Determination of ATP, ADP, AMP Levels by Reversed-Phase High Performance Liquid Chromatography in Cultured Cells

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Abstract

Cytoplasmic and mitochondrial Ca^{2+} signals couple cellular ATP production to activity related energy demand. In order to accurately determine the bioenergetic effect of Ca^{2+} signals, cellular energy charge, i.e. the compound ratio of the phosphorylated adenine nucleotides AMP, ADP and ATP should be estimated. Reversed-phase high-performance liquid chromatography (RP-HPLC) allows the rapid separation and quantitation of these molecules. Here we describe a protocol applied in our laboratories to quantify ATP, ADP and AMP nucleotides in cellular extracts.

Keywords: reversed-phase high performance liquid chromatography (RP-HPLC), ATP/ADP ratio, energy charge, glycolysis, oxidative phosphorylation, Ca^{2+} signal.

1. Introduction

Cellular Ca^{2+} signals are generated in response to a wide variety of extracellular signals in virtually all cell types and trigger responses ranging from muscle contraction, neuronal activity, hormone secretion to oocyte fertilization and cell growth (1). Overall, these cellular responses increase cellular energy demand by consuming ATP. It has been postulated that Ca^{2+} signals, in parallel to increasing cellular activity related energy demand, also signal ATP production via increased oxidation of nutrients, thus function as signals coupling cellular energy production to demand (2). The main sources of ATP production in cells are glycolysis and mitochondrial oxidative or substrate level phosphorylation (3–5). Glycolysis is either a direct source of ATP (produced by phosphoglycerate kinase and pyruvate kinase), or NADH produced by glyceraldehyde 3-phosphate dehydrogenase can be transferred to mitochondria via the malate-aspartate shuttle. Glycolysis is also the direct source of pyruvate, which is

terminally oxidized in the mitochondria by coupling TCA generated NADH to the respiratory chain. Importantly, several components (enzymes and transporters) of this catabolic machinery are directly regulated by Ca^{2+} (6).

While measuring cellular ATP levels (e.g. by luciferase luminescence-based methods) can provide some limited information on cellular bioenergetic state, it is the ratio of ATP and ADP which directly determines the rate of ATP dependent reactions (7). Moreover, phosphotransfer systems, involving ATP and AMP generation from ADP via adenylate kinases, maintain balance between ATP producing and consuming reactions (8), thus ultimately the compound ratio of the high free energy phosphate bonds in ATP and ADP over the total level of the three phosphorylated adenosine nucleotides is the accurate measure of cellular energy charge (9, 10), requiring the simultaneous determination of all three adenosine nucleotides.

Currently, reversed-phase high-performance liquid chromatography (RP-HPLC) is the method of choice for the most reliable, rapid simultaneous analytical determination of ATP, ADP and AMP (11). RP-HPLC separates molecules on the basis of their hydrophobic interaction in the mobile phase (the solvent) with the stationary phase of the column (the sorbent). Higher polarity (increase phosphorylation) leads the less interaction with the column and thus shorter retention times. The technique allows both the separation and identification of a large number of nucleotides (qualitative analysis), and the exact quantification of nucleotide concentrations (quantitative analysis). Here, we describe the application of this technology by using a reversed-phase fully porous silica (C18) column to identify and quantify adenine nucleotides (ATP, ADP and AMP).

2. Materials

Prepare all the solutions using ultrapure water and analytical grade reagents. When filtration is required, use filter bottles provided with PES membrane (pore size 0,22 µm).

2.1 Buffers for sample preparation

1. *Extraction Buffer*: 0,3 M perchloric acid ($HClO_4$), 1 mM ethylenediaminetetraacetic acid disodium salt (Na_2EDTA). Based on the MW and density of the 70% $HClO_4$ the extraction buffer is prepared by adding 258 µL $HClO_4$ and 20 µL of 0,5 M Na_2EDTA stock solution to 8 mL of water, then made up to 10 mL. For preparing 10mL of 0,5 M Na_2EDTA stock solution, weigh 1,86 g, dissolve in a beaker containing 5 mL water adding NaOH; adjust the pH at a pH 8.00; then using a graduated cylinder make it up to 10 mL. 10 mL of Extraction buffer is sufficient for processing around 10 or 20 samples. Each sample is prepared using 950µL or 475µL of Extraction buffer. This buffer is also used to prepare the blank reference for the RP-HPLC.
2. *Neutralization buffer*: 2 M potassium hydroxide (KOH). Weigh 2,24 g KOH, transfer it in a beaker containing 15 mL water to dissolve in; then, using a graduated cylinder, make up to 20 mL. This buffer is also used to prepare the blank reference for the RP-HPLC.

