

instructions

CodeLink Gene Expression System: Single-Assay Bioarray Hybridization and Detection

Warning

For research use only. Not recommended
or intended for the diagnosis of disease
in humans or animals. Do not use internally
or externally in humans or animals.



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CodeLink™ Expression Bioarray System

System description

CodeLink™ Expression Bioarray System comprises a set of bioarray products and tools for gene expression profiling experiments that allows monitoring of the mRNA levels of multiple genes simultaneously. The system includes:

- sets of carefully designed and validated bioarrays with integrated hybridization Flex Chambers that cover a wide range of discovery genes for several organisms
- target preparation and hybridization reagents
- optimized target preparation and processing protocols
- hybridization and post-hybridization parallel processing tools and fixtures
- bioarray quantitation analysis software

This user manual provides the protocol for single-assay bioarray processing, including hybridization, post-hybridization processing, and scanning of CodeLink™ Expression Bioarrays.

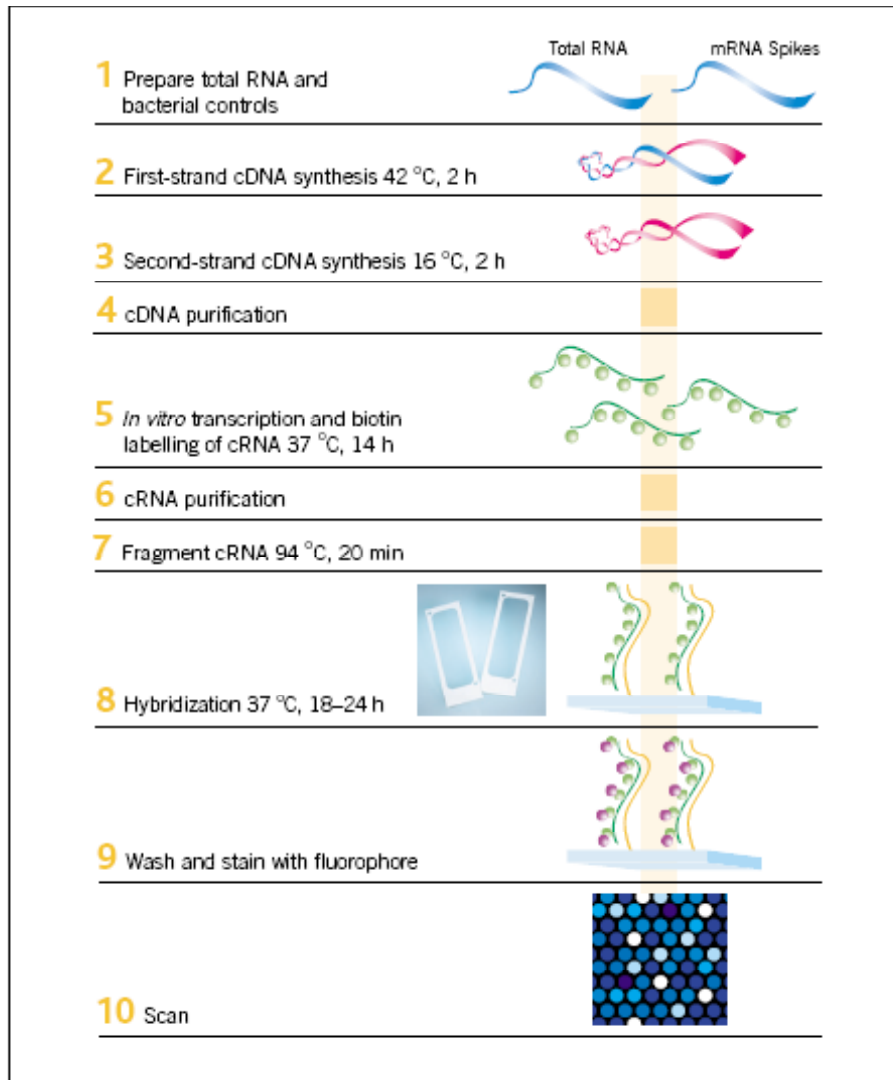


Fig. 1. CodeLink™ Expression Bioarray signals are produced by the hybridization of biotin-labelled complementary RNA (cRNA) target(s) to DNA oligonucleotide probes attached to a gel matrix followed by secondary labeling and signal detection.

Safety warnings and precautions

Consider all chemicals as potentially hazardous. Only persons trained in laboratory techniques and familiar with the principles of good laboratory practice should handle these products. Wear suitable protective clothing such as laboratory coats, safety glasses, and gloves. Exercise caution to avoid contact with skin or eyes; if contact should occur, wash immediately with water. See Material Safety Data Sheet for specific recommendations.

For each reagent required but not provided, follow the manufacturer's safety requirements.

Handling

These procedures involve working with RNA; therefore, exercise great care to avoid RNase contamination. All solutions must be RNase-free and pipette tips must be aerosol-resistant and changed before each step. Use commercially prepared nuclease-free water (rather than water treated with diethylpyrocarbonate (DEPC)) for all nucleic acid steps.

Storage

The CodeLink™ Expression Assay Reagent Kit components used in hybridization and detection should be stored as follows:

- Hybridization Buffer A: ambient temperature
- Hybridization Buffer B and 5× Fragmentation Buffer: 4 °C

Biotin-labelled cRNA target is prepared by a linear amplification method. The poly(A)⁺ RNA subpopulation (within the total RNA population) is primed for reverse transcription by a DNA oligonucleotide containing the T7 RNA polymerase promoter 5' to a d(T)₂₄ sequence (Fig 1). After second-strand cDNA synthesis, the cDNA serves as the template for an in vitro transcription (IVT) reaction to produce the target cRNA. The IVT is performed in the presence of biotinylated nucleotides to label the target cRNA. This method produces approximately 1000-fold to 5000-fold linear amplification of the input poly(A)⁺ RNA.

