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# Optimization of the cryopreservation of biological resources, *Toxoplasma gondii* tachyzoites, using flow cytometry

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ABSTRACT

The conservation of *Toxoplasma gondii* strains isolated from humans and animals is essential for conducting studies on *Toxoplasma*. Conservation is the main function of the French Biological *Toxoplasma* Resource Centre (BRC *Toxoplasma*, France, http://www.toxocrb.com/). In this study, we have determined the suitability of a standard cryopreservation methodology for different *Toxoplasma* strains using the viability of tachyzoites assayed by flow cytometry with dual fluorescent labelling (calcein acetoxymethyl ester and propidium iodide) of tachyzoites. This method provides a comparative quantitative assessment of viability after thawing. The results helped to define and refine quality criteria before tachyzoite cryopreservation and optimization of the cryopreservation parameters. The optimized cryopreservation method uses a volume of 1.0 mL containing $8 \times 10^6$ tachyzoites, in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% foetal calf serum (FCS). The cryoprotectant additive is 10% v/v $Me_2SO$ without incubation. A cooling rate of ~1 °C/min to −80 °C followed, after 48 h, by storage in liquid nitrogen. Thawing was performed using a 37 °C water bath that produced a warming rate of ~100 °C/min, and samples were then diluted 1:5 in IMDM with 5% FCS, and centrifuged and resuspended for viability assessment.



## 1. Introduction

*Toxoplasma gondii*, the causative agent of toxoplasmosis, is a protozoan parasite that is estimated to infect more than one-third of the world's human population [19]. Its life cycle comprises a series of five developmental stages in the intestinal epithelium of the feline definitive host and three stages relevant to infection in intermediate hosts which include all other species of mammals. These stages are sporozoites (contained in oocysts), and the obligate intracellular stages tachyzoites, and bradyzoites [19]. The population structure of *T. gondii* consists of three main clonal lineages that correlate with virulence expression in mice [9,23].

The early studies on conservation of protozoa (including *T. gondii*) were conducted in the mid-20th century. Despite the absence of reliable methods for assessing parasite viability after thawing, these initial experiments were used to optimize storage conditions and particularly to determine the ideal method for conservation [6]. A consensus were developed on the need to conserve *T. gondii* strains by cryopreservation and to store at the temperature of liquid nitrogen [3,22], with $Me_2SO$ as the cryoprotectant additive (CPA) [14,20,25]. Later, Hubálek [10] and Miyake et al. [18] sought to determine the optimal CPA concentration for

*Abbreviations:* BRC *Toxoplasma*, Biological *Toxoplasma* Resource Centre; Calcein-AM, calcein acetoxymethyl ester; CPA, cryoprotectant additive; FCS, foetal calf serum; IMDM, Iscove's Modified Dulbecco's Medium; PBS, phosphate buffered saline; PI, propidium iodide.

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cryopreservation mainly of extracellular protozoa. Viability assessment of these protozoa was based on observations of parasite mobility. This method of assessing viability, however, is not suitable for the intracellular parasite stages of *T. gondii*.

Booth et al. [1] described a mechanism for optimizing the cryopreservation of bradyzoites with viability assessment after thawing determined from cell cultures. Tachyzoites are the easiest parasite stage to obtain and to use in research. The method defined for bradyzoite cryopreservation is, however, not optimal for tachyzoites. Moreover, in the study of Booth et al., post-thaw viability assessment was semi-quantitative using microscopical enumeration of parasites after five days of culture, compared to the number of parasites prior to cryopreservation. With this method it is not possible to accurately determine percent viability after thawing, due to the time interval between thawing and parasite assessment.

Nevertheless, these studies have provided a consensus on some of the cryopreservation steps: cooling at −1 °C/min to −80 °C, followed by storage in liquid nitrogen. However, the published reports have used different CPA concentrations, different incubation times and temperatures following CPA addition, and different concentrations of foetal calf serum (FCS) in the cryopreservation medium. Differences could be ascribed mainly to the lack of a technique sensitive enough to determine the viability of the parasites for the purpose of optimizing the protocol steps. For example, Smith [25] showed that a loss of 1 log of tachyzoites could be observed after thawing compared to pre-cryopreservation.

