DNA Microarray Preparation by Light-Controlled In Situ Synthesis

Although array technology has been used in genomic research for only a few years, it has already become a standard tool in molecular biology laboratories, and it is expected to reshape the way in which future genomic research is conducted. Because a lot of sequence information is already available, and because even more will be available from public databases within the next few years, oligonucleotide arrays that require only sequence data for setup will become especially important. With such microarrays, produced by in situ synthesis using straightforward phosphoramidite chemistry, it is possible to address many applications, including resequencing, typing of single-nucleotide polymorphisms (SNPs), transcriptional profiling, and the investigation of variations in gene splicing. Spatial control during synthesis can be achieved either by light-directed activation or by using a robotic inkjet printer. This unit presents the light-directed activation process for producing oligonucleotide microarrays.

STRATEGIC PLANNING

With regard to materials and equipment, there are four requirements for performing light-directed in situ synthesis of DNA microarrays: (1) a solid support (preferably a glass substrate); (2) phosphoramidite building blocks carrying photolabile protecting groups; (3) a DNA synthesizer; and (4) an irradiation apparatus. Furthermore, for the subsequent use and analysis of the in situ–synthesized DNA microarrays, a hybridization chamber and a fluorescence scanner (preferably a laser- or CCD-based system) are needed.

Solid Support

In principle, any flat solid support that is suitable for oligonucleotide synthesis via phosphoramidite chemistry may be used. Although plastic surfaces have been used successfully, nearly all of the more widely used supports are made of glass. Ideal anchoring groups for starting the oligonucleotide synthesis process include hydroxyl and amino groups, with the former being preferred. This preference arises because phosphoramidite building blocks form more stable phosphodiester linkages to hydroxyl linkers compared with amino linkers.

If desired, a linker may be placed between the anchoring group and the starting monomer in the oligonucleotide synthesis. It has been shown that long linkers lead to increased yields from in situ syntheses on solid supports. Aside from solid supports consisting of dendrimeric linker systems (Beier and Hoheisel, 1999; see *UNIT 12.4*), quite a few other solid support media that are suited to in situ synthesis can be found in the literature (e.g., Southern et al., 1994; McGall et al., 1997).

Phosphoramidite Building Blocks Carrying Photolabile Protecting Groups

Several types of photolabile protecting groups have been introduced for the light-controlled synthesis of DNA microarrays. The more prominent ones include [(2-nitroveratryl)oxy]carbonyl (NVOC; Fodor et al., 1991; Pease et al., 1994), [(α-methyl-2-nitropiperonyl)oxy]carbonyl (MeNPOC; Pirrung and Bradley, 1995; McGall et al., 1997), dimethoxybenzoincarbonate (DMBOC; Pirrung et al., 1998), [2-(2-nitrophenyl)propoxy]carbonyl (NPPOC; Hasan et al., 1997; Giegrich et al., 1998), and [2-(2-nitrophenyl)ethyl]sulfonyl (NPES; Bühler et al., 2004). Nearly all of these photolabile protecting groups are typically employed at the 5′ position, thereby enabling

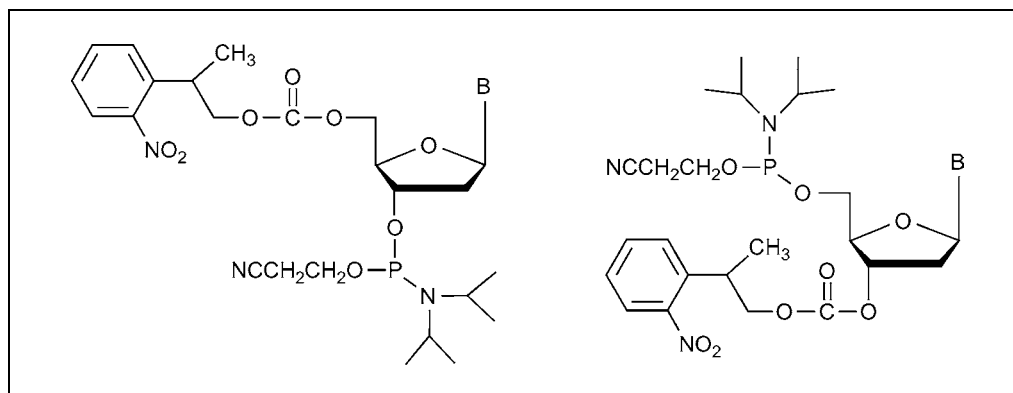**Contributed by Markus Beier and Jörg D. Hoheisel**

