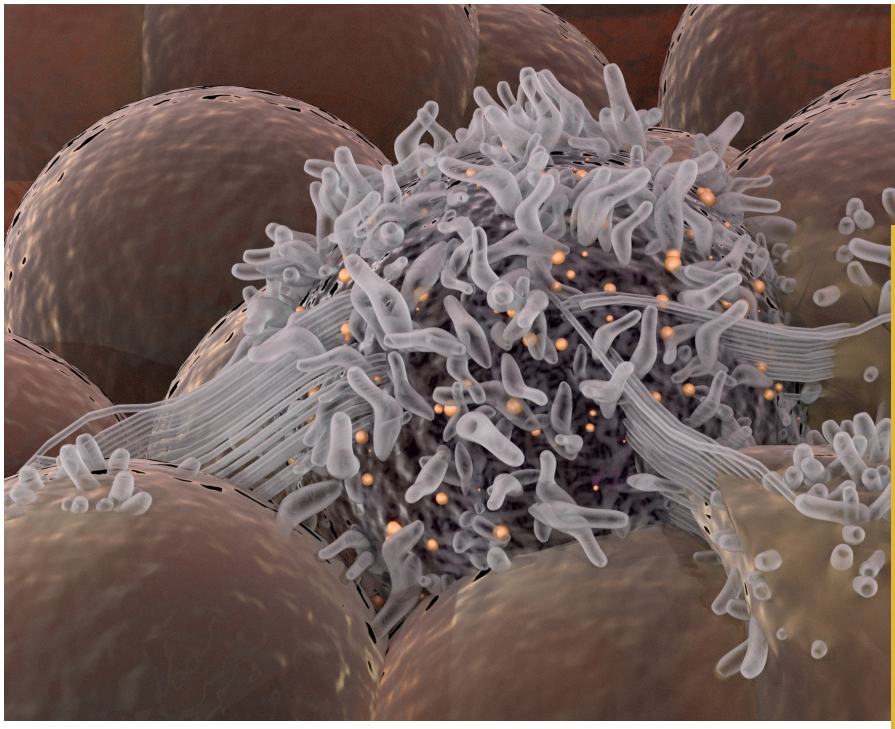

APPLICATION NOTE

AlphaLISA Technology

Authors:

Philippe Roby

Jeanine Hinterneder

PerkinElmer, Inc.
Hopkinton, MA

Protocol Optimization for Detection and Quantification of Membrane-Bound Proteins Using AlphaLISA Technology

Introduction

Membrane proteins are of high interest in both research and pharmaceutical studies. They are the principal mediators of signaling between cells, acting through binding with extracellular ligands to produce intracellular signals by either directly or indirectly activating other molecules, usually by enzymatic action. Their functions make studying their modulation essential for understanding biochemical processes, and they are often very useful biomarkers for detecting and evaluating disease states. However, working with cells can introduce its own set of difficulties, including the following:

- Various biomarkers of interest may be present in cells in widely varying amounts, from the ultra-abundant actin, to very rare transcription factors.
- Proteins of interest may be present in various cellular compartments including the nucleus, cytoplasm, organelles and the plasma membrane.
- Expression levels and ease of protein extraction can vary across different cell lines and origins.
- Significant optimization may be needed to develop a successful cell-based detection assay.

Therefore, in order to study membrane proteins in cells, an efficient detection platform is needed.

PerkinElmer®
For the Better

The Amplified Luminescence Proximity Homogenous Assay (Alpha) technology platform has proven to be a versatile, efficient, and sensitive method in detecting specific molecules using a no-wash format. One of its solutions, AlphaLISA®, has emerged as a powerful ELISA-type assay replacement that offers high sensitivity, wide dynamic range, and the ability to work with molecules of all sizes over a wide range of affinities. AlphaLISA is based on the association of two beads by an analyte. Figure 1 shows an example of AlphaLISA technology, whereby a streptavidin-conjugated Donor bead is bound to a biotinylated anti-analyte capture antibody, and Acceptor beads are directly conjugated to an anti-analyte detection antibody. When the specific analyte is present, the antibodies bring the Donor bead and Acceptor bead within close proximity of each other. Upon excitation with a 680 nm laser, the Donor beads generate singlet oxygen molecules that transiently diffuse in solution to activate nearby Acceptor beads, which through a series of reactions emits light at 615 nm. The light emission (AlphaLISA signal) is then detected on an Alpha-enabled instrument.