Conservation of strains is essential for toxoplasmosis research, and is the primary function of the French Biological *Toxoplasma* Resource Centre (BRC *Toxoplasma*, France, http://www.toxocrb.com/). BRC *Toxoplasma* was created in 2002 to preserve, under strict guidelines, those strains of *T. gondii* isolated from *Toxoplasma* infections in humans or animals. Currently the BRC *Toxoplasma* collection comprises 903 human strains and 374 animal strains. Since January 2010, the quality management at BRC *Toxoplasma* has received certification to the French standard NF 78 S96-900.

For BRC *Toxoplasma*, the establishment of optimal procedures for strain conservation is essential. Consequently, we have evaluated the performance of flow cytometry as a quantitative method to assess the viability of tachyzoites, and we have used this method to optimize conservation by cryopreservation. Because a number of the steps in cryopreservation are generic, our study has focused on optimization of the concentration of $Me_2SO$ and the incubation time in CPA, and on the concentrations of FCS used in the cryopreservation medium. Our studies have defined a protocol that can be used to cryopreserve the tachyzoites of the different strains of *T. gondii*.

## 2. Materials and methods

### 2.1. Toxoplasma gondii strains

Tachyzoites of three *T. gondii* strains, belonging to three different genotypes, were used: RH strain (genotype I) and ME-49 strain (genotype II), are reference strains that represent >10% of samples ordered by research teams from BRC *Toxoplasma*; and the TgH 00002 strain (atypical) [4], cryopreserved for several years, as control. All strains were provided by BRC *Toxoplasma*.

### 2.2. Cell and parasite cultures

*T. gondii* tachyzoites were maintained on Vero cell monolayers (ATCC, CCL-81) at 37 °C in a 5% $CO_2$-humidified incubator. Cells and parasites were grown in Iscove's Modified Dulbecco's Medium/Glutamax® (IMDM, Invitrogen, France), with 2% (v/v) FCS (Biowest, France) and antibiotics (100 IU/mL penicillin and 0.1 mg/mL streptomycin) (GIBCO®) in a culture flask (75 $cm^2$). This culture method optimizes Vero cell adhesion, thereby facilitating proliferation of tachyzoites. Cells and tachyzoites were counted in a Kova® Slide counting chamber in the presence of trypan blue [26]. Parasite cultures were determined to be free of *Mycoplasma* spp. contamination before cryopreservation by real-time PCR [11].

### 2.3. Freezing and thawing experiments

Cryopreservation medium and cooling methods were essentially as commonly used for cell storage [16,17,24]. The pellet from centrifugation of one culture flask was diluted in 10 mL of complete IMDM, with 5% (v/v) FCS and antibiotics (as above). Tachyzoites were counted in the presence of trypan blue. To $8 \times 10^6$ tachyzoites, pelleted by centrifugation, was added IMDM/Glutamax® with FCS and dimethyl sulfoxide ($Me_2SO$) and 1 mL aliquots were loaded into sterile cryovials (system 100™, Nalgene®).

In the first experiment, using the RH strain tachyzoites, 18 conditions were evaluated in triplicate, with variations in $Me_2SO$ concentration (10%, 15% and 20%) and either 0 or 30 min incubation together with different concentrations of FCS (5%, 10% and 20%). The second experiment used the ME-49 strain, and tested eight cryopreservation conditions, in triplicate: 10% and 15% $Me_2SO$ with incubation times between 0 and 15 min, in the presence of 5% or 10% FCS.

All cryovials were loaded into a freezing container (Nalgene®, Mr. Frosty) which was placed into a −80 °C freezer. This method produced a cooling rate of approximately −1 °C/min. After 48 h in the freezer, the cryovials were transferred to liquid nitrogen storage for one month.

Cryovials were thawed by immersion in a circulating 37 °C water bath, and then the cryovial contents were added to 5 mL IMDM/Glutamax® medium supplemented with 5% FCS and antibiotics. After centrifugation to remove $Me_2SO$, 1 mL of complete medium was added and parasites were labelled.

