidase activity

In 73 of the 158 papers, Nonidet P-40 was used as detergent in the lysis buffer, while 34 studies used NP-40. Nonidet P-40 and NP-40 were used at concentrations between 0.1% and 0.5 %, with 0.125 % being the most commonly used concentration for both detergents (n=68 for Nonidet P-40, and n=29 for NP-40). No other detergent was reported in any of the 158 papers.
We tested the effect of using either 0.125 % or 0.5 % Nonidet P-40 as compared to 0.125 % NP-40 or 0.2 % Saponin in a lysis buffer with 3 mM CPRG, 20 mM MgCl$_2$, and 5 mM DTT in Hi-S-PBS. Saponin was chosen due to its ability to permeate the cell membrane, but not organelle membranes, as to avoid releasing factors from organelles that could possibly influence the expression of β-galactosidase or its activity.

At optimal conditions of cell lysis and β-galactosidase enzyme activity, reactions between β-galactosidase and its substrate (here CPRG) should be linear with time. Interestingly, lysis buffers containing 0.5 % Nonidet P-40 (Fig. 2D) or 0.125 % NP-40 (Fig. 2B) resulted in partially non-linear absorbance recording of enzymatic activity, which contrasted with linear recording when instead using Saponin (Fig. 2A). The detergents might interrupt the interactions between β-galactosidase and its substrate, or they might permeate nuclear membranes, interfering with measurements of β-galactosidase activity.
**Fig. 2: Time-dependent monitoring of enzymatic activity after cell lysis with different detergents.** The graphs depict increasing absorbance with time from between 0 to 4 hours after cell lysis with a higher absorbance reflecting a higher concentration of a colored product due to enzymatic hydrolysis of the CPRG-substrate. The lysis buffers consisted of 3 mM CPRG, 20 mM MgCl$_2$, 5 mM DTT, and either 0.2 % Saponin, 0.125 % NP-40, 0.125 % or 0.5 % Nonidet P-40. Generally, higher $r^2$ values were found when using 0.2 % Saponin as the detergent. mT2 = murine TREM2. The measurements were made in triplicates in three independent experiments. The symbols represent mean ± S.E.M. Please notice that for clarity the scales of the y-axis differ between the four graphs.

Since not all signals were linear with time, simply showing a representative plot to compare the buffers is incorrect. Therefore, the mean of the absorbance measured at 4 hours after cell lysis in three different experiments is shown in Fig. 3. The absorbance readings were affected by the choice of detergent. 0.2 %
Saponin gave significantly higher signals compared to the three other detergents when stimulating with ApoE ε3 (p<0.0001 when comparing to either of the other detergents). When stimulating with anti-mTREM2 antibody, 0.2 % Saponin gave significantly higher signal than 0.5 % Nonidet P-40 and 0.125 % NP-40 (p=0.0030 and p<0.0001), while it was not statistically significant for the two other detergents (p>0.0036). The PMA and anti-goat backgrounds were not higher for the 0.2 % Saponin-containing lysis buffer than for the other detergents.

Fig. 3: Saponin generates a more sensitive reading of enzyme activity when included in the CPRG-containing lysis buffer. The lysis buffers consisted of 3 mM CPRG, 20 mM MgCl₂, 5 mM DTT in Hi-S-PBS, but they varied in choice of detergent (0.125 % Nonidet P-40, 0.5 % Nonidet P-40, or 0.125 % NP-40). We found significantly higher signals with 0.2 % Saponin in response to an anti-mTREM2 antibody compared to when 0.5 % Nonidet P-40 or 0.125 % NP-40 was used. The 150 nM mTREM2-ligand, ApoE ε3-stimulus also gave higher signals with 0.2 % Saponin compared to 0.125 % Nonidet P-40, 0.5 % Nonidet P-40, and 0.125 %
NP-40. The background signals did not vary significantly between the detergents. For all detergents, the signals for stimulation with antibody or ligand were significantly higher than the corresponding backgrounds. The number of planned comparisons were 14, and with a $\alpha$ level at 0.05, statistical significance level was set at $p<0.0036$. Small letters indicate statistical significance level: a: $p=0.0030$ compared to column A, aaaa: $p<0.0001$ compared to column A, bbbb: $p<0.0001$ compared to column B. mT2 = murine TREM2. The graphs depict mean ± S.E.M. from three independent experiments each performed with triplicates. Data shown was obtained by reading enzyme activity at 4 hours after cell lysis.

