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Contents

Simple detergent screening for optimal solubilization and purification of histidine-tagged membrane proteins

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Membrane proteins are not soluble in aqueous solution. For purification, they have to be removed from the lipid bilayer of the cell membrane. This is achieved by solubilization using detergents. The complex formed between a membrane protein and a detergent is water-soluble and such complexes can be purified using standard chromatography techniques. Finding a detergent suitable for the membrane protein of interest in a particular study is a vital part of the workflow in membrane protein research. Here we describe a rapid two-step procedure for detergent screening including solubilization followed by an affinity purification step using His Mag Sepharose™ Ni magnetic beads. The two-step procedure allowed flexible analysis and easier evaluation for final choice of detergent.

Introduction

Membrane proteins play major roles in many fundamental biological processes, acting in the transport of solutes and proteins, signaling (receptor for a hormone), metabolism, and in maintaining cell and tissue structure. Membrane proteins can be seen as the communicators between the cell (or organelle) and its environment.

Today, integral membrane proteins are the main targets for development of new pharmaceuticals. Increased structural and functional information on this group of proteins will help to improve the efficiency of drug discovery. Membrane proteins are often expressed as recombinant, tagged proteins for further study. Immobilized metal ion affinity chromatography (IMAC) purification using polyhistidine tagged proteins has become the prime choice for capture of recombinant membrane proteins.

Since membrane proteins are not soluble in aqueous solution, the use of detergents is essential in the workflow for membrane protein purification. The choice of detergent is a key factor for successful purification of an integral membrane protein to avoid protein losses and inactivation and detergent screening is therefore often necessary to find the optimal detergent for each protein and purpose. Standard procedure for detergent screening and choice of detergent is solubilization, in different detergents, followed by semiquantitative evaluation using Western blotting techniques.

Here we describe an approach for detergent-screening using a two-step procedure consisting of solubilization followed by affinity purification using His Mag Sepharose Ni magnetic beads.

Sample proteins and sample preparation

TatCy-$(His)_6$ and cytochrome bo_3 ubiquinol oxidase-$(His)_9$ were used as model membrane proteins. They were expressed in *Bacillus subtilis* and in *Escherichia coli*, respectively. After cultivation and harvest, cells were disrupted by bead-beating or high-pressure homogenization. Cell membranes were prepared by centrifugation at 100 000 × g for 1 h. The cell membrane pellet was collected.

Screening protocol

The cell membrane pellet was resuspended in 20 mM phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4. The total protein concentration was adjusted to 10 mg/ml. Solubilization was achieved by mixing 900 µl of membrane suspension with 100 µl of a 10% detergent solution, giving a final detergent concentration of 1%. Different detergents were tested (Table 1). After 30 min incubation followed by centrifugation at 50 000 × g for 30 min, the supernatants were collected.

IMAC purification was performed using a 5% slurry of His Mag Sepharose Ni beads equilibrated in 20 mM phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4, containing 0.1% of detergent. A volume of 1 ml of detergent-solubilized cell membranes was incubated with 100 µl of the beads, end-over-end, for 30 min. After washing in the above buffer, elution was performed by increasing the imidazole concentration to 500 mM. Solubilized membrane proteins and collected eluates (after IMAC purification) were analyzed by Western blotting using an anti-His antibody in combination with enhanced chemiluminescence for detection/quantitation, SDS-PAGE, Lowry assay, and/or gel filtration (GF).

Membrane Protein Purification Kit, which contains His Mag Sepharose Ni (IMAC magnetic beads precharged with Ni^{2+} ions), detergents at 10% stock solutions, and the above protocol were used for the experiments described.

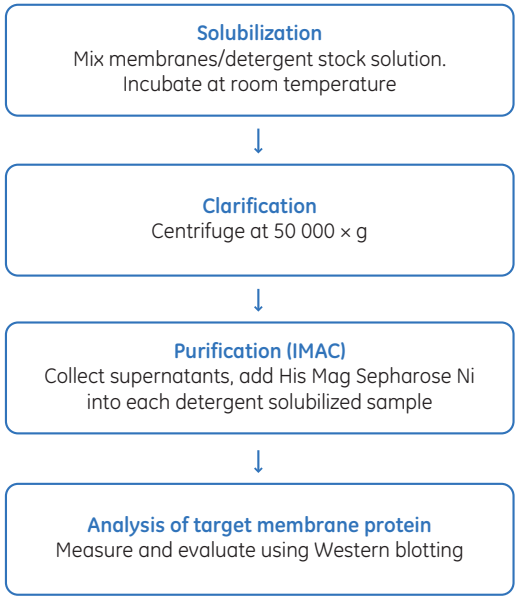


Fig 1. Two-step (solubilization and purification) detergent screening protocol.

Evaluation of the two-step (solubilization and IMAC purification) protocol

The two-step protocol was compared with solubilization only (one step).

For Western blotting, 1 ml aliquots of TatCy-$(His)_6$ containing membranes were solubilized in the different detergents (Table 1). Western blotting quantitation was performed after solubilization (Fig 2A) and indicated that all detergents could be considered with DDM showing the highest yield. Quantitation data for IMAC of TatCy-$(His)_6$ showed the highest yield for OG (Fig 2B). SDS-PAGE of the purified TatCy-$(His)_6$ showed high purity in the presence of FOS12, LDAO, and OG (Fig 2C).

From this comparison, it was evident that including a purification step provides more information on the performance of the detergents. Using one or more analytical methods allows for a more stringent evaluation and hence an optimal final choice of detergent. While DDM would have been the detergent of preference based on the analysis after solubilization (Fig 2A), a high yield of very pure TatCy-$(His)_6$ was achieved using the two-step protocol (Figs 2B and C).

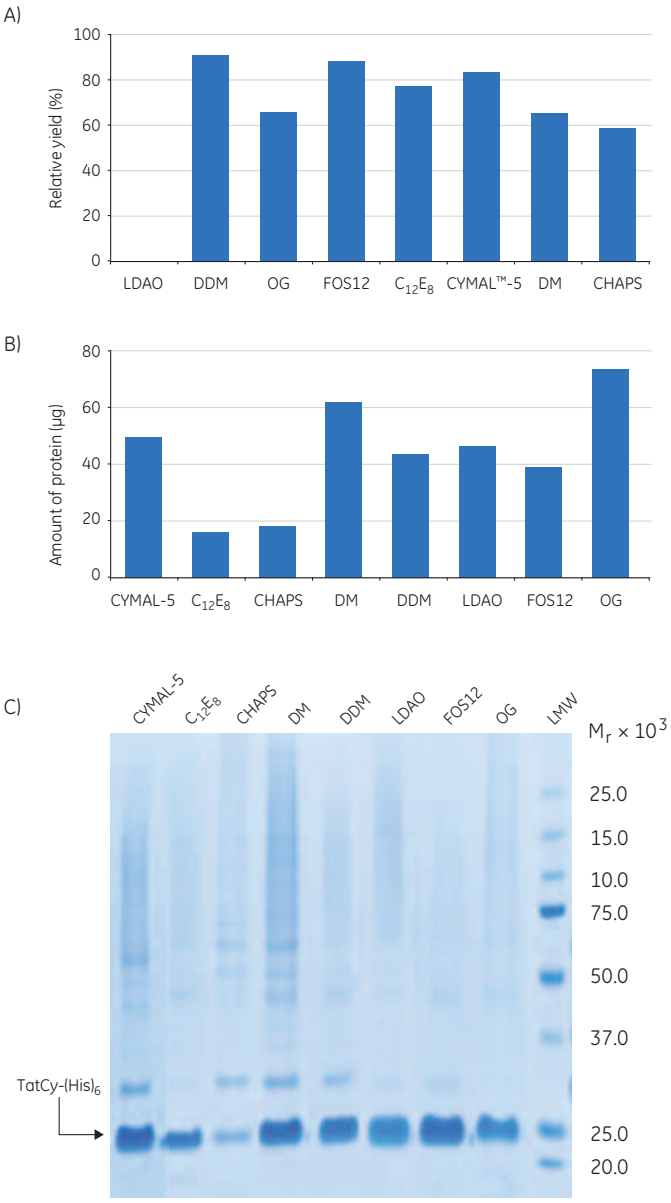


Fig 2. Western blot quantitiation of TatCy-$(His)_6$ **A)** after solubilization only and **B)** after Lowry assay quantitation following solubilization and IMAC purification. Purity **C)** was analyzed by SDS-PAGE and Coomassie™ staining.