### 2.4. Alive and dead tachyzoites

Control live tachyzoites were obtained from the medium following lysis of Vero cell cultures. Control dead tachyzoites were prepared by heating live tachyzoites to 90 °C, a method that kills tachyzoites without altering their morphology [8].

### 2.5. Fluorescence microscopy and flow cytometry analysis

To assess membrane integrity, a dual label of calcein-AM (2 μM) and propidium iodide (PI; 1 μM) was used (Molecular Probes®, Invitrogen™ detection technologies, USA) according to the manufacturer's instructions. Calcein-AM stains living tachyzoites green, PI stains dead tachyzoites red, while double labelling identifies apoptotic parasites [2,5,12,21]. Probes were selected according to the characteristics of laser flow cytometer ($\lambda_{excitation} = 488$ nm). Moreover, calcein-AM also reflects metabolic activity of tachyzoites (cytosolic marker for the presence of active esterases).

Tachyzoites were washed in cold PBS (Phosphate Buffered Saline), pH 7.2 (Invitrogen, France), then the probes were added and the samples incubated in the dark for 20 min prior to imaging. Labelled parasites were observed using a Zeiss Axiovert 200 video microscope (Zeiss, 150 Germany).

Labelled tachyzoites were also evaluated by flow cytometry. After thawing, tachyzoites were washed in cold PBS pH 7.2, the calcein-AM and PI probes added and samples incubated in the dark for 20 min. Comparable numbers of parasites were used for each independent test. Flow cytometry was performed using a

FACSCalibur™ Instrument (BD Biosciences, USA). The tachyzoite population was gated based on forward and side light-scattering properties. At least 10,000 gated events were evaluated for each condition. Analysis was performed on a logarithmic scale using CellQuest™ software (BD Biosciences). A control sample (without labelling) was included in each run with acquisition and analysis gates set accordingly. Each condition (triplicate) was expressed as mean ± standard deviation.

## 3. Results

### 3.1. Flow cytometry

Before testing the samples from BRC *Toxoplasma*, we used control samples to verify that the live and dead tachyzoites properly assimilated the calcein-AM and PI probes by fluorescence microscopy. Fig. 1 shows the uptake of probes by live and dead tachyzoites. Then, flow cytometry (Fig. 2) was used to test samples from four cryovials that had been stored for several years at BRC *Toxoplasma* (Table 1). We determined that the cryovial of RH strain tachyzoites stored in liquid nitrogen for 17 years was 11% viable (assuming viability before cryopreservation was 100%), that RH strain tachyzoites stored in liquid nitrogen for 13 years were 22% viable, and TgH 00002 strain tachyzoites stored for 9 years were between 42 and 54% viable depending on their concentration prior to cryopreservation. These results suggest a decrease in viability over time and/or the importance of the condition of the tachyzoites before cryopreservation. Higher viabilities were observed from samples with larger numbers of tachyzoites. These findings prompted us to test the efficiency of the flow cytometry viability assessment with tachyzoites cryopreserved using different conditions.

### 3.2. Optimization of the cryopreservation protocol

Optimization of the cryopreservation protocol was conducted with the RH reference strain (genotype I). A concentration effect of $Me_2SO$ was observed: viability using 5% FCS and 10% $Me_2SO$ was on average 94% compared to non-cryopreserved samples (by flow cytometry) and was approximately 7% lower when the $Me_2SO$ concentration was increased to 15% and approximately 27.5% lower with 20% $Me_2SO$ (Table 2). Increasing the concentration of FCS from 5% to 10% had little effect, but use of 20% resulted in a 26.5% lower recovery of viable tachyzoites with 10% $Me_2SO$. The effects of increasing the concentration of $Me_2SO$ and FCS together were cumulative, so that the combination of 20% FCS and 20% $Me_2SO$ resulted in an approximately 39.5% reduction in viability compared to 5% FCS with 10% $Me_2SO$. There was essentially no effect of increasing the incubation time in $Me_2SO$ from 0 to 30 min at any concentration of $Me_2SO$ or FCS (Table 2).

