Production of a genome-wide RNA interference library for functional gene characterization in cultured human cells

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RNA interference (RNAi) is a mechanism that gives researchers the ability to investigate gene function quickly and efficiently by specifically silencing the expression of the individual gene in question. Although quite easy in some species, RNAi initially proved more difficult in cultured mammalian cells because the long double-stranded RNA activated the cells' natural antiviral defence. To an extent, this has been overcome by using chemically synthesized small interfering RNAs (siRNAs) around 21 nucleotides long but this technique is still not always effective. Although there are now good algorithms available to predict sequences that will function well with a reasonable probability, only experimentation can show whether the selected siRNA actually degrades the mRNA in the cell efficiently.

To address this problem, scientists at the Max Planck Institute in Dresden are using Tecan equipment to create a human genome-wide library of endoribonuclease-prepared, so-called, esiRNAs[1,2] to use for a range of scientific applications. At the same time, they have developed a robust and automated transfection method that introduces the esiRNA molecules efficiently into the cell under constant conditions.

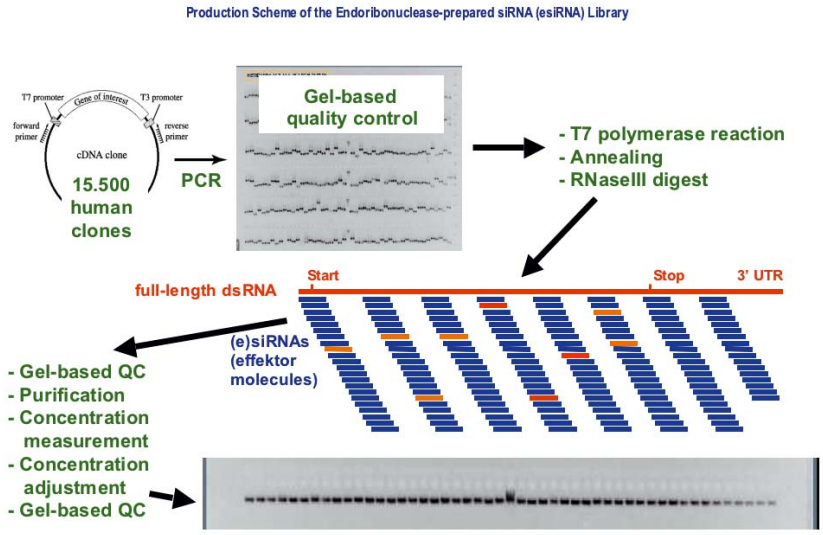

Figure 1: Production scheme of the esiRNA library[3]

The method for esiRNAs involves cleaving long double-stranded RNA molecules with Dicer or bacterial RNaseIII *in vitro* into overlapping siRNA molecules to introduce into the mammalian cell. The starting material (see figure 1; reproduced with permission from reference 3) is a collection of 15,500 *E. coli* bacterial clones containing a genome-wide cDNA library which covers, to the greatest possible extent, all known and predicted human genes. For each individual gene, the cDNA insertion fragments from the plasmids are amplified in 96-well microplates using PCR, and T7 polymerase promoters are attached on both sides. The T7 polymerase reads off mRNA on both sides from these promoters and it can then be hybridized to the RNA double strand. After adding RNaseIII from *E. coli*, the double-stranded RNA is cleaved into short overlapping siRNA fragments which contain the highly active effector molecules in the "pool", in addition to the less active or completely inactive molecules. This effector molecule mixture is purified through columns and, finally, the esiRNA concentrations for all molecule mixtures are measured and adjusted to the same concentrations in new microplates (normalization).

Figure 2: The Freedom EVO system to produce and normalize the genome-wide esiRNA libraries. For UV measurements to determine the RNA concentration, the RoMa arm lifts the plates down from the work table to the GENios Plus reader, which is placed on the level below, as seen in the lower right corner.

Gel-based quality control steps are included at all important stages in order to verify the lengths of the fragments, their purity and concentration. All data obtained are fed into a database, and all steps of the operation monitored by means of a LIMS. So far, a library has been obtained with over 14,000 molecule mixtures available in the same concentrations in 384-well microplates, in order to switch off a corresponding number of human genes in cultured cells and to investigate the effect on biological processes. A genome-wide library has subsequently been produced for mice.

The fundamental precondition for a well-adjusted and usable library is high precision aliquoting and subsequent fully integrated measurement in terms of both hardware and software. For these reasons, a Freedom EVO® Workstation (figure 2) with eight-channel LiHa tips is used for the final step - the standardization of the library. An integrated 96-channel Te-MO™ pipetting system rapidly and precisely aliquots for UV measurement of the esiRNA concentration using optional disposable pipette tips or a 96-channel Teflon® needle head. 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 which usually have differing degrees of static charge.

The workstation includes a fully integrated Tecan GENios™ Plus photometer to conduct the measurements at 260 and 280 nm, so that both the concentration and the purity can be determined by means of the quotients from the two measurements. For high throughput, the samples are aliquoted into 384-well microplates (Corning UV-Star) specially manufactured for this application. The best measurements are obtained when the samples are thoroughly mixed in the diluting medium, by what is known as 'sandwich' pipetting (14 µl of diluting medium, 2 µl of sample, 14 µl of diluting medium) and by means of a separate mixing pipetting step.

