

FairPlay III Microarray Labeling Kit

INSTRUCTION MANUAL

Catalog #252009 (10 reactions) and #252012 (30 reactions)

Revision C

Now Includes
Random Primers



For In Vitro Use Only

252009-12

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FairPlay III Microarray Labeling Kit

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FairPlay® III Microarray Labeling Kit

MATERIALS PROVIDED

Materials Provided	Quantity	
	Catalog #252009 10 reactions	Catalog # 252012 30 reactions
10× AffinityScript RT Buffer	50 µl	150 µl
20× dNTP mix with amino allyl dUTP	10 µl	30 µl
Oligo d(T) ₁₂₋₁₈ primer	20 µl (500 ng/µl)	60 µl (500 ng/µl)
Random primers (9-mers)	30 µl (500 ng/µl)	30 µl (500 ng/µl)
0.1 M DTT	30 µl	90 µl
RNase block (40 U/µl)	5 µl	15 µl
AffinityScript HC Reverse Transcriptase	30 µl	90 µl
DEPC water	500 µl	1.5 ml
20 mg/ml Glycogen	10 µl	30 µl
2× Coupling Buffer ^a	100 µl	300 µl
DMSO (high purity)	500 µl	1.5 ml
DNA-binding solution	2.5 ml	2 × 5.0 ml
Microspin cups ^b and 2-ml receptacle tubes	10	30

^a A visible precipitate may be seen in the 2× Coupling Buffer. Incubate the buffer at room temperature or 37°C to resolubilize the precipitate before use.

^b The capacity of the microspin cup is ~0.8 ml.

STORAGE CONDITIONS

DNA-Binding Solution, Microspin Cups and Receptacle Tubes: Room Temperature

All Other Components: –20°C

ADDITIONAL MATERIALS REQUIRED

GE Healthcare CyDye™ Reactive Dyes

CyDye™ Cy3 mono-Reactive Dye 5-Pack (Catalog #PA23001)

CyDye™ Cy3.5 mono-Reactive Dye 5-Pack (Catalog #PA23501)

CyDye™ Cy5 mono-Reactive Dye 5-Pack (Catalog #PA25001)

CyDye™ Cy5.5 mono-Reactive Dye 5-Pack (Catalog #PA25501)

ADDITIONAL MATERIALS REQUIRED (CONTINUED)

Invitrogen STP-Ester Dyes

Alexa Fluor® 555 and Alexa Fluor® 647 Reactive Dye Decapacks (Catalog #A32755)

Alexa Fluor® 555 Reactive Dye Decapacks (Catalog #A32756)

Alexa Fluor® 647 Reactive Dye Decapacks (Catalog #A32757)

Alexa Fluor® 488 Reactive Dye Decapacks (Catalog #A32750)

Alexa Fluor® 594 Reactive Dye Packs Decapacks (Catalog #A32751)

1 M NaOH

1 M HCl

100% Ethanol, molecular biology grade, DNase/RNase free

95% Ethanol, molecular biology grade, DNase/RNase free

75% Ethanol, molecular biology grade, DNase/RNase free (see “Microspin Cup Wash Buffer”)[§]

70% Ethanol, molecular biology grade, DNase/RNase free

Sulfolane [Sigma (Catalog #T22209)]

RNase/DNase-free H₂O [Invitrogen, Catalog #10977-015]

Tris-acetate agarose gel (see *Appendix*)

Minimal dye loading buffer[§]

DNA size standard, useful range 400–1000 bp

Ethidium bromide

UV transilluminator

10 mM Tris base, adjust pH to 8.5 with HCl (see “Microspin Cup Elution Buffer”)[§]

3 M Sodium Acetate, pH 4.5

ADDITIONAL MATERIALS REQUIRED FOR HYBRIDIZATION TO MICROARRAY

20× SSC buffer, high-quality, RNase/DNase-free[§]

10% (w/v) SDS solution, high-quality, RNase/DNase-free[§]

RNase/DNase-free H₂O, [Invitrogen, Catalog #750023]

Poly d(A)_{40–60}, 8 µg/µl [GE Healthcare, Catalog #27-7988-01]

Yeast tRNA, 4µg/µl [Invitrogen, Catalog #15401-011]

Microarray hybridization chambers

Microarray hybridization oven (with rotator)

Microarray scanner

Human COT-1 DNA®, 10 µg/µl[§] [Invitrogen Corporation, (Catalog #15279-011)] or

Mouse COT-1 DNA® [Invitrogen Corporation, (Catalog #18440-016)]

NOTICE TO PURCHASER

Some uses of the FairPlay III microarray labeling kit may require licenses from third parties in certain countries.

[§] See *Preparation of Reagents*.

INTRODUCTION

An increasing trend in identifying differentially expressed genes is the use of nucleic acid microarrays that contain hundreds or thousands of probes corresponding to genes of interest.¹ In these experiments, test and control mRNAs (targets) are converted by reverse transcription into cDNAs with labeled nucleotides. The labeled test and control cDNAs are then hybridized to probe genes on microarrays, and after unhybridized cDNA is removed, signal from the hybridized cDNA is detected. Differences in hybridization signals on the microarray correlate with differences in abundance of the mRNA used to prepare the labeled cDNA.

