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Retrovirus Molecular Conjugates: A Novel, High Transduction Efficiency, Potentially Safety Improved, Gene Transfer System

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Abstract: Two significant barriers limit the use of amphotropic retrovirus for human gene transfer protocols: 1) Low transduction efficiency in cells with low receptor expression and 2) safety concerns originating from the risk of formation and propagation of replication competent virus *in vivo*. In principle, if ecotropic retrovirus, which is incapable of infecting human cells, could be transiently modified to effectively transduce human cells, this safety risk could be alleviated. Here we demonstrate that formation of amphotropic retrovirus polylysine molecular conjugates (aMMLV-PL) enhanced gene transfer up to 10-fold in a variety of human cell lines over the equivalent of unconjugated vector (aMMLV). The polylysine modification and formation of ecotropic retrovirus molecular conjugates (eMMLV-PL) permitted effective and stable transduction of different human cell lines as well as primary human bone marrow stroma cells at frequencies of greater 80%. It is conceivable that this novel ecotropic based conjugate retrovirus vector could also potentially provide enhanced safety characteristics not only over amphotropic retrovirus vectors, but also over genetically tropism modified recombinant ecotropic vectors. In contrast to genetic modifications, physical or chemical modifications are not propagated. Thus, formation of replication competent eMMLV from conjugates would be self-limited and would not result in virus propagation in humans.

Introduction:

Gene therapy is emerging as a promising novel therapeutic approach to treat human disease (1-5). However, technical barriers related to the biology of vector cell interaction and patient safety remain the most critical endpoints in clinical trials. Therefore, the success of human gene therapy depends on effective transfer and expression of the desired genes into the target cell at the lowest possible risk for the individual. Gene therapy approaches most commonly utilize recombinant viral vectors, in which both cellular uptake and transgene expression depend on interactions between the viral vector and the target cell (3;4;6). The best established vector system for achieving stable transduction in human cells are amphotropic retrovirus vectors, with their infectivity or cellular uptake being at least in part determined by the presence of their natural receptors on the target cells. Since the use of retrovirus vectors is restricted to specific receptor expressing cells, transduction efficiency is largely determined by cell surface receptor density (7) (8;9). Due to the lack of ecotropic receptors on human cells, unmodified ecotropic retrovirus cannot be used for transduction of human cells.

Currently, all clinical approaches for stable transduction of human cells rely on amphotropic retrovirus. The rare event of formation of replication competent amphotropic retrovirus could result in systemic viremia of a treated individual, as amphotropic retrovirus can infect and replicate in most

human cells. If technology would permit the effective use of ecotropic retrovirus for human gene therapy protocols, this crucial safety concern could be alleviated. Uncontrolled virus replication in humans with ecotropic virus would be unlikely, the lack of natural receptors prevents infection of human cells. Here, we have developed and evaluated a strategy to accomplish this goal by synthesizing ecotropic retrovirus molecular conjugates (eMMLV-PL). To prove feasibility for this concept, the first step would require physical or chemical modifications of ecotropic retrovirus not only permitting infection of human cells, but also the efficient cellular transduction with the transferred gene. The development of strategies to overcome ecotropic/amphotropic receptor dependent vector uptake and enhancement of cellular transduction efficiency through circumventing the virus's natural pathway of cell entry has become a major goal of gene therapy research.

We previously described that recombinant adenovirus molecular conglomerate vectors (recMCV) substantially enhanced vector uptake and transgene expression after formation of polylysine based conglomerates over recombinant adenovirus particles alone (10). Here, we investigate the hypothesis that polylysine conjugated retrovirus results in increased uptake and gene expression over the equivalent of unconjugated virus (Figure 1A). Furthermore, we hypothesized that through this procedure, the tropism restriction of ecotropic virus could be overcome and human cells would become amenable to transduction with ecotropic retrovirus vectors (Figure 1B). If successful, this conjugate ecotropic vector would have an additional significant safety advantage over currently employed amphotropic systems.

Materials and Methods:

Cells and culture conditions. The human embryonic kidney cell 293 line was provided by Dr. Robert Gerard, UT Southwestern, Dallas, TX (also available from ATCC) (11). The human retinoblastoma cell line 911 was provided by Dr. Bout (12). The human pulmonary epithelial carcinoma-derived cell line A549 was purchased from ATCC (Manassas, Virginia). All cell lines were cultured in DMEM (Gibco-Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Gibco-Life Technologies) and penicillin/streptomycin (P/S) (Cellgro Mediatech, Herndon, VA) and maintained at 37^0C under a 5% CO_2 atmosphere in a humidified incubator (standard cell culture conditions). Primary human bone marrow (BM) cells were obtained by standard aspiration from the iliac crest of healthy voluntary donors and collected in MyeloCult H5100 medium (StemCell Technologies, Vancouver, BC), supplemented with 250 U/ml heparin. The Institutional Review board at LSUHSC of New Orleans, LA had approved this study involving human subjects. For erythrocyte lysis, specimens were incubated for 1 min in 0.747% NH_4Cl solution. After two washing steps with phosphate-buffered saline (PBS), 1×10^7 cells were seeded in 10-cm dishes (Nunc, Naperville, IL) and cultured in DMEM supplemented with 15% FBS and P/S and maintained under standard conditions. Non-adherent cells were decanted with media exchange, 2-3 times/week. A near confluent bone marrow stroma layer of fibroblast morphology was established after 2-3 weeks. Gene transfer experiments in these cells were

performed at 70-80% confluence.

Retroviral vectors. The amphotropic retroviral helper cell line PA317 or the ecotropic retroviral packaging cell line GP+E86 were transfected with the plasmid pLZ12 using calcium phosphate (13) (14). Plasmid pLZ12 was described by Galileo et al. and is a modification from plasmids originally made by Casadaban (15;16). Plasmid pLZ121 encodes the lacZ reporter gene under control of the RSV promoter and has a 5'nuclear localization signal (Figure 1).

The retroviral helper cell line was cultured under standard conditions (14). Supernatants were collected from stable vector producing cells and retrovirus purification was performed following a procedure previously described by Akatsuka et al (17). Briefly, a 30% (w/w) stock solution of polyethylene glycol-8000 (PEG-8000) (Sigma, St. Louis, MO) was prepared in double-distilled water and stored in aliquots at 4 ^{0}C. Viral supernatants were gently mixed with polyethylene glycol in 250 ml polystyrene tubes to achieve a final 8% PEG solution. The mixture was maintained overnight at 4^{0}C. After centrifugation at 1500$\times$G for 45 min, the precipitate was dissolved in 3ml of TES buffer (10 mM Tris-HCl, pH 7.2, 2 mM EDTA, 150 mM NaCl). The titer of virus preparations was determined as previously described on NIH3T3 monolayers (18;19).

Chemical linkage of retrovirus to polylysine (Ret-PL). Retrovirus was covalently linked to

Poly-L-Lysine (PL) (Sigma), molecular weight 30-70 kDa, using the heterobifunctional chemical linker EDC {1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride} (Pierce, Rockford, IL). Briefly, 3.6 ml of retrovirus (7×10^7 cfu/ml) was resuspended in HBS (5mM HEPES pH 7.8, 150mM NaCl) and 0.4 ml of PL (6mg/ml in HBS). 0.04ml of freshly made EDC solution (250mg/ml in dH_2O) was added and the reaction mixture was incubated on ice for 4 hours. This ratio of reagents was found to be optimal as determined in several pilot experiments in different human and murine cell lines (data not shown). The product was then dialyzed over night at 4^0C against a large volume of TES using 12-14 kDa cutoff membrane tubing (Spectra/Por, Laguna Hills, CA). The final product, Retrovirus-Polylysine (eMMLV-PL) was aliquotted in TES and stored at -80^0C.

Chemical linkage of Avidin to Polylysine. NeutrAvidin (NA) (Pierce, Rockford, IL) was covalently linked to Poly-L-Lysine (PL), molecular weight 30-70 kDa, using the heterobifunctional chemical linker EDC. Briefly, 10mg/ml NA was resuspended in HBS (5mM HEPES pH 7.8, 150mM NaCl). 1.5 ml of NA solution was gently mixed with and 0.5 ml of PL (6mg/ml in HBS), before 0.04ml of freshly made EDC solution (250mg/ml in dH_2O) was added. The reaction mixture was incubated on ice for 4 hours. The product was then dialyzed over night at 4^0C against a large volume of PBS using 12-14 kDa cutoff membrane tubing (Spectra/Por, Laguna Hills, CA). The final product, Avidin-Polylysine was aliquotted in PBS and stored at -20^0C.

