APPLICATION NOTE

Automating the construction & maintenance of PAC & cDNA libraries

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The Institute of Cancer Research (ICR) is actively supporting genomics research in a range of human cancers. The automation of library preparation is helping to accelerate this research so enhancing its potential to discover the genes involved in causing cancers.

The Need for Automation

Automation within the ICR was driven by a need for higher throughput and improved quality results. The automation of library preparation has been tested using the Flexys® system (**Genomic Solutions Ltd.**, Huntingdon, UK). This system has been seen to play an important role in the construction and maintenance of PAC and cDNA libraries by speeding up the processes of picking and gridding out libraries. This system takes just 20 hours to pick 10,000 clones generated by a cDNA library coupled with an accuracy of typically better than 99% — a scale of work that would be virtually impossible to perform manually. Automated handling improves the level of accuracy by reducing the degree of human error inevitable with such repetitive manual picking and gridding tasks.

Library Preparation

The construction of PAC and cDNA libraries involved subcloning and cDNA synthesis followed by picking and gridding (*Figure 1*). Human DNA was subcloned into PACs using the methods defined by Shepherd *et al.*[1], while for a shotgun PAC library, the methods of Smedly *et al.*[2] were used. For PAC libraries, a total of 200 bioassay plates were generated, each containing 1000 clones, and for the shotgun libraries, two bioassay plates or eight 100-mm Petri dishes were produced. A cDNA synthesis kit (**Stratagene**, La Jolla, CA) was used to make cDNA[3]. A total of 10 bioassay plates were generated for the cDNA libraries, each containing approximately 1000 clones.

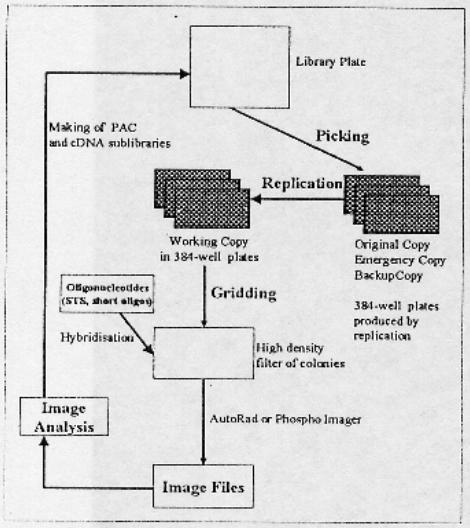

Fig. 1 An automated approach to the generation and maintenance of PAC and cDNA libraries.

Picking into 384-well Plates

Colony picking was controlled by Flexys Picker Software (Version 2.03). The Flexys picked from bioassay plates into 384-well plates (*Figure 2*) using six distinct image-processing steps: image acquisition, smoothing, edge detection, size selection, circularity and grey level selection (*Figure 3*). Following the image processing steps, coordinates were passed onto the six-pin head controller, which physically picked the colonies.

Parameters used for image processing were:

- Smoothing: 5 pixels
- Edge detection: 5 threshold of 5 pixels
- Size selection: min 200, max 2000 microns
- Circularity: 3 pixels
- Grey level: min 30, max 220 pixels

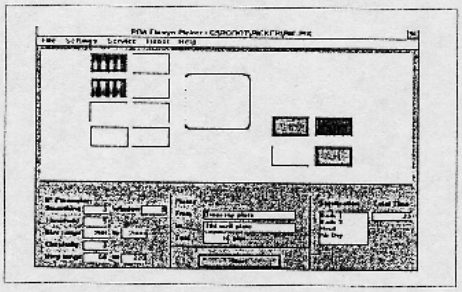

Fig. 2 Main template for picking from bioassay plates into 384-well plates.