A set of bacterial control mRNAs is included in CodeLink™ Expression Assay Reagent Kit as controls for the cDNA synthesis and the IVT reactions. These controls are added to the total RNA sample during target preparation. Each step of the CodeLink™ Expression Bioarray processing procedure, including target preparation and hybridization, can be monitored using these control mRNAs. Additionally, the bacterial control mRNAs can be used to estimate the sensitivity of RNA detection.

Hybridization is performed overnight in a temperature-controlled shaking incubator. Optimized hybridization buffer components are also included in CodeLink™ Expression Assay Reagent Kit for use at this step.

Post-hybridization processing includes a stringent wash to remove unbound and non-specifically hybridized target molecules, a staining step with a CyTM5-Streptavidin conjugate, and several non-stringent washing steps to remove unbound conjugate. Following a final rinse, the bioarrays are dried by centrifugation and scanned. Analysis of the bioarrays with the CodeLink™ Expression Analysis software is described in the Help accessible in the software.

CodeLink™ Gene Expression System: Single-Assay Bioarray Hybridization and Detection

Protocol overview

- 1. Fragment cRNA.** In 25 µl total volume, add 10 µg of cRNA to 5 µl of 5× fragmentation buffer and incubate at 94 °C for 20 minutes.
- 2. Prepare hybridization reaction mixture.** Bring 10 µg of fragmented cRNA, 78 µl of hybridization buffer component A, and 130 µl of hybridization buffer component B to a final volume of 260 µl with water. Incubate at 90 °C for 5 minutes and immediately chill on ice for 5–30 minutes.
- 3. Loading reaction mixtures into array chambers.** Slowly inject 250 µl of hybridization reaction mixture into array input port and seal ports with sealing strips.
- 4. Hybridization.** Set the shaker speed to 300 rpm and incubate slides for 18–24 h at 37 °C, maintaining a consistent hybridization time for comparative experiments.
- 5. Post-hybridization wash.** Remove the Flex Chamber using the hybridization removal tool to hold the bioarray in place while peeling the Flex Chamber off at a 60° angle. Then, place the bioarrays into the bioarray rack while it sits inside the medium reagent reservoir containing 0.75× TNT. Transfer the bioarray rack to the large reagent reservoir containing preheated 0.75× TNT, and incubate at 46 °C for exactly 1 h. Do not exceed 1 h incubation.
- 6. Detection with streptavidin-dye conjugate.** Fill each slot of the small reagent reservoir with 3.4 ml of Cy5-Streptavidin working solution. Transfer the bioarray rack from the large reagent reservoir into the small reagent reservoir and incubate bioarrays at ambient temperature for 30 minutes. Wash the bioarrays four times with 1× TNT (5 minutes each wash) in large reagent reservoirs. Rinse the bioarrays in 0.1× SSC/0.05% Tween™ for 30 seconds. Immediately follow the rinse with centrifugation to dry bioarrays, and store dried bioarrays in the dark.
- 7. Bioarray scanning and analysis.** Scan bioarrays and analyze with CodeLink™ Expression Analysis software. Scanning with GenePix™ 4000B is described in this booklet. For use of CodeLink™ Bioarrays with other scanners, visit the CodeLink™ website (www.appliedmicroarray.com).

Products used for processing

CodeLink iExpress Assay Reagent Kit, Manual Prep, 24 reactions	67601000
5× fragmentation buffer	
hybridization buffer component A	
hybridization buffer component B	
Cy5-Streptavidin for Microarrays (staining solution is a 1:500 dilution in TNB; see Appendix 1)	28900224
CodeLink Expression Analysis software v5	310035
CodeLink Shaker Kit, 12 slides	310031
CodeLink Parallel Processing Kit, 12 slides	310010

Use only high-quality cRNA generated with a CodeLink Expression Assay target preparation protocol for the following steps to achieve optimal results.

Avoid physical contact with the probe-containing area of the bioarray. Handle bioarrays on the edges throughout the procedure.

Exercise caution to avoid RNase contamination. All solutions must be RNase-free; pipette tips must be changed before each step. Use nuclease-free water for all nucleic acid steps. Use aerosol-resistant tips for all pipetting steps.

To avoid fluorophore photobleaching, prepare the Cy5-labelled Streptavidin conjugate in opaque or dark tubes. In addition, minimize bioarray exposure to ambient light, particularly after completing step 6.1.

Ensure that the ambient temperature in the laboratory is 23 °C ± 2 °C.

Minimize exposure to particulate matter. Clean all work areas prior to hybridization and analysis.

The list of reagents and equipment is recommended for best performance. Contact Applied Microarrays, Inc. technical support for information on use of alternatives.

Caution: Gently vortex and briefly centrifuge all reagent tubes prior to use.

Other materials required for processing

All reagents used in the protocol should be molecular biology grade or higher.

- 0.5% NEN blocking reagent (PerkinElmer, FP1020)
- nuclease-free water
- 1 M Tris-HCl, pH 7.6
- 5 M NaCl
- Tween 20
- 20× SSC (Ambion, 9763)
- Isopropanol (to wash reagent container for TNT preparation)
- GenePix 4000B Array Scanner (Axon Instruments) and computer configured for GenePix 4000B Array Scanner or other CodeLink™ validated scanner (see www.appliedmicroarrays.com for CodeLink™ validated scanner application notes.)
- centrifuge, 4-15C (QIAGEN Corp, 81010 or similar) with appropriate centrifuge plate rotor, 2 × 96 (QIAGEN Corp, 81031). For alternative centrifuges, contact Applied Microarrays, Inc. technical support or visit our Web site.
 - pipette tips, sterile, RNase-free, and aerosol-resistant
 - 1.7-ml microcentrifuge tubes, sterile and RNase-free
 - 15- and 50-ml conical tubes: sterile, nuclease-free
 - wide bore pipette tips: sterile, RNase-free, and aerosol-resistant
 - vortex mixer
 - water bath (settings 90 °C and 46 °C)
 - pipettors
 - microcentrifuge
 - thermal cycler
 - powder-free gloves
 - microtiter plate lid, black (Corning Corp, 3935)

Storage

Store hybridization buffer component A at room temperature. If a precipitate forms upon storage, briefly vortex prior to use to ensure solution homogeneity. If necessary, warm at 37 °C to redissolve.