Biotinylation of retrovirus (Ret-Biotin). Retrovirus was covalently linked to Sulfo-NHS-Biotin (Biotin) following the instructions of the manufacturer (Pierce). Briefly, 0.2ml of Biotin (10mg/ml in DMSO) was mixed with 2.8ml of retrovirus in PBS (7×10^7 cfu/ml). The reaction mixture was incubated on ice for 2 hours. The product (eMMLV-B) was then TES buffer exchanged (0.01M Tris-HCl, pH 7.2, 0.002 M EDTA, 0.15M NaCl) over EP 10 DG Columns (Bio-Rad, Hercules CA). EMMLV-B was aliquotted and stored in TES at -80^0C**.** The titer of eMMLV-B was determined as previously described on NIH3T3 monolayers (20).

Gene transfer analysis. lacZ staining was performed as previously described (21). Briefly, cells were fixed in 0.05% glutaraldehyde phosphate-buffer (150mM sodium chloride, 15 mM sodium phosphate, pH 7.3) (Sigma) for 10 min at room temperature, and rinsed with PBS. Cells were stained for lacZ expression overnight at 37^0C using freshly made x-gal solution buffer (5-bromo-4-chloro-3-indolyl β-galactosidase 1mg/ml, 20 mM potassium ferrocyanide, 20mM potassium ferricyanide and 2 mM $MgCl_2$ in PBS). After rinsing with 70% ethanol, cells were analyzed by microscopy for blue cells.

Polymerase Chain Reactions (PCR). Genomic DNA was extracted from cell pellets using a DNA extraction kit (Qiagen, Valencia, CA). A unique and specific 1,036bp sequence of the *E. coli* lacZ gene

was amplified using the primer pair: sense, 5'-GCC-GAC-CGC-ACG-CCG-CAT-CCA-GC-3', and anti-sense, 5'-GCG-GCG-CGG-TGA-CCA-CAC-CC-GG-3', using taq-polymerase (Promega, Madison, WI). Amplification conditions consisted of a 1 min denaturing cycle at 94^0C, a primer annealing step at 65^0C for 1 min and a primer extension step at 72^0C for 2 min for a total of 35 cycles. A final elongation cycle was performed over 10 minutes at 72^0C (MJ Research, Peltier Thermal Cycler, Watertown, MA). The product was analyzed after electrophoresis on a 1 % agarose gel after ethydiumbromide staining under UV fluorescence.

For quantitative REAL-time PCR, a unique and specific 84bp sequence of the *E. coli* lacZ gene was amplified using the primer pair: E. coliB-gal-f (sense), 5'-ATG-CGC-CCA-TCT-ACA-CCA-A-3', and E.coliB-gal-rev (anti-sense), 5'-AGA-AAC-AAC-CCG-TCG-GAT-TCT-C-3'. Product labeling was performed using the fluorochrome SYBR® Green following the instructions recommended by the manufacturer (PE Biosystems, Foster City, CA). Amplification conditions consisted of a 10-min AmpliTaq Gold polymerase activation step at 95^0C, followed by a denaturing step at 95^0C for 15 seconds and a combined annealing and extension step at 60^0C for 1 min in a thermocycler (GeneAmp®5700 sequence detection system, PE Applied Biosystems). A total of 40 cycles of sequential denaturing/annealing and extension were performed. A standard curve was established by concomitant amplification of DNA extracted from a retrovirally stably transduced NIH3T3 clone. The samples were analyzed with the 5700 software detection system and corrected for total DNA content using a Spectrophotometer, (DU640B, Beckman, Fullerton, CA). An R of > 0.99 was required for standard curves. The sensitivity of the assay was determined to be 10 copies per sample. Copy number per cell was determined by determining sample lacZ copy from a standard curve.

Southern blot analysis. Genomic DNA was extracted form different lacZ expressing 911 single cell clones previously transduced with eMMLV-PL, using a commercial DNA extraction kit (Qiagen, Chatsworth, CA) 40 μg of restriction digested genomic DNA and standards established with the lacZ gene derived from the *Not I* digested control plasmid pACCMVLacZ (21) were loaded on a 0.8% agarose gel and separated by electrophoresis following previously described procedures (22). The DNA was transferred to positively charged nylon membrane (MSI, Westborough, MA), which was subsequently hybridized with a ^{32}P-radiolabeled probe specific for the lacZ sequence (5'-GCC-GAC-CGC-ACG-CCG-CAT-CCA-GC-3'). After removal of non-hybridized probe by washing, the membrane was exposed to radiographic film and developed. Restriction digestion of pACCMVLacZ with *EcoR* I linearized the plasmid yielding a 12 kb fragment, digestion with *Not* I released a 4.3 kb cassette encoding the lacZ gene (21). For demonstration of integration by junction fragments, genomic DNA was digested with *Xba I.* There is a unique *Xba I* site in the retrovirus construct just 3' of the lacZ reporter gene (15;16). The frequency of *Xba I* restriction sites in human genomic is approximately one per 4 kb. For evaluation of lacZ copy number integrated into host cells, genomic DNA was digested with *Xho I* and *Xba I* to release a 4.4 kb fragment encoding the lacZ gene within the retrovirus construct (15;16). Genomic *Xho I* and *Xba I* digested DNA was analyzed by Southern together with different dilutions of purified *Not I* digested pACMVLacZ plasmid DNA fragments to establish a standard. The membranes were probed with the above described lacZ specific probe and the radiographic intensities were determined using a chemiluminescent 4000 Low Light Imaging System (Alpha Innotech Corporation, San Leandro, CA). Copy number per cell was calculated using previously published formulas where the band intensity of the transduced clones were compared to intensities from the standard curve (22-24).

Results

Formation of polylysine based retrovirus molecular conglomerates (aMMLV-PL) results in enhanced gene expression. To test the hypothesis that formation of polylysine based retrovirus molecular conglomerates would improve transduction efficiency over retrovirus particles, biotinylated aMMLV encoding the lacZ reporter gene (aMMLV-B) and avidinylated polylysine (PL-NA) were synthesized. The biotinylation procedure did not significantly reduce the infectivity and transgene expression of aMMLV-B over unmodified virus (aMMLV) in murine NIH3T3 cells (data not shown). Human 911 cells were infected with aMMLV-B with or without Polybrene at increasing moi (0.05 – 1.5) or the equivalent amount of aMMLV-B as molecular conglomerate by adding PL-NA (aMMLV-PL). Formation of retrovirus conglomerates significantly increased transgene expression at low moi's by up to 5-fold. At higher moi, transduction efficiency reached 100% for all vectors, as expected (Figure 2A). To investigate the ubiquity of this transduction enhancement, other cell lines, human 293, A549 and murine NIH3T3 cells were infected with aMMLV-B (with or without Polybrene), or the equivalent amount of retrovirus as aMMLV-PL. At low moi (0.2), formation of aMMLV-PL resulted in significant, 5-fold increased transgene expression in the examined cell lines (Figure 2B).

Formation of polylysine based retrovirus molecular conglomerates (eMMLV-PL) overcomes

tropism restriction of ecotropic retrovirus. To study the hypothesis that formation of polylysine-based conglomerates overcome the tropism restriction of ecotropic retroviruses, biotinylated eMMLV encoding the lacZ reporter gene (eMMLV-B) was joined to PL-NA to form eMMLV-PL. Similar to aMMLV-B, the biotin modification did not significantly decrease infectivity of eMMLV (data not shown). Human embryonal kidney 293 cells were infected with different vector and control constructs at the moi of 0.4 or 1.5 and analyzed for reporter gene expression. As expected, no transgene expression was observed with unmodified ecotropic virus (eMMLV) or eMMLV-B. However, physical linkage of eMMLV-B to PL-NA with conglomerate formation of eMMLV-PL resulted in significant transgene expression of 25% and 84%, respectively (Figure 3).

Polylysine based ecotropic retrovirus molecular conglomerates (eMMLV-PL) transduce various human cell lines and primary human bone marrow stroma cells at high efficiency. The human retinoblastoma cell 911, the human lung cancer cell line A549 and freshly isolated primary human bone marrow derived adherent stroma cells were transfected with eMMLV or eMMLV-PL at the moi of 0.5 and 1.5. Cells were not cultured under selective conditions favoring proliferation of transduced cells. Transgene expression was only seen in cells transfected with eMMLV-PL with 32% and 87% for 911 cells, 25% and 82% for A549 cells, and 30% and 92% for primary human stroma cells (Figures 4 and 5).

