Establishment of Imageable Model of T-cell Lymphoma Growing in Syngenic Mice

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Abstract. *Background: Cancer research is focused on processes which influence in vivo tumor growth dynamics, tumor microenvironment and antitumor immune responses. Recently, it was documented that some cytostatics, including their polymeric derivatives, were able to trigger an anticancer immune response. Such interactions are studied mainly in vitro but relevant in vivo studies are necessary and were only recently started. Whole-body imaging of fluorescently labeled tumors, which enables visualization of the whole-body down to single cell-cell interactions, is therefore a promising tool in understanding these processes. Materials and Methods: EL-4 T-cell lymphoma cells were transfected with plasmid containing either fusion construct of enhanced green fluorecent protein (EGFP) with H2B histone or pure EGFP gene under cytomegalovirus promotor and resistance to neomycin. Stability of expression was determined by flow cytometry and cellular localization of green fluorescence signal was tested using fluorescent microscopy. An in vivo whole-body imaging system was used to evaluate growth in vivo. Results: EL-4 cells were successfully transfected and established stable transfectants with a proliferation rate comparable to that of wild-type EL-4. Clone 12, with very strong whole-cell expression, enables tracking of metastatic spreading, whereas clone 3, with EGFP within the cell nucleus, allows frozen section analysis and observing of interaction with immunocompetent cells. Conclusion: Established imageable EL-4-EGFP$^+$ cell lines are a magnificent tool for the study of tumor growth and the tumor microenvironment.*

Interactions of the immune system with tumors have been studied for a long time but there are still many unknown processes. Contemporary anticancer research is focused on the positive modulation of anticancer immune response by cytostatics (1, 2). This cannot be investigated *in vivo* using human tumors growing in immunodeficient mice. Therefore an imageable cancer model, expressing suitable fluorescent protein, growing in imunocompetent animals should be a helpful tool for researchers.

Many different colors of fluorescent proteins have recently been described (3-5) and used to establish imageable cancer cell lines. Among them green fluorescent protein and its enhanced form (EGFP) are the most commonly used reporter genes (6), with an excitation peak at 488 nm and an emission peak at 508 nm. These fluorescent imageable tumor models can be used to visualize primary tumor growth, reaction to treatment, metastatic spreading, tumor cell motility, neoangiogenesis, and also tumor host interaction (7-12). For example, the behavior of cancer cells with low metastatic potential labeled with GFP and highly metastatic cancer cells labeled with red fluorescent protein (RFP) can be studied *in vivo* directly together (13). Alternatively, trafficking of fluorescent tumor cells within blood vessels and lymphatic channels can be visualized in real time to reveal the hidden aspects of metastatic spreading in living animals (14).

Growth of either primary tumor or metastasis labeled with fluorescent protein can be monitored noninvasively in real time (7, 12). For images of deeper tumors and/or metastasis with better resolution, or even for single-cell level of resolution, reversible skin flaps over many parts of the body (*e.g.* skin, brain, lung, liver) can be used (15).

We have recently established EGFP-expressing murine EL-4 T-cell lymphoma with either whole-cell expression or nuclear localization of mature fusion protein of EGFP with H2B histone. Here, we characterize two different EGFP transfectants with numerous possibilities for contemporary lymphoma research.

Materials and Methods

Materials. PmaxGreen-C plasmid containing *Pontellina plumata* GFP gene under cytomegalovirus promotor and cassete with neomycin resistence gene (Amaxa, Germany) was purchased from KRD Molecular Technologies (Czech Republic), and pEGFP-H2B

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Key Words: Imageable model, EL-4 T-cell lymphoma, EGFP, whole body imaging.

