profile

Chromos Molecular Systems Inc. is a public, Canadian-based biotechnology company leading the development of innovative therapies using a proprietary chromosome-based gene delivery and expression system for the production of therapeutic proteins and cell-mediated gene therapies.

Referred to as ***The ACE System,*** Chromos' artificial chromosome technology provides a powerful platform for ***cellular protein production*** and ***cell-mediated gene therapy***. The ACE System provides a number of significant advantages over traditional approaches including:

- Speed – Permits the rapid, efficient and reproducible insertion of selected genes.
- Expression – Facilitates high level and long-term expression of therapeutic proteins.
- Stability – Provides a non-integrating and stable system to selectively and predictably control therapeutic protein expression.

Chromos is focused on applying its ACE System for the commercial manufacture of proteins in mammalian ***cellular production systems.*** Given that current protein manufacturing capacity is not sufficient to support the demand for protein products, industry experts are looking for new and innovative technologies to (1) rapidly advance products into production and (2) increase the yield of proteins. Chromos' ACE System has been designed to address these two critical factors through unparalleled speed in engineering stable cell lines for high levels of recombinant protein expression.

Chromos is also applying its ACE System to develop ***cell-mediated gene therapies.*** The ACE System addresses the limitations of existing gene therapy systems by enabling a neutral non-integrating gene delivery and expression platform for therapeutic proteins. Chromos has generated proof of principle animal data that provides evidence of its safety and stability for gene therapy.

Chromos' Business Strategy is designed to create value based on its proprietary ACE System. This strategy works in two ways:

(1) By out-licensing Chromos' ACE System to multiple partners for use in protein production to generate short and medium term revenues, as well as long term royalty streams.

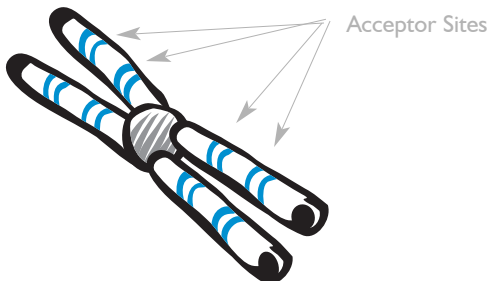
(2) By developing cell-mediated gene therapy products, alone and with partners, to generate medium and long term revenues.

Globally, Chromos is the only company that can produce and isolate mammalian artificial chromosomes. The Company began operations in 1996, has raised over $60 million to date, and employs 32 people. The ACE System is currently covered by 17 patents and 35 pending applications worldwide.

technology

The ACE System

The ACE System is a unique and proprietary gene expression platform technology that functions as a vehicle for transporting genes into the nucleus of a target cell where they can be expressed to produce one or more proteins in a controlled, predictable, and stable manner. The system consists of three components: (1) a neutral, functional mammalian artificial chromosome called the Platform ACE, (2) a vector for loading genes onto the Platform ACE referred to as the ACE Targeting Vector, and (3) a site-specific, Unidirectional Integrase



A pre-engineered platform chromosome (Platform ACE) containing multiple sequence-specific recombination "acceptor" sites.

Platform ACE

The Platform ACE is a pre-engineered mammalian artificial chromosome containing multiple sequence-specific recombination acceptor sites. The Platform ACE behaves as a fully functional chromosome in a variety of mammalian cells (including mouse, rat, hamster, chicken, rabbit, bovine, and human) and plant cells. Its composition primarily consists of heterochromatic DNA originating from the pericentric regions of acrocentric chromosomes [1, 2, 3]. These neutral sequences are typically devoid of functional genes and have no phenotypic effect on a host cell, making the ACE an ideal platform for carrying genes of interest in a normal chromosomal context. The Platform ACE also contains a functional centromere and telomeres derived from a natural mammalian chromosome. Thus, the Platform ACE typically exhibits the same mitotic and genetic stability as host chromosomes in a given cell.

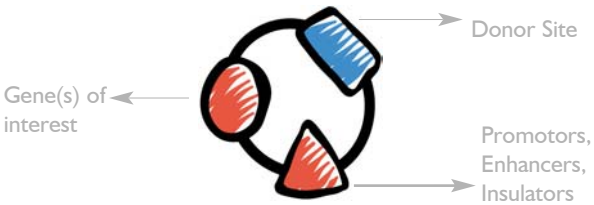
The Platform ACE can function in different mammalian cell types, including immortalized cell lines, protein production lines (CHO, NS0), primary cells, and adult-derived stem cells [4, 5, 6]. The presence of multiple recombination acceptor sites enables single or multiple insertions of several different genes or gene complexes. Furthermore, the Platform ACE can be engineered with a gene of interest in one cell type and readily isolated and moved to another cell type.

technology

ACE Targeting Vector

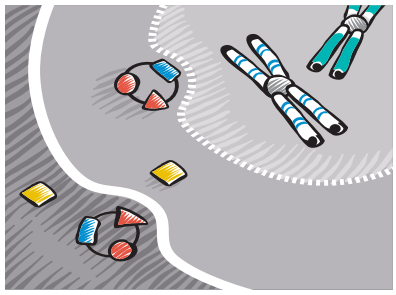
The ACE Targeting Vector is a proprietary vector that contains a specific "donor site" for integrase-mediated site-specific recombination into the Platform ACE. Genes or sequences of interest can be subcloned into the targeting vector to allow easy loading onto the Platform ACE. The ACE Targeting Vector has also been engineered to contain genetic elements to maximize gene expression, including promoters, enhancers, insulators, etc. The ACE Targeting Vector has been designed so that these expression-enhancing elements can be changed to suit the specific needs of the end user.