Various labeling protocols have emerged to convert test and reference mRNA into labeled cDNA in a reverse transcription reaction. One of the more popular methods is the direct incorporation of labeled nucleotides into the cDNA during the reverse transcription (RT) reaction. An inherent problem associated with this method is the biased incorporation of the different CY[™]-dyes into different samples, which can result in uneven distribution of fluorescence and/or overall low-level fluorescence in the resulting cDNA. This may be due to either the inability of the RT to efficiently incorporate the bulky CY-labeled dUTP or the insolubility of the CY-labeled dUTP under RT reaction conditions.

The FairPlay III Microarray Labeling Kit overcomes the dye incorporation bias problem, increasing the reliability and reproducibility of cDNA labeling of RNA targets, including those with secondary structure. The kit includes AffinityScript HC (high concentration) Reverse Transcriptase (AffinityScript HC RT), an engineered thermostable mutant derived from Moloney Murine Leukemia Virus (MMLV) reverse transcriptase. This improved reverse transcriptase exhibits enhanced RNA template binding capability which significantly improves reverse transcription on targets with secondary structure.

Dye bias problems are specifically avoided by dividing the labeling procedure into two parts: 1) preparing amino allyl modified cDNA and 2) chemically coupling the CY-dye to the modified cDNA. The kit contains components necessary to convert RNA to amino allyl modified cDNA, ready to be coupled to any fluorescent dye containing a NHS- or STP-ester leaving group. These include the fluorescent dyes in the Alexa Fluor[®] reactive dye decapack for microarray (Invitrogen) and in the CyDye mono-reactive dye packs (GE Healthcare).

When converting mRNA to fluorescence-labeled cDNA for use on microarrays it is often challenging to identify differentially expressed genes that are of low abundance and consequently low fluorescence intensity. The FairPlay III microarray labeling kit addresses this problem by doubling the yield of cDNA, as compared to the original FairPlay kit. This increase results in improved signal-to-noise ratios and increased hybridization signals with higher correlations. These improvements provide the highest cDNA yield, while maintaining an even representation of cDNA.

To generate fluorescence-labeled cDNA, total RNA is annealed to either oligonucleotide d(T) or random primers, and cDNA is synthesized in a reverse transcription reaction with AffinityScript HC RT, unlabeled dNTPs, and an amino allyl modified dUTP. The amino allyl labeled cDNA is treated with NaOH to hydrolyze the RNA strand. The labeled cDNA is then purified to remove unincorporated dNTPs, hydrolyzed RNA, and any Tris or other components of the labeling reaction that would inhibit the coupling reaction. The amino allyl labeled cDNA is then coupled to a fluorescent dye containing either a NHS- or STP-ester leaving group. The fluorescent dye-labeled cDNA is purified (using the DNA-binding solution and microspin cups provided) to remove uncoupled fluorescent dye prior to hybridization.

To prepare for the hybridization step, the labeled cDNA is then combined with blocking DNA, SSC buffer, and SDS (See *Preparation of Reagents*). The labeled cDNA is hybridized to the probe DNA on microarrays by incubating at 65°C for 14–18 hours. The unhybridized labeled cDNA is removed in two washing steps and the hybridized labeled cDNA is visualized using a microarray scanner. Specific DNA hybridization between the labeled cDNA and probe DNA on the microarray is promoted while undesired DNA hybridization is reduced by the addition of blocking DNA (see *Protocol Guidelines* for more information on blocking DNA sources).

The FairPlay III microarray labeling kit has been validated for use with ExonHit's SpliceArrays, with the minor modification of using random-hexamer labeling. A Technical Note describing this application is available through Stratagene Technical Support and at <http://www.stratagene.com/lit/notes.aspx>.

PROTOCOL GUIDELINES

- ♦ The quality and quantity of the RNA used is critical for successful preparation of labeled cDNA. The presence of cellular lipids, carbohydrates or proteins will significantly increase background fluorescence following hybridization. The guanidinium isothiocyanate method used in the Absolutely RNA miniprep kit is ideal for the isolation of total RNA for use in microarray applications. The OD 260/280 ratio of the RNA must be >1.8.
- ♦ We recommend analyzing the quality of the RNA based on the size distribution of recovered RNA molecules by microfluidics analysis using the Agilent 2100 Bioanalyzer. The Bioanalyzer uses electrophoretic separation on microfabricated chips to determine the quality of total RNA in a quantitative manner. The output provides an image of the RNA fragments including the 28S and 18S bands, similar to gel electrophoresis. Additionally, it calculates the RNA integrity number (RIN) using an algorithm. The instrument software uses the entire electrophoretic trace (all regions and peaks) to calculate the integrity of samples (RIN) using a scale of 1 to 10, with 1 being the most degraded and 10 being the most intact.