**DTT as an alternative to 2-mercaptoethanol**

In many applications, DTT has replaced 2-mercaptoethanol as a potent and less toxic reducing agent. In the literature reviewed by us, only 2-mercaptoethanol had been tested, and therefore we wanted to examine the effect of replacing it with DTT in the CPRG-assay. After finding the optimal DTT concentration (Supplementary Figure 2), we compared DTT to the commonly used 2-mercaptoethanol concentration (100 mM) (Fig. 4). There was no significant difference in the absorbance measured from samples lysed with DTT-containing buffer compared to 2-mercaptoethanol-containing buffer. When used together with Saponin and otherwise optimized lysis buffer components, DTT as well as 2-mercaptoethanol gave linear signals over time (Supplementary Figure 3). Especially when considering toxicity, DTT is a valid alternative to 2-mercaptoethanol in the CPRG-assay.
Fig. 4: Replacing 2-mercaptoethanol with DTT does not significantly affect β-galactosidase activity in the CPRG assay. When using a lysis buffer with DTT (5 mM) or 2-mercaptoethanol (100 mM), the enzymatic activity did not statistically differ. The lysis buffer consisted of 3 mM CPRG, 20 mM MgCl₂, 0.2% Saponin in Hi-S-PBS with either 5 mM DTT or 100 mM 2-mercaptoethanol. mT2 = murine TREM2. For both reducing agents, the signals for stimulation with antibody or ligand were significantly higher than the corresponding backgrounds. The number of planned comparisons were 6, and with an α level at 0.05, statistical significance level was set at $p<0.008$. The measurements were made in triplicates in three independent experiments. The bars represent mean ± S.E.M. The data shown was obtained 4 hours after cell lysis.

Increased reproducibility with a higher CPRG concentration

In most publications, the CPRG concentration in the lysis buffer was 0.15 mM (n=100 out of 158) consistent with what was originally reported (Sanderson & Shastri, 1994). When first using the lysis buffer described in the original report, we found a slightly decreased signal with time (after
approximately 20 hours). To ensure that the CPRG substrate concentration was not a limiting factor to the enzyme activity, we tested higher concentrations of CPRG (1-5 mM) and found that the enzymatic reaction was far from saturated with substrate. The signal reached a maximum at a 3 mM CPRG-concentration (Fig. 5), which was then used in the optimized lysis buffer. The signal for anti-mTREM2 antibody-stimulation and ApoE ε3-stimulation were both significantly higher when using 3 mM CPRG than 0.15 mM CPRG (p<0.0001 and p=0.0030, respectively). For 1, 3, and 5 mM CPRG, both stimulation with antibody and with ApoE ε3 gave significantly higher signals than the corresponding backgrounds. When 0.15 mM CPRG was used, these signals were not significantly higher than the corresponding backgrounds. The enzymatic reactions were linear over time (Supplementary Figure 4) indicating high enzymatic stability, minimal substrate depletion, and no product inhibition. In general we found that using substrate at a saturating concentration increased reproducibility.
Fig. 5: Effect of varying the CPRG substrate concentration. The enzymatic activity reached a ceiling at a 3 mM CPRG concentration when stimulating the receptor with a ligand. This concentration gave significantly higher signal of absorbance in response to stimulus with anti-TREM2 antibody or ApoE ε3 as compared to 0.15 mM CPRG (p<0.0001 and p=0.0030, respectively). Further increasing the CPRG concentration from 3 mM to 5 mM did not give higher absorbance measurements after the anti-mTREM2 stimulus. The backgrounds from cells stimulated only with PMA or from exposing cells to anti-goat antibody-coated wells did not vary significantly between the CPRG-concentrations. The lysis buffers consisted of 20 mM MgCl₂, 5 mM DTT, 0.2 % Saponin, and different CPRG concentrations in Hi-S-PBS. The number of planned comparisons were 14, and with an α level at 0.05, statistical significance level was set at p<0.0036. Small letters indicate statistical significance level: aaaa: p<0.0001 compared to column A, bb: p=0.0030 compared to column B. n.s. = not significant. mT2 = murine TREM2.

