TECHNICAL BULLETIN

Culturing Leukemic Stem & Progenitor Cells with StemSpan™ Medium

Background

Leukemias are a group of disorders of the blood and bone marrow and are characterized by malignant transformation and clonal expansion of hematopoietic stem and progenitor cells (HSPCs).

This Technical Bulletin describes the use of serum-free StemSpan™ medium and supplements for the culture of HSPCs isolated from patients with chronic myeloid leukemia (CML) or acute myeloid leukemia (AML). The protocols described below have been adapted from those originally developed for the isolation and culture of normal HSPCs, and enable the efficient in vitro expansion of $CD34^+$ cells isolated from CML and AML stem and progenitor cells, even when initial cell numbers are low or cell quality is poor.

CML is caused by a reciprocal translocation between chromosomes 9 and 22, yielding the Philadelphia (Ph) chromosome (Figure 1). The fusion gene encodes the BCR-ABL oncoprotein, a constitutively active tyrosine kinase that drives pathogenesis by perturbing multiple signaling cascades including the RAS/MAPK, PI3K/AKT, and JAK2/STAT5 pathways. Deregulation of these pathways leads to increased cell proliferation and reduced apoptosis, resulting in a massive accumulation of myeloid cells (predominantly granulocytes) in circulation.

Due to the identification of this single specific mutation, CML has served as a model disease for other cancer types, allowing for the study of cancer evolution and development of molecular therapies that target the affected cells. The survival and prognosis of CML patients has improved in recent years thanks to the introduction of specific tyrosine kinase inhibitors, such as Imatinib and Dasatinib, that target

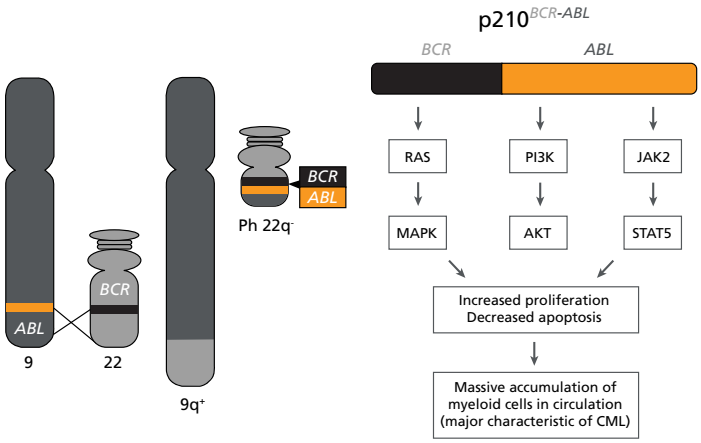

Figure 1. The BCR-ABL Fusion Gene

Schematic representation of the formation of the Philadelphia (Ph) chromosome encoding BCR-ABL and its biological effects. *Adapted from Lydon, 2009.*[6]

StemSpan™ Leukemic Cell Culture Kit

Table 1. Common Genetic Abnormalities in AML

GENETIC ABNORMALITIES	RISK PROFILE	5-YEAR SURVIVAL	RELAPSE RATE
t(8;21)(q22;q22); RUNX1-RUNX1T1	Favorable	70%	33%
t(8;21)(q22;q22); RUNX1-RUNX1T1 without mutated KIT			
inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11			
t(15;17)(q24;q21); PML-RARA			
Mutated NPM1 without FLT3-ITD (normal karyotype)			
Biallelic mutated CEBPA (normal karyotype)			
t(8;21)(q22;q22); RUNX1-RUNX1T1 with mutated KIT	Intermediate	48%	50%
t(9;11)(p22;q23); MLLT3-KMT2A			
Mutated NPM1 with FLT3-ITD (normal karyotype)			
Wild-type NPM1 with FLT3-ITD (normal karyotype)			
Other cytogenetic abnormalities not classified as favorable or adverse			
inv(3)(q21q26.2) or t(3;3)(q21;q26.2); GATA2-MECOM	Adverse	15%	78%
t(6;9)(p23;q34); DEK-NUP214			
t(v;11)(v;q23); KMT2A rearranged			
Mutated TP53 (regardless of abnormality)			
Mutated DNMT3A (normal karyotype)			
FLT3-ITD (normal karyotype)			
KMT2A-PTD (normal karyotype)			
–5 or del(5q); –7; abnl (17p); complex karyotype (3 or more chromosome abnormalities)			

Adapted from Dohner H et al. 2015.[1]

the BCR-ABL oncoprotein and suppress the survival and proliferation of leukemic cells.