If the Agilent Bioanalyzer method is unavailable, analyze the RNA sample using agarose gel electrophoresis. When RNA isolated from mammalian sources is viewed on a denaturing agarose gel, the ribosomal bands (28S and 18S) should appear as two bright bands at approximately 4.5 and 1.9 kilobases. The ratio of intensities of the 28S and 18S bands should be 1.5-2.5:1. Lower ratios may indicate that RNA degradation has occurred and that this RNA may not be suitable for the preparation of labeled cDNA. Additional bands, including low molecular weight bands corresponding to the 5S ribosomal RNA and tRNA may also be visible.

- ♦ The presence of EDTA and/or ethanol can inhibit reverse transcriptase activity in the labeling reaction. While we recommend that the RNA be ethanol precipitated, all ethanol must be removed prior to use in the labeling reaction.
- ♦ It is imperative to protect the RNA from any contaminating RNases until the cDNA synthesis is complete. Wear fresh gloves, use newly autoclaved pipet tips, and avoid using pipet tips or microcentrifuge tubes that have been handled without gloves. Ribonuclease A cannot be destroyed by normal autoclaving alone. Baking or DEPC treatment is recommended.
- ♦ PolyA mRNA can be used instead of total RNA in this protocol. Simply replace the 10 µg of total RNA with 0.4–1 µg of polyA mRNA.
- ♦ The amount of total RNA used in this protocol can be reduced from 10 µg to 5 µg, however, less labeled cDNA will be generated.
- ♦ The quality of the fluorescent dye is critical. The use of partially degraded fluorescent dyes may result in a higher fluorescent background following hybridization. Do not resuspend the NHS-ester fluorescent dyes in an aqueous solution such as water.

- No differences have been observed when using sulfolane instead of ethanol for fluorescent dye-labeled cDNA purification.
- The fluorescence intensities of CY3- and CY5-labeled cDNA hybridized to microarrays under competitive hybridization conditions have a high degree of correlation. If other fluorescent dye pairs are used to label cDNA, we highly recommend that the same cDNA be labeled with each of the fluorescent dyes, hybridized to a microarray under competitive hybridization conditions, and the correlation between the fluorescence intensities calculated. The ideal correlation is 1.0 and indicates that there is a 1:1 correlation between cDNA labeled with each of the fluorescent dyes. The use of fluorescent dyes resulting in labeled cDNA with high correlation results in more accurate identification of differentially expressed genes than the use of fluorescent dyes resulting in labeled cDNA with low correlation.
- The recommended blocking DNA sources are either human or mouse COT-1 DNA[®] (See *Preparation of Reagents*), yeast tRNA, and poly d(A)₄₀₋₆₀. If human DNA containing repetitive sequences is spotted on the microarray, use human COT-1 DNA in the blocking solution. Human COT-1 DNA is placental DNA that is 50 to 310 bp in size and is enriched for repetitive DNA sequences such as the Alu and Kpn family.^{2, 3} If mouse DNA containing repetitive sequences is spotted on the microarray, use mouse COT-1 DNA in the blocking solution. Mouse COT-1 DNA is mouse DNA that is 50 to 300 bp in size and is enriched for repetitive DNA sequences such as the B1, B2, and L1 family members.^{4, 5} Including human or mouse COT-1 DNA in the hybridization mixture reduces undesired hybridization between repetitive DNA sequences in the labeled cDNA and probe DNA. Yeast tRNA reduces undesired nonspecific DNA hybridization. Poly d(A) that is 40 to 60 bases in length promotes specific hybridization between the labeled cDNA and probe DNA by reducing hybridization of the polyA sequences in the probe DNA to the polyT tract in the labeled cDNA.
- The recommended volume of hybridization solution containing labeled cDNA to use for probing DNA arrays is 2 µl per cm² of coverslip surface area. This volume of hybridization solution, prepared according to the protocols provided, is sufficient even for high-density microarrays. The basic protocol produces 20 µl of hybridization solution, which is sufficient for a total microarray surface area of 10 cm². Increasing the volume of hybridization solution to 4 µl per cm² may facilitate coverslip manipulation in some cases. Additional volume increases are not recommended, however, since high solution volumes may cause the coverslip to drift on the slide surface during hybridization. Decreasing the volume of hybridization solution below 2 µl per cm² of coverslip surface area is not recommended.
- The basic protocols provided will result in the optimal concentration of cDNA during hybridization for most applications. Appropriate adjustments in the cDNA concentration for special applications must be determined experimentally.

PROTOCOL

cDNA Generation

Prepare separate cDNA labeling reactions for each fluorescent dye you wish to use. This protocol produces a sufficient amount of labeled cDNA per reaction to hybridize to a total microarray surface area of approximately 10 cm².

