Micro-Scale Engineering –II
Lab-on-a-Chip for DNA/RNA Analysis

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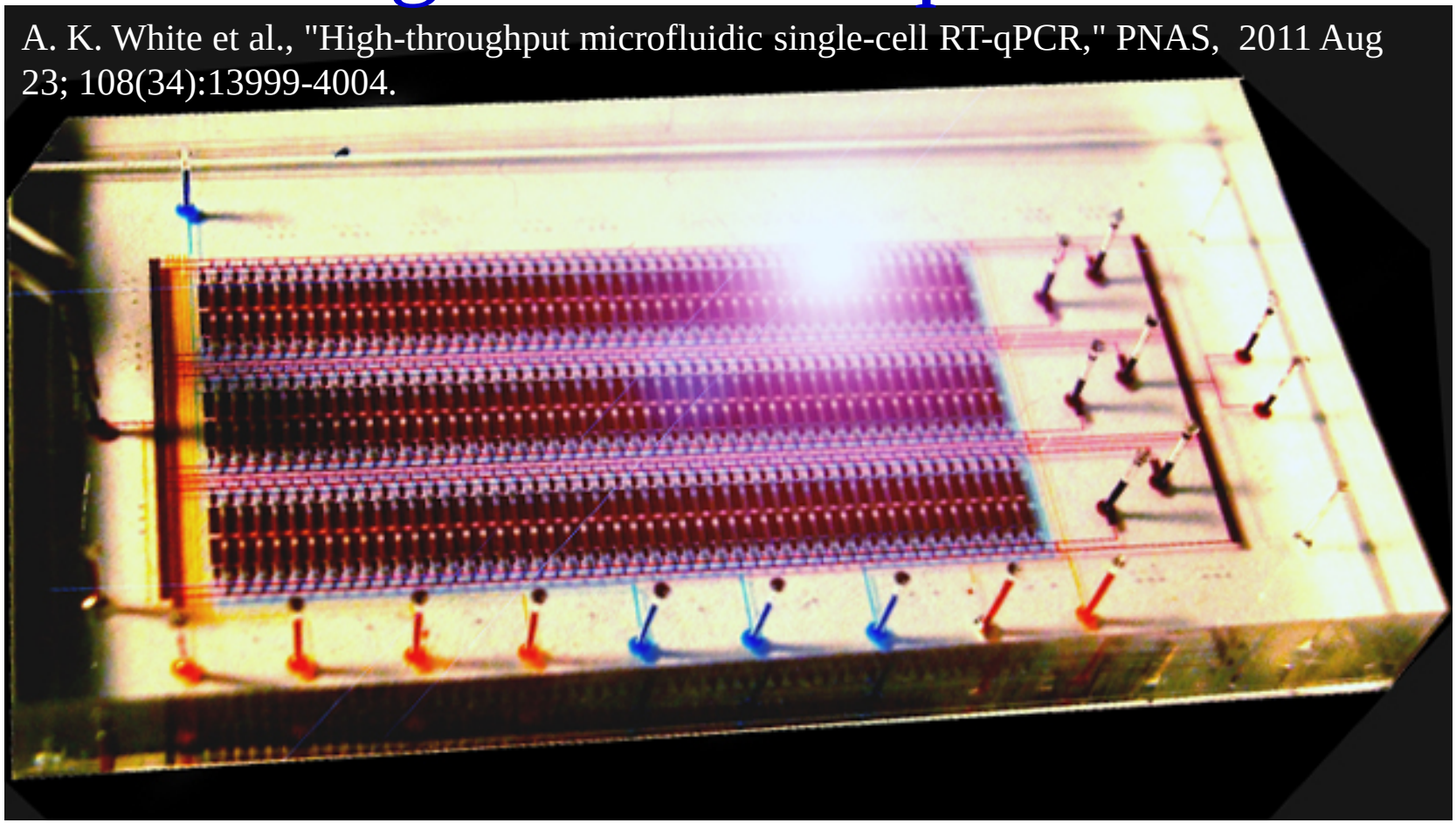
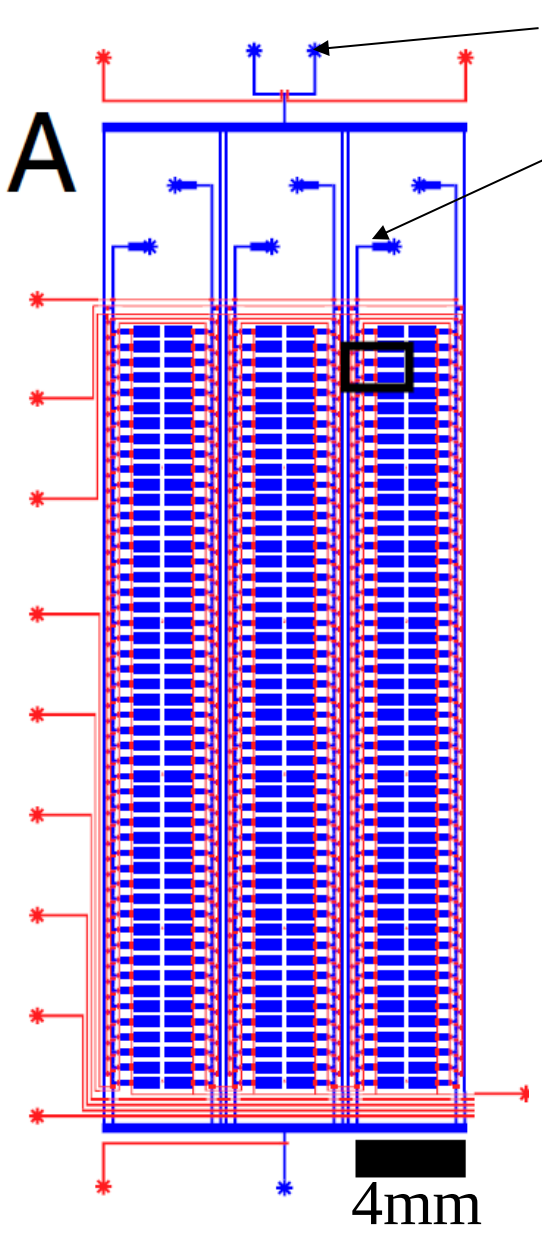
January 23, 2014

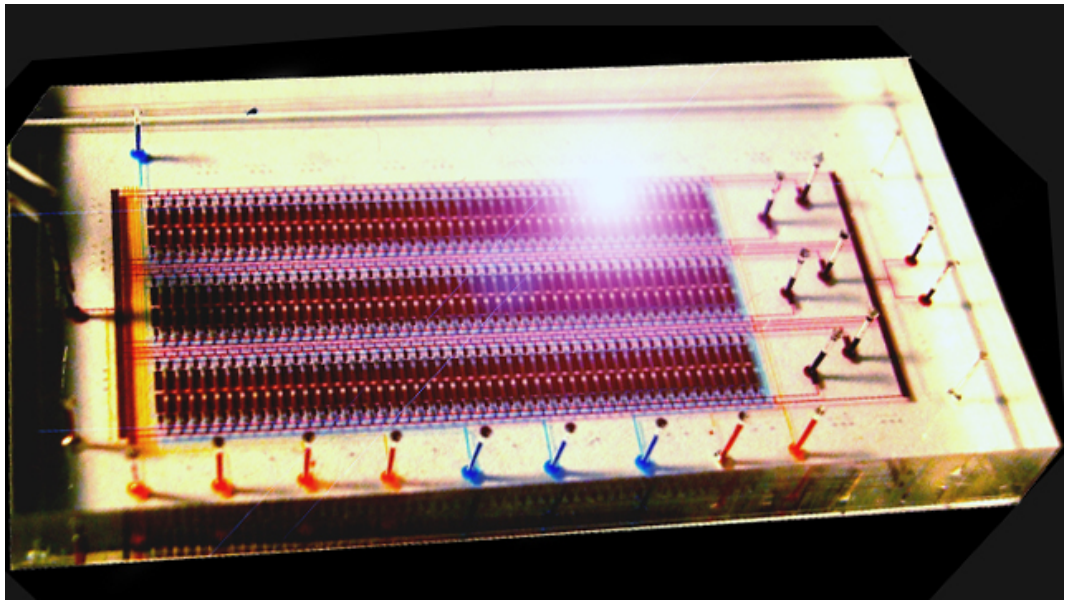
High-throughput microfluidic single-cell RT-qPCR

A. K. White et al., "High-throughput microfluidic single-cell RT-qPCR," PNAS, 2011 Aug 23; 108(34):13999-4004.



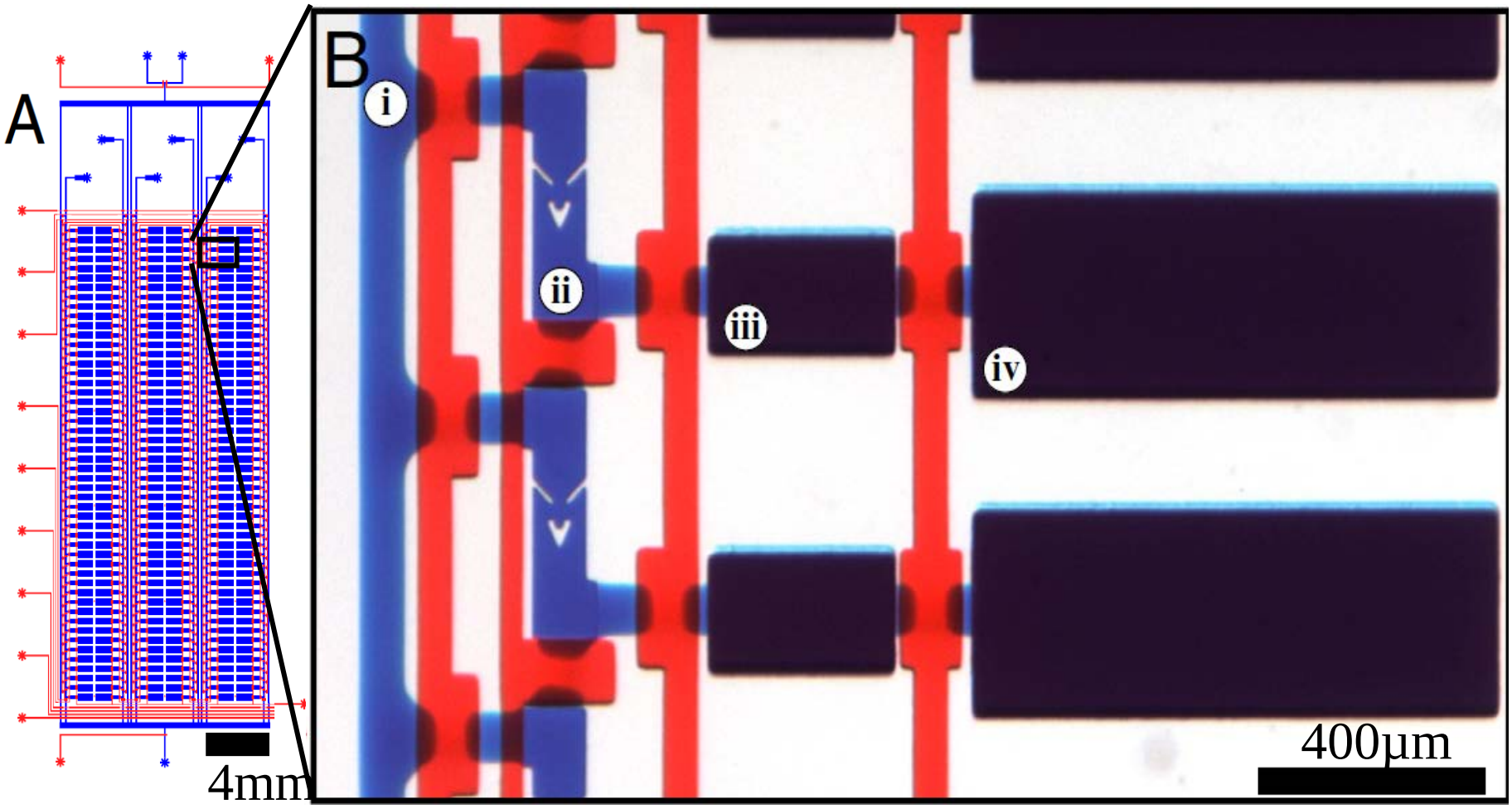
Microfluidic single-cell RT-qPCR





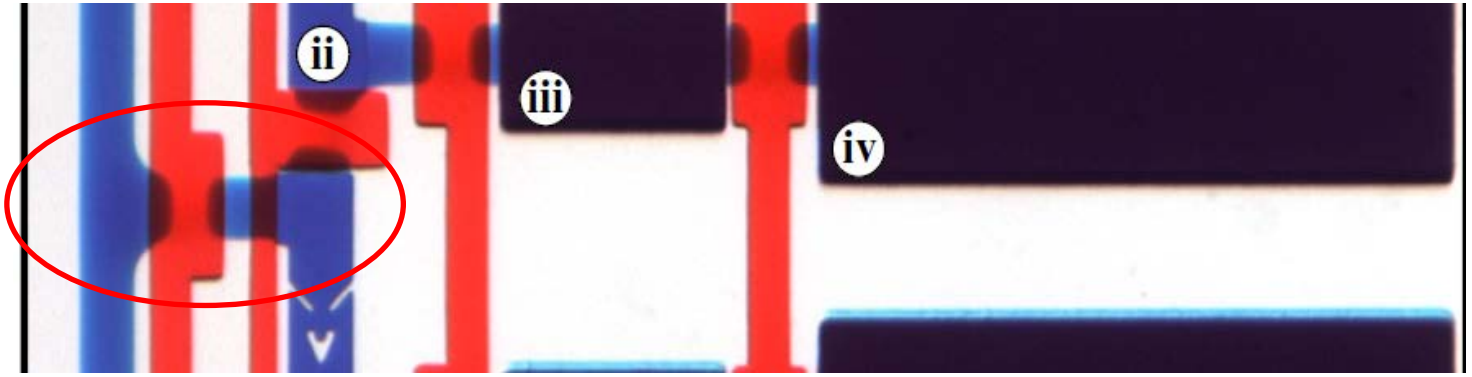
300 parallel RT-qPCR assays and executes all steps of single-cell capture, lysis, reverse transcription, and qPCR.