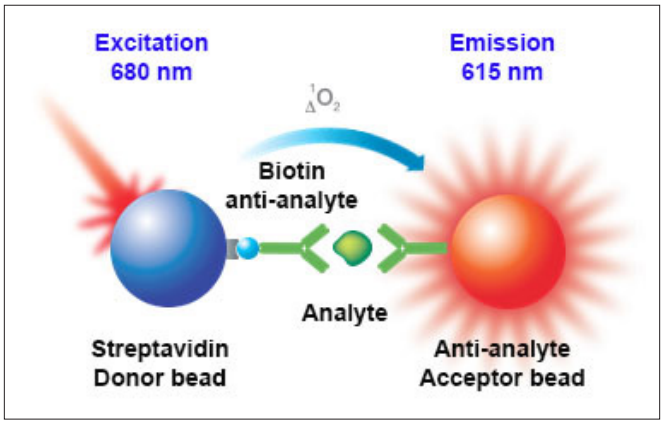

Figure 1. AlphaLISA assay schematic. In the presence of analyte, antibodies sandwich the analyte and bring the Donor beads and Acceptor beads within close proximity. Upon excitation singlet oxygen from the Donor beads diffuses and activates nearby Acceptor beads to generate light that is proportional to the amount of analyte.

AlphaLISA kits measure hundreds of targets, including cytokines, blood circulating proteins, and cellular proteins. This study uses AlphaLISA kits to analyze the various parameters involved in the detection and quantification of membrane-based receptors for the epidermal growth factor receptor (EGFR) in the human HeLa cell line. In parallel, the cytoskeletal protein vimentin was also assayed to evaluate how the protocol developed for detection of membrane proteins performs in the detection of an intracellular protein.

Note: The following protocol applies to membrane proteins that are either anchored or have a single trans-membrane domain. Proteins with many such domains (such as G-Protein Coupled Receptors or GPCRs with seven domains) are most often irreversibly denatured by attempts to extract them from the membrane unless special precautions are taken[1].

Materials and Methods

Cell Culture Methods

HeLa cells (ATCC® CCL-2™) were maintained in 75 mL cell culture flasks (Corning 430641U) in RPMI 1640 (ThermoFisher 11875-093) supplemented with 10% fetal bovine serum (FBS). Cells were harvested once they reached confluence by removing cell culture media, washing with 10 mL of PBS (ThermoFisher 10010-023) then detaching the cells with either 1.5 mL of 0.25% Trypsin-EDTA solution (ThermoFisher 25200-056) for three minutes or with 5 mL of Versene (ThermoFisher 15040-066) for 15 minutes (gentle tapping of the flask to detach cells might be required). The cells were then diluted to 10 mL with culture media and counted manually using 0.4% Trypan Blue solution (ThermoFisher 15250-061) and a hemacytometer.

For most assays, cells were plated in 96-well black ViewPlates (PerkinElmer 6005182) in 100 µL of culture media at seven seeding densities starting at 20,000 cells per well and titrating down by halves. The cells were allowed to settle and attach overnight in a 37 °C incubator supplemented with 5% CO_2. Each concentration of cells was plated in triplicate.

AlphaLISA Detection Assays

The following AlphaLISA assays were tested: EGFR (AL340), Vimentin (#AL377), beta2-microglobulin (#AL3067), heme oxygenase-1 (#AL3026), and PD-L1 (#AL344). AlphaLISA assays were performed on cellular lysates in 384-well white OptiPlates (#6007260) unless specified otherwise in the Results section.

In order to assess the presence and concentration of membrane-bound analytes, cells in culture must first be lysed in order for the proteins to be accessible. To do this, the supernatant was first aspirated out of the wells by vacuum. The cells were washed twice with sterile PBS to remove traces of media and then incubated for 30 minutes at RT in 50 µL of lysis buffer (usually AlphaLISA Lysis Buffer 1X; PerkinElmer #AL003C). A sample of the resulting lysate was transferred to a 384-well white OptiPlate for analysis.

The specific AlphaLISA protocols used for each biomarker are described on their respective Technical Data Sheets. In brief, a standard curve of the molecules to be tested is run with the analytes diluted in AlphaLISA Lysis Buffer, along with the samples of cell lysates generated, using 5 µL of analyte or sample. Results are read on an Alpha compatible reader, and analyzed using GraphPad Prism 6.0.

To analyze assay parameters, an assay with a typical cell membrane receptor (the EGF receptor) and an intracellular cytoskeletal protein (vimentin) was performed.

AlphaLISA Signal Detection

AlphaLISA assays must be read on an Alpha compatible reader, including the EnSpire™, the EnVision®, or the EnSight™. For this Application note, results were measured using a PerkinElmer EnVision multimode plate reader (Figure 2) using default values for standard Alpha detection. For high-throughput applications, when an even higher detection speed is required the Alpha HTS module is recommended. The system incorporates unique temperature control for sensitive Alpha and AlphaPlex™ assays,

