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Updates: http://wiki.oroboros.at/index.php/MiPNet21.14_Reference_sample_HRR

# Development of a reference sample for HRR

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## 1. Introduction

High-resolution respirometry (HRR) allows an efficient quantitative and qualitative analysis of mitochondrial energetics and the diagnostic assessment of function and dysfunction of oxidative phosphorylation (OXPHOS), known to be related to the occurrence of an increasing number of metabolic and other diseases. In the perspective of data harmonization between different laboratories and the establishment of a global data base on mitochondrial function (mtf), instrumental quality control combined with a standardized quality management system (QMSmtf) becomes crucial. This requires the availability of a reference sample which is functionally stable over time and across geographical space, as a basis of standard proficiency tests within and between reference laboratories.

## 2. HEK293T cells as reference sample

The reference sample for respirometry has to be suitable for prolonged storage and for large scale production in order to allow broad distribution to laboratories worldwide. Mammalian isolated mitochondria and tissue preparations do not match these requirements and were therefore ruled out. Preliminary tests were performed on several human immortalized cell lines. Three non-adherent human multiple myeloma cells, OPM-2, KMS-12-BM and NCI-H929 cells lines, were tested for their suitability as reference cell model. These cells can be readily prepared in large quantities in suspension cultures matching the criteria of large scale production. However, several attempts to establish protocols for assaying

permeabilized cells failed and we also observed a quite variable and rather low viability of cryopreserved cells, disqualifying these cells as reference samples. Similar disadvantages such as a decrease in respiratory rates during cryopreservation and high variability of respiration in cells from the same cryopreserved stock were observed in HeLa cells that were therefore also not appropriate as reference sample. In contrast, preliminary tests on HEK293T cells revealed that the viability of these cells was hardly affected even after prolonged storage and that several respiratory parameters slightly declined over time but did not show very dramatic reductions. HEK293T cells thus appeared to be suitable as reference samples and were chosen for further detailed analyses.

## 3. Instrumental setup

Instrumental setup followed standard procedures as described in detail elsewhere [1]. For each experiment, 4 instruments with two chambers each were run in parallel with chambers containing 2.3 ml of MiR05Cr. Medium was equilibrated to 37°C at a stirring rate of 750 rpm with stoppers in the 'open O2k-Chamber' position before closing the stoppers and thereby adjusting the final chamber volume to 2 ml. Every biological replicate was analysed in parallel in two chambers to produce two technical replicates.

## 4. Sample preparation

The respirometric studies described here were performed using 2 batches of HEK293T cells. A first set of experiments was performed with cells of a rather high passage. For a second set of experiments, cells were freshly purchased from a leading supplier of biological material, ATTC (cell collection code CRL-1573) and used after passage 3.

### 4.1. HEK293T culture and freezing procedure

HEK293T cells were cultured in 10 cm culture dishes in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS) (Biowest, Nuaillé, France) and 1% penicillin (50 units/ml) and streptomycin (50 µg/ml) (Gibco, Vienna, Austria). Cells at 90% confluence were washed once with 3 ml DPBS, then 1.5 ml Trypsin/EDTA solution (Gibco, Vienna, Austria) were added and the cells incubated at 37°C for 5 minutes. Cells detachment was monitored with a light microscope and cells were collected with a few ml culture medium, pooling cells from several dishes in 15 ml Falcon tubes. Cells were centrifuged for 5 min at 1500 rpm at RT, the supernatant aspired, and the pellet was resuspended in freezing medium (FBS with 10% DMSO). Cell concentration and viability were assessed using a Countess® II Automated Cell Counter and then adjusted to approximately $60*10^6$/ml by addition of freeze medium. 250 µl aliquots were transferred into cryo-tubes (Cryovial, Simport) and immediately placed at -80°C. The use of a Mr. Frosty™ Freezing Container that allows freezing to proceed at a

constant rate of cooling close to -1°C/minute did not significantly improve the viability and mitochondrial respiration of the cells compared to those frozen by placing the cryo-tubes directly at -80°C, therefore this freezing step was not included in the standard cryopreservation protocol.

