Sains Malaysiana 48(5)(2019): 1105–1110
http://dx.doi.org/10.17576/jsm-2019-4805-19

Optimization of Cryopreserved Single Cell Suspension of Gastric Epithelial Cells for Successful DotScan™ Antibody Microarray

(Pengoptimuman Pengkrioawetan Ampaian Sel Tunggal Sel Epitelium Gaster bagi Kejayaan Mikroatur Antibodi DotScan™)

ASIF SUKRI, ALFIZAH HANAFIAH*, NIK RITZA KOSAI, MUSTAFA MOHAMED TAHER, ISA MOHAMED ROSE & RAMELAH MOHAMED

ABSTRACT

Cell cryopreservation plays a pivotal role for successful immunological assays. DotScan™ antibody microarray employs live cells with satisfactory cell viability for detection of clusters of differentiation (CD) antigens in various diseases. Effective cryopreservation contributes to successful capture of cells on DotScan™ antibody microarray. The purpose of this study was to evaluate the effect of cells viability on cells binding to specific CD antigens. Cells with similar concentration but different percentage of cell viabilities were captured on DotScan™ slides. Cells stored for 5 days at -80ºC and added with freezing medium in stepwise manner had higher viabilities as compared to cells stored for 6 months at -80ºC and added with freezing medium in direct manner (p=0.012). Cells with 88% viability had higher binding to CD44/CD29 control antibodies as compared to cells with 33% viability. Short-term storage of cells and the technique for freezing medium addition to cells play a critical role for successful cryopreservation of single cell suspension.

Keywords: Cell surface antigens; cryopreservation; DotScan™ antibody microarray; epithelial cells

ABSTRAK

Pengkrioawetan sel memainkan peranan penting dalam kejayaan asai imunologi. Mikroatur antibodi DotScan™ memerlukan sel hidup yang memuaskan untuk pengesanan antigen kluster pembezaan (CD) dalam pelbagai penyakit. Keberkesanan pengkrioawetan menyumbang kepada kejayaan penangkapan sel ke atas mikroatur antibodi DotScan™. Tujuan kajian ini adalah untuk menilai kesan daya hidup sel ke atas pelekatan sel terhadap antigen CD yang khusus. Sel dengan kepekatan yang sama tetapi daya hidup yang berlainan ditangkap ke atas slaid DotScan™. Sel disimpan selama 5 hari pada suhu -80ºC dan ditambah media takat beku secara berperingkat mempunyai daya hidup yang lebih tinggi berbanding sel yang disimpan selama 6 bulan dan ditambah media takat beku secara langsung (p=0.012). Sel dengan daya hidup 88% mempunyai pelekatan yang lebih tinggi terhadap antibodi kawalan CD44/CD29 berbanding sel dengan daya hidup 33%. Sel yang disimpan untuk jangka masa singkat dan teknik menambah media takat beku terhadap sel memainkan peranan penting untuk kejayaan pengkrioawetan ampaian sel tunggal.

Kata kunci: Antigen permukaan sel; mikroatur antibodi DotScan™; pengkrioawetan; sel epitelium

INTRODUCTION

Long term storage of viable cells is important in tissue culture and immunology applications. In clinical works, cryopreservation can be applied for freezing cells and organs, blood transfusion, artificial insemination and in vitro fertilization. Obtaining viable cells after cryopreservation is pivotal to reduce cost of getting new cells and to ensure successful immunological assays. One of the immunological assays that rely heavily on high cells viability after cryopreservation is DotScan™ antibody microarray (Medsaic Pty Ltd, Eveleigh, NSW, Australia). DotScan™ array employs more than 140 clusters of differentiation (CD) antibodies on one slide for profiling of many CD antigens in a single assay. Current DotScan™ antibody microarray consists of 82 CD antibodies against established CD antigens (T and B cells markers and myeloid cell markers) and 40 CD antibodies selected against specific disease that can be customized (Sukri et al. 2016). CD antigens are cell surface molecules expressed mainly on leukocytes and other cells that are important in immune responses (Zhou et al. 2010). Hence, DotScan™ antibody microarray has been used to immunophenotype CD antigens in a number of diseases such as leukaemia (Belov et al. 2003), colorectal cancer (Zhou et al. 2010), liver diseases (Rahman et al. 2012) and gastric cancer (Sukri et al. 2016). DotScan™ antibody microarray enables rapid characterization of discrete leukemia subtypes whose CD antigens' expression may be correlated with leukemia typing. A study on leukemia has shown that DotScan™ antibody microarray can discriminate disease types from B-cell chronic lymphocytic leukemia, T-cell acute leukemia and prolymphocytic leukemia based on cell binding towards certain CD antibodies that are markers for T cells