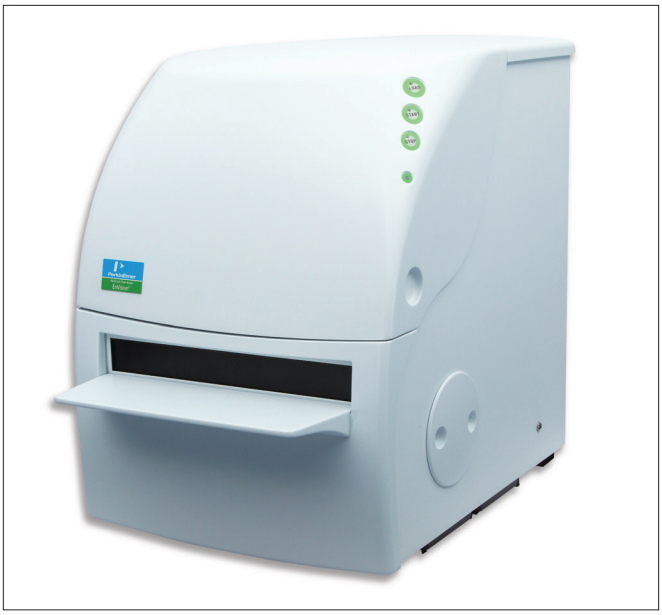

Figure 2. EnVision Multilabel plate reader image.

Standard Curves and Data Analysis

Standard curves for the AlphaLISA assays were performed in the same diluent as the samples being tested (AlphaLISA Lysis Buffer) using the recombinant standards provided in each kit. Curves were plotted using GraphPad Prism according to a nonlinear regression using the four-parametric logistic equation (sigmoidal dose-response curve with variable slope) and $1/Y^2$ data weighting. Protein levels in cellular lysates were quantitated by interpolation of AlphaLISA signal from the standard curve. Interpolated analyte concentrations represent the amount of protein in 5 µL of sample. All data shown (raw and interpolated signals) are the average of at least three wells.

Results and Discussion

Choosing an Appropriate Lysis Buffer

The first step was to test how efficient different lysis buffers were at extracting proteins from the cell membrane. The following buffers were tested on HeLa cells for the ability to extract and to measure EGFR and vimentin with AlphaLISA:

- AlphaLISA Lysis Buffer
- AlphaLISA *SureFire Ultra* Lysis Buffer (PerkinElmer, #ALSU-LB)
- Pierce RIPA Buffer (ThermoFisher 89900)
- Pierce M-PER Buffer (ThermoFisher 78501)
- Cell Lysis Buffer (10X) (Cell Signaling Technology 9803)
- MILLIPLEX MAP Lysis Buffer (EMD Millipore 43-040)

Before testing a specific lysis buffer on cells, it is important to first determine that the AlphaLISA assays, which are validated in PerkinElmer ImmunoAssay Buffer (IAB) or AlphaLISA HiBlock buffer, will also work when the analyte is diluted in the lysis buffer. This is a crucial step because the formulations of the various lysis buffers are different from one another and from the assay buffer and may contain something that affects the assay. Both EGFR and vimentin AlphaLISA assays were tested in the various lysis assay buffers and data from these tests presented in Figure 3.

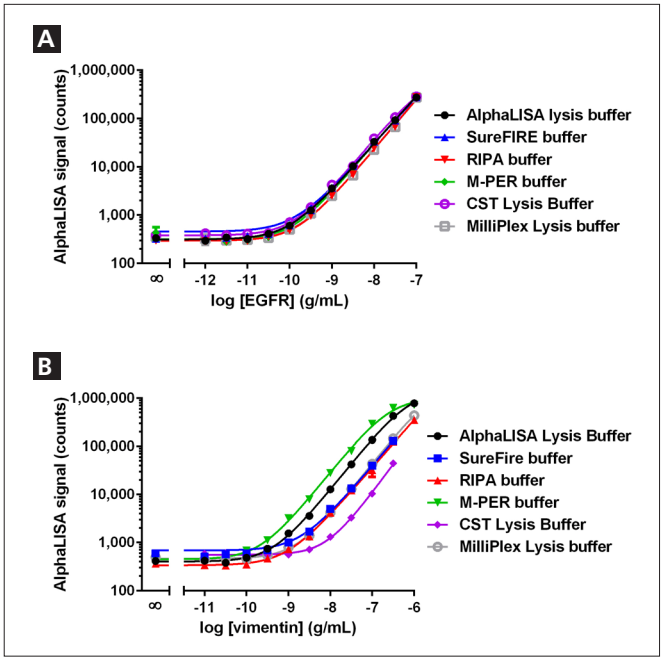

Figure 3. (A) Standard curves of human EGFR assayed using various assay buffers for analyte dilution. Antibody and bead additions were performed in AlphaLISA HiBlock buffer. (B) Standard curves of human vimentin performed using various assay buffers for analyte dilution. Antibody and beads additions were performed in IAB buffer.

Results indicate that for the EGFR receptor, the different lysis buffers have very little effect on EGFR detection (Figure 3A). Parameters such as sensitivity and EC_{50} are very similar. However, the situation for the vimentin assay is quite different. As illustrated by the curves in Figure 3B, both the sensitivity and EC_{50} values measured from the standard curves generated vary among the different lysis buffers tested. While the exact reason is uncertain, vimentin is a protein known to aggregate, and variations in buffer formulations may either make the buffer less efficient at preventing this, or may actually promote the phenomenon.