AML is much more heterogeneous than CML and is characterized by different chromosomal and genetic abnormalities with different risk profiles and different survival and relapse rates (Table 1).[1] Furthermore, AML cells in individual patients often carry multiple genetic abnormalities, which play unique roles in disease initiation and progression.

The study of both CML and AML has been greatly facilitated by the use of in vitro culture systems similar to those used to study normal hematopoietic stem and progenitor cells (HSPCs). In vitro culture has been used to elucidate the basic biology of these diseases, while allowing conditions to screen compounds such as tyrosine kinase inhibitors or other candidate therapeutics on primary patient-derived cells.[2-4] Studies on primary leukemic HSPCs are often impeded by the limited access to patient samples, which may be compounded by poor yield and viability of cryopreserved samples after thawing.

When culturing cells from valuable patient samples, it is important to choose a medium that is reliable, defined, and optimized for your cell type. The experiments presented in this Technical Bulletin were performed using the StemSpan™ Leukemic Cell Culture Kit (Catalog #09720), which contains StemSpan™ Serum-Free Expansion Medium II (SFEM II; Catalog #09605), StemSpan™ CD34+ Expansion Supplement (Catalog #02691), and UM729 (Catalog #72332).[5]

Other media and supplements, e.g. StemSpan™ SFEM (Catalog #09600), StemSpan™ CC100 (Catalog #02690) or CC110 (Catalog #02697), and small molecules, may also be used to generate similar culture conditions. The experiments in this Technical Bulletin were performed using the small molecule UM171, however similar results are expected when using UM729 prepared to a final concentration of 1µM.

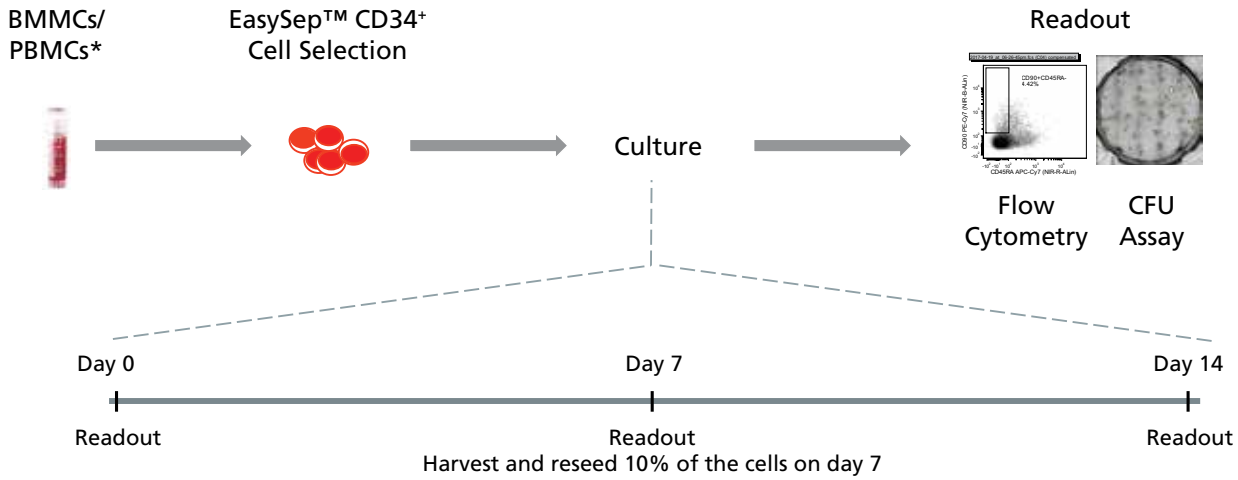

Figure 2. General Protocol for Culturing Leukemic CD34+ Cells

CD34+ cells isolated from CML or AML samples may first be purified using EasySep™ Human Cord Blood CD34 Positive Selection Kit II (Catalog #17896) and cultured for 14 days in StemSpan™ SFEM II supplemented with StemSpan™ CD34+ Expansion Supplement with or without addition of UM729. Cells may be analyzed on days 0, 7, and 14 with colony-forming unit (CFU) assays using MethoCult™ H4435 Enriched (Catalog #04435) medium, and by immunophenotyping for cell surface markers commonly expressed on HSPCs.

*The experiments in this Technical Bulletin were performed using cryopreserved cells, however similar results are expected when using fresh samples. Additionally in place of UM729, the small molecule UM171 was used to generate data in Figures 4 - 7. UM171 is no longer licensed for sale by STEMCELL Technologies, however similar results are expected when using UM729 prepared to a final concentration of 1 µM (data not shown). Further titration may be necessary to optimize cell fold expansion in specific conditions.