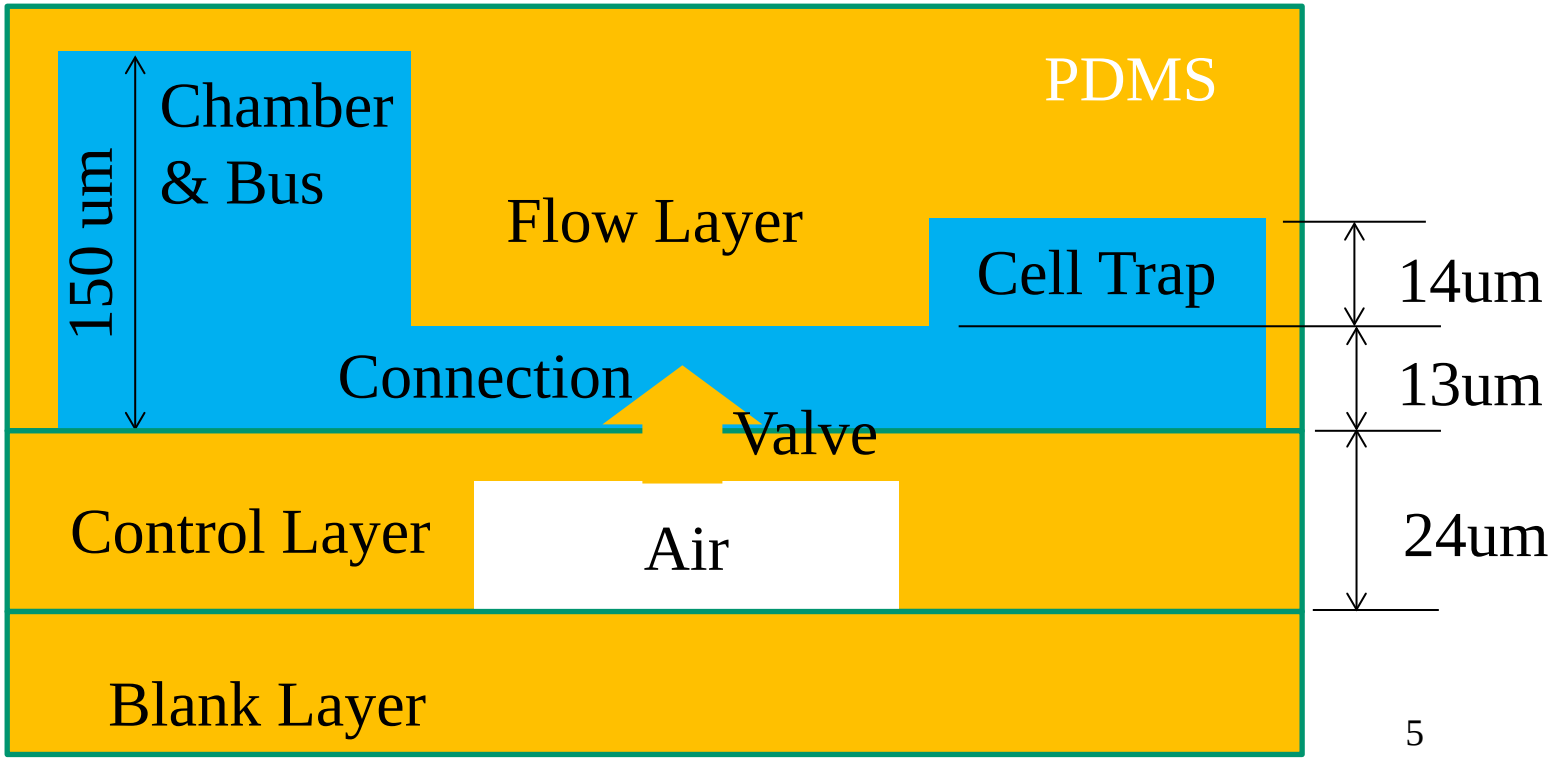
Cell Processing Unit



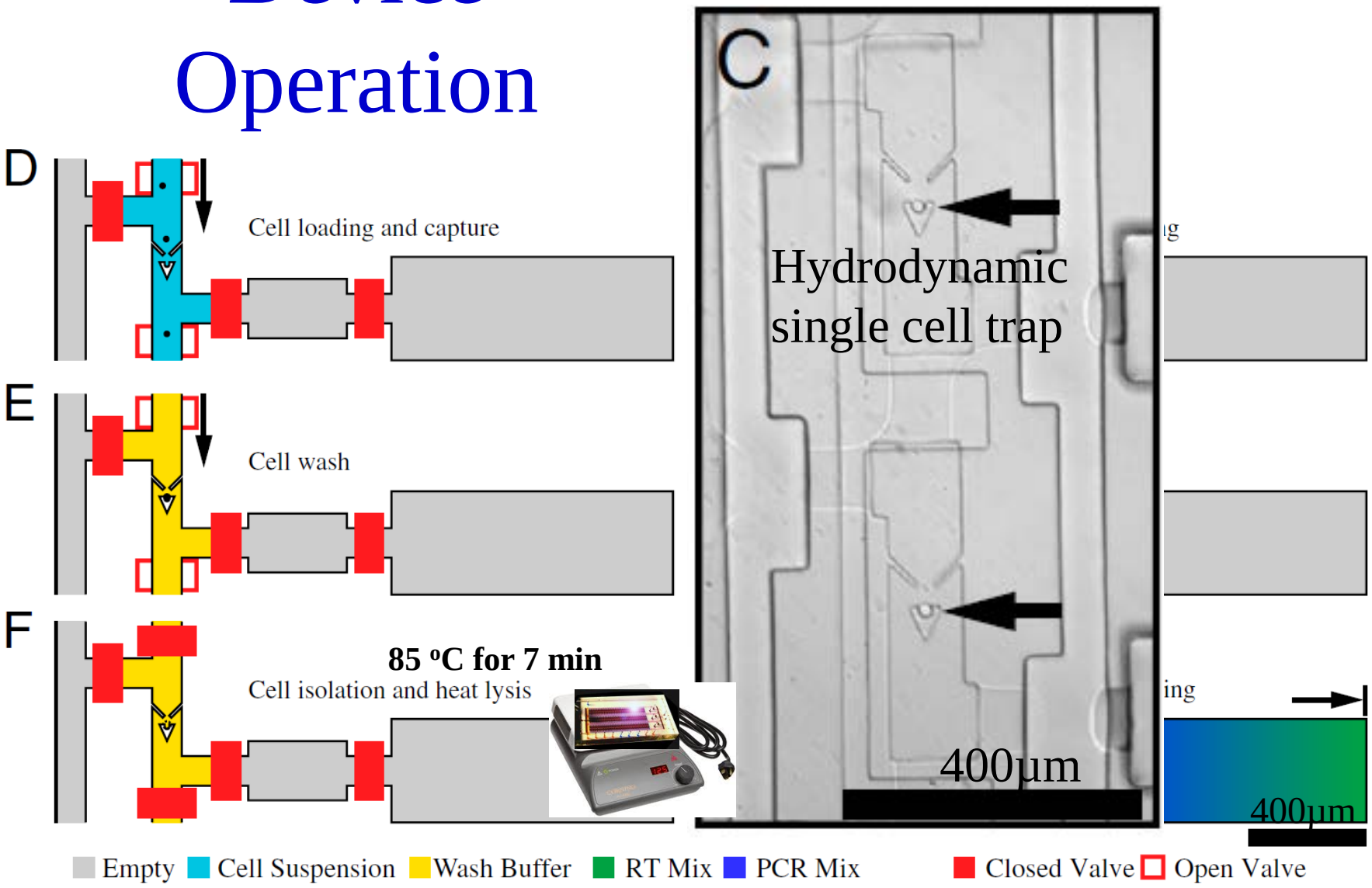
Each unit consists of (i) a reagent injection line, (ii) a 0.6-nL cell capture chamber with integrated cell traps, (iii) a 10-nL reverse transcription (RT) chamber, and (iv) a 50-nL PCR chamber.

Three Layers of the Device



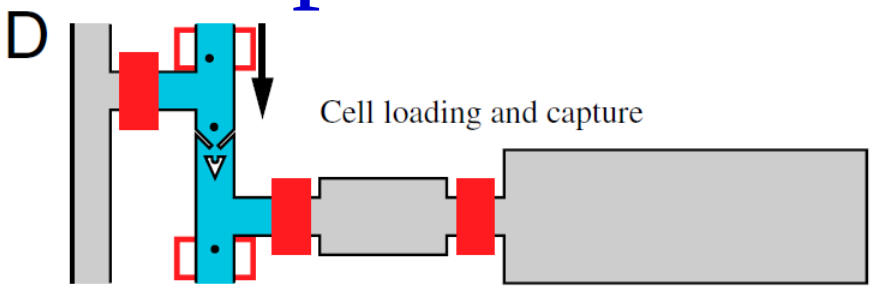


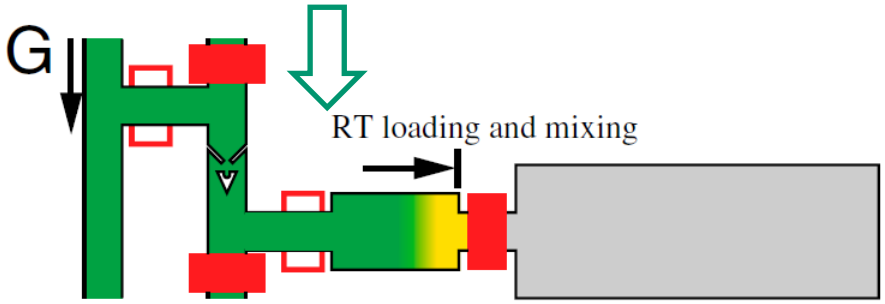
Device Operation

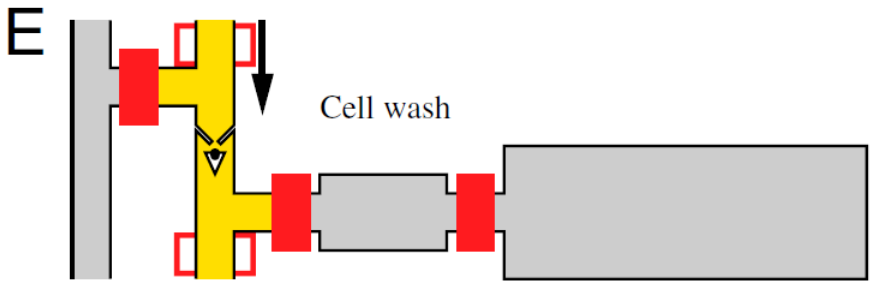


Device Operation

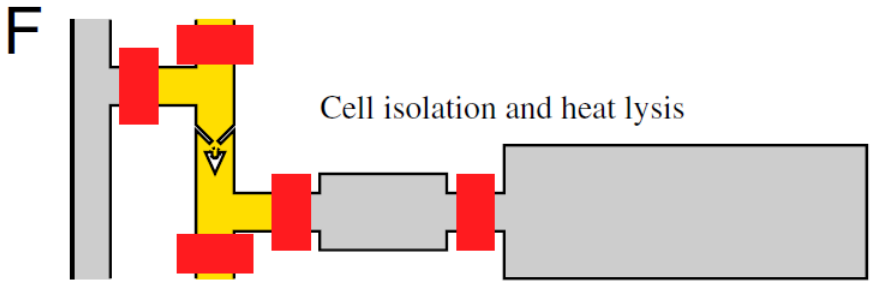
2 µL 10× Reverse Transcription Buffer, 4 µL 5× RTstem-loop miRNA primer from ABI, 1 µL 100mM dNTPs, 1.34 µL of 50 U⁄µL Multiscribe Reverse Transcriptase, 0.26 µL of 20 U⁄µL RNase Inhibitor, 2 µL 1% Tween 20, 9.4 µL PCR grade water.







A pulsed temperature RT protocol was carried out by placing the microfluidic device on a flatbed thermocycler (2 min at 16 °C, followed by 60 cycles of 30 s at 20 °C, 30 s at 42 °C, and 1 s at 50 °C). RT enzyme was inactivated at 85 °C (5 min), and then the device was cooled to 4 °C.

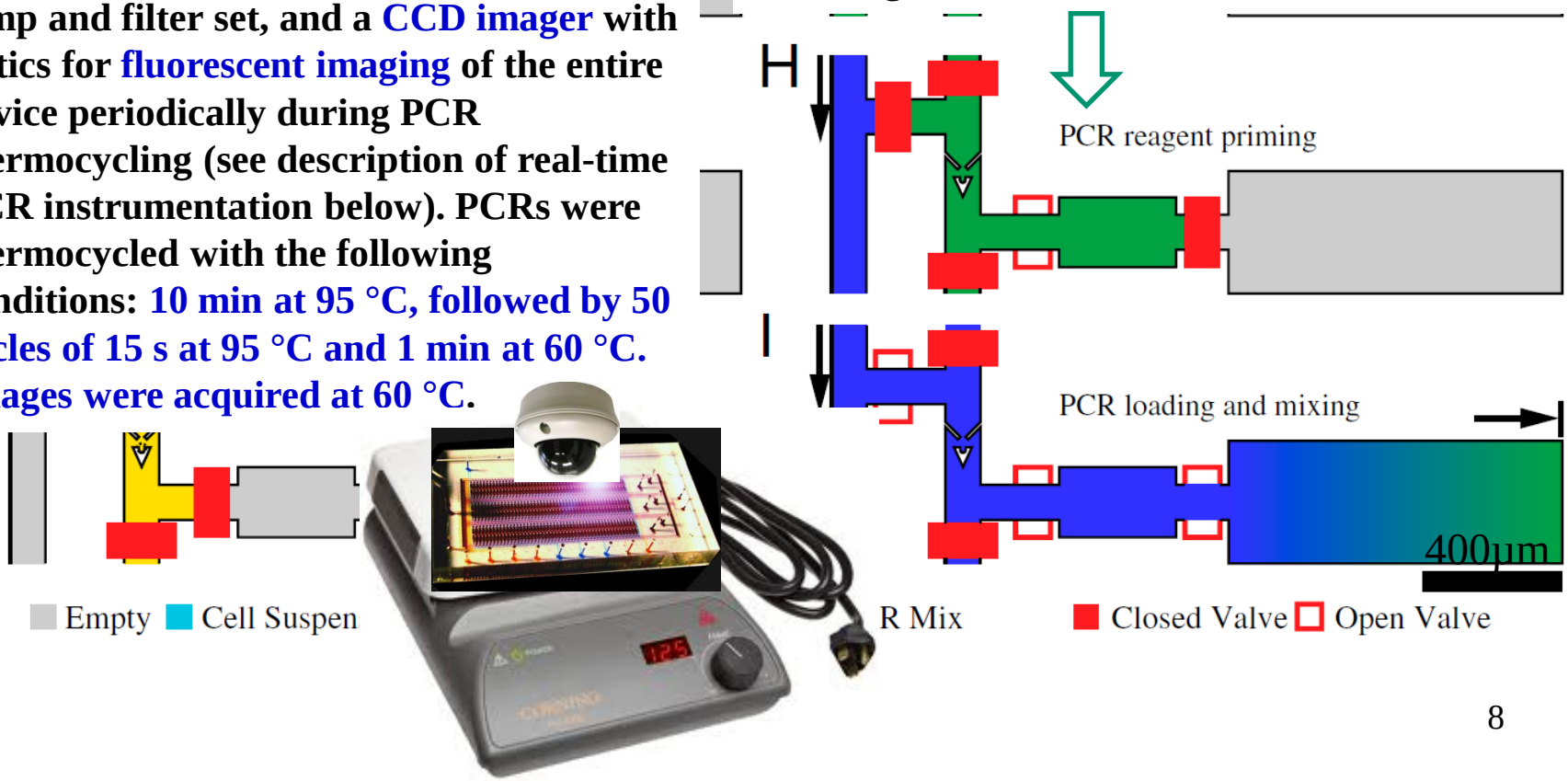


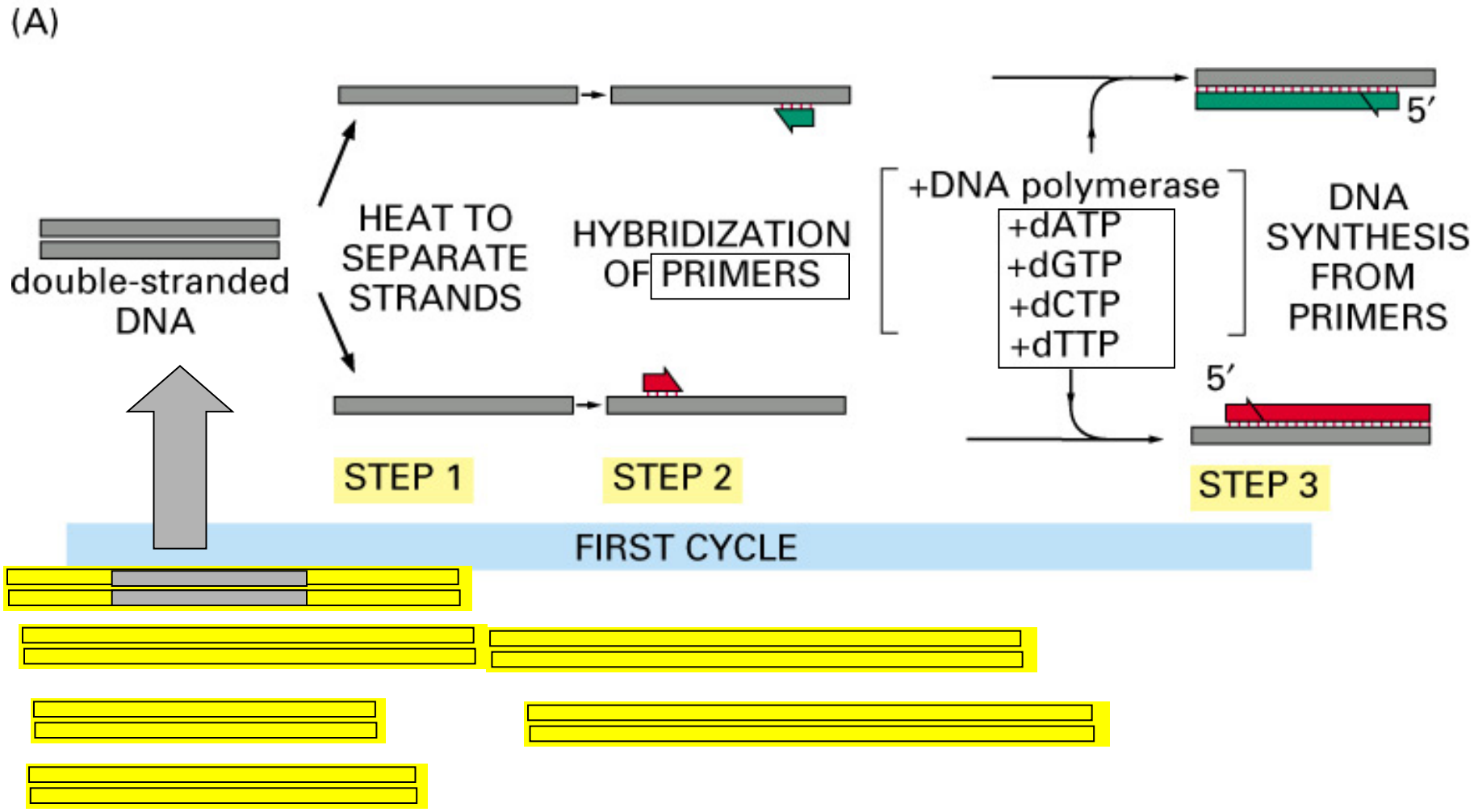
Empty Cell Suspension Wash Buffer RT Mix PC n Valve

Device Operation

The device was transferred to an enclosure for real-time PCR (Prototype version of Biomark™ Instrument, Fluidigm). The real-time PCR enclosure consists of a custom flatbed thermocycler, a xenon arc lamp and filter set, and a CCD imager with optics for fluorescent imaging of the entire device periodically during PCR thermocycling (see description of real-time PCR instrumentation below). PCRs were thermocycled with the following conditions: 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Images were acquired at 60 °C.

The PCR reagent was prepared with 25 µL of 2× TaqMan Universal Master Mix (ABI), 2.5 µL 20× Real-Time miRNA assays (primers and probe, ABI), 5 µL of 1% Tween 20, and 7.5 µL of PCR grade water.