Figure 12.5.1 Monomeric phosphoramidite building blocks. The photolabile [2-(2-nitrophenyl)propoxy]carbonyl (NPPOC) protecting group can be placed at the 5′ or 3′ position. B, nucleobase.

the preparation of DNA microarrays that expose a free 5′ end while leaving the 3′ terminus attached to the solid support. More recently, DNA microarrays that expose a free 3′ hydroxyl group have gathered special interest, opening the way for a new generation of applications that make use of enzymatic on-chip reactions (Beier et al., 2001). This unit focuses on experimental conditions that have been tested for the in situ synthesis of oligonucleotide microarrays using NPPOC-protected phosphoramidites (Fig. 12.5.1; Beier and Hoheisel, 2000; see *UNIT 12.3*). Conditions (e.g., buffer composition, irradiation time, power of the light source, irradiation wavelength) for cleavage of a different protecting group would have to be optimized specifically for that protecting group. The authors have also performed this protocol using MeNPOC (Affymetrix) as a protecting group. While NPPOC is best removed using an irradiation buffer containing a non-nucleophilic base such as acetonitrile, MeNPOC groups have to be removed under "dry" conditions. NPPOC also leads to a significantly higher yield than does MeNPOC.

DNA Synthesizer

Any conventional DNA synthesizer can be employed for light-directed synthesis of DNA microarrays. To perform an in situ synthesis on a flat surface, a flow cell–like holder for the solid support is needed (for an example, see Fig. 12.5.2). The inlet and outlet of this holder are attached to the DNA synthesizer in the same way a normal synthesis column is attached. To customize the DNA synthesizer for in situ synthesis on a solid support, the instrument's synthesis protocols have to be adapted to the flow cell holder. For high yields to be achieved, it has to be guaranteed that a sufficient volume of reagent solution is transported to the solid support and subsequently removed. Special care must be taken to ensure that no gas bubbles are generated on the solid support within the flow cell. In principle, all steps of the synthesis protocols presented here are the same as for standard DNA synthesis on a conventional synthesis column, except for the detritylation step. In light-controlled synthesis, detritylation is replaced by an irradiation step. In this step, the flow cell is flushed with an irradiation buffer, and defined regions of the solid support (where chain elongation is desired) are activated by irradiation.