Retrovirus molecular conglomerates (eMMLV-PL) integrate into the genome of human cells at one copy per cell. Integration of lacZ gene into the host genome was demonstrated by genomic DNA restriction digestion of five 911 single cell clones, using the enzyme *Xba I*. Different sized junction fragments were identified in all clones examined for presence of lacZ (Figure 6). The copy number of the transferred reporter gene in transduced 911 cells was measured by Real Time PCR and by Southern Blot. A copy number of 0.91/cell (0.87-0.95) was quantified by PCR and a copy number of 0.83/cell by Southern blot (Figures 7 A and 7 B). The combined evidence of these experiments indicates that eMMLV-PL results in integration of the transgene at 1 copy per cell.

Discussion

Our findings demonstrate that ecotropic retrovirus could be modified with conjugate technique in order to accomplish effective transduction of human cells. If successful, this procedure could potentially provide additional safety features for use in humans. Ecotropic virus is chemically linked within the conjugate, so it remains genetically unaltered. The theoretical risk of *in vivo* propagation is substantially less for a transient tropism modification compared to a genetically tropism modified ecotropic retrovirus vector, such as VSV pseudotyped envelopes. In the event that transiently modified ecotropic virus becomes replication competent, its progeny would be ecotropic retrovirus, which is incapable of propagating in humans. On the other hand, progeny arising from a genetically engineered, tropism-modified virus could continue to maintain its tropism-modified form and thus would remain infectious to human cells, posing a significant safety risk.

First, we had to proof that the polylysine conjugation does indeed enhance the transduction efficiency of retrovirus. Although previous studies showed in principle feasibility of retrovirus retargeting via alternate cell surface receptors, several retargeting studies were associated with significant reduction or even disappearance of cellular transduction (25) (26) (27) (28). In contrast, greatly enhanced transduction efficiency was reported for retrovirus vectors redirected by pseudotyping with the vesicular stomatitis virus (VSV) envelope (29;30).

To investigate the hypothesis that polylysine conjugation would enhance retrovirus transduction, retrovirus molecular conglomerates were synthesized by biotinylation and linking it to avidinylated polylysine. Our data demonstrate that at low moi, formation of molecular conjugates synthesized with amphotropic virus (aMMLV-PL) substantially enhances transgene expression over the equivalent amount of free amphotropic retrovirus (aMMLV-B). The human cell lines tested were permissive to infection with amphotropic retrovirus, thus increasing moi eventually resulted in nearly 100% transduction efficiency with both virus vectors. Therefore, the advantage in this particular experimental setting was limited to a transduction enhancement observed at low moi.

The mechanism of the increased transduction efficiency of amphotropic conjugates could be explained through enhancement of virus uptake via their natural receptors, or the utilization of an alternate pathway for virus uptake. The former concept has been proposed for fibronectin meditated retrovirus transduction augmentation (31;32). This model proposes that fibronectin facilitates colocalization of retrovirus particles and target cells with a large increase in local virus titer presented to the cell (31) (33;34). Similarly, the adjunct use of the positively charged polycations, such as polybrene, in retroviral gene transfer procedures has long been known to nonspecifically promote retrovirus transduction efficiency and is now used for many protocols (35).

To proof that the entry mechanisms of retrovirus-polylysine conjugates are independent of natural receptors and are entirely polylysine mediated, experiments were designed using ecotropic retrovirus

constructs in human cells. We found that only the physical binding of eMMLV to polylysine accomplished cellular transduction with efficiencies of 80-90% in different human cell lines and primary cells. Although polycations have previously been reported to facilitate infectivity and to enhance transduction efficiency (36), our data indicate that this effect can not only be greatly enhanced by chemical or physical linkage of virus and polycation, but this linkage allows to completely overcome receptor dependent virus uptake. This phenomenon could be explained by the ability of positively charged polylysine to nonspecifically penetrate cell surface membranes and enter the cytosol (37;38). Compared to individually retargeted retrovirus particles, conglomerates consist of multiple virus particles bound to polylysine thus providing a higher likelihood of delivering one intact virus to the nucleus. This explanation of an added survival benefit is supported by studies where DNA bound to polylysine was found to have significantly enhanced resistance to intracellular degradation over free DNA (39). Similar to DNA, the retrovirus particles may be relatively protected from nuclease digestion within the polylysine conglomerate formation. Therefore, the improved transduction efficiency could result as the combined effect of both polylysine mediated enhanced uptake and also improved retrovirus survival from cytosolic degradation.

Transduction with eMMLV-PL in human cells resulted in similar genomic copy number as eMMLV in the murine cell line NIH3T3. This implies that the formation of retrovirus conglomerates does not change the fundamental principal established for retrovirus/cell interactions of only a single

integration event with one retroviral copy per cell (40).

In summary, this novel retrovirus based gene transfer system has the potential to improve transduction efficiency that comes from low receptor expression, such as for instance in hematopoietic cells (8;9). Moreover, this system would provide additional safety features over amphotropic retrovirus vectors currently used in human trials. Even in the case of formation of replication competent ecotropic virus, it would be theoretically a self-limited problem, as progeny of transiently modified ecotropic virus could not further propagate in humans due to its tropism restriction. Lastly, the polylysine based retrovirus system may enable specific receptor retargeting by formation of receptor targeted retrovirus molecular conglomerate vectors (10;41).

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Figure 1

Retrovirus molecular conglomerates, proposed mechanism: Murine Moloney leukemia amphotropic retrovirus (aMMLV) or ecotropic retrovirus (eMMLV) encoding the lacZ reporter gene is directly linked to polylysine via the heterobifunctional chemical linker EDC or via biotin-avidin technology to from retrovirus-polylysine molecular conglomerates (aMMLV-PL and eMMLV-PL). aMMLV enters human cells via a specific receptor (Figure 1A, arrow 3). Polylysine enters cells via heparan receptors or nonspecifically via positive charges (Figure 1A, arrow 2). The conglomerate aMMLV-PL enters the cell via the polylysine component or via the amphotropic receptor (Figure 1A, arrows 1 and 2, respectively). eMMLV does not infect human receptors due to lack of ecotropic cell surface receptors (Figure 1B, arrow 3). Physically linking eMMLV to PL (eMMLV-PL) enables cellular uptake of eMMLV to human cells through the PL mechanism of cell entry (Figure 1B, arrow 2).

Figure 2

Enhanced gene transfer by formation of amphotropic retrovirus conglomerates (aMMLV-PL) to human and murine cell lines: Human 911 cells were infected using different amphotropic retrovirus constructs: Biotinylated lacZ encoding amphotropic retrovirus (aMMLV-B), aMMLV-B with polybrene, and aMMLV-B with avidinylated polylysine (PL-NA) as complete retrovirus conglomerates (aMMLV-PL). Near confluent cell monolayers were incubated with different constructs consisting of equivalent moi of aMMLV-B as shown beneath the X-axis (0.05 – 1.5). Cells were analyzed for lacZ transgene expression by X-Gal staining and the percentage of positive (blue-colored)

cells is indicated on the Y-axis (Figure 2A). Human 293, 549 and murine NIH3T3 cells were incubated with aMMLV-B, aMMLV-B with polybrene, and aMMLV-B with avidinylated polylysine (PL-NA) at the moi of 0.2 per construct and analyzed for lacZ transgene expression (Figure 2B). Each bar represents the mean of triplicates ± SE. Asterisks indicate a p-value of 0.05 or less. Depicted results are representative and consistent for a series of four independent experiments.

Figure 3

Gene transfer to human 293 cells with ecotropic retrovirus through formation of ecotropic retrovirus polylysine conglomerates: lacZ encoding ecotropic retrovirus (eMMLV), polylysine (PL), and different modifications thereof were synthesized using biotin-avidin technology. Near confluent human 293 cell monolayers were incubated with different constructs and compounds as shown beneath the X-axis. Equivalent moi of eMMLV (0.4 and 1.5) were used for each group. Cells were analyzed for lacZ transgene expression by X-Gal staining and the percentage of positive (blue) cells is indicated on the Y-axis. Depicted results are representative and consistent for a series of four independent experiments.