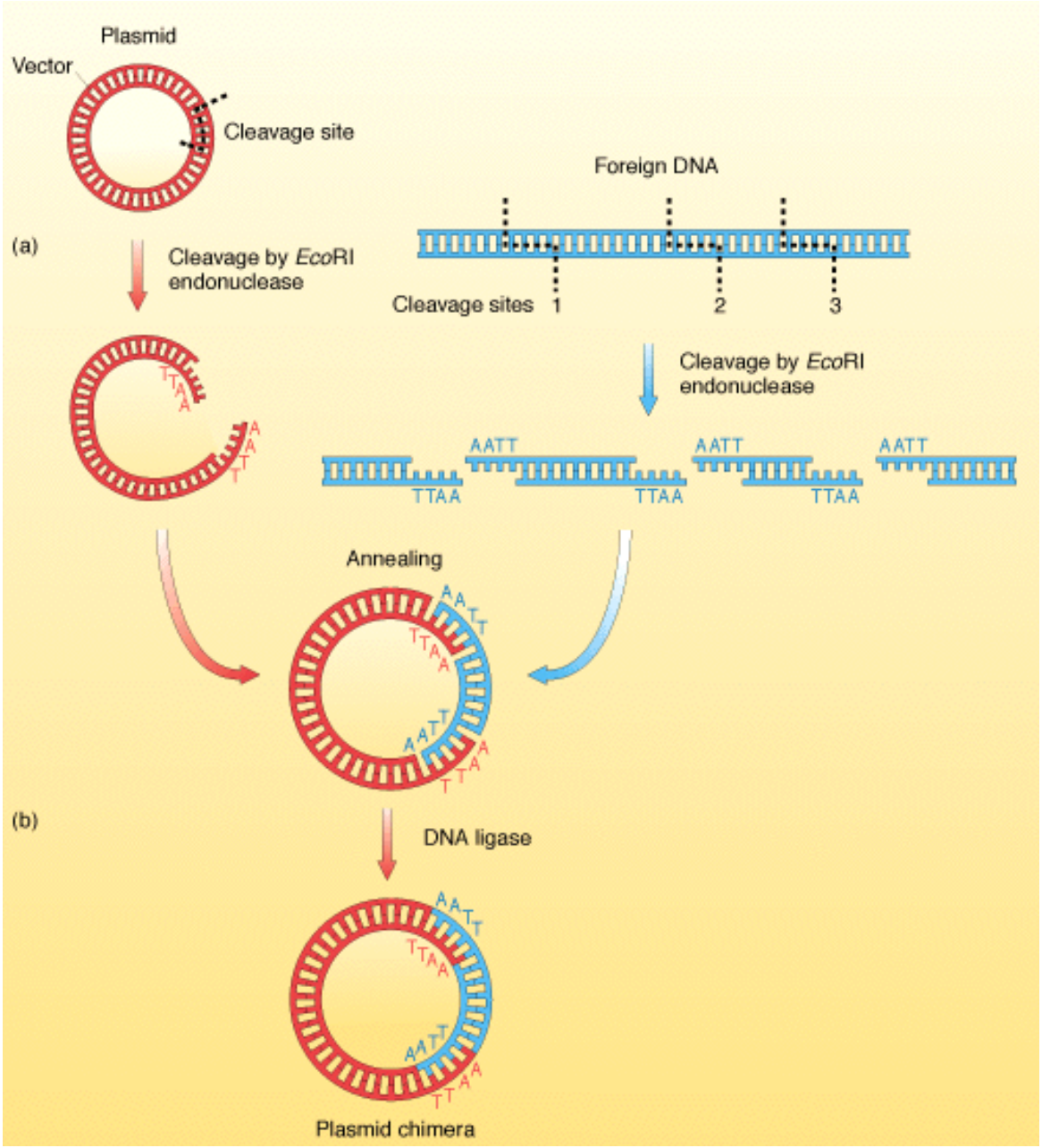
DNA fragments and oligonucleotides for Polymerase Chain Reaction (PCR)

Polyoma virus JC DNA detection by PCR

in CSF of HIV infected patients with suspected progressive multifocal leukoencephalopathy

CSF was obtained from lumbar puncture and stored at –70 °C until further analysis. CSF samples (500 µL) were thawed and centrifuged at 17,000 x g for 1 hr at 4 °C. The pellet and 180 µL of supernatant were mixed and digested with Proteinase K for 1 hr at 56 °C. **DNA was extracted** using a standard protocol that included phenol–choloform extraction and ethanol precipitation. The final pellet was resuspended in 50 µL of nuclease-free water. **Ten microliters** of each DNA extract was used in the PCR reaction using the **forward primer** AJ-1: 5'-A A AT G T T C C T C C A G T T C T-3' and the **reverse primer** A J - 2 : 5'- AT T C A C A AT G C T T T T C C C A – 3'. These primers amplify a fragment of **193 base pairs of the VP1 gene of J C V**. The cycling program consisted of **35 rounds of amplification**. Each amplification cycle consisted of denaturation for **30 sec at 94 °C, annealing for 30 sec at 55 °C, and elongation for 30 sec at 72 °C.** A final step of 72 °C was added. The PCR products were concentrated by ethanol precipitation, coated onto microplate wells, and **detected by hybridization with biotinylated probe** AJ-3 (5' biotin - C T T G AT G A AT T T G G T G T T G G G C – 3'). Absorbance was recorded in a microplate reader following an enzyme-mediated colorimetric reaction .

AMERICAN CLINICAL LABORATORY, November 2001



Restriction Nucleases

Bacteria
→ different enzymes
→ 4 to 8 nucleotides
→ to kill virus
→ methylation at an A or a C residue to protect itself.

Hundreds available.

Enzyme	Organism from which derived	Target sequence (cut at *) 5' -->3'
Ava I	Anabaena variabilis	C* C/T C G A/G G
Bam HI	Bacillus amyloliquefaciens	G* G A T C C
Bgl II	Bacillus globigii	A* G A T C T
Eco RI	Escherichia coli RY 13	G* A A T T C
Eco RII	Escherichia coli R245	* C C A/T G G
Hha I	Haemophilus haemolyticus	G C G * C
Hpa I	Haemophilus parainflenzae	G T T * A A C
Mbo I	Moraxella bovis	*G A T C
Pst I	Providencia stuartii	C T G C A * G
Sma I	Serratia marcescens	C C C * G G G
SstI	Streptomyces stanford	G A G C T * C
Sal I	Streptomyces albus G	G * T C G A C

- **http://www.firstmarket.com/cutter/cut2.html**

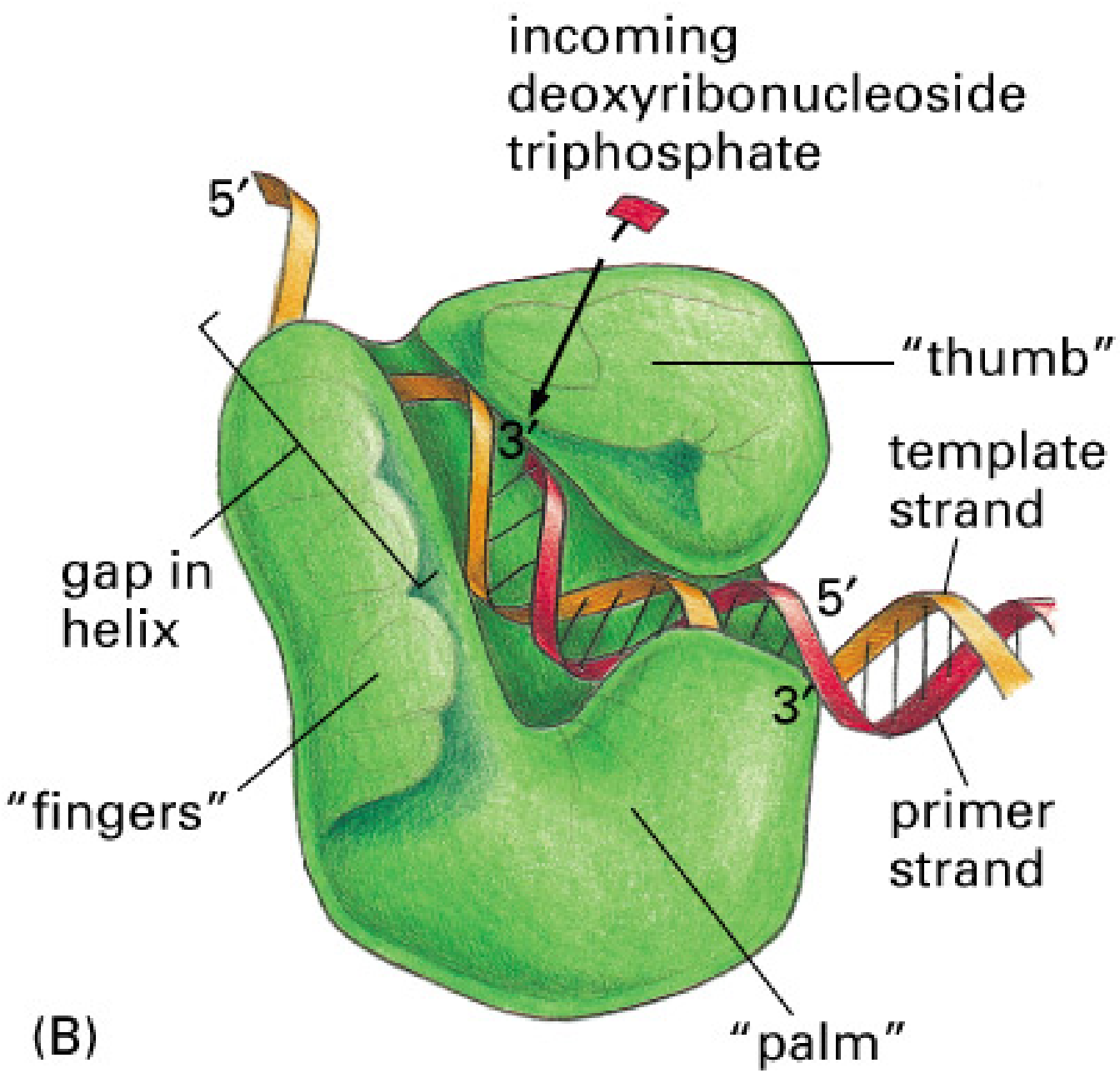
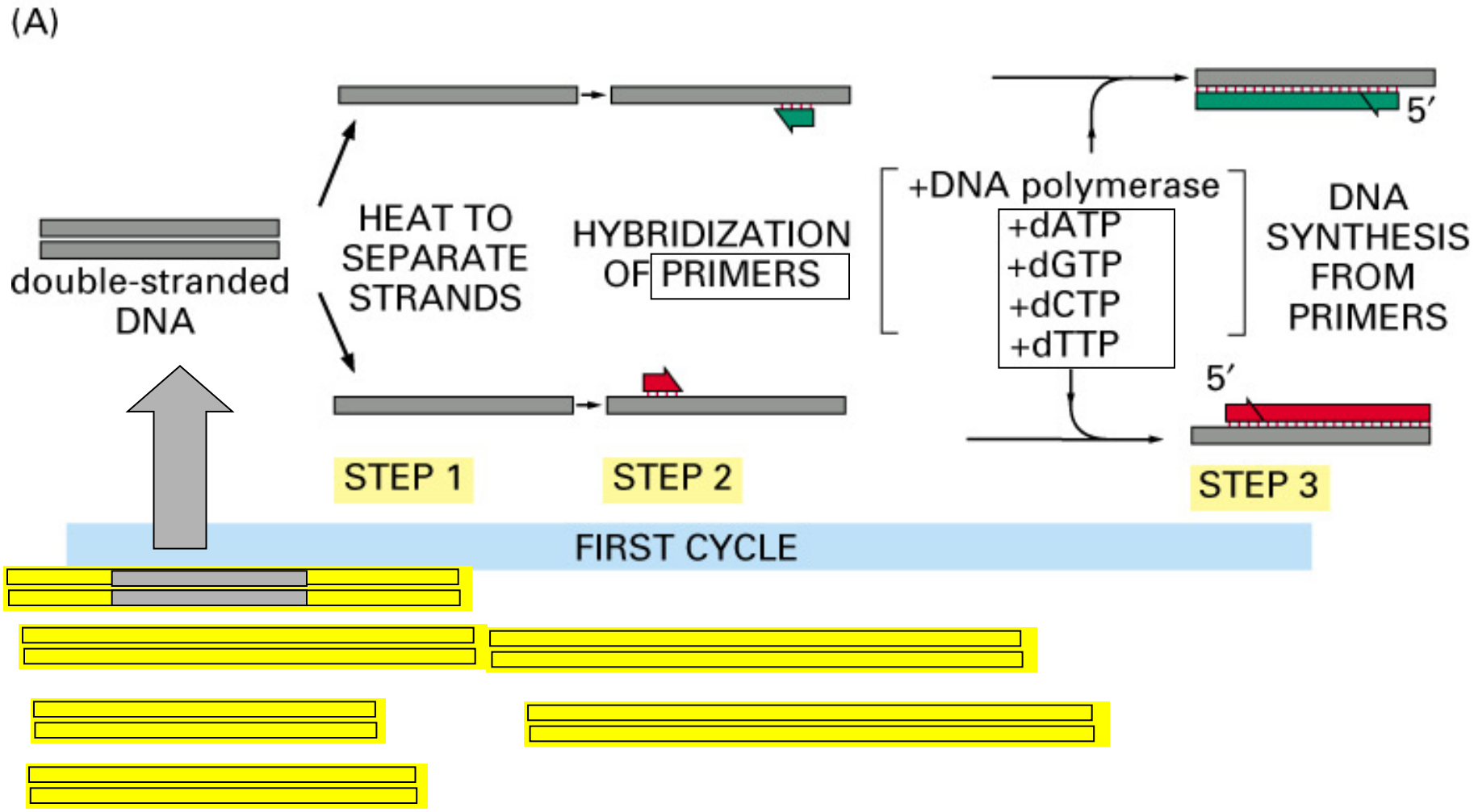
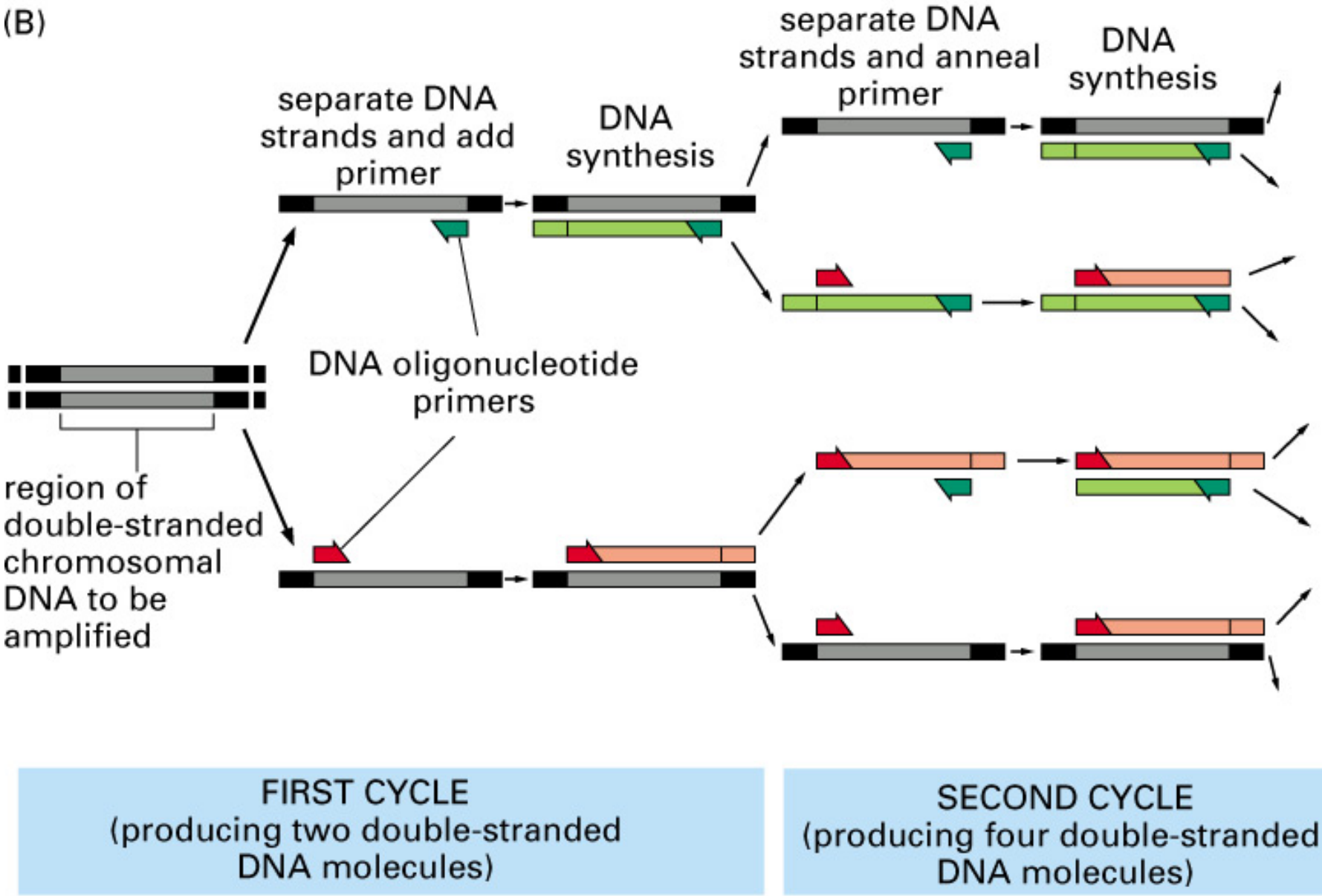


Figure 5–4

DNA Polymerase



DNA fragments and oligonucleotides for Polymerase Chain Reaction (PCR)



Polymerase Chain Reaction (PCR)

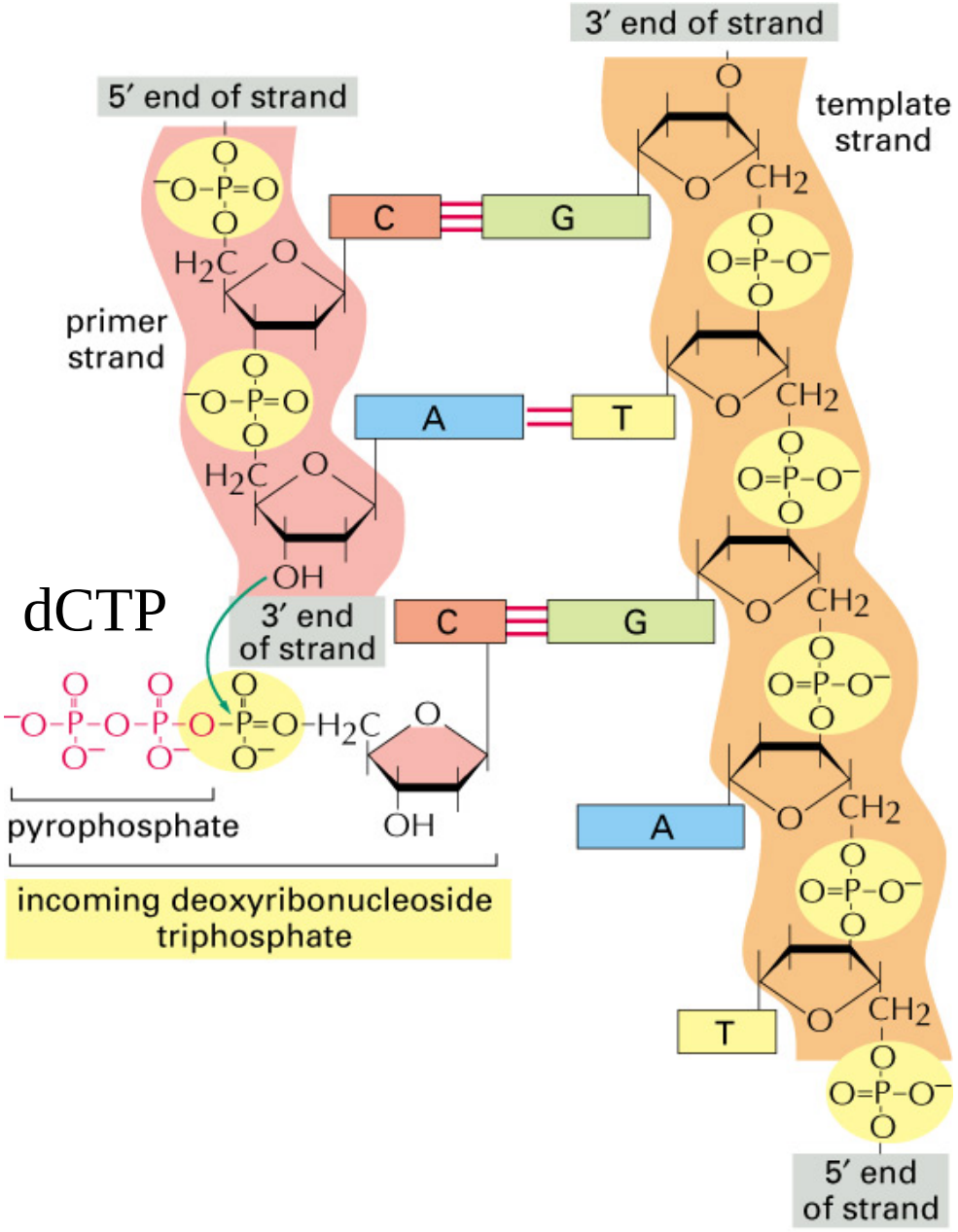


Figure 5–3. Molecular Biology of the Cell, 4th Edition.