Irradiation Apparatus

The irradiation device is responsible for illuminating defined regions of the solid support, thus removing the photolabile protecting groups present in those regions. At these deprotected positions, oligonucleotide chain elongation takes place during the next round of
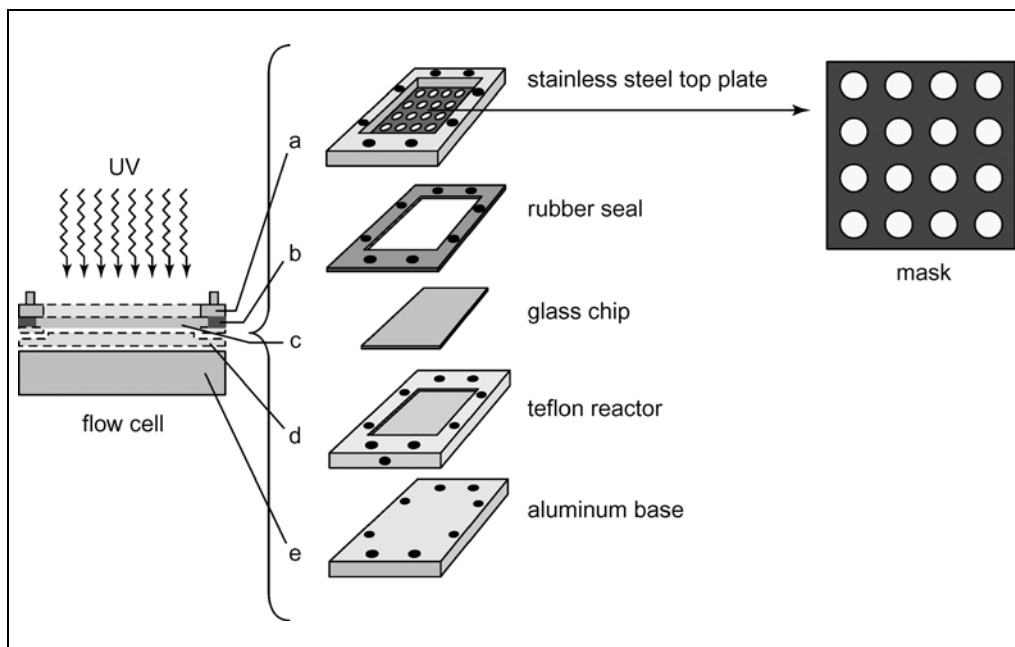
Figure 12.5.2 Flow cell apparatus for light-controlled in situ synthesis of DNA microarrays. The main components of the apparatus are the reactor and the mask. A rubber seal (**b**) is fitted around a glass chip (i.e., derivatized slide; **c**), which is then placed on top of a Teflon reactor (**d**) that sits on an aluminum base (**e**). The slide is positioned with its derivatized side facing down, such that it is illuminated through its "back" side. There will be a gap between the front (derivatized) side of the slide and the bottom of the reactor; its depth (∼0.5 mm) is defined by the thickness of the Teflon sealing (outer edge) of the reactor. The inner dimensions of the reactor should be smaller than the corresponding dimensions of the glass slide (so that the edges of the slide rest securely on the raised outer edges of the reactor), but larger than the area over which the oligonucleotide spots will be distributed. The inlet/outlet ports of the flow cell are drilled into the reactor itself at the front and back, and lead to the sealed space between the slide and the reactor. The actual ports at the outside of the reactor are made to fit the tubing of the DNA synthesizer that is used to control the flow of reagents. To complete the assembly of the apparatus, the stainless steel top plate (**a**), a single solid piece containing 16 to 64 holes, is placed on top of the seal and glass slide, and components **a** through **e** are fastened together. The mask will touch the back of the slide. For a full-color version of this figure, go to *http://www.currentprotocols.com/colorfigures.*

the synthesis cycle. The most important parameter is the spatial control of the irradiation pattern. Additional critical factors include intensity, wavelength, and duration of light exposure. Since most photolabile protecting groups absorb within the UV spectrum, the light source of choice is a mercury lamp. To set the irradiation wavelength for NPPOC removal, a 365-nm interference filter is typically employed.