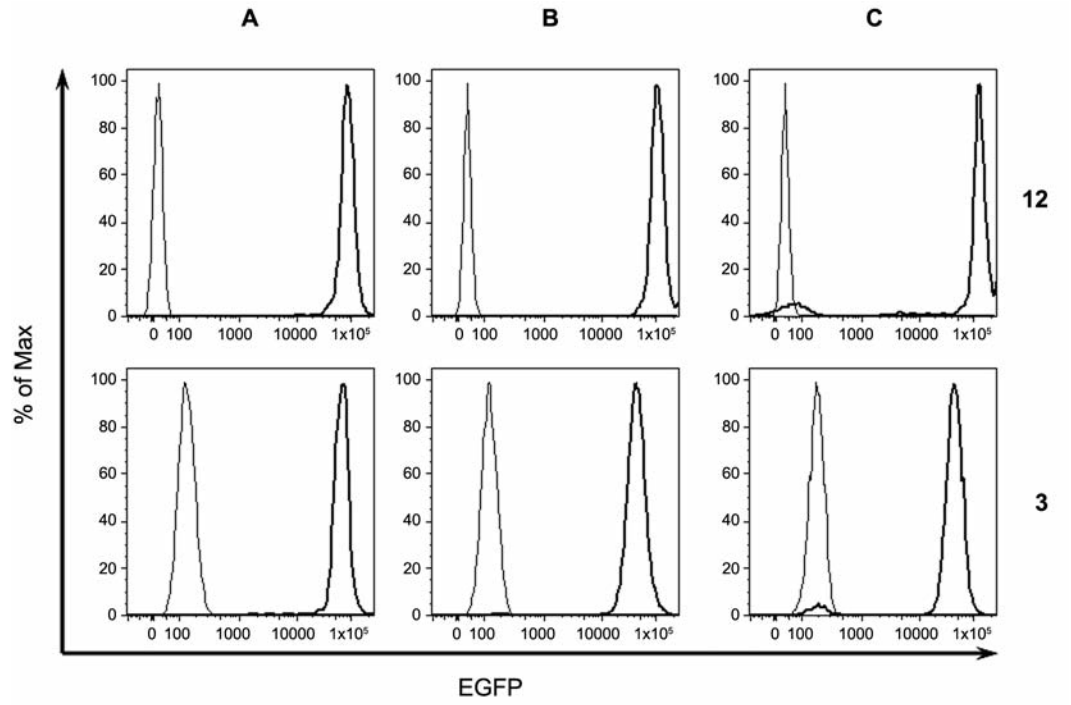

Figure 1. *Stability of EGFP fluorescence of clones 12 and 3. A, Four weeks after single-cell sorting; B, after devolution from deep frozen state; C, after ex vivo isolation.*

Table I. *Proliferation (MTT assay) of wild-type (WT) EL-4 cell line and EGFP transfected clones 3 and 12.*

	Proliferative activity *in vivo*		
	WT	Clone 3	Clone 12
Spontaneus proliferation	100%	90%±3.4%	84%±2.7%
IC_{50} (μg/ml of DOX)	0.031±0.008	0.009±0.002	0.016±0.004

plasmid (Clontech, USA) coding *Aequrea victoria* GFP gene fused with an H2B histone gene, and neomycin resistence gene was a kind gift of Dr. David Stanek (Institute of Molecular Genetics AS CR, v.v.i., Prague, Czech Republic). Neomycin (G418) and cultivation medium, together with required additives (L-glutamine, antibiotics, sodium pyruvate and glucose) were purchased from Sigma Aldrich (Czech Republic). Hoechst 33342 dye is a product of Invitrogen (USA) and nucleoporator solution GTporator M was purchased from GeneTrend (Czech Republic).

Cell lines. EL-4 cell line (TIB-39) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Both non-transfected and EGFP expressing EL-4 cell lines were cultivated in RPMI-1640 medium with extra L-glutamine (4 mM), sodium pyruvate (1.0 mM), 4.5 g l^{-1} glucose, penicillin (100 U ml^{-1}), streptomycin (100 U ml^{-1}), and 10% v/v fetal bovine serum.

Transfection. Nucleofection method (16) was used to stably transfect EL-4 cell line. Briefly, 2×10^6 EL-4 T-lymphoma cells underwent centrifugation (1200 rpm for 4 minutes) and the supernatant was completely discarded. The pellet was resuspended at room temperature in 100 μl of electroporation solution GTporator M and 2 μg of plasmid DNA were added into the cell suspension. The sample was carefully transferred into an Amaxa certified cuvette, avoiding air bubbles during pipeting. The nucleoporation was carried out on a Nucleofetor device (Amaxa), using program C-09. Pre-warmed cultivation medium (500 μl) was added immediately and the sample was transferred into 6-well plates with prewarmed medium. Cells did not stay in the electroporation solution longer than 10 minutes. Transfected cells were analyzed each day by fluorescence microscopy.