### 4.2. Thawing procedure for HRR

Cryo-tubes containing frozen cells were removed from the -80°C freezer, transferred to the lab packed in crashed ice within less than 5 minutes, 500 µl pre-warmed DPBS were immediately added, and the cells were quickly thawed by gentle pipetting to obtain a homogenous cell suspension. A small aliquot was removed and used for determination of viability and cell count as described above. The appropriate amount of the suspension was then injected into the O2k chambers containing MiR05Cr so as to obtain a final concentration of $1.5*10^6$ cells/ml. Resuspending cells in MiR05 instead of DPBS had no impact on respiration and may thus be used as an alternative, particularly when studying only permeabilized cells.

### 4.3. HEK293T storage in dry ice

To assess the effect of storage in dry ice on cell viability and mitochondrial activity, vials of HEK293T cells were prepared as described above (4.1. and 4.2.). Vials of cells previously stored at -80°C were transferred to a Styrofoam box filled with dry ice and the box was left at RT in order to reproduce shipping conditions. After 48h of storage in dry ice, 4 vials were immediately used for respirometry experiments and the remaining vials were transferred back to -80°C and tested after 7 days and after 1 month of storage.

## 5. The SUIT protocol for monitoring mitochondrial function in cryopreserved HEK cells

The instruments were adjusted as described in section 3. Cells were added and the SUIT protocols were followed as described below.

### 5.1. SUIT protocol for permeabilized HEK

For a first set of experiments the following SUIT protocol for permeabilized HEK was used:

Cells+Dig: NS_1PM 2D 3G 4c 5S 6U 7Rot 8Ama

| | | | | | |
|---|---|---|---|---|---|
| *E* | | | 6U | 7Rot | 8Ama |
| *P* | 2D | 3G+c | 5S | | |
| *L* | 1PM | | | | |
| | N | N | NS | S | ROX |
| | CI | CI | CI&II | CII | ROX |

Dig: digitonin, 10 µg/ml

P: pyruvate, 5 mM
M: malate, 2 mM
D: ADP, 2.5 mM
G: glutamate, 10 mM
c: cytocrome c, 10 µM
S: succinate, 10 mM
U: uncoupler CCCP, added in steps of 1 µM, from 0.5-5 µM
Rot: rotenone, 0.5 µM
Ama: antimycin A, 2.5 µM

## 5.2. SUIT protocol for intact HEK

In parallel to the protocol for permeabilized protocol described in 5.1, a protocol for intact cells was followed in the same set of experiments:

Cells: 1Omy 2U 3Rot 4Ama

| E | 2U | 3Rot | 4Ama |
|---|---|---|---|
| P | | | |
| L | 1Omy | | |
| | | | ROX |
| | | | ROX |

Omy: olygomicin, 2 µg/ml
U: uncoupler CCCP, added in steps of 1 µM, from 0.5-5 µM
Rot: rotenone, 0.5 µM
Ama: antimycin A, 2.5 µM

## 5.3. SUIT reference protocol 2 (RP2) for permeabilized HEK

For a second set of experiments the SUIT reference protocol 2 for permeabilized HEK cells was followed as outlined here:

Cells+Dig: NFSGpTm_1D 2Oct 3M 4c 5P 6G 7S 8Gp 9U 10Rot 11Ama 12Tm 13Azd

| E | | | | | | | 9U | 10Rot | 11Ama | 12Tm | 13Azd |
|---|---|---|---|---|---|---|---|---|---|---|---|
| P | 1D | 2Oct | 3M+c | 5P | 6G | 7S | 8Gp | | | | |
| L | | | | | | | | | | | |
| | ROX | F | F | NF | NF | NFS | NFSGp | SGp | ROX | Tm | ROX |
| | ROX | FAO | FAO | CI &FAO | CI &FAO | CI&II &FAO | CI&II &FAO&Gp | CII &Gp | ROX | CIV | ROX |

F refers to F-junction substrates, N to N-junction substrates, S to succinate, Gp to glycerophosphate, ROX to residual oxygen consumption and Tm to TMPD.