Once the targeting vector has been engineered with the gene of interest, it can be readily loaded onto the Platform ACE using a proprietary Unidirectional Integrase that catalyzes the site-specific recombination between the donor site of the targeting vector and the acceptor site(s) on the Platform ACE. The large recognition sequences at these sites increases the specificity of the targeting event, allowing for routine and predictable gene transfer. Moreover, unidirectional targeting facilitates multiple gene loading applications without loss of a previously loaded gene.

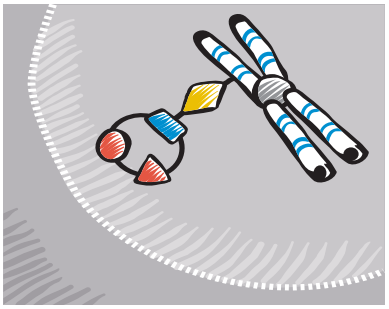


Unidirectional Integrase

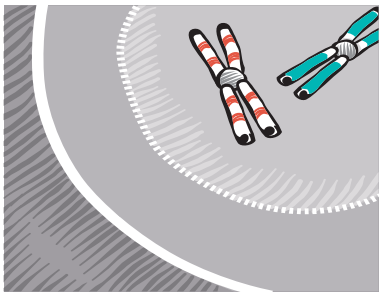
Recombination between the ACE Targeting Vector and the Platform ACE is catalyzed by a proprietary, Unidirectional Integrase enzyme derived from bacteriophage lambda. This integrase has been genetically modified to eliminate the need for bacterial accessory proteins that normally participate in the recombination reaction. Unlike systems such as Cre/lox, the Unidirectional Integrase does not catalyze excision reactions under the conditions used. This ensures reliable insertion of genes onto the Platform ACE and also allows for multiple rounds of gene loading if desired.



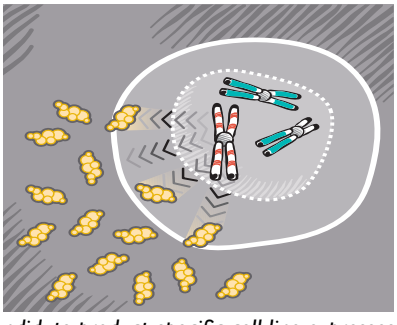
Using standard transfection techniques, targeting vector and integrase migrate into the cell containing the Platform ACE



Targeting vector recombines into multiple acceptor sites on Platform ACE, catalized by intergrase



Multiple copies of the product gene are incorporated into multiple acceptor sites in a single step



Candidate product-specific cell line expresses high levels of protein

engineering

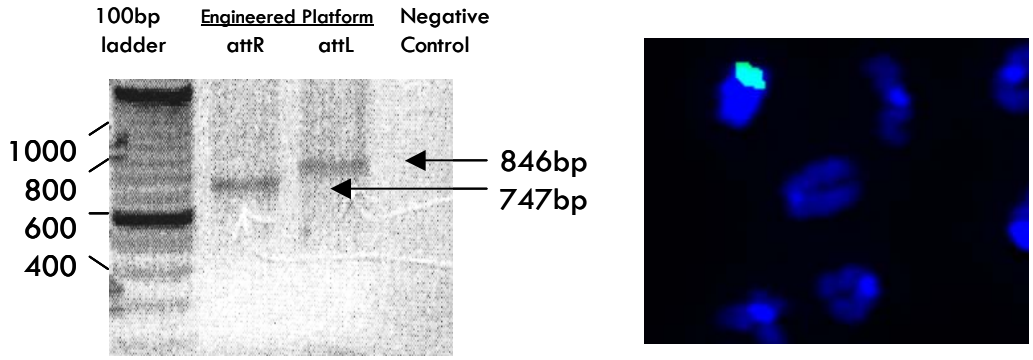
Engineering Genes onto the Platform ACE

Starting with the Platform ACE in a mammalian cell, it is simple and straightforward to engineer in one or more genes, gene complexes, or genomic sequences. Once loaded onto the platform, genes are stably maintained through subsequent cell divisions (see section on Stability). Engineering is performed as follows (see appendices for detailed information on engineering genes of interest onto the Platform ACE including information on Chromos' proprietary Unidirectional Integrase enzyme):

a) The gene of interest is subcloned onto the ACE Targeting Vector.
b) The resulting construct is transfected into a cell containing the Platform ACE, using standard transient transfection techniques.
c) A separate plasmid that transiently expresses the Unidirectional Integrase is simultaneously co-transfected.
d) The integrase catalyzes the unidirectional integration of the targeting vector into the Platform ACE, via recombination between the donor sequences on the targeting vector and the acceptor sequences on the Platform ACE.