Cell sorting and stability measurements. EGFP fluorescence was excitated by blue laser (Ex 488 nm) and passed through an emission filter (Em 530/30 nm). EGFP-positive cells were sorted by FACSort Vantage (Becton Dickinson, USA) to enrich the population and then were placed into selective medium containg 200 μg ml^{-1} of G418. After two weeks, the selective medium was replaced with fresh RPMI-1640 medium and selected cells underwent single-cell sorting onto 96-well plates

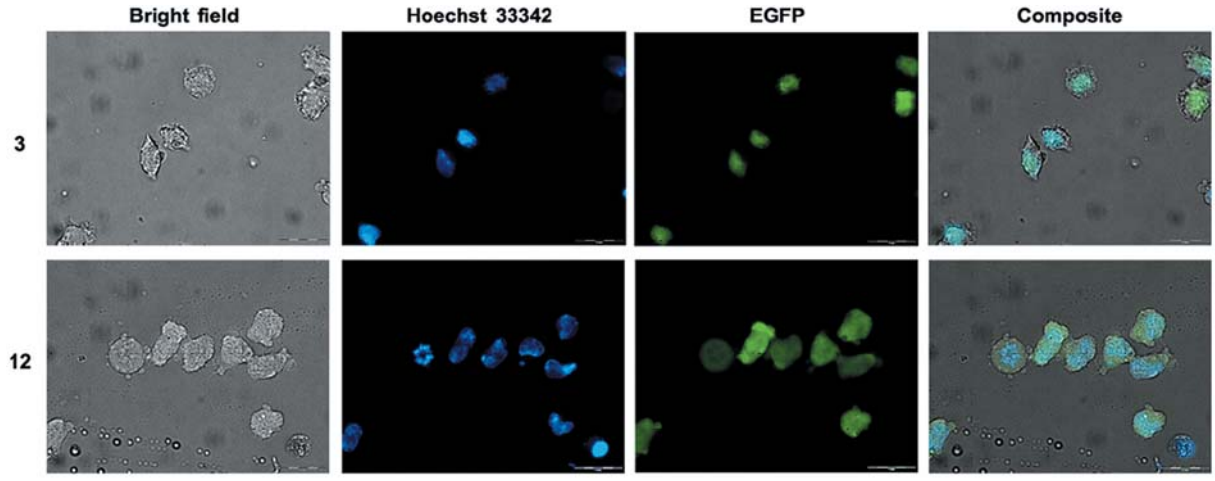

Figure 2. *Fluorescent microscopy images of clone 3 and clone 12 displaying cellular localization of mature EGFP-H2B and EGFP fusion construct, respectively. Hoechst 33342 dye was used for staining cellular nuclei.*

