Differential β2-adrenergic receptor expression defines the phenotype of non-tumorigenic and malignant human breast cell lines

Background

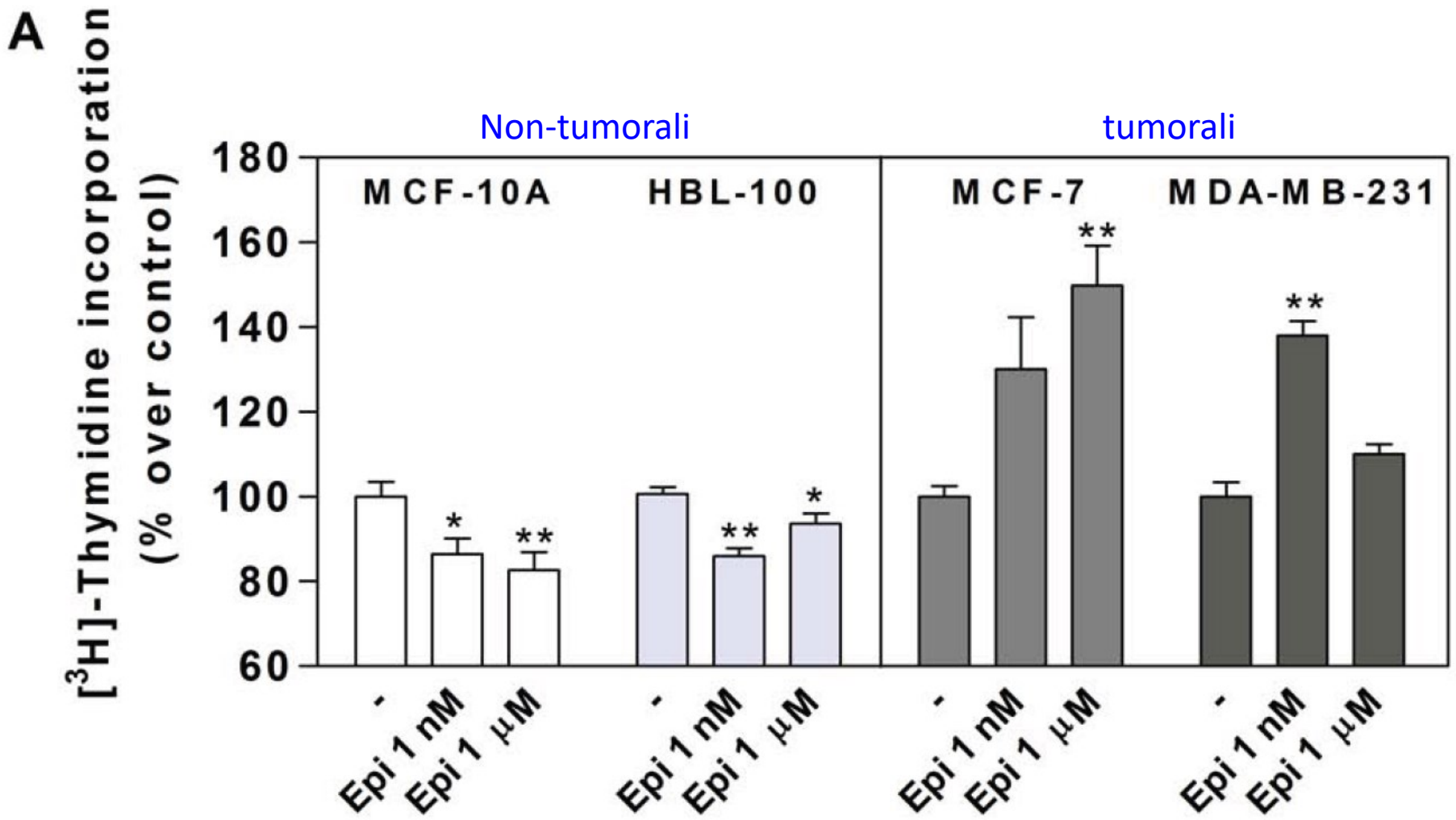
- Il tumore al seno rappresenta il 23% di tutti i tumori femminili ed è la prima causa di morte per tumore nelle donne

- I recettori adrenergici sono stati coinvolti sia nella stimolazione che nella inibizione della proliferazione tumorale

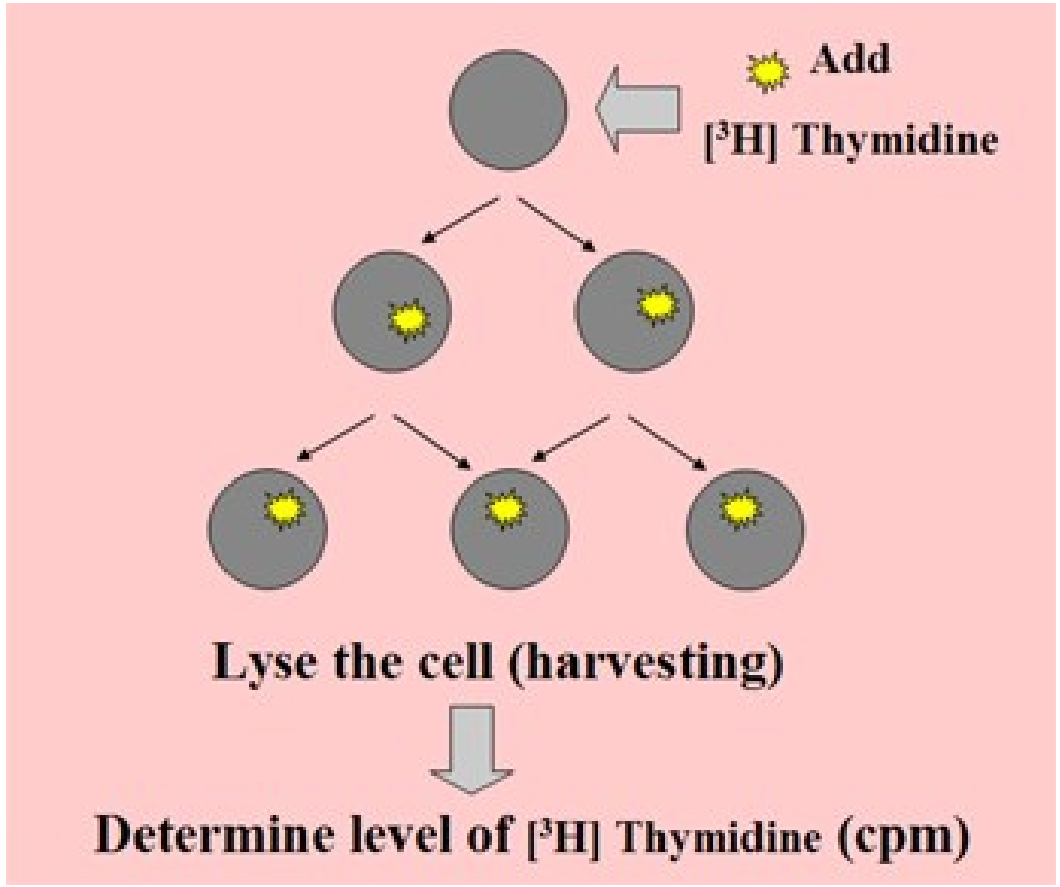
- Aderenalina e nor-nadrenalina attivano nove recettori: $\alpha_{1a,}$ $\alpha_{1b,}$ α_{1d} -Gq; $\alpha_{2a,}$ $\alpha_{2b,}$ $\alpha_{2c,}$-Gi; $\beta_{1,}$ $\beta_{2,}$ β_{3}-Gs

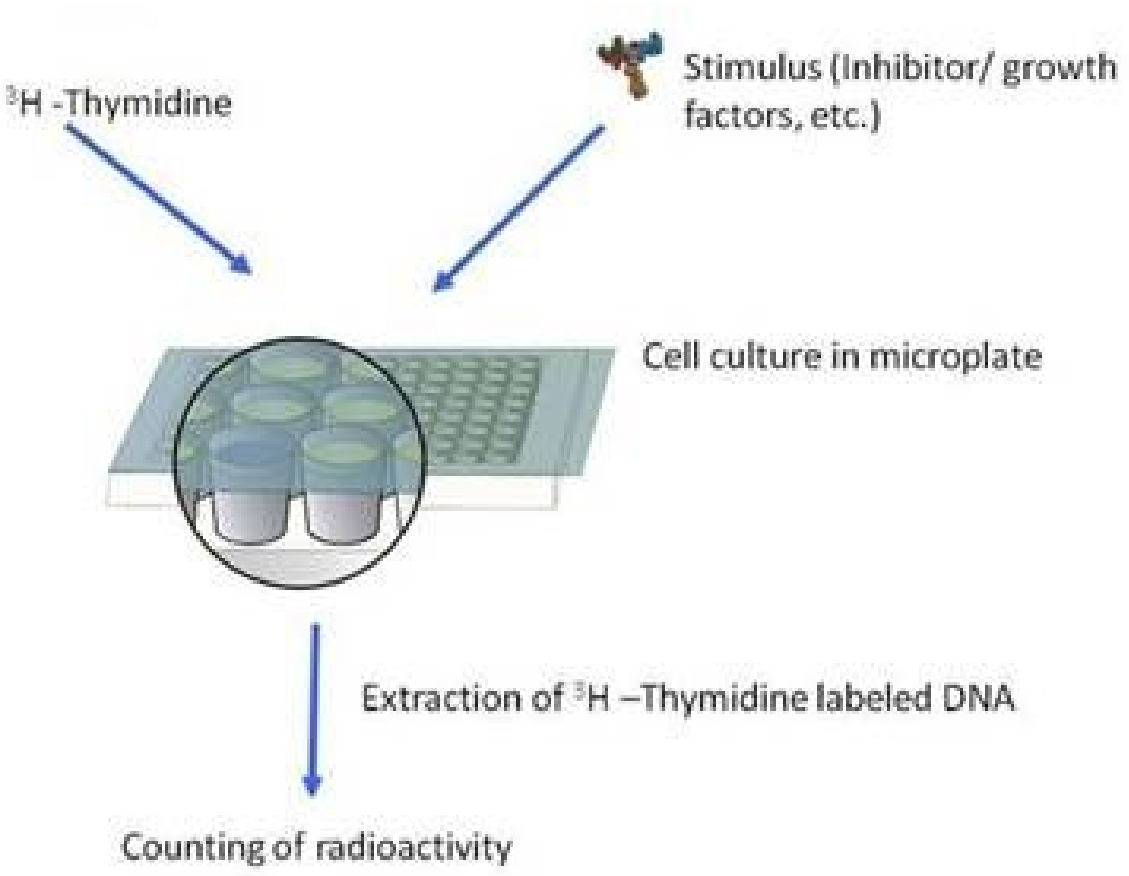
- Tra le caratteristiche peculiari di una cellula tumorale ricordiamo la **proliferazione**, la **ridotta adesione** e la **motilità**

Effect of epinephrine (Epi; attiva sui recettori α e β) on MCF-10A, HBL-100, MCF-7 and MDA-MB-231 cell proliferation.