Two conclusions can be drawn from these results. The first is that the effect of a specific buffer may differ from protein to protein. As such, each buffer should be tested for the protein of interest before performing the experiments. Also, a loss of performance for some buffers may decrease the sensitivity of the assay below detectable levels in cells. In both cases, AlphaLISA Lysis Buffer was quite efficient, offering the greatest sensitivity with the least effect on signal to background, making it a good choice. The second conclusion is that if attempts are made to multiplex the assay, a buffer that shows good results against all targets is required.

After testing the effects of lysis buffers on the standard curves, cellular lysates from HeLa cell cultures were tested. Cells were titrated from 20,000 cells per well in triplicate, grown overnight and lysed for 30 minutes using each of the lysis buffers tested. Concentrations of analyte were interpolated from standard curves run in the corresponding lysis buffer and average data presented in Tables 1 and 2.

Table 1. Concentration of EGFR protein present in lysates of HeLa cells produced with various lysis buffers. All amounts are in pg/mL and are an average of three wells.

Cells	AlphaLISA Lysis Buffer	*SureFire* Lysis Buffer	RIPA Buffer	M-PER Buffer	CST Lysis Buffer	MilliPlex Lysis Buffer
20,000	5,810	4,900	4,620	5,960	4,670	6,070
10,000	3,740	2,610	2,370	3,910	2,540	2,650
5,000	1,490	1,170	1,390	1,630	1,310	1,690
2,500	550	540	610	630	570	830
1,250	210	180	170	240	220	360
625	98	97	--	120	83	140
313	58	29	--	73	40	54

Table 2. Concentration of vimentin protein present in lysates of HeLa cells produced with various lysis buffers. All amounts are in ng/mL and an average of three wells.

Cells	AlphaLISA Lysis Buffer	*SureFire* Lysis Buffer	RIPA Buffer	M-PER Buffer	CST Lysis Buffer	MilliPlex Lysis Buffer
20,000	271	15.6	217	37.2	117	307
10,000	284	6.9	200	47.6	59.2	238
5,000	158	2.0	92.7	54.5	22.4	107
2,500	55.7	0.35	34.2	48.1	9.7	43.5
1,250	26.4	< LDL	14.5	26	3.8	17.9
625	11	< LDL	7.4	15.7	1.5	7.9
313	8.3	< LDL	2.9	7.6	< LDL	4

The results in Tables 1 and 2 illustrate several points:

1. The assay shows good sensitivity as both proteins can be measured with linearity down to 500 cells and up to 10,000 cells per well.
2. For abundant proteins, too many cells can create false results due to a combination of supersaturation (creating a "hook effect") or interference from cell materials such as DNA or protein aggregates.
3. For a typical cell membrane receptor (EGFR), all lysis buffers extract similar levels of protein, making them equal in performance (as long as the results are interpolated from a standard curve prepared in the same buffer).
4. For vimentin, the situation is different. Results vary significantly between buffers. The *SureFire* buffer is a soft detergent optimized to punch holes in membranes instead of destroying them and to protect phosphorylated proteins for detection. This makes it a good buffer for extracting free cytoplasmic proteins but is less efficient for extracting proteins from structures such as the cytoskeleton. Also noteworthy is M-PER, which appears to be unable to break protein aggregates in cells. AlphaLISA Lysis Buffer appears to be the most efficient buffer at extracting vimentin.

These results show that lysis buffer selection is very important when developing a cell-based AlphaLISA detection assay. This is especially true if your assay workflow requires the quantitation of proteins from both the plasma membrane and those located in intracellular compartments.

Choosing a Method for Cell Harvesting

Another important consideration in developing a cellular detection assay is the method used to harvest cells before transferring them to the assay plate (microplate). Adherent cells have to be detached from the flask in which they are grown in order to be plated and tested. The most common technique used by far is to incubate cultures for a short time with trypsin. This enzyme will cleave the proteins responsible for attachment of the cells to the flask, releasing them. However, since trypsin is a generic enzyme that cleaves after lysine and arginine residues irrelevant of the substrate, there is a risk that the protein of interest may also be cleaved. While such a protein might be regenerated by new synthesis, this takes time. Another popular method of detaching cells uses EDTA (as Versene solution). EDTA will remove metal ions that are involved in cell attachment. This should not alter the proteins on the cell surface.

To determine a method for cellular detachment, the harvesting of cells was performed with trypsin and Versene in parallel on cells from the same passage. Detached cells were plated, incubated overnight, and then tested for EGFR (membrane protein) and vimentin (intracellular protein). Results are presented in Table 3.

Table 3. Comparison of amounts of EGFR and vimentin present in HeLa cells harvested with trypsin or Versene. All results are in pg/mL.

