Washington University School of Medicine
Digital Commons@Becker

Open Access Publications

2007

Polony analysis of gene expression in ES cells and blastocysts

C. Rieger
Washington University School of Medicine in St. Louis

R. Poppino
Washington University School of Medicine in St. Louis

R. Sheridan
Washington University School of Medicine in St. Louis

K. Moley
Washington University School of Medicine in St. Louis

R. Mitra
Washington University School of Medicine in St. Louis

See next page for additional authors

Follow this and additional works at: https://digitalcommons.wustl.edu/open_access_pubs

Part of the Medicine and Health Sciences Commons
Please let us know how this document benefits you.

Recommended Citation

Rieger, C.; Poppino, R.; Sheridan, R.; Moley, K.; Mitra, R.; and Gottlieb, D., "Polony analysis of gene expression in ES cells and blastocysts." Nucleic Acids Research. 35, 22. e151. (2007).
https://digitalcommons.wustl.edu/open_access_pubs/140

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact vanam@wustl.edu.

Authors
C. Rieger, R. Poppino, R. Sheridan, K. Moley, R. Mitra, and D. Gottlieb

This open access publication is available at Digital Commons@Becker: https://digitalcommons.wustl.edu/open_access_pubs/140

Published online 10 December 2007 Nucleic Acids Research, 2007, Vol. 35, No. 22 e151
doi:10.1093/nar/gkm1076

Polony analysis of gene expression in ES cells and blastocysts

C. Rieger[1], R. Poppino[1], R. Sheridan[2], K. Moley[2], R. Mitra[3] and D. Gottlieb[1,*]

[1]Department of Anatomy and Neurobiology, [2]Department of Obstetrics and Gynecology and [3]Department of Genetics, Washington University School of Medicine, 660 S. Euclid Avenue, St Louis, MO 63110, USA

Received June 28, 2007; Revised October 24, 2007; Accepted November 15, 2007

ABSTRACT

Expression profiling of stem cells is challenging due to their small numbers and heterogeneity. The PCR colony (polony) approach has theoretical advantages as an assay for stem cells but has not been applied to small numbers of cells. An assay has been developed that is sensitive enough to detect mRNAs from small numbers of ES cells and from fractions of a single mouse blastocyst. Genes assayed include Oct3, Rex1, Nanog, Cdx2 and GLUT-1. The assay is highly sensitive so that multiple mRNAs from a single blastocyst were easily detected in the same assay. In its present version, the assay is an attractive alternative to conventional RT–PCR for profiling small populations of stem cells. The assay is also amenable to improvements that will increase its sensitivity and ability to analyze many cDNAs simultaneously.

INTRODUCTION

Stem cells are currently the focus of intense interest because of their importance in normal development and adult physiology as well as their potential application in clinical medicine. Expression profiling of stem cells poses a special challenge and lack of appropriate methods constrains progress in many branches of stem cell research. The challenge arises because stem cells occur as small populations surrounded by other cell types and because even the stem cell populations themselves are heterogeneous and encompass multiple cell populations. An ideal profiling method would have three capabilities. The first is the sensitivity to assay mRNAs in small populations and single cells and thus deal with heterogeneity. Because cell fate is determined by sets of genes rather than any single gene, the method must also allow parallel analysis of multiple genes. Finally the method must be quantitative since levels of expression rather than mere presence or absence of transcripts determines phenotype. While multiple expression analyses of stem cells based on PCR have been published no method fulfills all of these criteria. (1–5). The method of PCR colony ('polony') analysis differs in important ways from conventional PCR and has the potential to be very useful for profiling stem cells.

In polony [also called molecular colony (6)] analysis, individual DNA molecules are amplified clonally in a polyacrylamide gel matrix (7,8). Analysis is very efficient, with 80% of the input DNA molecules forming polonies so the method is inherently very sensitive (1). All polonies signify one starting template DNA molecule, so variations of amplification efficiency do not influence the final count of input templates. Cross-interference of different amplicons is largely avoided since the reactions are effectively isolated from one another by the gel matrix.

The DNA sequence of individual polonies can be ascertained by either sequence-specific fluorescent hybridization probes or an *in situ* sequencing procedure, thus opening the way for parallel multigene analysis (9). Because of these features, the polony method is an excellent candidate approach for profiling stem cells. However previous expression studies with polonies have used relatively large starting samples of cells (10) so it is not known if the technique can be applied to small numbers of cells and is useful for stem cell profiling.

In this report we demonstrate that the polony method can be used on small numbers of stem cells including ES cells and blastocysts. A method for isolating RNA and synthesizing cDNA from small samples was coupled with polony analysis and the sensitivity of the overall approach and the ability to do parallel analyses of multiple genes was evaluated. Our results represent significant progress towards the ideal profiling method described above and will encourage further technical developments of the polony approach.

MATERIALS AND METHODS

ES cell culture

All ES cell experiments were done with the RW4 line of ES cells derived from Sv129 mice. Undifferentiated (ES) were cells were grown on gelatin-coated tissue culture plastic in

*To whom correspondence should be addressed. Tel: +314 362 2758; Fax: +314 362 3446; Email: gottlied@pcg.wustl.edu

© 2007 The Author(s)
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Downloaded from nar.oxfordjournals.org at Washington University School of Medicine Library on July 17, 2011

the presence of leukemia inhibitory factor (LIF) using standard methods (11,12). In preparation for polony experiments, undifferentiated ES cells were trypsinized with 0.25% trypsin-EDTA (GIBCO) to detach cells from the surface and counted using a hemocytometer. For the 1000 ES cell isolation, cells were spun down and diluted to obtain a concentration of 500 000 cells/ml.

Embryo recovery and culture

Embryos were recovered as previously described (13). In brief, 3-week-old female mice (B6 × SJL F1, Jackson Laboratories; Bar Harbor, ME) were given free access to food and water and were maintained on a 12-h light/dark cycle. Female mice were superovulated with an intraperitoneal injection of 10 International Units (IU)/animal pregnant mare serum gonadotropin (PMSG, Sigma; St Louis, MO) followed 48 h later by 10 IU/animal of human chorionic gonadotropin (hCG, Sigma; St Louis, MO). Female mice were mated with males of proven fertility overnight following the hCG injection. Mating was confirmed by identification of a vaginal plug. Mice were sacrificed 96 h post-hCG injection to recover embryos at the blastocyst stage (3.5 d.p.c). Embryos were recovered by flushing dissected uterine horns and ostia with human tubal fluid medium (HTF, Irvine Scientific; Santa Ana, CA) containing 0.25% BSA (Bovine serum albumin fraction V, Sigma; St Louis, MO).