Several techniques can be used to control the accuracy of the light pattern during the irradiation step. Among them, photolithography, pioneered by Affymetrix (Fodor et al., 1991; Fodor et al., 1993), represents the most common technology for generating oligonucleotide microarrays. Spatial resolution is obtained by implementing a new photolithographic mask for each synthesis step; for a typical DNA microarray with 25-mer oligonucleotides, 75 to 85 photolithographic masks are commonly required (as many as 100 masks may be required). Another more flexible option is the use of a so-called spatial light modulator, such as a digital micromirror device (DMD). Using a DMD, the irradiation pattern can be controlled electronically via a computer interface, which is advantageous when a larger set of possible microarray designs is desired (Gao et al., 1998; Singh-Gasson et al., 1999). Both photolithography and DMD-aided irradiation represent high-end technologies, and only the latter can be performed using a commercially available benchtop instrument (Baum et al., 2003).
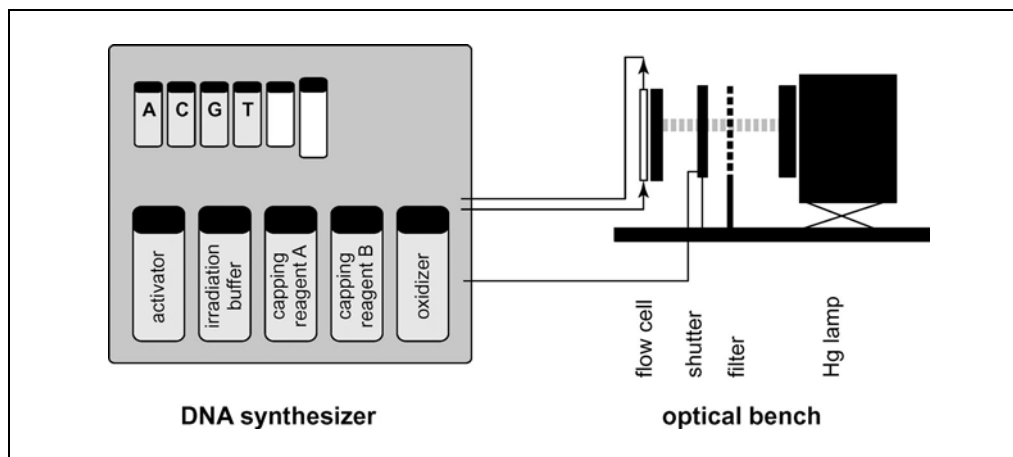
Figure 12.5.3 Schematic drawing of an assembly (consisting of a DNA synthesizer and an irradiation apparatus) for performing light-directed synthesis of oligonucleotide microarrays.

ASSEMBLY OF AN APPARATUS FOR LIGHT-DIRECTED SYNTHESIS OF OLIGONUCLEOTIDE MICROARRAYS

The low-tech apparatus described here (Fig. 12.5.3) represents a simple combination of the lithographic and DMD-based approaches and allows the production of oligonucleotide microarrays of low complexity (see below). Instead of replacing the photolithographic mask after every synthesis step, a single mask is directly mounted onto the flow cell and used throughout (Fig. 12.5.2). For the synthesis of a 16-feature microarray, for example, this unique mask would have 16 holes. The light pattern projected onto the microarray surface during each synthesis cycle is obtained by closing certain holes mechanically; this can be achieved by applying removable black tape to the desired holes to prevent irradiation of the surface underneath. As a result, the photolabile protecting groups at these positions (whether attached to the support or to the growing oligonucleotide) are not removed during the next synthesis step.

The irradiation apparatus consists of a mercury lamp within an air-cooled housing mounted on an optical bench. To select the right wavelength for removal of the NPPOC groups, a 365-nm interference filter is placed between the mercury lamp and the flow cell, which is also mounted on the optical bench.

The term *low complexity* refers to the low number of different sequences that can be synthesized on a single microarray (i.e., 16 to 64 distinct oligonucleotide probes). In principle, long oligonucleotides can be produced using this apparatus; however, because the method represents a low-tech approach, the overall yield corresponds directly to the precision of the instrumentation. This approach is not recommended for the synthesis of oligonucleotide probes longer than 25 bp. Although rather simple and not automated, this method permits the low-throughput production of oligonucleotide microarrays of lower complexity without the need for expensive equipment.

Materials

50 mM iodine in 7:1:2 (v/v/v) acetonitrile/pyridine/water

Flow cell apparatus (custom-made; Fig. 12.5.2), including the following:
    Teflon reactor
    Aluminum base plate
    Rectangular rubber seal

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Supplement 20

Current Protocols in Nucleic Acid Chemistry

Nonderivatized microscope slide (for optimization of DNA synthesizer program)
Derivatized microscope slide (for synthesis of microarray; see *UNIT 12.4*)
Stainless steel top plate with inner mask (16 to 64 holes)
DNA synthesizer (e.g., Eppendorf D200)
100-watt mercury lamp (e.g., Leica)
365-nm interference filter (e.g., Owis)
Optical shutter (e.g., Owis)
Optical bench (e.g., Owis) and assorted optical hardware

Assemble the chemical apparatus

1. Assemble a flow cell holder containing a derivatized microscope slide as follows (also see Fig. 12.5.2).

   a. Place the Teflon reactor on top of the aluminum base plate.

   b. Fit a rectangular rubber seal around a nonderivatized glass microscope slide. Place the slide on top of the Teflon reactor.

   c. Place the stainless steel top plate/mask on top of the slide.

   d. Secure the components of the flow cell apparatus to each other by tightening with thumbscrews, taking care not to break the glass slide.