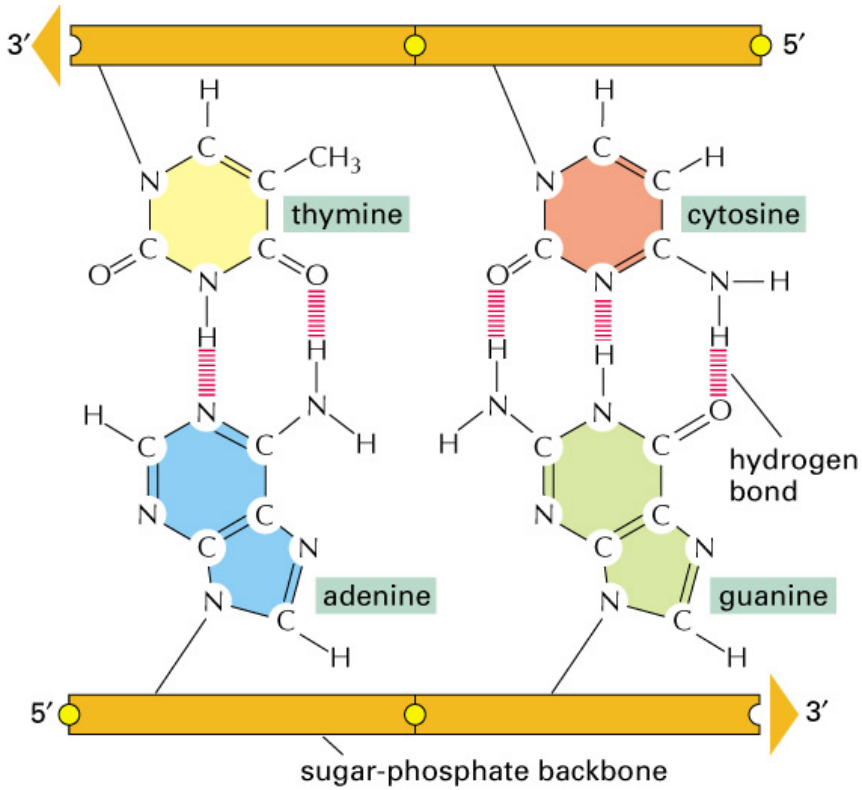
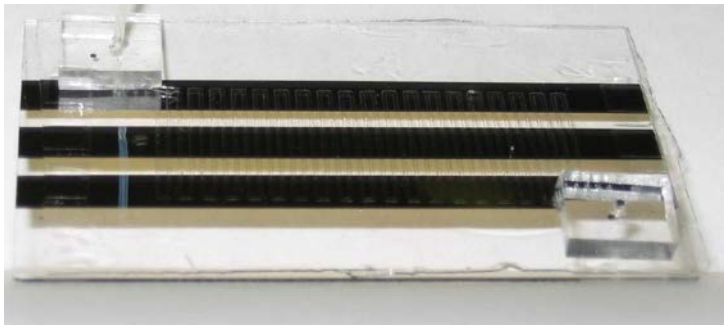
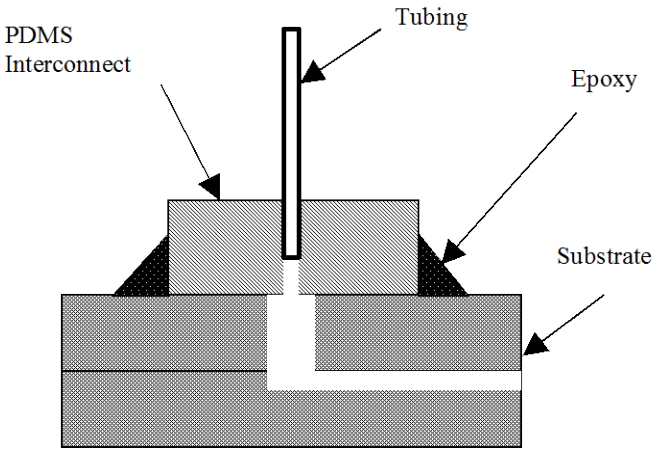


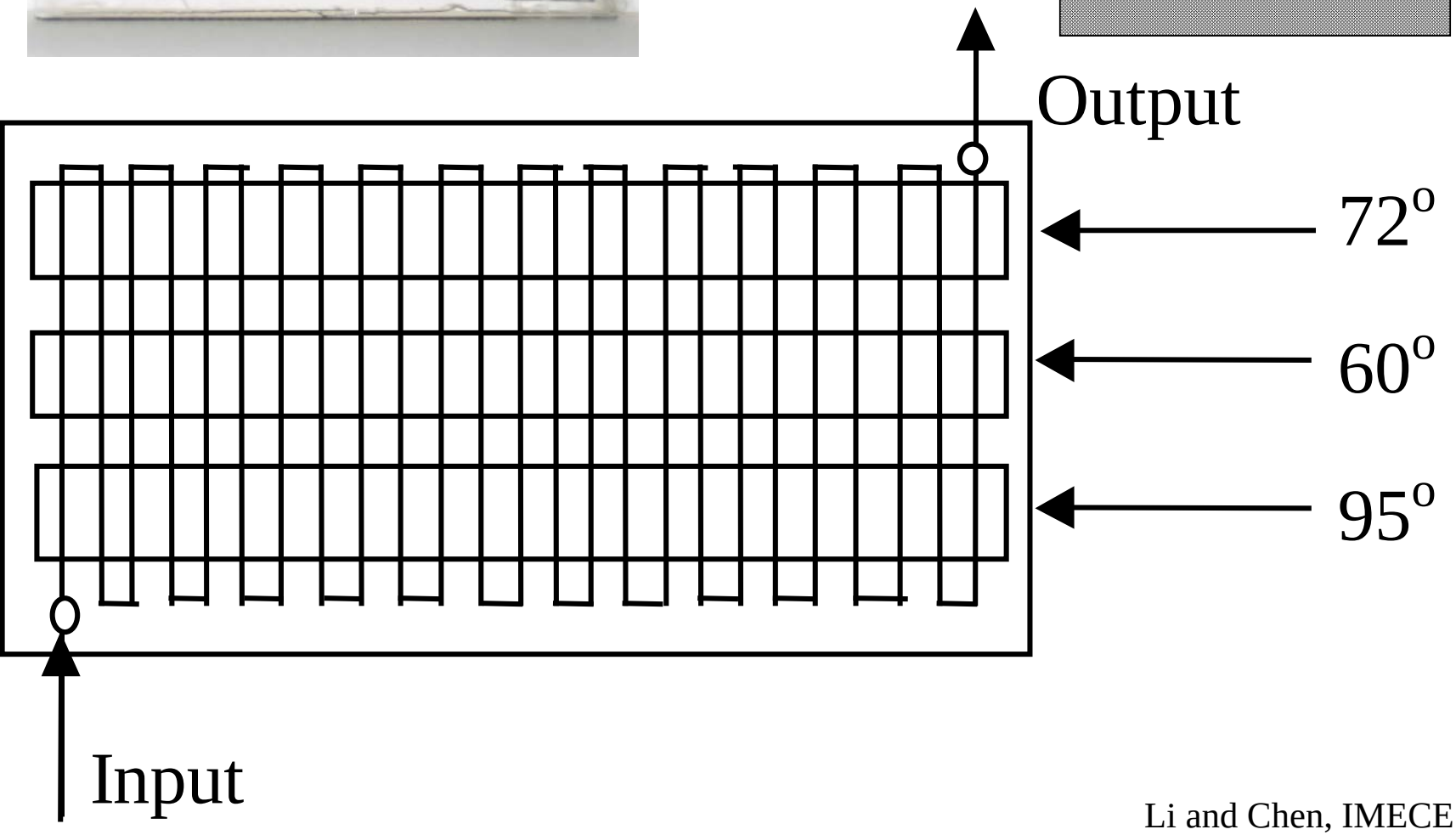
Figure 4–4. Molecular Biology of the Cell, 4th Edition.

dNTP: dATP, dCTP, dGTP and dTTP

PCR Chip

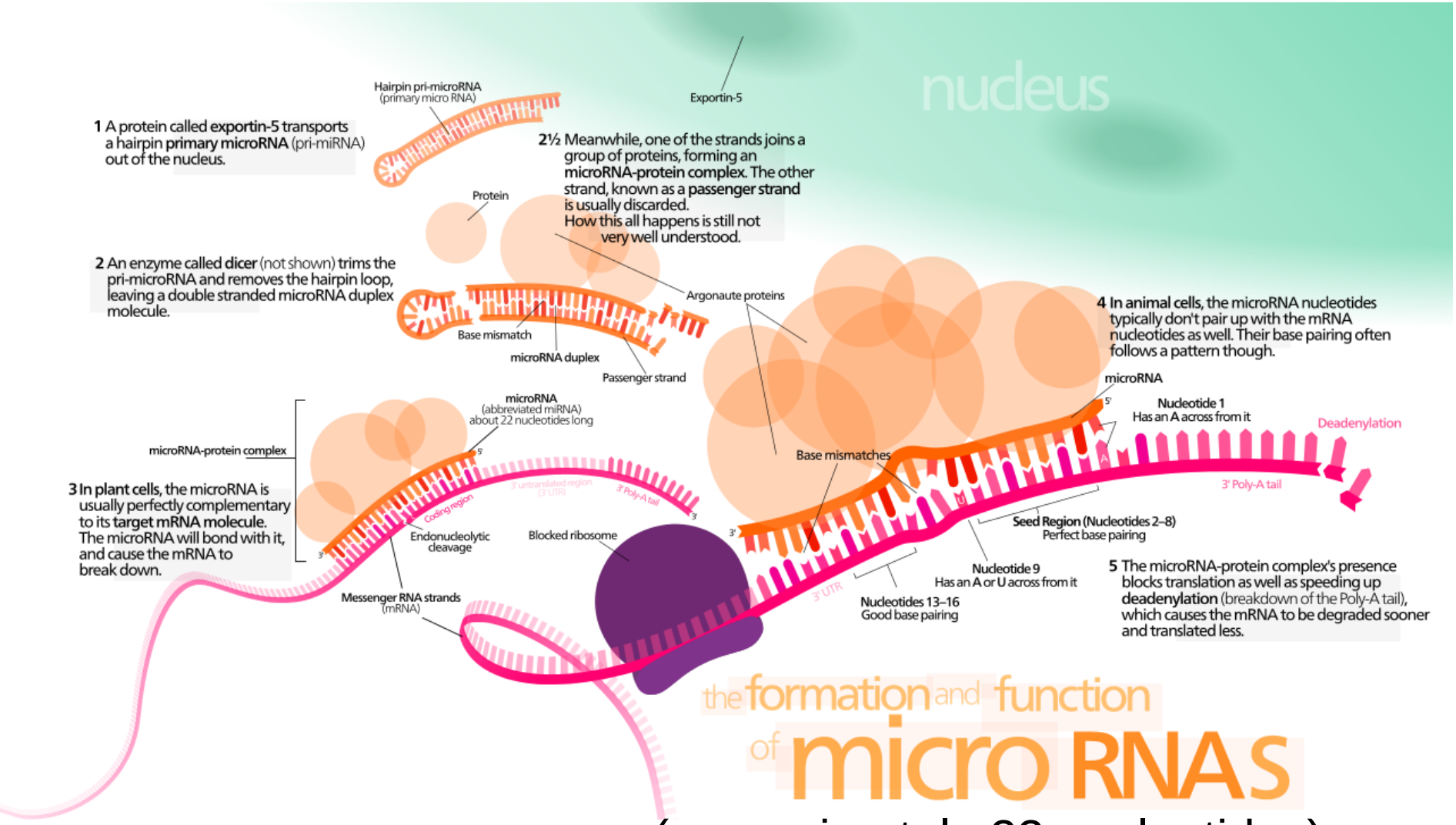




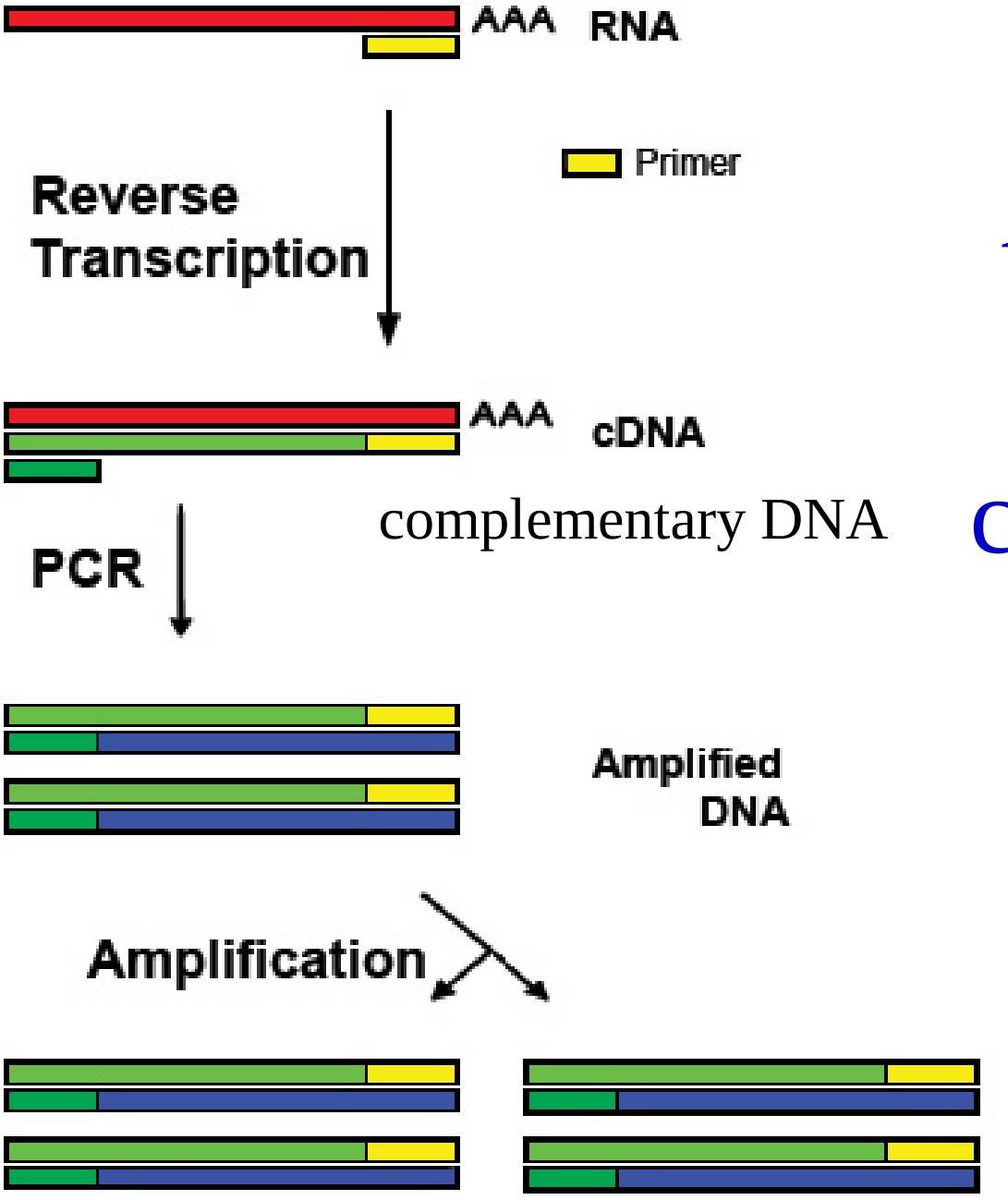


Li and Chen, IMECE 2002

MicroRNA: miRNA



(approximately 22 nucleotides)