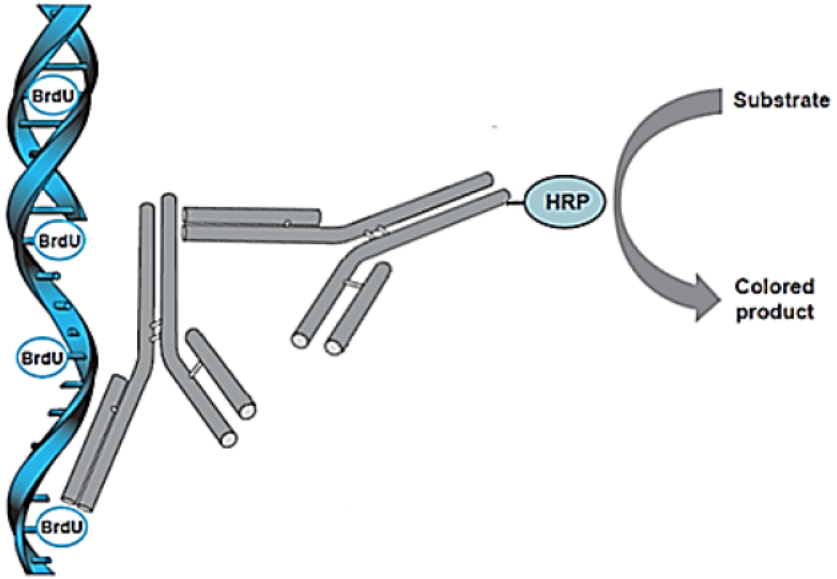


Proliferation assays based on DNA

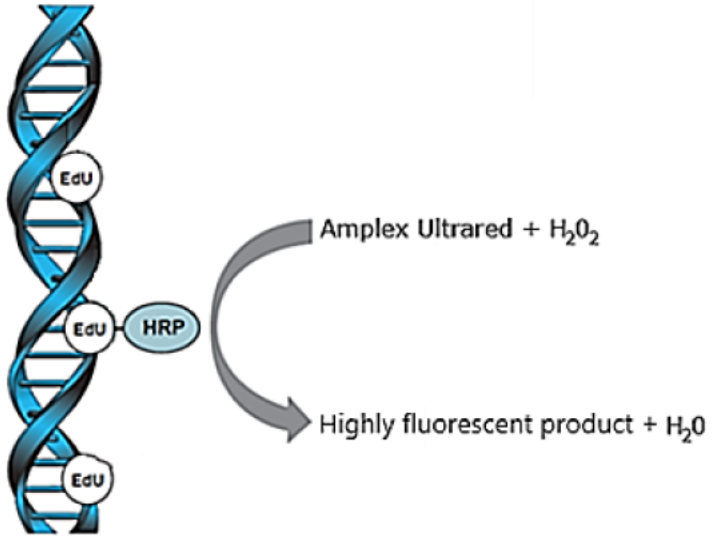




Cell proliferation assays



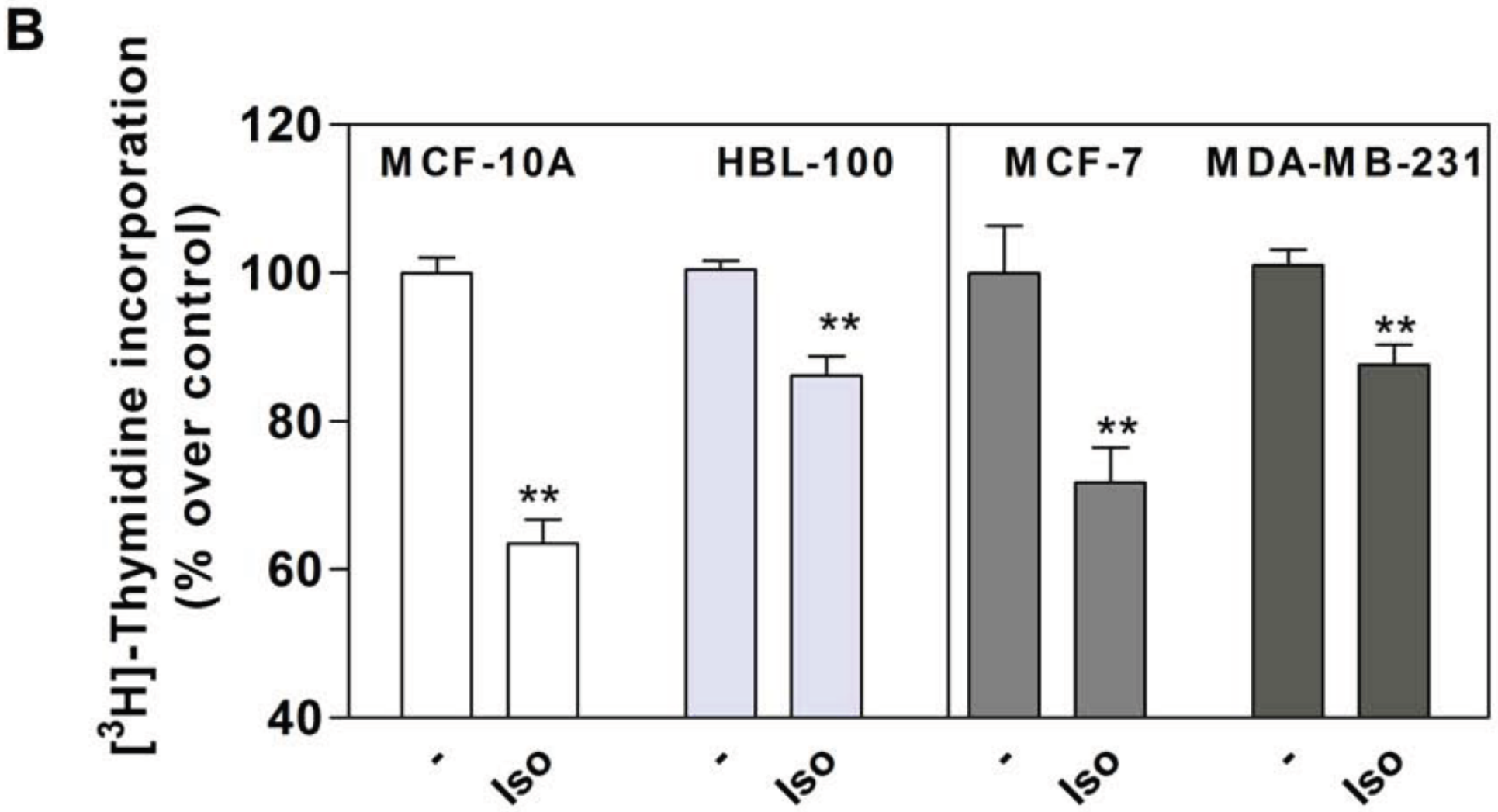
Antibody-based detection of BrdU



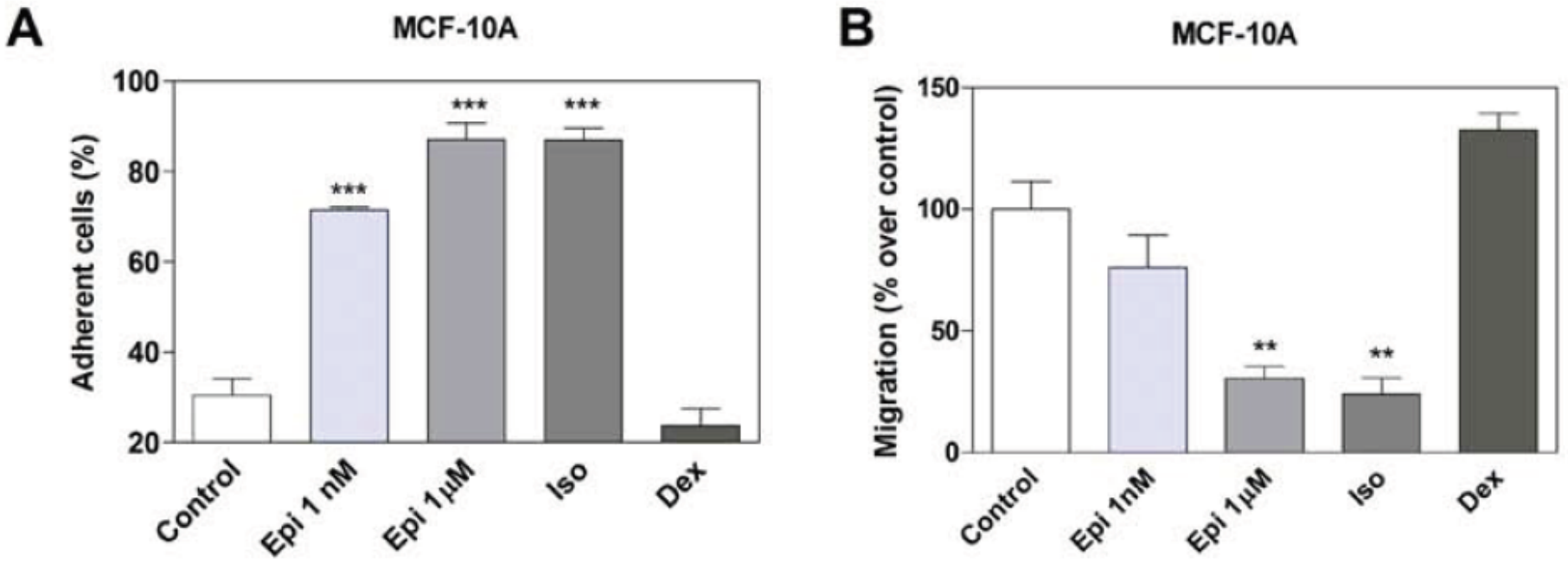
Click-iT EdU Proliferation Assay for microplates

Comparison of antibody-based BrdU and Click-iT™ EdU (5-ethynyl-2'-deoxyuridine) proliferation assays

Effect of isoproterenol (Iso; attivo sui recettori β) on MCF-10A, HBL-100, MCF-7 and MDA-MB-231 cell proliferation.