2.2 RP-HPLC buffers and standards

1. *Mobile phase*: 0,1 M Ammonium dihydrogen phosphate ($NH_4H_2PO_4$), pH 6,0, 1% methanol (*see* **Note 1**). Add approx. 100 mL water to a beaker or directly to a 1 L graduated cylinder, weigh 11,5 g $NH_4H_2PO_4$ and transfer it to the cylinder. Add water to reach a volume of 900 mL. Mix and adjust the pH with NaOH or KOH (*see* **Note 2**). Add 10 mL (1%) of methanol. Make up to 1L with water. Filter the solution using a filter

bottle. Filtration of the mobile phase is important to remove possible particulates that could enter the column.

2. *Adenosine 5'-triphosphate (ATP) standard solution*: 100 mM ATP, 10 mM Tris-HCl, pH 7,0 . Weigh 0,267 g ATP, dissolve it in 5 mL of 10 mM Tris buffer, pH 7,0 (*see* **Note 3**).
3. *Adenosine 5'-diphosphate (ADP) standard solution*: 100 mM ADP, 10 mM Tris-HCl, pH 7,0. Weigh 0,214 g ADP, dissolve it in 5 mL of 10 mM Tris buffer, pH 7,0 (*see* **Note 3**).
4. *Adenosine 5'-monophosphate (AMP) standard solution*: 100 mM AMP, 10 mM Tris-HCl, pH 7,0. Weigh 0,174 g AMP, dissolve it in 1 mL of 10 mM Tris buffer, pH 7,0 (*see* **Note 3**).

2.4 Equipment and Supplies

1. The HPLC equipment (Agilent 1100 series) is composed of the following modules: degasser, quaternary pump with manual injector, lamp and UV detector.
   The degasser module generates a partial vacuum allowing dissolved gases to permeate through a membrane for degassing. Alternatively, if the HPLC set up is not equipped with a degasser module, the mobile phase can be manually degassed using a vacuum pump.
   The quaternary pump ensures pulse free and stable solvent flow; in this model it is equipped with four solvent inlets with built-in gradient control. However, this protocol uses a constant mobile phase (isocratic elution), thus, gradient control is not necessary.
   The manual injector allows each sample to be manually loaded in the column using a syringe. While other HPLC setups can be provided with an autosampler, the solutions used in this protocol for the nucleotide extraction contain salt precipitates to be removed by a centrifugation step of each sample before loading, thus manual injection is preferable. The HPLC model used in this protocol is equipped with a deuterium and tungsten lamp with a wavelength range of 190-950 nm; the detection type is composed by 1024-element

photodiode array (DAD) (UV visible spectrophotometer). However, the protocol can be adapted to HPLC setups with other light sources and with a spectrophotometer recording only one wavelength.

2. Sample loop (20 µL). The sample loop is a small device for the injection volume control. Different loops can inject different volumes. For this protocol, using isocratic mode, small volumes are preferable.
3. Reversed-phase fully porous silica (C18) column (4.6 mm internal diameter x 250 mm length, 5µm particle size, 300 Å pore size (*see* **Note 4**).
4. Security guard. It is important for protecting the column and extending its lifetime, by preventing the entrance of sample particulate matter in the column (i.e. salts), that would cause increased pressure, thus affecting the baseline noise and consequently the outcome of the experiment. For this reason, if the pressure approaches the maximum limit of the column, substitute the security guard with a new one to prevent any damage to the column.

3. Methods

3.1 Sample preparation

1. Cell seeding: plate 10^6 cells in a 100mm plate. Prepare a number of plates corresponding to the number of treatments planned; prepare one more plate for protein quantification, which will be used for normalizing the results to protein content and thus allows the comparison of data obtained from different cell types.
2. Treatment of interest. After seeding, it is preferable let the cells recover for 24 h. At this point it is possible to proceed directly with the extraction protocol or apply the treatment of interest; type and timing of the treatment depend on the experimental plans. After the appropriate treatments, continue with the nucleotide extraction.

