GEN Genetic Engineering & biotechnology News

drug discovery Channel: Tutorial

Nov 1 2006 (Vol. 26, No. 19)

RNAi Libraries for Functional Genomics

Avoid the Difficulties of Predicting Efficient siRNAs and the Cost of Chemical Synthesis

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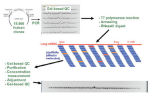

Figure 1

The RNAi mechanism provides researchers with the ability to specifically silence the expression of individual genes based on sequence-specific degradation of a selected mRNA population in the cell to prevent protein synthesis. The method can be quite straightforward when using the nematode Caenorhabditis elegans or cell lines from the fruit fly Drosophila melanogaster, for example.

In cultured mammalian cells, the method is slightly more difficult: long, double-stranded RNA activates the cell's natural antiviral defense. It was not until 2001 that this defense mechanism was circumvented by directly introducing chemically synthesized, small interfering (si)RNAs into the cell. Since then, RNAi has also been applied successfully in mammalian cells. Unfortunately, not all possible siRNAs are effective and, although there are now good algorithms available to predict sequences that will function well with a reasonable probability (50–85%), only experimentation can show whether the selected siRNA actually degrades the mRNA in the cell efficiently.

There is a need for RNAi libraries that cover all currently annotated genes of the organism and which can be acquired cheaply, or can be produced under standardized conditions in suitably equipped laboratories. There is also a need for robust and automated transfection methods for use in mammalian cells in order to be able to introduce the siRNA molecules efficiently into the cell under constant conditions.

To avoid the difficulties of correctly predicting efficient siRNAs and the huge costs of chemically synthesizing such molecules, the Technology Development Studio (TDS) and the Buchholz Group at the Max Planck Institute of Molecular Cell Biology and Genetics produced a genome-wide library of endoribonuclease-prepared (esi)RNAs. In addition, automated transfection of human cancer cells with esiRNA molecules is described.

Production of a Human Genome-wide RNAi Library

The method uses long, double-stranded RNA molecules that are cleaved with Dicer or bacterial RNaseIII in vitro into overlapping siRNA molecules, which can then be introduced into mammalian cells to degrade the desired mRNA specifically while avoiding the cell's antiviral defense mechanism. The starting material is a collection of 15,500 E. coli bacterial clones containing a genome-wide cDNA library (RZPD German Resource Center for Genome Research), which covers to the greatest possible extent all known and predicted human genes (Figure 1).

For each individual gene, the cDNA insertion fragments from the plasmids are amplified in 96-well plates using PCR with T7 polymerase promoters attached on both sides. The T7 polymerase reads off mRNA on both sides from these promoters and this is then hybridized to form double-stranded RNA. After adding recombinant RNaseIII from E. coli, the double-stranded RNA is cleaved into short overlapping siRNA fragments that contain the highly active effector molecules in the pool, in addition to the less active or completely inactive molecules.

This pool is purified through columns and, finally, the esiRNA concentrations for all esiRNAs are measured and adjusted to the same concentrations into new microtiter plates (normalization).

Gel-based quality-control steps are included at all important stages in order to verify the lengths of the fragments, purity, and concentration. All data obtained are fed into a database and all steps of the operation are monitored with a laboratory information management system. So far, a library has been obtained with over 14,000 molecule mixtures, available in the same concentrations in 384-well microtiter plates, in order to switch off a corresponding number of human genes in cultured cells and to investigate the effect on biological processes. Meanwhile, for certain parameters, an optimized human library has been produced, along with a genome-wide library for mouse.

The fundamental precondition for a well-adjusted and usable library is high-precision aliquoting and, subsequently, fully integrated measurement in terms of both hardware and software. For these reasons, a Freedom EVO Workstation with 8-channel liquid handling (LiHa) needles (Tecan) is used for the final step—the standardization of the library.

An integrated 96-channel Te-MO pipetting system with optional disposable pipette tips or a 96-channel steel needle head is used for rapid precision aliquoting, for the UV measurement of the esiRNA concentration. The Teflon-coated steel needles have the advantage that practically no serious retention of negatively charged siRNA occurs, as it does on the plastic surfaces of conventional interchangeable tips that usually have differing degrees of static charge and significantly greater surface roughness. Appropriate washing and sterilizing protocols were developed in order to avoid transferring substances onto the next plate or contaminating it with bacteria or fungi.