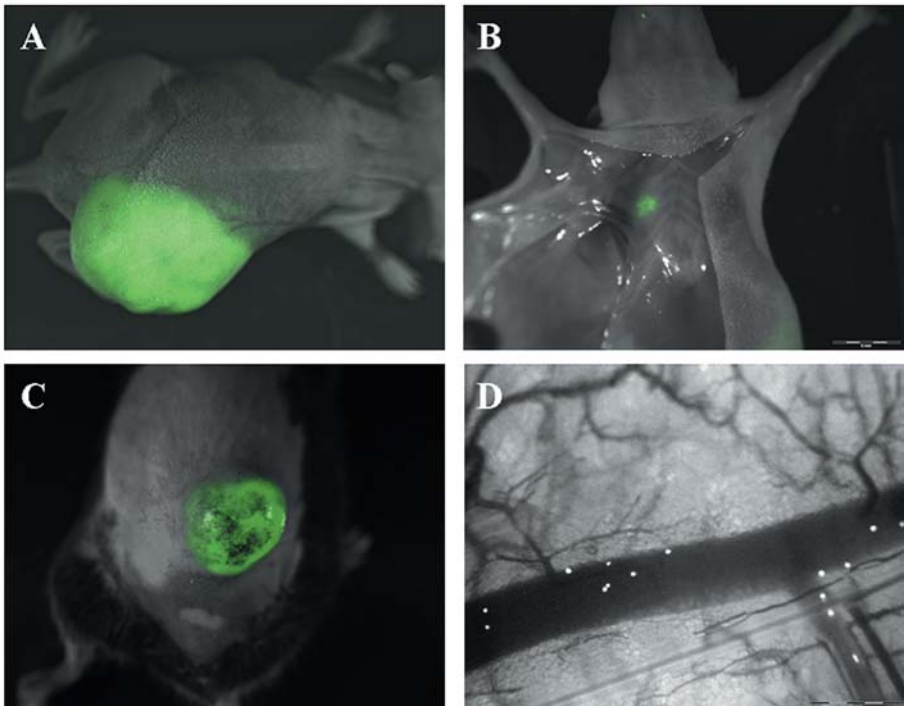

Figure 3. *In vivo imaging of A, tumor in a nu/nu mouse, clone 12; B, lung metastasis, clone 12; C, tumor in an immunocompetent C57/BL6 mouse, clone 3; D, single cells in blood vessel, clone 3.*

(NUNC, Denmark) to produce cell lines expressing EGFP at a stable level of expression. Stability of fluorescence was determined by flow cytometry LSR II (Becton Dickinson) following establishment and characterization of the transfectants, *i.e.* two, and four weeks after single cell-sorting, after devolution back into culture from deep frozen state, and finally after *ex vivo* isolation from well-established tumors. Analysis of obtained data was driven by FlowJo software (Tree Star, Ashland, OR, USA).

Fluorescent microscopy. Intracellular localization of GFP fluorescence was analyzed by fluorescence microscopy. DNA probe Hoechst 33342 (Invitrogen, USA) was used as a specific probe to confirm nuclear localization. Fluorescence channels were taken sequentially (GFP Ex filter 492/18, Em filter 510LP; Hoechst 33342 Ex filter 350/50, Em filter 420LP) on an Olympus Provis AX70-CellR System (Olympus Corp., Tokyo, Japan). Merging of separate images, image analysis, and co-localization were analyzed on AnalySIS 3.17 software (SIS System, Germany).

In vitro proliferative activity. The proliferative *in vitro* activity was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-lium bromide (MTT) conversion assay. 96-Well flat-bottomed microplates (NUNC, Denmark) were seeded with EL-4 cancer cells (5×10^3 cells in 200 μl). Some wells were exposed to doxorubicin (DOX) which was added in 50 μl (in triplicates) to achieve the desired concentrations (range 0.0016-0.8 μg ml^{-1}). Plates were incubated at 37˚C in a humidified 5% CO_2–95% air atmosphere for 48 h. After incubation, 150 μl of supernatant were carefully discarded and 20 μl of MTT solution (5 mg ml^{-1}) were added and plates were incubated at 37˚C for another 2 h. Subsequently 200 μl of DMSO were added and plates were kept 15-20 minutes in the dark. The absorbance was measured by an ELISA reader (Rainbow Thermo Tecan, USA), using a 540 nm filter. All 50% inhibitory concentration (IC_{50}) values are a mean of four independent experiments.

In vivo imaging. All animal studies were performed in accordance with the Act on Experimental Work with Animals (Decrees No. 311/97; 117/87, and Act No. 246/96) of the Czech Republic, which is fully compatible with the corresponding European Union directives. A total of 2×10^6 $EGFP^+$ EL-4 cells or 1×10^6 wild-type EL-4 cells were subcutaneously injected into the right flank of athymic *nu/nu* or C57/BL6 mice, respectively, and tumor growth was recorded. Alternatively, 2×10^6 cells were intravenously administrated *via* the tail or epigastrial vein, respectively. *In vivo* imaging was performed on an OV100 Whole Mouse System (Olympus Corp.) containing an MT-20 light source and an Orca II ERG (Hamamatsu, Japan) CCD camera. Animals were anesthetized by intraperitoneal injection of 300 μl per mouse of 1% Narkamon (Spofa, Czech Republic). Tumor growth imaging was non-invasive, observations of cell trafficking within blood vessels and lung metastases were carried out *via* a reversible skin flap window (7). EGFP fluorescence of the tumor cells was visualized with U-MWIB3 cube (Ex 460-495 nm, Em 510IF filters). Images were taken separately for green fluorescent channel and also for transmitted light, and merged and colorized in AnalySIS software 3.2.822 (SIS Systems).