Effect of adrenergic compounds on cell adhesion and migration in MCF-10A cells.

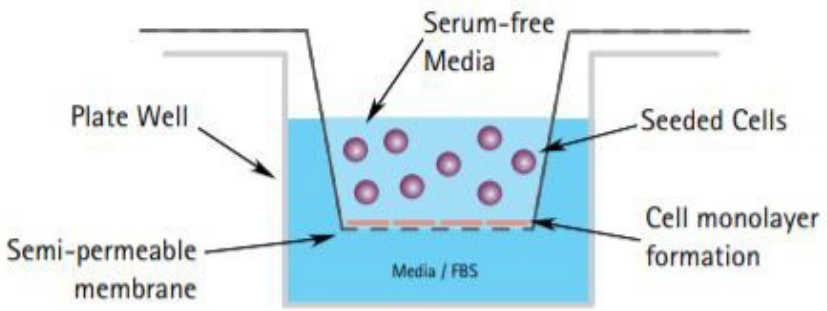


Dexmedetomidine=α_2-AR agonist

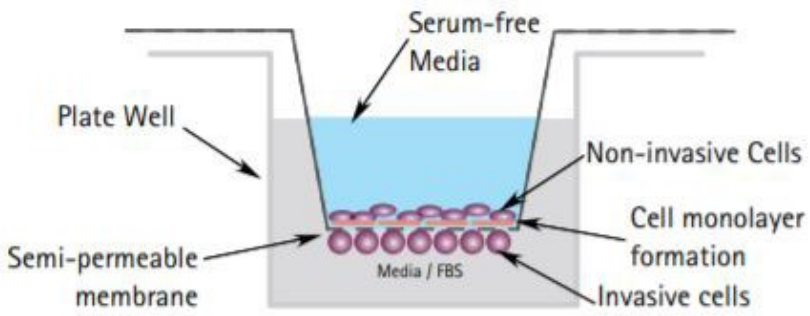
α_2-AR= Gi coupled receptor

Transwell migration assay

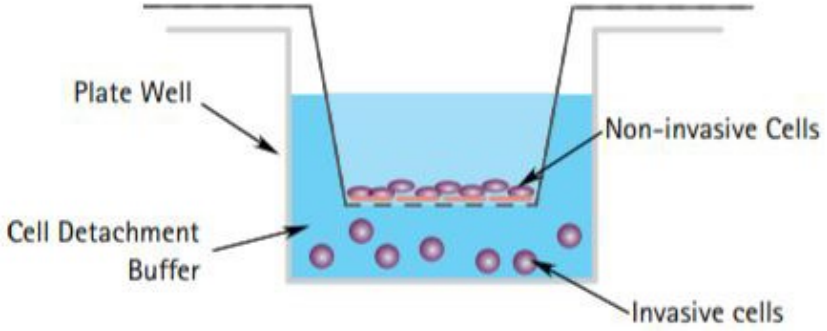
1. Load cell suspension into plate well insert



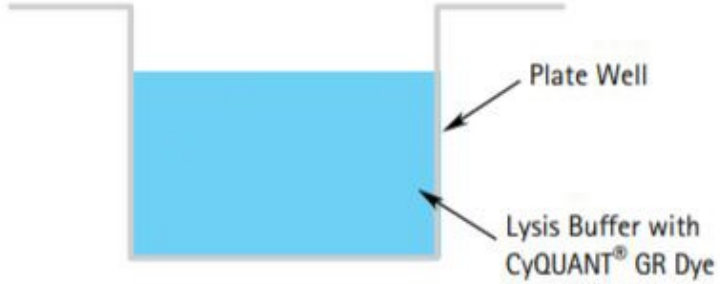
2. Invading cells migrate and attach to bottom of membrane. Non-invading cells remain above



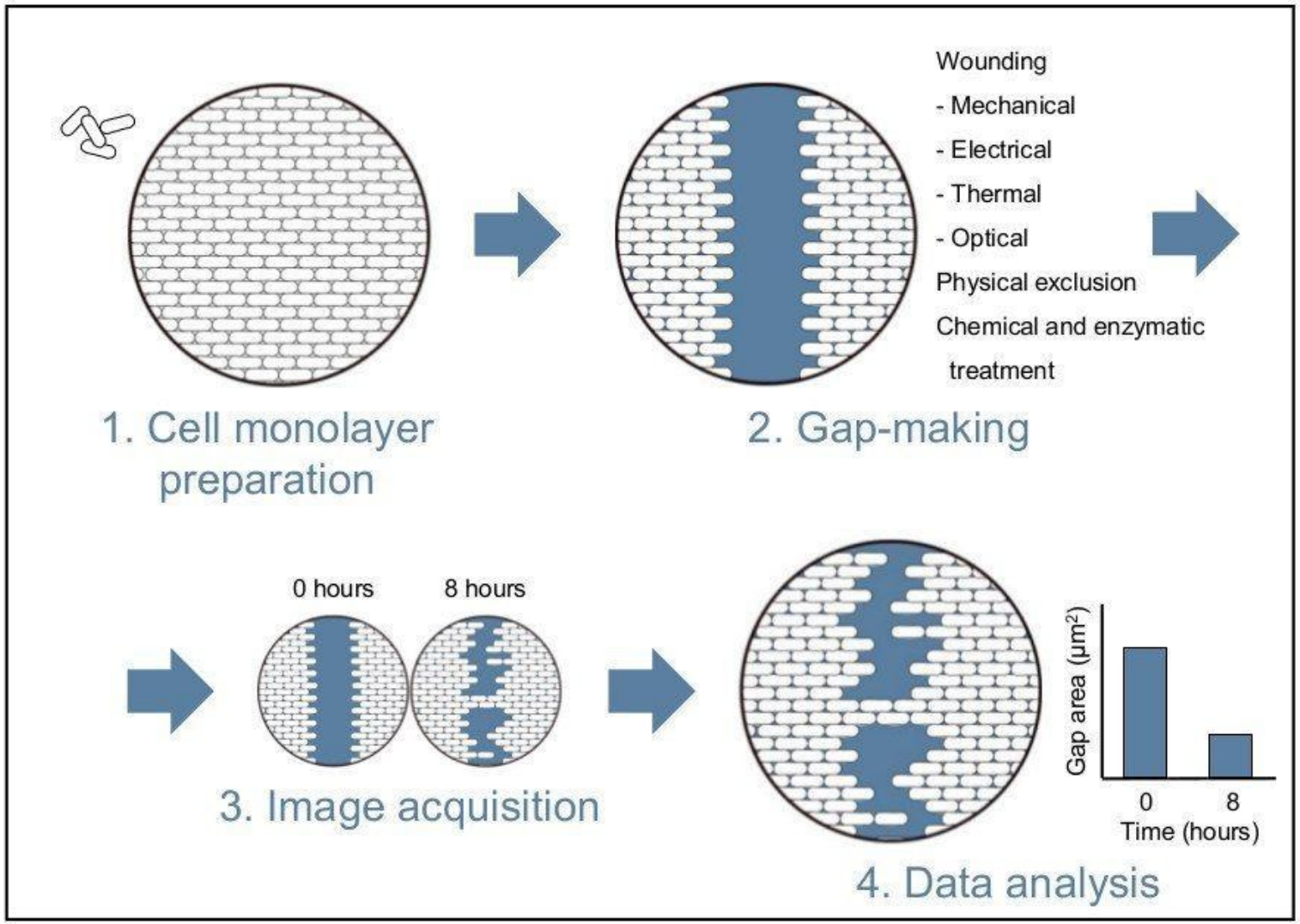
3. Detach invading cells in cell detachment buffer



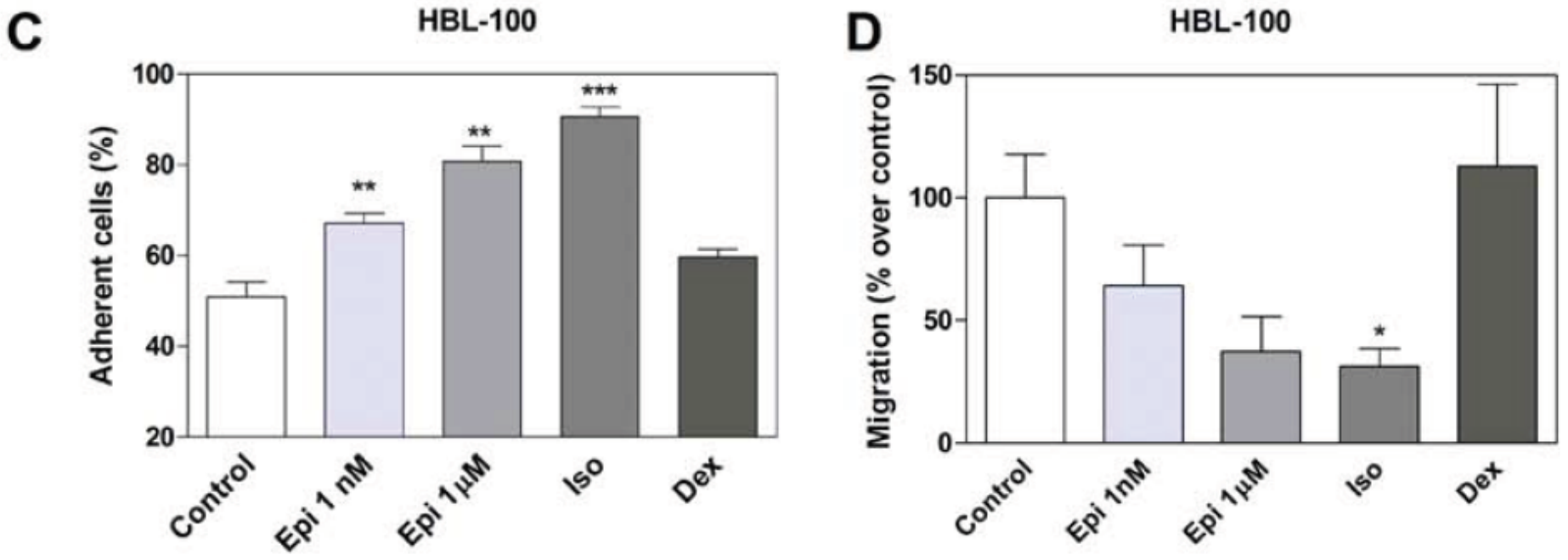
4. Lyse cells in cell lysis buffer and detect cell numbers with CyQUANT® GR Dye