Cells Seeded	Vimentin + Trypsin	Vimentin + Versene	EGFR + Trypsin	EGFR + Versene
20,000	271,000	265,000	5,810	12,560
10,000	284,000	280,000	3,740	6,730
5,000	158,000	196,000	1,490	2,740
2,500	55,700	75,000	550	1,260
1,250	26,400	37,000	210	460
625	11,000	17,600	98	280
313	8,300	8,700	58	140

Results show that vimentin concentrations appear to be unaffected by the type of harvesting method. This is expected, since the protein is intracellular and should not be exposed to protease activity. However, the amount of EGFR measured is significantly higher when the cells are harvested with Versene compared to trypsin (by a factor of ~ 2). This is not surprising since the EGFR extracellular domain has at least 52 cleavage sites for trypsin which indicates that exposure to trypsin for any length of time can degrade cellular membrane proteins into fragments too small for detection.

Optimizing Cell Lysis Conditions with AlphaLISA Lysis Buffer

Since proteases have been shown to hurt the EGFR protein in the harvesting phase, we tested if such proteases could have a similar effect when released by cells during cell lysis. To examine this, cells were lysed in AlphaLISA Lysis Buffer with or without cOmplete™ mini protease inhibitor cocktail (Sigma 4693124001), with one tablet added to 10 mL of buffer. Data from this experiment are shown in Table 4.

Table 4. Effect of protease inhibitor cocktail on the measurement of vimentin and EGFR proteins in HeLa cell lysates. All results are in pg/mL.

Cells Seeded	Amount Vimentin No Inhibitor	Amount Vimentin With Roche Cocktail	Amount EGFR No Inhibitor	Amount EGFR With Roche Cocktail
20,000	216,000	224,000	8,320	7,600
10,000	186,000	156,000	5,100	4,500
5,000	113,000	82,600	2,500	2,100
2,500	53,800	36,100	1,250	960
1,250	26,000	16,300	660	530
625	13,700	8,800	330	260
313	5,600	4,300	170	140

These results show that for both of these proteins (intracellular and membranous) proteases potentially released during cell lysis seem to have no negative effect on the AlphaLISA assays. However, this may not be true of all analytes and should be considered when investigating other targets. An attempt was also made to incubate the plated cells with culture media supplemented with the protease inhibitors, but these proved toxic to the cells (data not shown).

The next step was to test lysis buffer incubation times to verify that timing for lysis was optimized. Too long an exposure would be time-consuming and might degrade/aggregate some proteins, while too short a time would not release all the material present. This is particularly important in large screening campaigns, since the time of lysis of the first samples might actually be longer than those of the last. To measure the effect of incubation time on lysis efficiency, a time course of cell lysis on HeLa cells was performed, and both vimentin and EGFR were measured on lysate samples taken from the same well.

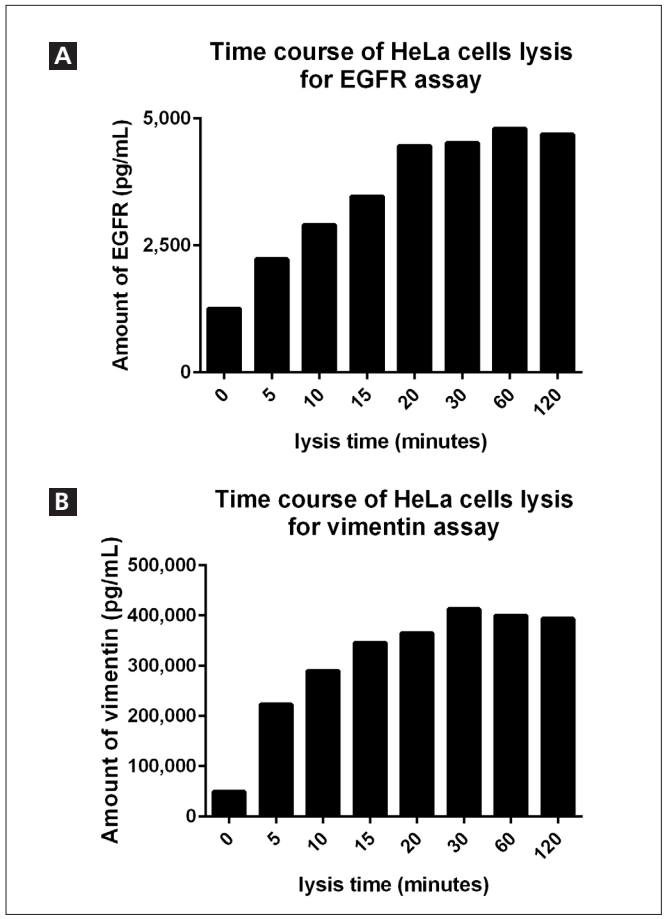

Figure 4. Time course of lysis of HeLa cells by AlphaLISA Lysis buffer and measurement of the amount of (A) EGFR and (B) vimentin present (in pg/mL). Time 0 is the addition of lysis buffer followed by immediate addition of beads and antibodies.

In both cases, the signal is stable after 30 minutes of incubation. If time is an issue, even 20 minutes of lysis gives results that are close to maximum. Longer incubations did not increase the results measurably. It is interesting to note that even "0" time (actually ~ 1 minute from adding the lysis buffer to taking out a sample for the assay) gives a significant signal. This indicates that the lysis buffer acts swiftly on cells.