RNA preps

Cells (either ES cells or blastocysts) were delivered to PCR tubes containing either 10 μg for blastocysts or 100 μg for ES cells of Dynabeads Oligo $(dT)_{25}$ in 20 μl or 100 μl lysis-binding buffer [100 mM Tris–HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA, pH 8.0, 1% LiDS, 5 mM dithiothreitol (DTT)]. Cells were lysed by pipetting up and down five times in the lysis-binding solution. Tubes were rotated for 10 min at room temperature to promote hybridization of the $poly(A)^+$ mRNA with the oligo(dT) tails of the Dynabeads. After hybridization of mRNA with Dynabeads, a series of washes was performed to prepare the mRNA for reverse transcription. Two washes were performed in wash solution A (10 mM Tris–HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS). Next, Dynabeads were incubated in 100 μl wash solution B (10 mM Tris–HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA) + 1 μl 1% Tween-20 for 5 min to allow the beads to equilibrate. This was followed by a second wash in wash solution B without Tween and a final wash in 10 mM ice-cold Tris–HCl, pH 7.5. In some experiments mRNA attached to the beads was used directly in an RT reaction. In others the mRNA was eluted in 10 μl Tris–HCl by heating at 90°C for 2 min.

cDNA synthesis

Reverse-transcription reactions were performed using the RETROscript kit (Ambion, Austin, TX). Final concentrations of components were as follows: 1× RT buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT), 5 μM oligo(dT_{18}), 500 μM each dNTP, 0.5 U/μl RNase Inhibitor, 5 U/μl MMLV reverse transcriptase, 0.05 μg/μl BSA was added as a carrier. For cDNA synthesis reactions performed on mRNA hybridized to Dynabeads the oligodT primer was omitted. cDNA synthesis reactions were carried out at 42°C on a roller for 1 h. An RT-minus reaction was always prepared in parallel by substituting water for MMLV RT-enzyme.

Polony reactions

Polony reactions were prepared according to Mitra and Church (7). Template cDNA was added to a liquid phase acrylamide gel mix containing PCR components. Templates were amplified using PCR within the gel. cDNA template or RT-minus suspension was added to a liquid-phase PCR mix (polony mastermix) [10 mM Tris–HCl pH 8.3, 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM forward primer, 1 μM primer reverse_Ac, 3.3 U or 3.8 U Jumpstart *Taq* (Sigma, St Louis, MO), 9% acrylamide, 0.05% bisacrylamide (Sigma)]. Then, 0.667 μl of degassed 5% ammonium persulfate (Sigma) and 0.667 μl 5% temed (Sigma) were added to the polony mix to a total volume of 28 μl or 40 μl. Nineteen microliters of this solution was pipetted underneath a clean No. 2 coverslip (18 × 30 mm Fisher) on a bind-silane (Sigma) treated Teflon-coated oval well slide (Erie Scientific, Portsmouth, NH). A Secure-Seal chamber (Grace Bio-labs) and mineral oil were added to the slide before cycling.

Slides were cycled using a PTC-200 thermal cycler (Bio-Rad, Hercules, CA) adapted for glass slides (16/16 twin tower block). The following program was used: denaturation (2 min at 94°C) followed by 43 cycles of denaturation, primer annealing and extension (30 s at 94°C, 30 s at 56°C, 30 s at 72°C). After cycling the Secure-Seal™ chamber was removed and slides were washed in hexane for 5 min to remove mineral oil and remaining adhesive. Coverslips were removed and slides were washed twice in solution 1E (10 mM Tris pH 7.5, 50 mM KCl, 2 mM EDTA, 0.01% Triton X-100) for 4 min with gentle shaking.

Hybridization for polony detection

Slides were incubated in 70% formamide in 1× SSC at 70°C on a roller for 2 min to denature double stranded DNA. Formamide was removed by washing with water for 3 min followed by washing with solution 1E. A blue Frame Seal chamber base (Bio-Rad) was applied to each slide and annealing mix was added (5.6 μM hybridization probe in 125 μl of 6× SSPE buffer with 0.01% Triton X-100). Slides were heated (2 min at 94°C, 7 min at 56°C). Frame seal chambers were removed quickly and slides were placed in wash1E to dilute away excess primer to limit non-specific binding. Slides were washed and stored in wash1E.

Primers

The primers used are listed in S1–S4; all primers are from IDT (Coralville, IA). Primers were selected using Primer 3 with the restriction of being within 800 bp of the 3′ end. All polony reverse primers include an acrydite group (Ac) on the 5′ ends (7). The 5′ end of the hybridization primers are covalently linked to a fluorescent dye (Cy5).

Downloaded from nar.oxfordjournals.org at Washington University School of Medicine Library on July 17, 2011

Visualizing and scoring polonies

Polony slides were coverslipped, and imaged using a GenePix 4000B (Axon Instruments, Union City, CA) microarray scanner and GenePix software. Optimal signal intensity for the Cy5 fluor was obtained for laser PMT gain of 700 (635 laser) and 82 (532 laser). Images were saved as TIF and JPEG files. Polonies were counted manually using ImageJ software and cell counter applet.

Competitive PCR

DNA competitors with a 50-bp deletion of the corresponding native amplicons were synthesized by standard methods. The competitors have the same terminal sequences as the native amplicons to ensure equal amplification. Forward primers, reverse primers and deletion primers are described in Table S4. For polony and competitive PCR analysis, RNA was extracted in a series of reactions containing 2000 ES cells and 100 μg of Dynabeads in 100 μl of lysis-binding buffer as previously described. RNA was eluted from Dynabeads in 20 μl DEPC H_20 and 4 μl oligo$(dT)_{18}$ and reverse transcription performed as previously described in a total volume of 42 μl. Competitive PCR reactions were carried out with a fixed amount of sample and varying amounts of competitor to determine the equivalence point.