Measurements were made in triplicates in three independent experiments. The bars represent mean ± S.E.M. The data shown was obtained at 4 hours after cell lysis.

Comparison of optimized lysis buffer to standard lysis buffer
We compared the effectiveness of the optimized lysis buffer with higher substrate concentration (3 mM CPRG) and an alternative detergent (0.2% Saponin) to that of the standard lysis buffer (Sanderson & Shastri, 1994) with 0.125% Nonidet P-40 and lower substrate concentration (0.15 mM CPRG). We also tested 0.125% NP-40 as this was used in 29 papers at the same concentration as Nonidet P-40.

Absorbance measured following specific stimulation of TREM2 was two-fivefold higher in the optimized lysis buffer when measured 4 hours after lysis, and three-sixfold higher when measuring 20 hours after lysis when compared to both standard lysis buffer and an NP-40-containing variant (Fig. 6). The signals after a stimulus with ApoE ε3 (300 nM) or an anti-mTREM2 antibody were significantly higher when using the optimized lysis buffer as compared to the standard lysis buffer with 0.125% Nonidet P-40 or 0.125% NP-40 (p<0.0001 for both stimulations at both time points). The 300 nM ApoE ε3 stimulation gave up to 4-5x higher signals using the optimized lysis buffer as compared to the standard lysis buffer, while the anti-mTREM2 gave up to a ~3x higher signal. Importantly, signals from anti-mTREM2- or 300 nM ApoE ε3-stimulation were significantly higher than the corresponding backgrounds in the optimized lysis buffer (p<0.0001 for both stimulations), while only the 300 nM ApoE ε3-stimulation gave significantly higher signals than the background when using the standard buffer with Nonidet P-40, but only at the 4-hour time point (p=0.002). In the NP-40-containing buffer neither the ApoE ε3- nor the antibody-stimulation gave signals that were significantly higher than the backgrounds. Backgrounds from PMA only or anti-goat only did not vary significantly between the three buffers at either time point.

When using the optimized lysis buffer, the absorbance was linear over time. Conversely, absorbance was not linear over time when using the standard lysis buffer or the NP-40 variant of it, as indicated by the increasing ratio from 4 hours (Fig. 6A) to 20 hours (Fig. 6B) of specific signals between the optimized lysis buffer and the standard lysis buffer including the NP-40 variant. The linearity of the signals when using the three different lysis buffers varied, as can be observed in Fig. 6C-E.
Fig. 6: Comparison of the optimized lysis buffer with the standard lysis buffer and an NP-40-containing variant of it. The optimized lysis buffer gave more linear time-dependent response and higher signals than the standard lysis buffer with either 0.125 % Nonidet P-40 or 0.125 % NP-40. **A:** Absorbance measured at 4 hours after lysis. **B:** Absorbance measured at 20 hours after lysis. **C:** Absorbance from start until 4 hours when using the optimized lysis buffer. **D:** Absorbance from start until 4 hours when using the standard lysis buffer containing 0.125 % Nonidet P-40. **E:** Absorbance from start until 4 hours when using the standard lysis buffer containing 0.125 % NP-40. The number of planned comparisons were 13, and with an α level at 0.05, statistical significance level was set at p<0.0038. Small letters indicate statistical significance level: aaaa: p<0.0001 compared to column A, bbbb: p<0.0001 compared to column B. n.s. = not significant. mT2 = murine TREM2. For the sake of clarity, there are different scales on the y-axis in graphs shown in A-E. The measurements were made in triplicates in three independent experiments. The bars and symbols represent mean ± S.E.M.