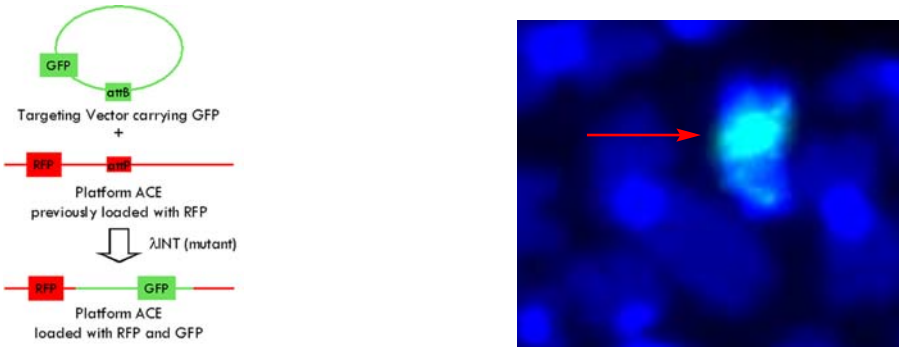
Note: Donor sequences are not present on the plasmid that transiently expresses the integrase, so it is not incorporated onto the Platform ACE and is quickly lost from the engineered cell.

Figure 1- Demonstration of precise targeting of the integration event.



A Platform ACE was loaded with a red fluorescent protein (RFP) gene as described. The left panel shows PCR analysis of the integration junctions (attR and attL) created by sequence specific recombination between the donor and acceptor sites. Fragments of the expected size for both junctions are clearly visible (arrows). The right panel shows FISH analysis of a metaphase spread from the resulting cell line. Hybridization of an RFP-specific probe to the Platform ACE appears in red.

Figure 2- Integration of a second gene onto a previously modified Platform ACE.



A Platform ACE chromosome containing multiple acceptor sites, which had been previously loaded with the RFP gene, was targeted for a second integration using a vector containing the green fluorescent protein (GFP) gene, as shown in the schematic (left). FISH analysis confirmed loading of the GFP gene onto the Platform ACE (arrow, right), resulting in the Platform ACE carrying both RFP and GFP.

speed

Cell line Production

A major advantage of Chromos' ACE System is the unparalleled speed in engineering stable cell lines for protein production. The steps noted below illustrates the dramatic difference in time and effort associated with generating production cell lines using the conventional approach of random integration compared with Chromos' ACE System.

ACE System (2 - 4 months)

1) Generate primary transfectants

2) Screen 50-150 colonies to identify best 5 - 10 lines

3) Identify lines expressing at 10-30 pg/cell*day ***without DHFR amplification or subsequent instability***

4) Adapt to serum-free suspension

Convential Methods (9 -12 Months)

1) Generate primary transfectants in CHO

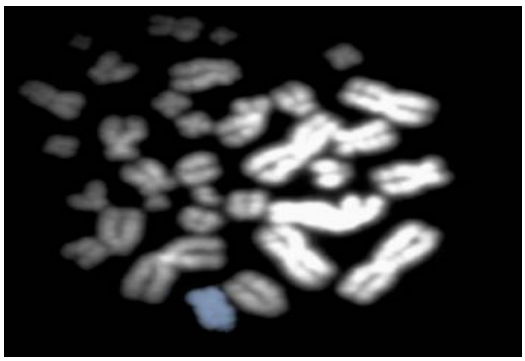
2) Screen up to 7000 colonies to identify best ~50 lines

3) 3-4 rounds DHFR amplification (4-6 months)

4) Identify best ~10 lines, giving 20-50 pg/cell*day

5) Adapt to serum-free suspension

Note: Due to instability of amplification, expression drops to 6-8 pg/cell*day



expression & stability

Expression

The ability to express genes at high levels from the Platform ACE and to maintain that expression for long periods of time through multiple cell doublings is an important feature of the ACE System.

Chromos has demonstrated that it can achieve industry competitive levels of recombinant protein expression on a per cell basis by loading multiple copies of a given gene on the Platform ACE. Results from a typical targeting experiment whereby genes encoding a humanized monoclonal antibody were introduced onto the Platform ACE are summarized below:

- 30 primary transfectants were screened for targeted integration and high antibody expression
- The best six antibody expressing clones were expanded for specific productivity testing (static culture conditions)
- Results: primary transfectants with specific MAb productivities at 10-30 pg/cell*day

Chromos has also demonstrated ***in vivo*** expression of a human gene in an animal model using our ACE System. In these experiments, rodent cells carrying a Platform ACE loaded with the human erythropoietin (huEPO) gene were implanted into the flanks of immunodeficient (SCID) mice. The huEPO secreted by the cells raised hematocrit levels from 52% to 65% 2-weeks post-implantation.

