

Semiautomated Clone Verification by Real-Time PCR Using Molecular Beacons

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ABSTRACT

Conventional, high-throughput PCR analysis of common elements utilizing numerous primer sets and template DNA requires multiple rounds of PCR to ensure optimal conditions. Laborious gel electrophoresis and staining is then necessary to visualize amplification products. We propose novel multi-color molecular beacons, to establish a high-throughput, PCR-based sequence tagged site (STS) detection system that swiftly and accurately confirms marker content in template containing common repeat elements. A simple, one-tube, real-time PCR assay system was developed to specifically detect regions containing CA and GATA repeats. Ninety-six samples can be confirmed for marker content in a closed-tube format in 3 h, eliminating product confirmation on agarose gels and avoiding crossover contamination. Multiple STSs can be detected simultaneously in the same reaction tube by utilizing molecular beacons labeled with multicolor fluorophores. Template DNA from 260 RPCI-11 bacterial artificial chromosome (BAC) clones was examined for the presence of CA and/or GATA repeats using molecular beacon PCR and compared with conventional PCR results of the same clones. Of the 205 clones containing CA and GATA repeats, we were able to identify 129 clones (CA, n = 99; GATA, n = 30) by using molecular beacons and only 121

clones (CA, n = 92; GATA, n = 29) by conventional PCR amplification. As anticipated, 55 clones that contained sequences other than CA or GATA failed molecular beacon detection. Molecular beacon PCR, employing beacons specific for tandem repeat elements, provides a fast, accurate, and sensitive multiplex detection assay that will expedite verification of marker content in a multitude of template containing these repeats.

INTRODUCTION

Expeditious technological progression in molecular genetics, primarily driven by the Mouse and Human Genome Project, has resulted in a multitude of high-throughput efforts to construct chromosome-specific maps. These maps are based on sequence-tagged sites (STSs) (6) and gene-based sequence tagged site markers (ESTs) (5). To date, a physical map consists of a series of well-spaced STSs and covers almost the entire human genome. By positioning ESTs relative to microsatellite markers, a human transcript map is being generated across the genome by the Radiation Hybrid Mapping Consortium (15). The bacterial artificial chromosome (BAC) has proved to be a good cloning vehicle to generate relatively stable libraries (16). Recently, by improving the construction of BAC libraries, Osoegawa et al. (13) were able to create a highly recombinant, 25.3-fold redundant human male BAC library (RPCI-11).

Currently, our laboratory is in the process of assembling a genome-wide arrayed BAC clone resource for fluorescence in situ sequence hybridization (FISH) analysis of chromosomes (12).

Individual positive BAC clones are identified through hybridization-based screening of high-density filter sets. At the present time, fluorescent fingerprint analysis (4) and PCR analysis (11) are being used as the standard methodologies to confirm positively selected BAC clones. However, an initiative like this requires technologies that simultaneously advance the output and reduce costs. Above all, the conventional agarose gel electrophoresis technique used to visualize individual PCR products leaves room for improvement because this procedure is very time consuming and often ambiguous. PCR products screened on agarose gels can be vague, and the use of different primer pairs under similar PCR conditions can easily result in the formation of nonspecific PCR products. Furthermore, an undesirable consequence of opening test tubes is the possible PCR product contamination of untested samples (8).

In this paper, we report the development of a semiautomated assay to achieve high-throughput screening. This extremely sensitive multiplex assay eliminates the labor-intensive and time-consuming product confirmation of gel electrophoresis. In a real-time PCR, STS/EST regions are amplified in selected positive BAC clones in sealed reaction tubes in the presence of fluorescent reporter probes. These fluorescent reporter probes, or molecular beacons, can identify specific STS regions. Additionally, an extra level of specificity is being introduced with these molecular beacons because the generation of a fluorescent signal is exclusively due to the detection of the intended PCR product.