3.2 Nucleotide extraction

1. In order to assure that all the steps will be quickly and accurately executed, all the materials need to be in place before starting the nucleotide extraction. It is important to stress the cells to the minimum possible, to get the most realistic picture of the cellular metabolic state.
2. Aspire completely the culture medium, do not rinse (*see* **Note 5**).
3. Add 475 µL extraction buffer directly to the plate. This volume has been optimized for this particular experimental setup to keep adenine nucleotide concentrations in the detectable range.
4. Scrape the plate and transfer the whole solution to an appropriately marked test tube.
5. Neutralize carefully with 85 µL 2M KOH, mix by inverting the tube, and from this point keep samples on ice.
6. Centrifuge 14000g for 10’ at 4°C. The neutralization step produces potassium perchlorate, a low solubility salt that precipitates. The centrifugation is fundamental to remove completely the precipitated salts before transferring the supernatant to a new test tube. For eliminating any precipitate formed or carried over, it is important to centrifuge the samples immediately before injecting in the column. Skipping the centrifugation step can block the security guard due to deposition of salts.
7. Transfer the supernatant to a new test tube and store the samples at -80°C.

3.3 RP-HPLC

Before starting the experiment switch on the HPLC and start the software according to the manufacturer’s instructions and set up a protocol for an isocratic separation. Set up the flow rate according to the column standard parameters (*see* **Note 6**) and the UV wavelength for detection at 254 nm (optimized in our system) or the closest available. Wait until the column

is equilibrated and the baseline of the chromatogram trace is stable. The centrifugation step in 3.2 has to be carried out immediately before injection.

1. Start with blank injection: inject the solvent in which samples and standards have been prepared in order to define the background signal. In this case the solvent is a solution composed by 475 µL extraction buffer and 85 µL KOH 2M. The blank injection is a negative control and produces a peak defined by its retention time, that indicates the time of which the solvent exits the column. This represents a reference point for the following runs of standards and samples, producing a series of peaks characterized by different retention times. The first peak appears at a time corresponding to the solvent, while all the others will exit after the solvent peak.
2. Prepare standard ATP, ADP and AMP samples at a concentration in the detectable range (*e.g. 40µM*) for setting up the ideal separation between the three species; inject separately each sample and check the retention times. If necessary, change the flow rate and load again, to optimize the separation of the peaks. When the retention times of each species are sufficiently distant to allow separation, inject a sample with a mix of the three nucleotides to check if the separation is accomplished also in a complex sample (**Fig 1**).

5. Inject the experimental samples. Identify the nucleotide peaks for comparison with standard runs (**Fig 2).**
6. Use the appropriate software (provided with the HPLC, or custom made) for calculation of peak areas (*see* **Note 7**). For quantitative analysis, define the working range of concentrations for each nucleotide species and separately build a standard curve injecting proper dilutions of pure nucleotides dissolved in the blank (mixture of extraction and neutralization) buffer (**Fig 3**). Analysis: using the calibration line built for ATP, ADP and AMP, extrapolate the three nucleotides concentration present in the sample; normalize the

results to protein content if comparing different cell lines. Then calculate the ATP/ADP ratio and the energy charge (EC= [ATP] + 0.5[ADP]/ [ATP]+[ADP]+[AMP]) (**Fig 4**).

7. Run each sample at least in duplicates. On each experimental day, both the standard and experimental samples should be measured to evaluate accuracy of each measure.

4. Notes

1. It is good practice to use the same batch of mobile phase for the entire duration of the experiment. Using a new batch might shift the peaks. However, experiments can be run on extended times, thus if it is necessary to use a new batch, make sure that column equilibration, blank run and a standard run are always performed to record precisely the retention time.
2. Dissolving a powder in a glass beaker, having water at the bottom, helps the magnetic stir bar to work easily and makes the mixing faster. Once the powder is dissolved, move to a cylinder and make up to the final volume. For adjusting the pH, use concentrated solutions of NaOH or KOH to narrow the gap between the starting and required pH. Then, use a dilution to avoid overshooting the pH.
3. For preparing 1 L of 1 M Tris-HCl pH 7.0, weigh 121,1 grams of TRIZMA base, dissolve it in a beaker containing 100 mL water; then transfer to a graduated cylinder and add water to a volume of 900 mL. Adjust the pH using concentrated HCl as described above. Use an analytical balance to properly weigh low amounts of powder, as in the case of ATP, ADP and AMP. Weigh directly into a small beaker after taring. Add 5 mL of Tris-HCL to dissolve the powder, mix and store at -20 or -80°C. It is good practice to verify concentrations by measuring the absorbance of these standard solutions.
4. The C18 silica column is most frequently used for separating peptides and proteins, but with the appropriate protocol it is capable of retaining and separating the adenosine