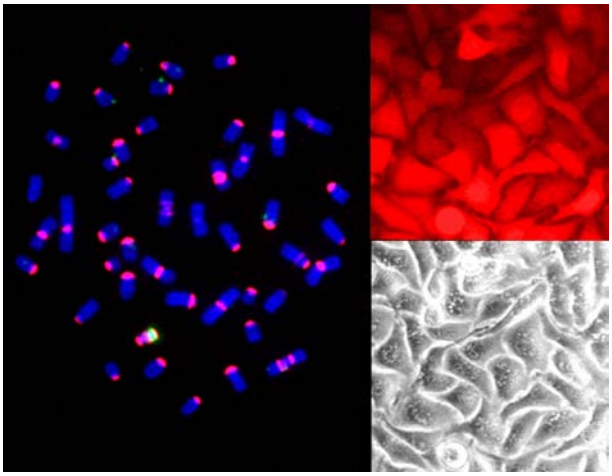
Stable Maintenance of the Platform ACE

The Platform ACE behaves as a natural chromosome and therefore is stably maintained through unlimited cell doublings. Unlike others systems, the Platform ACE does not integrate into the host chromosomes. This feature eliminates any concerns related to insertional modification of host genes. It also eliminates the unpredictability of context effects that plague expression in other systems.

Stability in Cultured Cell Lines and Transgenic Animals

In vitro and ***in vivo*** studies have demonstrated that the mitotic and genetic stability of the Platform ACE is comparable to that of the endogenous chromosomes in a given cell

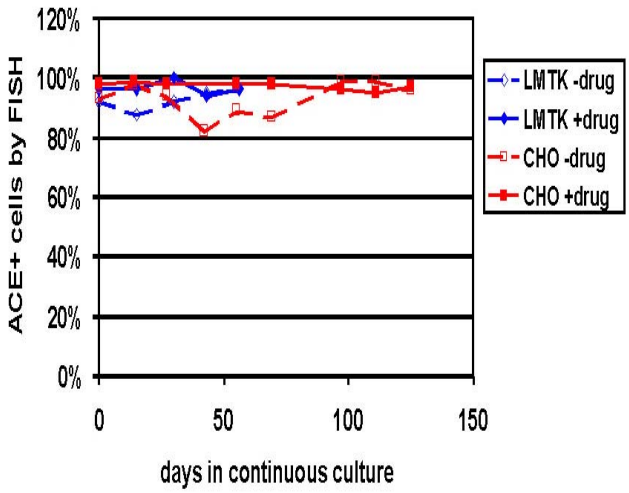
Figure 3- Expression of Red Fluorescent Protein in cultured cells containing a Platform ACE carrying the RFP gene



Left panel shows FISH analysis of a metaphase spread, probed for the RFP gene (green; specific for ACE) and for mouse major satellite sequences (red; common to ACE chromosomes and host chromosomes) and counterstained with DAPI (blue). Lower right panel shows a phase contrast image of ACE-containing cells in culture. Upper right shows a fluorescence image of the same field, demonstrating consistent expression of RFP.

expression & stability

Figure 4 - *Stable maintenance s in culture*



ACE platform cell lines (murine LMTK- and hamster CHO derived) were maintained in continuous culture in the presence or absence of selective antibiotic, and the presence of the ACE was assessed directly by fluorescenct *in-situ* hybridization (FISH) at the times indicated. Note that the ACE was stably maintained in over 90% of cells for periods up to 4 months, even in the absence of selective pressure.

Figure 5 - *Lucy, Chromos' first transgenic mouse to carry a prototype ACE chromosome*

Stability in Transgenic Animals

Chromos has demonstrated that ACEs were readily passed on through at least four generations of transgenic mice and expressed genes *in vivo* in a tissue-specific manner [7]. ACEs have also been successfully introduced into bovine embryos, cultured to at least the blastocyst stage, and shown to express a marker gene [8].

*Table 1 Stable maintenance of a prototype ACE in a Transgenic Mouse, and Transmision to Progeny **

Generation	% of Lymphocytes Containing ACE chromosome in Initial Sample	% of Lymphocytes Containing ACE chromosome in a Subsequent Sample
F0	98 (2 months)	99 (10 months)
F1	97 (2 months)	98 (6 months)
F2	96 (2 months)	ND

* Lymphocyte spreads were prepared from the indicated generations at the indicated times and analyzed by FISH for the presence of an intact ACE chromosome. A minimum of 100 spreads were analyzed for each sample.

purification

Purification of Platform ACE

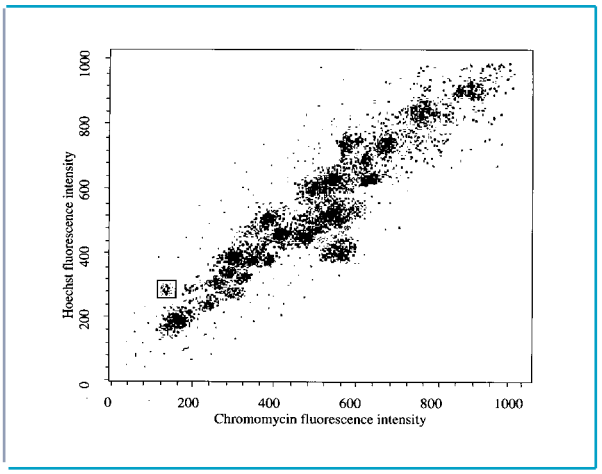
Chromos' ACE System is unique among mammalian artificial chromosomes in that full-size, intact Platform ACEs can be readily isolated in large numbers and subsequently delivered into a wide range of mammalian cells.