1. For each labeling reaction, resuspend 10 µg of total RNA in 12.0 µl of DEPC water.

Note *If the addition of control mRNA is desired, for example to gauge the success of the labeling reaction, add it at this step, but ensure that the final volume at the end of step 1 is 12.0 µl.*

2. Add 1 µl of 500 ng/µl oligonucleotide d(T)₁₂₋₁₈ or 1 µl of 500 ng/µl random primer. Incubate at 70°C for 10 minutes. Cool on ice until ready for use.
3. Combine the following components in a sterile, RNase/DNase-free microcentrifuge tube:

2 µl of 10× AffinityScript RT buffer
1 µl of 20× dNTP mix
1.5 µl of 0.1 M DTT
0.5 µl of RNase Block (40 U/µl)

4. Add the annealed primer and RNA (from step 2) to the mixture.
5. Add 3 µl of AffinityScript HC RT and incubate at 42°C for 60 minutes.
6. Add 10 µl of 1 M NaOH and incubate at 70°C for 10 minutes to hydrolyze RNA.
7. Cool to room temperature slowly; do not cool on ice.
8. Spin tube briefly to collect contents.
9. Add 10 µl of 1 M HCl to neutralize the solution.

cDNA Purification

The cDNA must be purified to remove unincorporated nucleotides, buffer components and hydrolyzed RNA. We recommend performing an ethanol precipitation to purify the cDNA. Incomplete removal of the Tris and ethanol will result in lower amino allyl-dye coupling efficiency. Care must be taken to ensure that the pellet is completely dry (at the end of step 6) indicating complete removal of the ethanol before proceeding to the dye coupling reaction.

1. Add 4 μ l of 3 M sodium acetate, pH 4.5 to the reaction.
2. Add 1 μ l of 20 mg/ml glycogen to the reaction.
3. Add 100 μ l of ice-cold 95% ethanol.
4. Incubate at -20°C for at least 30 minutes. The reaction can be stored at this point for several days or up to 2 months.
5. Spin the reaction stored in ethanol at $13\text{--}14,000 \times g$ for 15 minutes at 4°C . Carefully decant supernatant.
6. Wash with 0.5 ml ice cold 70% ethanol and spin at $13\text{--}14,000 \times g$ for 15 minutes at 4°C . Carefully decant supernatant and allow to air dry. A vacuum dryer can be used to speed up the process but DO NOT overdry.

If coupling to NHS-ester containing dyes from GE Healthcare (see *Additional Materials Required*) is desired, follow the protocol below (*NHS-Ester Containing Dye Coupling Reaction* and *Dye-Coupled cDNA Purification*) to couple the dye and purify the modified cDNA.

If coupling to STP-ester containing dyes from Molecular Probes (see *Additional Materials Required*) is desired, follow the user's guide provided with the dye to carry out this reaction. Use the reagents provided with that kit to resuspend, couple and purify the cDNA. Following coupling and cDNA purification, proceed to *Analysis of Fluorescence-labeled cDNA (Recommended)*.

NHS-Ester Containing Dye Coupling Reaction (For Use with GE Healthcare Dyes)

Note *Do not scale-down the following dye coupling protocol. Performing the reaction using reduced volumes results in a significantly reduced coupling efficiency.*

1. Resuspend cDNA pellet in 5 µl of 2× coupling buffer. If pellet was overdried it will be difficult to get the pellet back into solution. Gently heat at 37°C for 15 minutes to aid in the resuspension process.

Note *A visible precipitate may be seen in the 2× Coupling Buffer. Incubate the buffer at room temperature or 37°C to resolubilize the precipitate before use.*

2. The first time a tube of dye is used, resuspend in 45 µl DMSO. Use the high-purity DMSO provided in the kit. Do not substitute another DMSO. Vortex gently to ensure the pellet is completely solubilized. The unused dye can be aliquoted in single use aliquots and stored at –20°C in the dark for several months.

Note *DMSO is hygroscopic and will absorb moisture from the air. Water absorbed from the air will react with the NHS ester portion of the dye and significantly reduce or eliminate dye:cDNA-coupling efficiency. To reduce absorption, allow the dye to reach room temperature before opening and store the DMSO at room temperature. Do not leave either the dye or DMSO uncapped when not in use. During storage, tightly cap the resuspended dye and store at –20°C in the dark.*

3. Add 5 µl of dye to the cDNA. If the dye was stored at –20°C prior to use, allow the dye to reach room temperature before opening the container.
4. Mix by gently pipetting up and down.
5. Incubate for 30 minutes at room temperature in the dark.

Dye-Coupled cDNA Purification

The following dye-coupled cDNA purification method has been optimized for use with the DNA-binding solution and microspin cups provided in this kit. Briefly, in the presence of a chaotropic salt (introduced by the DNA-binding solution, included in this kit), the dye-coupled cDNA binds to the silica-based fiber matrix seated inside the microspin cup. Washing steps are employed to remove buffer salts and uncoupled fluorescent dye from the bound cDNA. Finally the cDNA is eluted from the matrix using a low-ionic strength solution. Use of the DNA-binding solution and microspin cups provided in this kit in conjunction with alternative purification protocols is not recommended.

Preparation of 80% Sulfolane

Prepare 80% (v/v) sulfolane by diluting 100% sulfolane with RNase-free water.

Preparation of 10 ml of 80% sulfolane is sufficient for processing 30 cDNA purifications. To prepare 10 ml of 80% sulfolane, add 2 ml of RNase-free water to 8 ml of 100% sulfolane.