Model RNA

To analyze the efficiency of RT a mimic mRNA was constructed. The mimic consists of the BNI5 yeast gene fused to a poly$(A)^+$ tail and was created by knitting PCR followed by cloning into the pBluescriptSKII(+) vector. RNA was transcribed from this plasmid by standard methods using T7 RNA polymerase. Model mRNA was purified by standard methods and quantified by OD260 absorption.

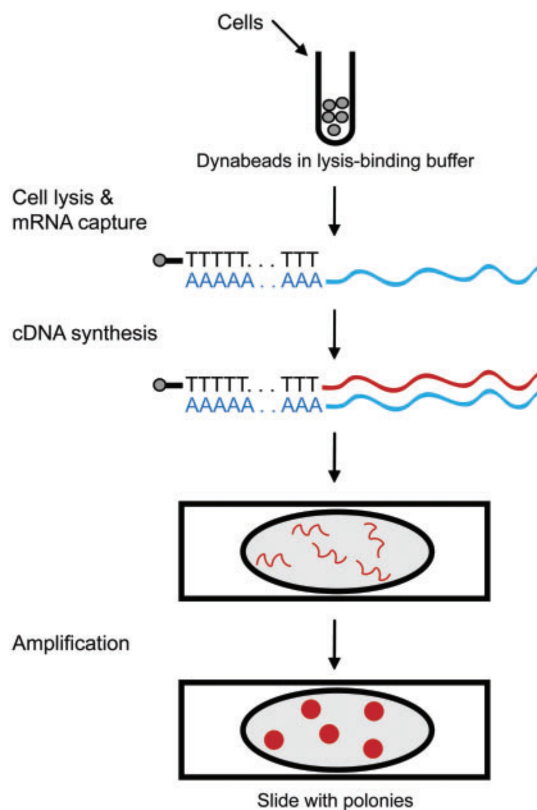

Figure 1. Flow chart of typical experiment. Cells (either 1000 ES cells, single blastocysts or multiple blastocysts) were delivered to a lysis-binding solution containing oligo$(dT)_{25}$ Dynabeads®. After cell lysis, mRNA was captured by hybridization with poly(A) tails on the beads and mRNA was reverse-transcribed into cDNA. cDNA was added to non-polymerized polyacrylamide gel mix containing PCR components and deposited in an oval well on a microscope slide. After polymerization of the gel, slides were thermocycled so that cDNA templates gave rise to polonies. Polonies were visualized by hybridization with a labeled gene-specific probe.

RESULTS

Polony technology has been used extensively to analyze genomic DNA and in a few instances cDNAs from large numbers of yeast or mammalian cells (8,10,14), but has not been used to profile gene expression from small numbers of cells. Our first question was whether polony analysis could be applied to small numbers of mammalian stem cells. Mouse ES cells were chosen because they offer a pure population of stem cells where the gene expression pattern is clearly related to cell fate choice (15,16). We also analyzed blastocysts, a stage of mammalian development comprised mainly of stem cells including a subset which corresponds to ES cells.

In the first experiment, 1000 ES cells were used as the starting sample for isolating mRNA. Several methods of RNA extraction were investigated and it was found that hybridization capture of mRNA on oligo $(dT)_{25}$ Dynabeads was particularly efficient (Figure 1). The mRNA from 1000 ES cells was captured on Dynabeads and added to a RT reaction with the oligo-dT of the beads serving as primer. After cDNA synthesis, a small fraction of the beads was delivered to a polony slide with primers designed to amplify Oct3, a transcription factor involved in maintaining the pluripotency of ES cells (17). Each polony slide received the equivalent of 10 cells worth of cDNA or an equal volume of a control reaction lacking RT. Slides were thermocycled and then stained with a labeled hybridization primer for Oct3. In this and all subsequent experiments hybridization probes are internal to the amplifying primers and are labeled with Cy5 coupled to the 5′ terminus. It is crucial that the assays be highly specific for the intended transcript and not show false positives. As with any PCR method, there is the potential of primer dimers and other unintended amplified sequences. Our results are very likely to be free of this sort of error for two reasons. All experiments include RT control samples and these do not produce polonies. Second, scoring polonies by hybridization of an internal primer which does not share sequence with the amplifying primers prevents signals from primer dimers and other unintended amplicons. Polonies were visualized on an Axon microarray scanner (Figure 2) and were abundant, evenly distributed and clearly distinguishable from background on the slides with cDNA. Importantly, polonies

Downloaded from nar.oxfordjournals.org at Washington University School of Medicine Library on July 17, 2011

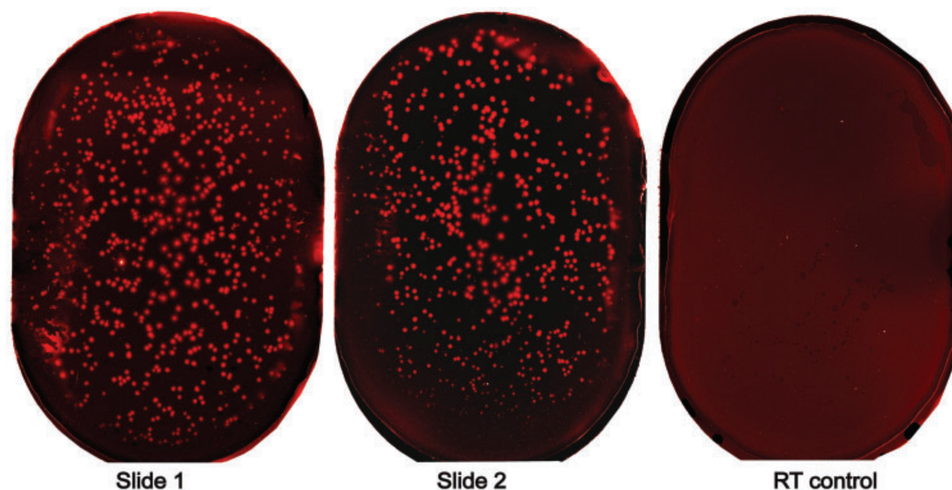

Figure 2. Oct3 polonies from ES cells. Slides 1 and 2 each received cDNA equivalent to 10 ES cells and were amplified to create Oct3 polonies. Polonies were visualized with a Cy5 gene-specific probe. The RT control slide is from a reaction without reverse transcriptase and has no polonies.