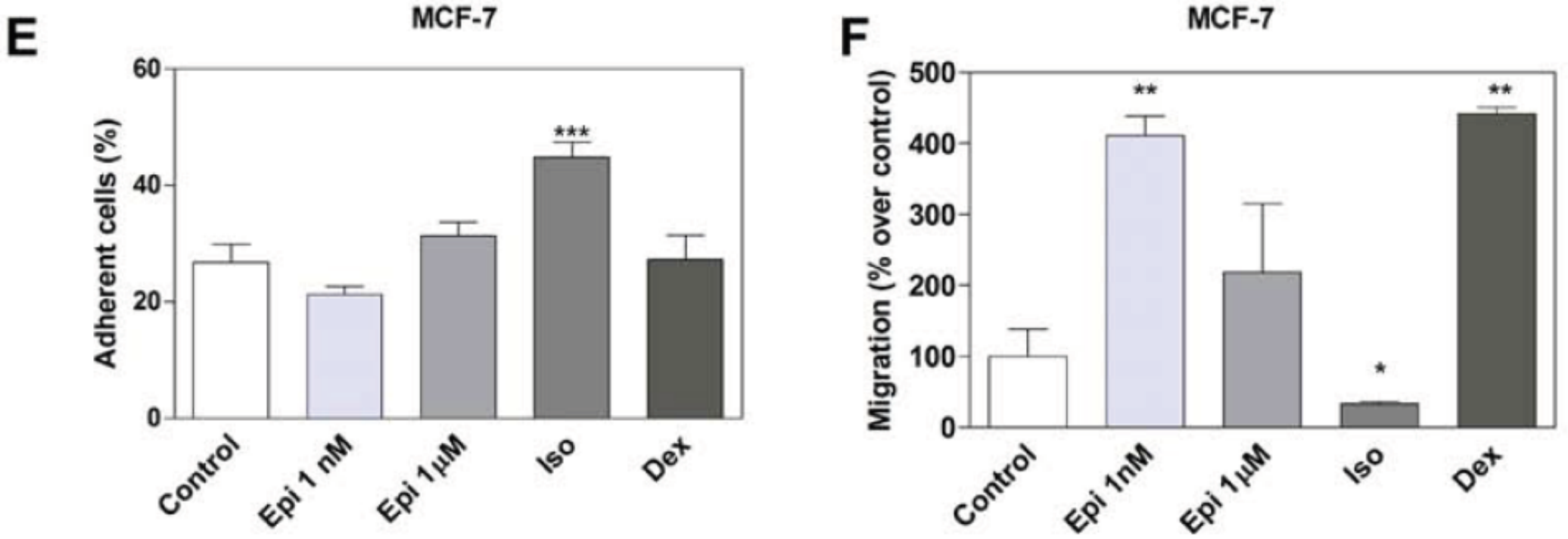
Wound-healing assay



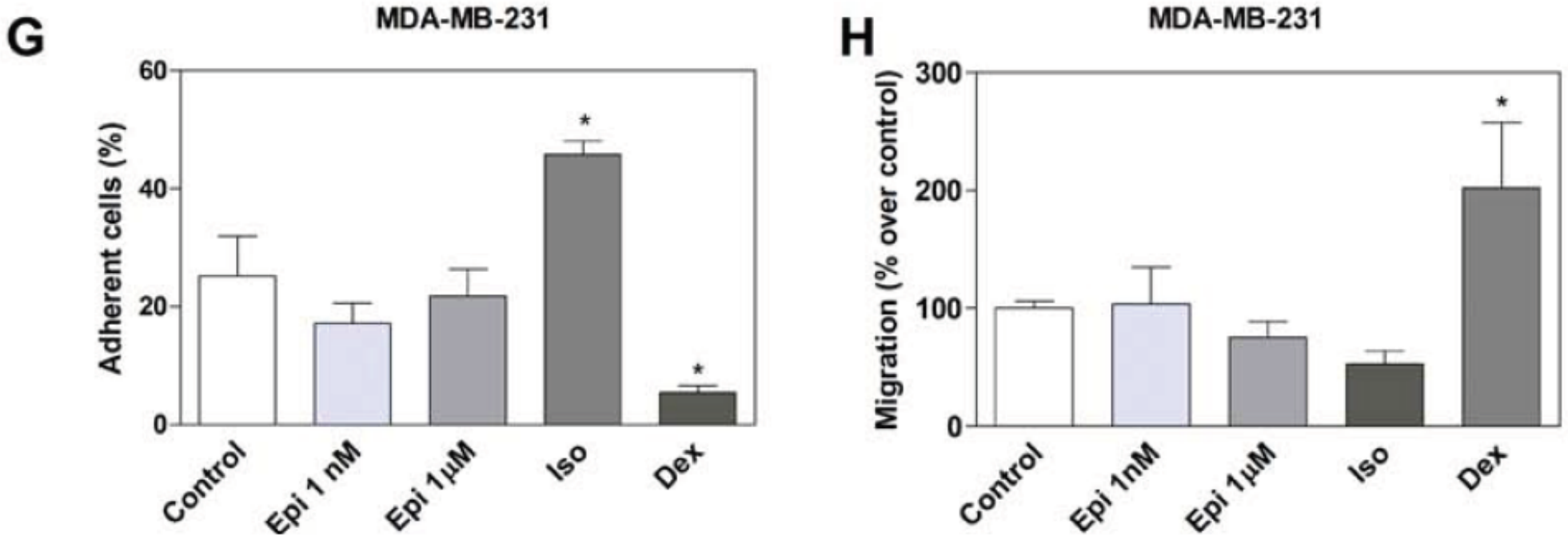
Effect of adrenergic compounds on cell adhesion and migration HBL-100 cells.



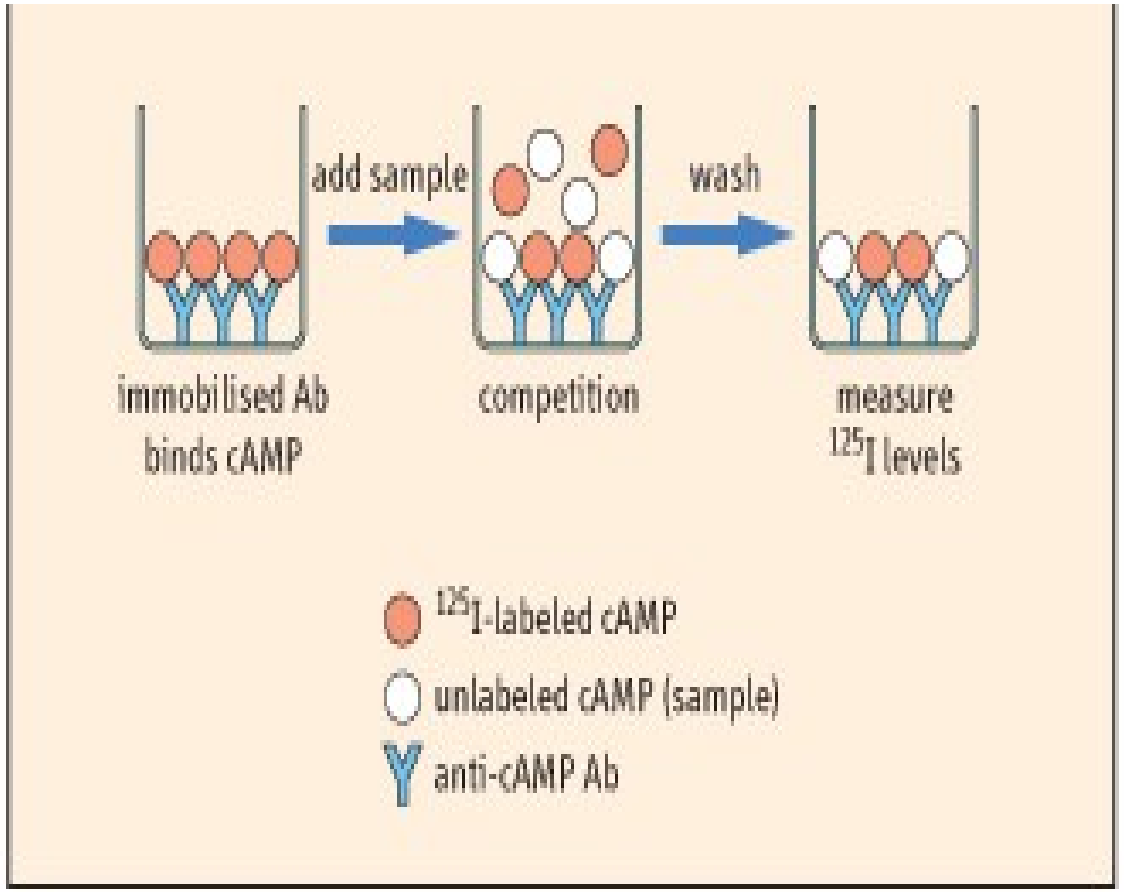
Effect of adrenergic compounds on cell adhesion and migration MCF-7 cells.



Effect of adrenergic compounds on cell adhesion and migration in MDAMB-231 cells.



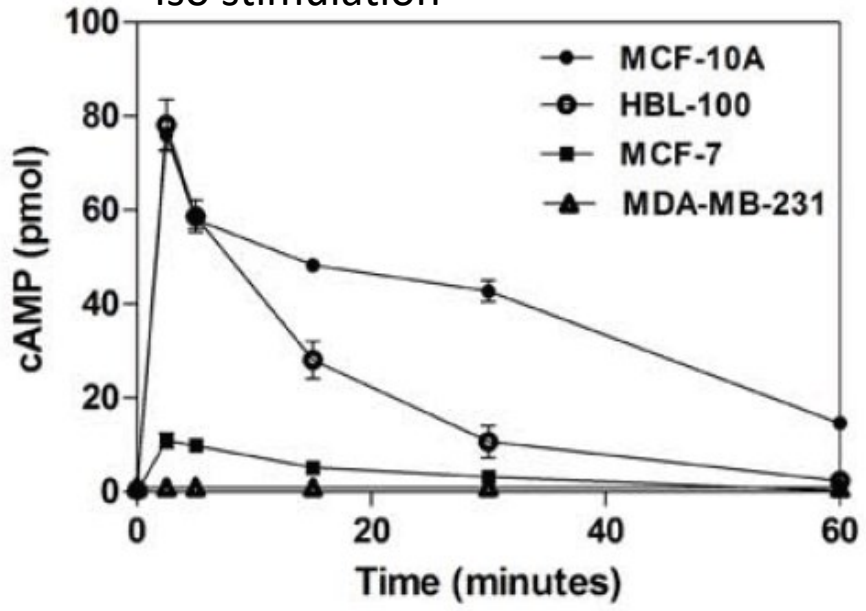
Measurement of cAMP by Radioimmunoassay (RIA)



cAMP levels in tumor and non-tumorigenic breast cells upon isoproterenol stimulation