Chromos uses a highly efficient method for purifying ACEs which exploits the unique composition of the platform ACE [9]. Briefly, dividing cells are blocked in metaphase and gently lysed to release the condensed chromosomes. These can be stained with fluorescent dyes that bind to AT or GC base pairs. Since they are AT-rich, ACEs preferentially bind to AT-specific dyes, allowing them to be readily distinguished from the host cell chromosomes. High performance flow cytometry is used to yield large quantities of pure, intact ACEs.

Tabel 2- Typical Platform ACE Sorting Results

Sort rates	10^6 ACE chromosomes per hour
ACE purity (by FISH)	>95% to >99% (depending on sort rate)
Shelf Life	7 - 10 days at 4 C°

Figure 6- Flow karyogram of a cell line containing a prototype Platform ACE.



Relative fluorescence intensity is plotted for Hoechst 33258 (AT base pairs) vs. chromomycin A3 (GC base pairs). Individual chromosomes appear as discrete clusters along a 45° axis, with smaller chromosomes at the lower left and larger ones at the upper right. Chromosomes with higher relative AT content appear above the 45° line, while those with higher GC content appear below. The Platform ACE is readily distinguished from the host chromosomes as a small, AT-rich chromosome (boxed).

delivery

Delivery of ACEs to Mammalian Cells

Platform ACEs are initially created in mammalian cells and can be subsequently loaded with genes within those cells. Broad utility requires the ability to move the ACE (with or without added genes) into cells which might be more suitable for specific applications, such as biopharmaceutical manufacturing. The ability to purify the chromosomes, as described previously, is the first key to achieving that. The second key is the ability to introduce purified ACEs into new cells.

Chromos has demonstrated that this can be readily achieved through various techniques, including microinjection, microcell fusion, and electroporation. More importantly, we have shown that purified chromosomes can be readily introduced into a wide variety of cells using simple, off-the-shelf transfection reagents such as cationic lipids and dendrimers [10]. Using labeled chromosomes, we have found that Platform ACEs can be delivered to as many as 25% of cells in culture [10, 11]. More importantly, the chromosomes appear to reach the nucleus in 5-10% of all cells, as evidenced by expression from a marker gene on the Platform ACE.

Table 3 - Delivery and Expression of ACEs in Cultured Cells

Cell Type *	V79 (hamster)	LMTK- (mouse)	Primary fibroblasts (rat)
Delivery **	11 - 15%	11 - 15%	16 - 25%
Expression ***	5%	5 - 7%	9%

* Cells were transfected with purified ACE Chromosomes using commercially available reagents, including cationic lipids or dendrimers.

** Percentage of cells containing an IdUrd-labeled ACE, 48 hours after transfection.

*** Percentage of cells expressing detectable green flouroescent protein, 72 -96 hours after transfection with an ACE carrying the GFP gene.

Table 4 - Partial list of cell types that have been successfully transfected with ACEs

Continuous cell lines	CHO-S (Chinese hamster ovary), DG44 (Chinese hamster ovary) V79 (Chinese hamster lung fibroblasts), LMTK - (mouse fibroblasts) A9 (mouse fibroblasts), P46-F1 (bovine), DT40 (chicken lymphoblasts) EJ30 (human bladder carcinoma), HepG2 (human hepatoma)
Primary cells	Skin fibroblasts (rat), L8 (rat myoblasts), 1043 SK (human fibroblasts)
Stem cells	Adult-derived mesenchymal stem cells (human bone marrow)
Embryos	Murine, Bovine

integration

Chromos' ACE System offers several ***key advantages*** over other site-specific integration systems that have been adapted for use in mammalian cells:

• Specificity

The attP was specifically chosen for use as the acceptor site on the Platform ACE because of its large size and complex sequence. Similar sequences are extremely unlikely to be present anywhere on the host cell chromosomes. Thus, integration of the targeting vector is highly specific for the acceptor sites on the platform.