Molecular beacons are single-

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lowed a previously described strategy (12). To improve the success rate of the marker to BAC correlation, we are employing overlapping oligonucleotide probes (“overgo”) based on the EST and STS sequences for the markers (10). The overgos are designed with the same average melting characteristics and are labeled by replicating the 5′ overhangs using ³²P nucleotide triphosphates. To increase the throughput of the screening process to high-density BAC colony membranes, the probes are pooled in mixtures of 36 probes each. Informative probe mixtures are prepared through the use of a 3-D (6 × 6 × 6) probe pooling strategy consisting of 216 distinct probes.

Selection of PCR Primer Sets

A Basic Local Alignment Search Tool (BLAST) search was conducted using a UNIX®-based script, which automates the sequence analysis to mask repetitive elements (Repeat Masker). PCR of STS/EST marker sequences present in BAC clone DNA was performed using established primer pairs (MapPairs®; Research Genetics, Huntsville, AL, USA) flanking these markers and generating products ranging from 127 to 299 bp.

Synthesis of Molecular Beacons

To confirm predicted probe-BAC

pairs, molecular beacons were constructed capable of recognizing CA or GATA STRs. Synthesized molecular beacons contain two complementary 5-nucleotide-long arms and a 20-nucleotide-long probe sequence. For the single-beacon assays (optimization experiments), fluorescein (FAM) was selected as the fluorophore. To distinguish the fluorescence of one molecular beacon from another in a duplex assay, tetrachloro-6-carboxyfluorescein (TET) and carboxytetramethylrhodamine (TMR) were selected on the basis that their emission maxima are well spaced from each other across the visible spectrum. The fluorophores FAM and TMR (Molecular Probes, Eugene, OR, USA) are joined to a sulphhydryl group at their 5′ terminal by removing the protective trityl moiety. The quencher 4-(4′-dimethylaminophenylazo)benzoic acid (DABCYL) (Molecular Probes) is coupled to a primary amino group at its 3′ end utilizing an amine-reactive derivative of DABCYL. The TET molecular beacon was synthesized completely on a DNA synthesizer (Applied Biosystems, Foster City, CA, USA) utilizing a controlled-pore glass column (Glen Research, Sterling, VA, USA) to introduce a 4-dimethylaminoazobenzene-4′-sulfonyl group (DABSYL) at the 3′ end of the oligodeoxyribonucleotide and a tetrachloro-6-carboxyfluorescein phosphoramidite (Glen Research) to introduce the fluorophore at the 5′ end of the molecule. Each molecular beacon was purified by HPLC. A previously described, detailed protocol for synthesizing molecular beacons is available at <http://www.molecular-beacons.org>.

Nucleotide Sequence of Molecular Beacons

CA repeat-specific molecular beacons: fluorescein-5′-CGAGCGTGTGTGTGTGTGTGTGTGTGCTCG-3′-DABCYL (CA-FAM), carboxytetramethylrhodamine-5′-CGAGCGTGTGTGTGTGTGGTGTGGCTCG-3′-DABCYL (CA-TMR); GATA repeat-specific molecular beacons: fluorescein-5′-CGAGCTATCTATCTATCTATCTATCGCTCG-3′-DABCYL (GATA-FAM), tetrachloro-6-carboxyfluorescein-5′-CGAGCTATCTATCTATCTATTATCGCTCG-3′-DABSYL (GATA-TET),