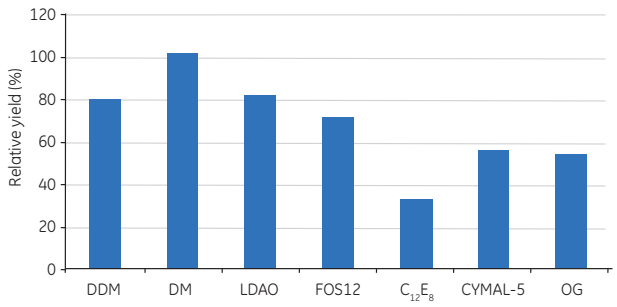
Table. 1. Detergents used for screening and their respective critical micelle concentrations. Abbreviations used, are given within parentheses

Detergents	CMC* (%)	CMC* (mM)
n-dodecyl-β-D-maltopyranoside (DDM)	0.009	0.17
n-decyl-β-D-maltopyranoside (DM)	0.09	1.8
n-dodecyl-N,N-dimethylamine-N-oxide (LDAO)	0.02	1.5
FOS-choline 12 (FOS12)	0.05	1.5
Octaethylene glycol monododecyl ether ($C_{12}E_8$)	0.005	0.09
5-cyclohexyl-1-pentyl-β-D-maltoside (CYMAL-5)	0.12	3.5
n-octyl-β-D-glucopyranoside (OG)	0.53	20
3-[(3-cholamidopropyl)-dimethyl aminio]-1-propane-sulfonate (CHAPS)	0.49	8

* Approximate critical micelle concentrations (CMC) in water at 20°C

Solubilization of membranes and purification was performed in approximately 1 h. Time for analytical evaluation depends on the methods that are needed to assay specific requirements. Yield, purity, and homogeneity are commonly included parameters.

A)



B)

Running conditions

Column: Superdex 200 5/150 GL
Sample load: 25 µl
Buffer: 10 mM phosphate buffer, 150 mM NaCl including the same detergent used for solubilization and purification
Flow rate: 0.3 ml/min
System: ÄKTAexplorer™ 10

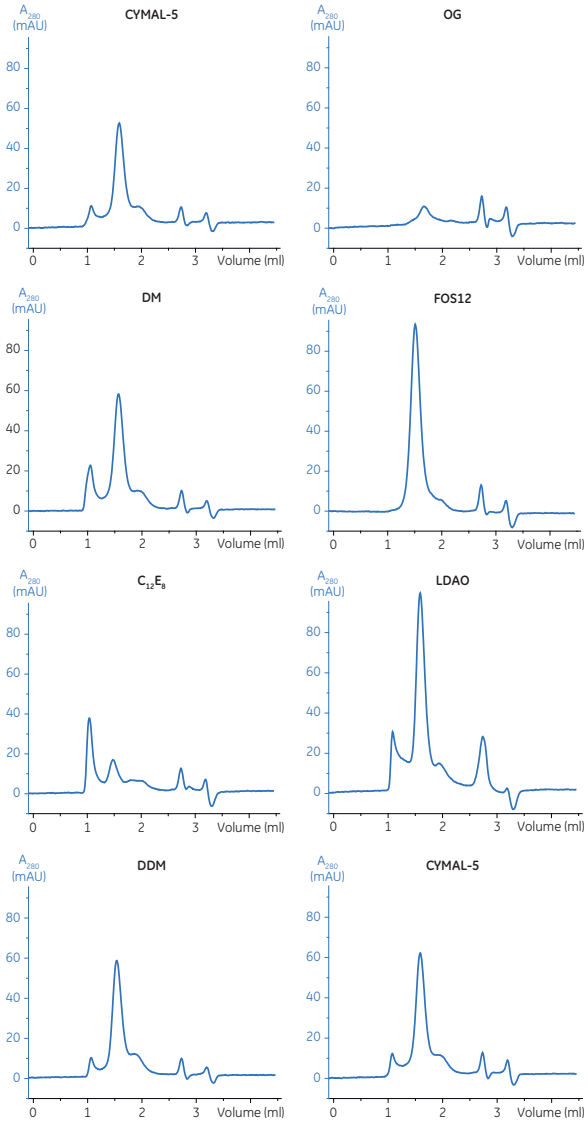


Fig 3. A) Western blot quantitation and **B)** homogeneity data for the cytochrome bo_3 ubiquinol oxidase-$(His)_9$ after detergent screening using the two-step protocol. All fractions analyzed were collected after IMAC purification (second step) in the screening protocol.

Screening for optimal detergents(s) for solubilization/purification of recombinant protein

Yield and homogeneity were set as the main parameters in a screening experiment to find an optimal detergent for cytochrome bo_3 ubiquinol oxidase-$(His)_9$ purification. The two-step protocol was followed using the seven detergents and the Mag Sepharose Ni beads in the Membrane Protein Purification Kit (Fig 1).

Antibody probing of the Western blotting procedure was performed with mouse anti-histidine and sheep anti-mouse antibodies as primary and secondary antibodies, respectively. Detection was performed using Amersham™ ECL™ Plus Western Blotting Detection Reagent. GF on a Superdex™ 200 5/150 column was used for assaying homogeneity. For this purpose, 25 µl of sample was applied at a flow rate of 0.3 ml/min. The Superdex 200 column was equilibrated in phosphate buffer and the respective detergent before each run.

This detergent screen shows that DDM, DM, LDAO, and FOS12 all gave good yields and could be considered for solubilization and purification of the cytochrome bo_3 ubiquinol oxidase-$(His)_9$ protein. The $C_{12}E_8$, CYMAL-5 (Affymetrix) and OG detergents all resulted in lower yields (Fig 3A).

The homogeneity analyses (Fig 3B) showed different levels of aggregation of the cytochrome bo_3 ubiquinol oxidase-$(His)_9$ protein for most of the detergents tested. However, application of FOS12 resulted in the most homogeneous material (*cf.* Fig 3B). For this detergent the homogeneity was calculated to be above 95%. For the requirements set in this study, FOS12 would be the detergent of choice.

Conclusions

Detergent screening is essential for optimizing the solubilization and purification of membrane proteins. The use of a two-step protocol, including solubilization and partial purification using the same detergent in both steps gave clear and easily obtained data for making the best choice of detergent. The use of the magnetic bead format is easy and rapid and in general a detergent screening including analyses and evaluation is done within less than one working day. The Membrane Protein Purification Kit facilitates rapid and easy screening procedure, giving time to include one or more analytical methods that allow for a more stringent evaluation and hence an optimal choice of detergent. Data from such screening experiments might be used for scaled-up membrane protein purifications.

Ordering information

Product	Code number
His Mag Sepharose Ni, 2 × 1 ml	28-9673-88
His Mag Sepharose Ni, 5 × 1 ml	28-9673-90
His Mag Sepharose Ni, 10 × 1 ml	28-9799-17
Membrane Protein Purification Kit	28-9805-82
Superdex 200 5/150 GL	28-9065-61
Amersham ECL Pus Western Blotting Reagent Pack*	RPN2124

* *Product replaced by Amersham ECL Prime Western Blotting Detection Reagent, RPN2232*

High enrichment of phosphorylated peptides from complex samples using TiO_2 Mag Sepharose™

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Protein phosphorylation is one of the most widespread regulatory mechanisms in nature. It is a key regulator of intracellular biological processes and at present, it is the most studied and best understood post-translational modification (PTM). Protein phosphorylation regulates cellular signaling and communication and is known to be involved in controlling diverse processes including metabolism, transcriptional, and translational regulation, degradation of proteins, proliferation, differentiation, and cell survival. Phosphorylation events frequently initiate and propagate signal transduction pathways. By incorporating TiO_2 Mag Sepharose beads in a phosphoproteomic workflow, more than 2000 phosphorylated peptides were identified from a complex sample and the enriched fraction contained 96% phosphorylated peptides.

Introduction

Cellular proteins in humans are predominantly phosphorylated on serine/threonine residues with a smaller extent of phosphorylation occurring on tyrosine residues. However, the overall low abundance of phosphoproteins complicates identification and characterization of this sub-proteome using standard mass spectrometry-based methods. In addition to the problem of low abundance, phosphorylated peptides are, due to their phospho-groups, generally more acidic than mainstream peptides, thereby complicating ionization in positive ion mode of mass spectrometry.

The challenges with global studies of phosphorylated peptides/proteins have spurred the development of molecular tools to preferentially enrich the phosphoproteome. The first enrichment strategy was based on interaction with ferric ions and was exploited by biochemists to isolate phosphoproteins and phosphopeptides using immobilized metal affinity chromatography (IMAC). Analogous to ferric ions, gallium (III) has also been shown to display affinity and selectivity to phosphopeptides. These enrichment strategies followed by mass spectrometry have convincingly proved to reveal the identity of the proteins and the sites of modification. An alternative to IMAC for selective enrichment of phosphopeptides is metal oxide affinity chromatography (MOAC) where media based on titanium dioxide (TiO_2) are most commonly used (1).

Comparative study to select kit for enrichment of phosphorylated peptides

While setting up a workflow for phosphoproteomic studies, we performed a comparative study of media based on TiO_2 to select the most convenient and efficient kit for enrichment of phosphorylated peptides from complex samples prior to mass spectrometry. Phos-trap™ Phosphopeptide Enrichment Kit (Perkin Elmer) and TiO_2 Mag Sepharose (GE Healthcare), which are two media with paramagnetic properties that are easily handled by use of a magnetic rack, were included in the study. Their ability to enrich phosphorylated peptides was analyzed by radiolabeling protein phosphates with ^{32}P in cell culture, precipitation of cellular proteins followed by trypsin digestion to peptides (Fig 1A).