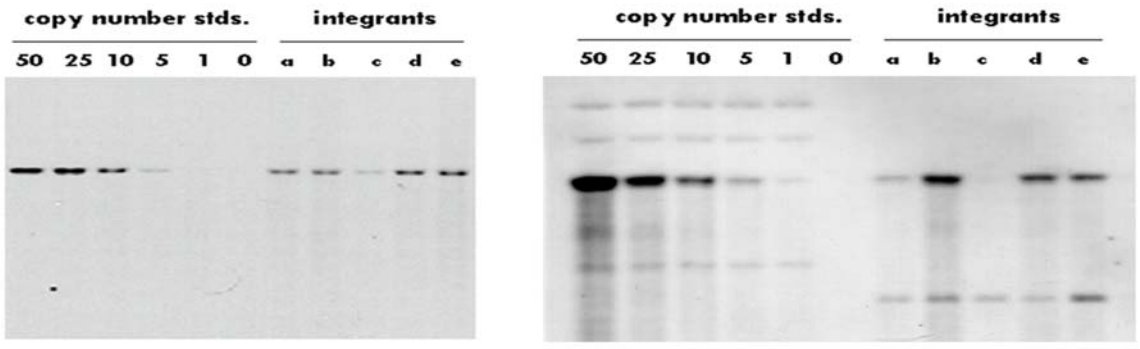
• Unidirectionality

In the absence of bacterial excision factors, the Unidirectional Integrase cannot catalyze excision of a previously integrated gene. This allows multiple different genes, or multiple copies of the same gene, to be loaded onto the Platform ACE during individual or successive operations. This contrasts with systems such as Cre/lox that catalyze excision as well as integration.

• Multiple Loading Capability

Since the Platform ACE carries multiple recombination acceptor sites (50-75), it is capable of multiple loadings of one or more target genes with no apparent limitation on the size of integrated genes. This feature is a significant advantage for increasing levels of recombinant gene expression (see section on Expression). In addition, the ability to load multiple genes at one time sequentially provides great flexibility in genetically modifying cells. For example, this feature enables sequential "re-engineering" of an existing production cell line by adding genes which improve its growth characteristics and/or provides enzymes required for specific post translational modifications that improve the quality and performance of the product protein.

Figure 7 - Copy Number of Integrants



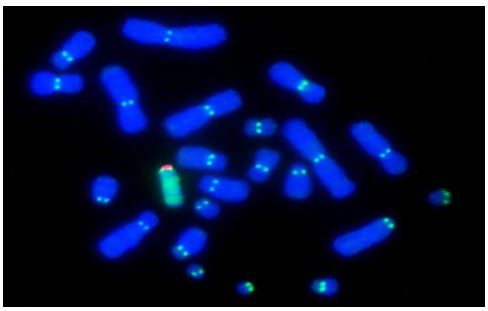
Murine cells carrying the Platform ACE and human fibroblasts carrying the human Platform ACE were each co-transfected with a DNA plasmid encoding the Unidirectional Integrase protein and an ACE Targeting Vector with a promoterless attB-blasticidin resistance gene. Blasticidin resistant colonies were picked at random, expanded, and processed for genomic DNA. Equivalent amounts of genomic DNA were digested with restriction endonucleases, fractionated on agarose gels, and transferred to nylon filters. The filters were probed with labeled DNA specific for the blasticidin^{r} gene. The data demonstrates that more than 25 copies of the Targeting Vector could be loaded onto different ACE Platforms.

summary

Summary of The ACE System

The ACE System offers a number of significant features that are ideally suited for cellular protein production and cell mediated gene therapy, including the following:

- Simple, rapid and predictable platform for gene expression and delivery.
- High levels of expression at high frequency without the need for drug-induced gene amplification.
- Mitotically stable system that does not depend on integration into host chromosomes.
- Very high carrying capacity.
- Ability to load multiple genes.
- Versatile system, portable to other mammalian systems.



Human artificial chromosome in a CHO cell: Hamster chromosomes (blue); human artificial chromosome (red/green); centromeres of all chromosomes are labelled (light green) with an anti-centromere antibody. The green colour on the human artificial chromosome is a probe for the foreign DNA. The red colour is a signal for a satellite DNA sequence which is specific for human chromosome 15, from which the human artificial chromosome was derived.

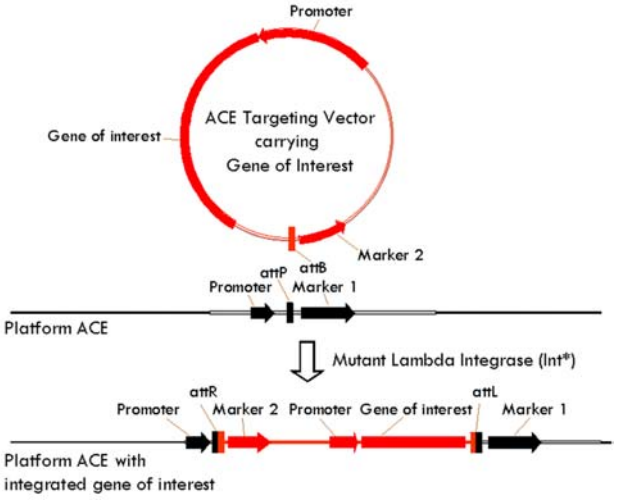
appendix a

Unidirectional Integration System

The system is derived from the well-characterized Int system used by bacteriophage lambda (λ) to integrate itself into the host chromosome of E. coli. The λ phage genome carries a specific integration site called attP, which is partially homologous to a smaller site on the bacterial chromosome called attB. The phage also encodes a site-specific integrase, Int, which catalyzes recombination between attP and attB, inserting the λ phage genome into the host chromosome. In the natural system, integration also requires certain protein factors encoded by the host bacterium.