Store hybridization buffer component B at 4 °C until use.

Keep 5× fragmentation buffer at 4 °C until use. Briefly vortex and spin down prior to use to ensure solution homogeneity.

For steps 1–2, use of a 10% volume mixture overage is recommended but not required.

Protocol for hybridization and detection

1

Fragmentation of cRNA

1.1 For each bioarray to be loaded, bring 10 µg of cRNA (from step 6.13 of CodeLink™ target preparation protocol) to a final volume of 20 µl with nuclease-free water in a thin-walled microcentrifuge tube.

1.2 Add 5 µl of 5× fragmentation buffer for each bioarray. Place tube in a thermal cycler and heat for 20 minutes at 94 °C using the heated lid feature.

1.3 Cool to 0 °C in the thermal cycler for at least 5 minutes.

2

Preparation of hybridization reaction mixtures

2.1 Set the temperature of the shaker-incubator to 37 °C for hybridization. Assemble bioarray tray posts from the CodeLink™ Shaker Kit onto the incubator platform.

2.2 Transfer the cRNA sample from the thin-walled tube to a 1.5-ml microcentrifuge tube. For each bioarray to be processed, prepare 260 µl of hybridization solution containing 10 µg of fragmented target cRNA in a 1.5-ml microcentrifuge tube:

78 µl hybridization buffer component A
130 µl hybridization buffer component B
27 µl nuclease-free water
25 µl fragmented cRNA (from Step 1)
260 µl total volume

2.3 Vortex the solution for 5 seconds at maximum speed. Incubate the hybridization solution at 90 °C for 5 minutes to denature the cRNA.

If performing replicate bioarrays, do not prepare more than 4 reactions (~1 ml) in a single microcentrifuge tube. Larger volumes will not allow good heat transfer.

2.4 Cool the tube(s) on ice for at least 5 and no more than 30 minutes. Load all bioarrays within 30 minutes of denaturing the cRNA.

3

Loading of reaction mixtures into bioarray chambers

3.1 Set the 12-slide shaker tray on a level surface. Place the bioarrays into the shaker tray with the input/output ports facing up. Load bioarrays in sets of 12 or less.

3.2 Vortex the hybridization reaction mixture for 5 seconds at maximum speed. Centrifuge briefly to gather the liquid at the bottom of the tube. Place the tube back on ice.

3.3 For each bioarray chamber, draw 250 µl into a 1-ml wide-bore pipette tip. Place the 1-ml pipette tip containing the hybridization solution over the array input port closest to the right edge of the slide label and press until the tip forms a seal with the chamber (see Appendix 5 for details).

Caution: Ensure that the target sample is loaded very slowly and carefully to avoid introduction of bubbles into the chamber.

3.4 Slowly inject the entire sample into the Flex Chamber without using the blowout feature of the pipettor. When the Flex Chamber is full, maintain thumb pressure on the pipettor, hold down the bioarray over the tab region with index finger of opposite hand, and remove the pipette tip from the input port. Discard any excess target mix remaining in the pipette tip. Aspirate any excess fluid

surrounding the outside of the port with a pipette tip and discard. Use a lint-free wipe to blot residual fluid from around the port, taking care not to actually touch the port.

3.5 After loading up to 12 bioarrays, seal the Flex Chamber ports using the sealing strips and port sealing tool as described in Appendix 5. Do not touch the Flex Chamber or directly depress the port.

4

Hybridization

4.1 Align the 12-slide shaker tray notches with the front and back posts fixed to the shaker-incubator platform to place the loaded shaker tray into the shaker-incubator. The Flex Chamber should be facing up.

4.2 Set the shaker speed to 300 rpm and incubate slides for 18–24 h at 37 °C. It is critical that arrays used in any form of comparison are hybridized for the same amount of time within the given range.

4.3 To prepare for the next step, fill a large reagent reservoir with 240 ml of filtered 0.75× TNT buffer. Cover the reservoir and incubate in a 46 °C water bath overnight.

Refer to Appendix 4 for identification of the processing fixtures.

Filter all TNT buffer solutions (Appendix 1) through a 0.2- μ m filter prior to use.

Prepare sufficient volumes of 1 \times and 0.75 \times TNT buffers (Appendix 1) for the entire procedure. Approximately one liter of 1 \times TNT, and 500 μ l of 0.75 \times TNT are needed for 12 bioarrays.

At the beginning of each wash step, gently agitate the bioarray rack.

Avoid contact with the probe-containing surface of the microarray throughout the post-hybridization steps.

Do not allow the bioarrays to dry out during any of the processing steps.

Clean the shaker incubator platform and the shaker tray with deionized water after each hybridization run.

Ensure that the temperature of the 0.75 \times TNT buffer placed in the large reagent reservoir is 46 $^{\circ}$ C.

Take care to prevent contamination of the fluid in the large reagent reservoir with water from the water bath.

5

Post-hybridization wash

5.1 Fill each slot in the medium reagent reservoir with 13 ml of filtered 0.75 \times TNT. Place the bioarray rack into the reservoir. Leave at ambient temperature.