### 3.3. Genotype

Using the ME-49 reference strain (genotype II), we observed similar levels of tachyzoite viability under the tested conditions as with the RH strain (Table 3). We cryopreserved samples of tachyzoites of genotype I and II strains using the same method (10% $Me_2SO$ without incubation, and 10% FCS) and demonstrated 95 ± 4% viability for genotype I and 92 ± 4% viability for genotype II after cryopreservation and storage for one month.

### 3.4. Cryopreservation protocol and cell culture

Six cryovials, RH and ME-49 strains in triplicate, prepared and cryopreserved using our optimized protocol (10% $Me_2SO$ with no incubation time, and 10% FCS), were thawed and tested in cell culture to verify tachyzoite infectivity. Tachyzoites from all cryovials were able to generate workable cell cultures after an average of 10 ± 1 days. By comparison, the time taken for non-cryopreserved control samples of tachyzoites to generate workable cell cultures was 7 ± 1 days. By this culture assay method, therefore, compared to non-cryopreserved controls, the cryopreserved/thawed tachyzoites were estimated to have lost a small proportion of viable tachyzoites. These data confirm the viability and infectivity of strains subjected to cryopreservation and thawing.

## 4. Discussion – conclusion

The purpose of this study was to optimize a protocol for the cryopreservation of *T. gondii* tachyzoites at BRC *Toxoplasma*. In parasitology, methods to determine parasite viability are generally difficult with intracellular parasites such as *T. gondii*. In order to optimize the cryopreservation protocol, the development of a method for assessing tachyzoite viability was therefore considered an essential step. Until now, most experimental models have provided an assessment of tachyzoite viability after thawing by inoculating mice – this provides a definitive assessment of infectivity

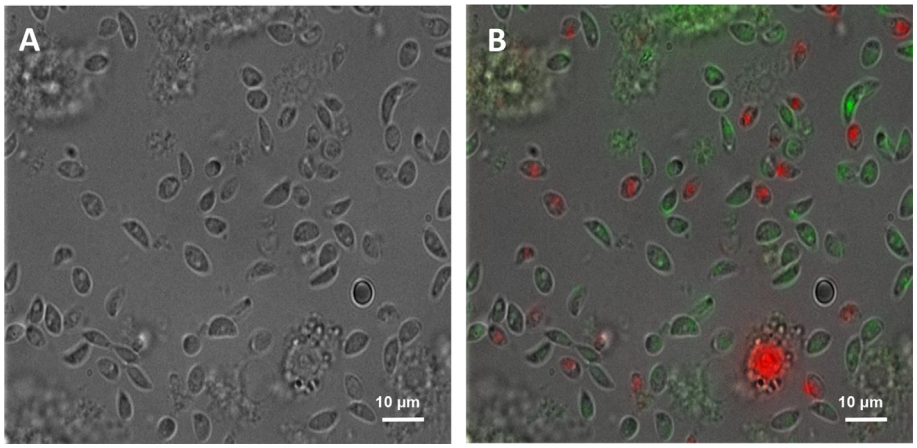


**Fig. 1.** Evaluation of tachyzoites by label incorporation and fluorescence microscopy. (A) Phase contrast image of RH strain tachyzoites post-thaw. (B) Localisation of propidium iodide (red fluorescence, dead tachyzoite) and calcein-AM (green fluorescence, viable tachyzoite) in RH strain tachyzoites.