Samples corresponding to 500 µg protein were used for the evaluation. The ^{32}P activity was measured in different fractions obtained from the enrichment procedure according to the instructions from the manufacturer. In our hands, as shown in Figure 1B, the TiO_2 Mag Sepharose had higher affinity for the phosphopeptides by reducing loss in the washing step as well as eluting a higher amount of phosphorylated peptides. TiO_2 Mag Sepharose was thus our medium of choice to include in further analyses.

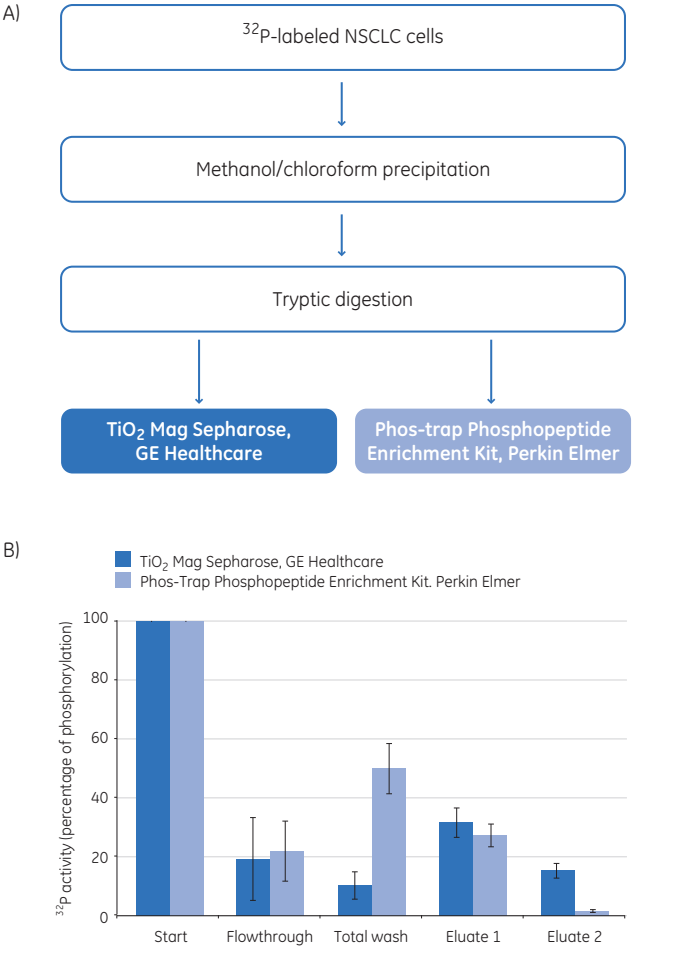

Fig 1. A) Workflow for comparative study of two media for phosphopeptide enrichment based on TiO_2. The efficiency was evaluated by use of ^{32}P-labeled peptides (NSCLC=Nonsmall cell lung cancer cell line). **B)** The diagram shows the ^{32}P activity (%) in fractions obtained from enrichment of phosphorylated peptides by the tested media. Error bars represent the standard deviation of the analysis (n=3). TiO_2 Mag Sepharose was selected for enrichment of phosphorylated peptides from complex samples.

Incorporating TiO_2 Mag Sepharose in a phosphoproteomic workflow

Inspired by work of Villén and Gygi (2) a prefractionation step was included prior to the enrichment by TiO_2 Mag Sepharose. By use of strong cation exchange (SCX) chromatography at low pH, phosphorylated peptides elute earlier in the gradient compared to a nonphosphorylated peptide of the same amino acid sequence due to the decrease of the net charge by the negatively charged phosphate group. Figure 2 shows the experimental workflow.

The total proteome (10 mg protein) from a complex cell lysate (lung cancer cell line U-1810) was extracted and digested by trypsin before prefractionation in this study. The SCX chromatography (polySULFOETHYL A column, PolyLC) was performed in phosphate buffer at pH 2.7 and 30% acetonitrile. Twelve fractions were collected during a salt gradient from 0 to 350 mM KCl.

The SCX-fractions were desalted by C18 clean-up (Sep-Pak™ Vac tC18 cartridge, Waters), lyophilized and dissolved in 120 µl binding buffer (1 M glycolic acid, 5% TFA, 80% acetonitrile). TiO_2 Mag Sepharose (240 µl) was equilibrated with 500 µl of binding buffer. Each fraction was incubated with 30 µl of media slurry for 40 min during mixing at room temperature followed by 3 washing steps and elution in 2 × 50 µl of 5% ammonium hydroxide according to the instruction protocol. Both elution fractions were pooled prior to lyophilization.

The phosphorylated peptides were analyzed in three replicates using a LTQ-Orbitrap™ Velos mass spectrometer (Thermo Fisher Scientific).

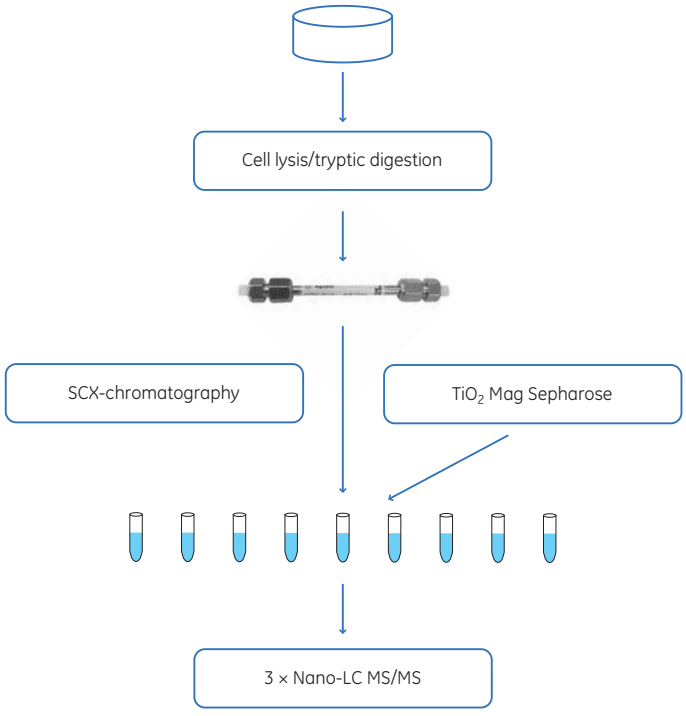

Fig 2. Experimental workflow for phosphoproteomics.

Protein identification

Using this workflow, 2324 peptides (corresponding to 1208 proteins) were confidently identified and as much as 2234 of them were proven to be phosphorylated, which gives an excellent enrichment ratio of 96%. A small part of the complete list of identified phosphorylated peptides and their phosphorylation sites is shown in Table 1; serine, threonine, and tyrosine are represented as phosphorylation sites. Mutliple phosphorylated fragments were also found, which indicates efficient capture of phosphorylated peptides by use of the described workflow. This workflow is now routinely used for signaling studies by phosphoproteomics in our lab (3).

Table 1. Part of the complete list of 2234 phosphopeptides identified in this study

Peptide	Modification	Position	AA
SVQEGENPDDGVRGSPPEDYR	Phospho	15	Ser
DSAIPVESDTDDEGAPR	Phospho	8	Ser
	Phospho	10	Thr
RGPNYTSGYGTNSELSNPSETESER	Phospho	16	Ser
LPSSPVYEDAASFK	Phospho	3	Ser
EEPLSEEEPCTSTAIASPEKK	Phospho	5	Ser
	Phospho	17	Ser
SSPARPPDVPGQQPQAAK	Phospho	1	Ser
DSMVMHNSDPNLHLLAEGAPIDWGEEYSNSGGGGSP-SPSTPESATLSEK	Phospho	37	Ser
GFGQSNSLPTAGSVGGGMGR	Phospho	7	Ser
DEILPTTPISEQK	Phospho	7	Thr
IAAPELHKGDSDSEEDEPTK	Phospho	11	Ser
	Phospho	13	Ser
GTMDDISQEEGSSQGEDSVSGSQR	Phospho	13	Ser
SGPPSPPSTATSFGGPRPR	Phospho	5	Ser
SRTSPVSR	Phospho	3	Thr
	Phospho	4	Ser
TLLPWDSDESPEASPGPPGPR	Phospho	7	Ser
	Phospho	10	Ser
IGEGTYGVVYK	Phospho	5	Thr
	Phospho	6	Tyr
NSFTPLSSSNTIR	Phospho	2	Ser
SPSTLLPK	Phospho	1	Ser
SQSIEQESQEK	Phospho	1	Ser
GNGSGGSRENSTVDFSK	Phospho	7	Ser
	Phospho	11	Ser
DDSLDLSPQGR	Phospho	7	Ser
TRSPLLSR	Phospho	1	Thr
	Phospho	3	Ser
QEKPAEKPAETPVATSPTATDSTSGDSSR	Phospho	18	Thr
DGVSLGAVSSTEEASR	Phospho	4	Ser
	Phospho	10	Ser
VGVEASEETPQTSSSSSARPGTPSDHQSQEASQFER	Phospho	6	Ser

Conclusions

High enrichment of phosphorylated peptides from complex samples was achieved by use of TiO_2 Mag Sepharose in a phosphoproteomic workflow. Analyzing phosphopeptides in a less complex sample increases the probability of successful analysis by MS.

