Counting Chromosome Copy Numbers Efficiently

DigC Is Designed to Analyze CNV in a Single Cell

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The analysis of copy numbers of genetic sequences is of fundamental importance in biology and medicine, and Digital Counting (DigC) is a new method to determine copy numbers at the single-cell level. Researchers at Olympus' (www.advalytix.com) Advalytix division have applied DigC to detect Trisomy 21 (Down syndrome) from single human fibroblasts.

DigC, a PCR-based method, is an alternative to FISH-based testing. It integrates with a molecular approach to creating single-cell populations from imperfectly enriched samples. This method has broad applications for noninvasive prenatal testing and other clinical tests that require the determination of copy numbers from single cells such as the detection of deletions or insertions that occur in tumors like retinoblastoma and lung cancer.

DigC counts the number of genetic copies contained in single cells, especially in the clinically important 0–5 range. The method is related to digital PCR in that the DNA sample is diluted and aliquoted into compartments such that, on average, there is less than one copy per compartment before PCRs are run in each compartment. That way, the traditional qPCR approach of quantifying the copy number of a genetic sequence from an analog amount of amplified material is converted into adding up the number of positive, digital PCR results from each compartment.

The key difference between DigC and digital PCR is that DigC starts with a single cell, while digital PCR starts with extracted DNA from a larger, unknown number of cells. This means that DigC requires few parallel reactions and results in a plain copy number, not a ratio from which a copy number can be inferred. A key requirement for the accuracy and efficiency of DigC is a sensitive amplification directly from single cells with a low drop-out rate. The platform used in this experiment to deliver the requisite low drop-out rate consists of Advalytix' AmpliGrid, a surface-structured glass slide with 48 individual 1 µl reaction sites, and AmpliSpeed, the thermal cycler for AmpliGrid slides.

Prenatal genetic testing (PGT) refers to all the techniques that screen or diagnose fetal genetic defects. PGTs fall into two groups: invasive tests such as amniocentesis and chorionic villus sampling and noninvasive tests based either on cell-free DNA or RNA or the direct analysis of fetal cells in maternal circulation (CFC). Given the medical risks associated with invasive PGTs, a lot of attention is focused on the development of noninvasive tests.

Overcoming Hurdles

The challenges that have to be resolved to bring a noninvasive PGT to market fall into two categories: the enrichment of fetal cells in maternal samples and the genetic analysis of a small number of fetal cells available in enriched samples that may also contain maternal background cells.

While recent advances have improved enrichment purity, consistently achieving 100% pure fetal cell samples remains a challenge. In order to produce 100% pure fetal cell samples from enriched samples that may contain up to 95% maternal cells, the Advalytix team is developing a "molecular last mile" that uses a variation of standard forensic identification techniques to precisely identify fetal cells. Using this

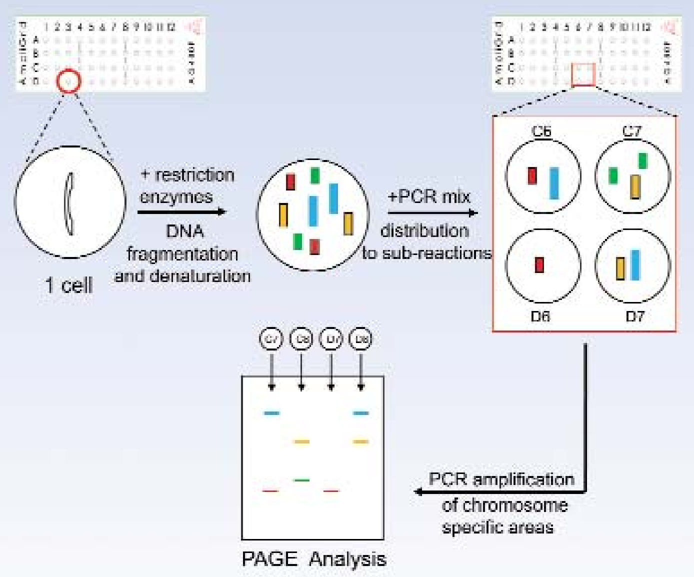

Figure 1. Description of the DigC workflow: One single cell is deposited on the reaction site, chromosomes are cut by specific restriction enzymes, and then denatured. PCR reaction mix is added, and the complete volume is distributed into subreactions. A specific multiplex PCR reaction is run, and products are analyzed by PAGE.