A

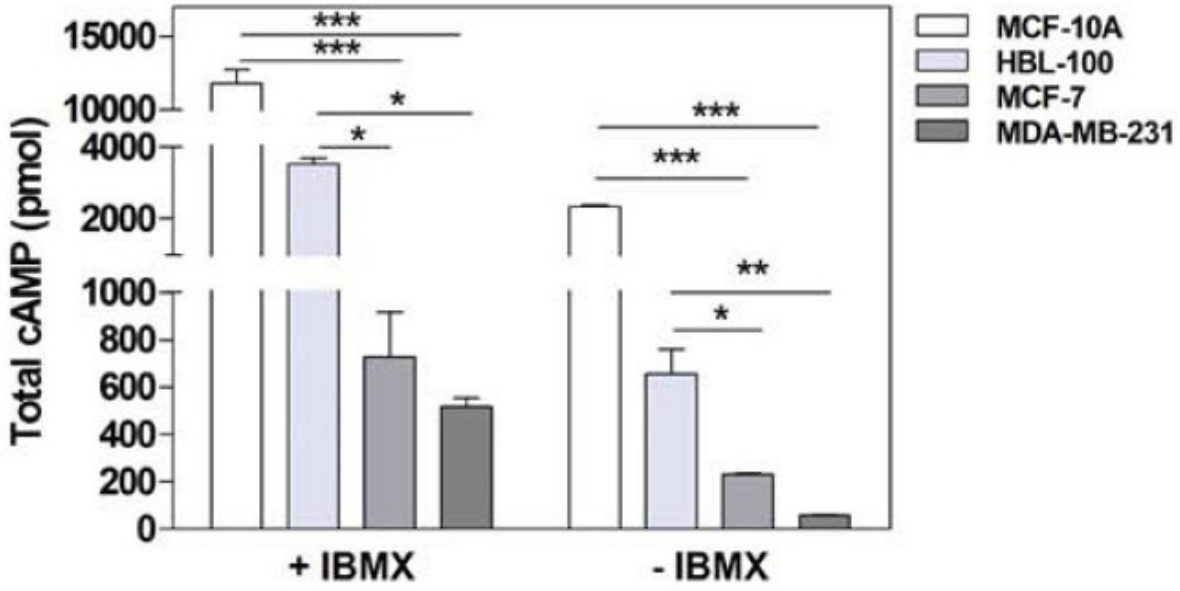
Iso stimulation



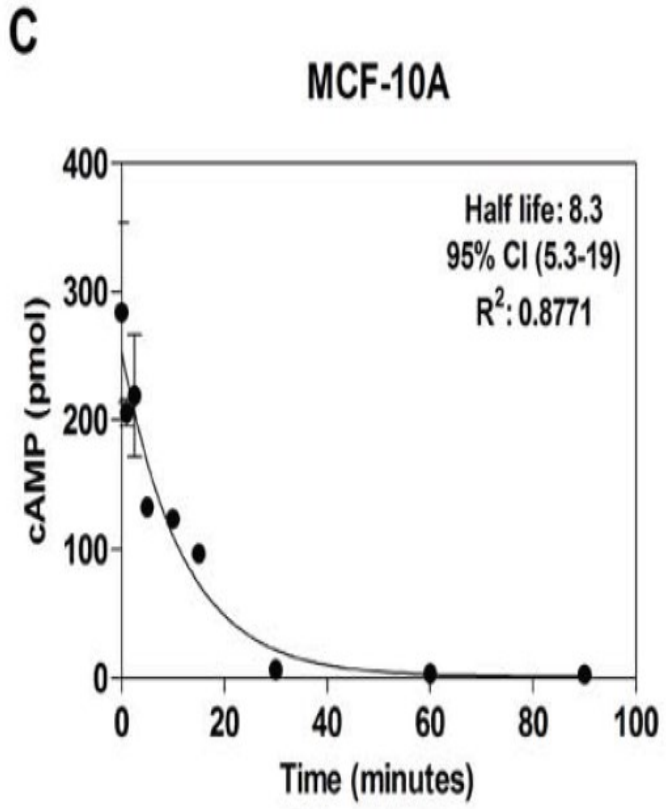
Without IBMX

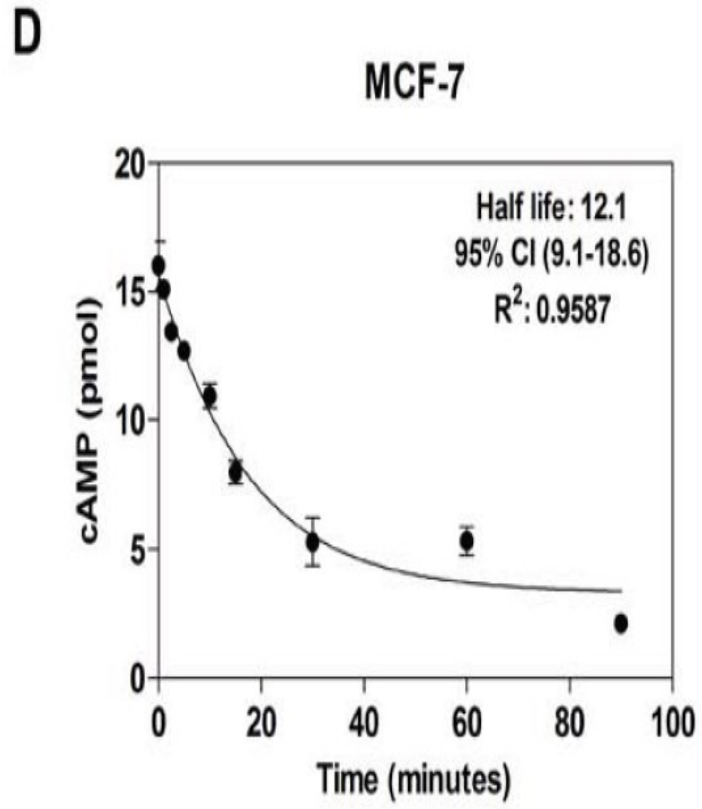
B

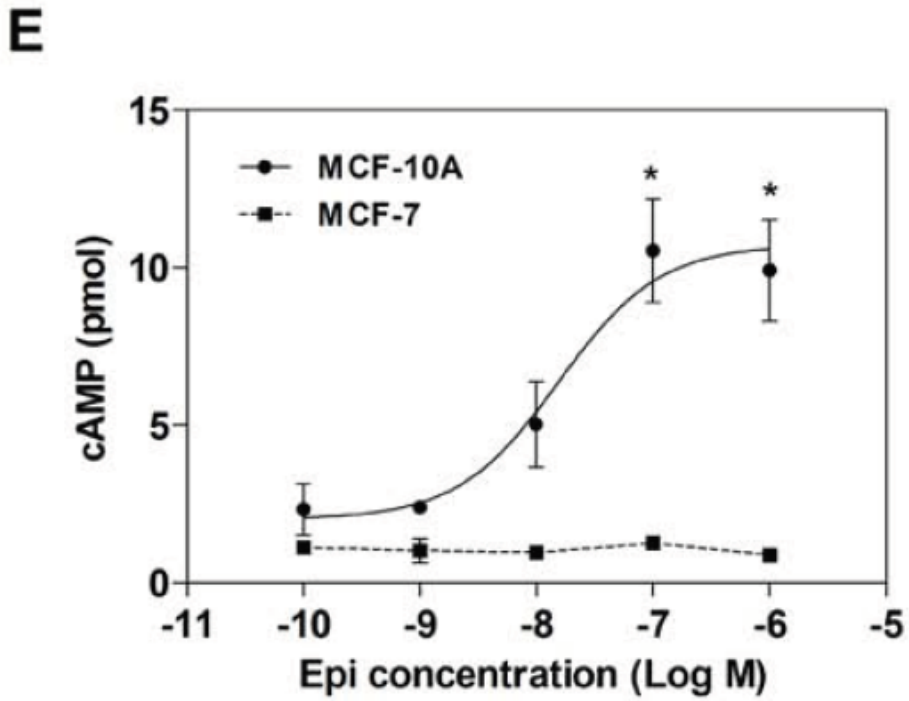
Iso stimulation



cAMP levels in tumor and non-tumorigenic breast cells upon isoproterenol stimulation