nucleotides. Other HPLC approaches, combining different columns and mobile phases, have been developed for the more specific role of nucleotide and small molecule detection (see e.g. (12, 13)), but laboratories equipped primarily for peptide and protein separation can also benefit from using the same C18 column for these diverse scopes (see e.g. (9, 14), adapted also for the present protocol).

5. The wash step can alter nucleotides levels and slows down the extraction procedure. We have also carried out tests to confirm that eventual medium residues do not interfere with the nucleotide separation in the RP-HPLC.
6. For the choice of the mobile phase flux rate, preliminary tests were performed to achieve the best flux for the separation efficiency and limiting the time required for each run. 0,6 mL/min initial flux led to a duration of 30 minutes, increasing the flux rate to 0,8 mL/min reduced the timing to 18 minutes without reducing separation efficiency. The working flux range should be reached gradually according to the manufacturer's instruction, to avoid damaging the column due to sudden increase in pressure.
7. The peak area corresponds to the amount of nucleotides present in the sample. Using the calibration curve, it is used to calculate the corresponding absolute concentration.

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References

1. Berridge,M.J., Lipp,P. and Bootman,M.D. (2000) The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.*, **1**, 11–21.
2. Szabadkai,G. and Duchen,M.R. (2008) Mitochondria: the hub of cellular Ca2+ signaling. *Physiology (Bethesda).*, **23**, 84–94.
3. Mookerjee,S.A., Gerencser,A.A., Nicholls,D.G. and Brand,M.D. (2017) Quantifying intracellular rates of glycolytic and oxidative ATP production and consumption using extracellular flux measurements. *J. Biol. Chem.*, **292**, 7189–7207.
4. Chinopoulos,C., Gerencser,A.A., Mandi,M., Mathe,K., Töröcsik,B., Doczi,J., Turiak,L., Kiss,G., Konràd,C., Vajda,S., *et al.* (2010) Forward operation of adenine nucleotide translocase during F0F1-ATPase reversal: critical role of matrix substrate-level phosphorylation. *FASEB J.*, **24**, 2405–16.
5. Brand,M.D. (2005) The efficiency and plasticity of mitochondrial energy transduction. *Biochem. Soc. Trans.*, **33**, 897–904.
6. Llorente-Folch,I., Rueda,C.B., Pardo,B., Szabadkai,G., Duchen,M.R. and Satrustegui,J. (2015) The regulation of neuronal mitochondrial metabolism by calcium. *J. Physiol.*, **593**, 3447–62.
7. Knowles,J.R. (1980) Enzyme-catalyzed phosphoryl transfer. *Annu. Rev. Biochem.*, **49**, 877–919.
8. Dzeja,P.P. (2003) Phosphotransfer networks and cellular energetics. *J. Exp. Biol.*, **206**, 2039–2047.
9. Yang,M.S. and Gupta,R.C. (2003) Determination of energy charge potential in the C6 glioma and the HepG-2 cell culture. *Toxicol. Mech. Methods*, **13**, 97–101.
10. Atkinson,D.E. (1968) The Energy Charge of the Adenylate Pool as a Regulatory Parameter. Interaction with Feedback Modifiers. *Biochemistry*, **7**, 4030–4034.

11. Aguilar,M.-I. (2004) Reversed-phase high-performance liquid chromatography. *Methods Mol. Biol.*, **251**, 9–22.

12. Bhatt,D.P., Chen,X., Geiger,J.D. and Rosenberger,T.A. (2012) A sensitive HPLC-based method to quantify adenine nucleotides in primary astrocyte cell cultures. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, **889–890**, 110–5.

13. Qian,T., Cai,Z. and Yang,M.S. (2004) Determination of adenosine nucleotides in cultured cells by ion-pairing liquid chromatography-electrospray ionization mass spectrometry. *Anal. Biochem.*, **325**, 77–84.