2. Using standard fittings provided with the DNA synthesizer, attach the synthesizer tubing to the inlet and outlet ports on the Teflon reactor.

   The DNA synthesizer tubing that is normally connected to the bottom of a synthesis column should be connected to the reactor's inlet port such that the inlet port is on the bottom when the flow cell apparatus is mounted on an optical bench, and the synthesizer tubing that is normally connected to the top of a synthesis column should be connected to the reactor's outlet port.

Modify DNA synthesizer protocols

3. Modify a standard, preprogrammed DNA synthesizer protocol by replacing the detritylation step with a waiting step of 300 sec, corresponding to the duration of irradiation. Ensure that this modified program also includes cleavage of the final photolabile protecting group.

   During the irradiation step, irradiation buffer is pumped into the flow cell until it is completely filled, without any bubbles. After the 300-sec waiting step, argon is pumped into the flow cell until the irradiation buffer is completely removed. This is followed by a washing step.

4. Fill all synthesizer reagent bottles with 50 mM iodine in 7:1:2 (v/v/v) acetonitrile/pyridine/water.

   The use of this colored solution allows the passage of liquid through the system to be monitored visually.

5. Run the modified DNA synthesizer program. Using the movement of the colored iodine solution as a guide, adjust the time and volume of each step (coupling, washing, capping, oxidation, and irradiation) to the volume of the flow cell.

Assemble the optical setup

6. Mount the mercury lamp, interference filter, and optical shutter onto the optical bench as shown in Figure 12.5.3.

   The distance between the lamp and the flow cell will depend on the type of lamp used. Generally, the lamp should be as close to the flow cell as possible. It is critical that the flow cell be illuminated completely.

7. To automate the illumination process, connect the optical shutter to the fraction collector port on the DNA synthesizer.

> *The synthesizer will trigger the opening of the shutter just before the flow cell is flushed with irradiation buffer, and will hold it open for the programmed irradiation period. The type of connector needed depends on the DNA synthesizer used; the authors use the DNA synthesizer's trityl cable for this purpose.*

8. Replace the nonderivatized slide in the flow cell apparatus with the derivatized slide on which the microarray is to be generated (installing the slide with its derivatized surface facing down), and then mount the flow cell onto the optical bench.

> *For this purpose, the authors use a slider that is mounted to the optical table and is fitted with a screw that couples to a hole drilled in the aluminum base of the flow cell.*

9. Align the mercury lamp, filter, shutter, and flow cell such that the illumination of the mask on the front of the flow cell is optimized.

LIGHT-DIRECTED IN SITU SYNTHESIS OF OLIGONUCLEOTIDE MICROARRAYS

In this protocol, the sequence programmed into the DNA synthesizer should resemble the sum of all of the different oligonucleotide probe sequences that are to be synthesized on the microarray. The simplest way to design the synthesis program is to perform a separate coupling step for each position of each sequence, uncovering the appropriate holes in order. As an example, for a simple "array" containing the two 4-bp sequences 5′-GTCG-3′ (sequence 1) and 5′-GATG-3′ (sequence 2), the program GGCTTAGG can be used, where monomers are added starting at the 3′ end with G for sequence 1, G for sequence 2, C for sequence 1, T for sequence 2, and so on. With careful planning, however, the program can be optimized to reduce the number of coupling cycles by coupling the same monomer on more than one sequence at a time. Thus, in the same example, the program used could be GCTAG, where both sequences start with a 3′-G, followed by C for sequence 1, T for both sequences, A for sequence 2, and finally G for both sequences. In this manner, three coupling cycles have been saved. In either approach, to avoid mistakes, it is advisable to make a careful plan for which holes are to be opened or closed in each step.