The eight tip LiHa pipetting system is used to assemble the standardized library. All eight tips can introduce the diluting medium first, independently of each other, and then add the appropriate quantities of the esiRNA mixture as a summand according to the instructions given by the LIMS. The sum results in equal final volumes of the various esiRNAs in the same concentrations. Samples are then taken from this newly assembled library on the 384-well scale, 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).

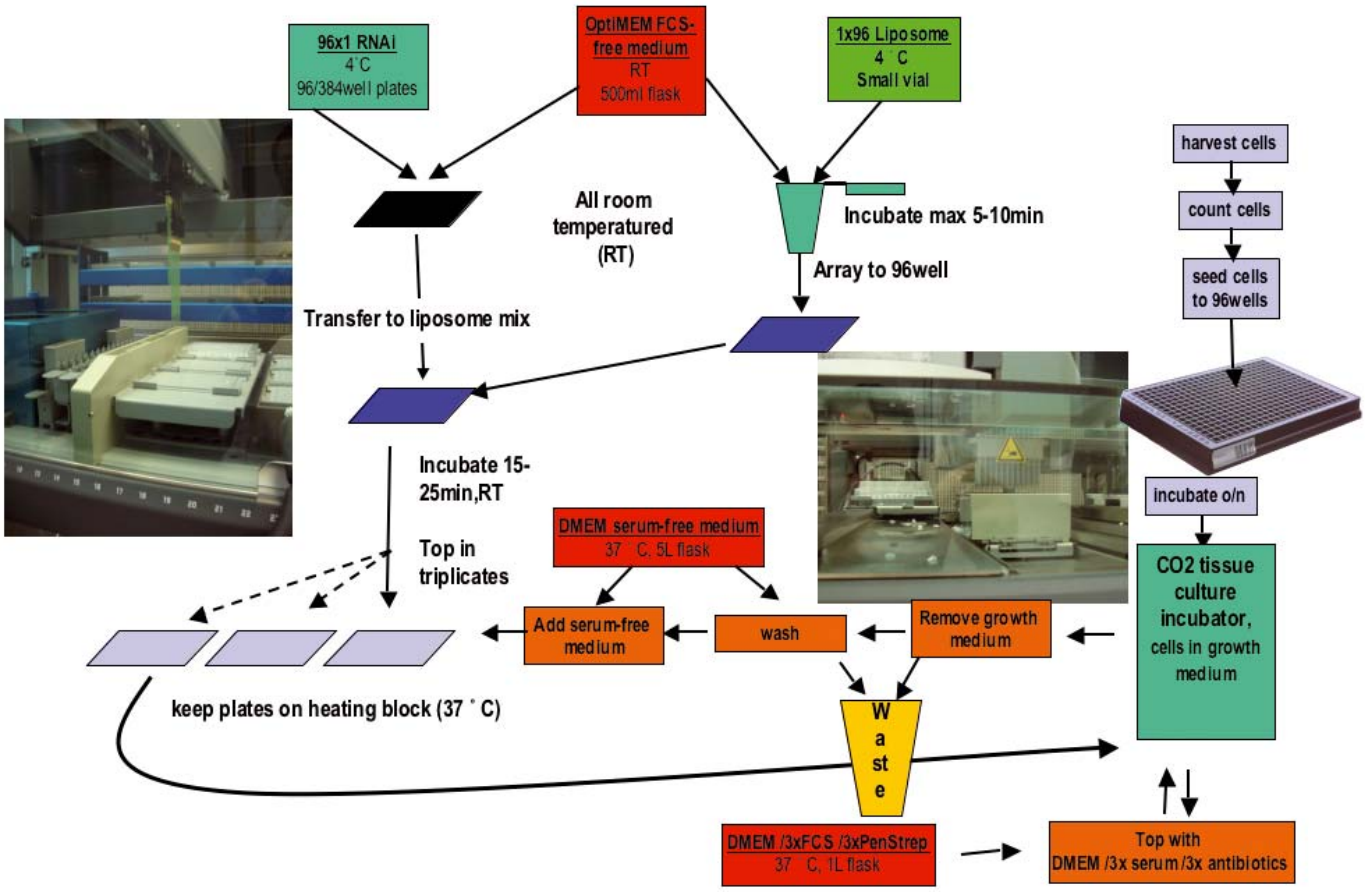

Figure 3: Transfection workflow[3]

Transfection in high throughput procedures

The next important step for this newly created library is automated transfection into human cancer cells. Positively charged liposomes are used to insert nucleotides of any type into the cells, compressing and "packaging" the nucleotide strands, helping to penetrate the cell membranes. The entire transfection process is standardized as far as possible, the CASY® cell counter (Schärfe System GmbH, Reutlingen, Germany) ensures a consistent way of counting, and variable distribution patterns resulting from pipette angle and other human errors are avoided by automated cell seeding (WellMate® Dispenser, MATRIX Corp., Hudson, USA).

Again, the Freedom EVO system is used to automate other stages of the transfection process (figure 3, reproduced with permission from reference 3, figure 3). The transfection unit is completely housed in a S2 safety cabinet (BDK Luft- und Reinraumtechnik, Sonnenbühl-Genkingen, Germany) to protect from external contamination (such as fungal spores, yeast or bacteria) during transfection and to allow working with genetically engineered viral vector-based RNAi libraries at biosafety level S2. Improvements of the shown workflow allowed a throughput of up to 30,000 samples in triplicate (90,000 in total) within 12 hours of pure robot time.

This genome-wide RNA interference 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 which enable our screening department to conduct gene characterization projects of this sort at a high throughput rate.

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References

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