14. Wang,J., Lin,T., Lai,J., Cai,Z. and Yang,M.S. (2009) Analysis of adenosine phosphates in HepG-2 cell by a HPLC-ESI-MS system with porous graphitic carbon as stationary phase. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, **877**, 2019–24.

Legend to figures

Figure 1. Chromatogram and UV spectrum of the adenosine nucleotide mixture.

a) Chromatogram of a standard mix composed of ATP, ADP and AMP (40μM each). The chromatogram shows the peaks recorded at a specific wavelength. X axis shows the retention time, Y axis indicates the absorption in mAU. Four peaks are recorded, representing solvent, ATP, ADP and AMP nucleotides, respectively. All peak parameters are shown in the table.

b) UV spectrum of the adenosine nucleotides of the standard mix. X axis shows the excitation wavelength, Y axis indicates the absorption in mAU. The 254 nm excitation peak corresponds to the absorbance of the aromatic ring of the molecule.

Figure 2. Chromatogram and UV spectrum of adenosine nucleotides in a cell extract.

a) Typical chromatogram of a sample. The adenosine nucleotides are identified by their retention time by comparing to the standard run (see Fig 1). The integration area of each peak is calculated for quantitative analysis. The relative amounts the three nucleotides in a sample is expressed by the area%.

b) The UV spectrum of the adenosine nucleotides identified in a sample following extraction. The absorbance reflects the amount of a nucleotides present in the sample.

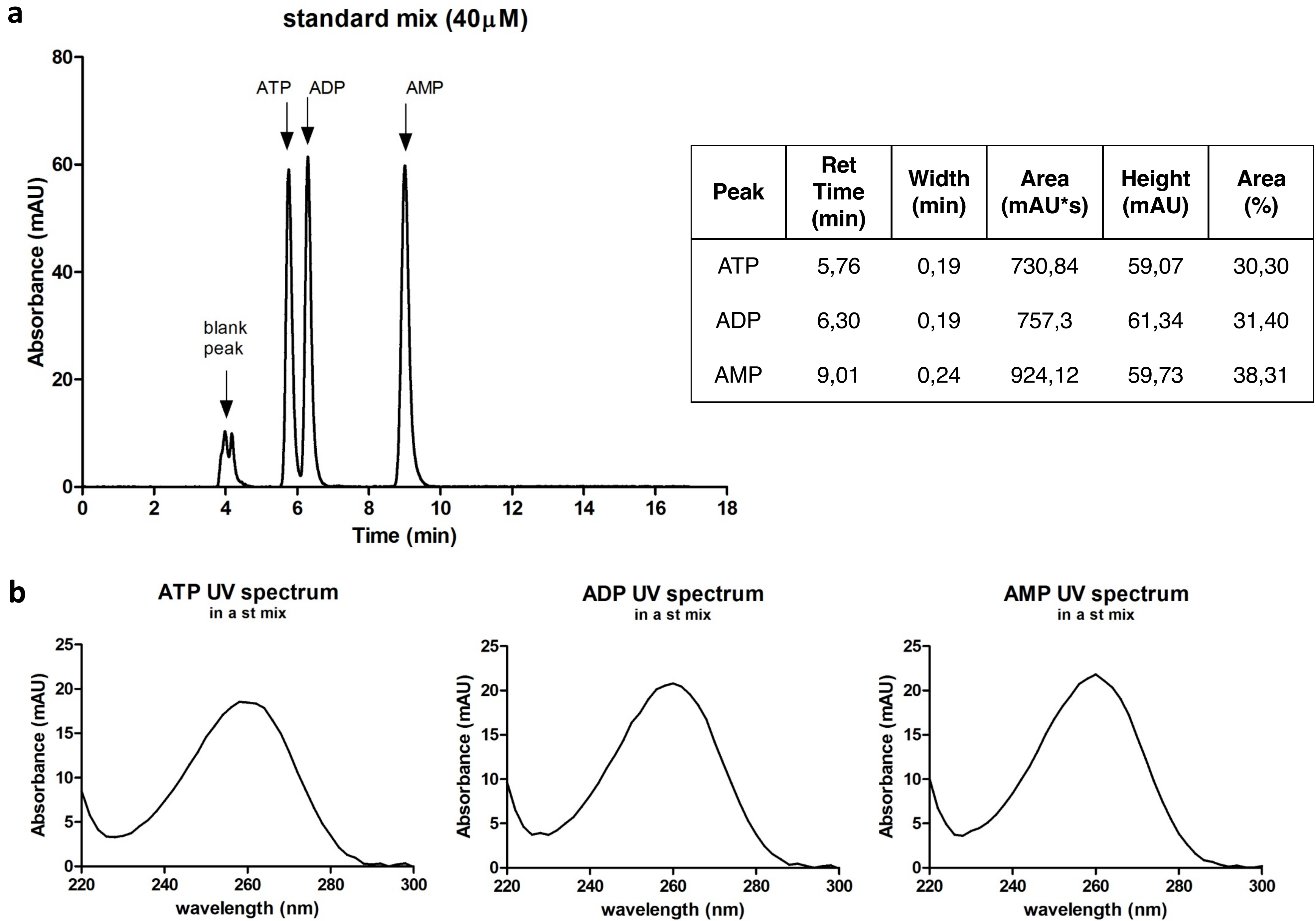
Figure 3. Quantitative analysis of adenosine nucleotides profiles in breast cancer cell lines. MCF7 and MDA-MB436 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM glucose and 2 mM glutamine.

