On-chip hybridization kinetics for optimization of gene expression experiments

Elena Khomyakova¹, Mikheil A. Livshits², Marie-Caroline Steinhauser³, Luce Dauphinot³, Sylvia Cohen-Kaminsky⁴, Jean Rossier³, Françoise Soussaline¹, and Marie-Claude Potier³

BioTechniques 44:109-117 (January 2008)
doi 10.2144/000112622

DNA microarray technology is a powerful tool for getting an overview of gene expression in biological samples. Although the successful use of microarray-based expression analysis was demonstrated in a number of applications, the main problem with this approach is the fact that expression levels deduced from hybridization experiments do not necessarily correlate with RNA concentrations. Moreover, oligonucleotide probes corresponding to the same gene can give different hybridization signals. Apart from cross-hybridizations and differential splicing, this could be due to secondary structures of probes or targets. In addition, for low-copy genes, hybridization equilibrium may be reached after hybridization times much longer than the one commonly used (overnight, i.e., 15 h). Thus, hybridization signals could depend on kinetic properties of the probe, which may vary between different oligonucleotide probes immobilized on the same microarray. To validate this hypothesis, on-chip hybridization kinetics and duplex thermostability analysis were performed using oligonucleotide microarrays containing 50-mer probes corresponding to 10 mouse genes. We demonstrate that differences in hybridization kinetics between the probes exist and can influence the interpretation of expression data. In addition, we show that using on-chip hybridization kinetics, quantification of targets is feasible using calibration curves.

INTRODUCTION

The ability to quantify the expression levels of all genes in a given tissue or cell with a single assay is an exciting promise. Two technologies are widely used: quantitative reverse transcription PCR (RT-PCR) and DNA microarray. While quantitative RT-PCR is easily applicable to a few hundred genes (1,2), DNA microarray technology has proven very useful for studying gene expression variations between many biological samples at the whole transcriptome scale. Although the successful application of microarray-based expression analysis was demonstrated in a number of applications, the main problem with this approach is the fact that hybridization signals do not necessarily correlate with target concentrations (3–5).

To get reliable microarray hybridization data, each probe should be specific for a unique transcript, and all probes on the same microarray should have similar thermodynamic stability. In addition, hybridization signals should be maximal in order to reach the highest sensitivity. During probe design, these

requirements can be considered using a theoretical sequence-based approach for prediction of duplex stability and hairpin formation. However, all predictions are based on hybridization models deduced from experiments in solution and not from experiments in which probes are immobilized on DNA microarrays. Even fully complementary oligonucleotide targets hybridized to immobilized probes of the same size might give different hybridization signals at equilibrium. Evidently, hybridization with complex cDNA targets will worsen the situation because of stable secondary structures of the targets themselves or interactions between targets. A possibility of competition between the targets can also complicate the hybridization data (6–8). Since expression levels can vary considerably from one gene to another, there will be high variations in the concentration of the different cDNAs hybridized to the microarray. According to standard protocols, hybridization is usually performed overnight (15–18 h) (6). For low-copy genes, reaching equilibrium might require hybridization times considerably longer.

Consequently for some probes, the measured fluorescent signals will not correspond to a plateau. There are several important consequences to this. First, as it is theoretically expected and experimentally proven (9,10), in non-equilibrium conditions, the proportion of cross-hybridized targets is higher as compared with equilibrium conditions. Moreover, other factors including thermodynamic stability of on-chip formed duplexes and the presence of secondary structures can change the kinetic properties of probe-target combinations.

If different oligonucleotide probes corresponding to the same cDNA, even of the same length and similar base composition, show different kinetic properties, this will have an impact on hybridization signals and sensitivity. In addition, for low-copy genes at typically non-equilibrium conditions, different expression results will be obtained with different probes corresponding to the same gene if the kinetic properties of the probes are different. Thus, depending on gene expression levels and kinetic properties of the

¹IMSTAR, Paris, France, ²EIMB, Moscow, Russia, ³CNRS UMR 7637, Paris, France, and ⁴CNRS UMR 8078, Le Plessis-Robinson, France

probes, contradictory results might be obtained.

Indeed this is what we have observed when studying mouse gene expression in various tissues using DNA microarrays probing neurotransmission genes, represented by two or three 50-mer probes each. Out of the 280 genes represented on the microarray, 29 corresponding to 14 genes were selected for detailed experiments. We performed complex analysis of hybridization including on-chip analysis of duplex thermostability and measurement of on-chip hybridization kinetics at different concentrations of targets. Antisense Texas Red-labeled oligonucleotide targets fully complementary to immobilized probes were used. Based on these experiments, we have analyzed some of the causes for the observed variations in cDNA hybridization to different probes and propose the recommendations for the improvement of the reliability of expression data, particularly for low-copy genes.

MATERIALS AND METHODS

Oligonucleotides

Twenty-nine oligonucleotide probes corresponding to 14 mouse genes involved in neurotransmission were purchased from Eurogentec (Liège, Belgium) as 5' end amino-modified 50-mer deoxyribonucleotides (Table 1). Oligonucleotide targets complementary to the 29 oligonucleotide probes were purchased from Eurogentec as well.