and B cells (Belov et al. 2005). In this assay, live cells with high expression of CD antigens will be captured and enumerated by using specialized software. Suspension of live cells derived from human mucosal tissue or blood in the forms of single cell suspension of mixed epithelial cells and leukocytes for the former or polymorphonuclear cells (PBMC) for the latter will be captured onto nitrocellulose layer of microarray slide containing more than 140 CD antigens. Upon capture on slide array, live cells form pseudopodia and subsequently bind to specific CD antibodies they highly express. Cryopreservation at -80ºC is essential to break mucous property of mucosal epithelial cells for successful cell capture. Four million cells with satisfactory cell viability are important for optimized cells binding (Zhou et al. 2011, 2010).

Recent advances in cryopreservation technique include slow freezing of cells in controlled freezer before storage in liquid nitrogen. However, this technique appears to be costly and unnecessary for short-term storage of cells. Conventional technique of cells cryopreservation follows the rule of freezing slowly to lessen formation of ice crystals that can lead to cell lysis (Güll et al. 2009). In this procedure, cells were cooled down at 1ºC/min to ensure proper freezing condition where ice crystal does not form. Nonetheless, this technique is still poorly developed and requires further evaluation. Preparation and technique to preserve the cells are paramount for successful cryopreservation. Long-term storage of cells includes cryopreservation in liquid nitrogen while short-term storage of cells may be achieved by storage at -80ºC. But how long can we store cells at -80ºC before storage in liquid nitrogen? Current knowledge suggests freezing cells slowly at -80ºC overnight before being transferred to liquid nitrogen. Many laboratories today opted to store cells at -80ºC than liquid nitrogen due to cost for maintenance of liquid nitrogen. It is suggested that short-term storage of cells in -70ºC dry ice for several weeks had satisfactory viability (Weinberg et al. 2011). Storage of cells at -80ºC or dry ice is important as cells are usually shipped from different countries and thus shipment takes usually days or weeks to arrive at intended destination (Weinberg et al. 2011). Nevertheless, it is not known exactly how long cells can be stored satisfactorily at -80ºC.

Apart from traditional rule of freezing slowly and thawing quickly, current techniques employed to improve cells viability after cryopreservation include slow addition of pre-warmed media to cells and use of pre-warmed media post thaw process (Ramachandran et al. 2012; Weinberg et al. 2009). Preparation technique of single cell suspension from tissue before cryopreservation is also important to maintain cell viability post freeze thaw process. This includes adequate incubation time of tissue with digestive enzyme and gentle handling of tissue (Zhou et al. 2011). To increase cells viability after cryopreservation, use of elevated levels of serum and stepwise addition of freezing medium have been applied. Primary cells are more sensitive to cryopreservation agent compared to established cell lines. Thus, proper handling to cryopreserve primary cells is important to maintain cells viability post freeze thaw process.

Despite the previously successful optimization of cryopreservation of cell suspension for colorectal epithelial cells using DotScan™ antibody microarray (Zhou et al. 2010), differences between physiology of gastric and colorectal epithelial cells required significant optimization. Thus this study was aimed to evaluate the effect of short- and long-term storage of single cell suspensions of gastric epithelial cells at -80ºC, to assess the importance of stepwise addition of freezing medium to sensitive gastric epithelial cells and to compare the effect of cells viability towards cells binding to CD antibodies on DotScan™ slides.

Materials and Methods

Sample Collection

Twelve gastric tissues from mucosal layer were collected from patients undergoing oesophagogastroduodenoscopy (OGDS) or gastrectomy procedure at Universiti Kebangsaan Malaysia Medical Centre from September 2013 to September 2014. Informed consents were obtained from patients before sample collection and the study was approved by the Medical Ethics Committee of the university (UKM1.5.3/244/02-01-02-SF0958). Tissues were placed in ice cold Hanks' Buffered Salt Solution (HBSS) with glucose (Gibco, USA) and stored at 4ºC for no longer than 12 h after collection.