The signal depends on the time of reporter cell stimulation
As time is important when studying interactions between receptors and their ligands, we were intrigued to find that most papers used overnight stimulation or equivalent (15-20 hours) independent on whether they were performing co-culture experiments (n=100 out of total 123 reports) or direct stimulations between a ligand and receptor-expressing cells (n=20 out of 31). Some papers reported stimulation times of 24 hours (n=18 for co-culturing, and n=4 for direct stimulation), while few reported direct stimulation of >24 hours (n=3). Likewise, <15 hours stimulation was rarely used (n=5 for co-culturing, n=4 for direct stimulation) (Supplementary Table 2). Some reports (n=15) did not specify stimulation time.

As many receptor/ligand-interactions are fast reactions and too prolonged stimulation can lead to desensitization, we investigated the optimal stimulation time between mTREM2 and its ligand, ApoE ε3. We explored stimulations in a range of 2-6 hours as well as 17 hours, and found that a short stimulation time of 2-4 hours gave a significantly higher signal, as shown by increased absorbance, than when the stimulation lasted for 17 hours (Fig. 7); for anti-mTREM2 stimulation (p<0.0001 for 2h vs. 17h, while n.s. for 4h vs. 17h) and for ApoE ε3-stimulation (p<0.0001 for both 2h and 4h vs. 17h). Conversely, stimulation with antibody cross-linking gave increasing absorbance over the same time-period (Fig. 7).

Thus, the optimal timing might be different for various receptor/ligand-interactions. It is likely that direct interactions between ligands and reporter cells are quicker than between reporter cells and ligand-bearing living cells. However, in general it is worth determining the optimal stimulation time when searching for and quantifying ligand stimulation of the reporter cells, and to report the stimulation time used.
Fig. 7: Effect of time of ligand/antibody stimulation on reporter signal transduction. The optimal time frame to expose the mTREM2-receptor in the BWZ-reporter cells to ApoE ε3 (300 nM) or an anti-mTREM2-stimuli was around 2-4 hours. The signal from an ApoE ε3-stimulation was lowest after 17 hours incubation. Anti-goat background was subtracted from the signal generated by the anti-mTREM2 (anti-mT2)-stimulus (the anti-goat background was equal among all stimulation times). PMA background remained at the same level irrespectively of the time of exposure to stimuli. Small letters indicate statistical significance level: aaaa: p<0.0001 compared to 17 hours of stimulation with antibody, bbb: p=0.003 compared to 17 hours of stimulation with ApoE ε3, bbbb: p<0.0001 compared to 17 hours of stimulation with ApoE ε3. mT2 = murine TREM2.

Measurements were made in triplicates from three individual experiments. The points represent mean ± S.E.M. The data shown was obtained at 4 hours after cell lysis.

Discussion and conclusions

When quantifying an enzymatic reaction, it is important to ensure optimal conditions to avoid inhibiting or limiting factors such as enzyme inhibitors or limited substrate availability. If conditions are designed to optimize β-galactosidase enzymatic activity measurement that is free from inhibitors and limiting factors,
the reaction between β-galactosidase and CPRG substrate should be linear with time. Optimal conditions were ensured by thorough testing of ideal pH, temperature, ion concentrations, and substrate concentration in the presence of different reducing agents and cell-lysing detergents. After an extensive literature review, we found a general lack of information about concentrations of lysis buffer components and sometimes even lack of information about time during which reporter cells were exposed to the ligand stimulus. Among the publications that did provide information about lysis buffer and stimulation time, the large majority referred to the lysis buffer used in the original report (Sanderson & Shastri, 1994) and stimulated cells 15-20 hours. With 277 citations, the original paper has laid the foundations for a vast number of experiments investigating ligands for specific chimeric antigen receptors in reporter cells. With 96 citations in recent years (2010-2017), the paper is still highly cited, and of the 96 citing papers, 61 of the papers used CPRG colorimetry. In 158 of the publications citing the original paper, the colorimetric CPRG assay was used to detect of stimulation of receptor signal transduction in the reporter cells. Presumably, most published results are valid, but unfortunately many experiments were likely done with suboptimal lysis conditions meaning that small signals or variations in signals might have gone unnoticed. We propose using a higher concentration of CPRG in the lysis buffer and to substitute Nonidet P-40 or NP-40 with Saponin. For decreased toxic exposure, 2-mercaptoethanol can be substituted with DTT. We also showed that it is important to test and report various times of ligand stimulation when using the reporter cells. Our suggested optimized method is provided as bullet points in Supporting Information.