Downloaded from nar.oxfordjournals.org at Washington University School of Medicine Library on July 17, 2011

Table 1. Oct3 polony counts from slides with 10 ES cell equivalents

Slide number	Sample	Oct3 polonies per slide	Oct3 polonies per cell
1	10 ES cell equivalents	656	66
2	10 ES cell equivalents	542	54
3	10 ES cell equivalents	618	62
4	10 ES cell equivalents	513	51
5	10 ES cell equivalents	403	40
6	10 ES cell equivalents	292	29
7	10 ES cell equivalents	509	51
8	10 ES cell equivalents	460	46
9	RT control	0	0
Average		499	50
SD		116	
SE			37
Slides 1–4 originated from the same RT reaction			
Slides 5–8 originated from the same RT reaction			

mRNA was isolated from 1000 ES cells and reverse transcribed to cDNA in two separate reactions. Ten ES cell equivalents of cDNA from each synthesis was delivered to four polony slides. After amplification, polonies were visualized by an Oct3 gene-specific hybridization probe. Scans of slides 1 and 2 appear in Figure 2. Oct3 polony counts ranged from 292 to 655 polonies per slide with an average of 499 per slide, equivalent to an average of 50 polonies per ES cell. Slide 9 is an RT control and does not contain polonies.

were absent from the RT control slide demonstrating that cDNA rather than genomic DNA is detected. To investigate reproducibility, an experiment with two independent RT reactions was performed (Table 1). Each RT reaction was assayed on four slides and the number of Oct3 polonies on each slide counted. The mean of all eight slides was 499 polonies with 116 SD; this is equivalent to a mean of 50 Oct3 polonies/cell. As discussed below this is a 'minimum' estimate of the number of mRNAs per cell as it does not take into account the efficiency of mRNA isolation and conversion to cDNA. We conclude that the polony approach allows the assay of expression from small numbers of ES cells.

Table 2. Oct3 polony counts from pooled and individual blastocysts

Slide number	Sample (number of blastocysts in starting sample)	Oct3 polonies per slide	Oct3 polonies per blastocyst
1	1/2 Blastocyst equivalent (10)	967	1868
2	1/2 Blastocyst equivalent (10)	901	
3	1/2 Blastocyst equivalent (1)	857	
4	1/2 Blastocyst equivalent (1)	871	1728
5	RT control (5)	0	
6	RT control (5)	0	0
Average		899	1798
SD		48.9	

mRNA was isolated from a pool of 10 blastocysts and diluted so that a half blastocyst equivalent was delivered to slides one and two. Next, mRNA isolated from a single blastocyst was divided between slides three and four. Two RT control slides contained mRNA from the equivalent of five blastocysts. Oct3 polony counts from the pooled blastocyst sample are in good agreement with the individual blastocyst sample. Data demonstrates that analysis from half of a blastocyst is feasible.

Having demonstrated that polonies can detect mRNA from small numbers of ES cells, we wanted to see if they could be used to detect mRNAs in a normal biological structure that contains stem cells and is made up of a small number of cells. We chose the mouse blastocyst since it is an intensely studied stage of mammalian development, is easily obtainable, and is comprised of only 75–100 cells (18,19). About 40% of the cells are in the inner cell mass (ICM) and phenotypically resemble ES cells. The transcription factor Oct3 is exclusively expressed in the ICM (20). In a range-finding experiment, 10 mouse blastocysts were pooled, their mRNA isolated and cDNA synthesized. Polony assays for Oct3 were conducted on two slides each containing cDNA equivalent to half of a blastocyst. There were 967 and 901 polonies on the two slides for a total of 1868 polonies/blastocyst (Table 2). Next, mRNA from a single blastocyst was isolated, reverse transcribed and two slides prepared. The average

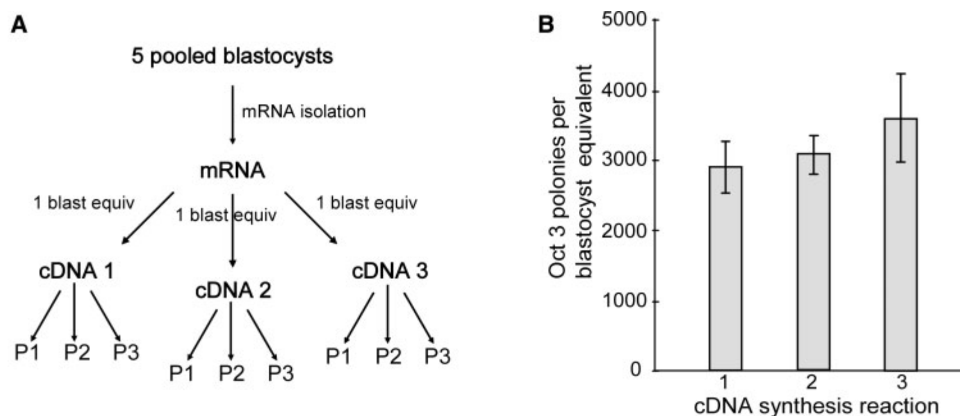

Figure 3. Analysis of variation of RT and polony generation steps. (**A**) Flow chart of the experiment. Five pooled blastocysts are used in a single mRNA prep and one blastocyst equivalent is used in three separate RT reactions. Each RT reaction is analyzed on three separate polony slides (P1-3) for Oct3. (**B**) Bar graph where each bar is the average number of polonies for three slides from the same cDNA synthesis. The error bars are the standard deviation. ANOVA indicates that independent cDNA preparations are indistinguishable ($P > 0.05$)

of these slides detected 1728 polonies/blastocyst (Table 2). Two RT controls were done with the mRNA equivalent of five blastocysts; no polonies were present. We conclude that the polony method is sensitive to the level of a single blastocyst and that the entire analysis from mRNA preparation through polony analysis is scaleable in the range of 1–10 blastocysts. The sensitivity of the polony assays compares very favorably with conventional RT–PCR analysis of expression in blastocysts, where multiple blastocysts are pooled to detect gene expression (21,22). However for some genes expression of multiple genes can be measured from a single embryo (23)