Preparation of Single Cell Suspension from Gastric Mucosal Tissues

Single cell suspensions from gastric mucosal tissues were prepared as described previously (Zhou et al. 2011). Tissues were minced into approximately 2 mm pieces and digested in an equal volume of 2% Collagenase Type IV in RPMI 1640 (Sigma Aldrich, USA) and 0.1% DNAse I (Sigma Aldrich, USA) in phosphate-buffered solution (PBS) for 1 h at 37ºC with gentle occasional shaking. Digested tissues were forced through 100 μm Filcon filter and filtered through 40 μm Filcon filter (BD Falcon, USA). Cells were washed once with HBSS and centrifuged at low speed (200×g). Supernatant was discarded as much as possible and freezing medium containing 10% dimethyl sulphoxide (DMSO) and 90% fetal bovine serum (FBS) was added to cell pellet either directly or in stepwise manner. In direct manner, 1 mL freezing medium (90% FBS and 10% DMSO) was added directly to cell pellet while in stepwise manner, cells were firstly resuspended in 0.5 mL FBS, followed by addition of an equal volume of 80% FBS and 20% DMSO. Cell concentration was adjusted to 10^7 cells/mL and were cryopreserved slowly in Mr. Frosty cryobox (Nalgene, USA) for overnight at -80ºC Ultra Low freezer (Sanyo). The freezer has temperature profile ranges from -80ºC to -85ºC. Cells were subsequently stored at -80ºC for 5 days and 6 months.

THAWING OF FROZEN CELLS

Cells were rapidly thawed in 37ºC waterbath for 2 min and immediately immersed in 9 mL pre-warmed HBSS. Cells were washed once with HBSS, centrifuged at 200×g for 5 min and treated with 0.1% DNAse I for 20 min. Cells count was performed using Trypan blue exclusion dye.

CAPTURING SINGLE CELL SUSPENSION OF GASTRIC EPITHELIAL CELLS ON DOTSCAN™ SLIDE

Capture of single cell suspension of gastric epithelial cells onto DotScan™ slides has been described in previous study (Sukri et al. 2016). The slides consist of more than 140 CD antibodies against CD antigens that have been customized to consist 82 established CD antibodies against T-, B-, and myeloid cells and another 42 CD antibodies were selected markers of gastric cancer cells and *Helicobacter pylori* infection (MedSaic Pty. Ltd., Eveleigh, NSW, Australia). Three hundred µL of single cell suspensions of gastric epithelial cells with similar cell concentration (2×10^6 cells/mL) but different cell viabilities (33% and 88%) were captured onto DotScan™ slide as described previously (Zhou et al. 2011). Briefly, cells were loaded onto nitrocellulose section of moistened slide and incubated for 1 h at room temperature. Slides were gently washed with 3 washes of fresh PBS after incubation and then fixed with 3.7% formaldehyde in PBS. Slides were then scanned and analyzed with a DotReader™ (Medsaic Pty Ltd., Eveleigh, NSW, Australia).

PREPARATION OF SINGLE CELL SUSPENSION FROM PERIPHERAL BLOOD

Preparation of single cell suspension from peripheral blood of a healthy subject was followed as described by the manufacturer (Sigma Aldrich, MO, USA). 10 mL blood was drawn from the subject and subsequently placed into ethylene diamine tetraacetic acid (EDTA) tube (BD, NJ, USA). Blood was carefully layered onto the Histopaque-1077 (Sigma Aldrich, MO, USA) and centrifuged at 400×g at room temperature for 30 min. Opaque layer containing mononuclear cells was transferred to conical tube and washed with PBS twice followed by centrifugation at 250×g for 10 min. Cells were immediately resuspended in 300 µL of PBS without cryopreservation for capture onto DotScan™ slide.

STATISTICAL ANALYSIS

Non-parametric Wilcoxon test was used to compare the effect of storage duration and steps in freezing medium addition to cell viabilities. *P* values less than 0.05 was considered as significant. All statistical analyses were performed using SPSS version 21.