References

1. Thingholm, T. E. et *al.* Analytical strategies for phosphoproteomics. *Proteomics* **9**, 1451–1468 (2009).
2. Villén, J. and Gygi, S. P. The SCX/IMAC enrichment approach for global phosphorylation analysis by mass spectrometry. *Nat. Protoc.* **3(10)** 1630–1638 (2008).
3. Stahl, S. *et al.* Phosphoproteomic profiling of NSCLC cells reveals that ephrin B3 regulates pro-survival signaling through Akt1-mediated phosphorylation of the EphA2 receptor. *J. Proteome Res.* **10**, 2566–2578 (2011).

Ordering information

Product	Code number
TiO_2 Mag Sepharose, 1 × 500 µl	28-9440-10
TiO_2 Mag Sepharose, 4 × 500 µl	28-9513-77

In vitro detection of cardiotoxicity using human embryonic stem cell-derived Cytiva™ Cardiomyocytes

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We evaluate the utility of human embryonic stem-cell (hESC)-derived Cytiva Cardiomyocytes as a potential screen for drug-induced cardiotoxicity using cytotoxicity and high-content biology (HCB). Our results indicate that an *in vitro* ATP cytotoxicity screen used in combination with cellular phenotypes in hESC-cardiomyocytes may be useful for flagging potential *in vivo* cardiotoxic structural liabilities. Furthermore, the data indicate that Cytiva Cardiomyocytes offer improved sensitivity in the detection of structural cardiotoxicity compared to the non-contractile rat cardiac H9c2 cell line.

Introduction

Cardiotoxicity is a major cause of drug attrition in preclinical and clinical development, serious adverse drug reactions (ADRs), and withdrawal from the market (1). Drug-induced cardiotoxicity is divided into two mechanistic areas: Functional and structural. Significant advances have been made in developing *in vitro* tools to identify the functional effects (e.g., prolongation of the QT interval) associated with novel therapeutics. In contrast, *in vitro* screens for drugs that are directly cytotoxic to the myocardium have not been developed. Here, we describe an *in vitro* physiological beating model system whereby the sensitivity and specificity of Cytiva Cardiomyocytes derived from the NIH-approved hESC H7 cell line and the non-contractile rat H9c2 cell line were determined and their ability to detect clinical structural cardiotoxins were compared (Table 1).

Methods

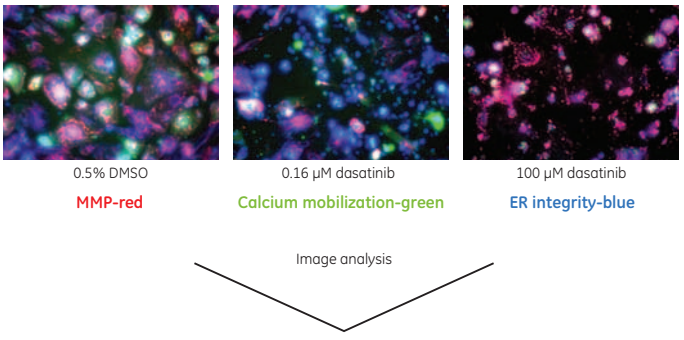
Cytiva Cardiomyocytes were cultured in BD Matrigel™ coated 384-well microplates in RPMI 1640, containing B27 supplement and incubated at 37°C for 72 h. Compound was then added (0.03 to 100 µM), and the incubation continued for a further 72 h. Cells were subsequently incubated for 1 h at 37°C in the presence of TMRE (mitochondrial membrane potential, MMP), Fluo-4 AM (calcium mobilization), ER-Tracker (ER integrity), and TOTO™-3 (membrane integrity) and then imaged using an ImageXpress™ High Content Screening System. Immediately following image acquisition, ATP depletion was assessed using the CellTiter-Glo™ Luminescent Cell Viability Assay to generate concentration response curves (Fig 1).

Compound classification

In order to evaluate the utility of Cytiva Cardiomyocytes to detect clinical cardiotoxins, compounds were classified based on information contained within their respective FDA approval packages. A panel of 15 clinically approved structural cardiotoxins, nine functional cardiotoxins, and eight non-cardiotoxins were subjected to live-cell imaging (MMP, calcium mobilization, and ER integrity) and cytotoxicity assessment (ATP depletion).

Results

Marketed drugs were categorized based on the occurrence and manifestation of cardiotoxicity reported in the FDA approval package. Not all parameters are modulated by each class of compound, however MMP was the most sensitive and specific indicator of structural cardiotoxicity in this model system (Fig 1). Correlation analysis among the different imaging parameters revealed correlation between MMP and calcium mobilization (r = 0.92), but little correlation among other parameters (r = 0.69 and 0.57) indicating that not all parameters are modulated in a similar manner.



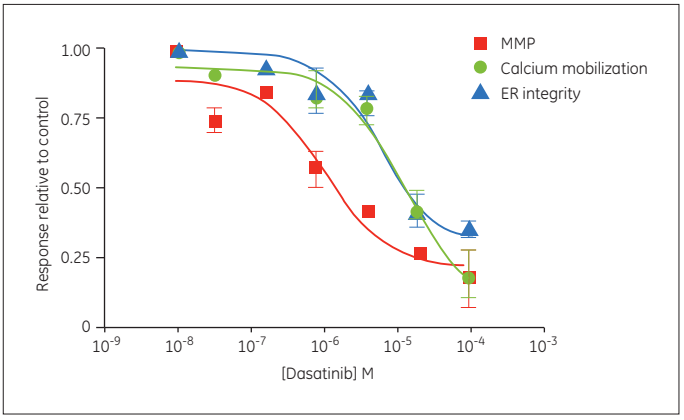


Fig 1. HCB parameters following reference compounds. Representative images in which Cytiva Cardiomyocytes were treated with vehicle, 0.5% DMSO, or the structural cardiotoxin dasatinib for 72 h followed by live-cell measurement of MMP, calcium mobilization, and ER integrity. Individual images were subsequently quantitated using automated image analysis algorithms: concentration curves and IC_{50} values were then calculated (not shown).

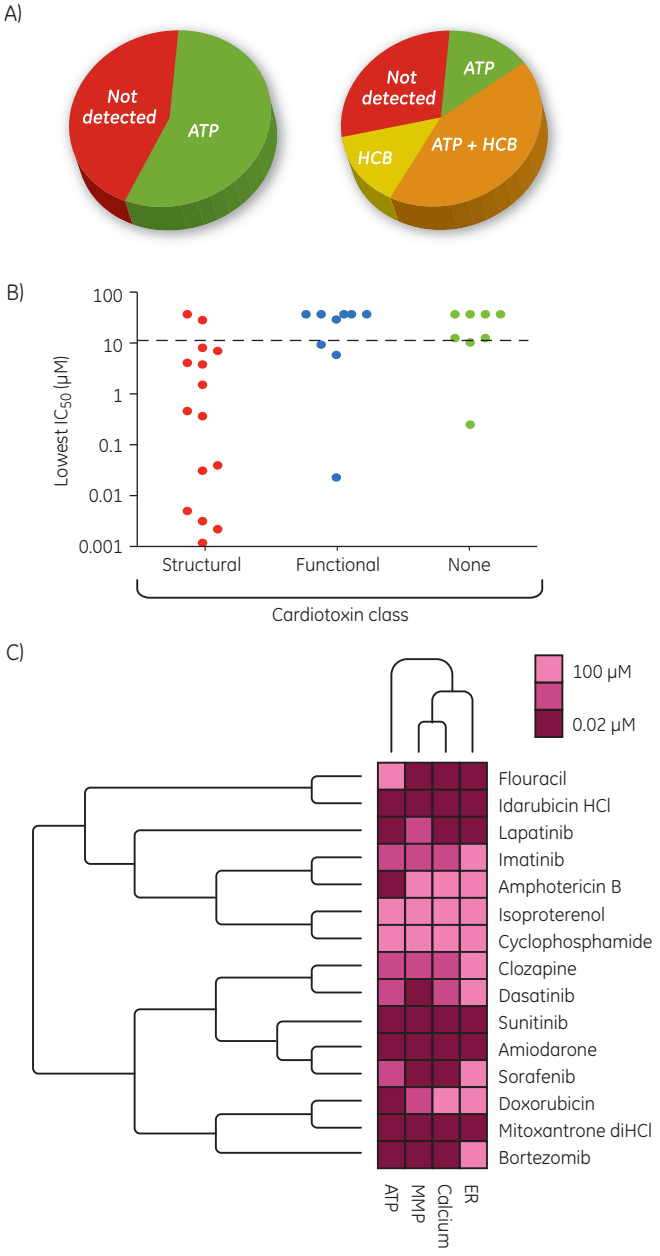


Fig 2. A) ATP and HCB parameters in combination offer improved detection of structural cardiotoxins, following 72 h exposure in hESC-CMs (10 µM cut-off). **B)** In addition to detection of structural cardiotoxins, this approach also detected one functional cardiotoxin. **C)** Cluster analysis revealed differences in detection of structural cardiotoxins between different parameters.