In order for the assay to be useful it is essential to know the sample-to-sample variability due to cDNA synthesis and polony reactions. In this and all subsequent experiments, we used two minor refinements of the previous protocol: mRNA was eluted from the beads prior to cDNA synthesis and the amount of *Taq* per slide was increased 3-fold. Taken together, these two steps increase polony counts by about 30% (data not shown). To measure variability, mRNA was isolated from a pool of five blastocysts and split into three sub-pools, each the equivalent of a single blastocyst (Figure 3). These were reverse-transcribed in parallel and cDNA analyzed for Oct3 transcripts in three polony reactions for each reverse transcriptase reaction. The variation between the polony numbers on replicate slides with the same reaction was acceptable, with the standard deviation being no more than 17.7% of the mean. There was also good agreement between the means for the three different cDNA syntheses, which differed by no more than 23%. An ANOVA analysis revealed that the different cDNA reactions were comparable to one another ($P > 0.05$) with an overall average value of 3213 ± 462 polonies/blastocyst. In conclusion, sample-to-sample variability is comparable to other widely used assays.

The ability to measure expression of multiple genes from a single sample is highly desirable and we next investigated whether the polony assay could detect expression of multiple genes from a single blastocyst. We chose two other transcription factors expressed in ES cells and the blastocyst ICM: Nanog and Rex1 (24,25). Gene-specific amplification and hybridization primers were designed for these mRNAs and validated with ES cells (data not shown). Next, individual blastocysts were assayed. RNA was extracted and cDNA synthesized by the same method as above and the cDNA from each blastocyst split and delivered to three individual slides with primers for either Oct3, Nanog or Rex1 and the slides assayed with the appropriate gene-specific hybridization probe. As shown in Figure 4, all reactions yielded polonies; counts from this experiment are given in Table 3. Oct3 gave the highest number of polonies; the number of Oct3 polonies/blastocyst was consistent with those of previous experiments. Nanog had the lowest number (~10% of Oct3) and Rex1 about twice as many as Nanog. The lower number of polonies for Nanog and Rex1 might mean that there are fewer mRNAs per blastocyst than Oct3. Alternatively, it could be because their isolation is less efficient or that cDNA synthesis is less efficient. We conclude that expression of at least three genes from a single blastocyst can be readily detected. This is in contrast with many current experiments with standard RT–PCR that require pooling multiple blastocysts (21,22).

Blastocysts contain two layers termed the ICM and the trophectoderm. Oct3, Nanog and Rex1 are all expressed in the blastocyst ICM. To test the generality of the polony method we assayed expression of Cdx2 a gene selectively expressed in the trophectoderm (26). Four individual blastocysts were analyzed for Cdx2 and Oct3 (Table 4). Cdx2 polonies are present in all four blastocysts and there is a large variation among the four blastocysts with a range from 809 to 2105 Cdx2 polonies. The range for Oct3 is 2268 to 4305 which is consistent with previous experiments. We conclude that the polony approach can detect expression of a gene that is specifically expressed in the trophectoderm lineage of the blastocyst. All of the genes assayed above are for transcription factors and it is desirable to show that polonies can detect another class of

Downloaded from nar.oxfordjournals.org at Washington University School of Medicine Library on July 17, 2011

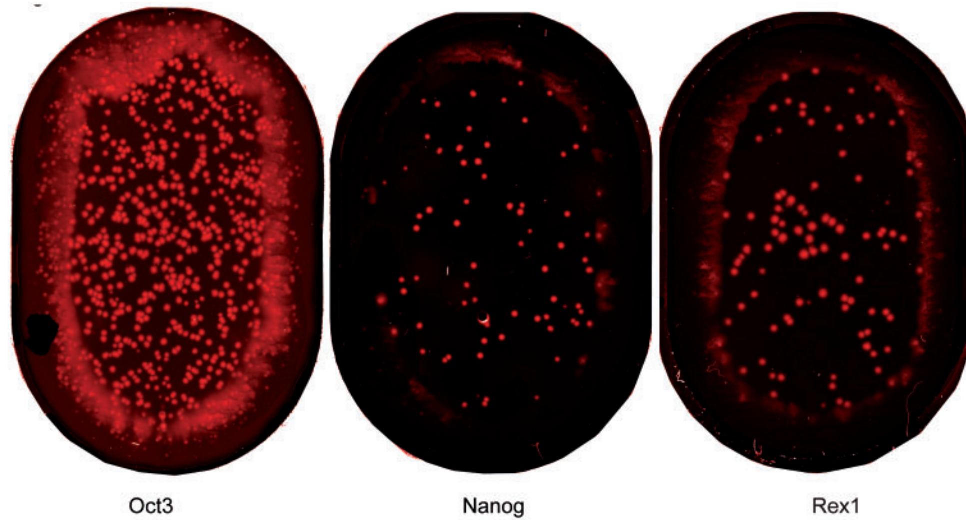

Figure 4. Detection of three genes from a single blastocyst. RNA and cDNA were prepared from a single blastocyst. One-fifth of the cDNA was assayed for each of three genes: Oct3, Nanog and Rex1.The polony method is sensitive enough to detect transcripts from only one-fifth of a blastocyst.

Table 3. Multigene analysis of a single blastocyst

Gene	Polonies per blastocyst
Oct3	3242 ± 189
Nanog	361 ± 168
Rex1	664 ± 210

Three individual blastocysts were analyzed for three genes: Oct3, Nanog and Rex1.Three separate polony reactions were performed using mRNA from each blastocyst. The numbers in the table represent average and SDs for each gene. Gene specific hybridization probes were used to detect and quantify polonies.