Optimal parameters for sterilisation (including gridding) were:

- Sonication: 5 seconds
- Ethanol bath: 10 seconds
- Heater: 5 seconds
- Air dry: 5 seconds

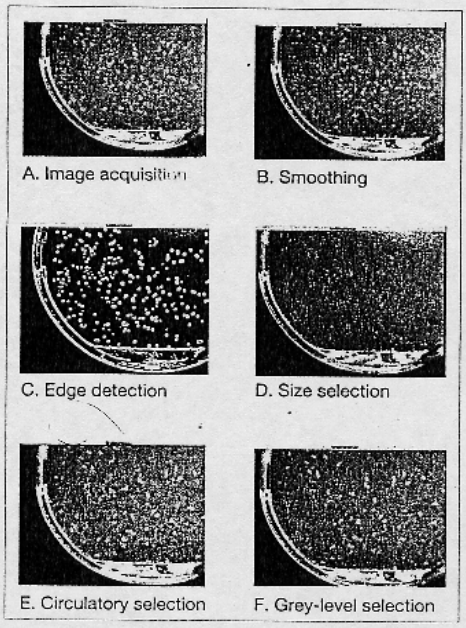

Fig. 3 (A) Image acquisition — this involves grabbing the plate image onto the CCD of the video camera. (B) Smoothing — this involves applying a mathematical 3 x 3-unit matrix to smooth out any image abnormalities such as jagged edges. (C) Edge detection (colony finding) — this involves applying a mathematical matrix such as the Laplacian 5 x 5 matrix together with pixel thresholding to eliminate false positive edges. (D) Size Selection — this involves applying a minimum and maximum size of colonies. (E) Circularity and grey level selection — this involves applying a shape descriptor algorithm to determine the circularity of the detected colony. A value of one indicates a uniform circle while the higher the value, the less circular the selected colony is. (F) Grey level selection — this involves applying a grey level pixel thresholding algorithm to the image. Pixel colour (grey level) is represented by a numerical value between -255 (black) and 255 (white) for eight-bit pixel representation. Therefore, by defining the low and high pixel thresholds, unwanted pixels (colonies) can be eliminated. For all figures, mainly white colonies are shown on a 100-mm Petri dish.

It took approximately 50 seconds to pick six colonies including a sterilisation step, 20 hours to pick 10,000 clones generated by a cDNA library and 400 hours to pick 200,000 clones generated by a PAC library without manual intervention. Using a 24-pin picking head will further increase throughput. A total of 520 384-well plates were generated from the PAC library and 26 384-well plates were generated from the cDNA library. Accuracy of picking was typically better than 99%, with 382 out of 384-wells showing culture growth. Experiments using the optimal sterilisation parameters indicated that clones were from isolated colonies with no cross contamination. The automatic system can also pick colonies from 150 mm Petri dishes and transport them into deep-well plates.

Colony Gridding

Gridding on filters and slides was performed using the Flexys Gridder Replicator Software (Version 2.02). This software defines a custom made pattern that allows easy cross referencing of the hybridisation results to corresponding 384-well plates without the need to use ink as a marker. Eight 384-well plates were gridded onto Hybond-N+ nitrocellulose filters (11.9 x 7.8 cm) with it taking just 30 minutes to grid eight copies of eight 384-well plates onto filters with only a single transfer.

Library Replication

When a library is generated it can only be used a maximum of eight times as viability decreases with each freeze/thaw cycle. To maintain library viability, it has to be replicated. Using the Version 2.02 software, each set of eight 384-well plates generated from the original picking stage were replicated onto three further sets of eight, fresh 384-well plates. The original copy of the library (constructed using picking) was replicated 24 plates at a time to make three copies of the original. Once a copy was depleted, another three copies were made to help maintain the viability of the library.

Optimal sterilisation parameters were:

Sonication:	• Not used because the pins were moving between cultures
Ethanol bath:	• 40 seconds
Heater:	• Not used because it kills some clones
Air dry:	•100 seconds to ensure ethanol had evaporated

This automated system can be used to reduce or increase the numbers of clones for screening by transferring them from a 384-well plate to 96-well plates, for example. The flexibility of the system means that it can also replicate libraries between standard 96- or 384-well plates to deep well microplates.

Conclusions

The Flexys system produces improved performance compared to manual methods in the construction and maintenance of PAC and cDNA libraries. The system's effective sterilisation eliminates carry-over contamination problems and manual picking errors are eliminated. The system has an improved throughput compared to manual methods and has the added advantage that it is easily adapted to suit different microplate types. The Flexsys software can also produce custom gridding patterns to help maximise gridding efficiency.

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References

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