Hybridization of cDNAs to the NeuroTrans Oligoarrays

Total RNA was extracted from thymus or cerebral cortex from B10D2 mice using the RNeasy kit (Qiagen, Valencia, CA, USA). The quality of RNA was checked on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Fifteen micrograms RNA were labeled with Cy5 by reverse transcription into cDNAs using 7.5 μM random hexanucleotides, 10 μM dithiothreitol (DTT), 125 μM dNTP, 25 μM dUTP-cyanine (Amersham plc,

Little Chalfont, Buckinghamshire, UK), and 400 U of Superscript II (Invitrogen, Carlsbad, CA, USA). Reference RNA (equimolar mixture of thymus, thymocytes, and cerebral cortex RNAs) was labeled with Cy3. Labeled targets were purified on QIAquick columns (Qiagen). Cy3 and Cy5 cDNAs were hybridized to NeuroTrans NT280M containing oligonucleotides for 280 genes involved in neurotransmission (two to three oligonucleotides per gene; GPL4746 on GEO; www.ncbi.nlm.nih.gov/geo). Hybridization was

performed at 42°C in 50% formamide, 4× standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), and 5× Denhart's overnight. Slides were washed for 2 min in 2× SSC, 0.1% SDS, for 1 min in 0.2× SSC, for 1 min in 0.1× SSC, and scanned on the ScanArray Lite (Packard Biochip, Billerica, MA, USA) using the same procedure for each slide. Fluorescence on each spot was extracted using the optical scan array (OSA) image analysis software package (www.imstarsa.com). Data are

Figure 1. The differences between hybridization signals for probe pairs corresponding to the same gene. (A) Expression levels (signal from a spot divided by the total signal from the microarray) after hybridizing cDNA targets from mouse cerebral cortex or thymus overnight. (B) Signal ratios for pairs of probes to mutual genes in cerebral cortex and in thymus. (C) Signal ratios between cerebral cortex and thymus for each probe, deduced from panel A. Values are from three independent microarray experiments.

Table 1. Probe Sets Corresponding to 14 Genes Selected for the Kinetic Experiments

Oligonucleotide number	Gene ID	Gene Symbol	Sequence
1	AF089730	slack.rat/Slack	AATGATCATCACCAGAACCCCTTTCTTACTGTACTCATCAACCCCGGCC
2	AF089730	slack.rat/Slack	CTGGTCGGGTGTTTATGATACAGCATGTTGGATACGCTGCTCACCAGTCC
3	AF109143	elk2/Kcnh3	CTCCAGGCTCTCTACTTTGTGTGCTCAGGTTCCATGGAGGTCCTCAAAGG
4	AF109143	elk2/Kcnh3	CATCTGCACCATGCTTATTGGAGCTCTGATGCATGCAGTGGTGTGGGA
5	D17584	subsp/Tac1	GCCCTGTTAAAGGCTCTTTATGGACATGGCCAGATCTCTCAAAAGGGA
6	D17584	subsp/Tac1	ATCGATGCCACGATGATCTAAATTATTGGTCGAGCTGGTCCGACAGTGA
7	J04509	jund1.2/Jund1	GCGCCTGGAGGAGAAAGTCAAGACCCTCAAAGCCAGACACCGAGCTGG
8	J04509	jund1.2/Jund1	GTTCGCTGTGAGTGTGTGTTCTTCTGTGTGTTTAAAGAAACCGG
9	J04509	jund1.2/Jund1	CGAGTCCACATTCTGTTCCGTAATCCTTGTTGCGCGGTTTGTGTTTT
10	NM_008421	kv3.1/Kcnc1	TCCCTCAATATCATTGACTTTGTGGCCATCTCGCCCTTCTACCTGGAGT
11	NM_008421	kv3.1/Kcnc1	CCCATCGTGAACAAGACCGAAATCGAGAACGTTTCGAAACGGCACCCAAAT
12	NM_008423	kv4.1/Kcnd1	GAGAAGACTACGTGTACAGAGTTACAGACGAGCTAATTTCACTGAGGC
13	NM_008423	kv4.1/Kcnd1	TCATTGCTTGCCTGTGCGCAGTCATTGTATCCCACTTCAGCCGATCTAC
14	NM_008791	pcp4/Pcp4	AGTCTCAGTTCAAGAAATCCAGAGAAAGAGCAGGATCAGCTCCATAG
15	NM_008791	pcp4/Pcp4	CATCTCACTCAATACACACCCTGCTACAAACCTGAAATGCATGAAGAGA
16	NM_009414	tph/Tph	AGTTTCTCAGAGACACCAAGACATAACTAGTCCATGAATGAGTTGCGG
17	NM_009414	tph/Tph	TCTTTGGGGCCGGCTTGCTTCTTCCATCAGTGAACCTCAACATGCACIT
18	NM_010611	konq2/Kcnq2	CTCAGGAACCATCTACGTTATCCCAAGTGTCTGTGAGGTAAAGTGTCTCA
19	NM_010611	konq2/Kcnq2	AAAAGGGTGAGAATGACCACTTTGACACCTACCGAGATGCACCTCGGTGG
20	NM_010777	MBP/Mbp	CGCTCAGCCTTCCCGAATCTCGCCCTCGGCTCTCTTAATAAAGTCCTTA
21	NM_010777	MBP/Mbp	AGAATTATCTGCTGAGAAGGCCAGTAAGGATGGAGAGATTCACCGAGGAG
22	NM_053405	task3/Kcnk9	CATTACACTGACTACTATAGGGTTGCGGCACTTTGTGGCCCTGCAATCCA
23	NM_053405	task3/Kcnk9	GCTATCACTGTATCAACAATCTGATATGACATGCTGCAACCTGGAAC
24	U51001	Dlx1/	CGTCCCCAGCTATACATCATGGTATCCCTCAGCGCACCAAGAGCTATGC
25	U51001	Dlx1/	AACCAGCAAATGTCTCCTTCTCCATGTCCCACGGGCACTACTCCATGCA
26	X15643	beta2/Adrb2	TGTTTGGGCTACGTCAACTCTGCTTCAATCCTCTTACTACTGTGCGGAG
27	X15643	beta2/Adrb2	CCAAAAGGCAGCTGCAGAAAGATAGACAAATCTGAAGGAAGATTCACAGCC
28	X80797	kv3.3/Kchc3	ACAAAAGGCCACAGAAACAGGATTCACAGTCAAGAGCTTGTGAACACGG
29	X80797	kv3.3/Kchc3	GGGGCTGATCTGACGACATCTCTGGGCTCCAAACCAACCTACTTCAAGAA