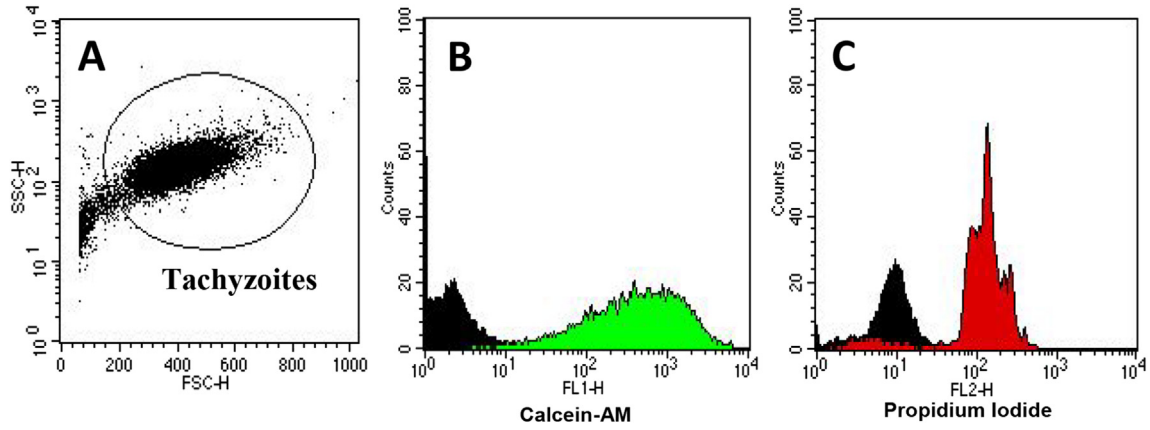


**Fig. 2.** Viability of *Toxoplasma* strains as measured by flow cytometry. (A) Dot-plot showing tachyzoite population based on parasite complexity (logarithmic scale, SSC-H) and parasite size (linear scale, FSC-H). (B–C) Histograms showing (B) the fluorescence intensity of calcein-AM in viable *Toxoplasma*, and (C) the fluorescence intensity of PI in dead *Toxoplasma*. The numbers of tachyzoites are represented on the y axis (counts).

**Table 1**
Tachyzoite viability of *T. gondii* RH and TgH 00002 strains with duration of cryostorage in comparison to the numbers of tachyzoites present before cryopreservation.

| *Toxoplasma* strains | Duration of freezing (years) | Count before freezing (tachyzoites/mL) | Cytometry viability (%) |
|---|---|---|---|
| RH | 17 | $0.5 \cdot 10^6$ | 11 |
| RH | 13 | $7.0 \cdot 10^6$ | 22 |
| TgH 00002 | 9 | $7.5 \cdot 10^6$ | 42 |
| TgH 00002 | 9 | $16.6 \cdot 10^6$ | 54 |

but is slow and expensive. In the mouse model the serology is monitored up to three to four weeks after inoculation [15]. Seroconversion confirms infectivity, and the titre of antibodies gives an indication of the percentage viability (the titre is considered to be proportional to the percentage of viable tachyzoites in the inoculum) [15]. Other models of infectivity look for the presence of parasites in the tissues or peritoneal fluid after inoculation [7,13,27]. Although the mouse model can provide an assessment of the strain viability, this animal method is inefficient and also inaccurate because the presence of even a small number of living tachyzoites is sufficient to induce infection of mice.

To carry out experiments with cell cultures (the most commonly used experimental model in studies of *T. gondii*), a small number of live tachyzoites is not sufficient to produce a useful tachyzoite culture. Aside from considerations related to animal experimentation, these essentially indirect determinations of viability are not appropriate to establish a method for optimal cryopreservation.

Flow cytometry, using dual fluorescent labelling of tachyzoites (calcein-AM and PI), has enabled us to develop a protocol for assessing tachyzoite viability, independent of the use of mice. Furthermore, this model has enabled us to accurately determine the mortality and viability of tachyzoites after thawing (more accurate, more reproducible, and less time-consuming than the trypan blue exclusion test). The advantages of this method are an accurate assessment of viability after thawing, ensuring integrity of the strains even after long periods of storage at liquid nitrogen temperatures.

Our results show that several cryopreservation conditions are approximately equivalent. We obtained 95 ± 4% viability for the genotype I strain and 92 ± 4% viability for the genotype II strain after cryopreservation and storage for one month. The optimized protocol combines 10% $Me_2SO$ without incubation, and 10% FCS.