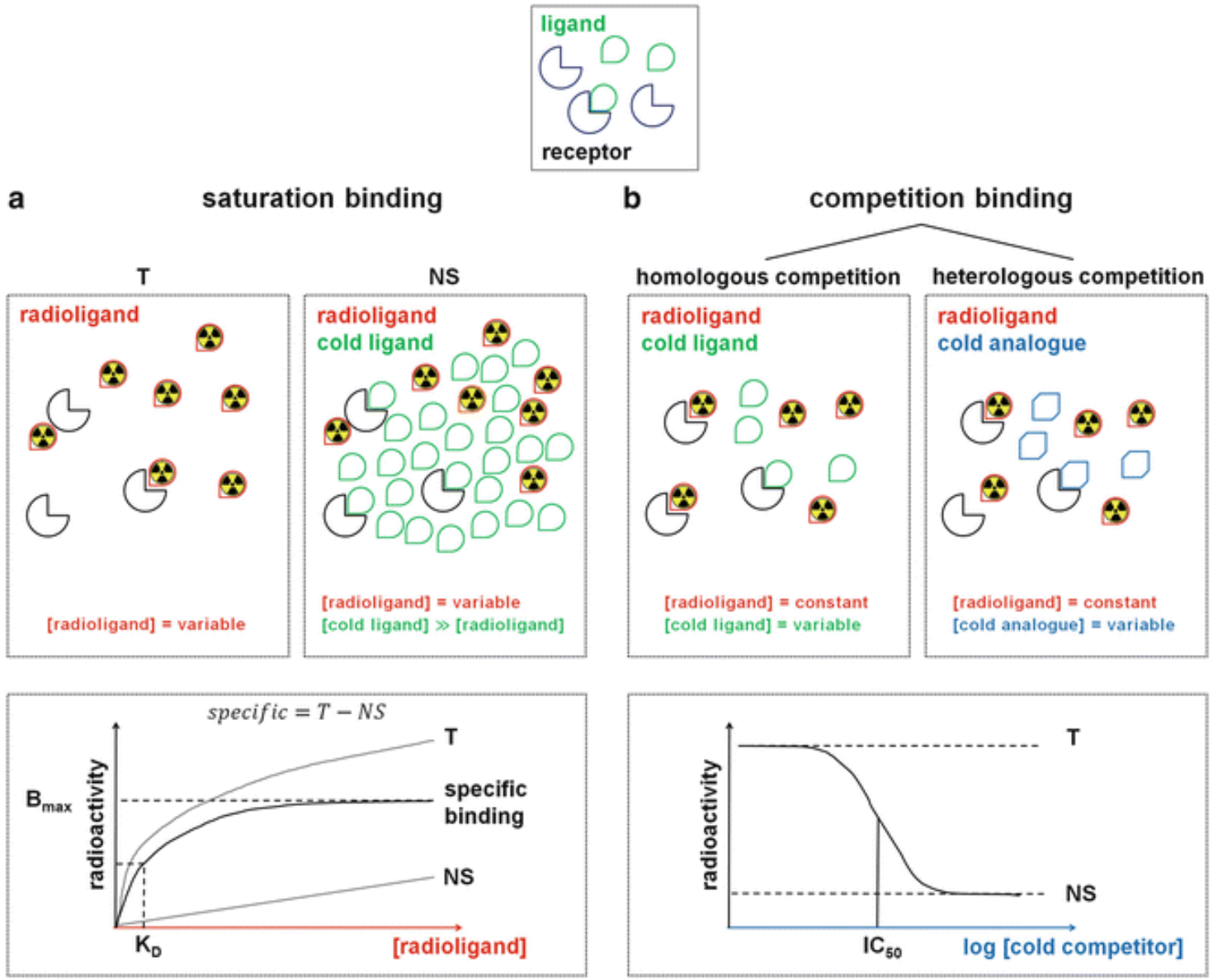




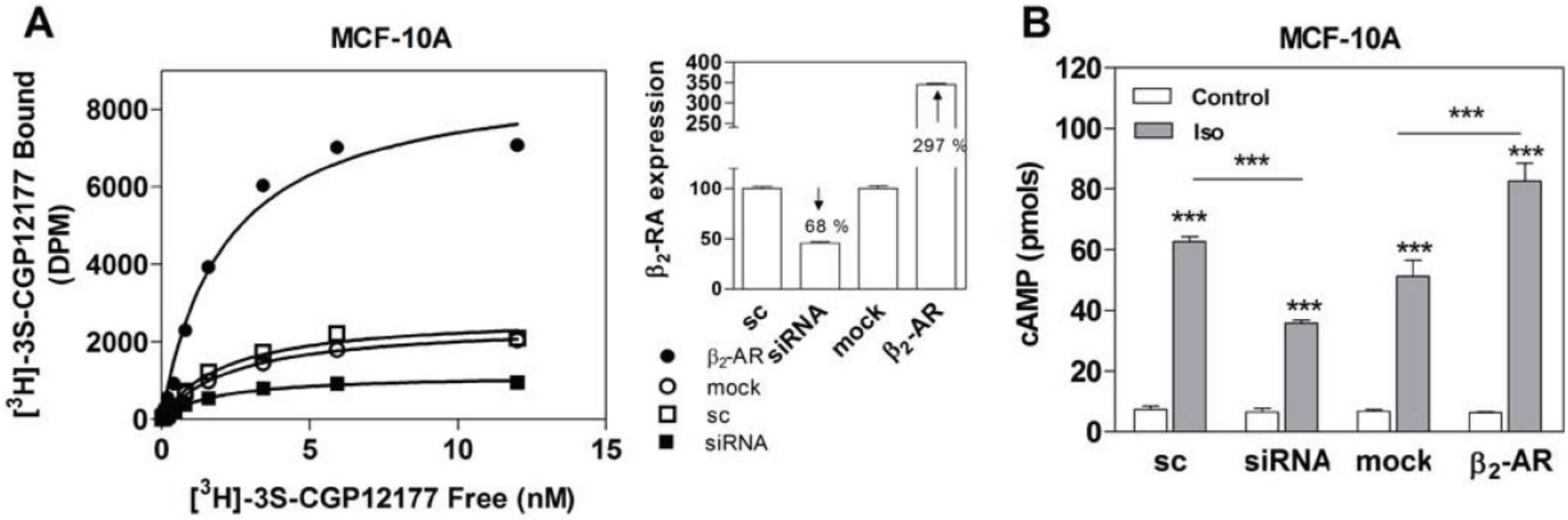


MCF-7 esprimono molti α2 AR

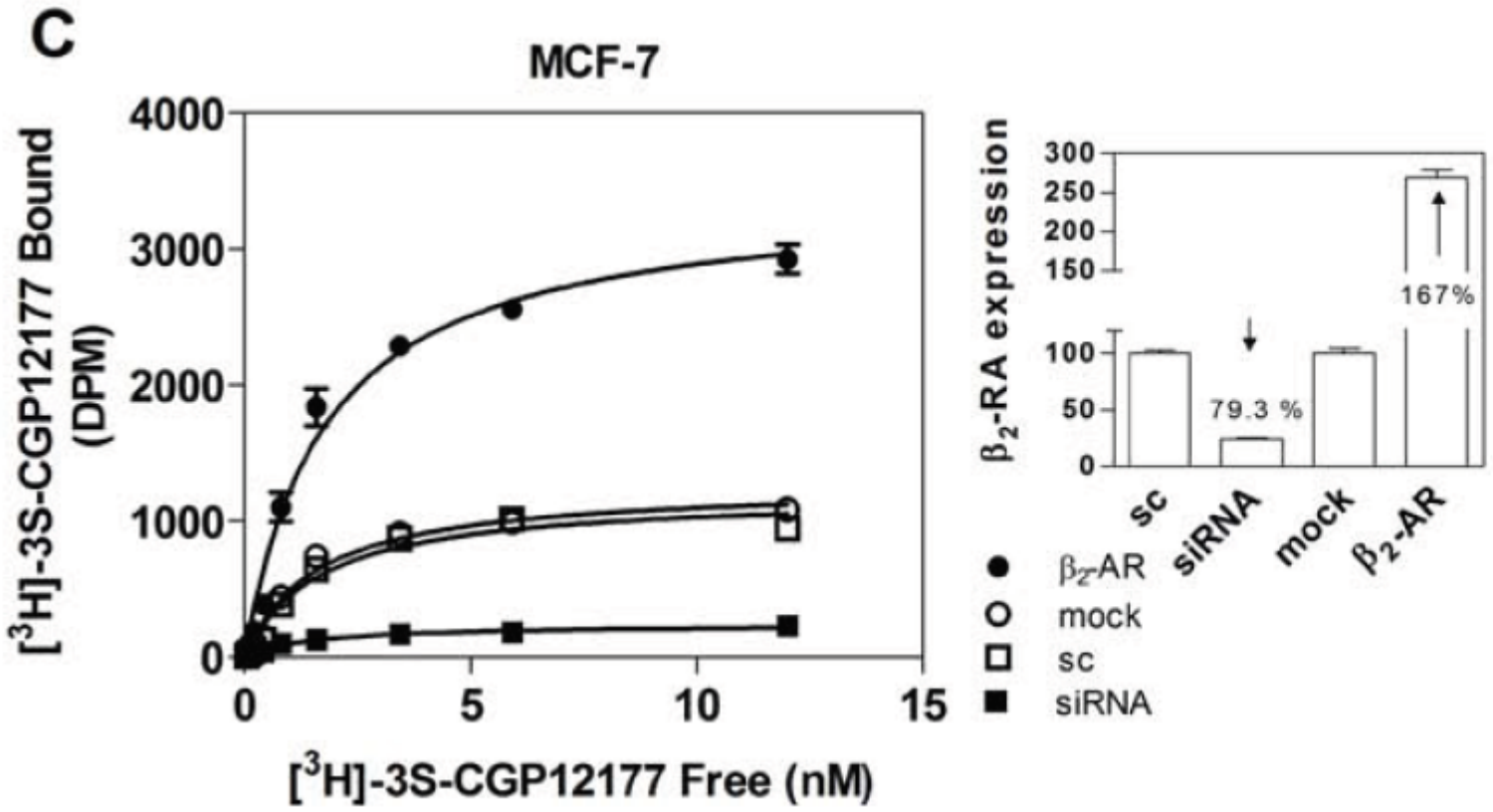
Radioligand binding assay

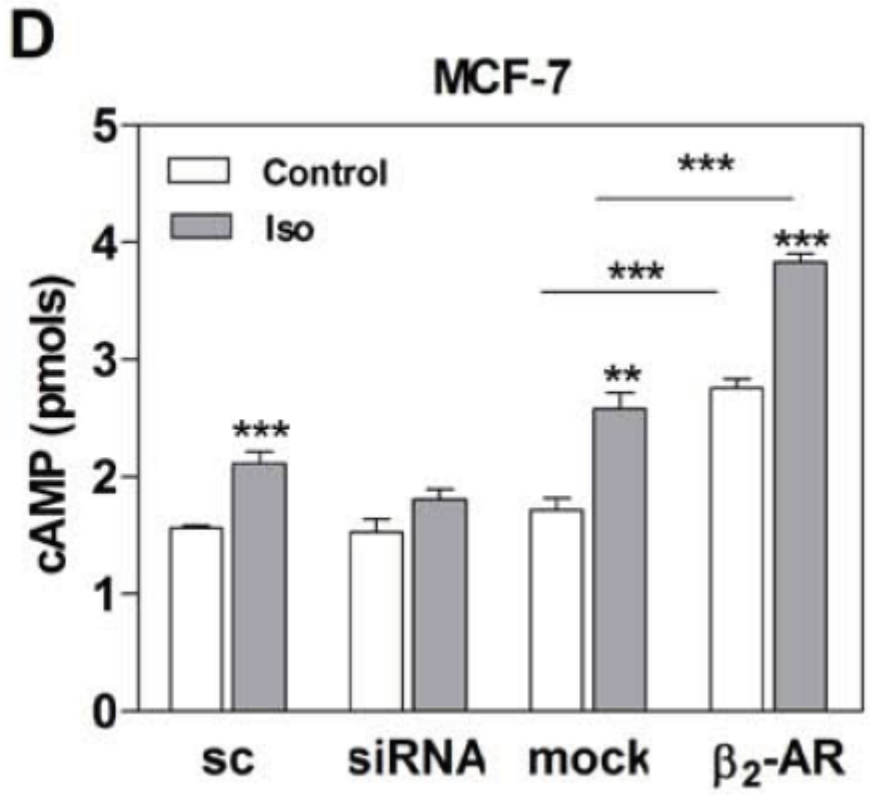


β2-AR overexpression and knock-down in MCF-10A cells

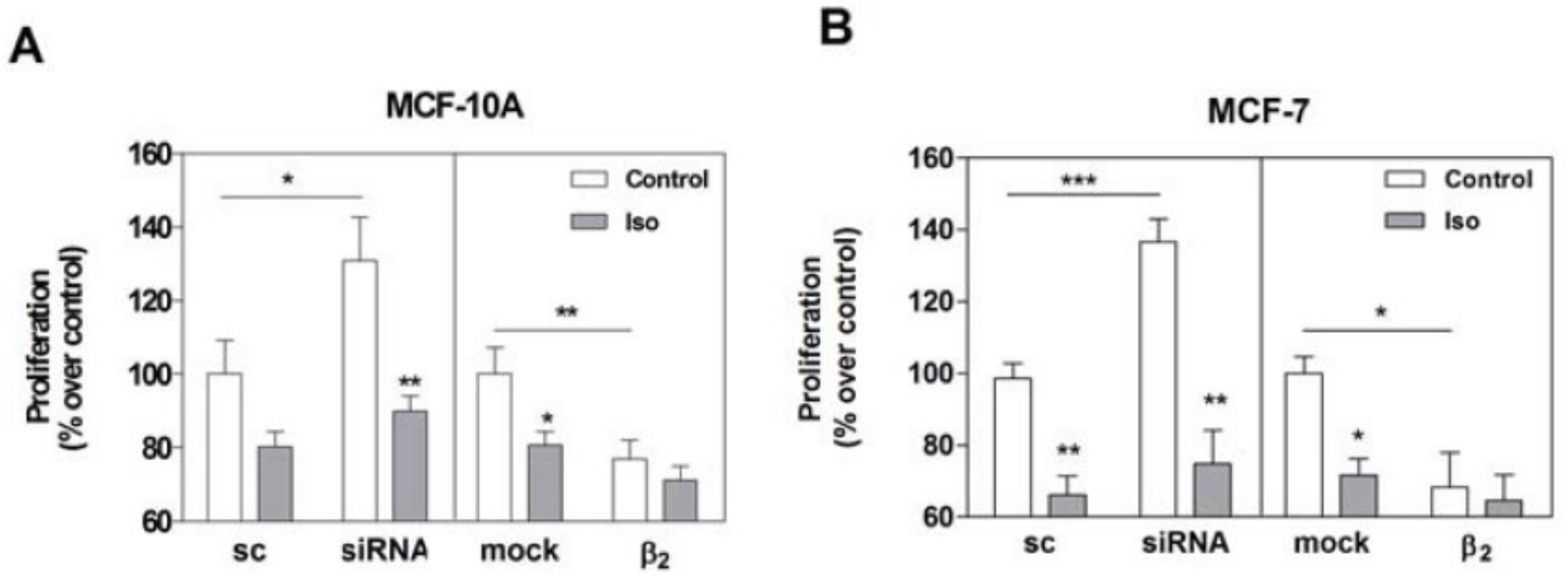


β2-AR overexpression and knock-down in MCF-7 cells

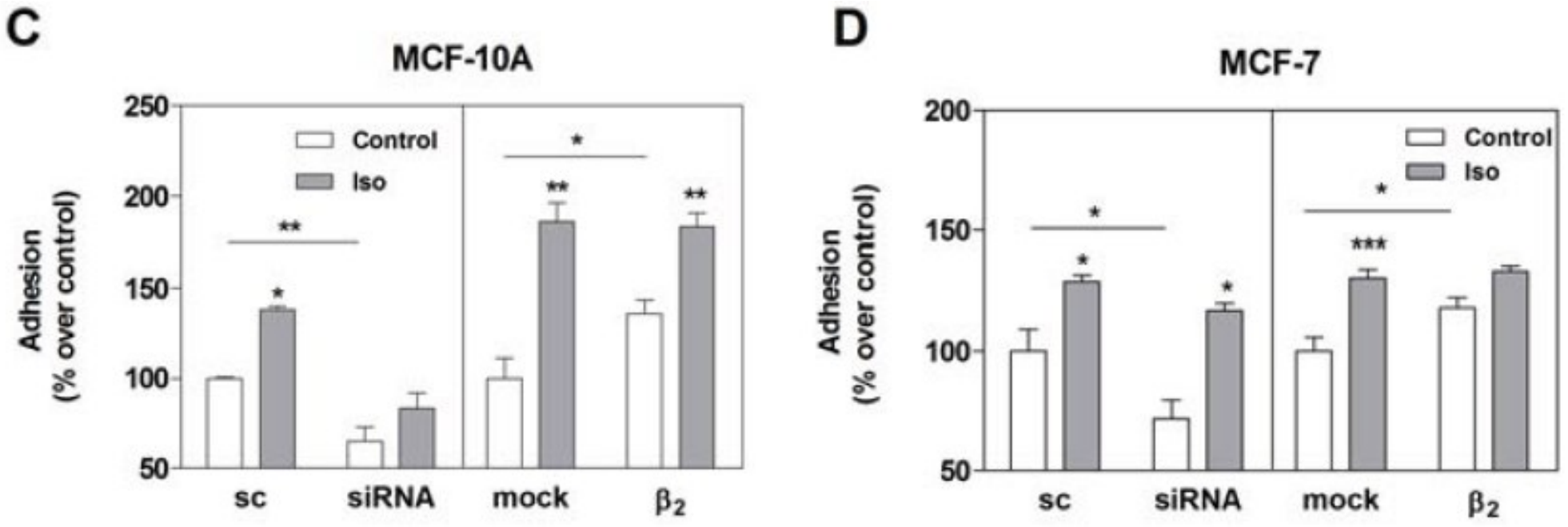




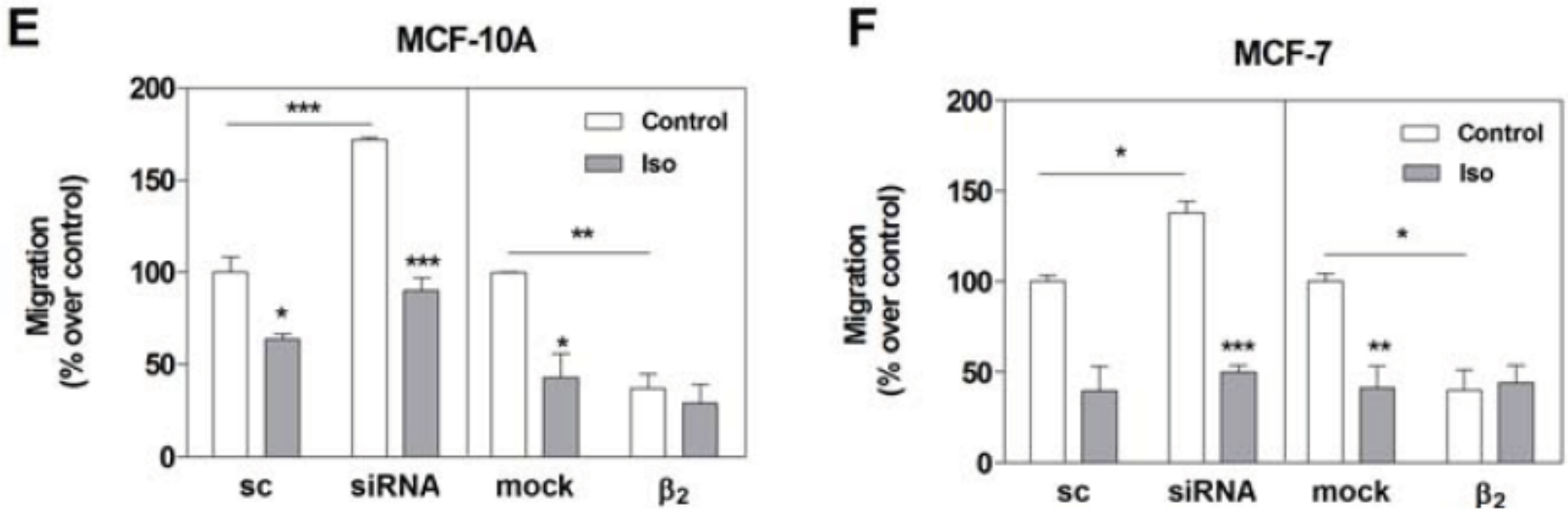