For more information including data comparing UM171 and UM729, see Fares et al. 2014.

Applications of Leukemic Cell Culture

- Research into the development of new therapies for CML or AML
- Study of disease progression and clonal evolution of leukemic stem and progenitor cells
- Elucidate mechanism of resistance and relapse of leukemic stem and progenitor cells to current therapies
- Identify biomarkers and develop assays to predict patient response to novel therapeutics

Protocol

Isolate

1. If starting with fresh peripheral blood mononuclear cells (PBMCs) or bone marrow mononuclear cells (BMMCs), proceed directly to step 2.

   If starting with frozen PBMCs or BMMCs, thaw a 1 mL vial of sample in a 37°C water bath. When just thawed, add 0.5 mL of DNase I Solution (Catalog #07900), gently mix, and incubate at room temperature (15 - 25°C) for 5 minutes. Washing is not recommended prior to isolation of CD34$^+$ cells as it may lower cell yield and viability. This thawing protocol increases cell yield and improves viability of the leukemic CD34$^+$ cells.

2. Isolate CD34$^+$ cells (from the DNase-treated thawed PBMCs/BMMCs or fresh PBMCs from step 1), using EasySep™ Human Cord Blood CD34 Positive Selection Kit II (Catalog #17896). This kit was originally designed for use with cord blood, but has been extensively tested with CML and AML samples.

3. Determine the number and percentage of viable cells using a hemocytometer with trypan blue or an automated cell counter. If enough cells are present, the percentage of CD34$^+$ cells can also be measured using flow cytometry. The number and/or concentration of CD34$^+$ cells can be determined by multiplying the total number of viable cells by the percentage of CD34$^+$ cells in the sample.

Culture

4. Prepare 100 mL of leukemic cell culture medium by combining the following:
   - 90 mL StemSpan™ SFEM II
   - 10 mL StemSpan™ CD34$^+$ Expansion Supplement
   - UM729 to a final concentration of 1µM*

5. Seed CD34$^+$ cells at 1000 viable cells per well in 100 µL of the prepared leukemic cell culture medium in 96-well plates, or 1000-10,000 cells in 1 mL medium per well in 12-well plates.

6. Incubate at 37°C for 7 days.**

7. On day 7, harvest the cells, wash, and resuspend in 1 mL of Iscove's MDM without any cytokines or supplements added.

8. Reseed 10% of the cell suspension into fresh leukemic cell culture medium, prepared as described in step 4, and incubate at 37°C for an additional 7 days.

9. On day 14, harvest cells, wash, and resuspend as in step 7.

*If using UM171, prepare to a final concentration of 175 nM

**Culture length can be modified based on the goals and requirements of the experiment. If culturing cells for >7 days, it is recommended to follow steps 7 and 8 at day 7, and as appropriate every additional 7 days.

Analyze

10. a. Determine the number and phenotype of cells expressing HSPC markers CD45, CD34, CD45RA, and CD90 on day 0 (if enough cells are available after CD34$^+$ cell isolation), and on day 7 and 14 after culture, using flow cytometry. Aldehyde dehydrogenase (ALDH) activity, which is high in primitive stem and progenitor cells, can also be determined using ALDEFLUOR™ (Catalog #01700) in parallel with HSPC marker staining.

    b. Measure the frequency and total number of hematopoietic progenitor cells on day 0, and on day 7 and 14 after culture, by performing colony assays with MethoCult™ H4435 Enriched medium (Catalog #04435) (refer to the StemSpan™ Leukemic Cell Culture Kit product information sheet (PIS) (Document #DX23137) for protocol and plating density). Count colonies after 14 days.

Optional: Colonies may be plucked from CML samples at day 14 for further analysis, including cDNA preparation and qPCR to determine BCR-ABL status. See Table 2 for primers. For AML samples, sequencing or qPCR may be performed to identify the presence of fusion genes or other mutations.