Figure 4:

Formation of retrovirus molecular conglomerates overcomes ecotropic tropism restriction in different human cell lines and primary human cells. Human cell lines

911, A549 and primary human bone marrow derived stroma cells were incubated with 0.4 or 1.5 moi of lacZ encoding eMMLV or the equivalent virus as molecular conjugate (eMMLV-PL). Cells were analyzed for lacZ transgene expression by X-Gal staining and the percentage of positive (blue) cells is shown on the Y-axis. Each bar represents the mean of triplicates ± SE. Asterisks indicate a p-value of 0.05 or less. Depicted results are representative and consistent for a series of four independent experiments.

Figure 5.

Transgene expression in human 293 and primary bone marrow stroma cells after eMMLV-PL LacZ gene transfer. Human 293 cells (panels A and B) and primary human bone marrow stroma cells (panels C and D) were transfected with eMMLV or eMMLV-PL, respectively. Cells were analyzed for LacZ transgene expression as outlined in the methods section and visualized by transmission light microscopy.

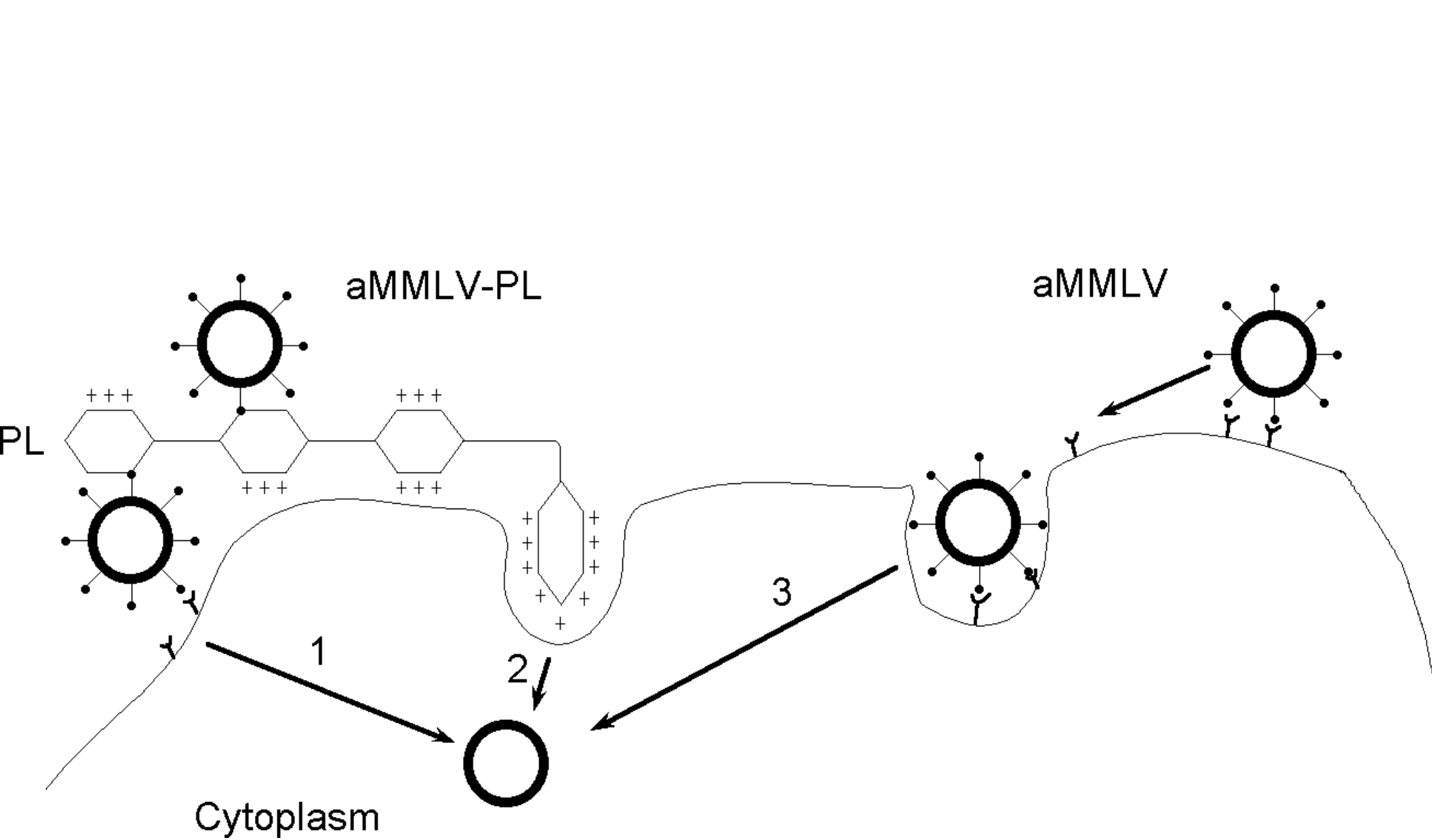
Figure 6:

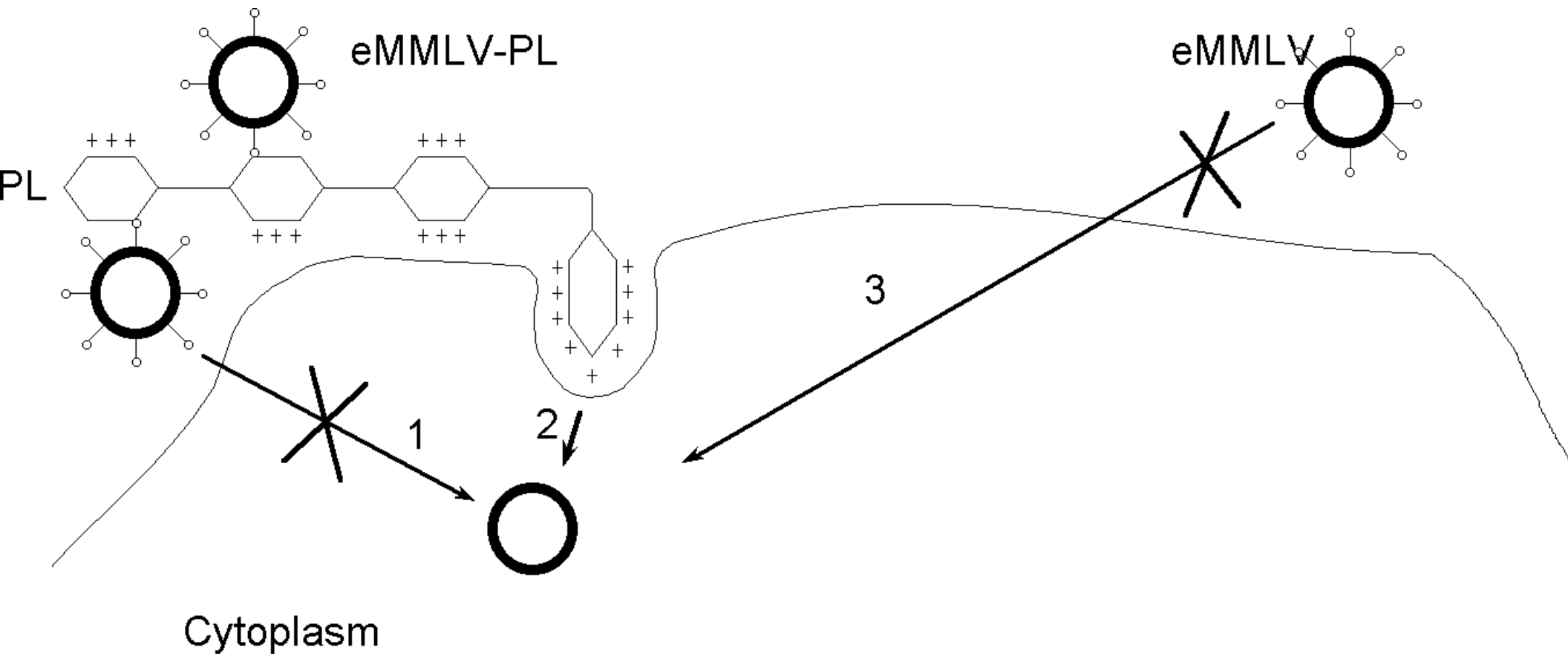
Ecotropic retrovirus molecular conjugates (eMMLV-PL) gene transfer to human cells results in genomic transgene integration. After transduction of 911 cells with eMMLV-PL, lacZ positive single cell clones cells were established. Genomic DNA was restriction digested with the enzyme *Xba I*, a single cutter within the retrovirus genome. For positive control, the plasmid pACCMVLacZ was *Not I* digested to release the 4.3-kb lacZ cassette. The DNA was then analyzed by Southern Blot using a probe specific for the lacZ gene.