Assay sensitivity and specifity were determined using cut-offs of 10 and 100 µM. The data in Table 1 show that Cytiva Cardiomyocytes combined with both ATP and HCB parameters offered improved sensitivity and were more predictive of cardiotoxicity compared to rat cardiac H9c2 cells.

Table 1. Comparison of hESC-derived Cytiva Cardiomyocytes and the non-contractile rat cardiac H9c2 cell line

Model/Assay	Sensitivity (%)	Specificity (%)	Cut-off (µM)
hESC-CM HCB + ATP	73	88	10
H9c2 HCB + ATP	31	94	10
hESC-CM HCB + ATP	93	53	100
H9c2 HCB + ATP	85	76	100

Conclusions

HESC-derived Cytiva Cardiomyocytes provide a physiological beating model system in which MMP was the most sensitive and specific imaging indicator of structural cardiotoxicity. Cytiva Cardiomyocytes offer improved sensitivity compared to the non-contractile rat cardiac H9c2 cell line and an *in vitro* ATP cytotoxicity screen in combination with cellular phenotypes (MMP, ER integrity, and calcium mobilization) in Cytiva Cardiomyocytes may be useful for flagging potential *in vivo* cardiotoxic structural liabilities. These combined approaches in hESC-CMs may allow insight into the mechanism of cardiotoxicity and provide a screening strategy for *in vitro* detection of novel therapeutics that cause structural cardiotoxicity.

References

1. Laverty, H. G. *et al.* How can we improve our understanding of cardiovascular safety liabilities to develop safer medicines? *Br. J. Pharmacol.* **163 (4)**, 675–693 (2011).

Ordering information

Product	Code number
Cytiva Cardiomyocytes (1E5), 1 × 10^5 cardiomyocytes	28-9774-35
Cytiva Cardiomyocytes (1E6), 1 × 10^6 cardiomyocytes	28-9763-98
Cytiva Cardiomyocytes (5E6), 5 × 10^6 cardiomyocytes	28-9763-99

Perfusion culture of natural killer cells in WAVE Bioreactor™ 2/10 system

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We present methods and results from two independent large-scale 21-day cultures of activated natural killer (NK) cells using WAVE Bioreactor 2/10 system. The described method supports significant expansion of activated NK cells although large donor-to-donor variability can be observed. The NK cells remain biologically functional and retain their cytolytic activity after expansion in WAVE Bioreactor 2/10 system.

Introduction

Natural killer (NK) cells are CD3-negative cytolytic lymphocytes that comprise between 5% and 20% of human peripheral blood mononuclear cells (PBMC). They mediate the first line of defense against virally infected cells or tumor cells. The tumor killing potential of NK cells has generated major interest in expanding high numbers of these cells *in vitro*.

NK cells are typically cultured in static bags or tissue culture flasks. To meet the need for large-scale expansion of functional NK cells, a scalable, cost-efficient, and simple production process is required. The WAVE Bioreactor 2/10 system allows cultivation of human white blood cells at very high viability, with cell densities of more than 10×10^6 cells/ml (1-4) while maintaining a functionally closed system.

Major advantages of the WAVE Bioreactor system compared to static culture are that the cells are cultured in a single disposable bag and that continuous perfusion of fresh media is possible, which supports high cell concentrations and reduces both labor and media costs. In addition, handling only one bag with high-density cell cultures reduces the risk of cross-contamination.

Materials and methods

Activation of NK cells in static culture

Human PBMCs (3H Biomedical) were counted and characterized by flow cytometry. The total number of NK cells ($CD16^+$ and/or $CD56^+$) was determined and cells were loaded into culture flasks (Nunc) at a concentration of 1×10^6 PBMCs/ml, with preheated NK cell media consisting of CellGro™ SCGM (CellGenix GmbH) supplemented with 10% human serum and 500 U/ml interleukin-2 (IL-2, Proleukin™, Novartis Pharmaceuticals). Anti-biotin MACSiBead™ particles (Miltenyi Biotec GmbH) were loaded with CD335 (NKp46)-biotin and CD2-biotin, and added to the PBMCs at a ratio of 1:2 beads; NK cells, and cultures were maintained at 37°C with a 5% CO_2 atmosphere. From day 5, the cells were counted daily and media was added to adjust the cell concentration to 1×10^6 cells/ml. When a minimal cell number of 200×10^6 cells was reached, the cells were transferred to the Cellbag™ bioreactor.

NK cell culture in the WAVE Bioreactor 2/10 system

WAVE Bioreactor 2/10 system (software version 2.62) with perfusion controller was set up according to the User manual. All connections between the Cellbag bioreactor, perfusion-tubing assemblies, feed and waste bags were performed in a sterile manner. At least 150 ml NK cell media supplemented with 0.02% Pluronic™ surfactant (Life Technologies) was transferred to the Cellbag bioreactor via the feed perfusion assembly. Heating (37°C) and rocking were started and the system was allowed to equilibrate for 2 h.

Cells from culture flasks were centrifuged and resuspended in 50 to 100 ml fresh media and transferred to the Cellbag bioreactor. The following conditions were used throughout the culture: temperature 37°C, CO_2 5%, airflow 0.1 to 0.2 lpm, angle of 6°, and rocking speed of 6 rpm. NK cell media supplemented with 0.02% Pluronic were prepared fresh every second day and added to maintain a cell density of approximately 1×10^6 cells/ml. Glucose, lactate, ammonia, and glutamine levels as well as pH, CO_2, and O_2 levels were measured daily. Viable cell counts were performed every 1 to 2 days and the percentage of cell subsets was determined weekly by flow cytometry. Perfusion was started when the maximum culture volume of 1 l and a cell density of 3 to 5×10^6 cells/ml was reached. Perfusion rate was then increased gradually to maintain the glutamine levels above 1 mM and the glucose levels above 1.5 g/l.

Analysis

Cellular subpopulations were analyzed weekly by flow cytometric analysis using standard procedures. In brief, 1×10^6 cells were washed and stained for 20 min in antibody cocktails and analyzed on a FACSCanto™ flow cytometer (BD Biosciences).

Degranulation of NK cells and cytokine release was measured by flow cytometry-based methods. Cytolytic activity of the expansion product was analyzed by flow cytometry using CellTrace™ CFSE Cell Proliferation Kit (Life Technologies).

Results

Three expansions of NK cells from PBMCs from healthy donors were evaluated, of which culture 2 and 3 are described here (full data available in ref. 5). Exceptional donor-to-donor variability was observed. The yields for the cultures were 0.7×10^9 and 30×10^9 cells, respectively (Fig 1), with viabilities above 90%. The total NK cell population increased in response to NK cell activation and expanded to 52% and 27% of the total cell number, giving a cumulative increase in NK cell number of 12- and 250-fold in cultures 2 and 3, respectively.

To evaluate the activity of the cells from the cultures, cytolytic activity, degranulation, and cytokine production against the NK target cell line K562 were measured. NK cells from culture 2 showed degranulation, produced high amounts of IFN-γ and TNF-α, and displayed cytolytic activity against target cells in a dose-dependent manner upon co-culture with K562 cells (Fig 2). In contrast, NK cells from culture 3 were not as active. No CD107a surface expression or cytolytic activity was detected after a 4 h co-culture with K562 cells (Fig 2A).

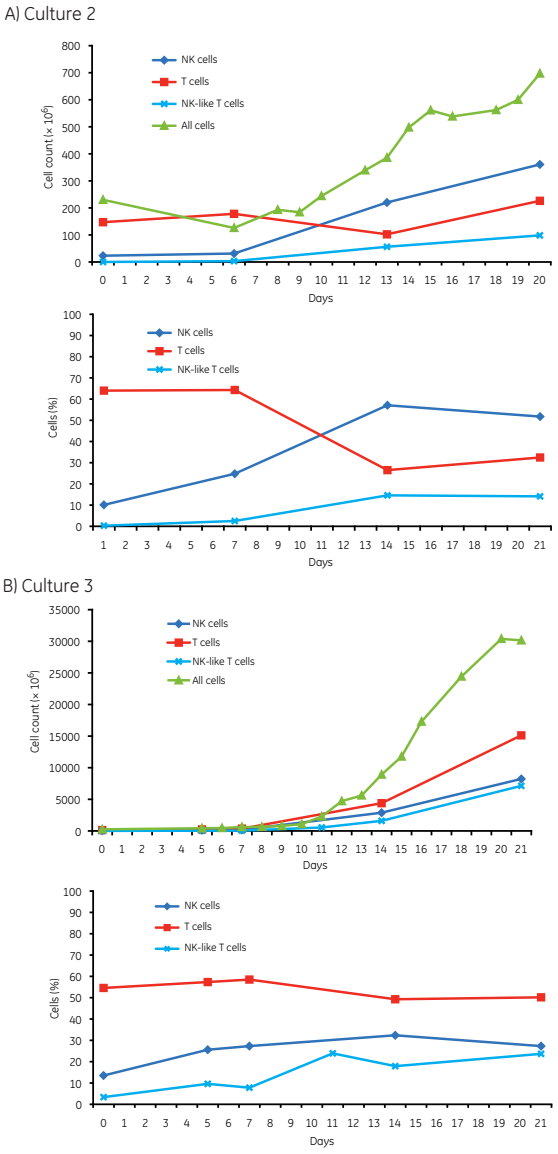


Fig 1. Cumulative cell count (× 10^6) and proportion of NK cells, T cells, and NK-like T cells for **A)** culture 2, and **B)** culture 3.