accessible on GEO database under the number GSE7574.

On-Chip Hybridization Kinetic Experiments

Microarrays specially designed for kinetic experiments were used. Amino-modified oligonucleotide probes (Table 1) were dissolved in 150 mM sodium phosphate buffer, pH 8.3, at 12.5 μ M and spotted on activated glass slides

according to patent no. WO03068712 using the BioRobotics MicroGrid II spotter (Genomic Solutions, Ann Arbor, MI, USA). Each oligonucleotide probe was spotted in quadruplet. Distance between spots was 400 μ m.

Oligonucleotide targets were labeled at the 3' end using terminal transferase. One hundred picomoles of an oligonucleotide were mixed with buffer, 5 mM CoCl_2 , 0.05 mM Texas Red-5'-ddATP (PerkinElmer, Waltham, MA, USA),

400 U terminal transferase (Roche, Basel, Switzerland), and incubated for 2 h at 37°C. Reaction was stopped by adding 2 μ L 0.2 M EDTA, pH 8.0, on ice. Labeled oligonucleotides were purified on DyeEx columns (Qiagen) and precipitated in 10 volumes of acetone and 2% LiClO_4 overnight at -20°C. After a 10-min centrifugation, the pellet was rinsed once with acetone, centrifuged again for 5 min, and dissolved in water at 12.5 μ M final concentration. The oligonucleotide concentration was adjusted after quantification using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 260 nm. Oligonucleotide labeling efficiency was 0.65 as estimated from the absorbance ratio measured at 260 nm (DNA) and at 612 nm (Texas Red).

Labeled oligonucleotide targets (equimolar mixture) were deposited on the microarray and covered with Gene Frame (Abgene, Epsom, UK). The volume of the hybridization chamber was 25 μ L (1 cm $\times$ 1 cm). Hybridization was carried out at 60°C in 2.5 $\times$ SSC, 0.2% SDS at different target concentrations from 0.05 to 1 nM for kinetic experiments and 10 nM for the thermostability measurement. For registration of on-chip kinetics and melting curves, OSA instrument (IMSTAR, Paris, France) equipped with a thermo-controlled microarray holder was used (Reference 11 and patent no. WO2004059302). Curves were recorded by measuring the fluorescence intensity on all the spots of the microarray after scanning, either at a fixed temperature (60°C for kinetic curves) or at 2°C intervals starting from 20° to 95°C (2°C/25 min at lower temperatures to 2°C/200 s at higher temperatures for equilibrium melting curves). Each experiment was started by the denaturation of probes and targets within the hybridization chamber at 95°C for 5 min. For kinetic experiments, after on-chip denaturation, the temperature was decreased to 60°C followed by the immediate start of hybridization signals registration at 60°C.