Effect of β2-AR expression on cell proliferation, in MCF-10A and MCF-7 cell lines



Effect of β2-AR expression on cell adhesion in MCF-10A and MCF-7 cell lines



Effect of β2-AR expression on cell migration in MCF-10A and MCF-7 cell lines



Conclusioni

- L'espressione dei β-AR e i livelli di cAMP sono più alti nelle cellule non-tumorali rispetto alle cellule tumorali.

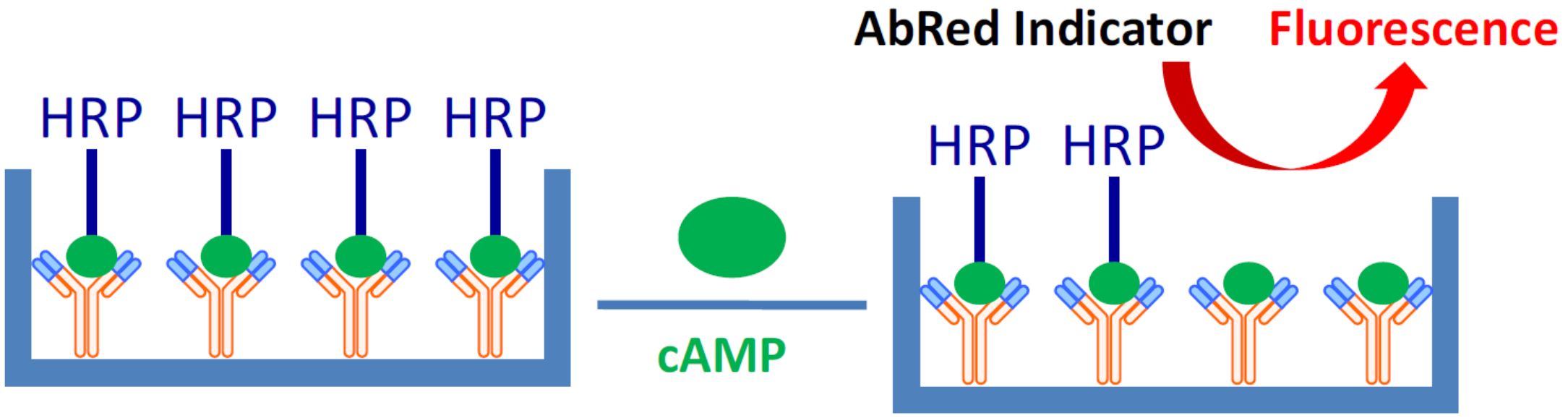
- La stimolazione o la modulazione dei livelli di espressione dei β-AR causa cambiamenti dei livelli di cAMP