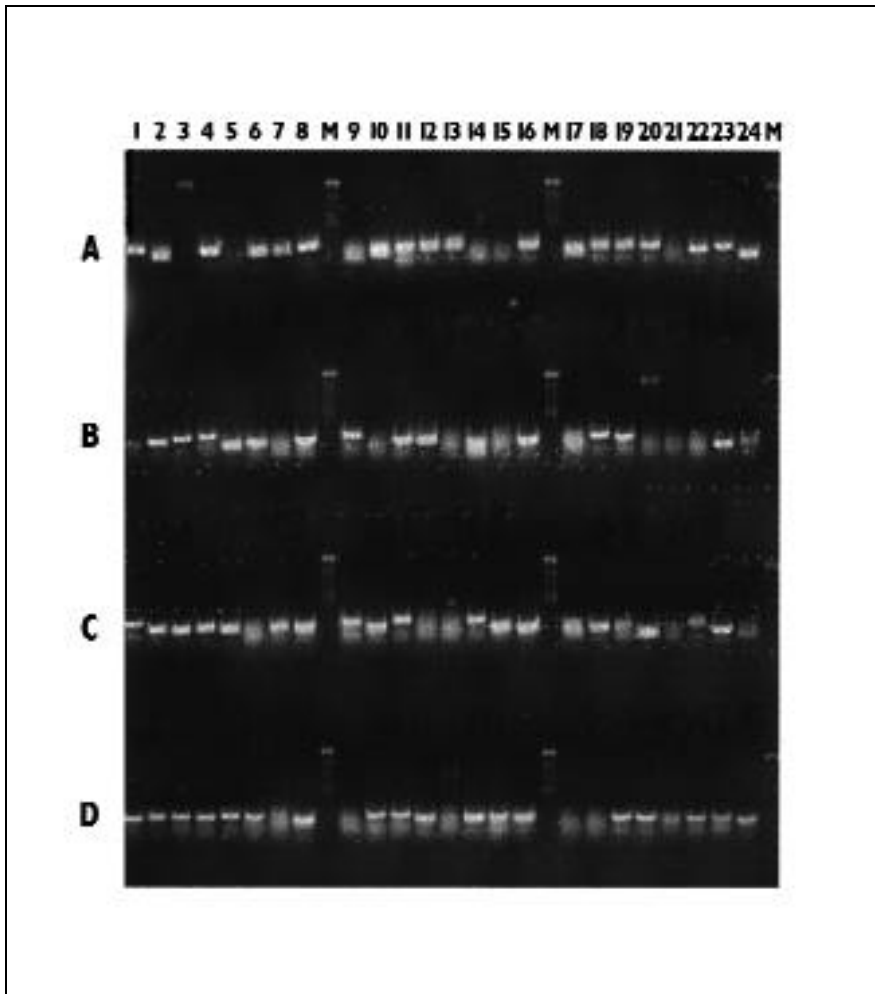


Figure 2. BAC clone confirmation through size assessment of PCR products by agarose gel electrophoresis. This figure shows conventional PCR products of 96 randomly selected samples on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV illumination. It illustrates that when clones are screened on agarose gels, bands can be rather vague (e.g., row A, lanes 15 and 21), or more than one product has been formed (e.g., row C, lane 9; row D, lane 15), which makes interpretation of the results difficult. Lanes M, 100-bp ladder (Life Technologies, Rockville, MD, USA).

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where underlines identify the complementary arm sequences of the stem.

Conventional PCRs

For the standard PCR analysis a PTC-200™ thermal cycler (MJ Research, Watertown, MA, USA) was used. PCR mixtures prepared for the conventional amplification reactions did not include molecular beacons. Each 50- μ L reaction contained 50 ng DNA, 0.20 μ M of a specific MapPairs primer set, 250 μ M deoxy-adenosine triphosphate (dATP), 250 μ M dCTP, 250 μ M dGTP, 250 μ M dTTP (Roche Molecular Biochemicals, Indianapolis, IN, USA), 0.3 U *Taq* DNA polymerase (Fisher Scientific, Pittsburgh, PA, USA), 1.75 mM MgCl₂, 50 mM KCl, and 10 mM Tris-HCl (pH 8.0). The thermal cycling program started with a 4-min denaturation at 95°C, followed by 30 cycles of amplification (denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and polymerization at 72°C for 45 s) and 1 cycle at 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis.