The attP and attB sites are neither perfectly homologous nor perfectly palindromic. Thus, recombination generates two hybrid sites called attR and attL. Int protein alone cannot catalyze recombination between these hybrid sites. An additional λ protein called Xis is required for the reverse reaction, excision, to occur.

Chromos has modified and adapted the λ Int system to create its proprietary unidirectional integration system depicted schematically below. In this system, the Int protein has been site-specifically mutated (Int*) to catalyze integration in the absence of any E. coli host factors. Integration remains unidirectional because of the absence of Xis.



appendix b

Generating the Platform ACE

Platform ACEs have been generated in multiple cells, including murine, human, and hamster cells. Depending on the details of the approach, a Platform ACE may have one or multiple copies of the recombination acceptor site, and can also be designed to carry selectable drug resistance markers, promoters, and other sequences that may enhance gene expression.

Platform ACEs are initially generated in cultured mammalian cells through a process that involves transfection of host cells, integration of selected elements near the centromere of an acrocentric host chromosome, and genetic amplification at the site of integration.

Details of this process have been described in the literature [1, 2, 3]. Briefly, the steps are as follows:

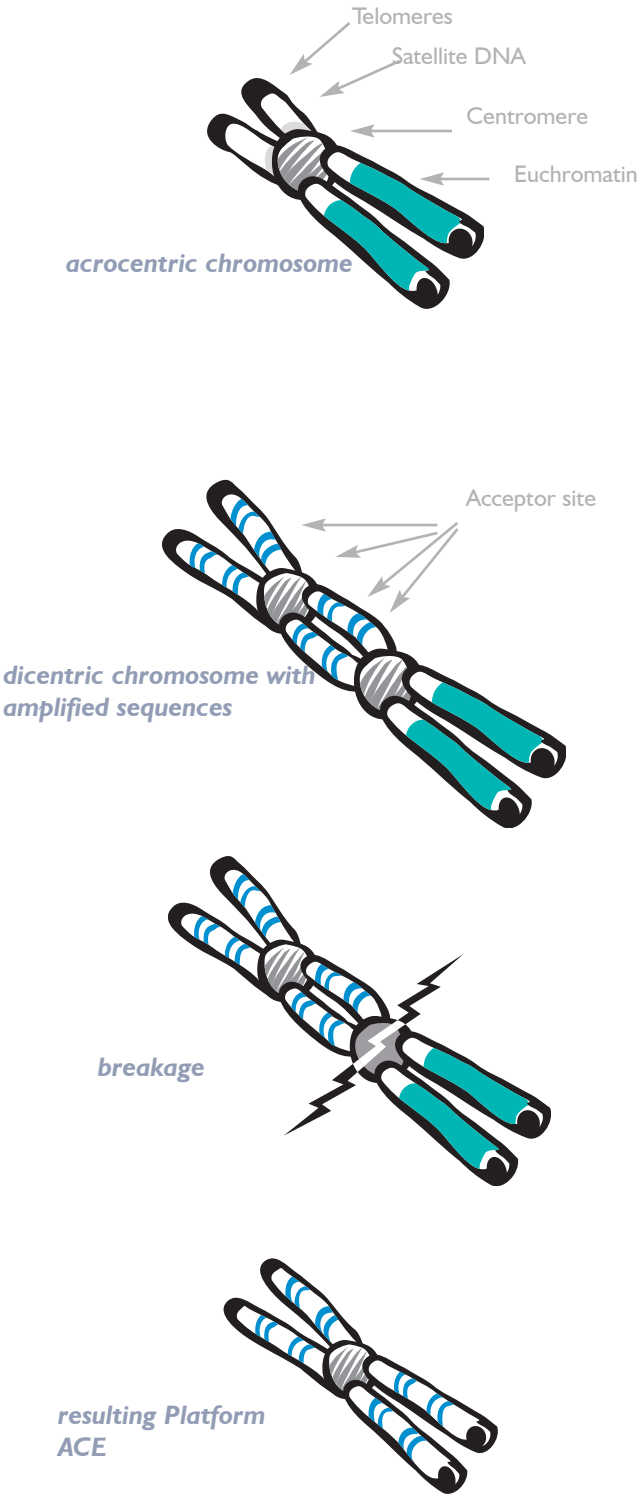
(1) The desired elements of the Platform ACE, including recombination acceptor site(s), marker gene(s), and appropriate expression elements, are first incorporated into a plasmid.

(2) The plasmid is co-transfected into an appropriate mammalian cell, along with specific DNA sequences that promote ACE formation.