Figure 7

Determination of transgene copy number after retrovirus molecular conjugates (eMMLV-PL) gene transfer.

After transduction of 911 cells with eMMLV-PL, lacZ positive single cell clone cells were established. Real time PCR was performed using a specific probe for lacZ on two clones (denoted by triangle and square) and compared to a standard curve established with the lacZ encoding plasmid pACCMVLacZ (dots). CT values are plotted against lacZ copy number (Figure 7A). For quantitative Southern Blot analysis, genomic DNA was restriction digested with *Xho I* and *Xba I* to release the 4.4-kb lacZ cassette from the integrated retrovirus construct. Plasmid pACCMVLacZ was restriction digested with the enzyme *Not I* releasing the 4.3 kb lacZ gene to establish a standard. The membrane was hybridized using a probe specific for the lacZ gene as outlined in the materials and methods section. Signal intensity was used to calculate copy number per cell (Figure 7B).


aMMLV-PL
aMMLV
PL
1
2
3
Cytoplasm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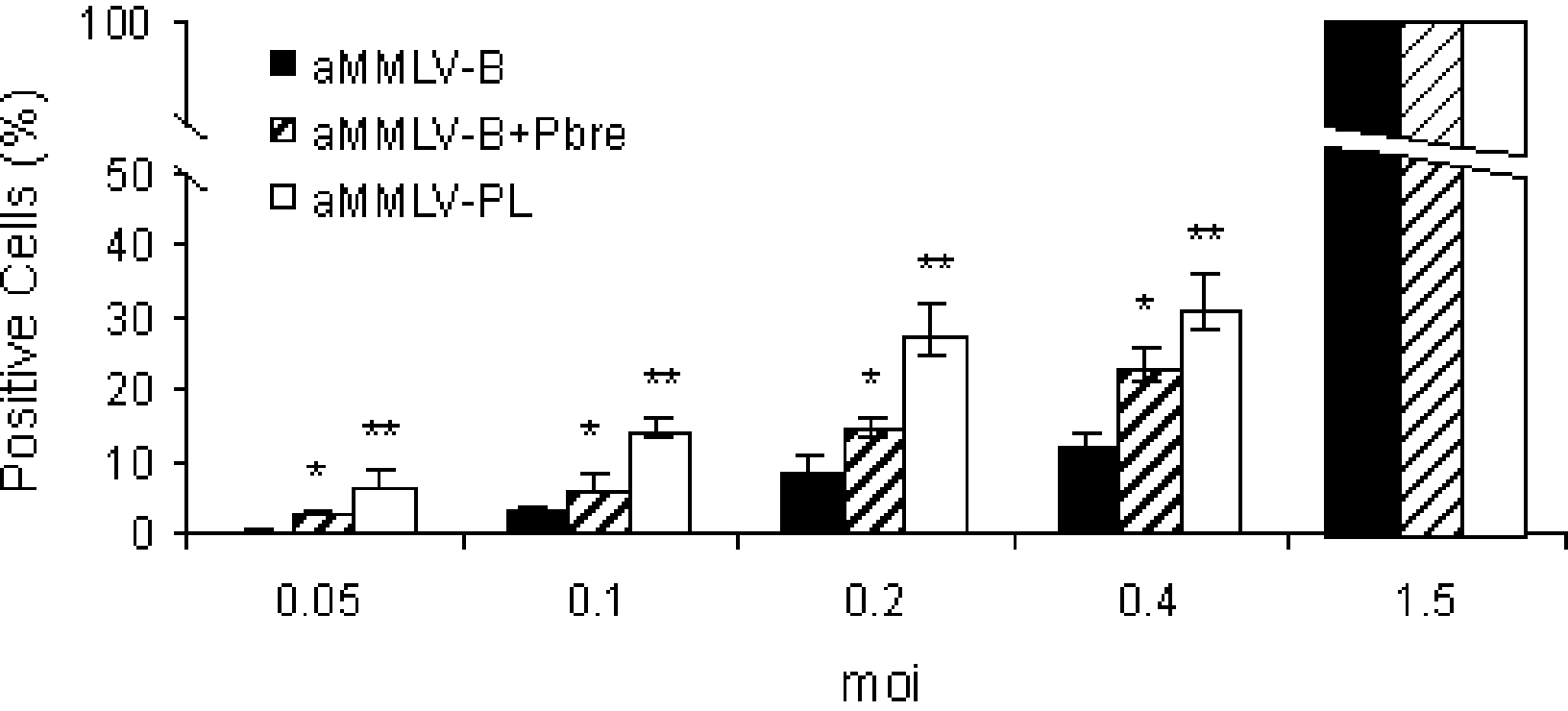
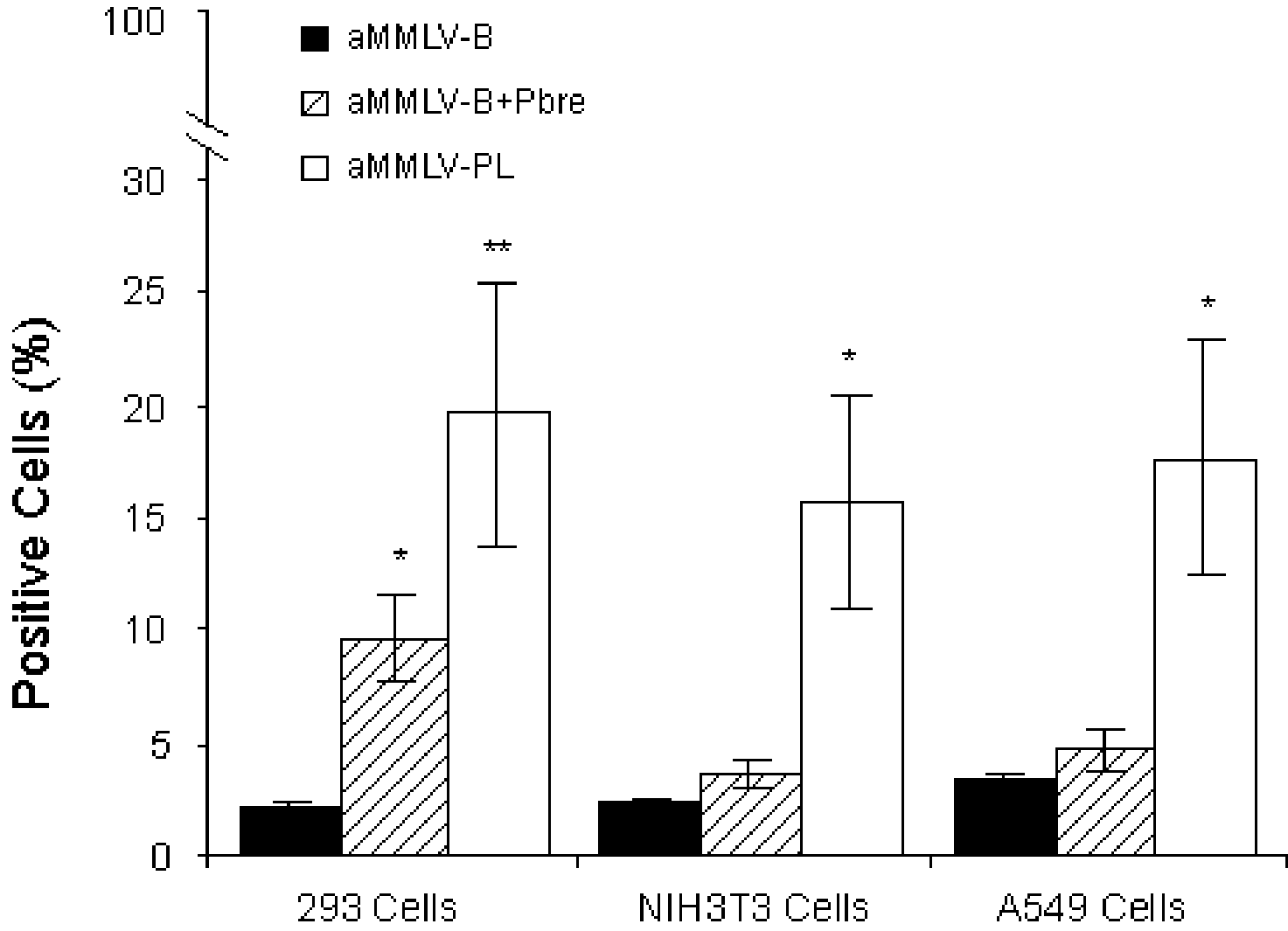
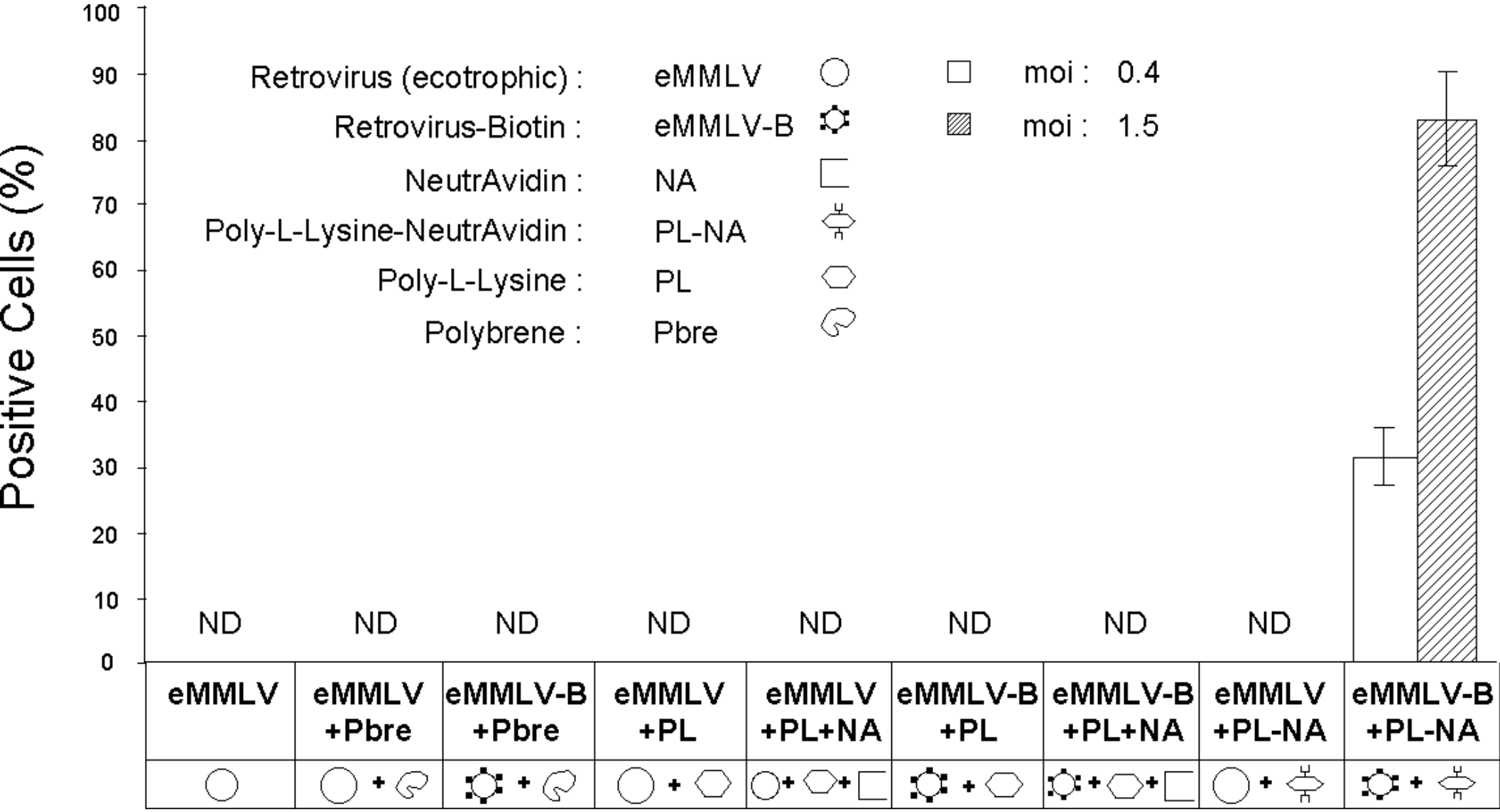
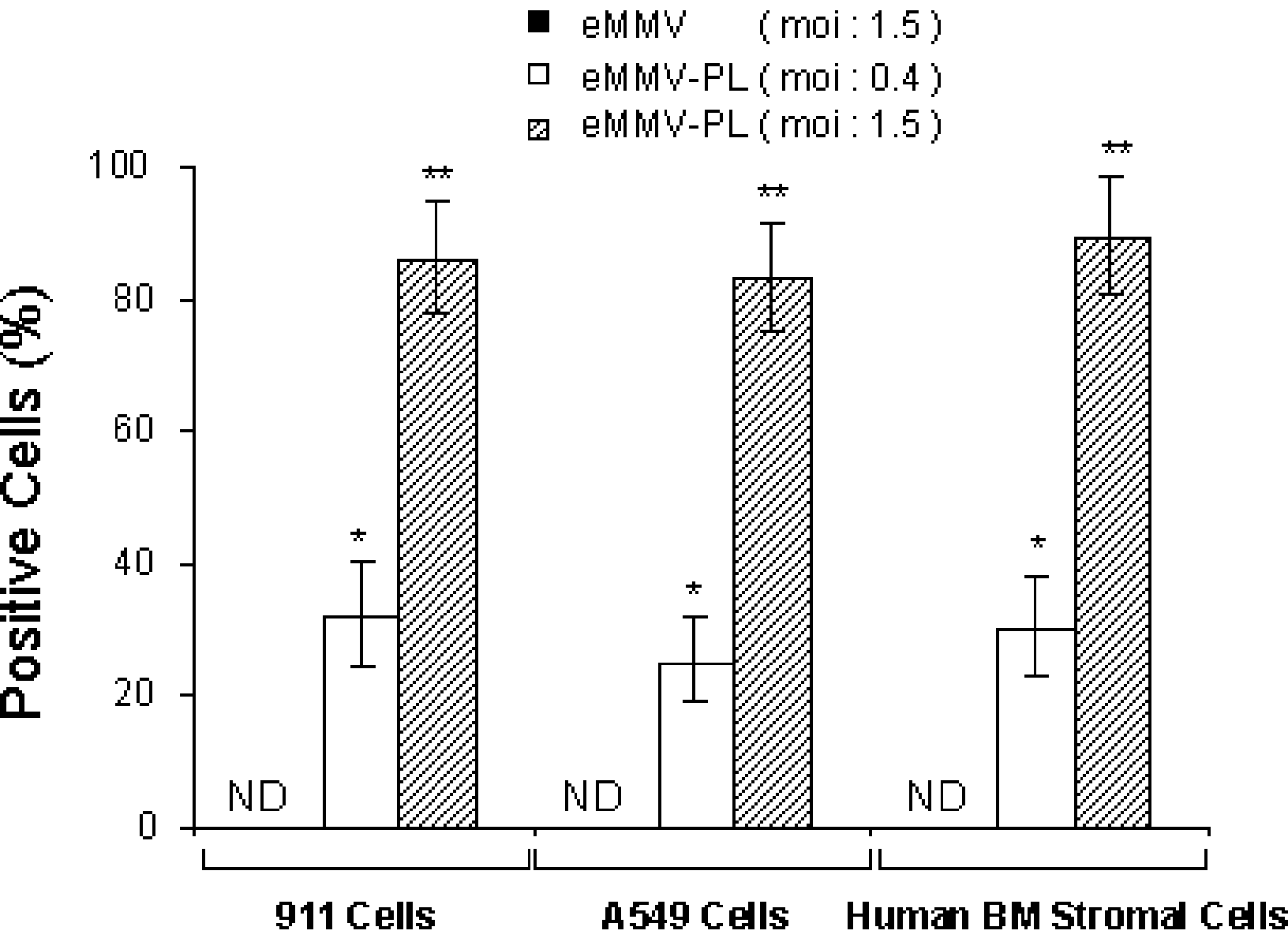
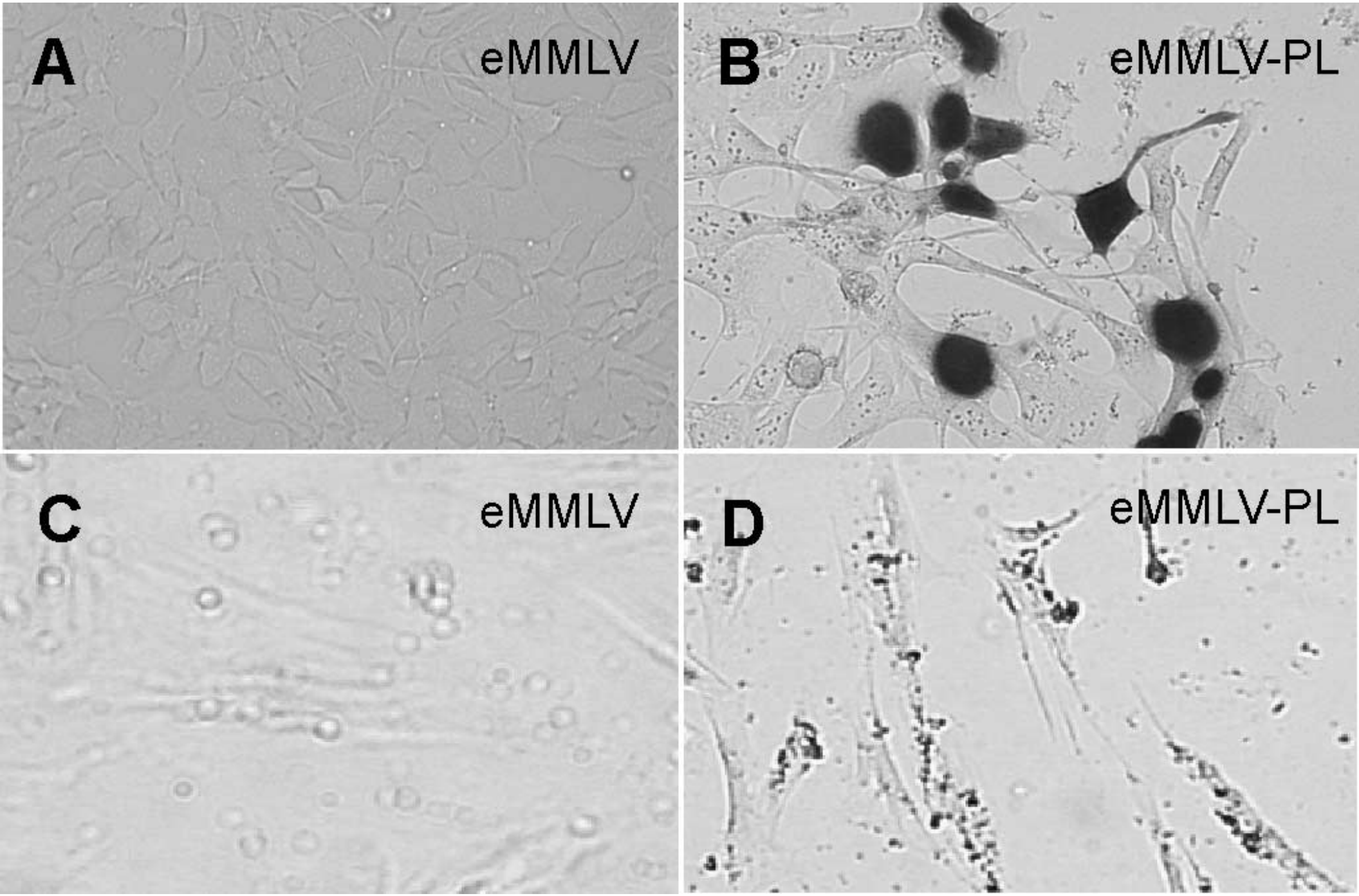
PL
eMMLV
3
1
2
Cytoplasm