With our studies, we provide evidence for an optimized protocol for the CPRG-based colorimetric assay in antigen-presenting reporter cell assays using the α/β BW5147-based cell lines as originally described (Sanderson & Shastri, 1994). With up to six-fold higher signals from stimulated cells and more stable, linear absorbance readout over time, the protocol should be valuable to many researchers examining
ligand-receptor interactions since it gives more consistent and reproducible results and enables
detection of ligands only exerting weak receptor stimulation.

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Fig. 8: Spectral absorbance analysis of optimized and standard lysis buffer. A: Absorbance spectrum after an ApoE ε3-stimulus and the optimized lysis buffer (3 mM CPRG, 20 mM MgCl₂, 5 mM DTT, and 0.2 % Saponin in Hi-S-PBS). B: Absorbance spectrum after the same stimulus with ApoE ε3 and the standard lysis buffer (0.15 mM CPRG, 9 mM MgCl₂, 100 mM 2-mercaptoethanol, and 0.125 % Nonidet P-40 in PBS). C: Absorbance spectrum after an ApoE ε3-stimulus and the optimized lysis buffer (3 mM CPRG, 20 mM MgCl₂, 5 mM DTT, and 0.2 % Saponin in Hi-S-PBS) measured with a separate spectrophotometer (EnVision). The optimal spectra were comparable between the two lysis buffers, and it was best to measure the absorbance at 560-570 nm. By measuring the unspecific signal at 700 nm the background was somewhat reduced. The wavelengths (in nm) are shown on the x-axis. OD: optical density is shown on the y-axis. The data shown was obtained 24 hours after cell lysis.

Fig. 9: Time-dependent monitoring of enzymatic activity after cell lysis with different detergents. The graphs depict increasing absorbance with time from between 0 to 4 hours after cell lysis with a higher absorbance reflecting a higher concentration of a colored product due to enzymatic hydrolysis of the CPRG-substrate. The lysis buffers consisted of 3 mM CPRG, 20 mM MgCl₂, 5 mM DTT, and either 0.2 % Saponin, 0.125 % NP-40, 0.125 % or 0.5 % Nonidet P-40. Generally, higher r² values were found when using 0.2 % Saponin as the detergent. mT2 = murine TREM2. The measurements were made in triplicates in three independent experiments. The symbols represent mean ± S.E.M. Please notice that for clarity the scales of the y-axis differ between the four graphs.

Fig. 10: Saponin generates a more sensitive reading of enzyme activity when included in the CPRG-containing lysis buffer. The lysis buffers consisted of 3 mM CPRG, 20 mM MgCl₂, 5 mM DTT in Hi-S-PBS, but they varied in choice of detergent (0.125 % Nonidet P-40, 0.5 % Nonidet P-40, or 0.125 % NP-40). We found significantly higher signals with 0.2 % Saponin in response to an anti-mTREM2 antibody compared to when 0.5 % Nonidet P-40 or 0.125 % NP-40 was used. The 150 nM mTREM2-ligand, ApoE ε3-stimulus also gave higher signals with 0.2 % Saponin compared to 0.125 % Nonidet P-40, 0.5 % Nonidet P-40, and 0.125 % NP-40. The background signals did not vary significantly between the detergents. For all detergents, the signals for stimulation with antibody or ligand were significantly higher than the corresponding backgrounds. The number of planned comparisons were 14, and with an α level at 0.05, statistical significance level was set at p<0.0036. Small letters indicate statistical significance level: a: p=0.0030 compared to column A, aaaa: p<0.0001 compared to column A, bbbb: p<0.0001 compared to
column B. mT2 = murine TREM2. The graphs depict mean ± S.E.M. from three independent experiments each performed with triplicates. Data shown was obtained by reading enzyme activity at 4 hours after cell lysis.