Another assay step examined more closely was the washing step introduced just before addition of the lysis buffer. This step is primarily used to remove traces of cell culture media, which could contain elements harmful to the assay (such as excess biotin found in media such as RPMI1640). Washing is a rather cumbersome step that requires addition and removal of liquid. This step has to be done carefully to avoid cross-well contamination, and adds to the time necessary to perform a screen. To determine if this step is necessary, assays for both EGFR (membranous) and vimentin (intracellular) assays were performed with the cells either unwashed or washed twice with sterile PBS.

Table 5. Effect of washing before lysis buffer addition on EGFR measurement.

Number of Cells Seeded	Washed (ng/mL)	Unwashed (ng/mL)	Unwashed % of Washed
20,000	8.2	4.6	55.7
10,000	4.8	2.7	54.9
5,000	2.5	1.3	51.4
2,500	1.2	0.7	58.1
1,250	0.6	0.3	53.8
625	0.28	0.17	61.9
313	0.13	0.09	67.7

Table 6. Effect of washing before lysis buffer addition on vimentin measurement.

Number of Cells Seeded	Washed (ng/mL)	Unwashed (ng/mL)	Unwashed % of Washed
20,000	460	422	91.7
10,000	412	336	81.5
5,000	191	141	73.8
2,500	94.4	69.8	73.9
1,250	48.1	33.9	70.4
625	23.1	14.8	64.8
313	10.0	7.00	70

Results indicate that washing the cells does increase performance of the assay, probably by removing interference from cell culture media and possibly removing cell debris that could contain denatured proteins. Also, the effect of washing is not identical between assays, suggesting that removing the washing step and applying a simple correction factor would probably not work.

All-in-One Well Assay Development

The final step in our assay optimization was to see if the assay can be performed *in situ*, or all-in-one well. The previous assays were all a two-plate transfer assay, where the cells were grown and lysed in one (96-well) plate, and material transferred to a 384-well plate for detection. To save time and materials, it is important to test assay performance in a 384-well plate. To do this, dilutions of cells were plated and allowed to adhere overnight in a 384-well CulturPlate (PerkinElmer 6007860) in 50 µL of RPMI1640 + 10% FBS. The next day, cells were washed twice with 50 µL of sterile PBS and then lysed with 5 µL of AlphaLISA Lysis Buffer followed by addition of anti-EGFR beads and antibody. A standard curve of EGFR in lysis buffer was also performed at the same time in the same plate (data shown in Figure 6).

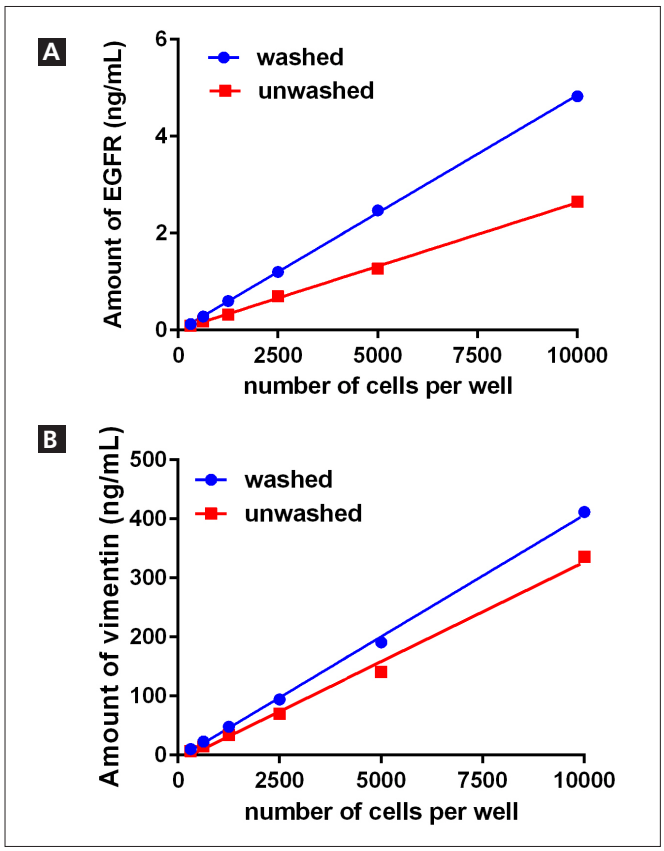

Figure 5. Comparison of assays for (A) EGFR and (B) vimentin for cells that were either washed 2X with sterile PBS (100 µL) or unwashed (and culture media removed by aspiration) prior to addition of lysis buffer.