Importantly, these results helped us refine the quality criteria before cryopreserving the tachyzoites by defining a percent viability of tachyzoites using flow cytometry. We were thus able to optimize the conditions to ensure improved conservation, in comparison with other protocols (Table 4): 1.0 mL final volume containing $8 \times 10^6$ tachyzoites per cryovial, 10% $Me_2SO$ with no incubation time, and 10% FCS. The results have enabled us to establish standards for biological quality for *T. gondii* samples stored at the BRC *Toxoplasma*.

As a result of these studies, we have introduced a stability program for the long-term evaluation of cryopreserved samples: cryopreservation of batches of cryovials of ME-49 strain tachyzoites and assessment of tachyzoite viability at 1, 2, 5, 8 and 10 years, in order to analyse the effects of long-term storage on the conserved samples. The flow cytometry method using dual fluorescent labelling, will also enable us to assess the viability of the existing strains cryopreserved by earlier nonoptimized methods and stored at the BRC *Toxoplasma* under different conditions (some strains have been stored for more than 25 years), in order to know their status, quantify their viability and determine interest in

**Table 2**
Percent viability of RH strain tachyzoites (type I) following cryopreservation using concentrations of $Me_2SO$ between 10% and 20% and FCS concentrations between 5% and 20% (mean ± standard deviation, results from triplicate experiments). Percent viability using flow cytometry expressed as the viability of thawed tachyzoites compared to tachyzoites prior to cryopreservation.

| | $Me_2SO$ 10% | | $Me_2SO$ 15% | | $Me_2SO$ 20% | |
|---|---|---|---|---|---|---|
| | 0 min | 30 min | 0 min | 30 min | 0 min | 30 min |
| FCS 5% | 95 ± 4% | 93 ± 2% | 87 ± 6% | 87 ± 8% | 66 ± 10% | 67 ± 8% |
| FCS 10% | 95 ± 4% | 93 ± 3% | 71 ± 1% | 78 ± 1% | 54 ± 8% | 55 ± 5% |
| FCS 20% | 67 ± 10% | 68 ± 11% | 70 ± 5% | 73 ± 5% | 54 ± 6% | 55 ± 7% |

**Table 3**
Percent viability of ME-49 strain tachyzoites (type II) following cryopreservation using concentrations of $Me_2SO$ of 10% and 15% and FCS concentrations of 5% and 10% (mean ± standard deviation, results from triplicate experiments). Percent viability using flow cytometry expressed as the viability of thawed tachyzoites compared to tachyzoites prior to cryopreservation.

| | $Me_2SO$ 10% | | $Me_2SO$ 15% | |
|---|---|---|---|---|
| | 0 min | 15 min | 0 min | 15 min |
| FCS 5% | 94 ± 3% | 92 ± 4% | 90 ± 3% | 86 ± 4% |
| FCS 10% | 92 ± 4% | 91 ± 5% | 86 ± 7% | 83 ± 5% |

**Table 4**
Comparison of reported protocols from key studies for the cryopreservation of *T. gondii*, in comparison with the protocol outlined in this study from the BRC *Toxoplasma*.

| | Smith [25] 1973 | Booth [1] 1996 | Miyake [18] 2004 | Zheng [27] 2012 | BRC *Toxoplasma* |
|---|---|---|---|---|---|
| Tachyzoites | Intracellular | Extracellular | / | Intratissular | Extracellular |
| Passage number in cell culture | 8 | / | / | / | <10 |
| Count before freezing (tachyzoites/mL) | $4–5 \cdot 10^6$ | / | / | / | $8 \cdot 10^6$ |
| % $Me_2SO$ | / | 12.5% | 10% | 12% | 10% |
| $Me_2SO$ contact time | / | / | 30 min | / | No |
| % FCS | / | 3% | / | 20% | 10% |
| Freezing step | −1 °C/min | −1 °C/min | −1 °C/min | / | −1 °C/min |
| Shortest storage time at −80°C | / | 24 h | / | 3 months | 48h |
| Preservation method | Liquid nitrogen | Liquid nitrogen | / | −80 °C | Liquid nitrogen |

maintaining their conservation, especially for those strains with no apparent viability but with potential for genetic support.

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## Conflict of interest

The authors declare that there are no conflicts of interest.

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