5.2 Remove bioarrays from the incubator one by one and keep the remaining bioarrays at 37 $^{\circ}$ C until ready for processing. This will ensure that the slides are still warm and aid with the ease of peel.

5.3 Place the bioarray to be processed into the Flex Chamber removal tool.

5.4 Remove the Flex Chamber by lifting the tab and slowly peeling the chamber from the bioarray at a 60 $^{\circ}$ angle.

Chamber Removal Hints: The peel angle prevents creasing of the Flex Chamber. Carefully observe the slide surface while peeling. If, as you peel, adhesive is separating from the Flex Chamber and sticking to the bioarray surface, then slow down the rate of peeling. A slow peel of approximately 3–5 s is critical to ensure that no adhesive remains on the bioarray after the chamber is removed. It should not take more than 5 minutes to process 12 slides during this step. If adhesive is visible on one edge of the bioarray, place that edge down in the bioarray rack slot (step 5.5).

5.5 Place the bioarray into a slot of the bioarray rack prepared in step 5.1. Use the bioarray position tool, tooth-side down, to ensure the bioarrays are properly seated.

5.6 To avoid potential cross-contamination, rinse the surface of the Flex Chamber removal tool with approximately 5 ml of ambient temperature 0.75 \times TNT buffer dispensed from a squirt bottle. Keep the medium reagent reservoir at ambient temperature until all bioarrays have been processed.

5.7 Repeat steps 5.3–5.6 for each hybridized bioarray.

5.8 Transfer the bioarray rack with bioarrays from the medium reagent reservoir into the pre-warmed, 0.75 \times TNT-filled large reagent reservoir from step 4.3. Replace the lid on the large reagent reservoir and then on the water bath. Incubate at 46 $^{\circ}$ C for exactly 1 h; longer incubation time may significantly reduce signal intensities.

If processing 12 bioarrays or less during secondary labeling, prepare a centrifuge balance unit (medium reagent reservoir with bioarray rack and blank glass slides) for the bioarray drying step. Recall that ambient temperature is $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

6.

Detection with streptavidin-dye conjugate

6.1 Fill each slot in the small reagent reservoir with 3.4 ml of Cy5-Streptavidin working solution (Appendix 1). Leave at ambient temperature. Cover the small reagent reservoir with a black lid to prevent photobleaching of the fluorophore by ambient light.

6.2 Remove the bioarray rack with bioarrays from the large reagent reservoir at 46°C , and place into the small reagent reservoir containing the Cy5-Streptavidin working solution. Cover with the lid and incubate bioarrays at ambient temperature for 30 minutes.

6.3 During incubation, prepare for the wash steps by filling three large reagent reservoirs each with 240 ml of ambient temperature $1\times$ TNT buffer.

6.4 After the 30 minutes incubation, remove the bioarray rack with bioarrays from the staining solution and place into one large reagent reservoir containing $1\times$ TNT buffer (prepared in 6.3). Do not drain the solution from the bioarrays. Following an initial gentle up-and-down agitation, incubate the bioarrays at ambient temperature for 5 minutes covered from light.

6.5 Remove the bioarray rack from the first large reagent reservoir with $1\times$ TNT buffer and place into a second large reagent reservoir containing $1\times$ TNT buffer. Again, do not attempt to drain the solution from the bioarrays and gently agitate the bioarray rack.

Incubate at ambient temperature for 5 minutes, covered from light. Repeat this step with two additional large reagent reservoirs containing fresh $1\times$ TNT buffer for a third and fourth wash.

6.6 During the third wash, thoroughly rinse a large reagent reservoir with distilled water and dry it. Completely fill this reservoir with the final rinse $0.1\times$ SSC/ 0.05% Tween 20 solution (Appendix 1).

6.7 Transfer the bioarray rack into the large reagent reservoir completely filled with $0.1\times$ SSC/ 0.05% Tween 20 at ambient temperature. Incubate the slides for 30 seconds while continually agitating mildly up and down.

6.8 Remove the bioarray rack from the large reagent reservoir and blot the bottom edge of the bioarrays briefly on an absorbent paper towel. Place the bioarray rack in a clean, dry medium reagent reservoir. Dry the bioarrays by centrifugation in the Qiagen Sigma 4-15C centrifuge with corresponding bucket rotor (2×96 -well plate) or similar system using following settings:

speed:	2000 rpm ($644 \times g$)
acceleration:	9
deceleration:	9
time:	3 minutes

Caution: Move into step 6.8 (the drying step) immediately because slides may dry non-uniformly in the air, leading to non-uniform signal loss.

Remember to use a centrifuge balance unit if processing less than 12 bioarrays.

6.9 To easily remove the bioarrays from the bioarray rack, place the bioarray rack with slides into the bioarray removal tool. Place the dry bioarrays into a light-protected slide box until they are scanned. Bioarrays should be scanned within two days of assay completion.

Note: On high density products (such as Whole Genome bioarrays), an outline of the removed Flex chamber will be visible on the bioarray surface; whereas the 10K and 20K products will not show this outline. This will not affect the bioarray performance.

6.10 Repeatedly rinse all reservoirs with deionized water to clean, and invert to dry.

6.11 Wash the rack with Alconox™ soap, scrubbing between the rails with a pipe cleaner style brush. Rinse thoroughly with deionized water to remove residual soap. Air-dry the rack.

Keep the bioarrays covered until they are scanned to protect them from dust and light and handle them with gloves.

For best results, scan bioarrays within two days of staining.

If bioarrays will be retained for future scanning, they should be stored in a dry box protected from light.

If regions of high background on the bioarray are noted upon scanning, the bioarrays may be rerinsed by repeating steps 6.7 and 6.8 from above.

Not all incidents of high background can be removed by rerinsing. Refer to the manufacturer's manual for use and maintenance of the scanner (1).