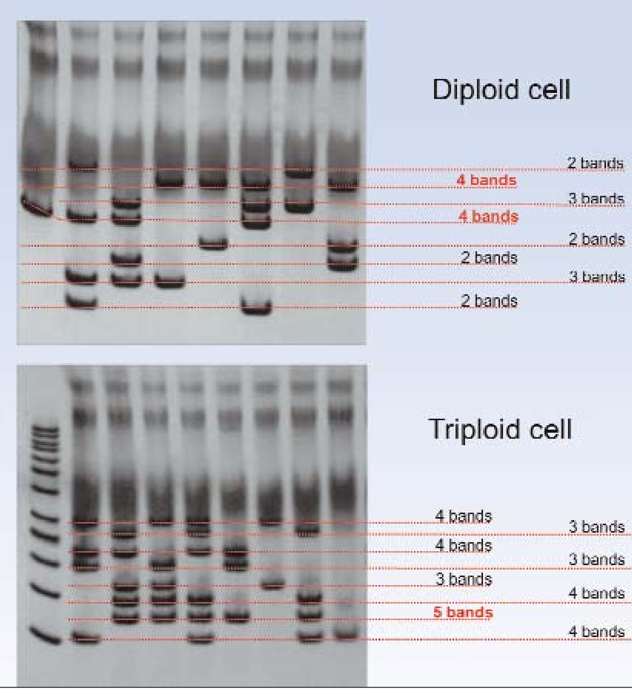

Figure 2. PAGE analysis 8-plex PCR reaction of diploid and triploid single cells: One single diploid cell was analyzed by PAGE after DigC. The maximum number of identical fragments detected is four, indicating a diploid genome (upper panel). The same analysis was run on a single triploid cell. In this case the maximum number of identical fragments detected is five, which indicates a triploid genome (lower panel).

method, 100% pure fetal samples will be available for DigC analysis.

How DigC Works

Using flow sorting or micromanipulation, cells are placed one-by-one on the AmpliGrid slide's reaction sites and analyzed by DigC. Figure 1 depicts a stable (nondividing) haploid cell such as a sperm cell that contains one set of chromosomes with one chromatid each. Restriction enzymes are pipetted onto the single cell to fragment its chromosomes. Then the AmpliSpeed thermocycler heats up the AmpliGrid slide to denature the DNA. This produces two single-stranded DNA templates from each sequence, depicted as red, yellow, blue, and green fragments. A 4-plex PCR reaction mix is added and the entire volume is aliquoted into four subreactions across the AmpliGrid.

The 4-plex PCR, specific for the red, yellow, blue, and green fragments, is run and then the products are analyzed by gel or capillary electrophoresis. Since the chromatid of the haploid cell has been denatured into two strands, studying the illustrative gel bands in Figure 1 should show 0, 1, or 2 bands of each color:

- 0 if none of the fragments were amplified correctly (they were drop-outs)
- 1 band, if either there was a drop-out or both colors happened to end up in the same reaction site
- 2 bands, if both reactions worked and the fragments took part in separate reactions.

Note, then, that if all of the bands corresponding to a color across all four subreactions are added up and more than two bands are obtained, there has to be more than one chromosome present in the cell. The chromosome corresponding to the surplus color must have been aneuploid. The inverse, however, is not true; observing two or fewer bands does not rule out aneuploidy. A drop-out of a surplus fragment would cause a false declaration of the corresponding cell as normal. This explains why an extremely low drop-out rate is required for accurate tests based on DigC.

Figure 2 shows actual results from analyzing stable (i.e., nondividing) diploid and triploid fibroblasts from a human cell line. An 8-plex reaction was used; that is eight independent sequences located on chromosome 21 were redundantly amplified and the reaction mix across eight subreactions was divided (one gel band per reaction).

A stable diploid cell has two copies of chromosome 21 with one chromatid each, that means that after denaturing researchers expect to see a maximum sum of four copies of each genetic fragment across all eight reaction sites (Figure 2, upper gel image). If more are seen, then the cell must have been triploid. This is exactly what is observed in Figure 2 (lower gel image); the second to last band occurs five times. Thus the corresponding chromosome is diagnosed triploid.

The stochastic probability of a false negative such as not finding five or six bands for a triploid cell is 20% for this 8-plex/8 sub-PCR setup. In an analysis of 40 triploid human fibroblast cells, a false negative rate of 23% was observed, very close to the stochastic prediction.

Experimental false negative rates are expected to be slightly higher than stochastic predictions due to a small amount of drop-outs. Increasing the degree of multiplex and/or number of subreactions quickly reduces the expected false negative rate. The same 8-plex reaction spread across 12 sub-PCRs theoretically achieves <2% false negatives; increasing the multiplex degree to 24 and spreading across eight sub-reactions predicts a false negative rate <1%.

DigC has been successfully applied to Trisomy 21 detection and can be extended to diagnose other genetic disorders. By varying the multiplex grade and the number of reaction sites, test accuracy can be engineered to a wide range of specifications. GEN

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