D: ADP, 2.5 mM

Dig: digitonin, 10 µg/ml
Oct: octanoylcarnitine, 0.5 mM
M: malate, 0.05-2 mM
c: cytocrome c, 10 µM
P: pyruvate, 5 mM
G: glutamate, 10 mM
S: succinate, 50 mM
Gp: glycerophosphate, 10 mM
U: uncoupler CCCP, added in steps of 1 µM, from 1-7 µM
Rot: rotenone, 0.5 µM
Ama: antimycin A, 2.5 µM
As: ascorbate, 2mM
Tm: TMPD, 0.5 mM
Azd: azide, >100 mM

For the analysis of dry ice cryopreservation tolerance, malate was titrated 3 times obtaining a final concentration of 0.05-0.1-2 mM.

# 6. Evaluation of cells respiration and viability

## 6.1. Cryopreservation of high-passage HEK

Figure 1 depicts the viability and the cell count of HEK 293T cells cultured and stored at -80°C for up to 3 months. Cells stored at -80°C for up to 1 month showed a similar viability compared to freshly cultured cells, while cells stored for 2 or more months showed a significant decrease in viability. Cell count was not affected significantly along the storage time evaluated. Figure 2 summarizes the results of mitochondrial respiration experiments performed with intact and permeabilized cells. The results of both protocols indicated a reduction in mitochondrial activity in cells cryopreserved at -80°C compared to the fresh cultured cells. After the initial drop in mitochondrial activity following cryopreservation, the cells appeared stable at -80°C for the 3 months storage time considered.