Images were captured and analyzed using OSA image analysis software package. The positions of the microarray spots were determined using

Figure 2. On-chip melting curves for the 21 probes. Each probe pair is represented on a separate graph. a.u., arbitrary units.

an integrated algorithm of automated spot finding on the hybridized microarray images. For kinetics and melting experiments, the hybridization signal S and local background fluorescence intensity B corresponding to each spot was calculated as described in Reference 11. To compensate for the temperature dependence of Texas Red and its bleaching after several measurements, the intensity $I(T)$ of each spot was calculated as described in Reference 12:

$$I(T) = \frac{S(T) - B(T)}{B(T)}$$

The number of binding sites ($N_{\text{binding site}}$) involved in duplex formation on the area of a microarray spot A_{spot} [cm^2] was estimated from the maximum value of the fluorescence intensity I_{max} (upper plateau of the melting curve), the concentration of oligonucleotide targets in solution (C_{target}), the thickness of hybridization chamber ($l = 500 \mu\text{m}$), and spot area A_{spot} [cm^2] as follows:

$$N_{\text{binding sites}} = 10^3 I_{\text{max}} A_{\text{spot}} l \cdot C_{\text{target}}$$

The maximum value $I_{\text{max}} = 0.5$ of hybridization signal registered in the experiment with 10 nM oligonucleotide target at 20°C corresponds to 5 amol (5×10^{-18}) of binding sites. Thus, the target quantity exceeds at least 10^2 - to 10^3 -fold the quantities of immobilized oligonucleotide probes, thus guaranteeing the constancy of target concentration during hybridization and melting. The invariability of background intensity at a fixed temperature during the kinetics measurements is an additional proof of the constancy of the target concentration during hybridization (data not shown).

RESULTS

Selection of Probes Giving Different Hybridization Signals for the Same Gene

Out of 22 hybridization experiments performed on the NeuroTrans oligoarray containing 50-mer probes with cDNAs from mouse forebrain or cerebellum, 14 genes showed signal differences of at least 10-fold between the two probes corresponding to the same gene in a minimum of four experiments (data not shown). Table 1 shows the list of the genes. We selected for detailed analysis 10 of these genes for which we obtained sufficient fluorescent signals in hybridization kinetics (Table 1, not in gray). For these 10 genes, signal intensities were determined from six additional hybridization experiments performed with either cerebral cortex or thymus cDNAs on NeuroTrans oligoarrays. Figure 1A shows the hybridization signals for each probe normalized to the total signal on the oligoarray. Figure 1B gives the signal ratios for the pairs of probes corresponding to the same gene. There are 11 ratios for 10 genes, because one gene was represented by three probes instead of two. As expected, the relative expression levels in cerebral cortex and thymus were contradictory depending on the oligonucleotide probe (Figure 1C). To analyze the impact of hybridization kinetics on gene expression data, we performed complex analysis, including on-chip study of duplex thermostability and on-chip hybridization kinetics at different target concentrations. Antisense Texas Red-labeled oligonucleotide targets fully complementary to immobilized probes were used for on-chip experiments in the same experimental conditions (buffer composition and temperature) as with hybridization of cDNA.

Theoretical Model of On-Chip Hybridization

The on-chip hybridization is a bimolecular reaction between 50-mers single-stranded oligonucleotide probes (P) immobilized to the microarray surface and the antisense target (T) molecules, either oligonucleotide or

Figure 3. An example of on-chip hybridization kinetics (probe pair 22/23). (A) Normalized fluorescent signals obtained after 9 h hybridization of targets complementary to probes 22 and 23. (B) Fluorescence ratios between probes 22 and 23 during hybridization kinetics deduced from panel A. (C) Dependence of the hybridization time (τ) on target concentration: example for probes 22 and 23. $1/\tau$, hybridization rate.

cDNA, present in the hybridization solution.

The kinetic equation of the corresponding reaction describes the increase of the quantity of target molecules hybridized to probes on a spot and the corresponding increase of the fluorescent hybridization signal (13):

$$J(t) \sim p \frac{Kc}{1 + Kc} \left(1 - e^{-t/\tau}\right) \quad [\text{Eq. 1}]$$

Figure 4. Simulation of hybridization kinetics for 10 probe sets corresponding to 10 different genes. The simulation is based on extrapolated oligonucleotide kinetics data. Hybridization signal is represented by the fraction of occupied probe molecules in a spot.

Here, p is the surface density of the immobilized probe, τ is the hybridization characteristic time, determined by the effective rate constants of association (k_{ass}) and dissociation (k_{diss}) and the target concentration (c):

$$1/\tau = k_{diss} + k_{ass}c = k_{diss}(1 + Kc)$$

[Eq. 2]

$K = k_{ass}/k_{diss}$ is the equilibrium binding constant for the considered probe-target. Equation 2 is true also in case of diffusion-limited kinetics in spite of a different interpretation of effective rate constants (14).

It is well known that the forward rate constants k_{ass} are very similar for perfect match (perf) and for mismatch (mism) duplexes, while reverse rate constants k_{diss} may differ considerably (9,15,16). Typically, $k_{diss}(\text{perf}) \ll k_{diss}(\text{mism})$, indicating that perfect duplexes are much

more stable than mismatch duplexes formed upon cross-hybridization (16,17). Thus, the equilibration time τ is longer for perfect duplexes than for mismatch duplexes (see Equation 2 and Reference 9). It was shown both theoretically (10,18) and experimentally (19) that at early stages of hybridization the highest concentration species dominate (either specific or non-specific), while at later stages the highest affinity species displace non-specific binding. Considerable amount of cross-hybridization (non-specific binding) is present under non-equilibrium conditions (9,20).

The possible formation of secondary structures in the probes and in the targets can influence both the rate and the extent of hybridization. The kinetic effects of secondary structures are documented in a number of studies (21,22).