7

Bioarray scanning and analysis

For scanning with a GenePix Array Scanner, use the following steps. For information on use of alternative scanners with CodeLink™ bioarrays, visit the Applied Microarrays, Inc. website (www.appliedmicroarrays.com).

- 7.1 Turn the scanner on 15 minutes prior to use.
- 7.2 Open the cover to expose the slide holder.
- 7.3 Lift the latch of the slide holder and lift the upper clip.
- 7.4 Wearing latex gloves, load the bioarray into the tray with the label side down and closest to the front of the scanner.
- 7.5 Pull the clip on the left of the slide out and let the bioarray fall into place. Release the clip to put pressure against the bioarray.
- 7.6 Grab bioarray by the edges and move the bioarray toward you.
- 7.7 Lower upper clip and press down on the latch until it clicks.
- 7.8 Slide the cover to the right to cover the slide holder.
- 7.9 Open the GenePix software and select the following settings:
- | | |
|-----------------|--|
| settings file: | product-specific .gps file |
| wavelength: | 635 nm |
| PMT voltage: | 600 V |
| laser power: | 100% |
| pixel size: | 5 μ m for high density bioarrays (such as Human Whole Genome), 10 μ m for 10K and 20K products |
| focus position: | 0 μ m |
- 7.10 In the Report tab, open the scanning script by clicking **Scan CodeLink Slide**.
- 7.11 Enter the bioarray serial number and click **Next**. The Experiment and Scan Information interface is displayed.
- 7.12 Type in the project name, experiment name, and sample name. The username is automatically captured. When opening this interface for the first time, a message box may ask whether to allow an ActiveX interaction to proceed. Click **Yes**.
- 7.13 Select a settings (.gps) file, which can be found on the product CD for high density products. If a settings file was previously selected, the name and path are displayed under Current Settings File. To select a new file, click **Browse** under Select New Settings File. The values for project name, experiment name, user name, and settings file that were entered for a previous bioarray are retained but may be changed.
- 7.14 In the Load and Scan Slide screen, the standard TIF file name for the current bioarray is displayed. If desired, the name may be modified, however, the slide number as the first part of the name cannot be altered.

7.15 Click **Browse** to select the image path or the location where the image files will be stored. If a Security Alert message box is displayed, click **Yes**.

7.16 Click **Scan Slide**. The Image tab will display, and the instrument will perform the scan.

7.17 When complete, the view will return to the Report tab.

7.18 Click **Save Image** to save the scanned image at the appropriate file location.

7.19 Slide the cover to the left and remove the bioarray.

7.20 To scan the next bioarray, click **New Slide** and enter the serial number for the next bioarray. The setting information previously entered will be retained.

7.21 Analyze the image from each bioarray using CodeLink™ Expression Analysis software.

Appendix 1: Stock solutions

1× TNT buffer (10 liters)

0.10 M Tris-HCl, pH 7.6
0.15 M NaCl
0.05% Tween 20

1. Prepare sufficient volumes of 1× TNT buffer for the entire procedure. Approximately 1 liter of 1× TNT is needed for 12 bioarrays.
2. Rinse a 10-liter reagent container with 150 ml isopropanol. Rinse the carboy twice with 2 liters of deionized water and completely drain the reagent container.
3. In a separate container, add the following:
1 liter 1 M Tris-HCl, pH 7.6
300 ml 5 M NaCl
5 ml Tween 20
8.7 liter deionized water
4. Mix well by swirling. Filter TNT through a 0.2 µm filter and transfer to reagent container. This solution can be stored up to two weeks at ambient temperature.

0.75× TNT buffer

1. Prepare a sufficient volume of 0.75× TNT buffer for the entire procedure. Approximately 500 ml of 0.75× TNT is needed for 12 bioarrays.
2. Add 25 ml of deionized water to 75 ml of 1× TNT buffer per 100 ml of buffer required.
3. This solution should be stored similarly to 1× TNT, in a isopropanol-rinsed reagent container.

TNB Buffer (0.6 liters)

0.1 M Tris-HCl, pH 7.6
0.15 M NaCl
0.5% NEN blocking reagent (PerkinElmer, FP1020)

1. To a clean 2-liter Erlenmeyer flask with a stir bar, add the following:
522 ml nuclease-free water
60 ml 1 M Tris-HCl, pH 7.6
18 ml 5 M NaCl
2. On a stir plate heated to 60 °C, slowly add 3 g of NEN blocking reagent in 0.5 g increments until all 3 g of blocking reagent are dissolved.
3. Turn off heat and continue mixing for 30 minutes. While the solution is still warm, filter through a 0.88 µm filter.

4. Aliquot the TNB buffer into 50- μ l tubes and store at -20 °C. TNB can be stored for 12 weeks at -20 °C.

5. For use, thaw TNB overnight at 4 °C. Thawed aliquots can be stored at 4 °C up to one week.

Minimize exposure of dye-conjugate reagents to ambient light.

Cy5-Streptavidin stock solution

1. Add 1 ml of nuclease-free water to the 1 mg bottle of lyophilized Cy5-Streptavidin. This reagent already contains buffer components. To mix, pipette gently up and down or vortex with moderate speed. Do not allow foam to form. Place on ice for 5 minutes. Repeat the mixing three additional times, placing on ice between mixings. Protect this solution from light at all times.

2. Aliquot the Cy5-Streptavidin solution for the number of bioarrays processed in a typical run. For example, dispense 90 μ l aliquots for a 12-bioarray run.

3. This solution can be stored up to six months (or until expiry date) at -20 °C, protected from light. Once thawed, unused portions of aliquots may be used for up to one week if stored protected from light at 4 °C. Avoid multiple freeze-thaw cycles.