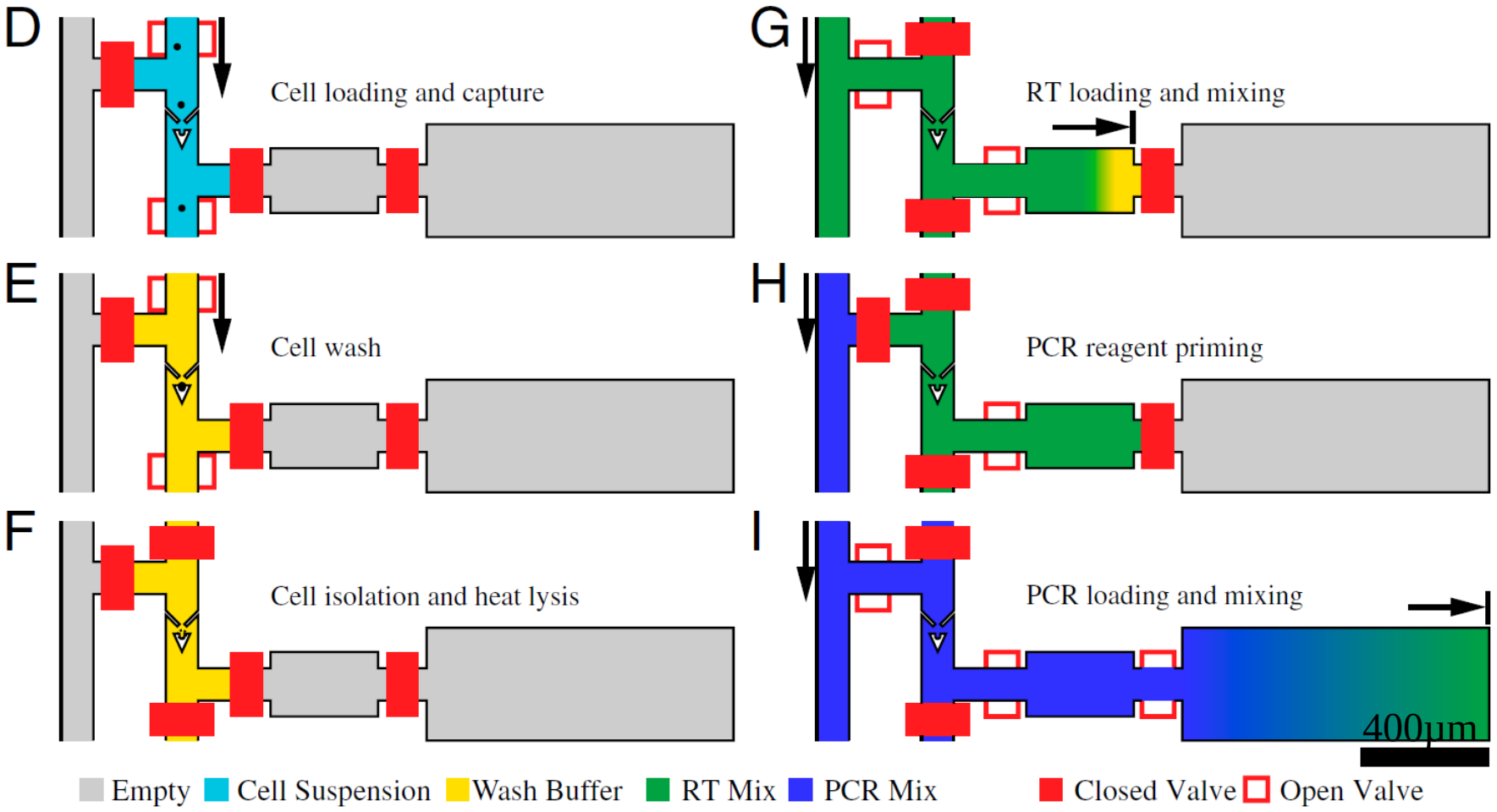


Reverse transcription polymerase chain reaction (RT-PCR)

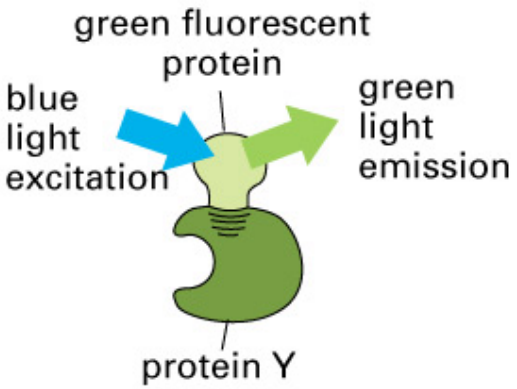
complementary DNA

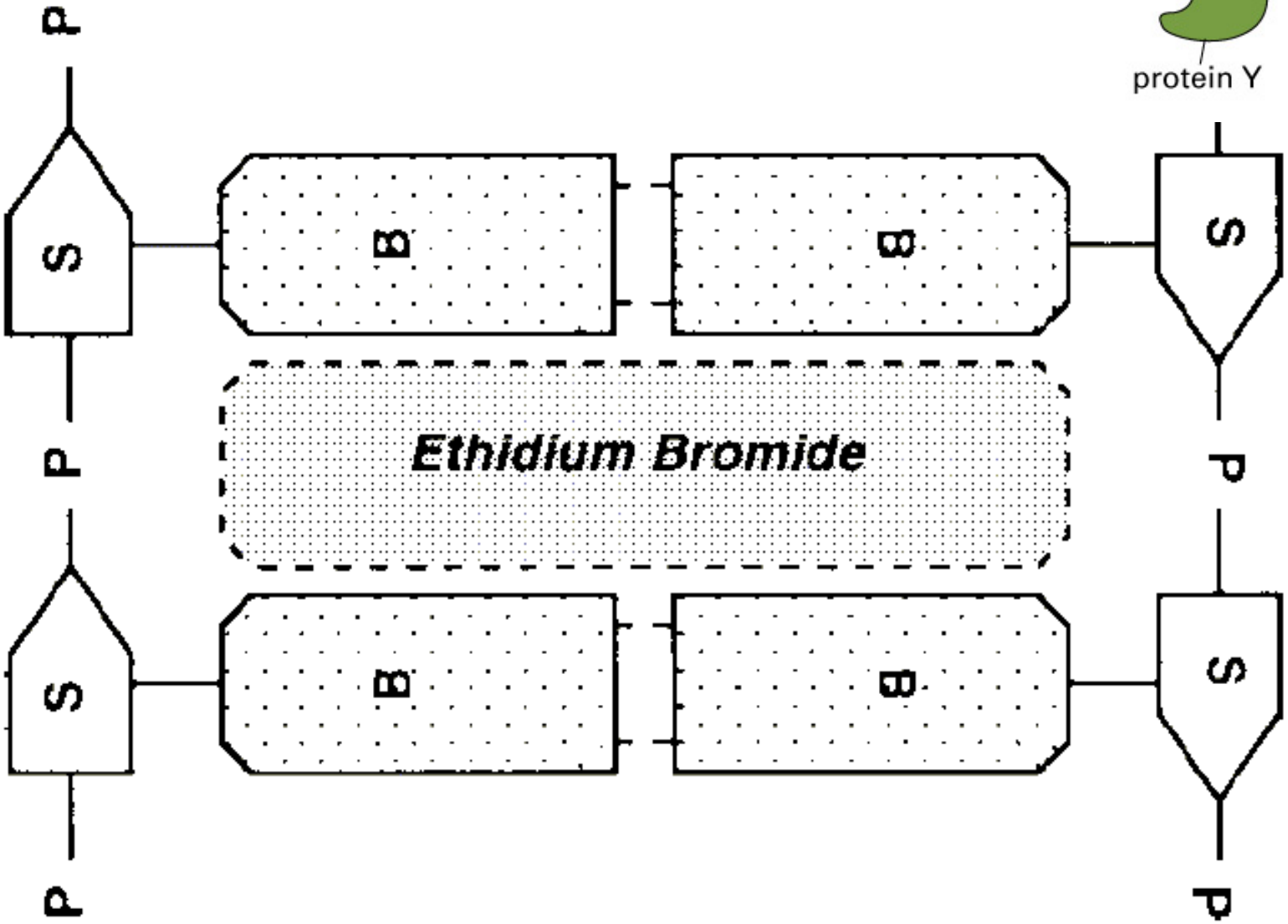
qPCR is referred to as quantitative PCR, quantitative real-time PCR, and real-time quantitative PCR.

Device Operation

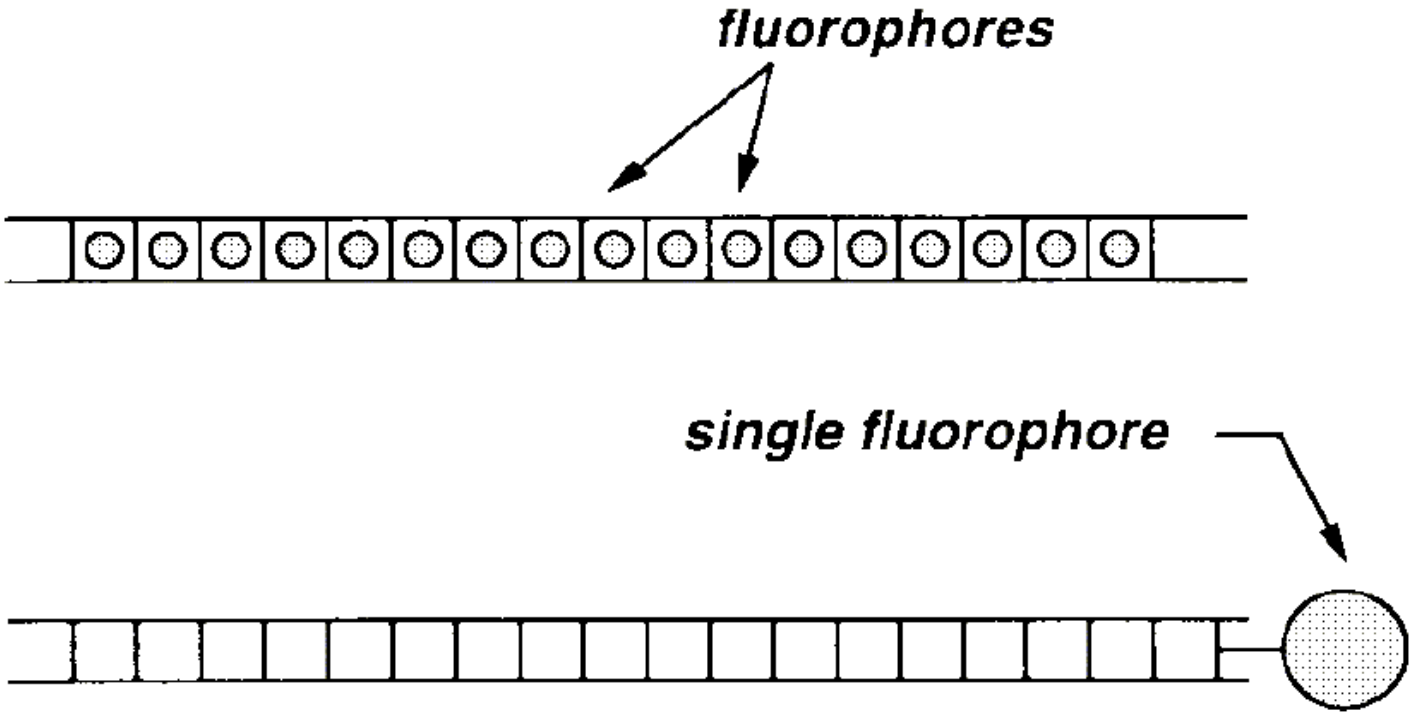


Intercalating Dyes



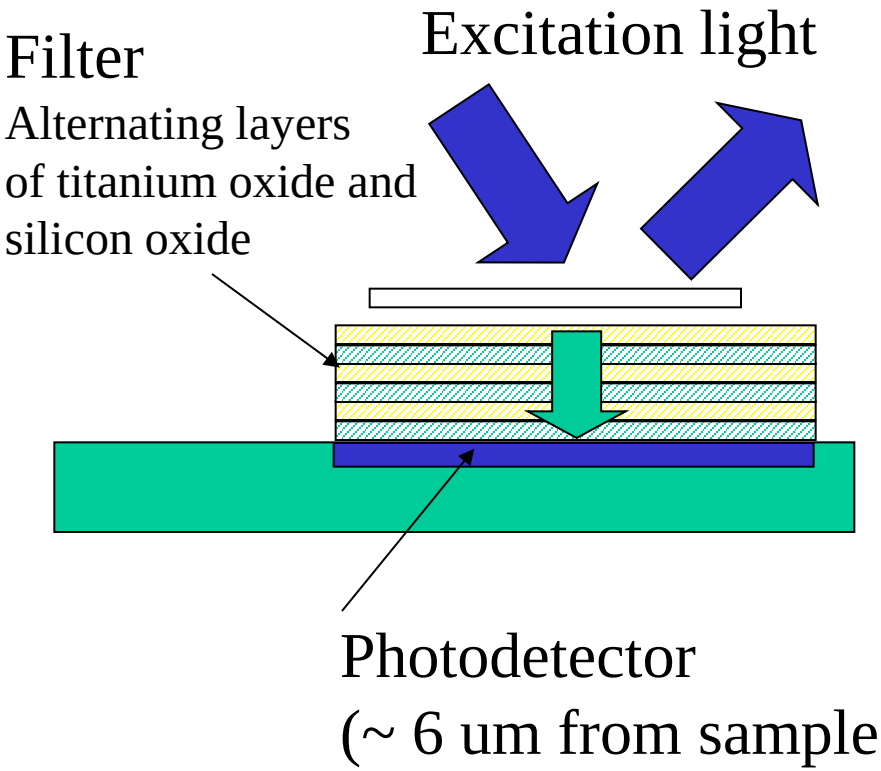


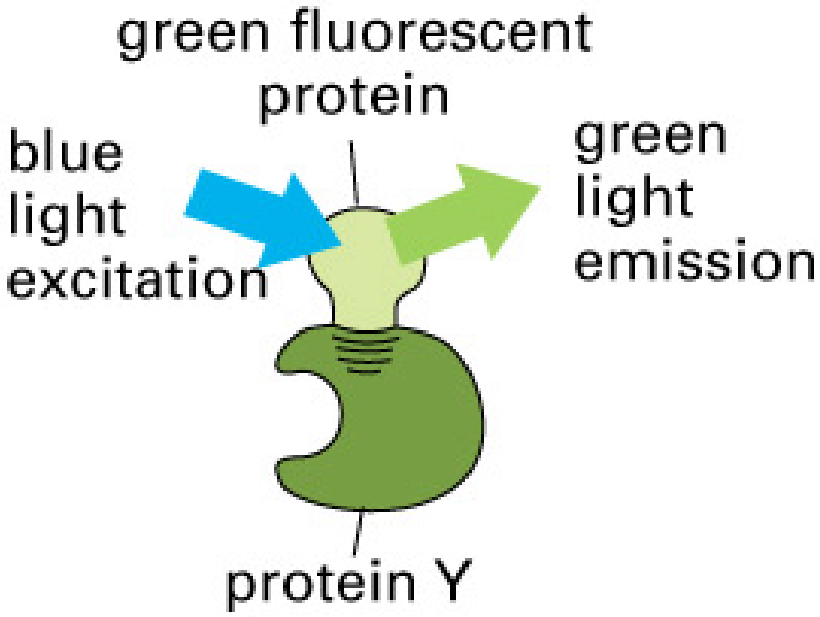
Fluorophores are between base pairs or bind to the end of the fragment



(b)

Green Fluorescent Protein (GFP)