Results

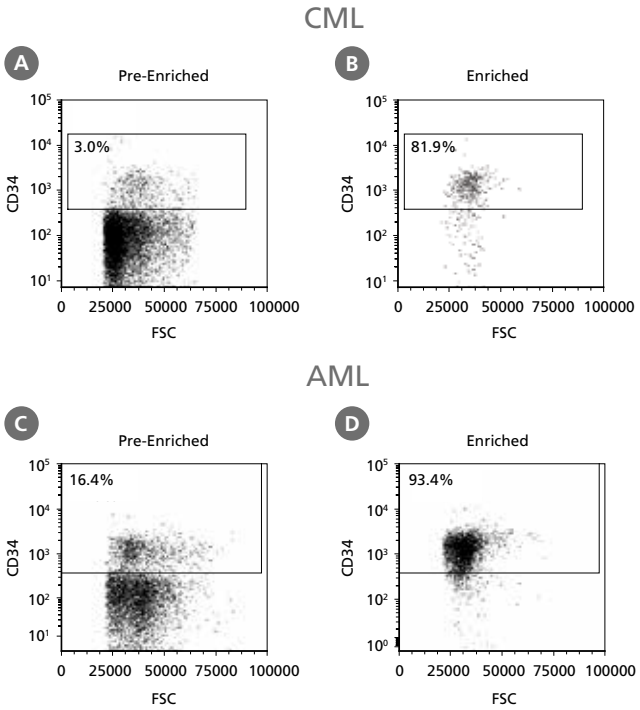

Figure 3. Isolation of Leukemic CD34$^+$ Cells

Cryopreserved CML or AML PBMCs and BMMCs were thawed and treated as described in Step 1 of the protocol. CD34$^+$ cells were isolated using EasySep™ Human Cord Blood CD34 Positive Selection Kit II. The percentage of CD34$^+$ cells before (A, C) and after (B, D) CD34$^+$ cell isolation was measured by flow cytometry. Dead cells were excluded by light scatter profile and viability staining. In this example the purity of CD34$^+$ cells increased from 3% to 82% (CML) and from 16% to 93% (AML).