The workstation includes a fully integrated GENios Plus photometer (Tecan) to conduct the measurements at 260 and 280 nm, so that both the concentration and the purity can be determined by means of the quotient from the two measurements. For high throughput, the samples are aliquoted into 384-well plates (Corning) specially manufactured for this application. These plates have practically no absorption in the UV range and are less fragile than quartz cuvettes.

The best measurements are obtained when the samples are thoroughly mixed in the diluting medium by 'sandwich' pipetting (14 µL of diluting medium, 2 µL of sample, 14 µL of diluting medium) and by means of a separate mixing step. The concentration and purity measurements are

exported automatically as a file in XML format straight into our LIMS at the TDS. Complete statistical analysis is carried out using this TDS-LIMS software and gel images are also imported into the TDS-LIMS. Samples of inadequate quality (no PCR band, PCR double-bands, incomplete RNaseIII digestion, etc.) or a too-low concentration are excluded.

The 8-needle LiHa arm is used to assemble the standardized library. Firstly, all eight needles can introduce the diluting medium, independently of each other and according to the instructions given by the LIMS, and then add the appropriate quantities of the esiRNA mixture as a summand, resulting in equal final volumes of the various esiRNAs in the same concentrations. Samples are then taken from this newly assembled library in 384-well plates, checked on a gel, and measured again in the UV spectrophotometer. The standard deviations after standardization lie within a range of less than 20% if effective use is made of the sample quantities (2 µL).

Transfection in High-throughput Procedures

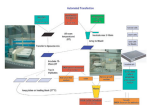

Figure 2

The Max Planck Institute of Molecular Cell Biology and Genetics has developed and optimized automated transfection into human cancer cells for use with this newly created library. Positively charged liposomes are used to pack and transport nucleotides into the cells. The entire screening process is standardized as far as possible.

The CASY cell counter (Scharfe System) ensures consistent counting, and variable distribution patterns resulting from human error are avoided by automated cell seeding (WellMate Dispenser, Matrix). The cells are seeded into microtiter plates on the day before transfection and incubated overnight at the transfection robot (Tecan Freedom EVO Workstation). The complex transfection program achieves a throughput of 40 transfections (>15 samples) in triplicate per day, i.e., 120 recipient plates with the cells. The protocol is illustrated in Figure 2.

First, the transfection reagent is prediluted in cell culture medium and pipetted into a 96-well plate with the 8-needle LiHa arm. An esiRNA library plate is diluted in medium, replicated, and mixed with the transfection reagent using the Te-MO 96-channel head. The cells are prepared while the transfection complex forms: three microtiter plates are taken from the incubator, washed, and filled with serum-free cell culture medium. The transfection complexes are placed on the cells in triplicate sets and the replicates are each dispensed into another cell plate. The three transfected microtiter plates are returned to the incubator. In parallel with the other transfection series, the transfected cells are taken out again after three hours and serum is added for optimal growth. The cells are then incubated for two to four days while awaiting analysis.

To optimize transfection, the human cervical carcinoma cell line, HeLa, was transfected with RNAi molecules against the kinesin-related mitotic motor protein Eg5, which plays an important role in spindle formation: switching it off causes mitotic arrest and subsequently apoptosis. The rounded mitotic cells can be counted simply under the microscope after 24–48 hours or, in the case illustrated here (Figure 3), the decrease in cell mass compared with the control-transfected plate can be measured after 72 hours by a light reaction using an ATP assay. The transfection efficiency is typically over 90% of cells and this leads to a pronounced loss of cells within 72 hours.

The Future

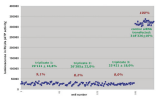

Figure 3

This genome-wide RNAi library places an extraordinary tool in the hands of researchers for the systematic investigation of individual genes of the human genome for their function in all elemental biological processes in the cell. In addition, we have developed transfection methods that allow our screening lab to conduct gene characterization projects of this sort at a high-throughput rate.

Multiparametric, cellular assays in the fields of oncology and infection biology are currently being developed at the TDS for use with the automated screening microscope OPERA (Evotec Technologies) and validated for genome-wide screening. Analysis of the several 10,000 or 100,000 images generated during a screening process is carried out by image-processing programs such as the Acapella software (Evotec Technologies) on-the-fly on the OPERA or off-line by the Cellenger software (Definiens). Automated fluorescence microscopy offers exceptional possibilities for analyzing the physiological status of individual cells at a high-throughput rate and with high information content.

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