RESULTS

EFFECT OF STORAGE DURATION AT -80ºC TO GASTRIC EPITHELIAL CELLS VIABILITY

To assess the effect of storage duration to cell viability for cells stored at -80ºC, cells were slowly frozen in Mr. Frosty cryobox overnight and subsequently stored at -80ºC for 5 days and 6 months. Freezing media were added stepwise in both cells stored either for 5 days or 6 months at -80ºC. Cell viability was counted by using Trypan blue exclusion dye to measure cell viability after freeze thaw process. As shown in Table 1, cell viability for cells stored at -80ºC for 6 months were ranged from 15% to 41% (median = 37%) while cells stored for 5 days had cell viability ranged from 76% to 88% (median = 84%). Increased cell viability (increment of 47%) was observed in cells stored frozen for 5 days compared to 6 months. Decreased cell viability and more cellular debris were observed in cells stored for 6 months as compared to cells stored for 5 days. Viability of cells stored for 5 days at -80ºC was significantly higher than cell viability stored for 6 months ($p = 0.012$; non-parametric Wilcoxon test). In addition, cells count using hemocytometer was difficult to perform in cells stored for 6 months compared to cells stored for 5 days due to the presence of more cellular debris of cells death.

EFFECT OF STEPS FOR FREEZING MEDIUM ADDITION TO CELLS VIABILITY OF GASTRIC EPITHELIAL CELLS

In order to assess the effect of addition of freezing medium to cells, cells were either added with freezing medium in stepwise or direct manner. After freeze and thaw process, freezing medium was added to cells in stepwise manner had viability ranged from 76% to 88% (median = 84%) while cells added with freezing medium in direct manner had cells viability ranged from 23% to 56% (median = 44.5%) as shown in Table 2. Cells viability added with freezing medium in stepwise manner was 45% higher than

TABLE 1. Cells viability of single cell suspensions of gastric epithelial cells stored for different time points: 5 days *vs* 6 months

Sample no.	Cells viability for 5 days storage	Sample no.	Cells viability for 6 months storage
1	87.9%	5	25.1%
2	76.0%	6	40.9%
3	86.9%	7	33.0%
4	81.2%	8	15.3%

TABLE 2. Cells viability of single cell suspension of gastric epithelial cells added with freezing medium in direct *vs* stepwise manners

Sample no.	Cells viability for direct addition of freezing medium	Sample no.	Cells viability for stepwise addition of freezing medium
9	23.0%	5	87.9%
10	34.8%	6	76.0%
11	55.2%	7	86.9%
12	55.9%	8	81.2%

cells added with freezing medium in direct manner (p = 0.012; non-parametric Wilcoxon test).

EFFECT OF CELL VIABILITY TO CELL BINDING ON DOTSCAN™ SLIDES

We sought to determine the effect of different cells viability with similar cells concentration to cells binding onto DotScan™ slide. Cells stored at -80ºC with different time storage duration were rapidly thawed and cell viability was assessed. Two samples with similar cell concentration, 2 × 10^6 cells/mL but different cell viabilities (33% and 88%) were subsequently captured onto DotScan™ slides. Polymorphonuclear blood monocytes cells (PBMC) with 95% cells viability and cells concentration of 4 × 10^6 cells/mL from healthy donor were also captured onto DotScan™ slide as a control. As shown in Figure 1, cells suspension with viability of 88% had higher binding to DotScan™ CD antibodies than cells suspension with 33% viability. In addition, cells with 88% viability had higher binding to common CD antigens expressed on cells such as CD44 and CD29 which are used as control antibodies in this assay. Meanwhile cells with viability of 33% showed almost undetectable binding to control antibodies (CD44 and CD29).

DISCUSSION

In the present study, we sought to determine the effect of storage duration at -80ºC to gastric epithelial cells viability. Gastric epithelial cells harvested from fresh tissues are sensitive to stress situation such as changes in pH, osmotic pressure, temperature and cryoprotectant. Therefore, proper handling of cells preparation and cryoprreservation techniques is needed to ensure healthy cells after freeze and thaw process. Cells viability stored at -80ºC for 5 days was significantly higher than cells stored for 6 months in similar condition. The results obtained were different from peripheral blood cells stored in liquid nitrogen which had unchanged cells viability after cryopreservation for 5 weeks or 10 years (Abbruzzese et al. 2013). At -80ºC temperature, cells are still able to undergo cellular metabolism while at temperature below -135ºC (liquid nitrogen), cell metabolism stops (Mazur 1984; Okamoto et al. 2001). In peripheral blood mononuclear cells isolated from HIV-infected patients, cells viability decreased slightly after cell storage at -70ºC or dry ice for 3 to 12 weeks compared to cells viability stored at liquid nitrogen (Weinberg et al. 2011). A study conducted for sperm cells in animal demonstrated that cell viability tends to decline upon storage at -80ºC after 4 months (Okamoto