Note *100% sulfolane is a solid at room temperature. Prior to diluting the sulfolane, melt by incubating in a 37°C waterbath until liquefied (overnight incubation is convenient for this purpose). The 80% sulfolane solution is a liquid at room temperature, and may be stored at room temperature for at least one month.*

If particulate matter is observed, the sulfolane solution may be filtered using a 0.2 µm nylon filter.

Microspin Cup Purification of Labeled cDNA

1. Add 90 µl of RNase/DNase-free H₂O to the 10 µl labeled cDNA.
2. Combine 100 µl of the provided DNA binding solution and 100 µl of 80% (v/v) sulfolane. Mix well by vortexing. Make sure that the two solutions are well mixed prior to use.
3. Add the 200 µl of DNA-binding solution and sulfolane mixture to the labeled cDNA and mix by vortexing.
4. Using a pipet, transfer the mixture to a microspin cup that is seated in a 2-ml receptacle tube. (Exercise caution to avoid damaging the fiber matrix with the pipet tip.) Snap the cap of the 2-ml receptacle tube onto the top of the microspin cup.

Note *To ensure proper sample flow, use the receptacle tube that is provided with the microspin cups. Do not substitute another tube.*

5. Spin the tube in a microcentrifuge at maximum speed for 30 seconds.

Note *The labeled cDNA is retained in the fiber matrix of the microspin cup.*

6. Open the cap of the 2-ml receptacle tube, remove and retain the microspin cup, and discard the DNA-binding solution containing the uncoupled dye.
7. Combine 100 μ l of the DNA binding solution and 100 μ l of 80% (v/v) sulfolane. Mix well by vortexing. Make sure that the two solutions are well mixed prior to use.
8. Add the 200 μ l of DNA-binding solution and sulfolane mixture to the microspin cup. Snap the cap of the receptacle tube onto the top of the microspin cup.
9. Spin the tube in a microcentrifuge at maximum speed for 30 seconds.
10. Open the cap of the 2-ml receptacle tube, remove and retain the microspin cup, and discard the solution in the receptacle tube.
11. Add 750 μ l of 75% ethanol to the microspin cup (see “Microspin Cup Wash Buffer” in *Preparation of Reagents*). Snap the cap of the receptacle tube onto the top of the microspin cup.
12. Spin the tube in a microcentrifuge at maximum speed for 30 seconds.
13. Open the cap of the 2-ml receptacle tube, remove and retain the microspin cup, and discard the wash buffer.
14. Repeat steps 11–13.
15. Place the microspin cup back in the 2-ml receptacle tube and snap the cap of the receptacle tube onto the microspin cup.
16. Spin the tube in a microcentrifuge at maximum speed for 30 seconds. On removal from the centrifuge, make sure that all of the wash buffer is removed from the microspin cup.
17. Transfer the microspin cup to a fresh 1.5-ml microcentrifuge tube^{||} and discard the 2-ml receptacle tube.
18. Add 50 μ l of 10 mM Tris base, pH 8.5 directly onto the top of the fiber matrix at the bottom of the microspin cup (see “Microspin Cup Elution Buffer” in *Preparation of Reagents*).

^{||} 1.5-ml flat snap cap microcentrifuge tubes from Continental Laboratory Products, Inc. are recommended.

19. Incubate the tube at room temperature for 5 minutes.

Note *Maximum recovery of the labeled cDNA from the microspin cup depends on the pH, the ionic strength, and the volume of the elution buffer added to the microspin cup; the placement of the elution buffer into the microspin cup; and the incubation time. Maximum recovery is obtained when the elution buffer is ≤ 10 mM in concentration with pH 7–9, when not less than 50 μ l of elution buffer is added directly onto the fiber matrix at the bottom of the microspin cup, and when the tube is incubated for 5 minutes.*

20. Snap the cap of the 1.5-ml microcentrifuge tube onto the microspin cup and spin the tube in a microcentrifuge at maximum speed for 30 seconds.
21. Open the lid of the microcentrifuge tube and recover the flow through containing the purified labeled cDNA.
22. Elute additional labeled cDNA by pipetting the flow through back onto the fiber matrix of the same microspin cup.
23. Re-seat the spin cup on the same 2-ml receptacle tube that contained the liquid from the first-pass elution.
24. Incubate the tube at room temperature for 5 minutes.
25. Snap the cap of the 1.5-ml microcentrifuge tube onto the microspin cup and spin the tube in a microcentrifuge at maximum speed for 30 seconds.
26. Open the lid of the microcentrifuge tube and recover the flow-through containing the purified labeled cDNA.
27. Harvest one final elution from the microspin cup by repeating steps 22–26.
28. Open the lid of the microcentrifuge tube and recover the flow through containing the purified labeled cDNA.
29. (Optional) Remove a 2- μ l sample of the labeled cDNA for analysis of frequency of dye incorporation using a small-volume spectrophotometer (such as a NanoDrop® instrument). See the *Spectrophotometric Analysis Method* section under *Analysis of Fluorescence-labeled cDNA* for more information on performing this analysis.