To model the on-chip hybridization kinetics, we used oligonucleotide targets that were strictly complementary to the oligonucleotide probes immobilized on the NeuroTrans microarray. The linear dependence (Eq. 2) of the hybridization rate ($1/\tau$) on target concentration c allows to measure k_{diss} and K and then to deduce τ at the cDNA target concentrations used during gene expression experiment.

Thermodynamic Analysis of On-chip Formed Duplexes

Thermodynamic stability of the hybridized duplexes was estimated in a separate experimental study. Oligonucleotides listed in Table 1 were spotted onto custom microarrays. These microarrays were hybridized with Texas Red-labeled 50-mer oligonucleotide targets complementary to immobilized probes until equilibrium was reached. Rather high oligonucleotide target concentration (10 nM) was chosen to ensure fast attainment of hybridization equilibrium. Hybridization was performed in the same buffer as the one used for cDNA hybridization. On-chip melting of the duplexes was then recorded. Normalized melting curves are shown in Figure 2.

Equilibrium melting temperatures (T_m) obtained by fitting the theoretical equation for the occupation level (12):

$$\eta(T) = \frac{1}{1 + e^{\frac{\Delta H}{R} \left(\frac{1}{T_m} - \frac{1}{T} \right)}}$$

to experimentally obtained melting curves are represented in Table 2.

However, the similarity of melting curves observed in the thermodynamic experiments does not guarantee the identity of hybridization kinetics. An example using the experimental data for the probe pair 22-23 is represented in Figure 3. Figure 3A shows the difference in on-chip normalized kinetic curves for probes 22 and 23 recorded at target concentrations 0.05, 0.1, 0.25, and 1 nM. The dependence of hybridization signal ratios between probes 22 and 23 on time is represented in Figure 3B. At high target concentrations (1 nM), the equilibration time was short, and the final value of the ratio between probe 22 and probe 23 was close to 1 (Figure 3B). For lower target concentrations (0.1 and 0.25 nM), because of the difference in hybridization kinetics between probes 22 and 23, at the beginning of hybridization, the signal ratio was high, especially at concentration 0.1 nM. However, when approaching equilibrium, the ratio decreased to smaller values close to 1. At the lowest target concentration (0.05 nM) even after 50,000 s of hybridization, the ratio 22/23 was only approaching 1 (Figure 3B).

On-chip Hybridization Kinetics

For a detailed study of the kinetic effects, we have measured the on-chip hybridization kinetics of Texas Red-labeled 50-mer oligonucleotide targets at four concentrations: 0.05, 0.1, 0.25, and 1 nM. Results of fitting (K and k_{diss} values) are listed in Table 2. These experiments allowed the calculation of τ as well as the modeling of hybridization kinetic curves at any target concentration.

Simulation of Kinetics Curves from the Theoretical Model

As an example we simulated the kinetic curves for 0.01 and 0.005 nM target concentrations, using Equation 1, and K and k_{diss} values listed in

Table 2. Evidently, such estimation of kinetic parameters K and k_{diss} has a limitation coming from experimental errors that are more essential at lower target concentrations. Figure 4 shows the curves obtained for all the probes tested. Figure 5 indicates the simulated hybridization signal ratios after 15 h of hybridization (panel A) and at saturation (panel B) for the analyzed probe pairs at four concentrations of oligonucleotide targets: 0.05, 0.01, 0.005, and 0.001 nM. For slow kinetics, duplex hybridization ratios are different depending on the hybridization time.

DISCUSSION

The discrepancy between hybridization signals obtained from different probes corresponding to the same gene is a problem for gene expression analysis. Except for the well-known alternative splicing and stable secondary conformation of cDNA targets, some

Figure 5. Comparison of pair ratios for simulated hybridization signals after 15 h of hybridization (A) and at equilibrium (B). Melting temperature (T_m) deduced from on-chip experiments.

Table 2. Kinetics Parameters and T_m Deduced from On-Chip Experiments

Probes	T_m (°C)	k_{ass}	K
3	60.8	1.64×10^6	3.23×10^{10}
4	62.1	1.45×10^6	2.67×10^{10}
5	59.3	9.74×10^5	3.97×10^{10}
6	56.4	4.96×10^5	1.46×10^{11}
7	58.3	7.93×10^5	1.01×10^{10}
8	55.7	1.55×10^6	1.23×10^{10}
9	57.1	1.19×10^6	1.03×10^{10}
12	57.8	9.37×10^5	3.25×10^{10}
13	56.7	1.71×10^6	2.63×10^{10}
14	62.3	1.65×10^6	4.95×10^{10}
15	64.4	1.05×10^6	2.17×10^{10}
18	59.3	1.10×10^6	5.24×10^{10}
19	59.6	1.72×10^6	4.85×10^{10}
20	58.4	1.53×10^6	2.62×10^{10}
21	64.6	9.44×10^5	1.17×10^{10}
22	68.7	1.30×10^6	4.46×10^{10}
23	67.0	8.23×10^5	9.90×10^{10}
26	62.3	7.51×10^5	1.39×10^{10}
27	56.6	1.35×10^6	1.63×10^{10}
28	65.6	1.14×10^6	2.58×10^{10}
29	63.7	7.79×10^5	8.70×10^9

T_m , melting temperature; k_{ass} , rate constant of association; K , equilibrium binding constant.

other factors could influence microarray expression results. To analyze these factors we performed thermostability study and analysis of on-chip hybridization kinetics at four concentrations of complementary oligonucleotide targets: 1, 0.25, 0.1, and 0.05 nM. Theoretical analysis applied to our on-chip kinetics data allowed estimation of kinetic behavior at target concentrations corresponding to cDNA concentrations in gene expression experiments.

