

Blastocyst production and microinjection technology: a reduction and refinement in the use of the mouse in a common laboratory procedure

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Abstract

A key step of chimera creation is the production of injectable blastocysts. To accomplish this, each lab is required to set up its own colony of breeder males and to purchase females at breeding age. This requires significant resources including competence, dedicated space, equipment, and time. Experienced researchers also recognize there can be variations of blastocyst quantity and quality that are difficult to control. We are reporting the development of commercial service to provide frozen morulae that are supported with technical recommendations and appropriate culture media (BlastoKit®, Charles River, Lyon, France). After embryo thawing and overnight culture, the BlastoKit® permits researchers to produce injectable blastocysts while using less space and other resources, including animals, whilst still maintaining the benefits of standardization. NB, because of centralized embryo production, the number of embryos produced per breeding male is higher. These two points participate to a reduction in animal use. In addition, the BlastoKit® helps control the quality of embryo production. The risk to euthanize females for no embryo production and to thaw valuable ES cells clones without blastocyst to inject is almost eliminated. Germ line transmission using BlastoKit® derived embryos has been validated by a range of users in private and academic laboratories for both C57BL/6Ncrl and BALB/cAnNcrl embryos. An analysis of blastocyst development rates (at least 70% for C57BL/6Ncrl and at least 50% for BALB/cAnNcrl embryos), microinjection efficiency and birth rates (up to 40% for C57BL/6Ncrl embryos) obtained during beta-testing will be presented and analyzed.

Blastokit®

When a targeted ES cell clone is identified, this clone needs to be injected into a recipient blastocyst in order to complete the process of knockout mice production. Thus, transgenesis lab needs to produce batches of blastocysts in a timely manner.

This step is technically easy, but producing enough embryos of good quality can be difficult. In particular, embryo quantity can vary a lot from one experiment to another. As the number of ES cells available for injection is often limited, this can be a problem.

Also, managing the breeder colony and the housing of the donor females requires time and space commitments in the animal facility. As a result of these factors, because every transgenesis lab needs to be sure that they can produce sufficient embryos, the number of animals that are used is unnecessarily high.

The BlastoKit® has been developed to solve most of these issues. Using the BlastoKit®, the amount of sufficient injectable blastocysts remains the same over the experiments. Also, when blastocyst production is outsourced in this way, the Transgenics lab is able to reassign cage space in the animal facility and better utilize the staff time that was traditionally dedicated to embryo production. There is also a reduction in the number of animals used in the facility.

The BlastoKit® has been developed to allow injection of 129 ES cells lines into C57BL/6Ncrl blastocysts and B6 ES cells lines into BALB/cAnNcrl blastocysts (1). The first version of the BlastoKit® offers frozen morulae straws and the protocol to thaw them and produce blastocysts. Each straw contains 40 frozen embryos. In the second version of the BlastoKit®, the media required to thaw the embryos and to produce blastocysts will be also provided.

Methods

BlastoKit® morulae production

C57BL/6Ncrl and BALB/cAnNcrl SPF females are superovulated at the age of 3 weeks and 8 weeks, respectively (2), (3). The females are mated with adult males of the same origin. At 2.5dpc, the females are euthanized by CO₂ inhalation and the oviduct recovered. The morulae are recovered from the oviduct by flushing 500µl of M2 medium per oviduct. Healthy morulae are washed twice in 200µl M2 medium drops and placed at 37°C in a 200µl M2 medium drop covered by mineral oil until freezing.

BlastoKit® embryo freezing and thawing

Embryo freezing is performed (according to (4)) in a controlled-rate freezer to -40°C, in 0.3ml straws. Thawing is performed according to (4). After thawing, morulae are washed twice in 200µl drops of M2 and twice in 200µl drops of KSOM.

BlastoKit® blastocyst production

Thawed morulae are incubated overnight at 37°C, in a 200µl drop of KSOM, covered with mineral oil. Starting recovery at 10:00am on Day 1, ready-to-inject blastocysts are obtained by 12:00pm on day 2.

BlastoKit® quality control

For each freezing batch, a QC straw of at least 10 embryos is frozen. The freezing batch is validated for shipment only if the blastocyst production rate (produced blastocysts/frozen morulae) is higher than 75% and 50% for C57BL/6Ncrl and BALB/cAnNcrl, respectively, and if, after reimplantation in a pseudopregnant female, at least one live pup is obtained.

Expected gains

The gains that can be made using the BlastoKit®, in relation to time and the number of animals required to produce 60 blastocysts are evaluated for both C57BL/6Ncrl and BALB/cAnNcrl strains in Table 3. The expected number of chimeras that could be obtained with 60 injected embryos is 5 and 4 (for C57BL/6Ncrl and BALB/cAnNcrl BlastoKit® embryos, respectively), using birth rate and chimera/pup rate indicated in table 1 (small labs for C57BL/6Ncrl embryos). Considering that 60-80% of these chimera are males (data not shown), 2-3 males chimera can be produced, enough for germ line transmission testing. Calculation details are available by contacting the authors. Gains expected from using the BlastoKit® are summarized in Table 4.