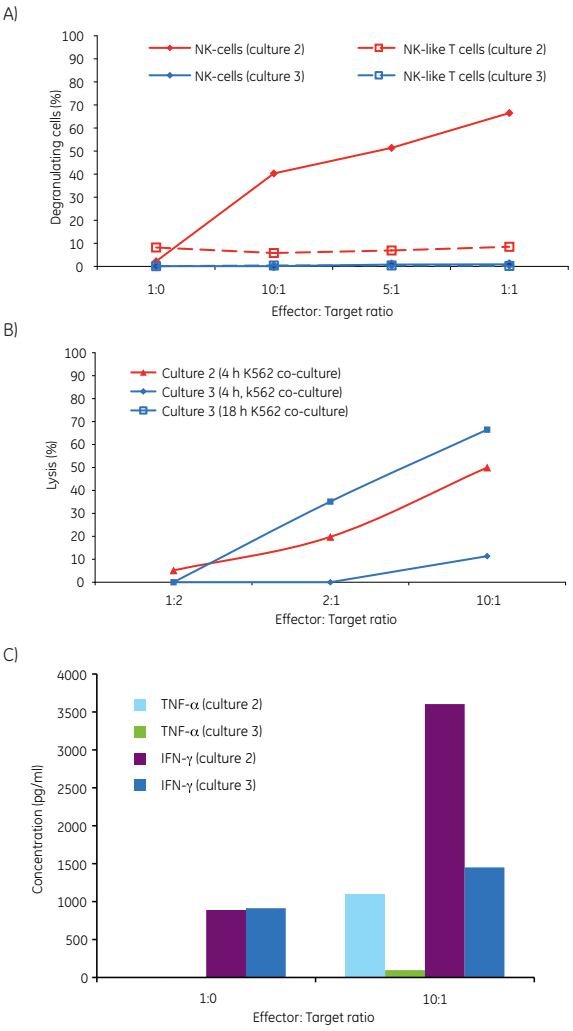


Fig 2. A) Percentage of degranulating NK cells against K562 target cells as measured by CD107a expression after co-culture with K562 cells at different effector:target ratios. **B)** Cytotoxic activity in response to K562 co-culture at different effector:target ratios. **C)** Cytokine secretion of NK cells before and after co-culture with K562 cells at a ratio of 10:1 for 5 h. The results are mean values from triplicate wells. Note that TNF-α was undetectable in the unstimulated effector cell controls.

Conclusions

Two independent NK cell populations were expanded using the WAVE Bioreactor 2/10 system. The cost-saving on labor by using the WAVE Bioreactor system is one of the biggest advantages due to the handling of only one bag and the automated media-exchange, which dramatically reduces the hands-on-time in large-scale expansions. The results confirm the well-known high inter-individual variations in NK cell expansion potential and activity. Culture 2 showed relatively slow expansion rate but had clear activity towards K562 cells. In contrast, the expansion rate of culture 3 was high but the NK cells showed modest activity. It is therefore recommended to monitor the cells frequently and carefully to obtain optimal cell concentration and cellular activity.

References

1. Hami, L. S. *et al.* GMP production and testing of Xcellerated T Cells for the treatment of patients with CLL. *Cytotherapy*, **6(6)**, 554–62 (2004).
2. Tran, C. A., *et al.* Manufacturing of large numbers of patient-specific T cells for adoptive immunotherapy: an approach to improving product safety, composition, and production capacity. *J. Immunother.*, **30(6)**, 644–54 (2007).
3. Hollyman, D., *et al.* Manufacturing validation of biologically functional T cells targeted to CD19 antigen for autologous adoptive cell therapy. *J. Immunother.*, **32(2)**, 169–80 (2009).
4. Sutlu, T. *et al.* Clinical-grade, large-scale, feeder-free expansion of highly active human natural killer cells for adoptive immunotherapy using an automated bioreactor. *Cytotherapy*, **12(8)**, 1044–55 (2010).
5. Application note: Perfusion culture of human natural killer cells in the WAVE Bioreactor 2/10 system. GE Healthcare, 28-9936-25, Edition AA (2011).

Ordering information

Product	Code number
WAVE Bioreactor System 2/10 (BASE2/10EH incl. Cellbag holder)	28-9377-86
Filter Heater (FLTHTR2)	28-4116-39
CO_2/Air Aeration Controller (CO2MIX20)	28-9377-95
Cellbag-2L (BioClear™ 10, Perfusion, DO)	28-9376-52
System 2/10 Perfusion Controller (PERFCONT2E CellTech)	28-9884-64

PAA Laboratories cell culture technologies now part of GE Healthcare Life Sciences

GE Healthcare has recently acquired PAA Laboratories, a developer and supplier of cell culture products for biomedical research and manufacture of biopharmaceuticals including recombinant proteins, antibodies and vaccines. PAA has manufacturing facilities registered with the FDA including a modern powder mill for manufacture of powdered cell culture media for the biopharmaceutical industry. The strategic fit between the two businesses enables us to provide an upstream to downstream integrated service to our customers and help optimize every stage of their manufacturing process.

PAA – The Cell Culture Company

PAA Laboratories, founded 1988 in Austria, specializes in the manufacture and worldwide distribution of cell culture products for research, development, diagnostic, and biopharmaceutical production. The product offering includes animal and human sera, liquid and powder media, reagents, and supplements. Production facilities in Austria, Canada, and Australia as well as sales subsidiaries and a comprehensive distributor network provide the highest service level to meet customer requirements. In 2009, PAA expanded its FDA registered and GMP manufacturing site in Europe with a modern powder production facility. The whole powder facility is strictly free of animal components (ACF) and designed to facilitate the regulatory submission process for biopharmaceutical production. The latest technologies in both milling and blending (including Process Analytical Technology; PAT) provide customers with true inter- and intra batch homogeneity of powder media up to 8000 kg batch sizes. In addition, PAA now offers a complete service from cell clone optimization and media adaptation to contract manufacturing.

Product portfolio

PAA's processes are set at pharmaceutical standards and GMP regulations defined by the European Union, the US- FDA, and the ICH. They use only carefully selected and screened raw materials and EP grade water in the manufacture of liquid cell culture products. The strict guidelines for the QA and QC procedures ensure that only high quality products are supplied to customers.

Liquid and powdered media

The product range includes classical media (DMEM, RPMI 1640, etc.), serum- and protein-free media, cell-type specific media for different applications such as stem cell, hybridoma and neuronal cultivation, as well as cytogenetic diagnosis. PAA offers a range of powdered basal media and buffer solutions. From raw material selection to the finished product, the whole process conforms to FDA and EU-GMP guidelines.

Sera

A core competence of PAA is the sourcing and manufacturing of a variety of sera products from a wide range of origins. Large "true pool" batches ensure high levels of batch-to-batch consistency. PAA's sera portfolio comprises fetal bovine sera, bovine sera, a variety of other animal sera, and human sera. Special treatments such as gamma irradiation, heat inactivation, UV treatment, dialysis and specific animal virus testings are available.

Reagents

PAA offers a range of amino acid solutions, vitamins, antibiotics, selection antibiotics, and cell detachment agents. In addition, cell-type specific growth supplements for hybridoma cloning, amniocyte cultivation, and serum-free cell culture are also available.

Buffer solutions

PAA supplies standard salt solution buffers (PBS, DPBS), balanced salt solutions (HBSS) and pH control buffers (sodium bicarbonate and HEPES). Cell culture grade water purified by ion exchange and reverse osmosis is also part of the product range.

hES Prime – for maintenance and expansion of hES cells

Human embryonic stem cells (hESC) are derived from blastocysts and defined by their ability to proliferate indefinitely while remaining undifferentiated. Often, hESC are cultured with the time-consuming use of mouse and human fibroblast feeder layers or feeder-conditioned medium (FBS included). This can lead to undefined conditions and moreover the feeder cells may interact in further experiments, leading to unreliable results.