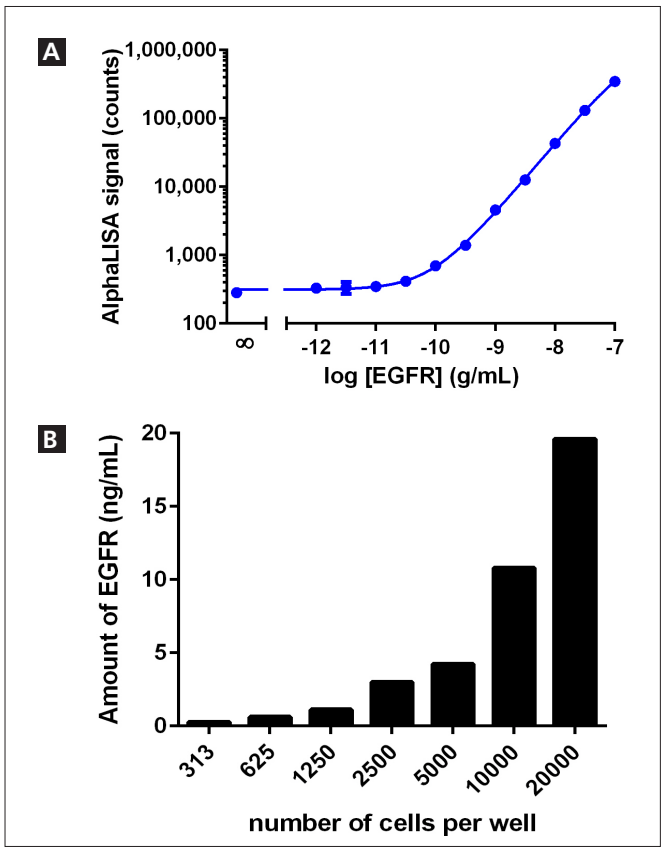

Figure 6. All-in-One well assay for EGFR. (A) Standard curve of EGFR prepared in AlphaLISA lysis buffer in a 384-well CulturPlate used to interpolate data collected from (B) HeLa cells prepared in the same kind of microplate.

Results presented in Figure 6 illustrate that the AlphaLISA EGFR assay is functional as an *in situ* assay in 384-well plates. It is important to run a standard curve alongside in the lysis buffer using the same plate as the assay. Some proteins may actually stick to the treated surface of a CulturePlate and give skewed results when a standard curve is run, compared to standard non tissue-treated assay plates. Since this is impossible to predict in advance, the standard curve should be re-run when a new plate type is selected for accurate quantification of analyte concentration.

Testing the Assay Protocol With Other Analytes

Once the cell-based AlphaLISA assay protocol was optimized, it was tested on other proteins in our HeLa cell cultures. Beta2-microglobulin is a membrane-associated protein that is coupled to the plasma membrane by interactions with other transmembrane proteins, without having a transmembrane section of its own. Heme oxygenase-1 is a protein bearing a transmembrane sequence, but is expressed in intracellular organelles, mainly in the endoplasmic reticulum. Detection of this enzyme would require lysing two sets of membrane. AlphaLISA assays for both these analytes were performed by plating and lysing the cells in a 96-well plate and transferring samples to a 384-well plate as described in the methods section. Data from this experiment are presented in Table 7.

Table 7. Quantities of two other proteins in HeLa cells (all amounts in pg/mL).

Number of Cells	Amount of Beta2-Microglobulin	Amount of Heme Oxygenase-1
40,000	2,300	30,900
20,000	3,200	15,400
10,000	3,200	6,630
5,000	2,000	2,900
2,500	1,100	1,440
1,250	660	700
625	310	< LDL

The beta2-microglobulin protein is known to be expressed on HeLa cells, is membrane associated, but binds to other transmembrane proteins. Data presented in Table 7 suggest our protocol is sufficient to disrupt those interactions and release the protein. Assays with high amounts of cells seem to show a signal plateau, which could be due to hooking effects, aggregation of the protein, or decreased expression in denser cultures due to increased cell-to-cell surface interactions. Heme oxygenase can also be detected with high efficiency. It is known to be present in almost all cells, including HeLa cells. It is a protein associated with the endoplasmic reticulum (ER). This means that its detection implies breaking both the plasma membrane and the ER membrane to release the protein.

Finally, to verify that our protocol works on a membrane protein in another cell line, we measured PD-L1 receptor expression levels in the HCC38 human basal breast cancer cell line. PD-L1 is a plasma membrane receptor upregulated in this cell line by stimulation with IFN-γ. For this experiment, cells were seeded into 96-well ½ Area ViewPlates (PerkinElmer #6005760), allowed to attach and grow overnight, and treated with 20 ng/mL of IFN-γ. After 24 more hours, cells were lysed with 25 µL of AlphaLISA Lysis Buffer, and 5 µL of lysate was taken for sample measurement with AlphaLISA in 384-well OptiPlates. Data from this experiment, shown in Table 8, indicate that we can detect PD-L1 present on HCC38 cells and confirm the assay protocol can be applied to multiple cell types.

Table 8. Amounts of PD-L1 detected in HCC38 cells after using the optimized protocol.