a) Standard curves of the adenosine nucleotides, that are used to calibrate the adenosine nucleotides concentrations (in μM) of the whole cellular extract.

b) Relative ATP, ADP, AMP levels in MCF-7 and MDA-MB436 cells were expressed as the peak area normalized to the protein content. The values are mean +/- SD of two technical runs of the same sample. ctrl is the control; oligo indicates that cells before the harvest were treated with oligomycin, the inhibitor of ATP synthase, for 30 minutes .

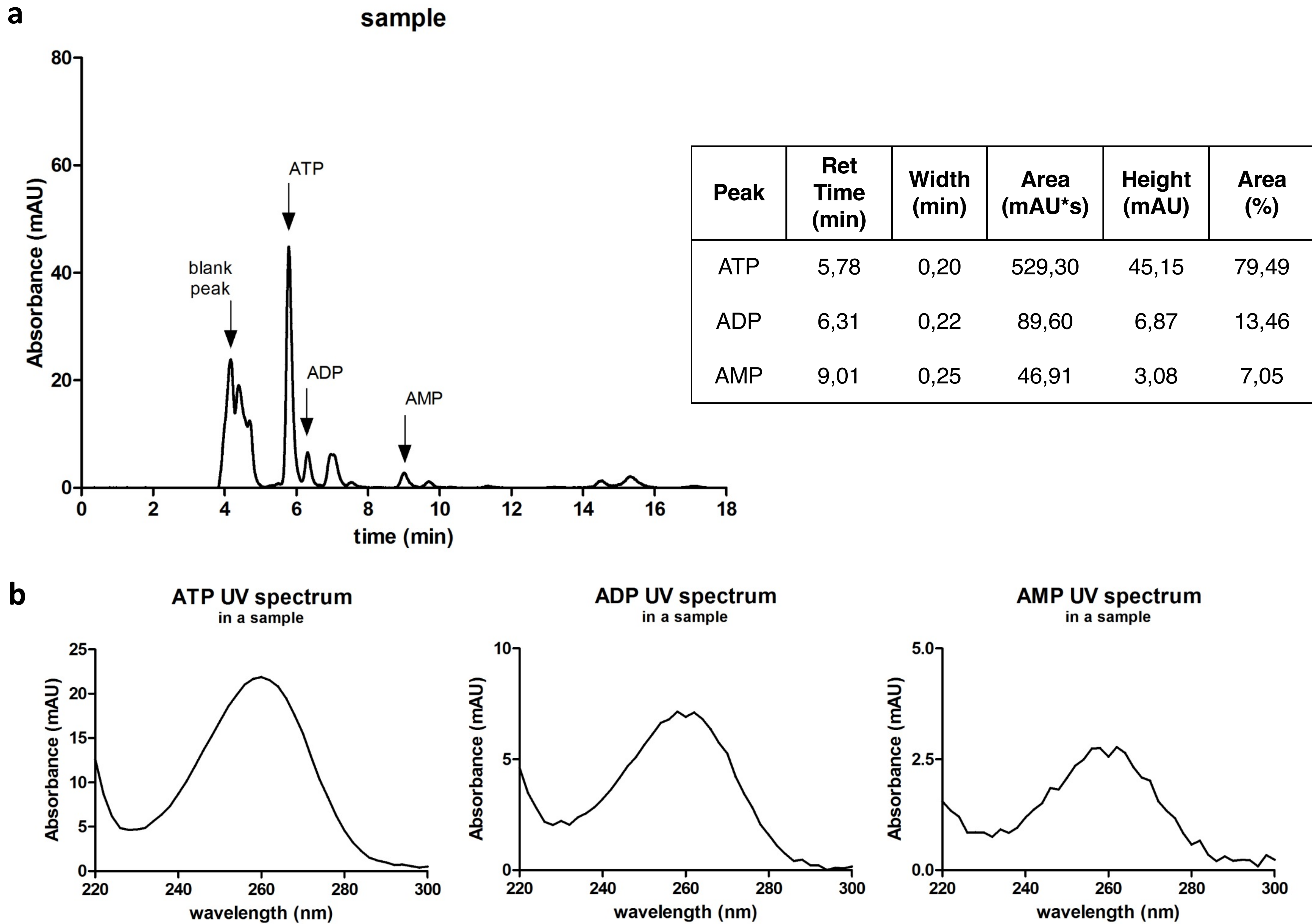
c) ATP/ADP ratio is calculated using the data of the peak area normalised to the protein content.