30. To reduce the volume of the labeled cDNA, place the labeled cDNA in a centrifuge and apply a vacuum. Continue to apply a vacuum until the volume has been reduced to approximately 14 µl.

Note *If a higher concentration of labeled cDNA is desired, the volume may be reduced further by continued centrifugation under vacuum. Scale down the volumes of other hybridization solution components accordingly (see the Hybridization section).*

31. Adjust the volume of the labeled cDNA to a final volume of 14 µl by adding RNase/DNase-free H₂O.

Analysis of Fluorescence-labeled cDNA (Recommended)

Although the labeled cDNA may be visibly colored, serving as an indicator of a successful dye-coupling reaction, most researchers prefer to analyze the extent of dye incorporation directly. The efficiency of cDNA labeling may be analyzed by the following three methods: 1) spectrophotometric analysis to determine the dye-to-base ratio, 2) capillary electrophoresis and 3) slide-gel electrophoresis analysis.

Spectrophotometric Analysis Method

Determine the frequency of dye incorporation (number of dye molecules incorporated per 100 bases cDNA) directly using a small-volume spectrophotometer such as a NanoDrop instrument. Using a 2- μ l aliquot of the labeled cDNA sample eluted from the microspin cup (50- μ l sample), collect the absorbance spectrum at 240–800 nm.

The yield of cDNA may be calculated using the formula:

$$\text{cDNA (ng)} = A_{260} \times 37 \text{ ng}/\mu\text{l} \times 50 \mu\text{l sample volume}$$

The yield value may be converted to the quantity of cDNA bases using the molecular weight 324.5 g/mole bases.

To calculate the quantity of dye molecules in the sample, follow the dye manufacturers' recommendations for the emission wavelength and extinction coefficient values to use for quantification of each specific labeling dye. Calculate the number of dye molecules incorporated per 100 bases; greater than 1 dye molecule per 100 bases cDNA is expected.

Capillary Electrophoresis Analysis Method

CY5-labeled cDNA can be analyzed using the Agilent 2100 Bioanalyzer with the RNA 6000 LabChip® kit. This chip-based analysis allows the comparison of labeled samples according to their separation patterns and peak areas. In addition, a rough size estimate can be obtained and failed cDNA labeling reactions can be identified. This method allows for the rapid analysis of multiple samples through a single separation channel with excellent reproducibility. Details for the method can be found in the Agilent Application Note *Analysis of CY5-labeled cRNAs and cDNAs using the Agilent 2100 Bioanalyzer and the RNA 6000 LabChip® Kit* available at <http://www.chem.agilent.com/scripts/LiteraturePDF.asp?iWHID=28050>.

Note *This analysis is appropriate only for CY5-labeled cDNA. CY3-labeled cDNA cannot be detected with the Agilent 2100 Bioanalyzer.*

Slide-Gel Electrophoresis Method

For this analysis, the cDNA is electrophoresed on an agarose gel. To determine if fluorescent dye has been coupled, the cDNA is visualized with a laser scanner. This method also allows verification that the RNA template strand has been completely removed, using ethidium bromide staining and nucleic acid visualization with a UV transilluminator following electrophoresis.

1. Remove 0.5 μ l of each labeled cDNA (from the final 14- μ l preparation) and analyze by slide gel electrophoresis (see *Appendix I*). Use a 2% agarose Tris-acetate gel. Prepare CY-labeled cDNA with minimal dye loading buffer (see *Preparation of Reagents*). Prepare a DNA size standard using standard dye loading buffer.

Note *The addition of ethidium bromide to the gel or running buffer will affect detection of the CY3- or CY5-labeled DNA during scanning. To prevent this, include a DNA size standard in a separate lane. After electrophoresis, separate this lane from the others and stain with ethidium bromide.*

2. Detect CY5-labeled DNA by scanning the gel with a 635-nm laser with a 700-nm emission filter and CY3-labeled DNA by scanning the gel with a 532-nm laser with a 580-nm emission filter. Compare to ethidium bromide-stained DNA size standard to determine length of labeled cDNA.

Note *Labeled DNA should be 400–1500 bp in length. Any uncoupled dye will appear as a bright band at the leading front of the gel.*

3. Stain the gel containing the cDNA with ethidium bromide and visualize with a UV transilluminator.

Note *Complete removal of the RNA template is indicated by very poor or no staining of the cDNA with ethidium bromide.*

Hybridization

Array hybridization and washing protocols are provided below. Other standard microarray hybridization/washing protocols may also be used.

Note *The DNA blocking and hybridization solutions should be tested for autofluorescence prior to use by spotting on a clean glass microscope slide and scanning using the microarray scanner.*

1. Add 1 μ l of 10 μ g/ μ l human or mouse COT-1 DNA, 1 μ l of 8 μ g/ μ l poly d(A)_{40–60}, and 1 μ l of 4 μ g/ μ l yeast tRNA to each concentrated labeled cDNA.