Table 4. Analysis of the trophectoderm gene Cdx2

Sample	Cdx2 polonies per blastocyst	Oct3 polonies per blastocyst
Single blastocyst	809	2268
Single blastocyst	2105	4305
Single blastocyst	1682	3614
Single blastocyst	1986	4027
Average	1646	3554
SD	585	903

RNA was extracted from each of four blastocysts and reverse transcribed separately. Each cDNA was split and analyzed for Cdx2 (2 slides) and Oct3 (1 slide). Polonies per blastocyst and the average and SD are indicated.

genes. We therefore assayed the expression of GLUT-1, a membrane protein that is one of the primary glucose transporters in blastocysts (Figure 5) (27). GLUT-1 assays were done on six individual blastocysts and Oct3 was measured as a control. GLUT-1 polonies are present in each blastocyst with an average of 348 ± 84 polonies/blastocyst. The blastocysts had 3340 ± 674 polonies for Oct3, in accordance with previous experiments. We conclude that all the blastocysts tested express GLUT-1

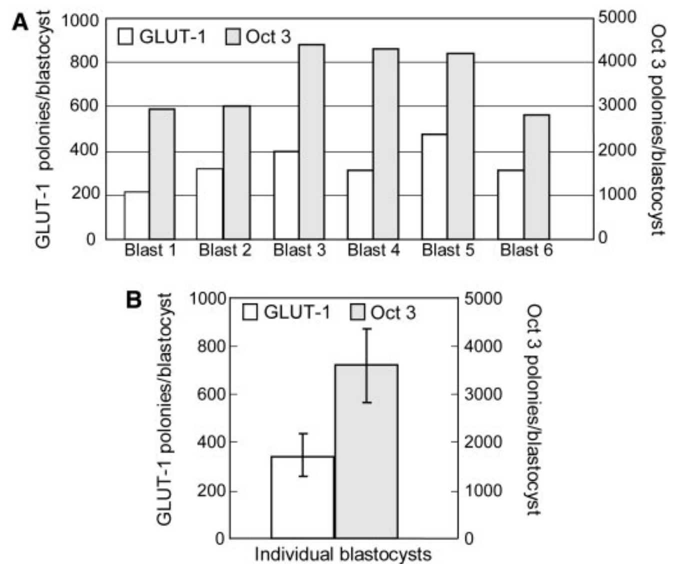

Figure 5. GLUT-1 assayed by polonies. (**A**) GLUT-1 and Oct3 polonies per slide for six individual blastocysts. Each data point represents an average of two replicate slides for GLUT-1 and one slide for Oct3. All slides contain one-fifth of the cDNA from a single blastocyst. (**B**) Comparison of GLUT-1 and Oct3 polonies/blastocyst for averaged individual samples.

and that the polony method is suited for analysis of this gene.

In order to further validate the use of polonies for small numbers of cells a direct comparison with an established PCR method was performed. Competitive PCR was chosen as the standard method because of its sensitivity and rigorous quantitative design (28). Expression assays were done on ES cells for Oct3, Nanog and Rex1 by polonies and competitive PCR and the results compared. Polonies were counted on slides containing cDNA from 10.4 ES cell equivalents for the three genes. Average and

Downloaded from nar.oxfordjournals.org at Washington University School of Medicine Library on July 17, 2011

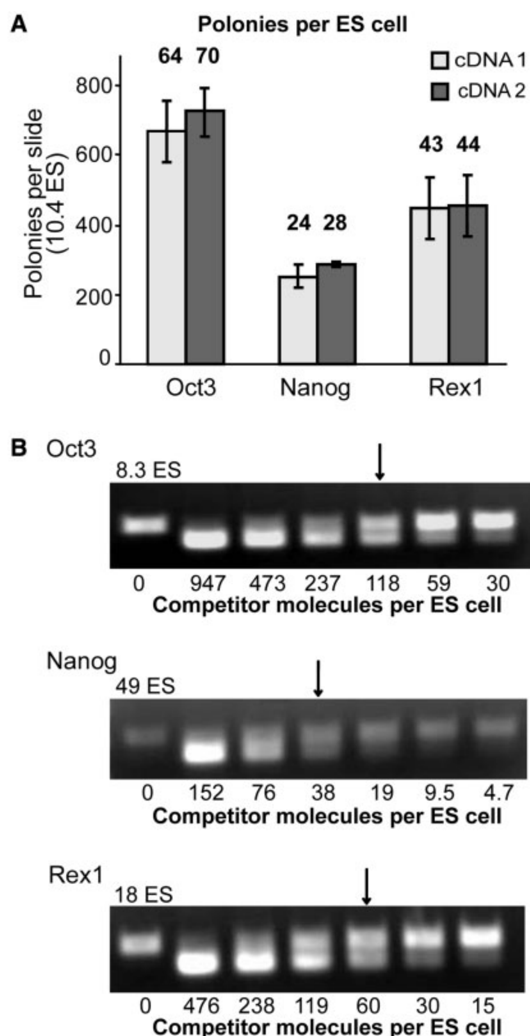

Figure 6. Polonies and competitive PCR for three genes. (**A**) Polonies per slide and per ES cell equivalent for Oct3, Nanog and Rex1. The average number of polonies and standard deviation of three replicate slides containing 10.4 ES cell equivalents are shown. Calculated number of polonies per cell for each set of slides is indicated. (**B**) Competitive PCR for three genes. DNA competitors with 50-bp deletions were generated for Oct3, Nanog and Rex1. Competitive PCR reactions with the indicated number ES cell cDNA equivalents and varying amount of competitor are shown.

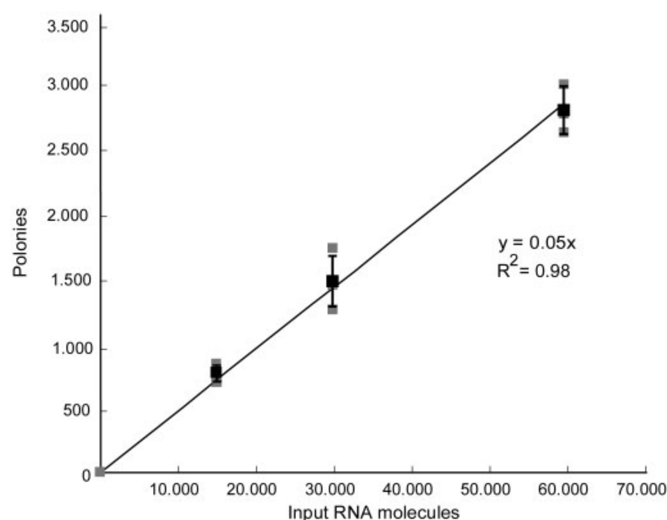

Figure 7. Efficiency from RNA to polony. RNA input is plotted against polony output for three levels of RNA input. The number of polonies increased linearly with the number of RNA molecules added to the polony reaction. Polony counts from each slide are shown by a gray box. Mean values + standard deviation for each set of slides at a particular dilution are shown in black.

standard deviation of polony counts for three replicate slides and calculated polonies per ES cell equivalent are shown in Figure 6A. The polony method shows an average of 67 Oct3 cDNAs per cell, 26 Nanog cDNAs per cell and 43 Rex1 cDNAs per cell. Competitive PCR gels for each of the three genes are shown in Figure 6B. Note that the number of ES cell equivalents used to obtain an equivalence point using PCR differed for each of the three genes. Using competitive PCR we obtain an estimate of 118 Oct3 cDNAs per cell, 38 Nanog cDNAs per cell and 60 Rex1 cDNAs per cell. The number of polonies per ES cell is thus similar to the number of cDNAs measured by competitive PCR for each of the three genes. RT controls for each gene using competitive PCR and polonies showed no background. In summary, polony assays and competitive PCR assays give comparable results.