**Fig. 11: Replacing 2-mercaptoethanol with DTT does not significantly affect β-galactosidase activity in the CPRG assay.** When using a lysis buffer with DTT (5 mM) or 2-mercaptoethanol (100 mM), the enzymatic activity did not statistically differ. The lysis buffer consisted of 3 mM CPRG, 20 mM MgCl₂, 0.2 % Saponin in Hi-S-PBS with either 5 mM DTT or 100 mM 2-mercaptoethanol. mT2 = murine TREM2. For both reducing agents, the signals for stimulation with antibody or ligand were significantly higher than the corresponding backgrounds. The number of planned comparisons were 6, and with an α level at 0.05, statistical significance level was set at p<0.008. The measurements were made in triplicates in three independent experiments. The bars represent mean ± S.E.M. The data shown was obtained 4 hours after cell lysis.

**Fig. 12: Effect of varying the CPRG substrate concentration.** The enzymatic activity reached a ceiling at a 3 mM CPRG concentration when stimulating the receptor with a ligand. This concentration gave significantly higher signal of absorbance in response to stimulus with anti-TREM2 antibody or ApoE ε3 as compared to 0.15 mM CPRG (p<0.0001 and p=0.0030, respectively). Further increasing the CPRG concentration from 3 mM to 5 mM did not give higher absorbance measurements after the anti-mTREM2-stimulus. The backgrounds from cells stimulated only with PMA or from exposing cells to anti-goat antibody-coated wells did not vary significantly between the CPRG concentrations. The lysis buffers consisted of 20 mM MgCl₂, 5 mM DTT, 0.2 % Saponin, and different CPRG concentrations in Hi-S-PBS. The number of planned comparisons were 14, and with an α level at 0.05, statistical significance level was set at p<0.0036. Small letters indicate statistical significance level: aaaa: p<0.0001 compared to column A, bb: p=0.0030 compared to column B. n.s. = not significant. mT2 = murine TREM2. Measurements were made in triplicates in three independent experiments. The bars represent mean ± S.E.M. The data shown was obtained at 4 hours after cell lysis.

**Fig. 13: Comparison of the optimized lysis buffer with the standard lysis buffer and an NP-40-containing variant of it.** The optimized lysis buffer gave more linear time-dependent response and higher signals than the standard lysis buffer with either
0.125 % Nonidet P-40 or 0.125 % NP-40. **A:** Absorbance measured at 4 hours after lysis. **B:** Absorbance measured at 20 hours after lysis. **C:** Absorbance from start until 4 hours when using the optimized lysis buffer. **D:** Absorbance from start until 4 hours when using the standard lysis buffer containing 0.125 % Nonidet P-40. **E:** Absorbance from start until 4 hours when using the standard lysis buffer containing 0.125 % NP-40. The number of planned comparisons were 13, and with an α level at 0.05, statistical significance level was set at p<0.0038. Small letters indicate statistical significance level: aaaa: p<0.0001 compared to column A, bbbb: p<0.0001 compared to column B. n.s. = not significant. mT2 = murine TREM2. For the sake of clarity, there are different scales on the y-axis in graphs shown in A-E. The measurements were made in triplicates in three independent experiments. The bars and symbols represent mean ± S.E.M.

**Fig. 14: Effect of time of ligand/antibody stimulation on reporter signal transduction.** The optimal time frame to expose the mTREM2-receptor in the BWZ-reporter cells to ApoE ε3 (300 nM) or an anti-mTREM2-stimuli was around 2-4 hours. The signal from an ApoE ε3-stimulation was lowest after 17 hours incubation. Anti-goat background was subtracted from the signal generated by the anti-mTREM2 (anti-mT2)-stimulus (the anti-goat background was equal among all stimulation times). PMA background remained at the same level irrespectively of the time of exposure to stimulus. Small letters indicate statistical significance level: aaaa: p<0.0001 compared to 17 hours of stimulation with antibody, bbb: p=0.003 compared to 17 hours of stimulation with ApoE ε3, bbbb: p<0.0001 compared to 17 hours of stimulation with ApoE ε3. mT2 = murine TREM2. Measurements were made in triplicates from three individual experiments. The points represent mean ± S.E.M. The data shown was obtained at 4 hours after cell lysis.