Molecular Beacon PCRs

Amplification reactions employing molecular beacons were performed in an ABI PRISM® 7700 spectrofluorometric thermal cycler using sealed optical tubes in a 96-well microplate (both from Applied Biosystems). Preceding the PCRs with molecular beacons in duplex, reference emission spectra of CA-TET and GATA-TMR were stored in the spectrofluorometric thermal cycler by hybridizing each molecular beacon to an excess of perfectly complementary oligonucleotides at 55°C. This enables the generated fluorescent emission spectra to be separated into the contributions from each of the two differently colored molecular beacons present in the same reaction tube.

Each 50- μ L reaction contained 0.20 μ M of a specific MapPairs primer set, 250 μ M dATP, 250 μ M dCTP, 250 μ M dGTP, 250 μ M dTTP, 2 U AmpliTaq Gold® DNA polymerase (Applied Biosystems), 4 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 50 ng target DNA, 0.2 μ M CA-FAM, or 0.2

μ M GATA-FAM. When assays were performed in duplex, both beacons were added to the same reaction (0.2 μ M CA-TET and 0.4 μ M GATA-TMR). The thermal cycling program started with 10 min at 95°C, by which the AmpliTaq Gold DNA polymerase is activated, followed by 35 cycles of 30 s at 95°C, 60 s at 55°C, and 30 s at 72°C. Fluorescence was monitored with a 100-ms exposure time during the annealing steps.

RESULTS

Clone Confirmation through Conventional PCR

Positive clones from segment 1 of the RPCI-11 human male BAC Library that had been identified on high-density filter sets were subsequently individually confirmed by PCR analysis. PCRs were performed using unique established primer pairs flanking each of the positive screened STS/EST markers. Clones that contained an EST, a CA, GATA, or GGAA repeat were verified. A total of