The probes selected for the study showed high hybridization signals as well as a significant difference between probes corresponding to the same gene (Figure 1). Figure 1B indicates at least a 2-fold difference in the hybridization signals for each pair of probes in cerebral cortex and/or thymus. When the difference is the same for both tissues, it is most probably caused by different secondary structures of the probes or/and of the targets. Such a case makes no confusion in the interpretation of the expression data. However, several probe pair ratios were found to differ in cerebral cortex and thymus (probes 3-4, 7-8, 7-9, 14-15, 18-19, 20-21, 22-23, 28-29) (Figure 1B). This entailed a difference in expression level ratios between cerebral cortex and thymus depending on the probe (Figure 1C). This difference could be due to the presence or absence of alternative transcripts in cerebral cortex or thymus that would correspond to one probe or to the other. Indeed probes from pairs 19-18, 20-21, 22-23, and 29-28 map either to different exons or to alternative 5' or 3' untranslated regions (UTRs) in two databases (www.ensembl.org; genome.ewha.ac.kr). However, for pairs 3-4, 14-15, and 27-28, the two probes mapped to the same transcript. Alternatively, the observed tissue-dependent variations in signal ratios between two probes corresponding to the same gene could result from differences in hybridization kinetics at different cDNA target concentrations. In addition, kinetic properties of the probes could be different within the same microarray. Thus, if a gene is highly expressed, hybridization signals at the end of the experiment may reach saturation levels. In this condition, signal ratio between oligonucleotide pairs will only depend on the equilibrium binding constant K of on-chip duplex formation

(see above). However, for low-copy genes, hybridization signals might be rather far from saturation, and thus hybridization signal ratios will depend on the hybridization time and target concentration.

To illustrate this, we performed on-chip kinetics analysis at different target concentrations and modeled the behavior of the duplexes. We first analyzed the temperature stability of on-chip formed duplexes. The experimental data (Figure 2) demonstrate that thermodynamic stability of the duplexes, including also the enthalpy ΔH of duplex formation, reflected by the shape of the melting curves, is similar for all immobilized probes tested. The minor differences observed cannot explain the difference in hybridization signals of oligonucleotide probes belonging to the same gene. In addition, the experimental data confirmed the correct design of oligonucleotide probes and the absence of degradation of either probes or targets. However, the similarity of melting curves does not guarantee the identity of hybridization kinetics. Indeed, probes 22 and 23 have similar melting curves but different hybridization curves (Figure 3, A and B). This suggests that signal ratios between the two probes depend on target concentration and hybridization time.

Based on the kinetic data obtained at higher concentrations of targets using Equations 1 and 2, it was possible to model the kinetics behavior of the duplexes at target concentrations corresponding to real cDNA hybridization experiments. DNA target concentration in biological samples was estimated using the data of Reference 23. Approximately 10 μ g RNA are typically used for microarray experiments. This amount corresponds to RNA quantities contained in 10^6 cells. The copy number of single genes in a cell varies from 1 to 10^4 , and the efficiency of reverse transcription is about 20% (24). Thus, the expected concentration of cDNA in 20 μ L hybridization solution is 10^{-14} to 10^{-10} M. Unfortunately, the on-chip kinetic measurements at concentrations lower than 5×10^{-11} M (0.05 nM) are hardly feasible because the fluorescent hybridization signals are too low as compared with the fluorescence of the

hybridization solution. Thus, according to Equation 2, using the linear dependence of $1/\tau$ value measured at concentrations 0.05, 0.1, and 0.25 nM, we can estimate the K and k_{diss} values for each probe-target pair (Figure 4 and Table 2). Based on these data, we were able to extrapolate the kinetics behavior at low target concentrations after 15 h hybridization and at saturation (Figure 5, A and B). Figure 4 shows that 15 h (54,000 s) hybridization time was not always sufficient for reaching at least 70% of maximal hybridization signal, especially at 0.005 nM target concentration (probes 6, 12 14, 18, 23, and 28). Obviously, for target concentration below 0.005 nM, the hybridization kinetics would be even slower. Thus, one can expect that after hybridization times used in classical experiments (roughly 15 h), the majority of hybridization signals is rather far from saturation level.