Feeder-free and feeder-dependent cultivation of hES cells

PAA Laboratories has developed hES Prime, a new xeno-free medium that enables both the maintenance and expansion of human embryonic stem cells without differentiation. Whether you require feeder-dependent or feeder-independent culture conditions, hES Prime can meet your specific requirements. Successful cultivation under feeder-independent conditions is possible on BD Matrigel™ and further supplementation of hES Prime HSA. Under feeder-dependent conditions with mouse or human feeder cells, hES Prime can be used as a ready-to-use medium that requires no additional supplementation. All components are defined and from non-animal origin. The optimized base medium contains recombinant insulin, bFGF, TGFβ, and human transferrin.

PAA's hES Prime has been extensively tested on several hES cell lines under feeder-free and feeder-dependent conditions. Cultivation studies concerning growth kinetics demonstrated that hES Prime medium is highly suitable for human embryonic stem cells, independent from the coating matrix or cell layer. Comparing BD Matrigel and human foreskin fibroblasts (HFF), no significant difference in cell growth could be detected (Fig 1). The microscopic analyses in Figure 2 show similar cell morphology on both matrices.

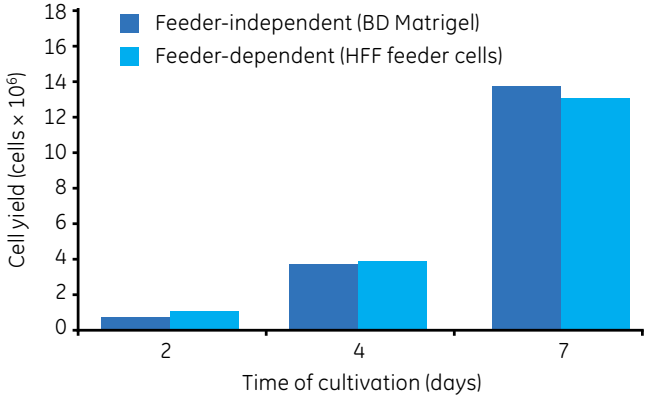
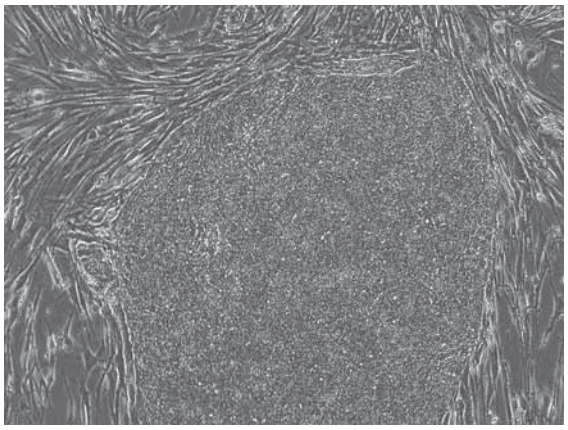


Fig 1. Comparison of feeder-dependent (on HFF feeder cells) and feeder-independent (on BD Matrigel matrix) growth of human embryonic stem cells (HuES6) cultured in hES Prime medium. Cell counts are reported for days 2, 4, and 7, respectively.

A)



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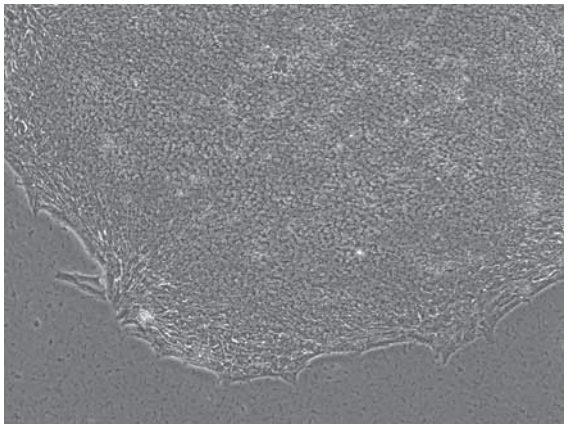


Fig 2. Microscopic documentation of **A)** feeder-dependent (on HFF feeder cells and **B)** feeder-independent (on BD Matrigel matrix) growth of human embryonic stem cells (HuES6) cultured in hES Prime Medium.

hES Prime delivers:

- Feeder-free and feeder-dependent culture of hESC
- Effective proliferation of hESC (> 50 passages)
- No differentiation of hESC
- Maintenance of pluripotence
- Xeno-free and chemically defined
- Superior expansion of hESC (i.e., h9.2, I6, I3.2, H1, huES6)

Ordering information

Product	Code number
hES Prime with Stable Glutamine, 100 ml	U11-847
hES Prime with Stable Glutamine, 500 ml	U15-847
hES Prime HSA, 10% Solution, 5 ml	C005-031
hES Prime HSA, 10% Solution, 25 ml	C025-031

Improve the basics in Western blotting using Amersham™ ECL™ Prime

Amersham ECL Prime is a highly sensitive Western blotting reagent for chemiluminescent detection of proteins. The reagent enables the use of highly diluted primary and secondary antibodies without compromising sensitivity and the stable signal generated allows multiple exposures and makes the reagent suitable for large experimental series. To support you in your Western blotting with Amersham ECL Prime and other reagents from GE Healthcare, we provide a number of useful online resources as well as our comprehensive handbook.

A new video describing the step-by step approach to successful Western blotting using Amersham ECL Prime is available. This video will help you get started with the reagent and provides useful hints and tips as well.

G

The Amersham ECL Prime forum gives you access to comments posted by other scientists using the reagent in Western blotting. Here you have a chance to win a T-shirt either by uploading your own Amersham ECL Prime results or publishing a comment. Sign up to gain access to the forum at

The Western blotting discussion forum is another online resource that allows you to share any problems you may be experiencing in Western blotting and provides troubleshooting advice. The forum is monitored by GE Healthcare experts who will help you solve typical issues with Western blotting.

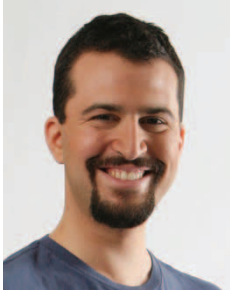
Finally, the handbook Western blotting – Principles and Methods is the definitive guide to chemiluminescent, chemifluorescent, and fluorescent Western blotting techniques using the wide range of products from GE Healthcare. This handbook is an excellent resource for experts and novices alike, and provides a wealth of information for the entire workflow, from sample preparation through to detection and analysis.

Ordering information

Product	Code number
Amersham ECL Prime Western Blotting Detection Reagent for 1000 cm^2 membrane	RPN22232
Western blotting handbook – Principle and Methods	28-9852-56

GE & Science Prize for Young Life Scientists

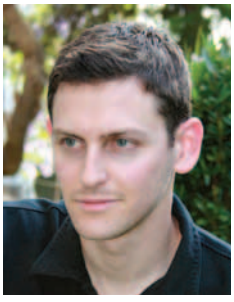
Congratulations to our 2011 winners!



Dr. Erez Lieberman Aiden

Grand Prize Winner and Regional Winner, North America

Essay: Zoom!



Dr. Eran Eden

Regional Winner, All Other Countries

Essay: Proteome Dynamics and Fate the of Individual Cancer Cells in Response to a Drug

Dr. Felipe Karam Teixeira

Regional Winner, Europe

Essay: Mechanisms of Transgenerational DNA Methylation Inheritance

Dr. Tatsuya Tsukahara

Regional Winner, Asia

Essay: CDK Directs the Chromosome Passenger Complex to Centromeres for Chromosome Bi-Orientation

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Typhoon™ FLA 9500

- **Versatility:** Imaging of multifluorescent-, chemifluorescent-, radioisotope-labeled, and colorimetric samples
- **High resolution and quantitation:** Pixel resolution of up to 10 µm and a linear signal response over five orders of magnitude provides precise quantitation in gels, blots, tissue sections, and arrays
- **High sample throughput:** Scanning area of 40 × 46 cm enables simultaneous imaging of up to 20 gels or blots, measuring 10 × 8 cm in size
- **Flexibility:** Optimized performance for new applications by adapting the system with stages, detectors, filters, and lasers
- **2-D DIGE imaging:** Simultaneously image two 2-D DIGE gels for differential expression studies
- **Visible and infrared fluorescence imaging:** Optional near infrared excitation for imaging infrared dyes

Typhoon FLA 9500 is a robust and versatile laser scanner that is suited to multiuser environments. Biomolecular imaging applications include sensitive and quantitative measurements of Western blots, multiplex fluorescence, and radioisotopic labels by storage phosphor as well as digitization of colorimetric stains. The system is flexible and can be customized with lasers, filters, and detectors for multiplex near infrared fluorescence.

Ordering information

Product	Code number
Typhoon FLA 9500	29-0040-80

PBS-P+ 10× running buffer for Biacore™ systems

The present recommendations for interaction analysis of low-molecular weight analytes in Biacore systems are that the running buffer should contain detergent in order to ensure problem-free use of systems heavily used for compound analysis.