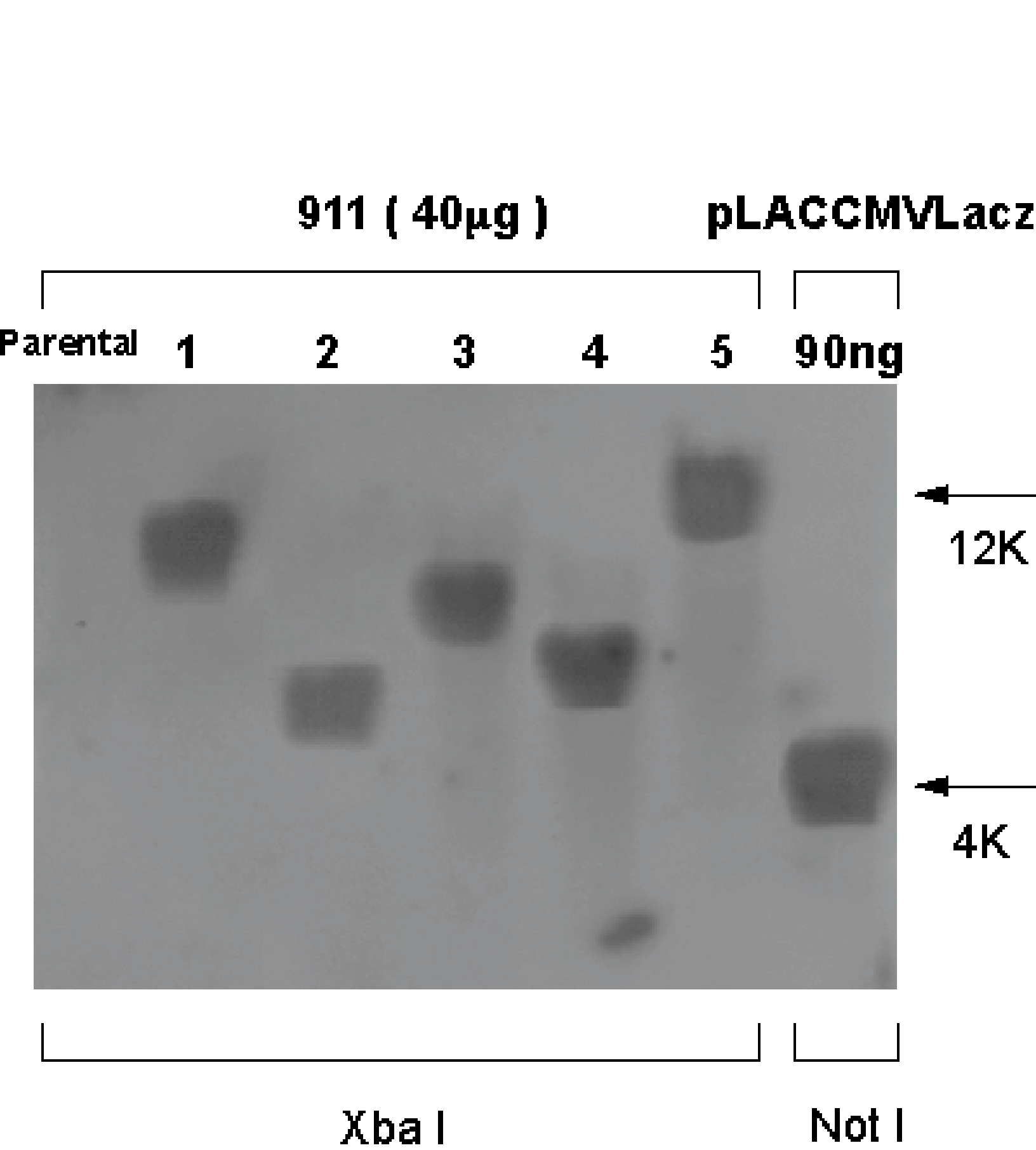

100
50
40
30
20
10
0
Positive Cells (%)
aMMLV-B
aMMLV-B+Pbre
aMMLV-PL
*
**
*
**
*
**
*
**
0.05
0.1
0.2
0.4
1.5
moi