1. Excitation using blue light emtting diode, < 500 nm
2. Fluorescent light at 515 nm

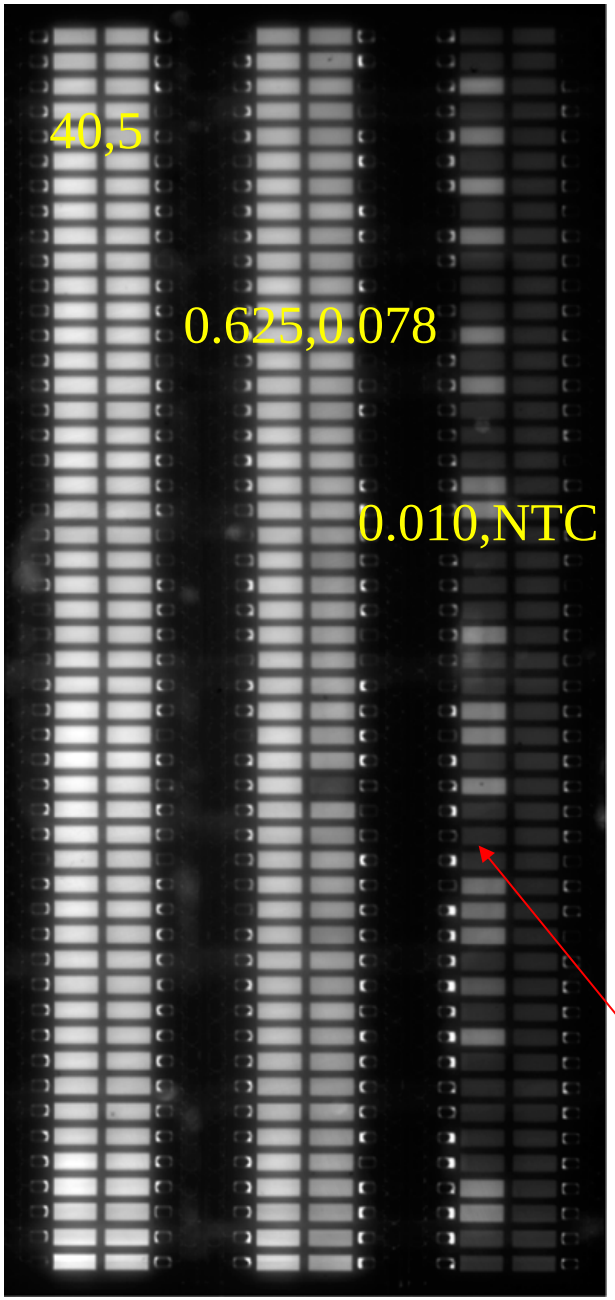
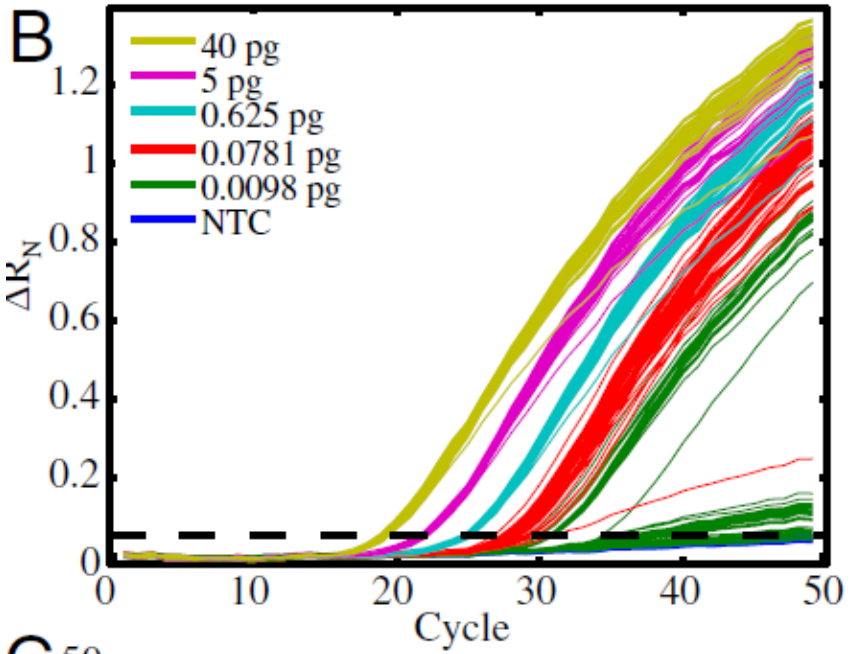
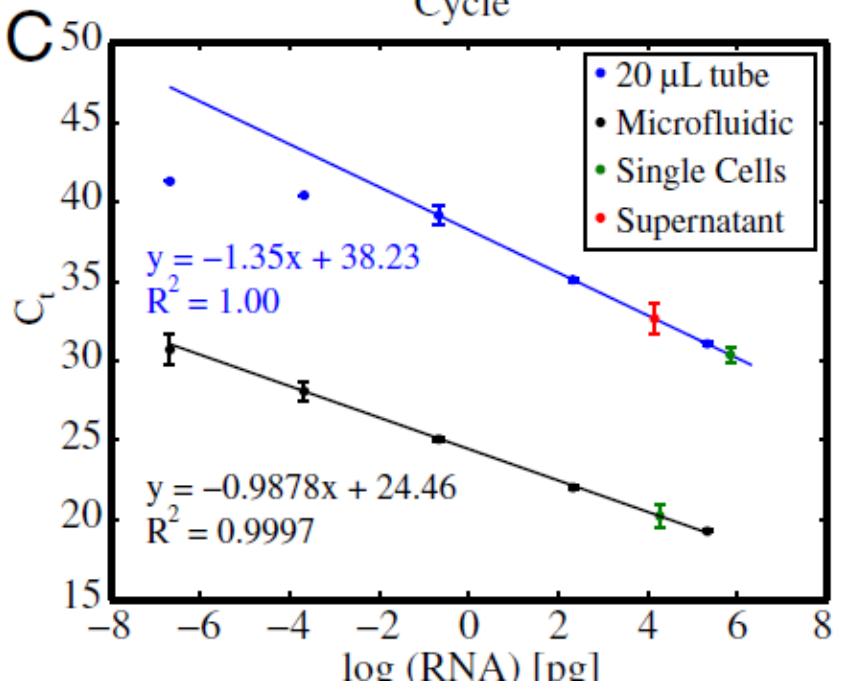


Image taken after 40 cycles of PCR from dilution series of purified total RNA (GAPDH housekeeping gene expression; no cells)

Total RNA from K562 cells
6 lanes (left to right): 40 pg⁄chamber, 5 pg⁄chamber, 625 fg⁄chamber, 78 fg⁄chamber, 10 fg⁄chamber, and no-template control (NTC)

Digital pattern → single molecule detection → average copy number of GAPDH to be 979 +/- 240 transcript copies per single-cell equivalent (20 pg)



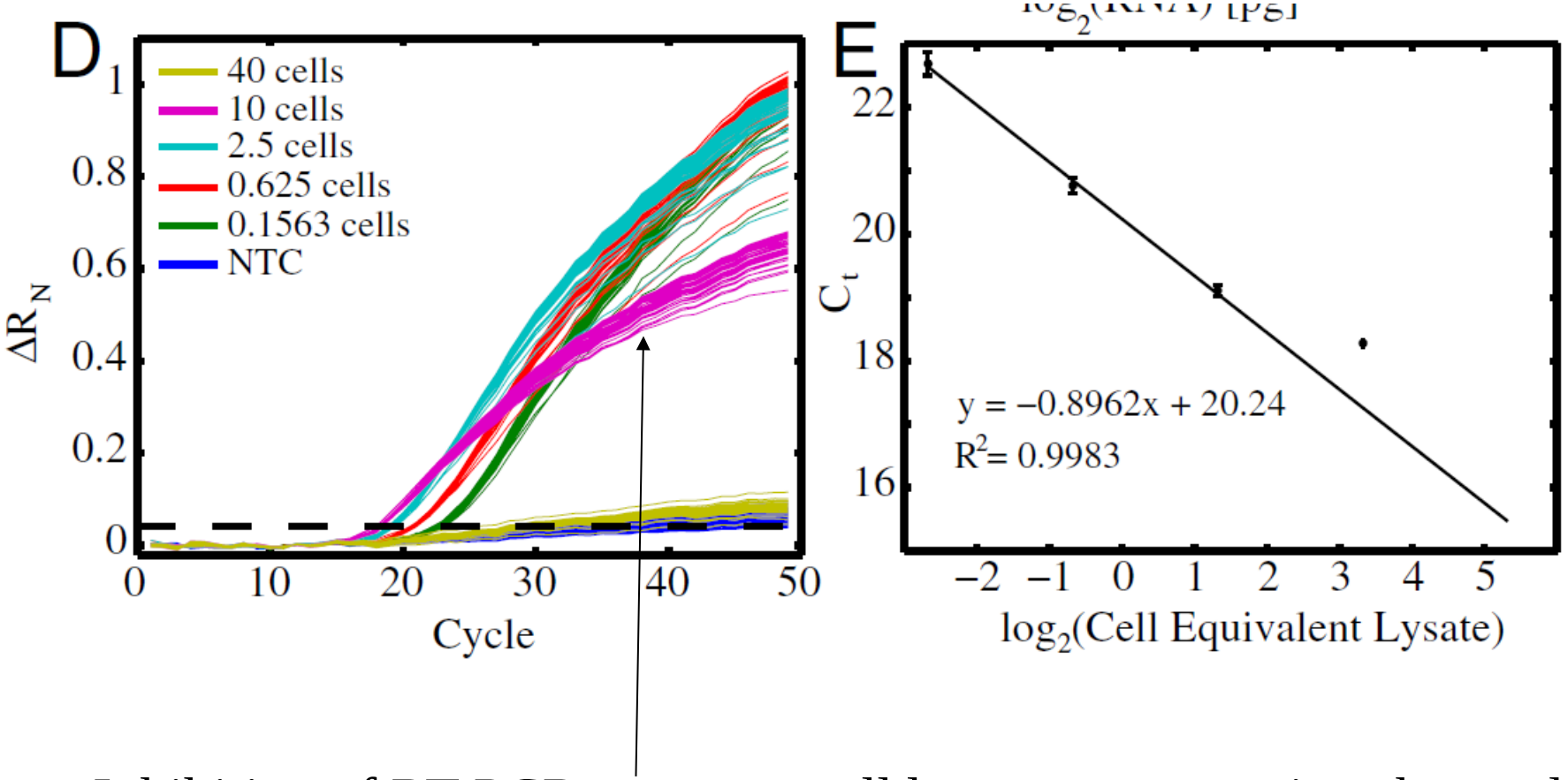


Real-time amplification curves

Efficiency of Amplification: uniform amplification across the array and technical error of less than 10% in absolute concentration, near the limit of qPCR precision.

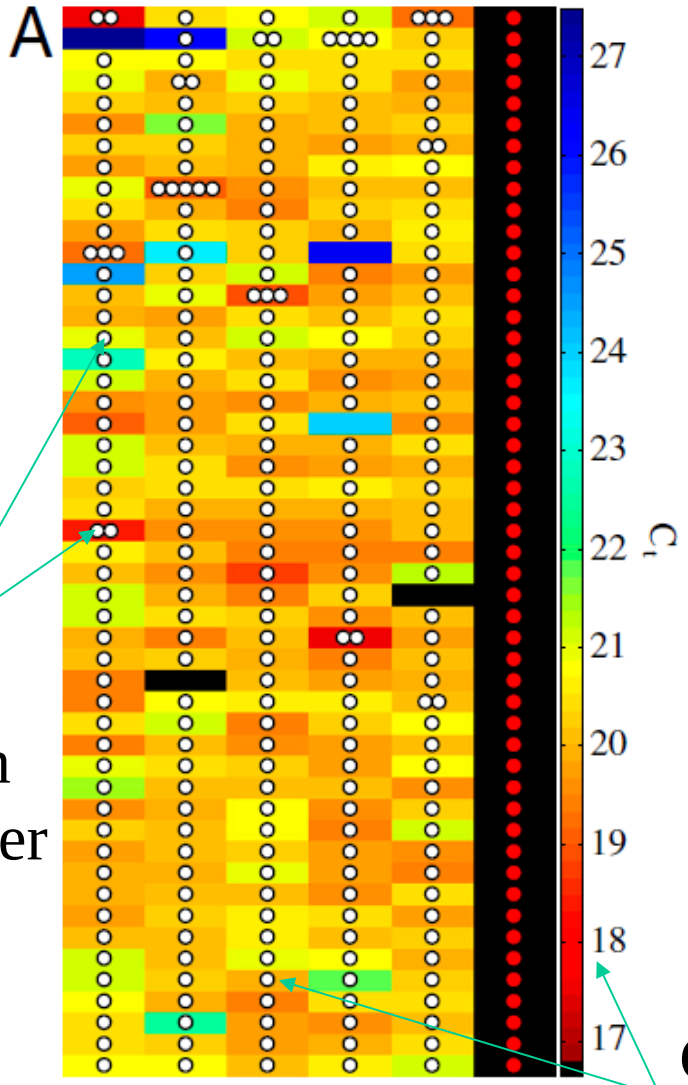
C_t: Cycle Threshold

Real-time amplification curves of GAPDH in K562 cell lysate dilutions

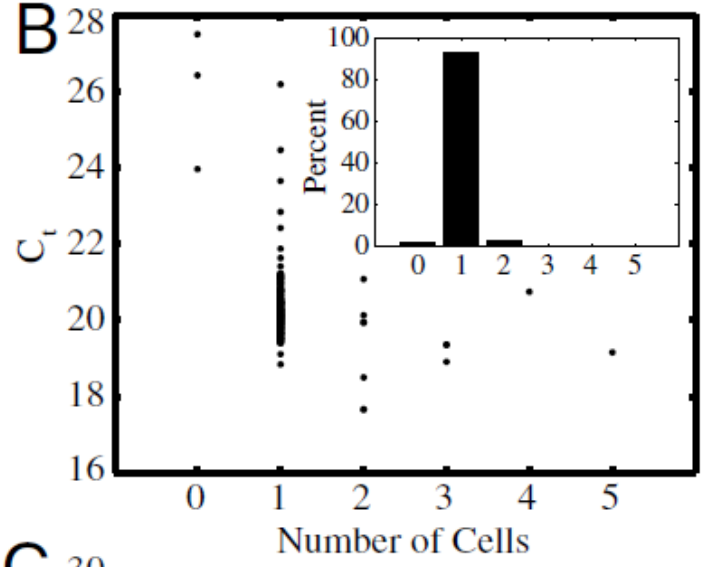


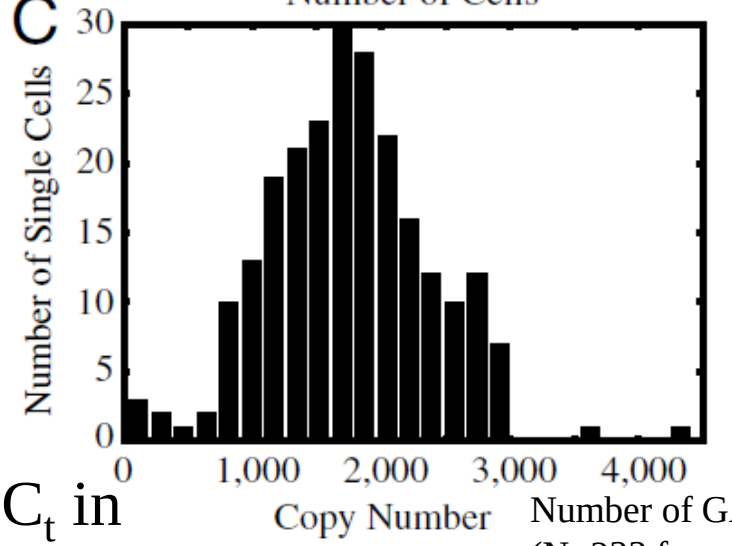
Inhibition of RT-PCR occurs at cell lysate concentrations beyond 10 beyond 10 cell equivalents per 50 nL reaction.

Ct of Cell(s) in Each Unit



Cells in each chamber

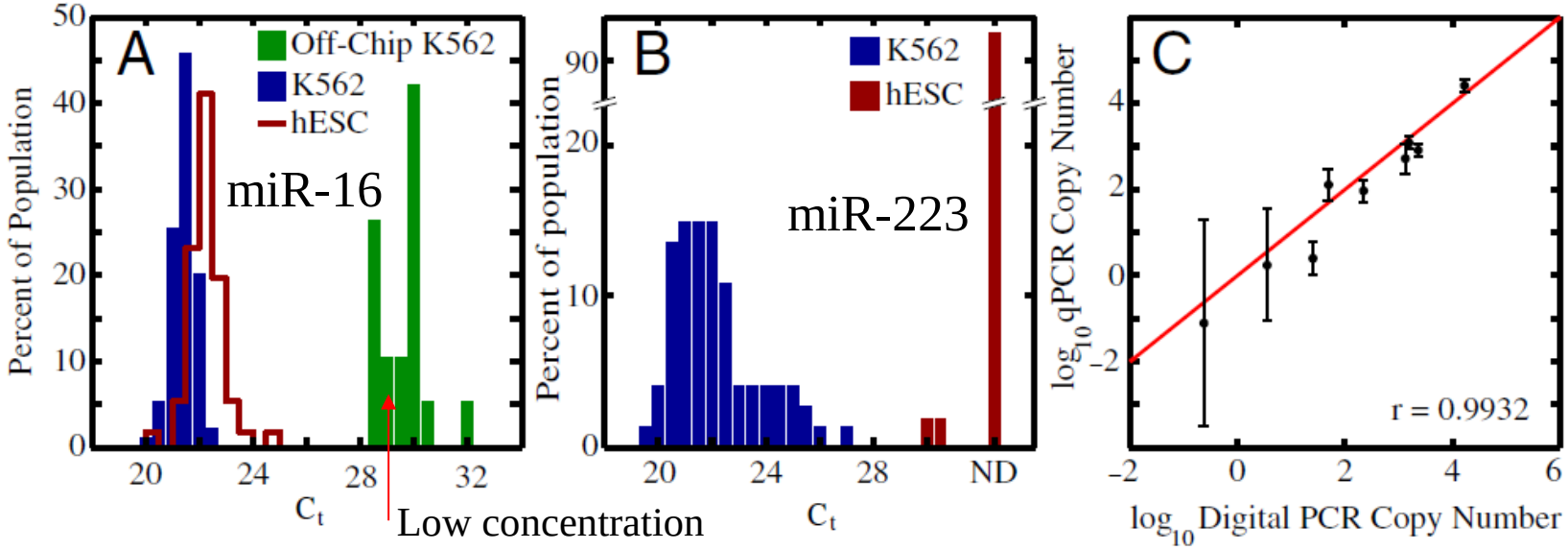


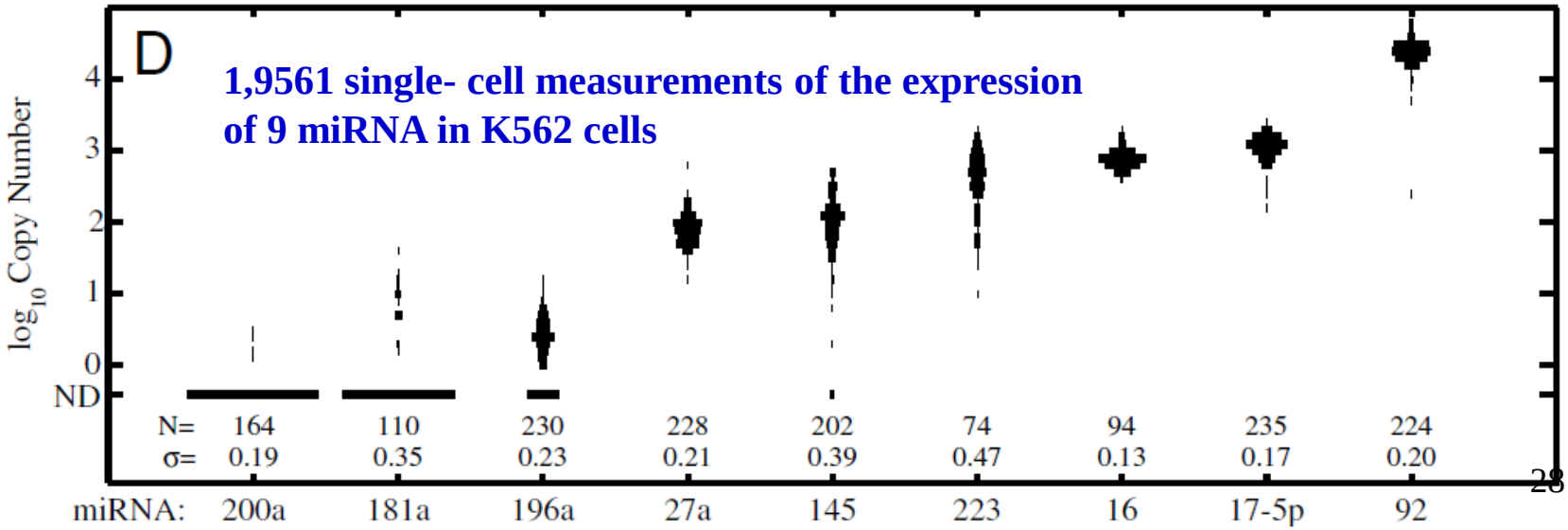


C_t in each chamber

Number of GAPDH transcripts (N=233 for a single cell)

miRNA expression in K562 cells and hESCs





An Integrated Nanoliter DNA Analysis Device (Mark Burns et al., Science, 1998)