The numbers of polonies per cell is less than the actual number of mRNAs per cell due to inefficiencies in extracting mRNA and reverse transcription of mRNA to cDNA. Determining the efficiency from RNA to cDNA (reverse transcription) is a step toward extrapolating polony counts to actual number of mRNAs present in a cell. To this end, a model RNA was constructed, a known amount reverse transcribed and the efficiency of the reaction determined with polonies (Figure 7). A plasmid for generating model RNA was constructed by joining a yeast gene (BnI5) to the poly(A)$^+$ rich region from the *Xenopus* elongation factor-1α gene. The model RNA (1.6 kb containing A_{70}) was synthesized by T7 polymerase. For three dilutions of model RNA, the number of polonies increased linearly with increasing amount of template (Figure 7). RNA template conversion to polony ranged from 4.8–6.1% on individual slides and averaged 5%. Control polony slides without RNA did not produce polonies proving that the polony reaction is specific to the model RNA; RT controls were also negative. To explore the generality of this finding, polonies for other regions of this model RNA were tested. Efficiency from RNA to polony for these other amplicons was similar (data not shown). These data are in good agreement with measurements of RT efficiency in the literature (29). Recently, differences in the efficiency of reverse transcription among templates have been shown (30), although the reasons for the variability of the RT step have not been discovered.

DISCUSSION

The polony method of analysis was adapted for use with small numbers of stem cells. The method is sensitive,

Downloaded from nar.oxfordjournals.org at Washington University School of Medicine Library on July 17, 2011

can be applied to most genes and allows a degree of multiplexing; it gives comparable results to competitive PCR, an established method for quantifying cDNAs (28). The approach is also amenable to future refinements that will extend its powers.

The method is sensitive enough to detect mRNAs in fractions of a single mouse blastocyst which is comprised of only 75–100 cells. Specifically, we have detected mRNAs in as little as one-fifth of a single blastocyst. In the case of Oct3, expression is confined to the ICM which is comprised of about 35 cells demonstrating the method is sensitive to seven cells (1/5 of 35 cells) for this particular RNA. It is significant that the number of Oct3 polonies/ES cell ($\sim$50) predicts that there would be about 1750 polonies/blastocyst (50/cell $\times$ 35 ICM cells/blastocyst) a number close to what is measured. The generality of the method was demonstrated by performing assays on five separate genes representing two classes: transcription factors and a membrane transporter. They also include genes exclusive to the ICM (Oct3 and Nanog), an mRNA expressed in both ICM and TE (Rex1) and an mRNA expressed specifically in the TE (Cdx2) (26). Taken together, these results suggest the method will be applicable to most genes of interest. The number of mRNAs present per cell is likely to be greater than the number of polonies due to losses of mRNA in extraction and inefficiency in conversion of mRNA to cDNA by reverse transcriptase. Future developments of the method are needed to discover the efficiencies of the steps leading up to polonies.

In this study we measured the mRNA from three genes from individual blastocysts by performing parallel assays on fractions of the cDNA from a single blastocyst. Polonies for multiple templates can be analyzed on the same slide by including multiple primer pairs (31) so it is likely that as many as 10 genes can be amplified by a simple extension of the method we used. Much greater increases in the number of genes that can be assayed might be achieved by using universal amplifying primers and applying fluorescence *in situ* sequencing of the polonies (9). Thus future enhancements of our method could easily assay dozens of genes per blastocyst.

In summary, the results of these studies show that the polony approach may be applied to the problem of stem cell expression profiling and should encourage efforts to further develop this system for the special needs of stem cell biology.

ACKNOWLEDGEMENTS

We thank Deany Delaney for help with cell culture and Beryl Ojwang for development of the competitive PCR assays. This work was supported by National Institutes of Health Grant P50-HG003170 to George Church (Harvard) through subcontracts to D.G. and R.M. Funding to pay the Open Access publication charges for this article was provided by P50-HG003170.

Conflict of interest statement. None declared.