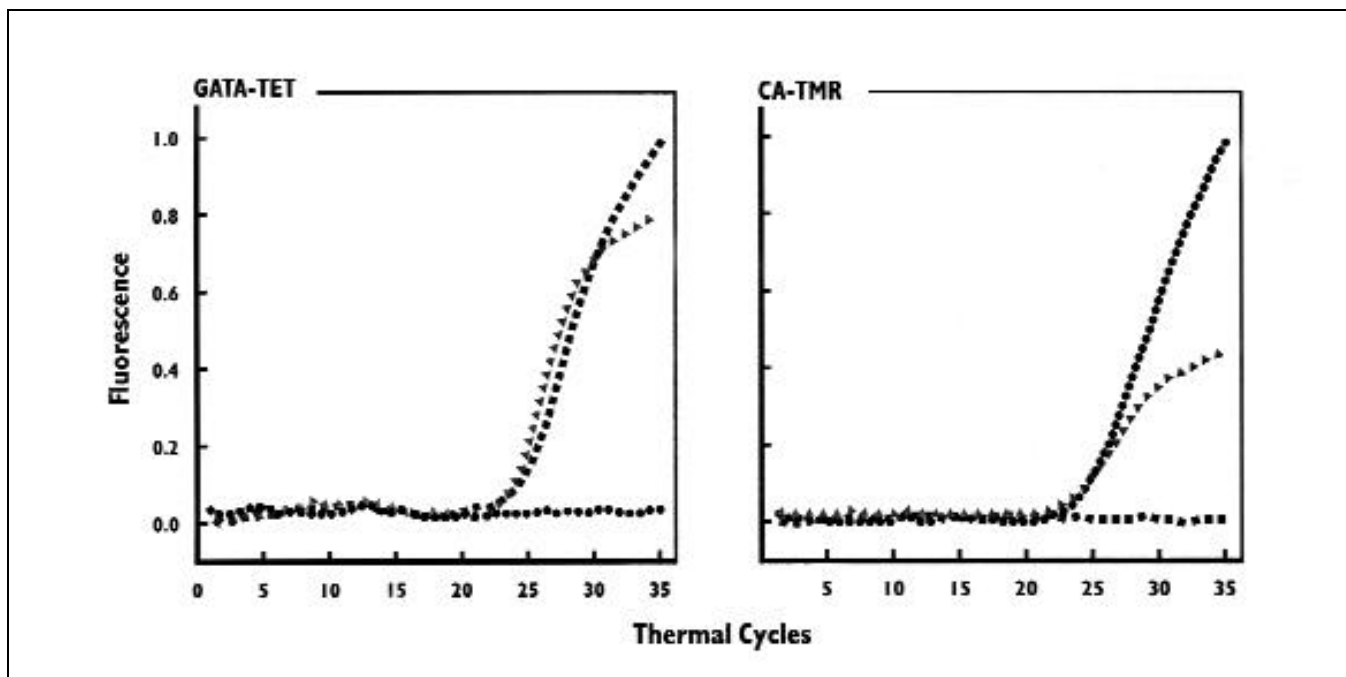


Figure 3. Specificity of real-time multiplex detection of CA and GATA repeat products. Two sets of five assays were carried out in parallel, each initiated with BAC clone DNA containing CA repeats (●), GATA repeats (■), CA/GATA repeats (▲), BAC clone DNA without any CA/GATA repeats (not shown), and a nontemplate control (not shown). Every tube contained a set-specific established primer pair to amplify either GATA or CA repeats, respectively. All tests were carried out in the presence of two differently colored, STS-specific molecular beacons [GATA-TET, left-hand panel (reports emission at 535 nm); CA-TMR, right-hand panel (reports emission at 575 nm)]. Only the molecular beacon whose probe sequence was complementary to a product formed a hybrid and emitted a fluorescent signal corresponding to either a CA or GATA repeat. Fluorescence was measured every cycle and plotted versus the cycle number. Fluorescent signals were absent in the template with no repeats and nontemplate control assays (not shown).

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Table 1. Comparison of BAC Clones Confirmation through Size Assessment of the PCR Product and Specific Binding of Molecular Beacons

LOCi	Internal Marker Type	BAC Clones Confirmed Positive by		BAC Clones Not Confirmed Positive by	
		Molecular Beacons and Agarose Gel	Molecular Beacons Only	Agarose Gel Only	Molecular Beacons and/or Agarose Gel
D7S517	CA	68d19 93a15	39g3 234n20		151m24
D7S531	CA	77o10 160p22 191p7	70d12		102o8 182i15
D7S499	CA	37i12 97p11 197b22 166d23 237c10			
D7S502	CA				132o23 222g12
D7S545	CA	170h15 232f14			37i12 69H 222g12 82a15 98i11 186d23 237c10
D7S559	CA	232n11 186d23 237c10			
AFMB296YE5	CA	80i6			
D7S527	CA	21n11 32c15 59k14 86d22 164b8 154d8			
D7S530	CA	46o13 63g17 70m2 119e8 205l23 206c15			
D7S544	CA	144o13 154k19 198a24			
WI-2901	CA	70g4 183b11			61c18
D7S587	CA	1n24 128o1 142o11 220k4 243d15	78c11		41o4 54p11 140c21 150k10 267o17 148c1
D7S522	CA	211e22	85h5		175n20 189e18 274b15
D7S480	CA	23i15 147p1 181d23 183b10 197g20	254f9	3b12	
D7S550	CA	250c14 286l2 78l2 103h10			
D7S550	CA	254f9			104k19 105j11 111m6 210b22 11h21 11n19
AFMC016YC9	CA	9c22 11h21 78l2 103h10 65h21 264g16	11n19 201b4 225f7		55h21 78l2 103h10 201b4 264g16
AFMA099WE5	CA	118j20 204m6 71k5	279e23	209g24	32h13 46b4 77e2 226c1 228j18 237e7 104k19
D7S509	CA	39c7 45g1 80j18 115n3 148f17 205l5 228i4			105j11 111m6 210b22
D7S1779	CA	249d23 4m2 19i13 22g20 46k1 168h5			54p11 140c21 150k10 236o18 237e23 267o17
D7S551	CA	4h2 59j19 64g19 188a12 256c7			279c21 111m6 210b22
AFMA082XO9	CA	178f1 222h2 249c6			27f20 59j19 64g19 78o18 148g21
D7S588	CA				186o24 231p11 256c7
D7S513	CA/GATRa	37a13 66g13 142k21 154e10 155a14			
CHLC.GATA21D08	GATA	157f9 169i11 208k23		113k24	89p11
CHLC.GATA73F12	GATA	97h7 177l20		115o3	141i24
CHLC.GATA4E02	GATA	3i18 64b19 64i9 94j2 98m4 104g2			
		141e24 239l22			
		148j6 172n6 218d2 218n2			