47mm X 5mm X 1mm

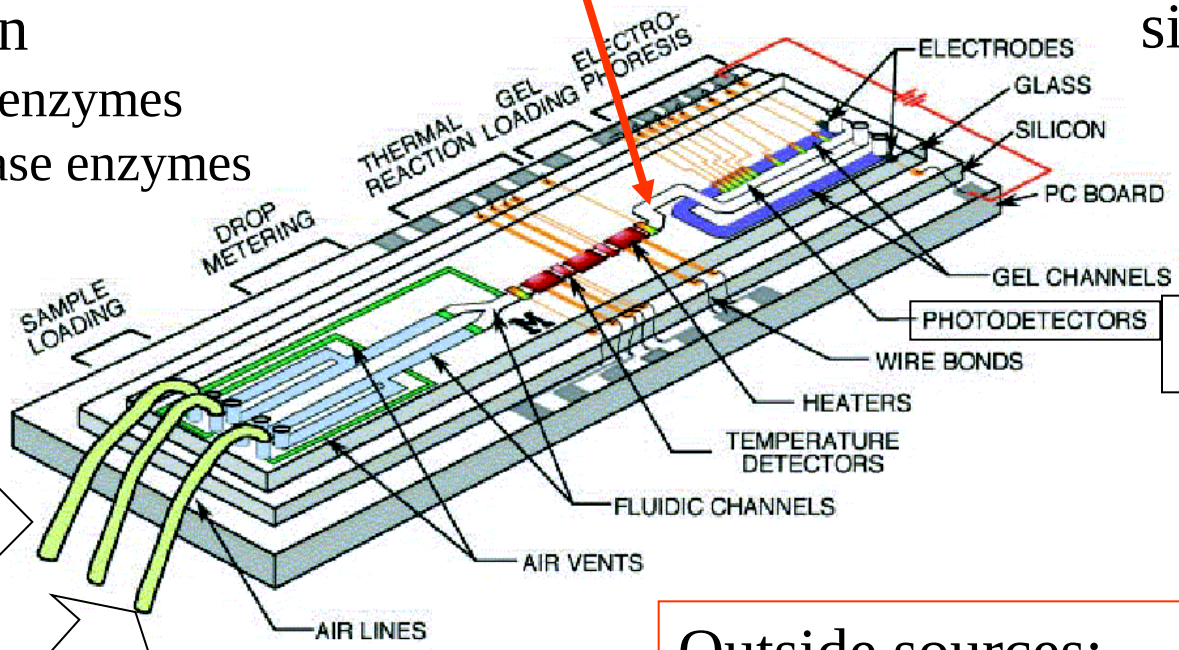
Intercalatng dye

Electronic signals

Reagent solution

Bso B1 restriction enzymes

Bst DNA polymerase enzymes



DNA solution

DNA fragments

amplification site-specific oligonucleotides

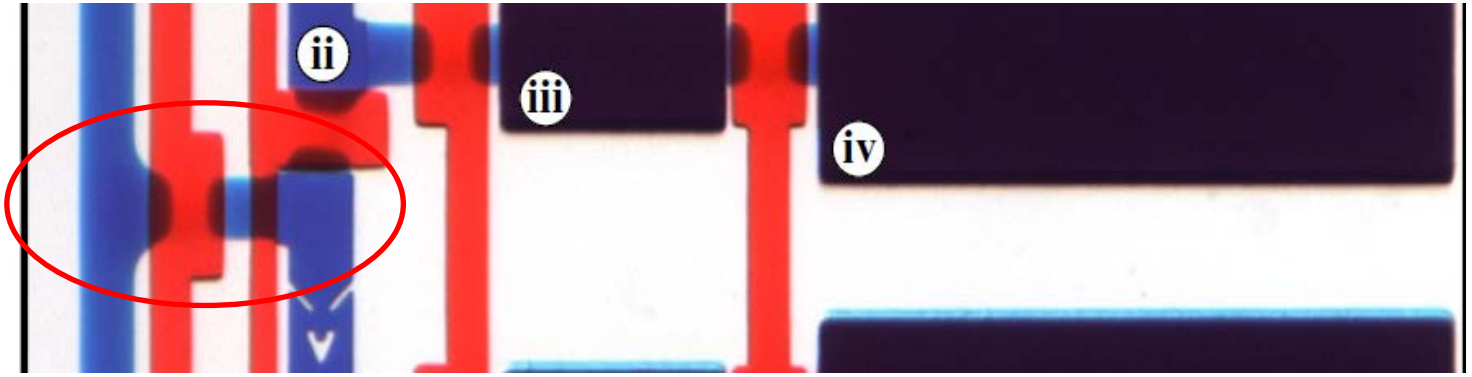
Outside sources:

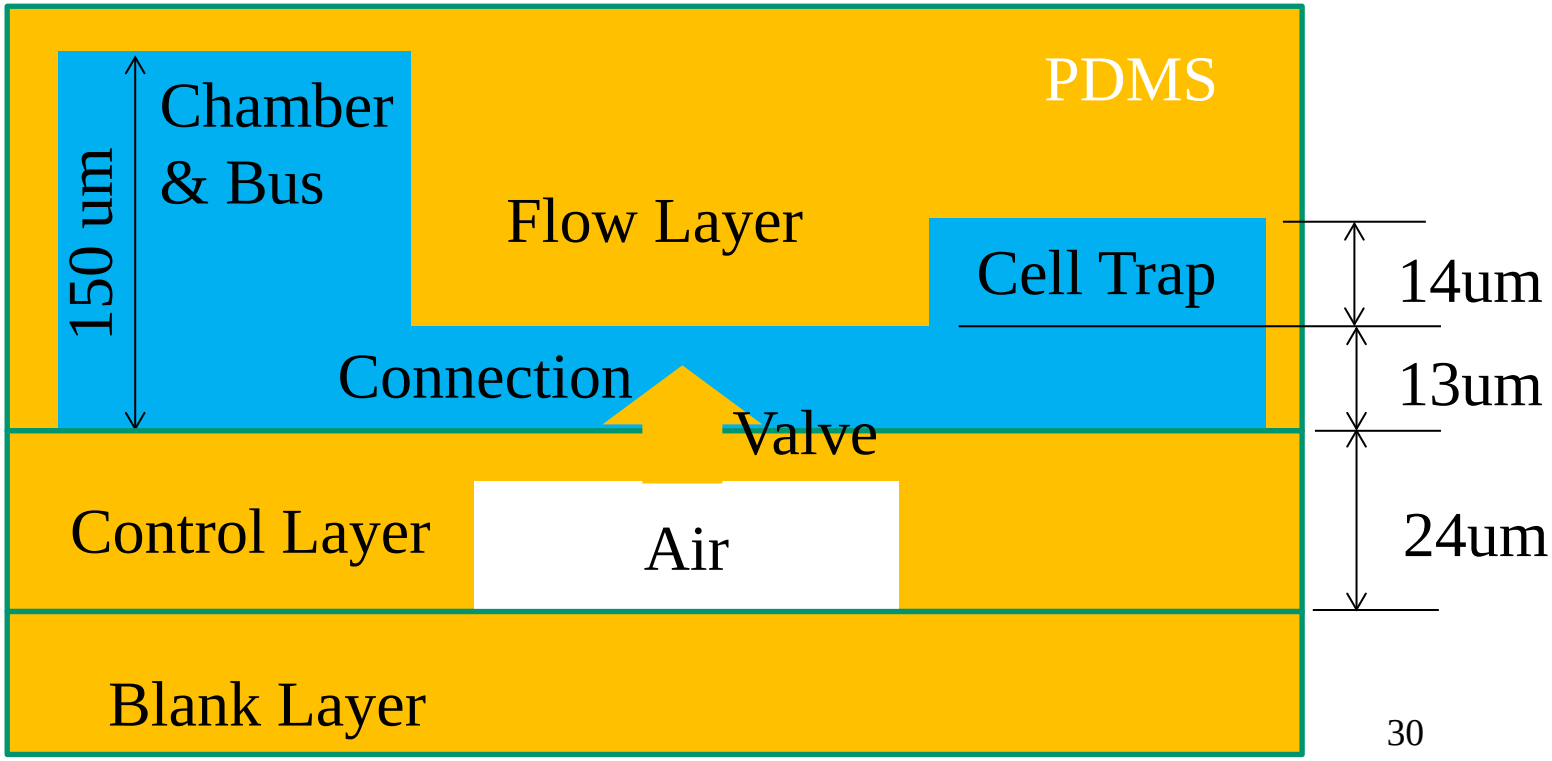
Pressure

Electronic control

Optical excitation source

Three Layers of the Device

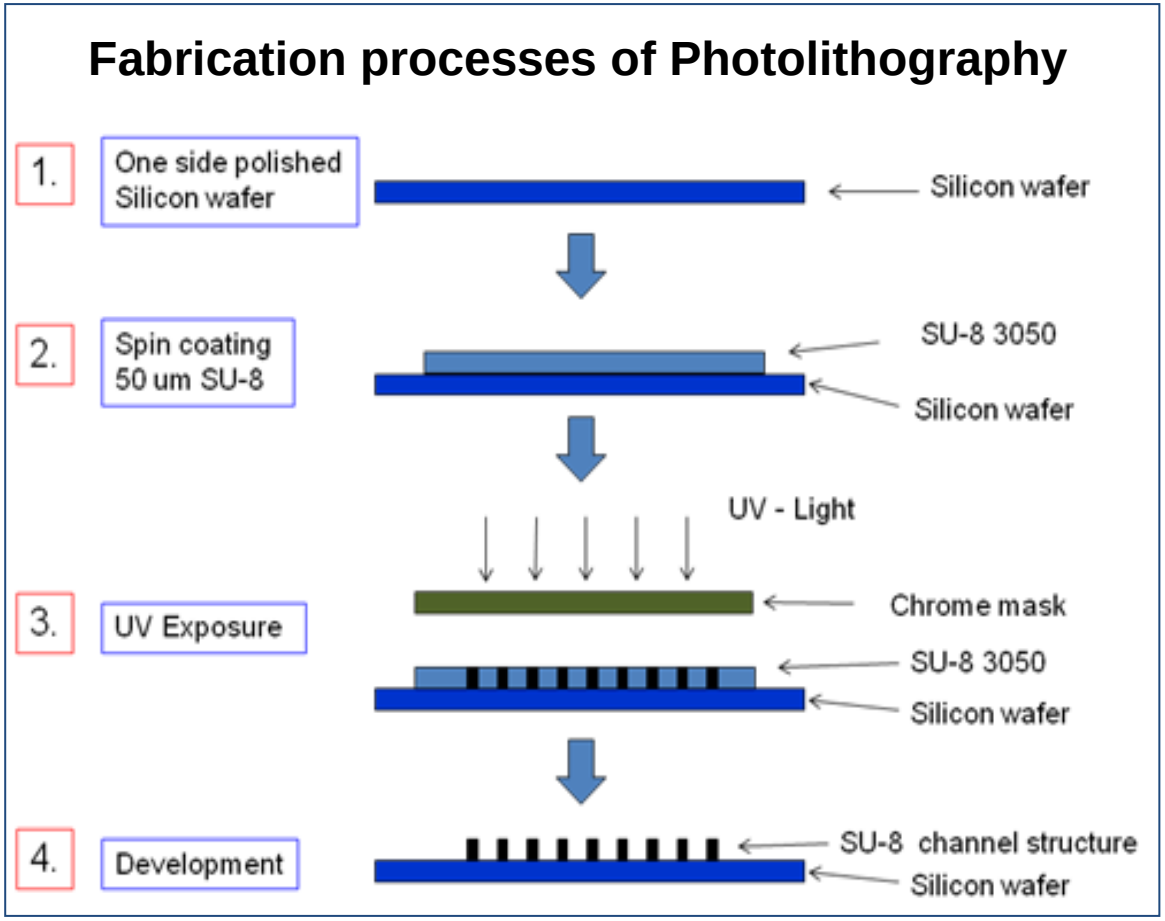


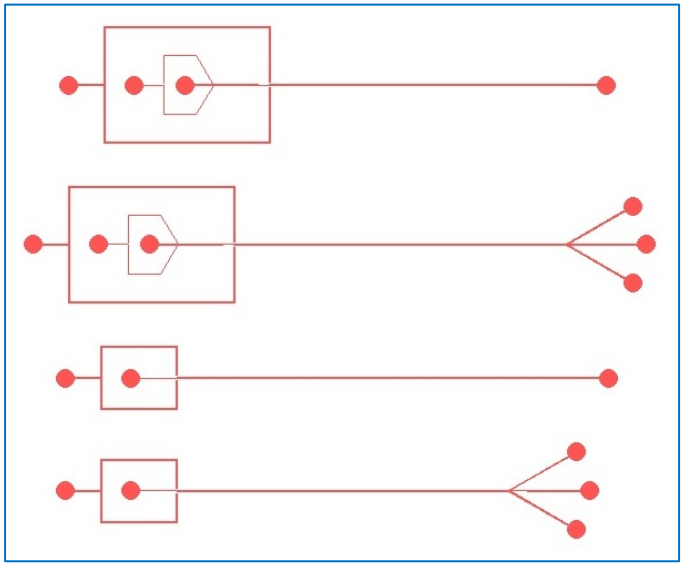


Fabrication Process for a Microfluidic Device

Photolithography:

Photo resist: Microchem SU-8 3050
Thickness: 50 um, at 3000rpm 45 seconds





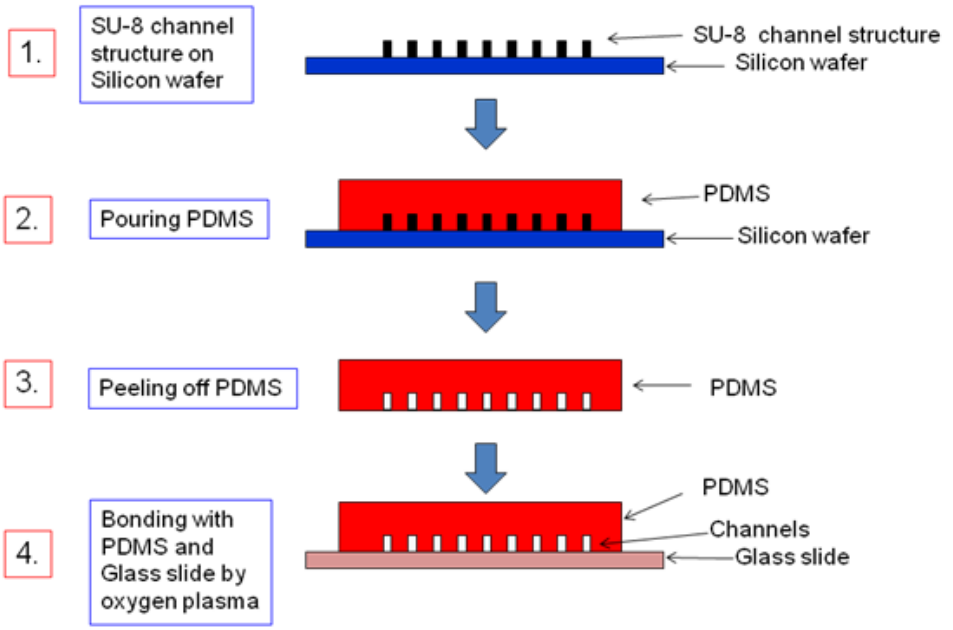
Masks

SU-8 structure

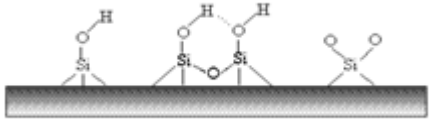
Soft lithography :