Materials

> Standard DNA synthesizer reagents (Proligo):
>> Activator (e.g., pyridine hydrochloride, dicyanoimidazole, tetrazole)
>> Anhydrous acetonitrile (for wash steps)
>> Oxidizing reagent
>> Capping reagents
> Irradiation buffer: 50 mM diisopropylethylamine (DIPEA) in acetonitrile
> NPPOC-protected phosphoramidite solutions in acetonitrile (dA, dC, dG, and T; concentration, 0.1 M for each; see *UNIT 12.3*)
> 25% (v/v) ammonia in water
> Nitrogen stream
>
> Assembly for light-directed oligonucleotide microarray synthesis (see Basic Protocol 1)
> Removable black tape (e.g., standard laboratory tape): one precut piece of sufficient size to cover the entire stainless steel mask, plus precut pieces to cover each individual hole in the mask
> Polypropylene jar with lid
> Orbital shaker
>
> Additional reagents and equipment for automated DNA synthesis (*APPENDIX 3C*)

12.5.6

NOTE: Immediately before being used in this protocol, phosphoramidites (prepared as in *UNIT 12.3*) should be dried and dissolved in acetonitrile. Care should be taken to prevent phosphoramidite solutions from absorbing humidity from the atmosphere.

Perform light-directed synthesis

1. Fill each reagent bottle of the DNA synthesizer with the appropriate standard DNA synthesizer reagent, irradiation buffer (to replace detritylation solution), or NPPOC-protected phosphoramidite solution (see Fig. 12.5.3).

2. Program the desired nucleotide sequence into the DNA synthesizer (see above), using the program modified for irradiation (see Basic Protocol 1).

3. Cover all holes in the flow cell mask using a single piece of removable black tape.

4. Turn on the mercury lamp.

   The lamp remains on throughout the entire synthesis, with the shutter being triggered to open just before the flow cell is flushed with irradiation buffer. Thus, each irradiation step starts with the opening of the shutter and ends 300 sec later with the closing of the shutter.

5. Start the DNA synthesizer program.

6. After the first coupling step (but before the first irradiation step begins), remove the single piece of black tape covering the flow cell mask. Use a separate, precut piece of black tape to cover each microarray spot that is to be protected from irradiation during the first synthesis cycle, and leave all other holes uncovered. At the conclusion of the first irradiation step, carefully cover each uncovered hole in the mask with a separate, precut piece of removable black tape.

7. In each subsequent synthesis cycle, remove the tape from each microarray spot at which chain elongation is desired during that cycle. Continue until all desired sequences have been completed.

   The accurate removal and attachment of tape can be complicated with a 16-hole setup, and even more challenging when a 64-hole setup is used. Careful experimental planning is necessary, and it is particularly advisable to make notes in advance regarding each irradiation pattern to be used.

Perform final deprotection

8. After the synthesis program has been completed, remove the microscope slide from the flow cell holder.

9. Fill a polypropylene jar with 25% (v/v) ammonia. Immerse the microscope slide in the ammonia solution, cover with a lid, and agitate 2 hr using an orbital shaker.

   This removes both the base-protecting groups and the cyanoethyl groups.

10. Remove the microscope slide from the deprotection solution, wash with water, and dry under a stream of nitrogen.

HYBRIDIZATION TO OLIGONUCLEOTIDE MICROARRAYS

This protocol describes the hybridization of target oligonucleotides to oligonucleotide microarrays produced by light-directed in situ synthesis. Hybridization is performed using a simple coverslip procedure, which is easy to perform and cost-effective.

Materials

Target oligonucleotide sample
SSARC hybridization buffer (see recipe)
Microscope slide containing oligonucleotide microarray (see Basic Protocol 2)
Nitrogen stream

Coverslip
Polypropylene vessel with lid
Fluorescence scanner, preferably laser or charge-coupled device (CCD), and image analysis software

1. Prepare the hybridization solution by dissolving the target oligonucleotide sample in SSARC hybridization buffer at room temperature.