Table 1. Continued

LOC1	Internal Marker Type	BAC Clones Confirmed Positive by		BAC Clones Not Confirmed Positive by	
		Molecular Beacons and Agarose Gel	Molecular Beacons Only	Agarose Gel Only	Molecular Beacons and/or Agarose Gel
CHLC.GATA67A05	GATA	104k19 105j11 111m6 210b22			469 108b5 179h13 214j1 254i14
CHLC.GATA64H06	GATA	111m6 210b22 105j11			3e19 254i14 84g8 247f23 271a11
CHLC.GATA42A01	GATA	24j13 72d16 78o18 127c19 249i10			
CHLC.GATA84E04	GATA	244p13			43h19
CHLC.GATA2C08	GATA		84f2 103e18		25f5 108c10
CHLC.GGAA22F06	GGAAP			60g20 84f16 87h10 92i13 108k16 133n9 156a14	20e12 20m17 24f23 51a20 104g10 122a8 125d21 139o1 151b6 167o19 164h9 169g24 182i15
WI-9592	ESTp			98f5 187c8 151m24 234n20	33g17 44c18 142f17 213c2 237j10 237m11 240n11
WI-5575	EST			151m24 234n20	117i8 135k12 143i5 237j10 237m11
WI-4829	EST			44c18 142f17 240m11	
WI-4637	EST			20g8 43p15 108j15	33g17 213c2
WI-4152	EST			124p14 194m4 216j8	
WI-3234	EST			30e11	205b3
WI-9804	EST			69k 82a15 98h11	
NIB1997	EST			150a15	

^aBAC clones anchored to locus D7S513 were all confirmed positive by molecular beacons for both CA and GATA STRs.

^bGGA/EST markers undetermined by the molecular beacon approach.

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260 BAC clones were tested utilizing 40 different specific oligonucleotide sets derived from as many STS/EST markers available through the Whitehead Center for Genome Research (Cambridge, MA, USA). Out of the 260 hybridization positive clones, 79.2% ($n = 206$) were unique clones from which 145 clones were verified by conventional PCR and agarose gel electrophoresis (i.e., 70.4% of the unique clones) (Table 1). Figure 2 shows products of the final confirmation achieved through size assessment of PCR products by agarose gel electrophoresis. As a consequence of the high-throughput screening, the PCR analyses for all STSs were carried out under identical conditions, resulting sometimes in rather vague bands and/or multiple products. This makes interpretation of the results often difficult, and repetitive screening of these clones is therefore inevitable.

Real-Time Detection of PCR Products

To overcome the imperfections described above of the BAC clone verification through size assessment of PCR products by agarose gel electrophoresis, a semiautomated assay was developed with the ability to identify specific STRs. In a real-time PCR, we detected these STS/EST regions utilizing molecular beacons. First, we had to determine that molecular beacons could easily discriminate between the different STRs. Therefore, two fluorescein-labeled molecular beacons were synthesized, each with internal loop sequences of 20 bp long, complementary to either 10 CA repeats or 5 GATA repeats. The arm sequences of the beacons were designed to form a hybrid at the PCR annealing temperature. To confirm the hairpin structure, a computer program (22) further analyzed the complete sequence of each molecular beacon. For this occasion, established primer pairs for each of the two STRs were selected on the basis of their PCR product expediency, as determined in previously performed conventional PCR assays. Two human DNA samples were used as templates for PCRs in which the CA or GATA repeats were amplified in separate tubes in the presence of the specific molecular beacons.