2. Add 3 μ l of 20 $\times$ SSC and 0.5 μ l 10% SDS to each labeled cDNA for a final volume of 20 μ l.

Note *The minimum recommended amount of hybridization solution is 2.0 μ l per cm² of coverslip. The size of the coverslip should exceed the size of the microarray by 2–4 mm in each dimension. Store unused labeled cDNA at 4°C in the dark for up to 2 weeks.*

3. Remove an appropriate amount of labeled cDNA (from step 2) to hybridize to the microarray. Heat at 99°C for 2 minutes, then cool to 45°C. Spin down condensation. Incubate at 45°C for 20 minutes before continuing to step 5.
4. To identify the position of the microarray on a slide, align a template with the microarray.
5. Apply labeled cDNA prepared from test RNA to one microarray and labeled cDNA prepared from control RNA to another microarray. Bubbles between the coverslip and the microarray must be avoided. Carefully pop large bubbles in the hybridization solution with a pipet tip before placing coverslip on the microarray but do not touch the microarray with the pipet tip. Add coverslips. Small bubbles will disappear when the microarray hybridization chamber is placed in the water bath. Do not attempt to reposition the coverslip once it is on the microarray.
6. Add 30 μ l of 3 $\times$ SSC (diluted with the DEPC water provided) to the indentations on each side of the microarray hybridization chamber. Place slides in separate chambers and seal according to the manufacturer's instructions. Be careful not to move coverslip. Do not invert the chamber.
7. Submerge the chambers in a water bath at 65°C for 14–18 hours. Do not exceed 18 hours.

Washing

Prepare wash solutions using RNase/DNase-free water. Do not reuse wash solutions. Use a fresh wash solution each time. Do not allow slides to dry between wash steps. Transfer slides directly from one wash solution to the other.

1. Prepare 500 ml of 0.2 μ m–filtered 0.5 $\times$ SSC, 0.01% SDS (diluted with RNase/DNase-free H₂O) and place in a clean 2 liter beaker (first wash solution). Prepare an additional 50 ml of the first wash solution for each slide and place in separate 50-ml conicals. Prepare 500 ml of 0.2 μ m–filtered 0.06 $\times$ SSC and place into a clean 2 liter beaker (second wash solution). Place a clean magnetic stirbar into each beaker containing wash solution. 500 ml of each wash solution is sufficient for up to 10 slides. If more than 10 slides are being washed, increase the wash volume by 50 ml for each additional slide.

2. Suspend a slide carrier in the first wash solution approximately 1 cm above the magnetic stirbar. Make sure that the slides will be completely covered with the wash solution when placed into the slide carrier. If needed, prepare and add more wash solution.
3. Remove slides from hybridization chamber. Do not expose microarray to air by removing coverslip.
4. Place slides into separate conicals containing 50 ml of the first wash solution. Remove coverslip by gentle agitation. If needed, the coverslip can be removed with forceps. If forceps are used, do not touch microarray with forceps. After removal of the coverslip, transfer the slide to the slide carrier that is suspended in the first wash solution. Wash at room temperature for 5–10 minutes at room temperature with gentle swirling of the wash solution.
5. Transfer the slide carrier containing the slides to the second wash solution and suspend the slides approximately 1 cm above the stirbar. Make sure that the slides are covered by the wash solution. Wash at room temp for 5–10 minutes with gentle swirling of the wash solution to ensure the complete removal of SDS.
6. Dry slides by blotting the slide holder briefly on paper towels and spinning at $1000 \times g$ for 1–2 minutes. If the slide holder cannot be used in the centrifuge, transfer each slide to a separate empty 50-ml conical prior to centrifugation. If the slide has a bar code, place the end of the slide with the bar code at the bottom of the conical. Do not air dry slide.

Hybridization Detection

Follow manufacturer's recommendations for use of a microarray scanner.