   A final oligonucleotide concentration ranging from 2 to 100 nM should be appropriate, depending on the microarray used and the specific assay being performed.

2. Apply 50 μL hybridization solution onto the microarray on the microscope slide and then cover with a coverslip, taking care to avoid bubble formation underneath the coverslip.

3. Place the microscope slide face-up on a damp tissue in a polypropylene vessel. Close the lid and allow hybridization to occur at the required temperature for the desired length of time (usually overnight).

   The damp tissue ensures a humid environment for hybridization.

4. Using tweezers, remove the coverslip from the slide.

5. Wash the slide carefully by briefly immersing in cold SSARC hybridization buffer, and then dry under a stream of nitrogen.

6. Insert the slide into a fluorescence scanner and analyze in accordance with the manufacturer's instructions.

7. To reuse the slide with a new target oligonucleotide, denature duplexes by incubating the slide for 30 sec at 95°C in 2.5 mM Na_2HPO_4 containing 0.1% (w/v) SDS. Repeat with fresh stripping solution if needed. Wash with water to remove salt, and dry the slide before storing.

   The reuse of slides after storage for >3 months is not recommended.

   The original target oligonucleotides must be completely removed before the slide is rehybridized. One or two incubations is usually sufficient; repeated or extended incubations have only been required for a strong positive charge on the solid support. Complete target removal should be verified by fluorescence scanning.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; *for suppliers, see* **SUPPLIERS APPENDIX**.

SSARC hybridization buffer

600 mM NaCl
60 mM sodium citrate
7.2% (v/v) sodium *N*-laurylsarcosine (Sigma)
Adjust pH to 7.0 using 1.0 M citric acid or 1.0 M NaOH
Store 6 months at room temperature

COMMENTARY

Background Information

There are two techiques for producing microarrays: (1) spotting/deposition of prefabricated DNA sequences (10 to 70 bp) or PCR products, and (2) in situ synthesis of oligonucleotides (0 to 70 bp). Whereas the spotting approach is used primarily when sequence information is not available, the in situ approach is superior due to its flexibility.

Microarrays generated by in situ synthesis are usually fabricated from the 3′ end to the 5′ end, yielding microarrays that are valuable

tools for straightforward hybridization-based assays. Building an oligonucleotide chain from the 5′ end to the 3′ end leaves a free 3′-OH group, which has an additional use in microarrays: with a free 3′-OH, enzyme-based assays (e.g., using primer extension) become possible. Synthesis in this "reverse" direction can be achieved using the appropriate NPPOC-protected monomers described here and in *UNIT 12.3*.

The low-tech approach presented here is not useful for applications such as gene expression profiling, where hundreds or thousands of genes—each represented by up to 20 oligonucleotides—must be investigated. However, the simplicity and low cost associated with the preparation of these low-complexity microarrays make this approach ideal for applications in which the analysis of a small number of oligonucleotides can result in meaningful findings (e.g., SNP typing, virus typing, basic microarray technology studies).

Critical Parameters

The critical parameters for this method have been discussed elsewhere (see Strategic Planning and *UNIT 12.3* and *UNIT 12.4*).

Anticipated Results

The product yield is determined by the coupling efficiency as well as the efficiency of removal of the photolabile protecting groups. Whereas the observed coupling efficiency is comparable to that for standard DNA synthesis methods (98% to 99%), the trigger for high yields—and, therefore, the primary factor that determines the rate of failure sequences—is the type of photochemistry used. It has been shown, for example, that NPPOC protecting groups are removed more efficiently than are MeNPOC protecting groups.

Time Considerations

For a 25-mer array, the maximum number of synthesis cycles is $4 \times 25 = 100$ (i.e., the number of possible nucleotide choices at a given position times the number of nucleotides in the synthesized oligomers). Typically, 40 to 70 cycles are needed to build a 25-mer array, depending on the choice of sequences. Assuming the use of 10-min irradiation steps and 5-min synthesis cycles, a complete microarray synthesis can be expected to take 10 to 17 hours.

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**DNA Microarray
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12.5.10