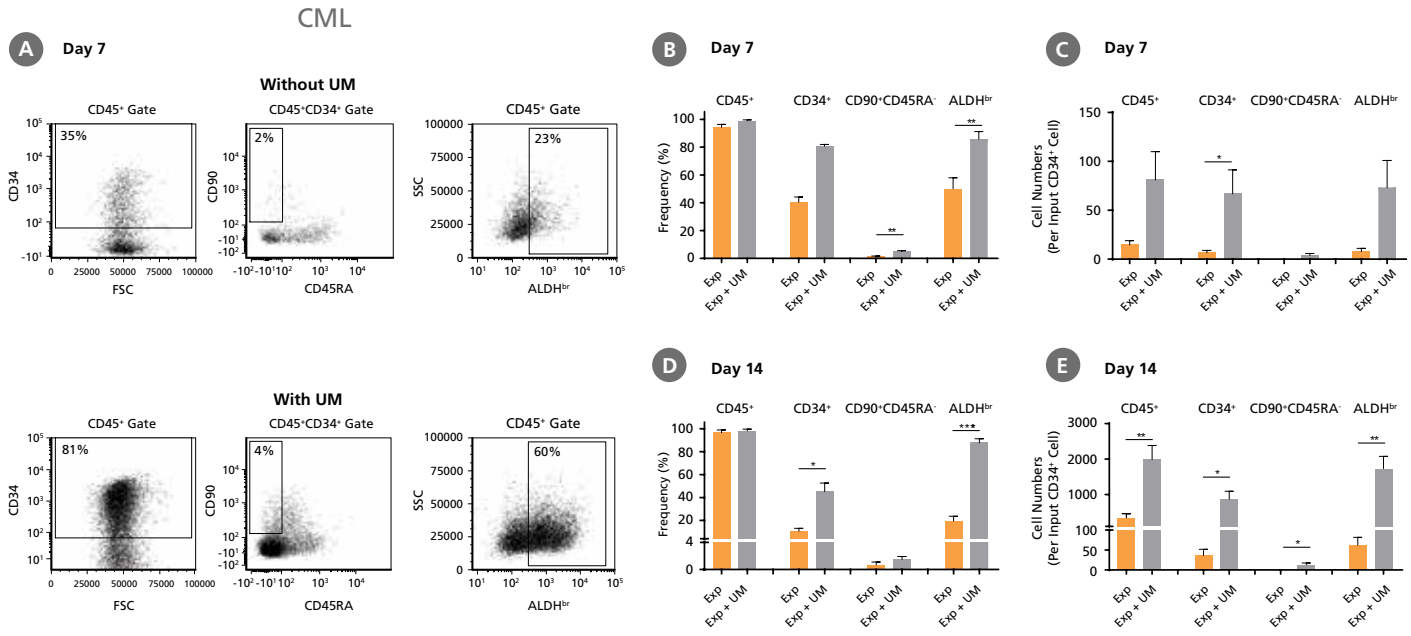

Figure 4. Expansion of CD34$^+$ CML Cells

CD34$^+$ CML cells were cultured in StemSpan™ SFEM II containing CD34$^+$ Expansion Supplement (Exp) without or with UM171 (UM) as described in the protocol on page 3. After 7 and 14 days, the cultured cells were stained with fluorescently labeled antibodies against CD45, CD34, CD90, CD45RA, and with ALDEFLUOR™ (Catalog #01700) to measure ALDH activity, and analyzed by flow cytometry. Sequential gates were used to determine the percentages of viable CD45$^+$, CD45$^+$CD34$^+$, and CD45$^+$CD34$^+$CD90$^+$CD45RA$^-$ cells (based on "Fluorescence Minus One" (FMO) controls), and ALDH^{br} cells (based on DEAB control). (A) Representative flow cytometry profiles at day 7 are shown. The (B,D) frequency and (C,E) cell numbers of these subsets per initial CD34$^+$ cell on (B,C) day 7 and (D,E) day 14 are shown. StemSpan™ SFEM II supplemented with CD34$^+$ Expansion Supplement supports the expansion of CML cells in culture. The addition of UM171 enhances expansion of all subsets shown (~10-fold expansion of CD34$^+$ progenitor cells at day 7 and ~20-fold at day 14 compared to cultures without UM171). UM729 is expected to provide similar results when used at a final concentration of 1µM. Data shown are mean ± SEM (n = 6). P-values were calculated using a two-tailed paired Student's t-test (*P < 0.05; **P < 0.01; ***P < 0.0001). All six CML samples tested were able to expand in culture.