(3) The plasmid integrates into the pericentric, heterochromatic region of an acrocentric chromosome.

(4) This integration event leads to large-scale genetic amplification of the surrounding sequences (including the nearby centromere), creating a dicentric chromosome.

(5) Subsequent breakage of the unstable dicentric chromosome, followed by healing of the broken ends by telomeres, results in an independent, genetically and mitotically stable new chromosome, referred to as the Platform ACE.



scientific publications

1. ***Evidence for a megareplicon covering megabases of centromeric chromosome segments.*** Hollo, Gy., Kereso, J., Praznovsky, T., Cserpan, I., Fodor, K., Katona, R., Csonka, E., Fatyol, K., Szeles, A., Szalay, A., and Hadlaczky, Gy. ***Chromosome Research*** 4:240-247 (1996)

2. ***De novo chromosome formations by large-scale amplification of the centromeric region of mouse chromosomes.*** Kereso, J., Praznovsky, T., Cserpan, I., Fodor, K., Katona, R., Csonka, E., Fatyol, K., Hollo, Gy., Szeles, A., Ross, A., Sumner, A., Szalay, A., and Hadlaczky, Gy. ***Chromosome Research*** 4:226-239 (1996)

3 ***Novel generation of human satellite DNA-based artificial chromosomes in mammalian cells.*** Csonka, E., Cserpán, I., Fodor, K., Holló, G., Katona, R., Kersesö, J., Praznovszky, T., Szakái, B., Telenius, A., de Jong, G., Udvardy, A., and Hadlaczky, Gy. , ***Journal of Cell Science*** 113: 3207-3216 (2000)

4 ***Stability of a Functional Murine Satellite DNA-Based Artificial Chromosome Across Mammalian Species.*** Telenius, H., Szeles, A., Keresö, J., Csonka, E., Praznovszky, T., Imreh, S., Maxwell, A., Perez, C., Drayer, J., and Hadlaczky, Gy. ***Chromosome Research*** 7:3-7 (1999)

5. ***In-Vitro transfer of a 60 Mb mammalian artificial chromosome into primary cell lines using cationic lipids and a dendrimer***. de Jong, G., Babich, S., Meitz, A., Vanderbyl, S. and Dreyer, J. (Abstr. 550) ***Molecular Therapy*** 3(5): 194-195 (2001)

6. Efficient transfer of an artificial chromosome platform into adult human mesenchymal stem cells. Vanderbyl, S., MacDonald, N., de Jong, G., Sidhu, S., Gung, L., Perez, C. and Perkins, E (Abstr. 27). ***Molecular Therapy*** 5 (5): 10 (2002)

7. Generation of transgenic mice and germline transmission of a mammalian artificial chromosome introduced into embryos by pronuclear microinjection. Co, D., Borowski, A., Leung, J., van der Kaa, J., Hengst S., Platenburg, G., Pieper, F., Perez, C., Jirik, F., and Drayer J., ***Chromosome Research***, 8:3-7 (2000)

8. ***Expression of a Reporter Gene After Microinjection of Mammalian Artificial Chromosomes into Pronuclei of Bovine Zygotes.*** Wang, B., Lazaris, A., Lindenbaum, M., Stewart, S. Co, D., Perez, C., Drayer, J., and Karatzas, C., ***Molecular Reproduction and Development*** 60: 433-438 (2001)

9. ***Mammalian Artificial Chromosome Pilot Production Facility: Large-Scale Isolation of Functional Satellite DNA-based Artificial Chromosomes.*** deJong, G., Telenius, A., Telenius, H., Perez, C., Drayer, J., Hadlaczky, Gy., ***Cytometry*** 35:129-133 (1999)

10. ***Efficient in-vitro transfer of a 60-Mb mammalian artificial chromosome into murine and hamster cells using cationic lipids and dendrimers.*** de Jong, G. , Telenius, A., Vanderbyl, S., Meitz, A., & Drayer, J., ***Chromosome Research*** 9:475-485 (2001)

11. ***A Flow Cytometry Technique for Measuring Chromosome-Mediated GeneTransfer***. Vanderbyl, S., Macdonald, N., and de Jong, G., ***Cytometry*** 44: 100-105 (2001)

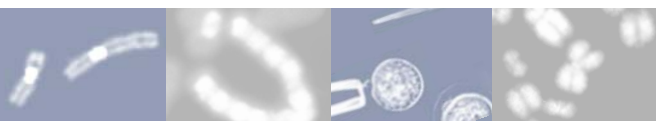
For more information, please contact:

Joseph Zendegui, Ph.D.
Vice President Business Development
jzendegui@chromos.com

Shafique Fidai, Ph.D
Director, Business Development
sfidai@chromos.com

Tel: 604 415 7100
Fax: 604 415 7151

Website: www.chromos.com



CHROMOS

Leading the development and commercialization of chromosome technologies

FISH image of murine cells containing the platform ACE carrying a gene of interest

CHROMOS MOLECULAR SYSTEMS INC.