d) The energy charge of each cell line calculated as EC= [ATP] + 0.5[ADP]/ [ATP]+[ADP]+[AMP]).



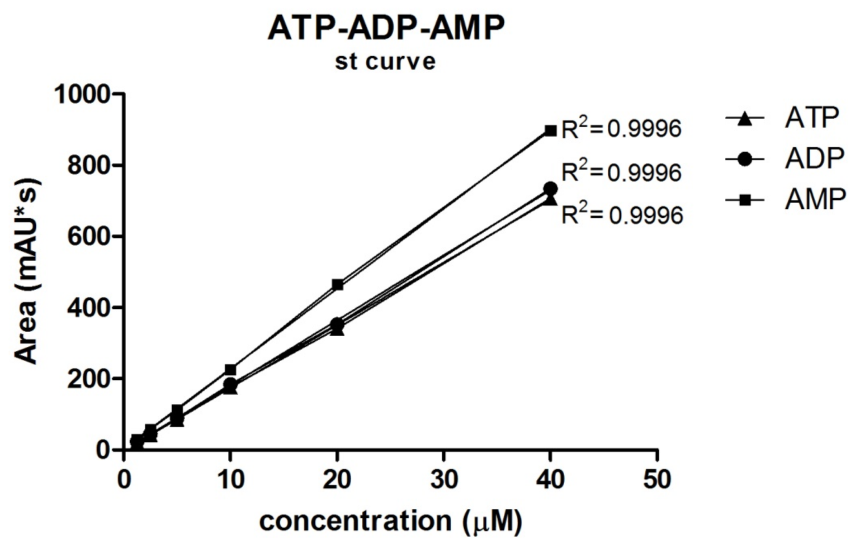
Peak	Ret Time (min)	Width (min)	Area (mAU*s)	Height (mAU)	Area (%)
ATP	5,76	0,19	730,84	59,07	30,30
ADP	6,30	0,19	757,3	61,34	31,40
AMP	9,01	0,24	924,12	59,73	38,31