- La stimolazione dei recettori β-AR o l'aumento della loro espressione causa una riduzione della proliferazione e migrazione

- La riduzione dei livelli di β-AR causa una aumento della proliferazione e migrazione

- L'azione farmacologica sui recettori adrenergici potrebbe coadiuvare la terapia antitumorale

Dosaggio cAMP con metodo ELISA

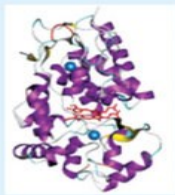


Maximum HRP activity
(no cAMP)

Decreasing HRP activity
(increasing free cAMP)

6. Materials Supplied

Item	Quantity	Storage condition (before prep)	Storage condition (after prep)
	1 plate		
cAMP Standard (lyophilized, 33 µg)	1 vial	4°C	4°C
Assay Buffer	20 mL	4°C	4°C
HRP-cAMP Conjugate	1 vial	4°C	4°C
10X Wash Solution	10 mL	4°C	4°C
Cell Lysis Buffer	10 mL	-20°C	-20°C
Hydrogen Peroxide Solution (3% H_2O_2)	50 µL	4°C	4°C
AbRed Indicator (lyophilized)	1 vial	-20°C	-20°C
Substrate Buffer	10 mL	4°C	4°C
Anti-c-AMP coated 96 well plate (black, flat bottom)	1 plate	4°C	4°C



Horseradish peroxidase (EC 1.11.1.7)

$$Substrate_{reduced} + H_2O_2 \longrightarrow Substrate_{oxidized} + 2H_2O$$

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring fluorescence at Ex/Em = 540/590 nm
- MilliQ water or other type of double distilled water (ddH_2O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- Sterile 96 well plate with flat bottom for cell culture
- Reagents and instrumentation necessary to perform cell culture
- Dounce homogenizer (if using tissue)
- DMSO
- (Optional) Forskolin (ab120058) – to induce cAMP release

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 Assay Buffer:
Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

9.2 cAMP Standard (33 µg):
Reconstitute standard in 1 mL of Assay Buffer to generate a 100 µM cAMP Standard stock solution. Aliquot cAMP standard so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.3 HRP-cAMP Conjugate:
Reconstitute in 55 µL of Assay Buffer to generate a 50X HRP-cAMP Conjugate stock solution. Aliquot 50X HRP-cAMP conjugate stock solution so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.4 10X Washing Solution:
Prepare **1X Washing Solution** by diluting 1 mL of 10X Washing Solution in 9 mL ddH_2O. Keep at room temperature while in use. Store unused 10X Washing Solution at 4°C.

9.5 Cell Lysis Buffer:
Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

9.6 Hydrogen Peroxide Solution:
Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

9.7 AbRed Indicator:
Reconstitute in 50 µL of DMSO (not included) to generate a 200X AbRed stock solution. Aliquot 200X AbRed stock solution so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.8 Substrate Buffer:
Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

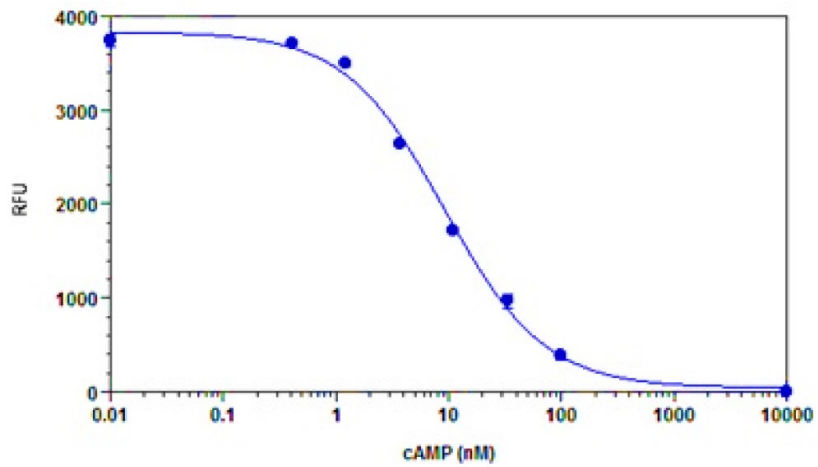
9.9 Anti-cAMP coated 96 well plate:
Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

10.1 Use the 100 µM (100,000 nM) cAMP standard, prepare a standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Sample to dilute	Volume standard in well (µL)	Assay Buffer (µL)	End conc cAMP in well
1	100 µM stock	20	180	10,000 nM
2	Std #1	5	495	100 nM
3	Std #2	150	350	30 nM
4	Std #3	150	300	10 nM
5	Std #4	150	350	3 nM
6	Std #5	150	300	1 nM
7	Std #6	150	350	0.3 nM
8	Blank (none)	0	150	0 nM

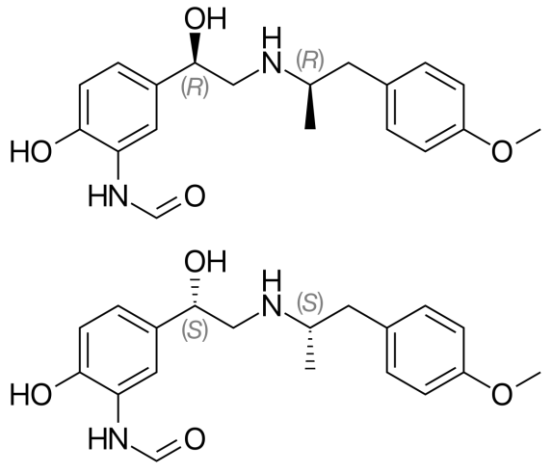


Each dilution has enough amount of standard to set up duplicate readings (2 x 75 µL).

11. Sample Preparation

General sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C. When you are ready to test your samples, thaw them on ice and proceed with the Sample Preparation step. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.
- Each cell line should be evaluated on an individual basis to determine the optimal cell density. Cells may be seeded the day before or on the day of the experiment depending on the cell type and/or the effect of the test compounds.



Formoterol

11.1 Adherent cell samples:

11.1.1 Grow 3 x 10^4 – 10^6 cells/well in a sterile 96-well plate. Incubate overnight in a 37°C/ 5% CO_2 incubator.

11.1.2 The following day, treat cells as required with test compound(s)of interest.

11.1.3 Aspirate cell solution after incubation.

11.1.4 Add 100 µL/well of Cell Lysis Buffer and incubate at room temperature for 10 minutes.

11.1.5 Centrifuge samples for 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.1.6 Collect supernatant and transfer to a new tube.

11.1.7 Keep lysate on ice.

12.1 Plate loading:

12.1.1 Add standard and samples to wells of the anti-cAMP coated 96-well plate (Step 9.9) as follows:

- Standard wells = 75 µL standard dilutions.
- Sample wells = 5 – 75 µL samples (adjust volume to 75 µL/well with Assay Buffer).

12.1.2 Incubate plate at room temperature for 5 – 10 minutes.

12.2 cAMP Assay procedure:

12.2.1 Prepare 1XP HRP-cAMP conjugate working solution by diluting 1: 50 the 50X HRP-cAMP conjugate stock solution (Step 9.3) in Assay Buffer and mixing well. Keep on ice.

12.2.2 Add 25 µL/well of 1X HRP-cAMP conjugate to each standard and sample well.

12.2.3 Incubate plate at room temperature for 2 hours on a plate shaker.

12.2.4 Aspirate plate contents and wash plate 4 times using 200 µL/well of 1X Wash solution.

12.2.5 Prepare AbRed Working Solution by diluting 50 µL of 200X AbRed Stock solution (Step 9.7) and 11.5 µL of Hydrogen Peroxide solution (Step 9.8) into 10 mL of Substrate Buffer (Step 9.8).

Δ Note: AbRed Working solution is not stable. Do not store for future use.

12.2.6 Add 100 µL AbRed Working Solution into each standard and sample well. Mix well.

12.2.7 Incubate at room temperature for 10 min – 2 hours protected from light.

12.3 Plate measurement:

12.3.1 Monitor fluorescence increase at Ex/Em = 540/590 nm (cutoff 570 nm) using a microplate reader in top read mode.

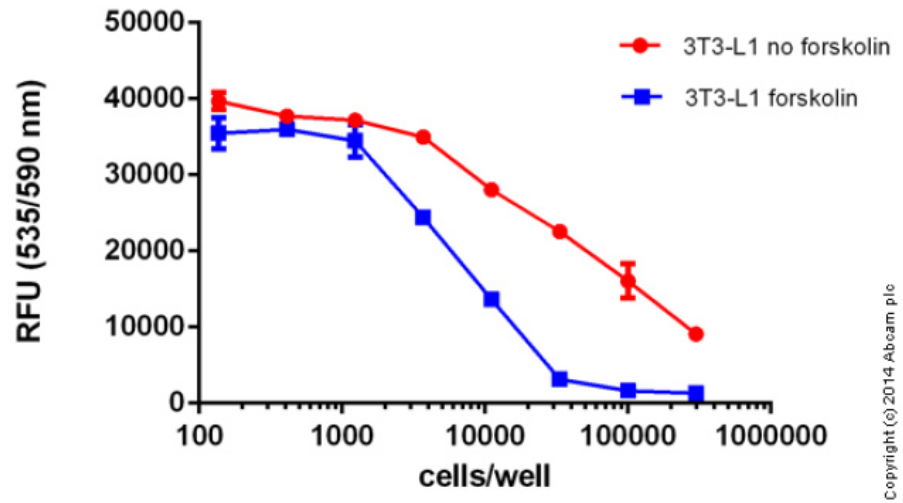


Figure 3. cAMP dose response. In increase amount of 3T3-L1 cells untreated or treated with 100 µM Forskolin (ab120058) for 15 minutes.

13. Calculations

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

13.1 Calculate the mean value of the duplicate readings for each standard and sample.

13.2 To generate a Standard value, plot the graph using the standard concentrations on the x-axis and the corresponding mean fluorescence on the y-axis.

13.3 The best-fit line can be determined by regression analysis using a four-parameter logistic curve-fit.

13.4 Determine the unknown sample concentration from the standard curve and multiply the value by the dilution factor.

Protocol Summary

Standard curve preparation

Sample preparation

Add HRP-cAMP conjugate to samples & standards

Incubate RT 2 hours

Wash wells

Add detector (AbRed Working solution)

Incubate RT 15 – 60 minutes

Measure fluorescence (Ex/Em = 540/590 nm)

Grazie per l’attenzione