While at equilibrium (saturated signals) a decrease in target concentration leads to an increase of the absolute value of the hybridization signal ratio (Figure 5B), at non-equilibrium conditions (15 h of hybridization), and at low concentrations, some probe pairs (5-6, 12-13, 18-19, and 22-23) had ratios inverted as compared with the one obtained at equilibrium. Moreover, two pairs (5-6 and 28-29) had lower ratios after 15 h as compared with equilibrium conditions. Since probes 6 and 28 are characterized by slow hybridization kinetics (Figure 4), 15 h is definitively not enough for reaching equilibrium.

In pairs of probes addressed to mutual genes, the highest hybridization signals were obtained with probes 4, 6, 8, 13, 14, 19, 20, 23, 27, and 29 (Figure 1A). Only some of these probes (14, 19, 20, and 27) showed hybridization signals that differed more than 4-fold for cDNA targets from cerebral cortex and thymus (Figure 1C). Simulation of kinetics indicated that for these probes, a decrease in target concentration is expected to induce an increase of the hybridization signal ratio between probes within the same pair (Figure 5A). Indeed for probe 14, which corresponds to a gene that is expressed more in cerebral cortex than in thymus (Figure 1A), a decrease in target concentration induced a decrease in hybridization signal (Figure 5A). However, for probes

19 and 20, a decrease in target concentration in cerebral cortex as compared with thymus induced an increase in hybridization signal, which is opposite to what was predicted. Thus for these two probes, the kinetics model did not comply with the expression data. The most likely explanation may be the presence of splicing variants in cerebral cortex but not in thymus.

The tissue-dependent alternative splicing could be proposed as an alternative explanation for the low hybridization signal for probe 22 as well. However, since a very low hybridization signal was observed for probe 22 both in cerebral cortex and thymus, the most probable explanation seems to be the hairpin formation in cDNA fragment corresponding to probe 22. Stable secondary structure in cDNA could also explain the hybridization results obtained for probes 5, 6, 9, 12, 26, and 28.

Finally, for probe pair 3-4, there was no difference in expression levels of the corresponding gene between cerebral cortex and thymus (Figure 1A). Thus, the comparatively lower intensity of hybridization signal corresponding to probe 3 for both thymus and cerebral cortex could also be the consequence of cDNA secondary structure formation.

Based on our results and to improve microarray gene expression results, we can now make several recommendations. First, one should apply the best experimental conditions for reaching equilibrium for the majority of cDNAs hybridized on the microarray. One way would be to increase the hybridization time at least up to 24 h. Another solution, which seems to be more appropriate, would be to decrease the volume of the hybridization solution. Using microfluidic stations will greatly accelerate the hybridization process and essentially improve the expression data analysis (6). Secondly, fragmentation of cDNA targets will result in destabilization of the secondary conformations. Also, more attention should be brought to oligonucleotide probe design, including minimization of hairpin formation. It should be noted, too, that the kinetic properties of oligonucleotides are difficult to predict a priori. Performing experimental studies of on-chip kinetics for all the immobilized probes is rather complicated. Thus, the optimal solution could be to study on the

same microarray several oligonucleotide probes (at least 3, ideally 5–10) complementary to different regions of the gene and to select for expression analysis only the probes showing the highest hybridization signals. This should be helpful in avoiding the misinterpretation coming from slow-kinetics probes as well as in excluding the influence of alternative splicing and of secondary structures of DNA targets on the hybridization results.

Finally, we demonstrate that following the linear dependence between the characteristic hybridization kinetics and the target concentration, it is possible, using calibration curves, to deduce the target copy numbers from on-chip hybridization experiments.

ACKNOWLEDGMENTS

The authors wish to thank E. Tatarinova for very helpful discussion, C. Mikonio, D. Le Clerre, and F. Richard for spotting the oligoarrays, and S. Ursulet and E. Dreval for technical help.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests

REFERENCES

- Dolganov, G.M., P.G. Woodruff, A.A. Novikov, Y. Zhang, R.E. Ferrando, R. Szubin, and J.V. Fahy. 2001. A novel method of gene transcript profiling in airway biopsy homogenates reveals increased expression of a Na⁺-K⁺-Cl⁻ cotransporter (NKCC1) in asthmatic subjects. *Genome Res.* 11:1473-1483.
- Czechowski, T., R.P. Bari, M. Stitt, W.-R. Scheible, and M.K. Udvardi. 2004. Real-time RT-PCR profiling of over 1400 *Arabidopsis* transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. *Plant J.* 38:366-379.
- Hekstra, D., A.R. Taussig, M. Magnasco, and F. Naef. 2003. Absolute mRNA concentrations from sequence-specific calibration of oligonucleotide arrays. *Nucleic Acids Res.* 31:1962-1968.
- Held, G.A., G. Grinstein, and Y. Tu. 2006. Relationship between gene expression and observed intensities in DNA microarrays—a modeling study. *Nucleic Acids Res.* 24:E70.
- Kuo, W.P., F. Liu, J. Trimarchi, C. Punzo, M. Lombardi, J. Sarang, M.E. Whipple, M. Maysuria, et al. 2006. A sequence-oriented comparison of gene expression measurements across different hybridization-based technologies. *Nat. Biotechnol.* 24:832-840.
- Bishop, J., S. Blair, and A. Chagovetz. 2006. A competitive kinetic model of nucleic acid surface hybridization in the presence of point mutants. *Biophys. J.* 90:831-840.
- Horne, M.T., D.J. Fish, and A.S. Benight. 2006. Statistical thermodynamics and kinetics of DNA multiplex hybridization reactions. *Biophys. J.* 91:4133-4153.
- Fish, D.J., M.T. Horne, R.P. Searles, G.P. Brewood, and A.S. Benight. 2007. Multiplex SNP discrimination. *Biophys. J.* 92:L89-L91.
- Dai, H., M. Meyer, S. Stepaniants, M. Ziman, and R. Stoughton. 2002. Use of hybridization kinetics for differentiating specific from non-specific binding to oligonucleotide microarrays. *Nucleic Acids Res.* 30:E86.
- Bhanot, G., Y. Louzoun, J. Zhu, and C. Delisi. 2003. The importance of thermodynamic equilibrium for high throughput gene expression arrays. *Biophys. J.* 84:124-135.
- Khomyakova, E.B., E.V. Dreval, M. Trandang, M.-C. Potier, and F.P. Soussaline. 2004. Innovative instrumentation for microarray scanning and analysis: application for characterization of oligonucleotide duplexes behavior. *Cell. Mol. Biol.* 50:217-248.
- Fotin, A.V., A.L. Drobyshev, D.Y. Proudnikov, A.N. Perov, and A.D. Mirzabekov. 1998. Parallel thermodynamic analysis of duplexes on oligodeoxyribonucleotide microchips. *Nucleic Acids Res.* 15:1515-1521.
- Lauffenderberger, D.A. and J.J. Linderman. 1996. *Receptors*. Oxford University Press, New York, NY.
- Sorokin, N.V., V.R. Chechetkin, S.V. Pan'kov, O.G. Somova, M.A. Livshits, M.Y. Donnikov, A.Y. Turugin, V.E. Barsky, and A.S. Zasedatelev. 2006. Kinetics of hybridization on surface oligonucleotide microchips: theory, experiment, and comparison with hybridization on gel-based microchips. *J. Biomol. Struct. Dyn.* 24:57-66.
- Livshits, M.A. and A.D. Mirzabekov. 1996. Theoretical analysis of the kinetics of DNA hybridization with gel-immobilized oligos. *Biophys. J.* 71:2795-2801.
- Wang, S., A.E. Friedman, and E.T. Kool. 1995. Origins of high sequence selectivity: a stopped-flow kinetics study of DNA/RNA hybridization by duplex- and triplex-forming oligos. *Biochemistry* 34:9774-9784.
- Ikuta, S., K. Takagi, R.B. Wallace, and K. Itakura. 1987. Dissociation kinetics of 19 base paired oligo-DNA duplexes containing different single mismatched base pairs. *Nucleic Acids Res.* 15:797-811.
- Zhang, Y., D.A. Hammer, and D.J. Graves. 2005. Competitive hybridization kinetics reveals unexpected behavior patterns. *Biophys. J.* 89:2950-2959.
- Bishop, J., C. Wilson, A.M. Chagovetz, and S. Blair. 2007. Competitive displacement of DNA during surface hybridization. *Biophys. J.* 92:L10-L12.
- Sartor, M., J. Schwanekamp, D. Halbleib, I. Mohamed, S. Karyala, M. Medvedovic, and C.R. Tomlinson. 2004. Microarray results improve significantly as hybridization approaches equilibrium. *BioTechniques* 36:790-796.
- Sekar, M.M.A., W. Bloch, and P.M. St. John. 2005. Comparative study of sequence-depen-

- dent hybridization kinetics in solution and on microspheres. *Nucleic Acids Res.* 33:366-375.
22. **Gao, Y., L.K. Wolf, and R.M. Georgiadis.** 2006. Secondary structure effects on DNA hybridization kinetics: a solution versus surface comparison. *Nucleic Acids Res.* 34:3370-3377.
 23. **Carter, M.G., A.A. Sharov, V. VanBuren, D.B. Dudekula, C.E. Carmack, C. Nelson, and M.S. Ko.** 2005. Transcript copy number estimation using a mouse whole-genome oligonucleotide microarray. *Genome Biol.* 6: R61.
 24. **Tsuzuki, K., B. Lambolez, J. Rossier, and S. Ozawa.** 2001. Absolute quantification of AMPA receptor subunit mRNAs in single hippocampal neurons. *J. Neurochem.* 77:1650-1659.

Received 29 June 2007; accepted 18 September 2007.

M.-C.S.'s present address is Max Planck Institute of Molecular Plant Physiology, Am Muehlenberg 1, 14476 Golm, Germany.

Address correspondence to Elena Khomyakova, IMSTAR, 60 rue Notre Dame des Champs, 75006 Paris, France. e-mail: elena.khomiakova@imstar.fr; or Marie-Claude Potier, CNRS UMR 7637, ESPCI 10 rue Vauquelin, 75005 Paris, France. e-mail: marie-claude.potier@espci.fr

To purchase reprints of this article, contact: Reprints@BioTechniques.com