TROUBLESHOOTING

Observation	Suggestion
High background signal	Isolate RNA using a method that removes carbohydrates, lipids and cellular proteins.
	Purify labeled cDNA to remove uncoupled fluorescent dye and fluorescence-labeled proteins.
	Do not handle slides with bare hands; handle slides by the edges with gloved hands or forceps.
	Follow the blocking procedure recommended by the microarray manufacturer for reducing nonspecific hybridization to the microarrays.
	Avoid the presence of large bubbles in hybridization solution during hybridization; if large bubbles are present in the hybridization solution after placing on the microarray, carefully pop the bubble with a pipet tip but do not touch the microarray with the pipet tip.
	Clean microarray hybridization chamber prior to use.
	Add $3 \times$ SSC to hybridization chamber during hybridization to prevent slide from drying out.
	Do not remove coverslip prior to placing slide in wash solution.
	Prepare fresh washing solutions each time.
	Follow washing procedures correctly; do not allow the microarray slides to dry out until the very last drying step using centrifugation.
	Spin slide to dry.
	Verify that DNA blocking reagents (COT-1 DNA, polyA and yeast tRNA) do not autofluoresce.
Smeared DNA spots	Use correct concentration of SSC and SDS during hybridization.
	Do not move coverslip after placing on the array.
	Do not wash above room temperature or below a concentration of $0.06 \times$ SSC.
Low signal or none at all	Isolate the RNA in the presence of a ribonuclease inhibitor, and ensure all labware and reagents are free of RNases.
	Remove any inhibitors of AffinityScript HC RT (SDS, EDTA, guanidinium chloride, formamide, Na_2PO_4 , or spermidine) with an additional 70% (v/v) ethanol wash following ethanol precipitation; remove residual ethanol.
	Verify the integrity of the RNA by denaturing agarose gel electrophoresis.
	Verify the incorporation of fluorescent dyes and integrity of the labeled cDNA by agarose gel electrophoresis.
	Verify that the RNA template strand was completely removed by hydrolysis.
	Use correct concentration of SSC and SDS during hybridization.
	Verify that the hybridization solution was placed on the microarray.
	Verify that the correct side of the slide was scanned for the microarray.
	Verify that the correct region of the microarray was scanned.
Low recovery of the labeled cDNA from the microspin cups	Use a low-ionic-strength (≤ 10 mM) buffer, pH 8.5 as the elution buffer.
	Do not add less than 50 μl of elution buffer to the microspin cup.
	Add the elution buffer directly onto the fiber matrix of the microspin cup to ensure complete coverage of the membrane.
	Incubate the tube for 5 minutes after adding the elution buffer prior to centrifugation.
	Make sure that all the wash buffer is removed from the microspin cup before adding the elution buffer.

PREPARATION OF REAGENTS

20× SSC Buffer, High-Quality RNase/DNase-free 175.3 g of NaCl 88.2 g of sodium citrate 800.0 ml of RNase/DNase-free water Adjust pH to 7.0 with a few drops of 10 N NaOH Add RNase/DNase-free water to a final volume of 1 liter	Human or Mouse COT-1 DNA, 10 µg/µl Concentrate the 1 µg/µl solution of COT-1 DNA by 10-fold using either of the following methods: - Dehydrate DNA under vacuum - Precipitate DNA with 0.1 volume of sodium acetate and 2 volumes of 100% ethanol. Wash with 1 volume of 70% ethanol Resuspend DNA in DEPC-treated water to a final concentration of 10 µg/µl Store at –20°C
10% (w/v) SDS, high-quality RNase/DNase-free 5.0 g SDS Add RNase/DNase-free water to a final volume of 50 ml	6× Minimal Dye Loading Buffer Combine the following and mix until dissolved: 30 ml 100% glycerol 2.5 mg bromophenol blue 70 ml dH₂O
Microspin Cup Elution Buffer 10 mM Tris base Adjust pH to 8.5 with HCl If the elution buffer is made by diluting a higher molarity Tris base, pH 8.5 to a final molarity of 10 mM, verify that the pH is still 8.5. If not, adjust the pH to 8.5 using either HCl or NaOH.	Microspin Cup Wash Buffer 75 ml 100% (v/v) ethanol 25 ml dH₂O, molecular biology grade, DNase- and RNase-free

APPENDIX I: THE SLIDE GEL ELECTROPHORESIS TECHNIQUE FOR ANALYZING LABELED cDNA

The easiest method to prepare a thin gel for this analysis is to use a 5 × 7.5-cm glass slide, position a minigel comb over it with high tension clips, and add 10 ml of 2% (w/v) molten agarose near the upper center of the slide. The surface tension of the solution will prevent overflow and produce a small, thin gel, which can be scanned without further drying. Do not allow the teeth of the comb to overlap the edge of the plate or the surface tension may be broken. Use Tris-acetate buffer in the agarose gel solution and during the electrophoresis. Include a DNA size standard in a lane of the gel, prepared with standard dye loading buffer. For the electrophoresis, use standard agarose gel tanks.

For fluorescence-labeled cDNA detection, stain the size standard separately in ethidium bromide following electrophoresis. After scanning the gel containing the fluorescence-labeled cDNA to detect fluorescence, stain the entire gel in ethidium bromide to verify complete removal of the RNA template.

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ENDNOTES

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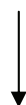
An Agilent Technologies Division

FairPlay III Microarray Labeling Kit

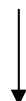
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QUICK-REFERENCE PROTOCOL

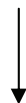
Generate cDNA from an RNA sample using the reverse transcriptase reagents provided, including the dNTP mix with amino allyl dUTP, which facilitates the downstream fluorescent dye labeling.



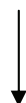
Purify the cDNA to remove unincorporated nucleotides, buffer components and hydrolyzed RNA, preventing inhibition of the coupling reaction.



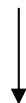
Couple the amino allyl-labeled cDNA to a fluorescent dye containing either a NHS- or STP-ester leaving group.



Purify the fluorescent dye-labeled cDNA to remove uncoupled fluorescent dye prior to hybridization using the purification reagents provided.



Analyze the labeled cDNA to determine if the fluorescent dye has been successfully coupled, if the uncoupled dye is completely removed, and if the RNA template strand has been completely hydrolyzed, ensuring optimal hybridization of the labeled cDNA and probe DNA.



Hybridize the labeled cDNA to the DNA probe, perform washes and detect hybridization using a microarray scanner.