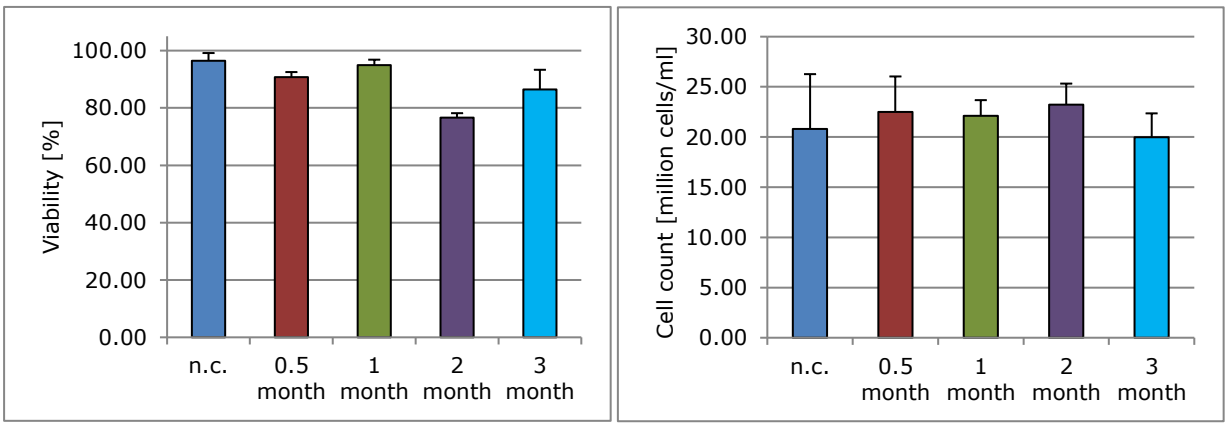
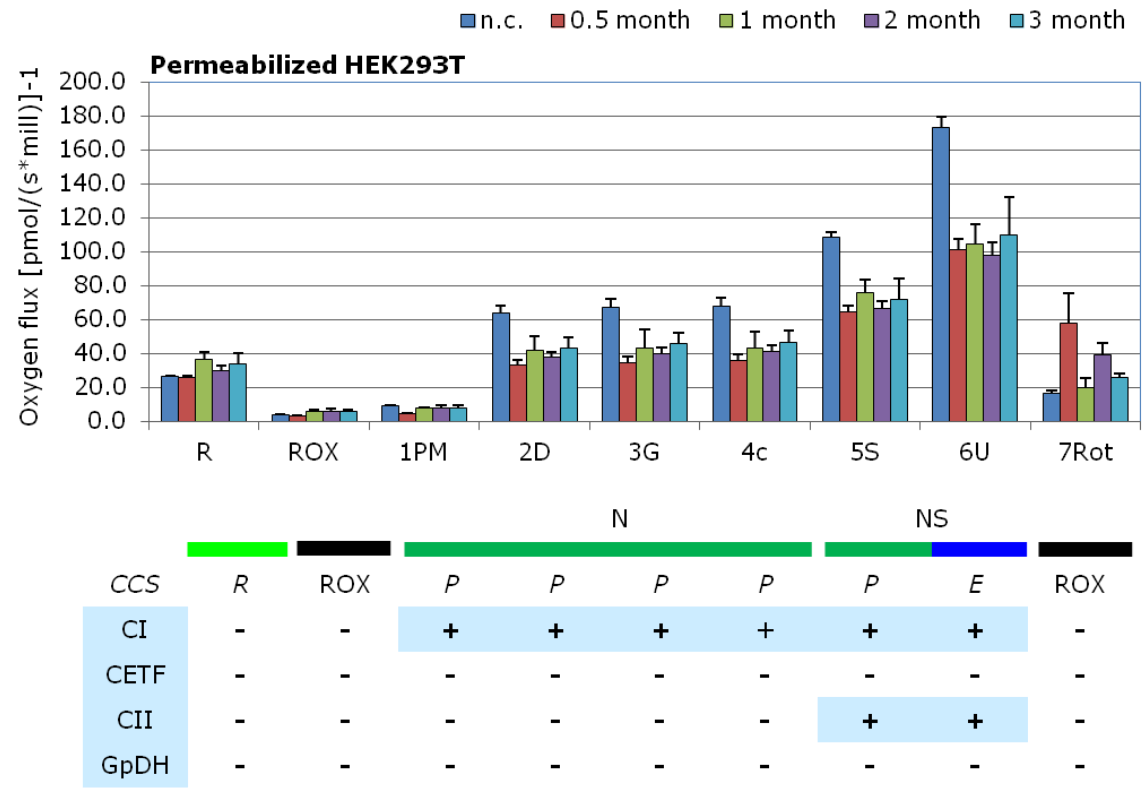



Fig. 1. Viability (left panel) and cell count (right panel) of HEK 293T cells not cryopreserved (n.c.) and cryopreserved at -80°C for 0.5 to 3 months. Data shown are means ± SD of 4, 2, 8, 4 and 11 cultures respectively.



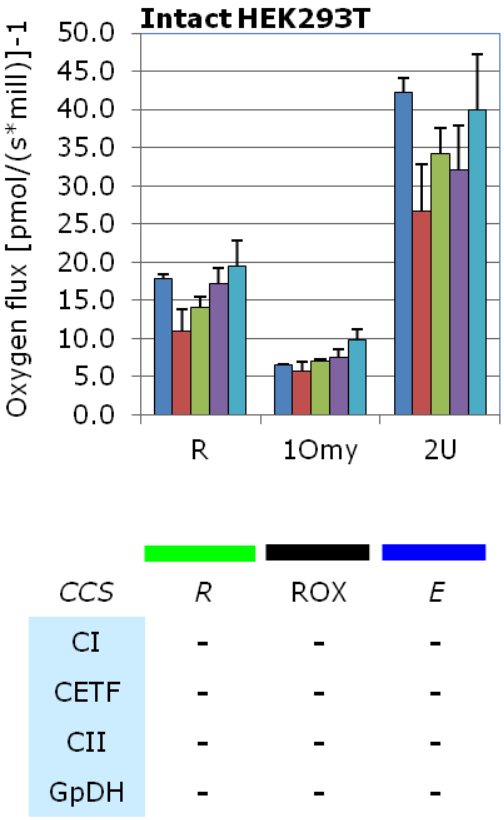




Fig. 2. Respiration rates in different coupling and substrate states of permeabilized (upper panel) and intact (left panel) HEK 293T cells not cryopreserved (n.c.) and cryopreserved at -80°C for 0.5 to 3 months. Data shown are means ± SD of 2 experiments for the 0.5 months time-point and 4 experiments for the other time-points, each measured in duplicate.