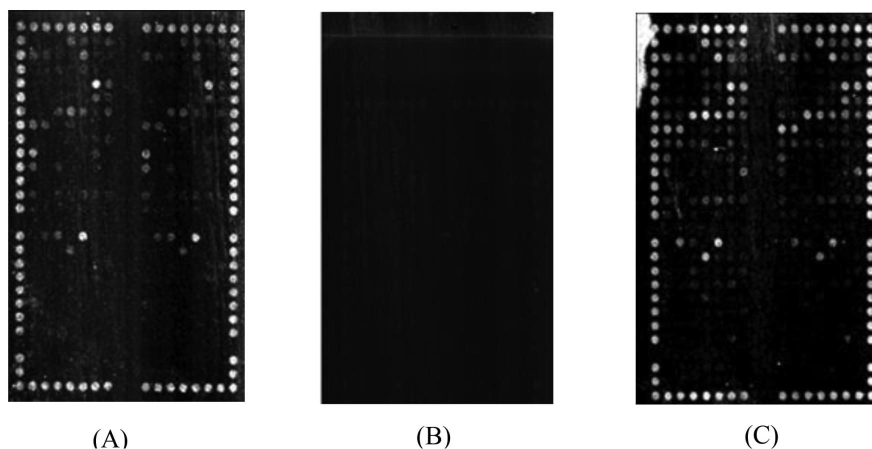

FIGURE 1. Images of gastric epithelial cells binding to CD antibodies on DotScan slide. The image A showed strong binding of live cells to control CD antibodies (CD44/ CD29 as labelled by white rectangular shapes) while in B, cells binding was weak and almost undetectable. The image A was comparable to fresh PBMC captured on slide as shown in image C. Cells concentration for both images were adjusted to 2 × 10^6 cell concentration in 300 µL RPMI 1640 and 10% FBS with different cell viabilities; 88% and 33% for images A and B, respectively. PBMC was captured on DotScan™ slide in image C as a control

et al. 2001). This can be further inferred that cells viability irrespective of cell type decreases after being stored up to 4 months at -80ºC. Therefore, liquid nitrogen should be used for long-term cryopreservation especially if storage exceeds 4 months. Mucous property of gastric epithelial cells was reduced once cells cryopreserved in -80ºC. Cryopreservation also significantly reduced red blood cells concentration in cryopreserved cells. Cell clumping was observed after freeze and thaw process especially in cells stored for 6 months at -80ºC. Addition of DNAse I significantly reduced cell clumping property.

Freezing medium containing 10% DMSO and 90% FBS was used to reduce the effect of cryoprotectant agent i.e., DMSO is toxic to cells. Higher concentration of FBS in freezing medium had been shown to increase cells viability of primary hepatocyte cells cryopreserved at -80ºC (Stevenson et al. 2004). Protein and nutrient contents in serum can lessen the impact of toxic DMSO agent to cells and 'cushion' cells recovery upon freeze and thaw process. Currently, American Type Culture Collection (ATCC) suggested the use of 95% complete medium and 5% DMSO to cryopreserve gastric adenocarcinoma cell line. However, primary gastric cells (whether normal or carcinoma) harvested using enzymatic digestion are sensitive to DMSO and thus correct freezing medium which consists of higher serum concentration can facilitate cells during stressful event of cooling and thawing processes.

Viability of cells cryopreserved using stepwise addition of freezing medium was higher compared to cells cryopreserved in direct addition of freezing medium. Stepwise addition of freezing medium to fragile gastric epithelial cells reduces osmotic shock that occurs during introduction of DMSO to cells before cryopreservation. Slow addition of DMSO to hepatocytes cells significantly increased cells viability (Terry et al. 2010). DMSO is a cryoprotectant agent that prevents formation of ice crystal in cells during cooling process. However, long exposure of DMSO to cells are detrimental as DMSO absorbed to cells is toxic and can subsequently lead to cells death upon long exposure to DMSO. Therefore, the technique for addition of DMSO prior to cryopreservation is paramount for high cell viability post freeze and thaw process as demonstrated in this study.