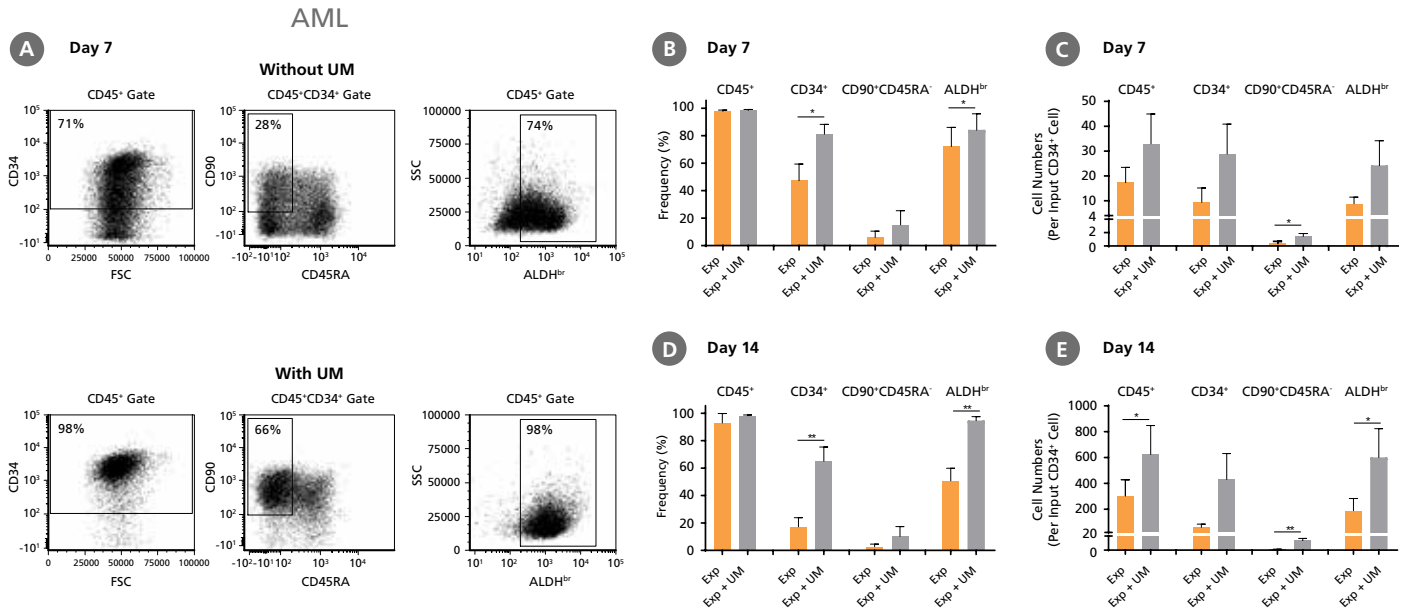

Figure 5. Expansion of CD34$^+$ AML Cells

CD34$^+$ AML cells were cultured in StemSpan™ SFEM II containing CD34$^+$ Expansion Supplement (Exp) alone, or with UM171 (UM) as described in the protocol. After 7 and 14 days, the cultured cells were stained with fluorescently labeled antibodies and with ALDEFLUOR™ Reagent as described in Figure 4. (A) Representative flow cytometry profiles at day 7 are shown. The (B, D) frequency and (C, E) cell numbers of these subsets per initial CD34$^+$ cell on (B, C) day 7 and (D, E) day 14 are shown. SFEM II supplemented with CD34$^+$ Expansion Supplement supports the expansion of AML cells in culture. The addition of UM171 further enhances expansion of all subsets shown (~3-fold expansion of all subsets at day 7 and ~7-fold at day 14 compared to cultures without UM171). UM729 is expected to provide similar results when used at a final concentration of 1µM. Data shown are mean ± SEM (n = 6). P-values were calculated using a two-tailed paired Student's t-test (*P < 0.05; **P < 0.01). Six out of ten AML samples tested were able to expand in culture.

When cultured with UM171, the frequencies of CD34$^+$ cells, CD34$^+$CD90$^+$ CD45RA$^-$ cells, and ALDH^{br} cells in AML cultures at day 7 and day 14 (Figure 5) were similar to those of CML samples (Figure 4) (~80% CD34$^+$ cells for both CML and AML samples at day 7); whereas the numbers of CD34$^+$ cells, CD34$^+$CD90$^+$ CD45RA$^-$ cells, and ALDH^{br} cells in AML samples (Figure 5) were 2.5-fold lower than those of CML samples (Figure 4) (~70-fold expansion of CD34$^+$ cells in CML samples vs ~30-fold expansion of CD34$^+$ cells in AML samples at day 7).

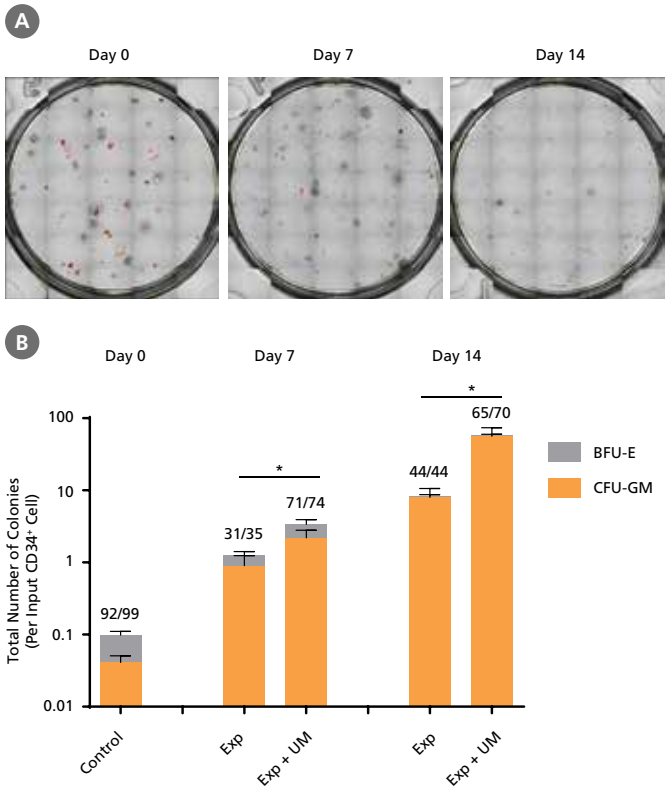

Figure 6. Colony-Forming Potential of CD34$^+$ CML Cells is Maintained During Culture

CML cells were assayed in colony assays using MethoCult™ H4435 Enriched medium directly after CD34$^+$ cell isolation (day 0) or after 7 or 14 days of expansion without or with UM171 (UM; as described in Figure 4). After 14 days of culture in StemSpan™ SFEM II with CD34$^+$ expansion supplement (Exp) without or with UM171, colonies were (A) imaged with STEMvision™ and counted manually from digital images. (B) CFU output expressed as the total number of colonies per original input CD34$^+$ cell. Numbers above each of the individual bars indicate the proportion of BCR-ABL positive colonies, measured by qRT-PCR on individual plucked colonies across 6 different samples (8-12 colonies were plucked for each sample per condition, see Table 2 and Figure 8 for details on the primers and procedure). SFEM II supplemented with CD34$^+$ Expansion Supplement (Exp) supports the expansion of colony-forming progenitor cells in culture. UM171 further promotes colony forming progenitor cell output (~3.5-fold expansion at day 7 and ~8-fold at day 14). UM729 is expected to provide similar results when used at a final concentration of 1µM. Single-colony qRT-PCR analysis revealed that colonies generated from samples on day 0, and colonies generated from cells expanded for 7 and 14 days, were predominantly BCR-ABL$^+$ but also that normal BCR-ABL- progenitor cells were present at low frequencies. Data shown are mean ± SEM (n = 6). P-values were calculated using a two-tailed paired Student's t-test (*P < 0.05).