## 6.2. Cryopreservation of low-passage HEK

A second set of experiments was performed using HEK293T cells directly obtained from a supplier of biological material (ATTC), propagated to a large population up to passage 3 and then collected as described above and cryopreserved at -80°C for up to 3 months. Figure 3 depicts the viability and the cell count of this batch of cells. Cells stored at -80°C show a slight time-dependent decline in cell viability compared to samples assaysed prior to cryopreservation. Cell count was not affected significantly over storage time considered. Figure 4 summarizes the results of mitochondrial respiration experiments performed with permeabilized cells. The mitochondrial activity was not significantly reduced in cells cryopreserved for 0.5 and 1 month, the latter samples actually displaying a slight increase in various substrate states in OXPHOS and in ETS capacity and complex IV activity. In contrast, a moderate reduction in mitochondrial activity was subsequently observed in cells stored at -80°C for 2 and 3 months. The FCR data obtained by normalizing respiratory rates to ETS capacity were largely comparable in freshly cultured cells and cells cryopreserved for up to 3 months.

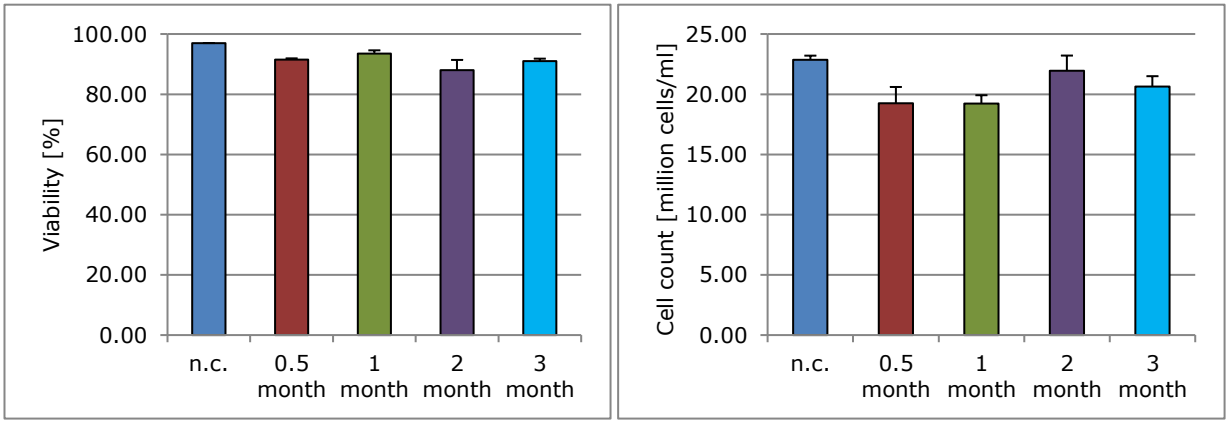



Fig. 3. Viability (left panel) and cell count (right panel) of HEK 293T cells not cryopreserved (n.c.) and cryopreserved at -80°C for up to 3 months. Data shown are means ± SD of 2 cultures for the cultured cells examined prior to freezing and of 4 cultures for the other time-points.