In this study, we also assessed the effect of similar cells concentration with different cells viability for capturing of CD antibodies onto DotScan™ slides. DotScan™ antibody microarray can be used to immunphenotype more than 140 CD antigens in parallel at one time. Previous studies suggested the use of 4×10^6 cells/mL for optimized cells capture onto DotScan™ slides[2,5]. DotScan™ antibody microarray is designed consisting of more than 80 CD antibodies including control CD antibodies namely CD44 and CD29 that are expressed mostly on any cells surface. These CD antigens function as positive binding for detection of any cell type. We found that 2×10^6 cells/mL of gastric epithelial cells concentration with viability of 88% had comparable cell binding to CD44/CD29 antibodies upon comparison with cell binding of fresh control PBMC (cells concentration and cells viability of 4×10^6 cells/mL and 95%, respectively). Good cells viability (more than 80%) for cells less than 4×10^6 cells/mL plays pertinent role for successful cell capture onto DotScan™ slides. As demonstrated by Zhou et al. (2011), cells viability less than 50% was excluded from DotScan analysis. This can be further inferred that cells viability less than 50% contains more dead cells and can impede binding of CD antigens on cells to antibodies. Dead cells also release DNA leading to higher cell clumping. As single cell suspension is important for successful cell capture, cell clumping contributes to failure of live cells to bind to antibodies.

This study also has limitations. Firstly, we did not assess necrosis and apoptosis in cells that might occur during cell processing steps and after thawing cells from cryopreservation. Apoptosis and necrosis processes that occur during cryopreservation steps might play role in viability of cells during pre- and post-cryopreservation. Secondly, this study only focused on the use of DMSO and FBS as freezing medium. We did not assess the possibility of using other cyroprotectant and freezing medium such as serum-free medium for successful cryopreservation of gastric epithelial cells.

This work represents an advance in biomedical science because it shows that successful optimization of gastric epithelial cell cryopreservation is essential for successful DotScan™ antibody microarray experiment. This study not only has clinical importance for successful cryopreservation technique in DotScan™ microarray experiment using gastric epithelial cells, but can also be applied for other types of cells harvested from mucosal tissue layer i.e., gastrointestinal tract. As cryopreservation technique is paramount in transporting cells from a medical center to another medical center for research collaboration purpose, the results from this study can be applied for successful transportation of cells that requires short-term cell cryopreservation.

ACKNOWLEDGEMENTS

This research is funded by the Ministry of Science, Technology and Innovation of Malaysia (grant no. 02-01-02-SF0958) and Universiti Kebangsaan Malaysia (GUP-2016-075). We are grateful to staffs at Endoscopy and Surgery Units at the Universiti Kebangsaan Malaysia Medical Centre for their technical assistance.

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Asif Sukri & Alfizah Hanafiah*
Department of Medical Microbiology and Immunology
Faculty of Medicine
Universiti Kebangsaan Malaysia
Jalan Yaacob Latiff, Bandar Tun Razak
56000 Cheras, Kuala Lumpur, Federal Territory
Malaysia

Nik Ritza Kosai & Mustafa Mohamed Taher
Department of Surgery
Faculty of Medicine
Universiti Kebangsaan Malaysia
Jalan Yaacob Latiff, Bandar Tun Razak
56000 Cheras, Kuala Lumpur, Federal Territory
Malaysia

Isa Mohamed Rose
Department of Pathology
Faculty of Medicine
Universiti Kebangsaan Malaysia
Jalan Yaacob Latiff, Bandar Tun Razak
56000 Cheras, Kuala Lumpur, Federal Territory
Malaysia

Ramelah Mohamed
Biotech Diagnostics
UKMTech
Universiti Kebangsaan Malaysia
43600 UKM Bangi, Selangor Darul Ehsan
Malaysia

*Corresponding author; email: alfizah@ppukm.ukm.edu.my

Received: 9 September 2016
Accepted: 5 March 2019