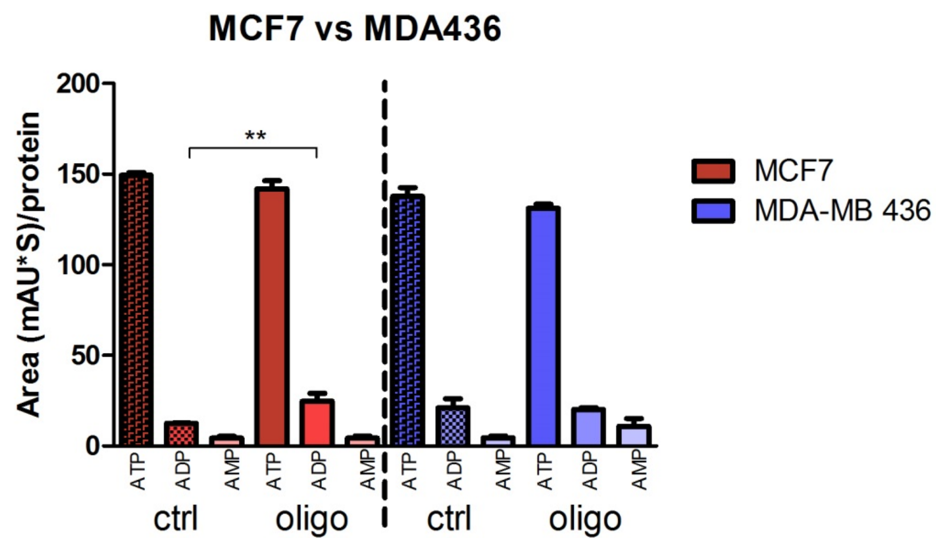
Figure 1

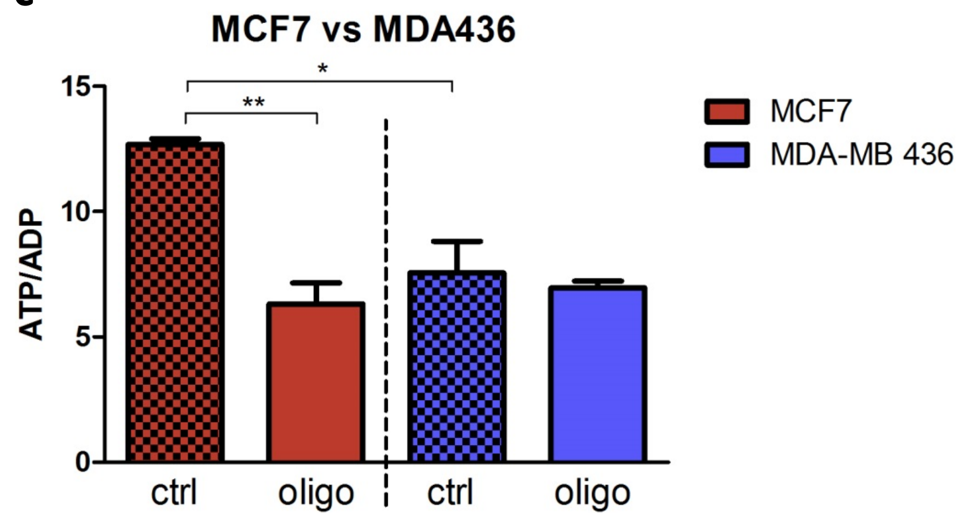


Peak	Ret Time (min)	Width (min)	Area (mAU*s)	Height (mAU)	Area (%)
ATP	5,78	0,20	529,30	45,15	79,49
ADP	6,31	0,22	89,60	6,87	13,46
AMP	9,01	0,25	46,91	3,08	7,05

Figure 2







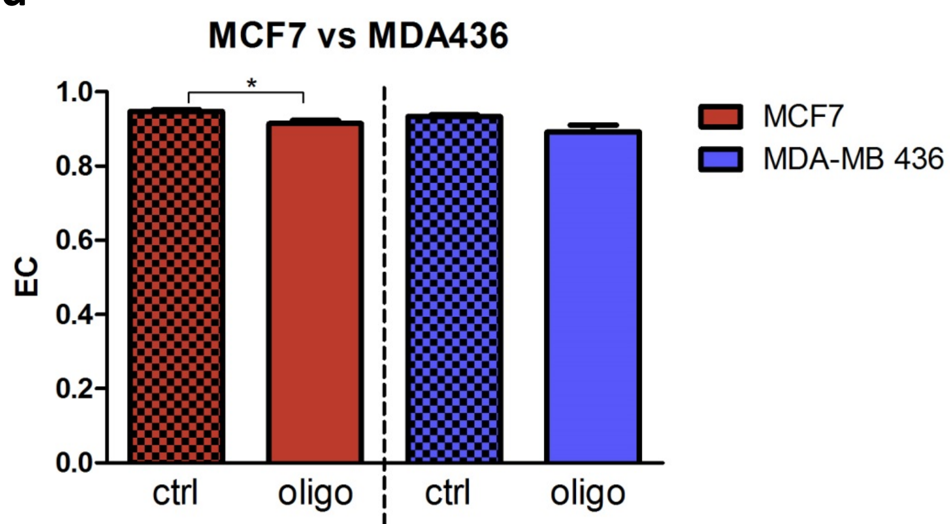


Figure 3