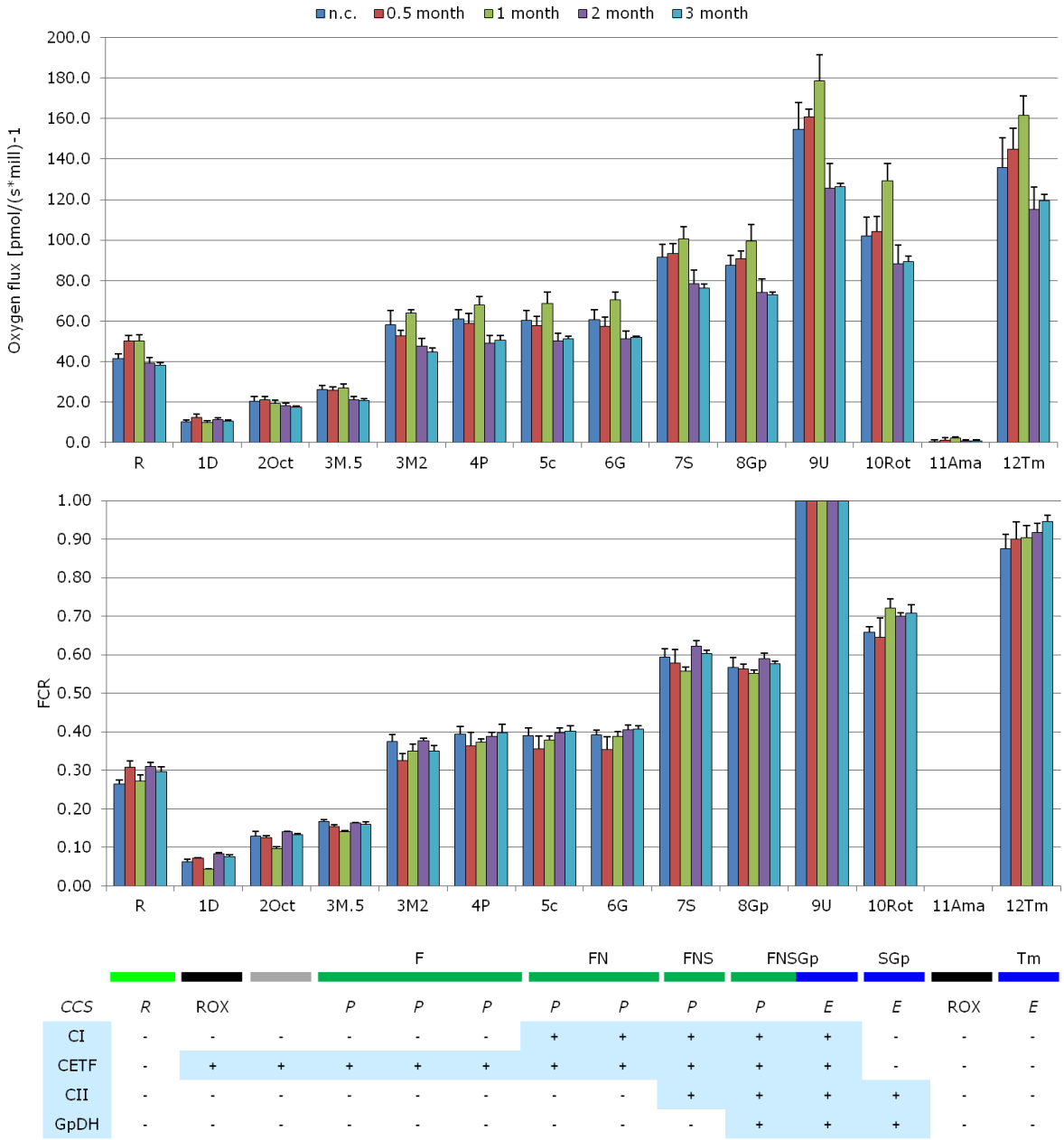



Fig. 4. Respiration rates (upper panel) in different coupling and substrate states of permeabilized HEK 293T cells not cryopreserved (n.c.) and cryopreserved at -80°C for 0.5 to 3 months. Data shown are means ± SD of 4 experiments, each measured in duplicate. The lower panel shows FCR data.