A spectrofluorometric thermal cycler possessing a 488-nm laser light source was used to determine the elicited fluorescence in real-time. In all the tested samples, fluorescent signals were generated with molecular beacons complementary to the products containing a specific STR, whereas a noncomplementary beacon does not generate a signal. In control reactions in which a DNA template is absent, no increase in fluorescence was observed (data not shown). Furthermore, agarose gel electrophoresis disclosed the presence of PCR products in all tested samples, except for the samples without any DNA template (control). Our results demonstrate that these molecular beacons bind specifically to their intended target product of either CA or GATA repeats.

Real-Time Multiplex Detection of CA and GATA Repeat Products

The final stage of our study was to establish a high-throughput application in which BAC clones containing CA and/or GATA repeats are distinguished in the same sealed reaction tube. This can be achieved by applying CA-TMR and GATA-TET molecular beacons simultaneously in a single PCR assay. The emission spectra of TET (emission maximum 535 nm) and TMR (emission maximum 575 nm) are reported during the annealing stage of all 35 thermal cycles. In the spectrofluorometer thermal cycler, the stored reference emission spectra of TET and TMR were utilized to decompose the recorded spectra into individual spectral contributions of GATA-TET and CA-TMR molecular beacons. The manifestation of green-yellow and orange fluorescence identifies the GATA repeat and CA repeat, respectively.

To examine the sensitivity of our newly developed detection assay, the 260 hybridization positive BAC clones, previously characterized by conventional PCR, were put to the test in a comparative molecular beacon assay. To preserve objectivity, a blind study was performed. BAC clone DNA, control human DNA, and nontemplate samples were mixed with the corresponding established primer pairs in Roswell Park Cancer Institute. These samples were then coded and shipped

overnight to the Public Health Research Institute, where the molecular beacon genotyping data were generated.

Figure 3 shows the specificity of the real-time multiplex detection of CA and GATA repeat products. Five assays were carried out in parallel, initiated with BAC clone DNA containing CA and/or GATA repeats, BAC clone DNA without any CA/GATA repeats, and a nontemplate control. The result shows that individual clones are easily distinguished from each other by the color of the fluorescent signal generated by molecular beacons complementary to a CA or GATA repeat. No fluorescent signals were generated in both the template with no repeats and nontemplate control assays (data not shown).

The results in Table 1 show that molecular beacons detected 30 out of 43 GATA repeat clones (conventional PCR, 29/43) and 99 out of 162 CA repeat clones (conventional PCR, 92/162). With the CA-TMR and GATA-TET molecular beacons, we additionally verified 10 BAC clones containing CA repeats and two BAC clones containing GATA repeats. However, one GATA repeat and three CA repeat clones, which were initially positively confirmed by the conventional method, were tested as negative. Unexpectedly, all eight positive clones anchored to locus D7S513, which is reputed to be a CA repeat marker, generated fluorescence with both TET and TMR fluorophore labeled beacons. A sequence query for marker D7S513 at the Whitehead Institute (<http://www.genome.wi.mit.edu>) showed that there were also 11 repeated GATA sequence blocks present within the product area of this locus. PCR products of BAC clones and positive control DNA containing ESTs or GGAA repeats, as well as the nontemplate controls, remained negative for TMR and/or TET fluorescence. Amplified positive control DNA, which contained CA and/or GATA repeats, did generate the expected TMR and/or TET fluorescence.