Results

Cell sorting and stability measurements. Single-cell sorting efficiency and purity was very high, as aproximately 70% of sorted cells gave rise to a clonal cell line and all sortings were accompanied by a very low rate of contamination. Stability of EGFP expression was at first step determined four weeks after single-cell sorting. Five out of 65 clones with cytoplasmatic localization (EGFP-Cyt) and 10 out of 140 clones with EGFP-H2B fusion construct (EGFP-H2B) showed the desired stability. Stabile clones were transfered to deep frozen state. Expression of EGFP determined after devolution was stabile in all tested clones, *i.e.* in 5 EGFP-Cyt clones and 10 EGFP-H2B clones, respectively. After testing of transfected clone growth in mice, the best performing clone 12 EGFP-Cyt and clone 3 EGFP-H2B were chosen as potential candidates and their perfect stability of fluorescence was proven again after *ex vivo* isolation (Figure 1). Throughout the testing, clone 12 performed with much brighter fluorescence compared to clone 3.

Fluorescent microscopy. As expected, green fluorescence of clone 3 expressing fusion protein EGFP-H2B showed clear nuclear co-localization with DNA staining by Hoechst 33342 (Figure 2); EGFP fluorescence of clone 12 was detected throughout the whole cell (Figure 2).

Proliferative activity in vitro. Proliferative activity *in vitro* of both transfected clones, which was measured by MTT conversion assay, demonstrated that the rate of spontaneous proliferation is slightly lower compared to wild-type EL-4 cells (Table I). Transfected lines are up to 3 times more sensitive to treatment with DOX (Table I) compared to the original cell line.

In vivo imaging. Clone 12 does not exhibit tumor growth in syngeneic conventional C57/BL6 mice. However, tumor growth at a rate similar to wild-type EL-4 cells (Figure 3A) was observed in immunodeficient nude mice using the Olympus whole-body imaging system (OV-100). Lung metastases, which were induced by intravenously administered clone 12 were visualized in nude mice through the chest wall (Figure 3B). In contrast, clone 3 is able to grow in immunocompetent mice (Figure 3C) at the same rate as parental EL-4 cells and thus enables studies of the response of tumor to the treatment, tumor microenvironment, inflammation reaction reflecting tumor growth, *etc.* This clone can also be used for tracking cells in the bloodstream or those attached to the wall of blood vessels (Figure 3D) at a single cell resolution *via* the skin flap.

Discussion

Various types of cancer cells have recently been labeled using fluorescent proteins and have become a useful tool to track processes related to cancer growth (7). The important task is to prepare and select imageable transfectants with *in vitro* and *in vivo* behavior similar to that of the parental cancer cell line.

The reasons for metastatic dissemination of lymphomas, and their prevention and treatment represent basic questions to be solved. The EGFP transfected T-lymphomas we have developed can provide insight to enable us to reveal the secrets of these problems. The very bright clone 12 enables the growth of the established metastases in various organs (brain, liver, lymph nodes, spleen *etc.*) to be observed noninvasively, or with the help of only mildly invasive surgery enables the formation and fate of micrometastases to be followed using reversible skin flaps. An obstacle for valuable imunohistochemical studies based on frozen tissue sectioning is free cytoplasmatic EGFP, which disperses while adhering the section to the glass slide. This may be overcome by establishing a transfectant with EGFP fused to

a protein that is not lost during sectioning (for example histone). Moreover, fusion with histone (H2B in our case) brings the advantage of nuclear localization of the EGFP signal. Such condensed occurrence of the fluorescent signal opens the way for other research techniques such as laser scanning cytometry of frozen tissue sections. Fluorescent tumor cells can easily be detected and a dense nuclear signal enables simple segmentation.

Another field of anticancer research is focused on the interaction of tumor cells with the immunocompetent cells of the host and on therapeutical approaches *via* manipulation of immune system. This can be done using fluorescent models and whole-body imaging techniques, which allow the interaction site to be investigated at single-cell resolution. Such processes cannot be examined in human cancer models in immunodeficient athymic or SCID mice, which do not have a fully developed adaptive immune system. Therefore, it is an advantage that we have developed an $EGFP^+$ clone growing in a conventional host with a well-developed immune system, as this is a crucial point for future immuno-interventional studies.