## 6.3. Tolerance of dry ice preservation of low-passage HEK

Figure 5 depicts the viability of the HEK cells stored at -80°C before and after 48 h storage in dry ice. Storage in dry ice did not significantly affect cell viability, even if a slight decrease in cell viability was noted in cells stored for 1 month at -80°C after 48 h of storage in dry ice. Figure 6 summarizes the results of the mitochondrial respiration experiments performed on 4 independent runs using 4 separate cell stocks in duplicate. These experiments indicate that storage in dry ice did not

significantly alter mitochondrial respiration of cells stored up to one month at -80°C after dry ice storage. Respiration appeared to be slightly but not significantly diminished after one month at -80°C following dry ice storage.

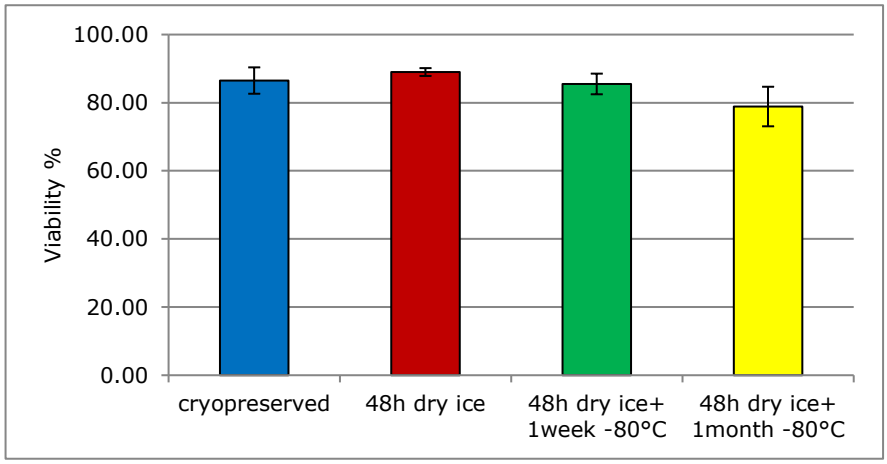
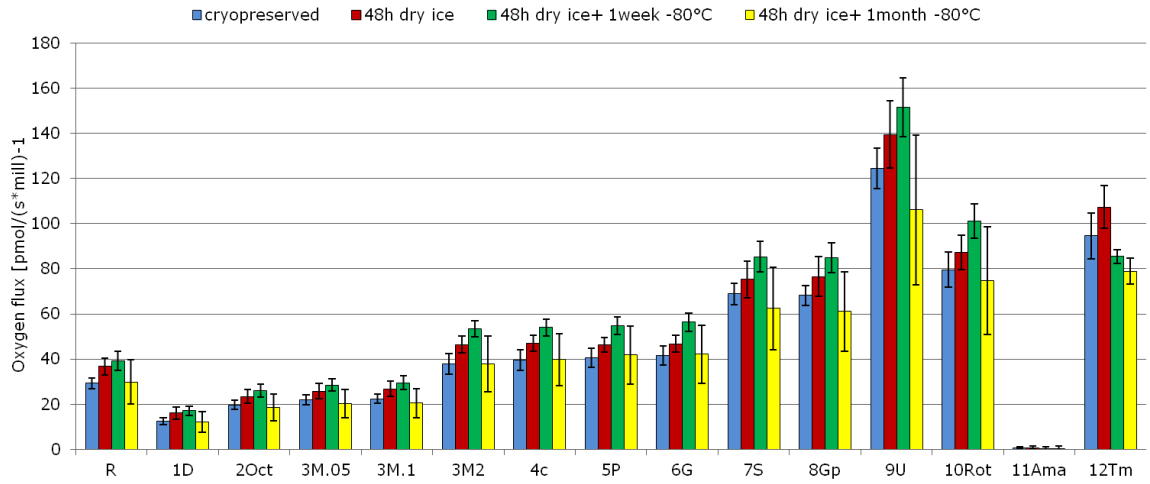
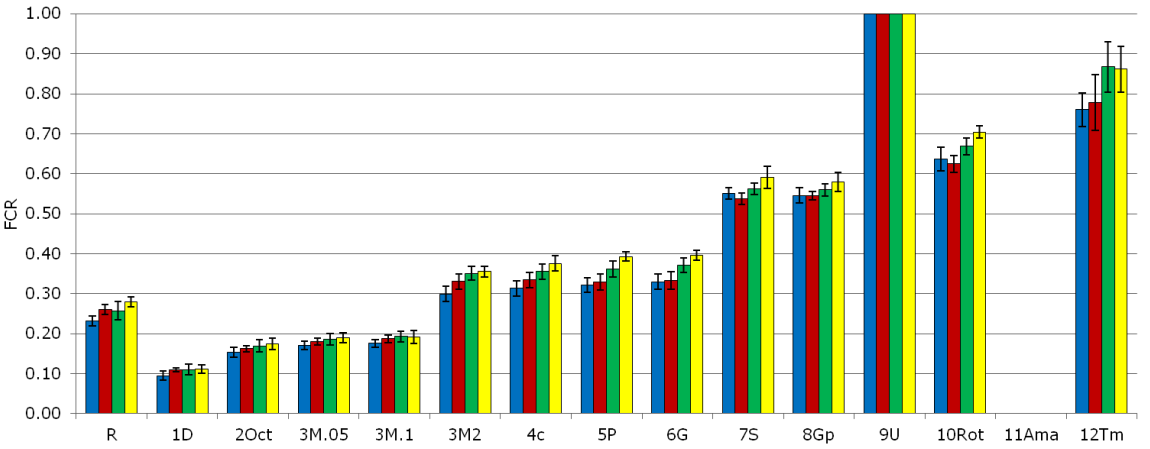