Polydimethylsiloxane (PDMS)
DOW CORNING -- SYLGARD 184

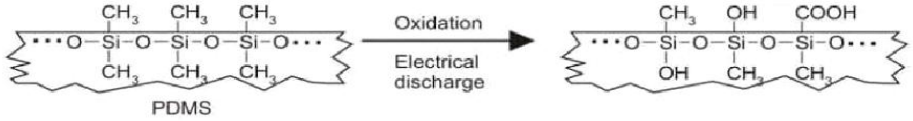
Fabrication processes of Softlithography



a. Oxygen plasma treatment on Glass slide



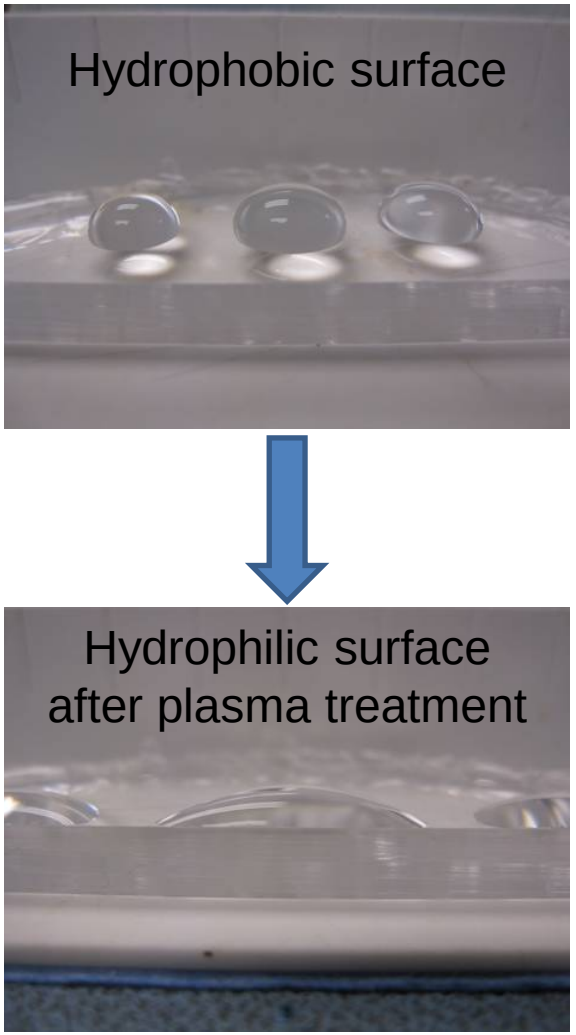
b. Oxygen plasma treatment on PDMS

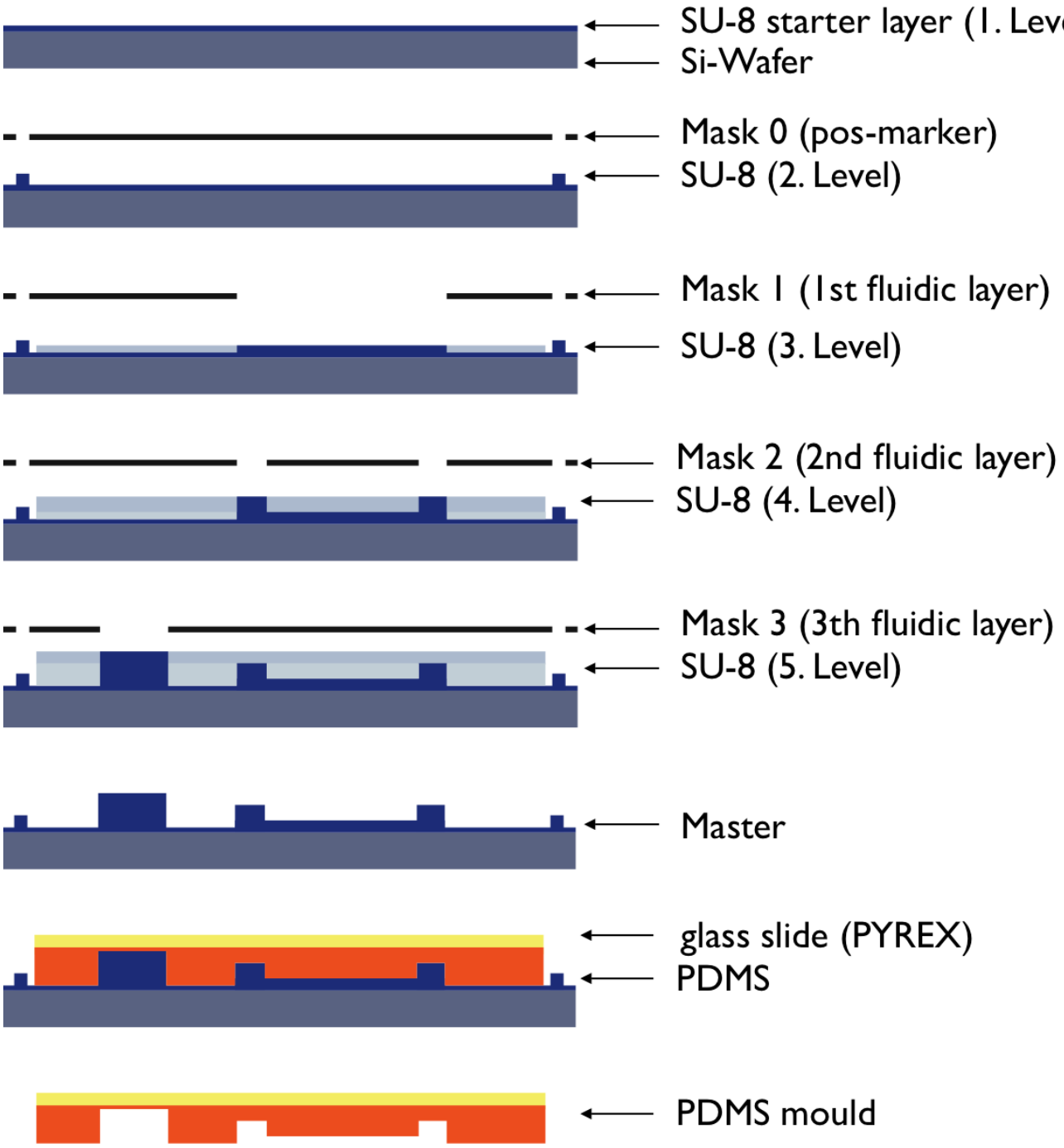


Surface oxidation is believed to expose silanol groups (OH) at the surface of the PDMS layers that when brought together form covalent siloxane bonds (Si–O).

Oxygen plasma surface treatment

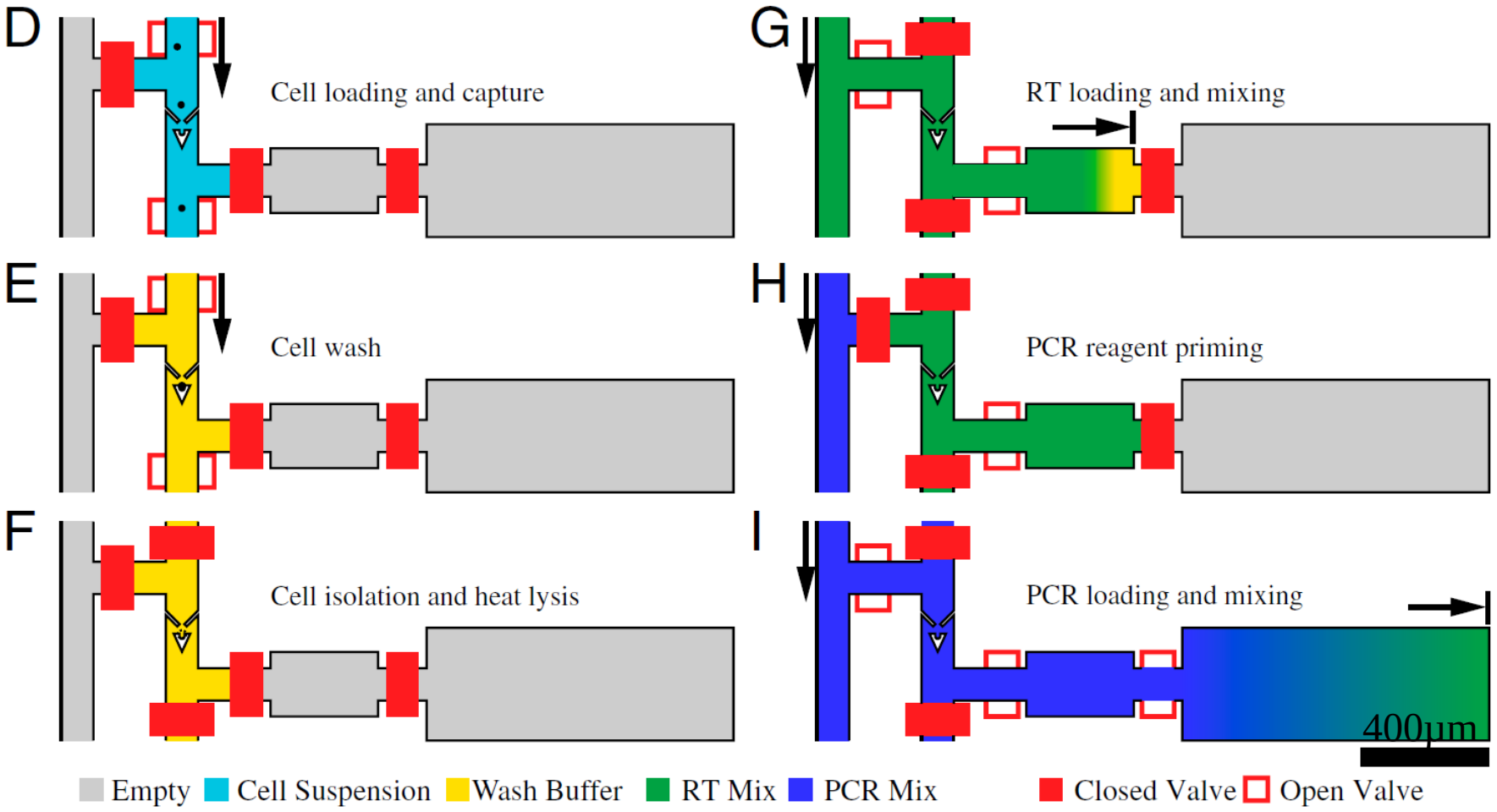
30 seconds in Oxygen Plasma chamber: PDMS surface property converts from hydrophobic to hydrophilic





Multilayer soft lithography

Device Operation



Cell trapping: no significant bias in selecting cells of different sizes

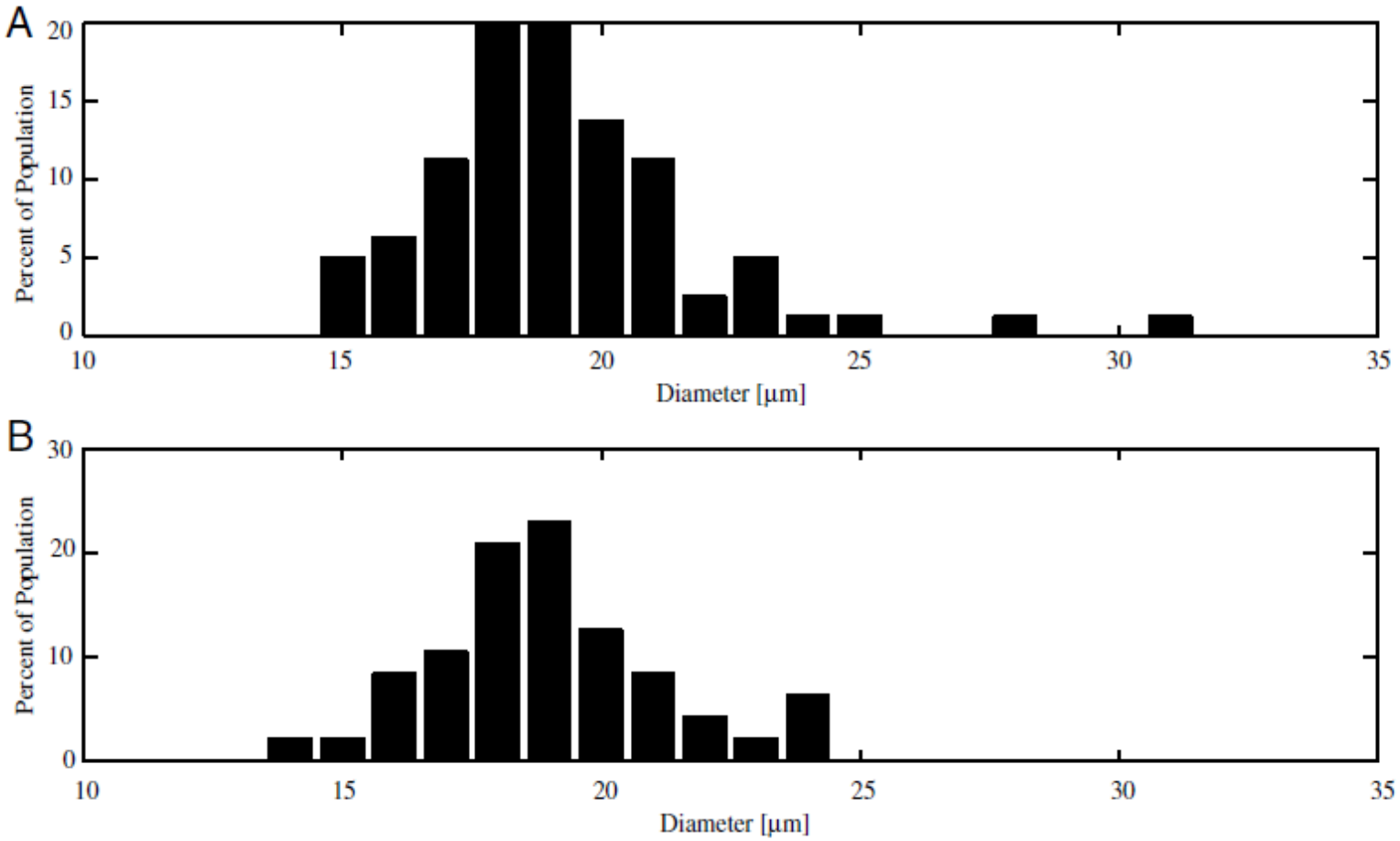


Fig. S1. Histograms showing the size distribution of cells in original sample as measured by Cedex (A) are consistent with the size distribution of cells isolated by microfluidic traps (B). Under the assumption of spherical cell shape the distribution of diameters of trapped cells corresponds to a mean volume of 4.2 pL with a standard deviation of 2.0 pL

Free RNA, cellular debris, and untrapped cells washed away

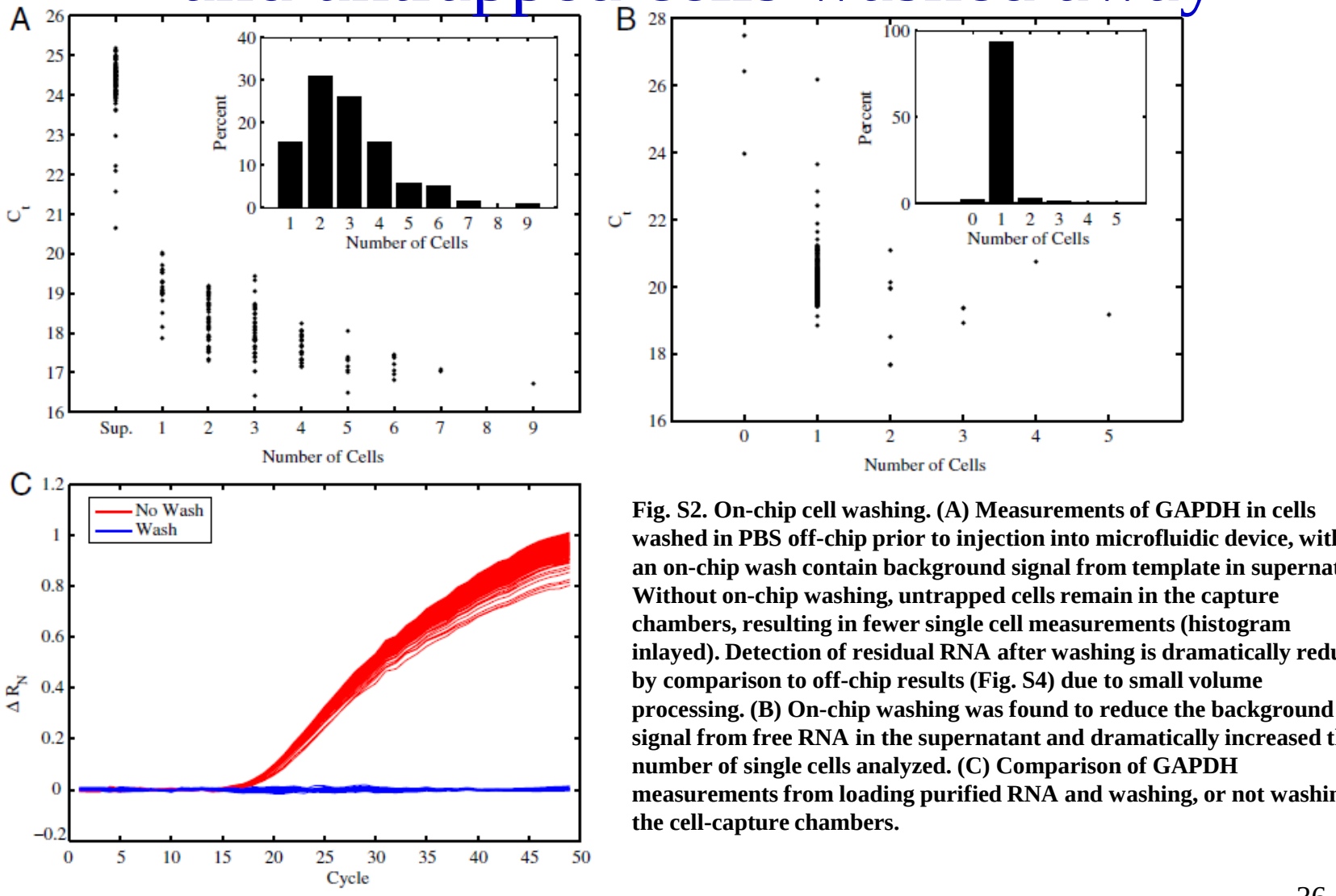


Fig. S2. On-chip cell washing. (A) Measurements of GAPDH in cells washed in PBS off-chip prior to injection into microfluidic device, without an on-chip wash contain background signal from template in supernatant. Without on-chip washing, untrapped cells remain in the capture chambers, resulting in fewer single cell measurements (histogram inlayed). Detection of residual RNA after washing is dramatically reduced by comparison to off-chip results (Fig. S4) due to small volume processing. (B) On-chip washing was found to reduce the background signal from free RNA in the supernatant and dramatically increased the number of single cells analyzed. (C) Comparison of GAPDH measurements from loading purified RNA and washing, or not washing, the cell-capture chambers.