Conclusion

Two different imageable murine models (clones 3 and 12) of EL-4 T-cell lymphoma were established and characterized. Stable transfectants showed a comparable proliferation rate to wild-type EL-4 cells. Moreover, one of them (clone 3) exhibits a tumor growth rate similar to that of the wild-type cell line in conventional C57/BL6 mice, which opens the possibility of evaluating the interaction of the immune system with the tumor in a fully immunocompetent environment. Clone 12, with very strong expression of EGFP, enables effective tracking of metastatic spread, whereas clone 3, with its nuclear expression of EGFP, allows frozen tissue section analysis, which together with the whole-body imaging technique could be a useful tool for understanding tumor-host interactions.

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References

1 Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, Castedo M, Mignot G, Panaretakis T, Casares N, Métivier D, Larochette N, van Endert P, Ciccosanti F, Piacentini M, Zitvogel L and Kroemer G: Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med *13*: 54-61, 2007.
2 Spisek R and Dhodapkar MV: Towards a better way to die with chemotherapy: role of heat-shock protein exposure on dying tumor cells. Cell Cycle *6*: 1962-1965, 2007.
3 Chalfie M, Tu Y, Euskirchen G, Ward WW and Prasher DC: Green fluorescent protein as a marker for gene expression. Science *263*: 802-805, 1994.
4 Shaner NC, Patterson GH and Davidson MW: Advances in fluorescent protein technology. J Cell Sci *120*: 4247-4260, 2007.
5 Hoffman, RM: A better fluorescent protein for whole-body imaging. Trends Biotechnol *26*: 1-3, 2008.
6 Dove A: Illuminating cancer models. Nat Biotechnol *18*: 249, 2000.
7 Hoffman RM: The multiple uses of fluorescent proteins to visualize cancer *in vivo*. Nat Rev Cancer *5*: 796-806, 2005.
8 Hoffman RM and Yang M: Subcellular imaging in the live mouse. Nat Protoc *1*: 775-782, 2006.
9 Hoffman RM and Yang M: Color-coded fluorescence imaging of tumor-host interactions. Nat Protoc *1*: 928-935, 2006.
10 Hoffman RM and Yang M: Whole-body imaging with fluorescent proteins. Nat Protoc *1*: 1429-1438, 2006.
11 Chishima T, Miyagi Y, Wang X, Yamaoka H, Shimada H, Moossa AR and Hoffman RM: Cancer invasion and micrometastasis visualized in live tissue by green fluorescent protein expression. Cancer Res *57*: 2042-2047, 1997.
12 Yang M, Baranov E, Li XM, Wang JW, Jiang P, Li L, Moossa AR, Penman S and Hoffman RM: Whole-body and intravital optical imaging of angiogenesis in orthotopically implanted tumors. Proc Natl Acad Sci USA *98*: 2616-2621, 2001.
13 Yamamoto N, Jiang P, Yang M, Xu M, Yamauchi K, Tsuchiya H, Tomita K, Wahl GM, Moossa AR and Hoffman RM: Cellular dynamics visualized in live cells *in vitro* and *in vivo* by differential dual-color nuclear-cytoplasmic fluorescent-protein expression. Cancer Res *64*: 4251-4256, 2004.
14 Hayashi K, Jiang P, Yamauchi K, Yamamoto N, Tsuchiya H, Tomita K, Moossa AR, Bouvet M and Hoffman RM: Real-time imaging of tumor-cell shedding and trafficking in lymphatic channels. Cancer Res *67*: 8223-8228, 2007.
15 Yang M, Baranov E, Wang JW, Jiang P, Wang X, Sun FX, Bouvet M, Moossa AR, Penman S and Hoffman RM: Direct external imaging of nascent cancer, tumor progression, angiogenesis, and metastasis on internal organs in the fluorescent orthotopic model. Proc Natl Acad Sci USA *99*: 3824-3829, 2002.
16 Tervo HM, Allespach I and Keppler OT: High-level transfection of primary rabbit T lymphocytes. J Immunol Methods *336*: 85-89, 2008.

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