1:500 Cy5-Streptavidin working solution

1. Prior to dilution, centrifuge the thawed Cy5-Streptavidin stock for 1 minutes at = 8000 \times g to remove any precipitates.

2. Add 6.8 μ l of the Cy5-Streptavidin stock solution to 3393.2 μ l of filtered TNB buffer for each bioarray to be processed. Mix gently by inversion. Use this working solution within 15 minutes of preparation.

0.1 \times SSC / 0.05% Tween 20 (1 liter)

20 \times SSC (Ambion, 9763)

Tween 20

1. Make sure all solutions are molecular biology grade. Prepare sufficient volume of 0.1 \times SSC/0.05% Tween buffer for the entire rinsing or re-rinsing procedure. Approximately 250 ml of 0.1X SSC/0.05% Tween buffer is needed for 12 slides.

2. In a clean 1-liter container, add the following:

994.5 ml	deionized Water
0.5 ml	Tween 20
5 ml	20 \times SSC buffer

Mix well by swirling. This solution can be stored up to 2 weeks at ambient temperature.

Appendix 2: Bacterial mRNA control use interpretation

Extracting Positive Control Data from CodeLink™ Expression Analysis Software

Under the Expression Analysis menu button in the CodeLink™ Expression Analysis software version 4.0, select the Control Probes Report. Select to use the Positive Controls with spots of all quality flags and update the graph. The graph can be easily copy/pasted for record-keeping purposes.

Interpretation

The final mass ratios of the bacterial control mRNAs to the total RNA for the dilutions given are:

1:10 000 000 for *araB*, *entF*, *fixB*, *gnd*, *hisB* and *leuB*

For a typical mammalian cell, mRNA comprises 1–5% of the total RNA content. If the proportion of mRNA content within total RNA for a particular tissue is unknown, then an estimate of the mass ratios of the bacterial control mRNAs to the target mRNA within the total RNA can be made by assuming a midpoint of this range, or 3% mRNA content. The above total RNA mass ratio then corresponds to an estimated mRNA mass ratio of 1:300 000 for all the bacterial transcripts. The normalized signals from these controls can be graphed, generating sensitivity plots like that shown in Figure 2 below. The noise level for each probe is indicated by the red line and the positive control probe signal above that line shows that the ability to detect signal above noise is at an estimated sensitivity of one copy per cell. Good system performance is indicated if >70% of the positive controls probes exhibit signal above their local noise.