Number of Cells	Amount of PD-L1 (pg/mL)
15,000	425
7,500	217
3,750	140
1,875	74
940	42
470	7.6
235	2.7

Key Considerations for Assay Optimization

Results presented here and in numerous scientific research papers show that AlphaLISA is a versatile, sensitive, rapid and easy to optimize platform for detection and quantification of cellular proteins. Such a platform compares advantageously with more laborious methods such as Western Blotting and ELISA assays.

Our data show that optimizing AlphaLISA assays for use in different cellular models for the detection of membrane-bound proteins is a straightforward and simple process. We tested our protocol on both the membrane protein EGFR and the cytoskeletal protein vimentin in HeLa cells. Several elements of the procedure were examined:

1. **Efficiency of the detection assay in lysis buffer:** All standard curves must be generated in the matrix that is to be analyzed. If cell lysates are used, standard curves must be functional in the same buffer. Some buffers may be harsher than others, mainly those containing SDS or steroidal-based detergents (such as CHAPS). We found that AlphaLISA Lysis Buffer is an efficient buffer that has been optimized to have a minimal effect on detection assays.
2. **Seeding density:** Too few cells will not produce enough analyte for a good detection, while too many might hinder the assay, either by generating too much protein (creating "hook effects") or generating interference by their components such as DNA or opacity from lipids. For abundant proteins, even fewer than 1000 cells will generate results.

3. **Testing lysis buffers on cells:** Results with the internal protein vimentin were more variable than the membrane protein EGFR, showing that membranous proteins are easier to extract than internal proteins, especially those involved in structures such as the cytoskeleton. In both cases, the AlphaLISA Lysis Buffer from PerkinElmer performed the best and is recommended for such assays.
4. **Cell harvesting technique:** The classical method using trypsin was compared to a softer method using non-enzymatic solutions such as Versene (an EDTA buffered solution). For membranous proteins, Versene was shown to be more efficient, as trypsin, a non-specific cutter, can degrade the proteins on the surface of the cells while Versene does not. This was confirmed when vimentin was tested. This protein should not be exposed to trypsin and, as expected, shows no significant difference in detection between harvesting techniques. For membranous proteins, using non-enzymatic methods is recommended.
5. **Effect of protease inhibitor:** Cellular lysis can release various proteases that could alter the results of sampling. We tested if a protease inhibitor cocktail would improve results. No improvement was seen. However, no loss of efficiency was seen either, showing that these cocktails have no effect on the detection method. If literature shows that proteases would be a danger to the assay, inhibitor cocktails can be added without affecting the AlphaLISA assay. AlphaLISA will tolerate protease inhibitor cocktails if required by the user.
6. **Lysis time:** We tested the time necessary to lyse the cells. For both assays (EGFR and vimentin), results show that lysis begins quickly, as signal is observed even after one minute. A maximum is seen for both assays at 30 minutes. This maximum release stays stable up to at least two hours. This helps designing large screens, where treatment of many plates may generate longer exposure. A 30 minutes incubation in lysis buffer is recommended.
7. **Washing cells:** Cells are usually washed before performing lysis and detection. Our results confirm that for both EGFR and vimentin, washing the cells improve performance significantly. This may be due to the presence of elements in residual culture media that would interfere with detection assays such as biotin in RPMI 1640. Therefore, washing the cells twice using a buffered salt solution such as PBS is recommended.
8. **Transfer assay vs. all-in-one well assay:** We compared running the assay in the same plate in which the cells were grown vs. a conventional assay where samples are taken from the growth plate and seeded into a detection plate. The assay was successful in both conditions. An "all-in-one-well" assay allows for a simpler assay with fewer steps. It is important to first validate the detection assay in the plates used to grow cells since anecdotal evidence show that some analytes of interest may stick to these plates. Therefore, we recommend that the assay be performed in cell culture treated plates, but detection should be checked first.
9. **Target protein:** For further confirmation of our results, other types of targets were tested. Beta2-microglobulin is a protein associated to the membrane by interaction with other transmembrane proteins but contains no transmembrane section of its own. Heme oxygenase is on the other side of the spectrum. As it is associated to the endoplasmic reticulum by a transmembrane domain, releasing it for testing means that both the cell membrane and the endoplasmic reticulum must be lysed for detection. In both cases, the proteins were successfully detected. The assay will also work with membrane-associated proteins and with membranous proteins in cell compartments.
10. **Cell type:** The protocol was tested on a second cell line, the human breast cancer cell line HCC38 using the transmembrane receptor PD-L1 as the target. Results indicate that the assay can detect PD-L1 on these cells with high sensitivity.

Conclusion

In conclusion, developing an assay for detecting both transmembrane and intracellular proteins using AlphaLISA is a straightforward process. Such an assay is versatile, easy to perform, quite sensitive, and cost-effective, with a few parameters needing to be optimized.

Reference

1. Grisshammer, R. (2009).Purification of recombinant G-protein-coupled receptors. Methods in Enzymology, Vol 463, pp 631-645.

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®

013676_01 PKI