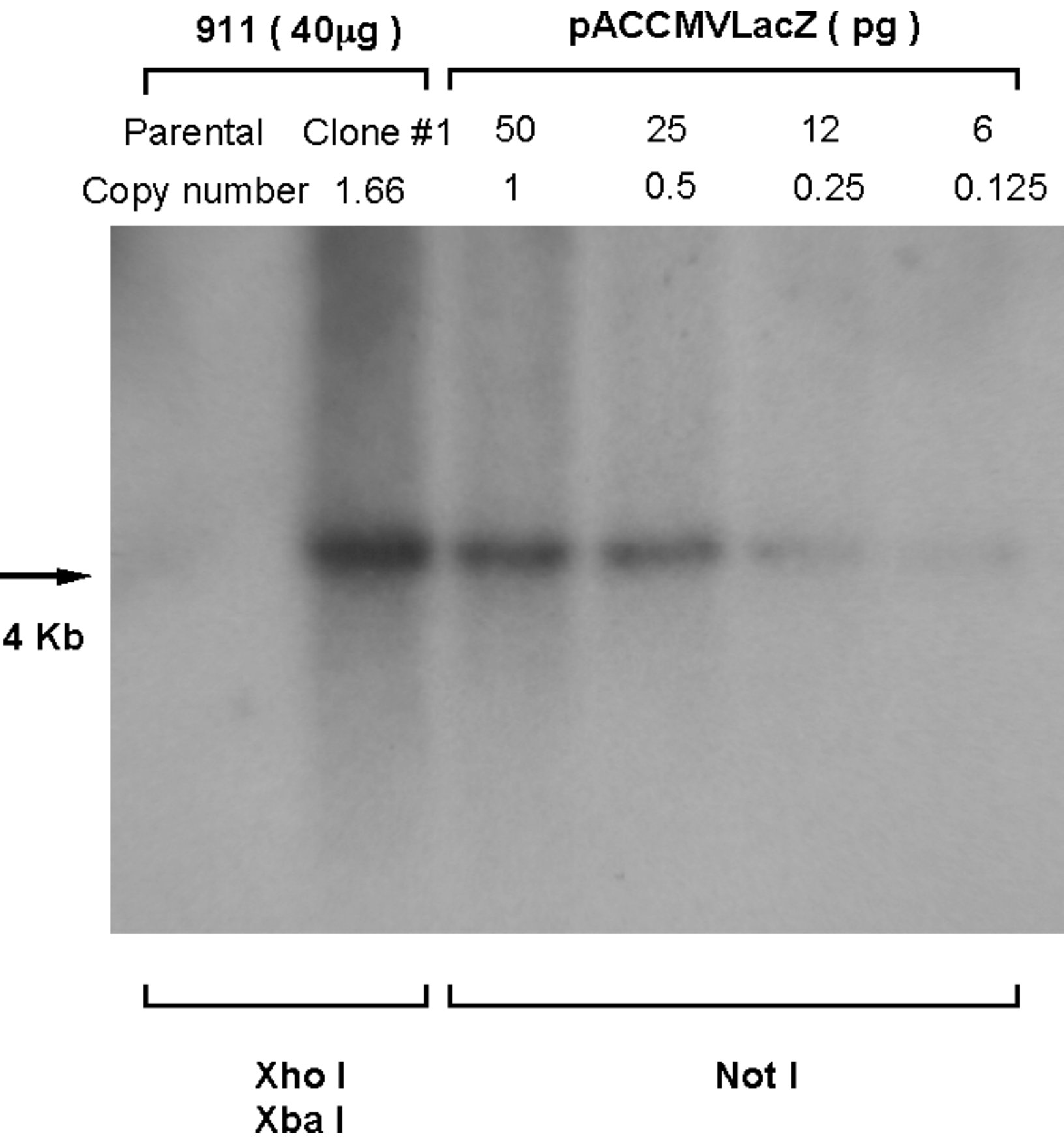

100
30
25
20
15
10
5
0
Positive Cells (%)
aMMLV-B
aMMLV-B+Pbre
aMMLV-PL
*
**
*
*
293 Cells
NIH3T3 Cells
A549 Cells

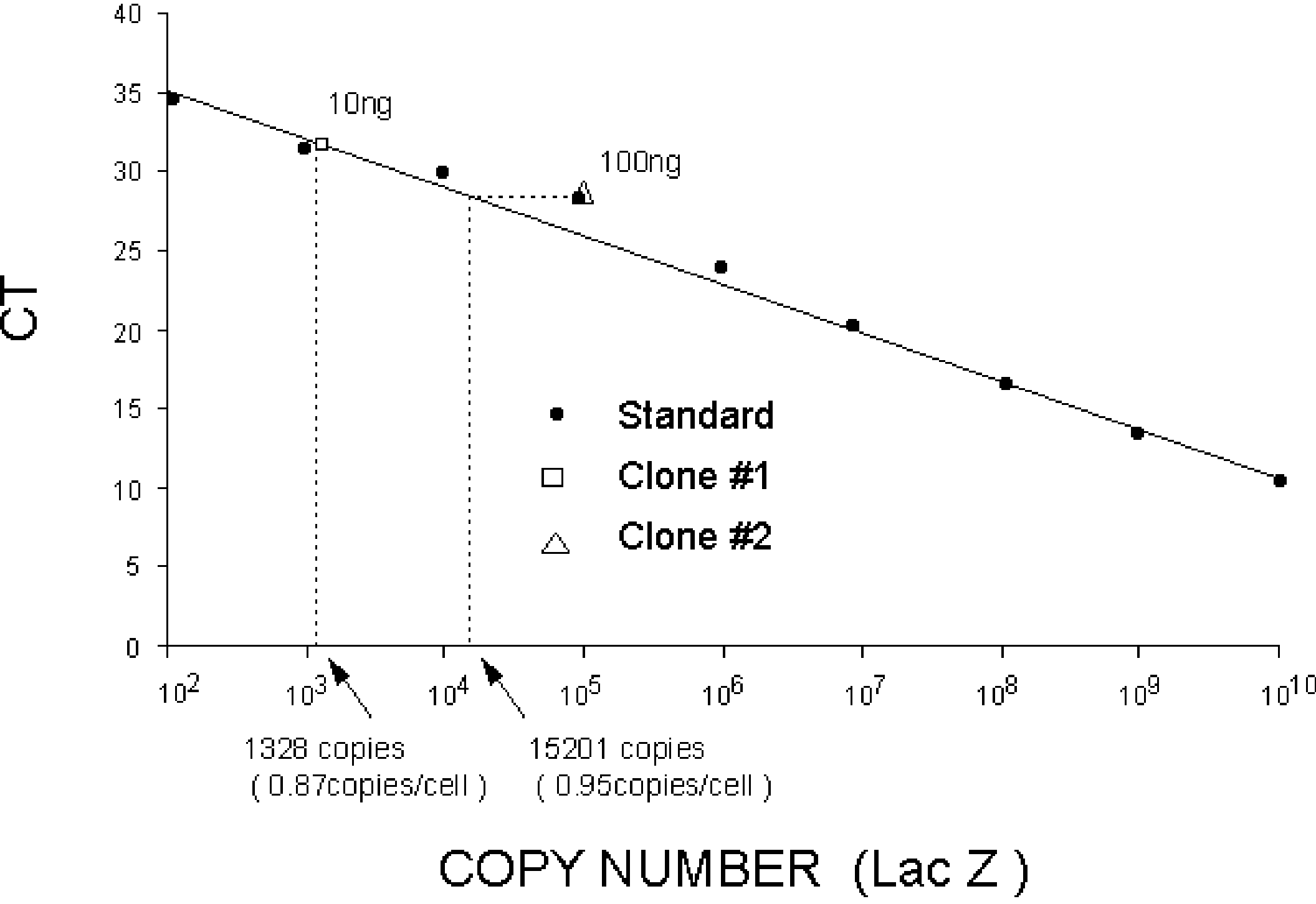

Positive Cells (%)
100
90
80
70
60
50
40
30
20
10
0
Retrovirus (ecotrophic) : eMMLV
Retrovirus-Biotin : eMMLV-B
NeutrAvidin : NA
Poly-L-Lysine-NeutrAvidin : PL-NA
Poly-L-Lysine : PL
Polybrene : Pbre
moi : 0.4
moi : 1.5
ND
ND
ND
ND
ND
ND
ND
ND
eMMLV
eMMLV +Pbre
eMMLV-B +Pbre
eMMLV +PL
eMMLV +PL+NA
eMMLV-B +PL
eMMLV-B +PL+NA
eMMLV +PL-NA
eMMLV-B +PL-NA


eMMV (moi : 1.5)
eMMV-PL (moi : 0.4)
eMMV-PL (moi : 1.5)
Positive Cells (%)
100
80
60
40
20
0
ND
ND
ND
*
*
*
**
**
**
911 Cells
A549 Cells
Human BM Stromal Cells


A
eMMLV
B
eMMLV-PL
C
eMMLV
D
eMMLV-PL


911 (40μg)
pLACCMVLacz
Parental
1
2
3
4
5
90ng
12K
4K
Xba I
Not I


911 (40μg)
pACCMVLacZ (pg)
Parental
Clone #1
50
25
12
6
Copy number
1.66
1
0.5
0.25
0.125
4 Kb
Xho I
Xba I
Not I


40
35
30
25
20
15
10
5
0
Ct
10ng
100ng
Standard
Clone #1
Clone #2
10^2
10^3
10^4
10^5
10^6
10^7
10^8
10^9
10^{10}
1328 copies
(0.87copies/cell)
15201 copies
(0.95copies/cell)
COPY NUMBER (Lac Z)