DISCUSSION

With the ambitious goals to complete high-quality full DNA reference sequences of human (2) and mouse,

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and ultimately those of other mammalian species (17), plants, and insects, there is a need to develop new supporting technologies. Resources of mapped BAC clones are prerequisites to sequencing entire genomes. Here, we describe a novel BAC clone confirmation assay by real-time PCR utilizing molecular beacons. This distinctive molecular beacon application is a high-throughput, PCR-based STS detection assay that will support the construction of a genome-wide arrayed BAC clone framework. So far, fluorescent fingerprint analysis (4) and PCR analysis (11) have always verified BAC clone eligibility. Visualization of individual PCR products by way of agarose gel electrophoresis is time consuming. Diametrically opposed to this technique is our newly developed PCR/hybridization test. This assay utilizing molecular beacons is simple, and it completely eradicates the labor-intensive product confirmation of gel electrophoresis. Furthermore, the specificity of the molecular beacon allows low-stringency amplification parameters, resulting in robust amplification of almost any primer/template combination. One of the many advantages of working with molecular beacons is that cross-contamination does not occur because tubes remain sealed while the data is being generated (19). The profound specificity of molecular beacons enables a multiplex one-tube detection assay because mixtures of multicolored molecular beacons can help discriminate between different targets (9,18).

When 260 positive clones from the RPCI-11 human male BAC library (identified on high-density filter sets) were put through the conventional PCR confirmation test, 145 clones were verified (Table 1). On first sight, these results might seem to be rather poor. However, high-density filter sets used for hybridization screening purposes are reused to reduce the cost of analysis. Under initial conditions, some positive signals appeared to be previous hybridization signals (unpublished results). In addition, we also found that for 10% of STS/EST markers, the primer pairs were not successful in generating a PCR product. Since PCR was performed using established primer pairs for microsatellite markers,

there is always a possibility that a marker sequence (derived from the Whitehead Center for Genome Research) either contains errors or polymorphic sites.

Due to the tremendous throughput of BAC clones that need confirmation, it is a necessity that PCR analyses for all the STSs were carried out under identical conditions. It is likely that optimizing the individual PCR conditions for markers that did not amplify could increase the percentage of positive clones. A consequence of the standard PCR conditions is the sometimes-ambiguous product production. Clone confirmation through size assessment of the PCR product by agarose gel electrophoresis often show vague or multiple bands (Figure 2). This makes interpretation of the results difficult, and repetitive screening becomes a necessity. This is in sharp contrast to molecular beacon assays. Due to the specific interaction of a molecular beacon with its complementary target, aspecific bands (PCR products) will be completely ignored.

The application described here of the real-time multiplex detection of STRs containing products in the same closed reaction vessel has proved to be an extremely sensitive and specific test system. This was accomplished by using two different molecular beacons simultaneously, each specific for a different STS region and each emitting light of a different color (TET, emission maximum 535 nm; TMR, emission maximum 575 nm). The results of this completely blind study (Table 1) show that with molecular beacons, 129 out of 205 BAC clones with CA or GATA repeats were detected, whereas with the conventional method, only 121 BAC clones could be verified. Furthermore, none of the 35 EST or 20 of the BAC clones containing GGAA repeats were scored as false positive. The unanticipated detection of a combined CA and GATA repeat in all eight BAC clones anchored to locus D7S513 demonstrates the highly specific interaction of molecular beacons with their complementary target.

Although we only screened the two most common repeats observed in the STSs utilized in this study (CA and GATA repeats), the construction of

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multicolor molecular beacons specific for each other minor repeat element observed in STSs (GGAA, ATA, GGAT, GCT, etc.) could allow for the rapid, multiplex detection of any STS, from any organism, in a single assay. Fortunately, the use of (multiple) molecular beacons can be highly cost effective. In particular, when used in large-scale projects, there will only be a marginal increase in expenses made per test as compared to the conventional agarose gel electrophoresis technique used to visualize individual PCR products. Recently, molecular beacons have been successfully applied in different mutation detection studies (3,7,14,20,21). In conclusion, this newly developed molecular beacon application is perfectly suited to expedite the BAC clone confirmation throughput.

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