PBS-P+ 10× is a running buffer stock solution containing 0.2 M phosphate buffer with 27 mM KCl, 1.37 M NaCl, and 0.5% Surfactant P20 (Tween™ 20). The buffer will yield pH 7.4 when diluted and supplemented with 2% DMSO. The instructions included in the product also provide information about obtained pH when other DMSO concentrations are used.

The running buffer is suitable for use with all Biacore systems except Biacore Flexchip.

Ordering information

Product	Code number
PBS-P+ 10×	28-9950-84

Biacore™ kits and reagents for capture of histidine-tagged proteins

Capture of histidine-tagged recombinant proteins is an attractive approach for protein attachment in Biacore systems for a number of reasons, one being that physiological conditions can be used during the whole procedure. In addition, capture of tags generates a directed structural orientation of the protein on the surface, potentially offering optimal site exposure.

Two capture approaches can be used for attachment of histidine-tagged ligands in Biacore systems: capture on nickel-chelated nitrilotriacetic acid (NTA) groups, and capture with immobilized anti-histidine antibodies. GE Healthcare now expands the product portfolio with products to support both approaches.

His Capture Kit

His Capture Kit is designed for capture of histidine-tagged proteins by an anti-histidine antibody and provides an alternative to capture on nickel-chelated nitrilotriacetic (NTA) groups. Depending on the nature of the interacting molecules, this approach might give lower nonspecific binding and/or higher binding stability. The anti-histidine antibody is a monoclonal antibody directed against polyhistidine tags in the C or N terminus of a protein. The optimized and verified assay keeps assay development to a minimum.

Sensor Chip NTA

Sensor Chip NTA is ready for nickel loading and capture in both sensor chip formats and different pack sizes, now also including Series S pack of 1.

Sensor Chip NTA has a carboxymethylated dextran pre-immobilized with nitrilotriacetic acid (NTA). Histidine-tagged molecules are immobilized via Ni^{2+}/NTA chelation. Simple, efficient surface regeneration is made by injection of EDTA to remove metal ions.

NTA Reagent Kit

Capture of histidine-tagged molecules using Sensor Chip NTA requires loading of the surface with nickel ions. After analyte injection the sample cycle is finished by regeneration of the surface, which removes the captured molecule. For these purposes, NTA Reagent Kit contains ready-to-use nickel solution and regeneration solution.

Ordering information

Product	Code number
His Capture Kit	28-9950-56
Series S Sensor Chip NTA*, pack of 3	BR-1005-32
Series S Sensor Chip NTA*, pack of 1	28-9949-51
Sensor Chip NTA†, pack of 3	BR-1000-34
Sensor Chip NTA†, pack of 1	BR-1004-07
NTA Reagent Kit	28-9950-43

* *For use in Biacore 4000, Biacore T200, Biacore A100, Biacore T100, and Biacore S51.*

† *For use in Biacore X100, Biacore 3000, Biacore 2000, Biacore 1000, Biacore Upgrade, Biacore X, Biacore C, and Biacore J.*

TALON® Superflow™ and prepacked formats

TALON Superflow is intended for purification of histidine-tagged recombinant proteins by immobilized metal affinity chromatography (IMAC). TALON Superflow is a cobalt-based chromatography medium (resin) that offers enhanced selectivity for histidine-tagged proteins compared with nickel-charged media.

The medium is also available in convenient prepacked formats—HiTrap™ TALON crude columns, His SpinTrap™ TALON microspin columns, His MultiTrap™ TALON 96-well plates, and His GraviTrap™ TALON gravity flow columns. The different formats allow rapid and convenient screening and purifications as well as easy scale-up, with a minimum of sample preparation and equipment.

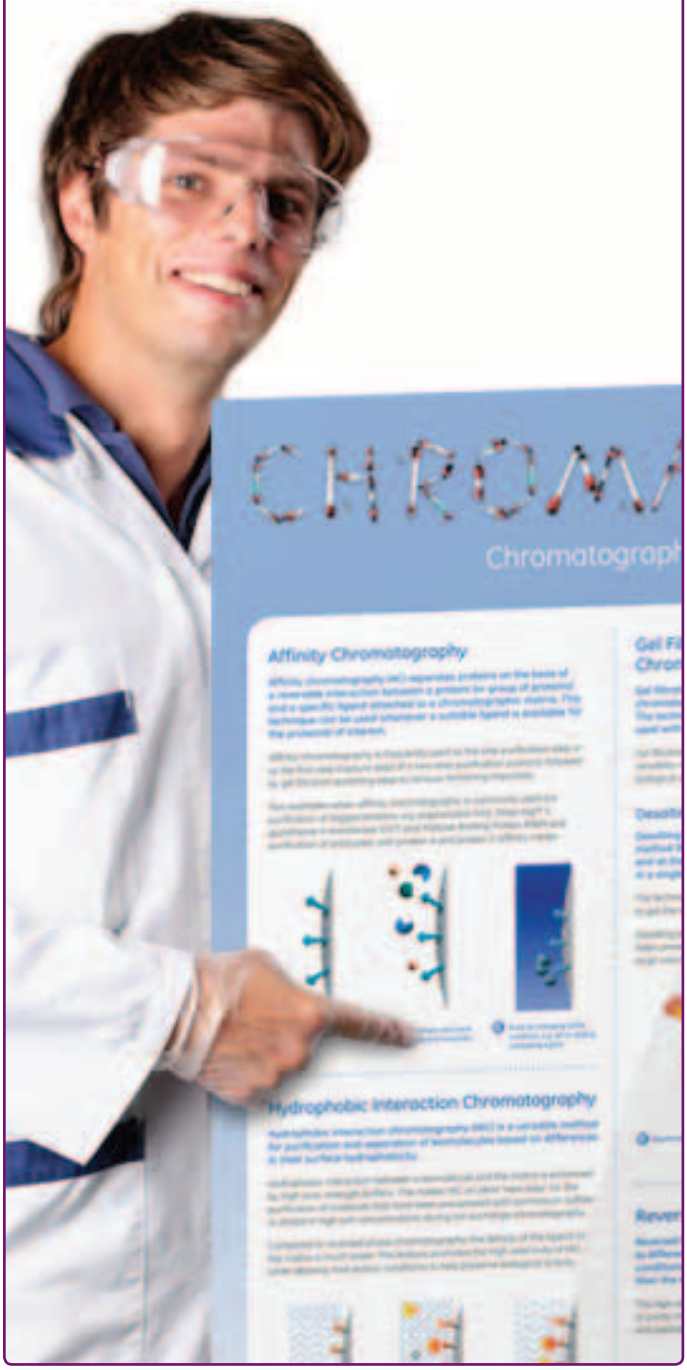
Key features and benefits:

- Suitable for purification of histidine-tagged proteins when Ni^{2+} is not the optimal choice of metal ion
- Compatible with commonly used IMAC reagents and appropriate for purifying proteins under native or denaturing conditions
- High purity in one step using a mild purification process that preserves structure and function of sensitive histidine-tagged proteins
- HiTrap TALON crude and His GraviTrap TALON columns save time and preserve target protein by allowing direct load of unclarified samples
- His SpinTrap TALON and His MultiTrap TALON enable screening with highly reproducible results

Ordering information

Product	Code number
HiTrap TALON crude, 5 × 1 ml	28-9537-66
HiTrap TALON crude, 100 × 1 ml*	28-9538-05
HiTrap TALON crude, 5 × 5 ml	28-9537-67
HiTrap TALON crude, 100 × 5 ml*	28-9538-09
TALON Superflow, 10 ml	28-9574-99
TALON Superflow, 50 ml	28-9575-02
His SpinTrap TALON 50 × 100 µl	29-0005-93
His GravTrap TALON 10 × 1 ml	29-0005-94
His MultiTrap TALON 4 × 96-well plates	29-0005-96

Chromatography educational poster



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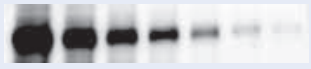
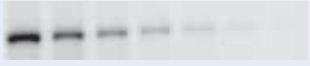
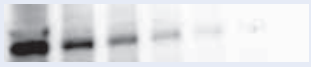
Thank you ECL™ Plus, you've been great.

For every success there's a successor. It's called progress. So while we knew we had something special in Amersham™ ECL Plus, our Western blotting team was quietly working on the next generation of detection reagent. The result: a new substrate that operates with higher levels of sensitivity, signal intensity and stability than even its famous predecessor, making it an excellent choice for CCD imagers. **Welcome to ECL Prime.**

Signal intensity sustained at higher antibody dilutions

Ab dilution		Amersham ECL Prime		Amersham ECL Plus	
Primary	**Secondary**	10 µg	156 ng	10 µg	156 ng
1:3000	1:300 000				
1:10 000	1:500 000				

Comparison of Amersham ECL Prime and Amersham ECL Plus detection of β-catenin in NIH 3T3 whole cell lysates using different dilutions of rabbit anti-β-catenin and HRP-conjugated anti-rabbit IgG.

imagination at work

11/2010