REFERENCES

1. Chiang,M.K. and Melton,D.A. (2003) Single-cell transcript analysis of pancreas development. *Dev. Cell*, **4**, 383–393.
2. Tietjen,I., Rihel,J.M., Cao,Y., Koentges,G., Zakhary,L. and Dulac,C. (2003) Single-cell transcriptional analysis of neuronal progenitors. *Neuron*, **38**, 161–175.
3. Hartmann,C.H. and Klein,C.A. (2006) Gene expression profiling of single cells on large-scale oligonucleotide arrays. *Nucleic Acids Res.*, **34**, e143.
4. Bengtsson,M., Stahlberg,A., Rorsman,P. and Kubista,M. (2005) Gene expression profiling in single cells from the pancreatic islets of Langerhans reveals lognormal distribution of mRNA levels. *Genome Res.*, **15**, 1388–1392.
5. Tang,F., Hajkova,P., Barton,S.C., Lao,K. and Surani,M.A. (2006) MicroRNA expression profiling of single whole embryonic stem cells. *Nucleic Acids Res.*, **34**, e9.
6. Chetverina,H.V., Samatov,T.R., Ugarov,V.I. and Chetverin,A.B. (2002) Molecular colony diagnostics: detection and quantitation of viral nucleic acids by in-gel PCR. *Biotechniques*, **33**, 150–152, 154, 156.
7. Mitra,R.D. and Church,G.M. (1999) In situ localized amplification and contact replication of many individual DNA molecules. *Nucleic Acids Res.*, **27**, e34.
8. Zhu,J., Shendure,J., Mitra,R.D. and Church,G.M. (2003) Single molecule profiling of alternative pre-mRNA splicing. *Science*, **301**, 836–838.
9. Mitra,R.D., Shendure,J., Olejnik,J., Edyta Krzymanska,O. and Church,G.M. (2003) Fluorescent in situ sequencing on polymerase colonies. *Anal. Biochem.*, **320**, 55–65.
10. Kim,J.B., Porreca,G.J., Song,L., Greenway,S.C., Gorham,J.M., Church,G.M., Seidman,C.E. and Seidman,J.G. (2007) Polony multiplex analysis of gene expression (PMAGE) in mouse hypertrophic cardiomyopathy. *Science*, **316**, 1481–1484.
11. Bain,G., Kitchens,D., Yao,M., Huettner,J.E. and Gottlieb,D.I. (1995) Embryonic stem cells express neuronal properties in vitro. *Dev. Biol.*, **168**, 342–357.
12. Bain,G., Yao,M., Huettner,J.E., Finley,M. and Gottlieb,D.I. (1998) *Culturing Nerve Cells*. MIT Press, Cambridge, MA.
13. Moley,K.H., Chi,M.M. and Mueckler,M.M. (1998) Maternal hyperglycemia alters glucose transport and utilization in mouse preimplantation embryos. *Am. J. Physiol.*, **275**, E38–E47.
14. Mikkilineni,V., Mitra,R.D., Merritt,J., DiTonno,J.R., Church,G.M., Ogunnaike,B. and Edwards,J.S. (2004) Digital quantitative measurements of gene expression. *Biotechnol. Bioeng.*, **86**, 117–124.
15. Lee,T.I., Jenner,R.G., Boyer,L.A., Guenther,M.G., Levine,S.S., Kumar,R.M., Chevalier,B., Johnstone,S.E., Cole,M.F. *et al.* (2006) Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell*, **125**, 301–313.
16. Boyer,L.A., Plath,K., Zeitlinger,J., Brambink,T., Medeiros,L.A., Lee,T.I., Levine,S.S., Wernig,M., Tajonar,A. *et al.* (2006) Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature*, **441**, 349–353.
17. Pesce,M. and Scholer,H.R. (2001) Oct-4: gatekeeper in the beginnings of mammalian development. *Stem Cells*, **19**, 271–278.
18. Chisholm,J.C., Johnson,M.H., Warren,P.D., Fleming,T.P. and Pickering,S.J. (1985) Developmental variability within and between mouse expanding blastocysts and their ICMs. *J. Embryol. Exp. Morphol.*, **86**, 311–336.
19. Johnson,M.H. and McConnell,J.M. (2004) Lineage allocation and cell polarity during mouse embryogenesis. *Semin. Cell Dev. Biol.*, **15**, 583–597.
20. Strumpf,D., Mao,C.A., Yamanaka,Y., Ralston,A., Chawengsaksophak,K., Beck,F. and Rossant,J. (2005) Cdx2 required for trophectoderm. Oct4 restricted to ICM. *Development*, **132**, 2093–2102.
21. Carayannopoulos,M.O., Schlein,A., Wyman,A., Chi,M., Keembiyehetty,C. and Moley,K.H. (2004) GLUT9 is differentially expressed and targeted in the preimplantation embryo. *Endocrinology*, **145**, 1435–1443.
22. Riley,J.K., Heeley,J.M., Wyman,A.H., Schlichting,E.L. and Moley,K.H. (2004) TRAIL and KILLER are expressed and induce

Downloaded from nar.oxfordjournals.org at Washington University School of Medicine Library on July 17, 2011

apoptosis in the murine preimplantation embryo. *Biol. Reprod.*, **71**, 871–877.
23. Zhang,J., Tam,W.L., Tong,G.Q., Wu,Q., Chan,H.Y., Soh,B.S., Lou,Y., Yang,J., Ma,Y. *et al.* (2006) Sall4 modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of Pou5f1. *Nat. Cell Biol.*, **8**, 1114–1123.
24. Mitsui,K., Tokuzawa,Y., Itoh,H., Segawa,K., Murakami,M., Takahashi,K., Maruyama,M., Maeda,M. and Yamanaka,S. (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell*, **113**, 631–642.
25. Rogers,M.B., Hosler,B.A. and Gudas,L.J. (1991) Specific expression of a retinoic acid-regulated, zinc-finger gene, Rex-1, in preimplantation embryos, trophoblast and spermatocytes. *Development*, **113**, 815–824.
26. Deb,K., Sivaguru,M., Yong,H.Y. and Roberts,R.M. (2006) Cdx2 gene expression and trophectoderm lineage specification in mouse embryos. *Science*, **311**, 992–996.
27. Heilig,C.W., Saunders,T., Brosius,F.C. III, Moley,K., Heilig,K., Baggs,R., Guo,L. and Conner,D. (2003) Glucose transporter-1-deficient mice exhibit impaired development and deformities that are similar to diabetic embryopathy. *Proc. Natl Acad. Sci. USA*, **100**, 15613–15618.
28. Zentilin,L. and Giacca,M. (2007) Competitive PCR for precise nucleic acid quantification. *Nat. Protoc.*, **2**, 2092–2104.
29. Dufva,M., Svenningsson,A. and Hansson,G.K. (1995) Differential regulation of macrophage scavenger receptor isoforms: mRNA quantification using the polymerase chain reaction. *J. Lipid Res.*, **36**, 2282–2290.
30. Warren,L., Bryder,D., Weissman,I.L. and Quake,S.R. (2006) Transcription factor profiling in individual hematopoietic progenitors by digital RT–PCR. *Proc. Natl Acad. Sci. USA*, **103**, 17807–17812.
31. Mitra,R.D., Butty,V.L., Shendure,J., Williams,B.R., Housman,D.E. and Church,G.M. (2003) Digital genotyping and haplotyping with polymerase colonies. *Proc. Natl Acad. Sci. USA*, **100**, 5926–5931.

Downloaded from nar.oxfordjournals.org at Washington University School of Medicine Library on July 17, 2011