Table 4: Expected gains by using BlastoKit®

Animal Use	Speed of Chimera production
Reducing number of females to sacrifice	Guarantee of ES cells injection (no ES cell thawing without blastocysts to inject)
Reduced number of males and females to mate	Stable embryo quality
Reduced number of females sacrificed without embryo production	Stable embryo quality
Reduced number of individual colonies at facility	Ability to plan an experiment only 24 hours in advance
Space Saving	Time Saving
Gain the space dedicated to male colony	No mating
Gain the space for the female stock holding	No vaginal plug checking
Gain the space for the production colony (to produce females and males)	No uterus collection
	No animal stock management

Conclusion

By offering ready-to-use embryos, the BlastoKit® allows transgenesis laboratories to simplify blastocyst production for ES cell injection. The efficiency of the system was assessed by the production of germ line transmitting chimeras over several laboratories. The injection tests showed that embryo quality, birth rates and chimera rates are lower with the BlastoKit® embryos than with fresh ones. This is linked to the use of superovulation and freezing. But, the loss of efficiency is in an acceptable range. With the same numbers of injected embryos (60 blastocysts), the BlastoKit® allows for the production of chimeras from a single line (5 and 4 for C57BL/6Ncrl and BALB/cAnNcrl respectively). The injection tests carried out at several laboratories showed that the BlastoKit® is sensitive to experimental conditions. Laboratories that perform multiple injection sessions per week show lower birth rates than laboratories that perform fewer injection sessions even if the BlastoKit® embryos are from the same origin. After discussion with the "large lab" teams, it seems that the productivity needs of these labs may lead to impaired quality of experimental conditions and to reduced control. Thus, if process quality can be controlled, the BlastoKit® can offer more flexibility and reactivity in the ES cell injection process, in particular in improving consistency in blastocyst quality and quantity. Also, by reducing the number of animals used for blastocyst production, the BlastoKit® allows for reduction and refinement in mouse use and allows some savings to be made in time and in animal facility space.

References

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Injection tests

Even though the full method was validated and published previously for C57BL/6 embryos (5), it is necessary to evaluate the efficiency of the BlastoKit®. The ability of BlastoKit® embryos to develop the blastocyst stage and to give birth to live pups is evaluated in the Charles River embryology lab. But, to evaluate blastocyst injectability and the ability to produce chimeras and conduct germ line transmission, ES cell injections need to be performed. A large program of injection tests was performed on both C57BL/6Ncrl and BALB/cAnNcrl models.

Method

Straws and medium were sent to laboratories that perform ES cell microinjection. The laboratories were from academic research institutions (n=2), pharmaceutical companies (n=3) and biotech companies (n=1). These laboratories thawed the straws according to the protocol sheet provided and they injected ES cells into BlastoKit® embryos in parallel with fresh blastocysts. Blastocyst production rates, embryo injectability, birth rate, chimera production and germ line transmission are assessed.

Results

Results for C57BL/6Ncrl and BALB/cAnNcrl BlastoKit® injection tests are summarized in Table 1. Blastocyst quality results are summarized in Table 2. More detailed results are available by contacting the authors.

Table 1: BlastoKit® injection tests results

		BLASTOKIT® BLASTOCYST PRODUCTION				
		Straws		Recovered embryos	Recovered blastocysts	
		Nbr of straws	Nbr of embryos		Blast./ recov.	Blast./ frozen
C57BL/6Ncrl		63	2520	94,7%	74,7%	70,8%
BALB/cAnNcrl		17	680	94,6%	52,7%	49,9%

		INJECTION TESTS				
		BIRTH		CHIMERAS		
		Implanted blastocysts	Birth rate %	Chimers / Pups	Germ line transmission	
C57BL/6Ncrl	Large Labs	BLASTOKIT®	1477	5,1%	39,5%	YES
		FRESH EMBRYOS	1451	19,9%	65,7%	YES
	Small Labs	BLASTOKIT®	200	24,5%	32,7%	YES
		FRESH EMBRYOS	186	33,9%	55,6%	YES
BALB/cAnNcrl	BLASTOKIT®	384	21,6%	32,5%	Under breeding	
	FRESH EMBRYOS	358	29,3%	49,5%	YES	

Table 2: BlastoKit® blastocysts quality results

		BLASTOKIT® BLASTOCYST QUALITY			
		Nbr of injected embryos	# Poor	# Medium	# High
C57BL/6Ncrl	BLASTOKIT®	1369	19,9%	34,2%	45,9%
	FRESH EMBRYOS	649	0,3%	25,3%	74,4%
BALB/cAnNcrl	BLASTOKIT®	397	5,0%	25,2%	69,8%
	FRESH EMBRYOS	259	3,1%	23,6%	73,4%

Table 3: Animal reduction, time saving and expected results by using BlastoKit®

	C57BL/6Ncrl			BALB/cAnNcrl		
	Fresh embryos	Blastokit®	GAINS	Fresh embryos	Blastokit®	GAINS
Nbre of animals used	30 females	6 females	80%	60 females	25 females	58%
	15 males	6 males	60%	30 males	25 males	16%
Time to produce ready-to-inject embryos	4h	40'	83%	6h	1h	83%