Fig. 5. Viability of HEK 293T cells stored at -80°C for 2 days (cryopreserved), right after 48 h storage in dry ice, and after 48 h storage in dry ice followed by 1 week and 1 month storage at -80°C.




| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | F | | | | FN | | FNS | FNSGp | | SGp | | Tm | |
| *CCS* | *R* | ROX | | *P* | *P* | *P* | *P* | *P* | *P* | *P* | *P* | *E* | *E* | ROX | *E* | |
| CI | - | - | - | - | - | - | - | + | + | + | + | + | - | - | - | |
| CETF | - | + | + | + | + | + | + | + | + | + | + | + | - | - | - | |
| CII | - | - | - | - | - | - | - | - | - | + | + | + | + | - | - | |
| GpDH | - | - | - | - | - | - | - | - | - | - | + | + | + | - | - | |

Fig. 6. Respiration rates (upper panel) of HEK 293T cells in different coupling and substrate states. Data shown are means ± SD of 4 experiments, each measured in duplicate. Respiration was measured after 2 days storage at -80°C (cryopreserved), right after 48 h storage in dry ice and after 48 h storage in dry ice followed by 1 week and 1 month storage at -80°C. The lower panel shows FCR data.

## 7. Conclusions

HEK293T cells appeared to tolerate cryopreservation reasonably well, maintaining cell viability almost constant and showing a decline of respiration which was not very pronounced. Importantly, this decline in respiration appeared to affect all coupling and substrate states in a uniform manner, thereby preserving the respiratory signature of the cells. From our two series of experiments, it appeared that the passage at which cells are cultured may be a critical parameter for ensuring the maintainance of good mitochondrial activity. In early-passage HEK293T cells even storage in dry ice for 48h appeared to be tolerated well. Thus, for a moderate period of time at -80°C, cell viability and respiration remained stable and comparable to that of freshly cultured cells. Therefore, we conclude that HEK293T cells can be considered as promising candidates to serve as a valuable respirometric reference sample. Further detailed analysis have to be performed in order to assess the tolerance that HEK293T cells have for prolonged cryopreservation periods in order to define a standard protocol for cryopreservation and for the distribution of the reference sample.

## 8. References

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