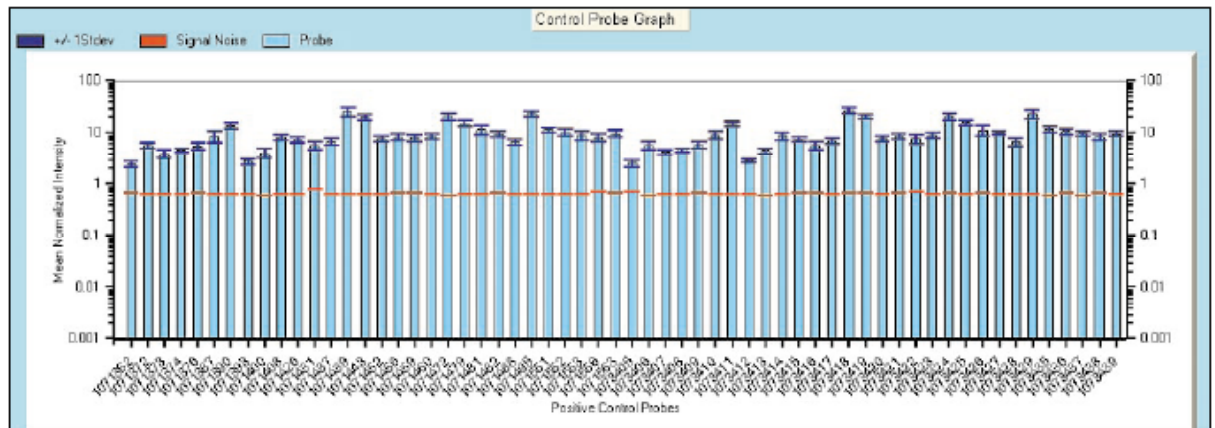


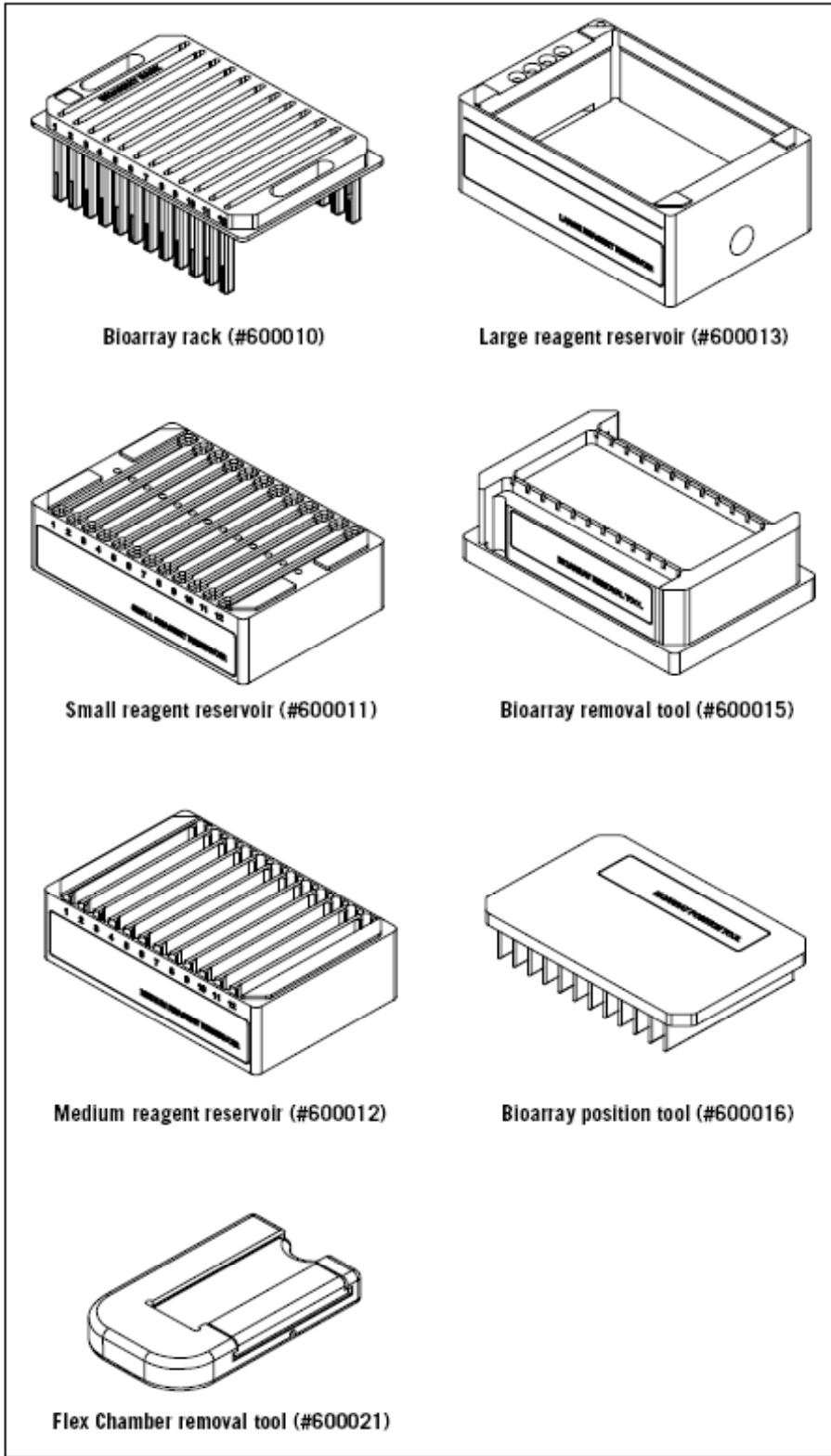
Fig 2. Typical sensitivity plot showing that normalized signal from each bacterial control probe is above noise for the set of control mRNAs spiked at an estimated mass ratio of 1:300 000.

Appendix 3: Precipitation of cRNA

It may be necessary to remove contaminants in the cRNA solution by ethanol precipitation.

1. Transfer the cRNA from step 6.13 of the target preparation protocol to a new 1.5-ml microcentrifuge tube. Add 50 μ l (0.5 volumes) of ice-cold 5 M ammonium acetate, and vortex for 5 seconds at maximum speed. Add 375 μ l (2.5 volumes) of ice-cold absolute ethanol to sample and vortex for 5 seconds at maximum speed. Incubate the sample at least 30 minutes at -20 $^{\circ}$ C.
2. Centrifuge at = 10 000 \times g for 15 minutes at 4 $^{\circ}$ C. Carefully remove and discard the supernatant.
3. Add 0.5 ml 70% ethanol and vortex for 5 seconds to wash pellet.
4. Centrifuge at 4 $^{\circ}$ C for 10 minutes at = 10 000 \times g. Carefully remove and discard supernatant. Remove any recalcitrant drops using a 200- μ l pipette tip. Air-dry the pellet.
5. Resuspend cRNA in 20 μ l nuclease-free water. Quantify the precipitated cRNA by UV spectrophotometry as detailed in section 7 of the manual target preparation protocol.
6. Store the cRNA at -70 $^{\circ}$ C.

Appendix 4: CodeLink™ Parallel Processing Kit



Appendix 5: Sealing the Flex Chamber port

1. Place the Sealing Strips to achieve the seals illustrated in Figure A for the 2-port Flex Chamber style and in Figure B for the 4-port Flex Chamber style.
2. Press the Sealing Strip against the port using the Port Sealing Tool (Figure C).

Adhesive will attach firmly at first contact. Use caution to ensure that the Sealing Strip is in the proper orientation before placing it on the chamber. **Do not remove a misaligned Sealing Strip.** Instead, place a second strip to cover any open ports.

Fig A. Proper placement of the Sealing Strips over the 2-port Flex Chamber ports.

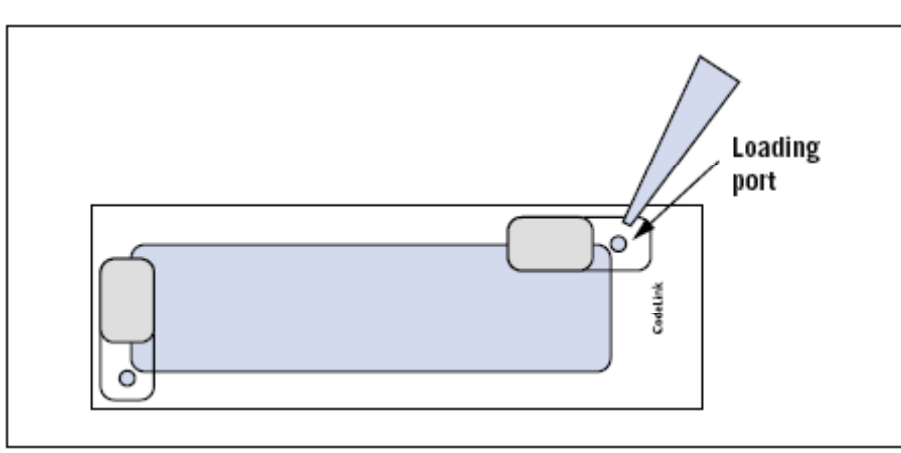


Fig B. Proper placement of the Sealing Strips over the 4-port Flex Chamber ports.