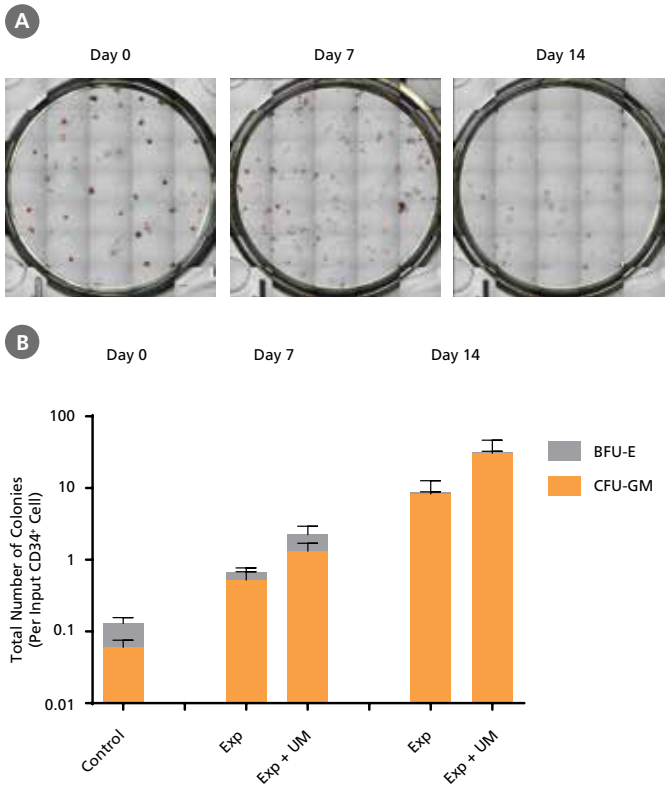

Figure 7. Colony-Forming Potential of CD34$^+$ AML Cells is Maintained During Culture

AML cells, after CD34$^+$ cell isolation (day 0) or after 7 or 14 days of expansion without or with UM171 (as described in Figure 5), were plated in colony assays with MethoCult™ H4435 Enriched medium. After 14 days of incubation, colonies were (A) imaged with STEMvision™ and counted manually from digital images. (B) CFU output expressed as the total number of colonies per original input CD34$^+$ cell. SFEM II supplemented with CD34$^+$ Expansion Supplement (Exp) supports the expansion of colony-forming progenitor cells in culture. Addition of UM171 further promotes colony-forming progenitor cell output (~3-fold expansion at day 7 and ~4-fold at day 14). UM729 is expected to provide similar results when used at a final concentration of 1µM. Data shown are mean ± SEM (n = 6). P-values were calculated using a two-tailed paired Student's t-test (*P < 0.05).

Table 2. Primer-Probe Sequences, Working Concentrations, and Primer Efficiencies

TARGET	PRIMER	5′-	FINAL CONCENTRATION (1X)	PRIMER EFFICIENCY AGAINST SYNTHETIC DNA; R^2 VALUE
BCR-ABL (generic)	FW	TCCGCTGACCATCAAYAAGGA	300 nM	97%; 0.9996
	REV	CACTCAGACCCTGAGGCTCAA	300 nM	
	Probe	CCCTTCAGCGGCCAGTAGCATCTGA	200 nM	
BCR-ABL (b3a2 specific)	FW	GGGCTCTATGGGTTTCTGAATG	400 nM	99%; 0.9995
	REV	CGCTGAAGGGCTTTTGAACT	400 nM	
	Probe	CATCGTCCACTCAGCCACTGGATTTAAGC	200 nM	
BCR-ABL (b2a2 specific)	FW	ATCCGTGGAGCTGCAGATG	400 nM	100%; 0.9996
	REV	CGCTGAAGGGCTTCTTCCTT	400 nM	
	Probe	CCAACTCGTGTGTGAAACTCCAGACTGTCC	200 nM	
BCR	FW	CCTTCGACGTCAATAACAAGGAT	500 nM	99%; 0.9999
	REV	CCTGCGATGGCGTTCAC	500 nM	
	Probe	TCCATCTCGCTCATCATCACCGAC	250 nM	