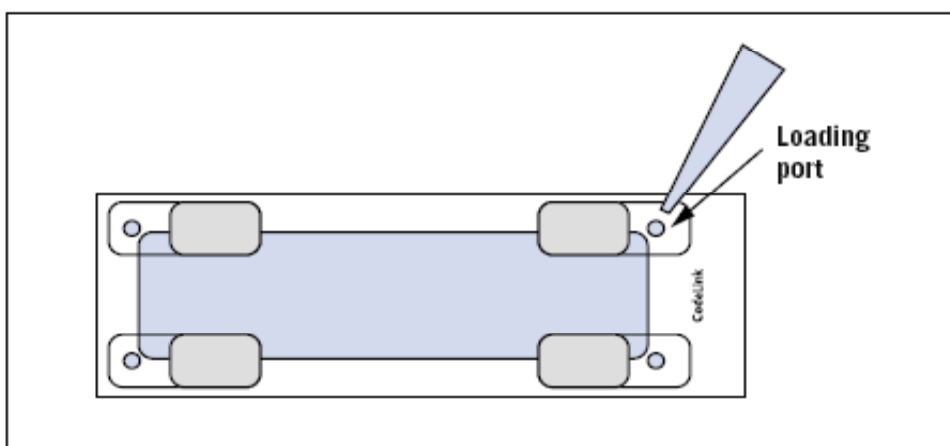
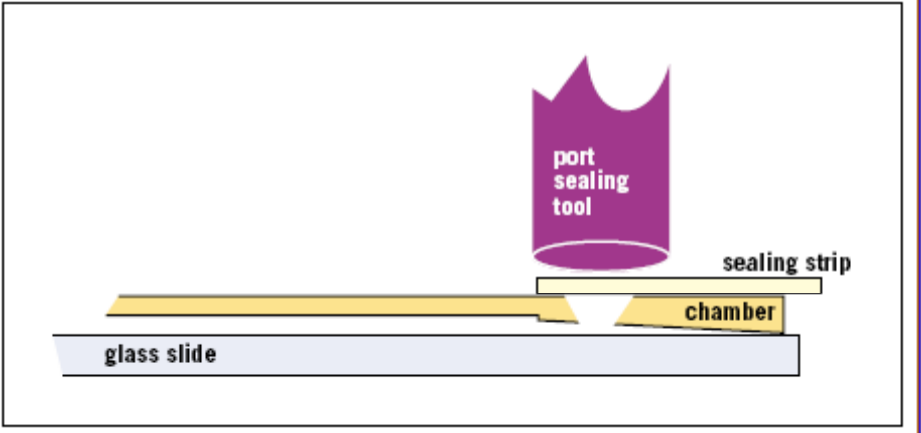
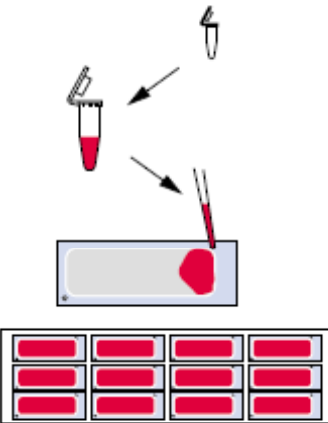


Fig C. Proper port sealing technique.



Appendix 6: Process Flow Charts
 Bioarray Hybridization and Detection

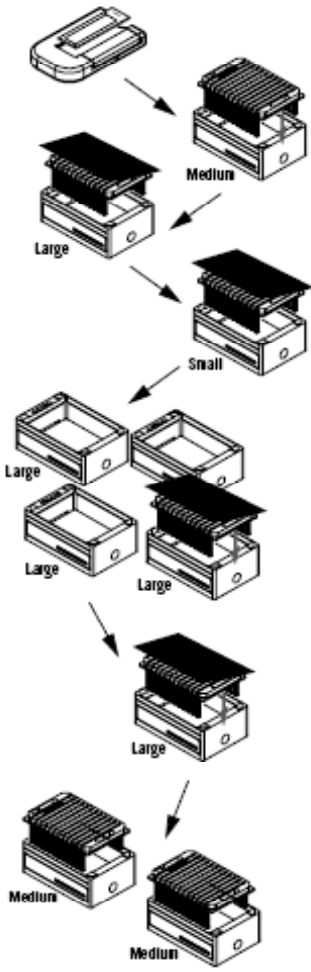


Fragment cRNA:
 add 5× Fragmentation Buffer to cRNA, 94 °C, 20 min

Prepare Hybridization Mix:
 mix fragmented cRNA, Hyb Buffer A, and Hyb Buffer B

Load Slides:
 Carefully pipette 250 µl into chamber;
 seal ports;
 300 rpm shaker-incubator, 37 °C, 18–24 h

CodeLink Bioarray Post-Hybridization Processing



Do not allow slides to dry out during the following steps.

After 18 h or 24 h hybridization at 37 °C, remove Hybridization Chamber from each slide and place slides in Holding Medium Reservoir.

Holding Reservoir:
 0.75× TNT, 23 °C ± 2 °C,
 ≤ ~10 min

Stringent Wash:
 0.75× TNT, 46 °C,
 EXACTLY 1 h

Secondary Labelling:
 SA-Cy5 Staining Solution,
 ambient temperature, 30 min

Wash:
 4 separate washes of 1× TNT,
 ambient temperature, 5 min each

Final Rinse:
 1 rinse of 0.1× SSC/0.05% Tween,
 30 s
Move immediately to next step.

Spin Dry:
 Centrifuge at 644 rcf, 3 min

References

1. *GenePix 4000 User's Guide*, Axon Instruments, Inc., 2500-136 Rev E (2001).

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