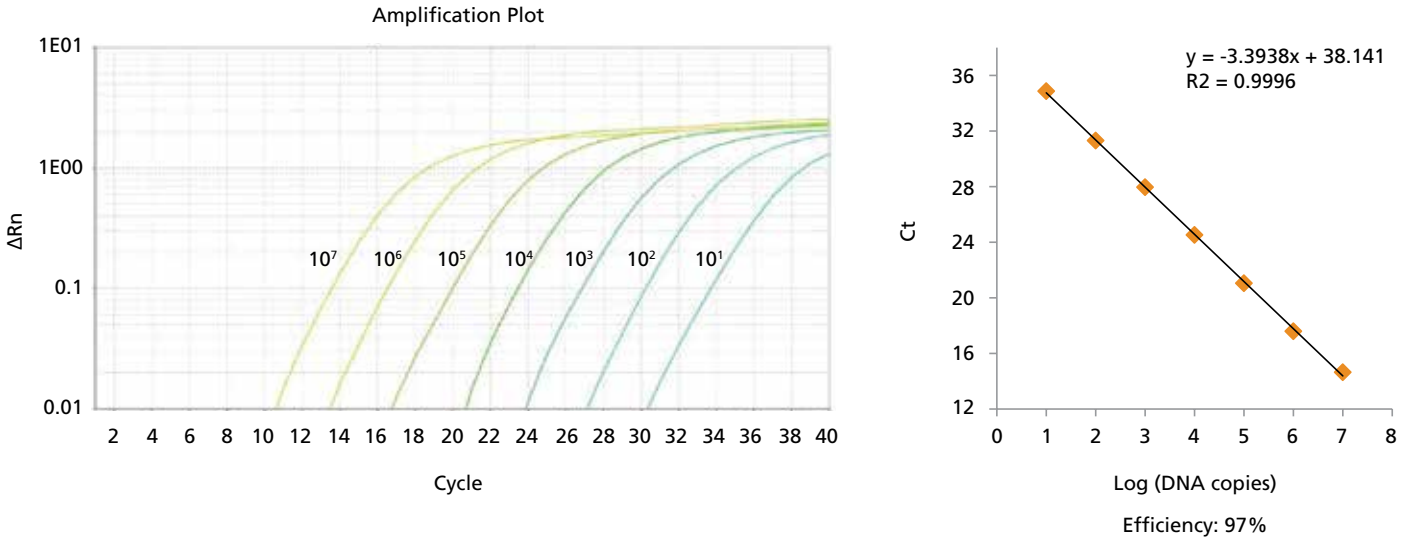

Figure 8. Probe-Based qPCR Assays Can Be Used to Identify Single BCR-ABL$^+$ Colonies Generated in CFU Assays of CD34$^+$ Cells Isolated from CML Samples

Probe-based qPCR assays were utilized to identify BCR-ABL$^+$ single colonies obtained from the non-expanded, 7-day-expanded, and 14-day-expanded CML cells as shown in Figure 6. All primer sets were able to detect as few as 10 copies of DNA with primer efficiencies > 90%. An amplification plot and a standard curve using BCR-ABL generic primer against b2a2 synthetic DNA fragments are shown. Numbers beside each amplification curve indicate number of DNA copies. Primer-probe sequences, working concentrations, and primer efficiencies are shown in Table 2.

Products

For Isolation and Culture of CD34+ CML or AML

PRODUCT	UNIT SIZE	CATALOG #
Human Cord Blood CD34 Positive Selection Kit II	1 Kit	17896
DNase I Solution (1 mg/mL)	1 mL	07900
StemSpan™ Leukemic Cell Culture Kit EasySep™	1 Kit	09720
Kit Components		
StemSpan™ SFEM II	100 mL 500 mL	09605 09655
StemSpan™ CD34+ Expansion Supplement (10X)	10 mL	02691
UM729	250 µg	72332

For Phenotyping by Flow Cytometry

PRODUCT	CATALOG #
Anti-Human CD45 PE Antibody, Clone HI30, PE-conjugated	60018PE
Anti-Human CD34 APC Antibody, Clone 581, APC-conjugated	60013AZ
7-AAD (7-Aminoactinomycin D)	75001
ALDEFLUOR™ Kit	01700

For Colony Assay and qPCR Analysis

PRODUCT	SIZE	CATALOG #
MethoCult™ H4435 Enriched	100 mL 24 x 3 mL	04435 04445
qPCR Master Mix Kit	1 mL Kit 5 mL Kit	07516 07517
Nuclease-Free Water	300 mL 2 mL	79001 79002

References

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